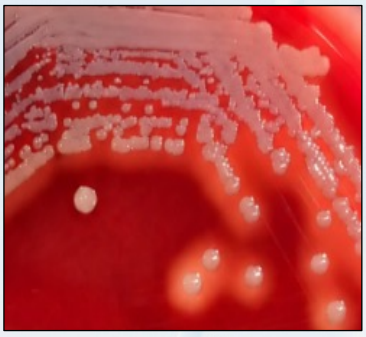
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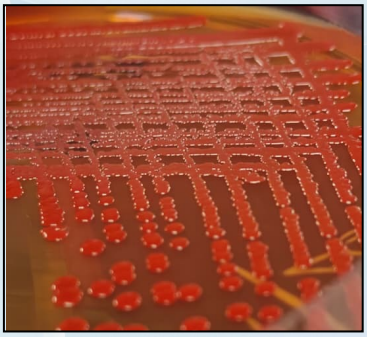
Standard Operating Procedures for Bacteriology

Laboratory Diagnosis of Bacterial Infections and Detection of Antimicrobial Resistance

Third Edition | 2026

Antimicrobial Resistance Surveillance and Research Network





Indian Council of Medical Research
New Delhi, India

STANDARD OPERATING PROCEDURES

FOR

BACTERIOLOGY

Laboratory Diagnosis of Bacterial Infections and Detection of Antimicrobial Resistance

3rd Edition 2026

Antimicrobial Resistance Surveillance and Research Network

Indian Council of Medical Research
New Delhi, India

General disclaimer: The Indian Council of Medical Research (ICMR) has taken all reasonable precautions to ensure the accuracy and reliability of the information presented in this SOP. The technical content has been provided by the ICMR Nodal Centres for Bacteriology and is intended for informational and scientific purposes. The procedures and recommendations should be implemented by appropriately trained personnel in accordance with applicable institutional protocols and biosafety requirements.

Standard Operating Procedures for Bacteriology, 2026
ICMR - Antimicrobial Resistance Research & Surveillance Network (AMRSN)
Third edition
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Foreword

Antimicrobial resistance is one of India's most serious public health threats, and the response to it depends on reliable laboratory data. ICMR's Antimicrobial Resistance Surveillance and Research Network (AMRSN) has, since its inception, generated nationally representative evidence that has shaped treatment guidelines, stewardship and policy. That evidence is only as good as the uniformity of the procedures behind it.

This revised edition updates the earlier manual to reflect recent advances in diagnostic microbiology. It now includes MALDI-TOF and syndromic molecular panels for identification, a single harmonised chapter on susceptibility testing aligned with current CLSI standards, molecular detection of resistance genes, and a strengthened External Quality Assurance Scheme. Reference to any commercial product is made only for standardisation and does not imply endorsement by ICMR.

I commend the editorial board, contributing experts and the ICMR team for their efforts. I am confident this manual will strengthen the quality of AMR data across the country and support India's National Action Plan on AMR.

My best wishes to all users of this manual.

Rajiv Bahl

Dr Rajiv Bahl
Director General, ICMR,
Secretary, Department of Health Research

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Abbreviations

AST	Antimicrobial susceptibility testing
ATCC	American Type Culture Collection
BAL	Bronchoalveolar lavage
BAP	Blood Agar Plate
BCC	*Burkholderia cepacia* complex
BHIB	Brain heart infusion broth
BPMM	Biphasic McConkey medium
BSL	Biosafety level
CAPD	Continuous ambulatory peritoneal dialysis
CHOC	Chocolate agar
CLSI	Clinical and Laboratory Standards Institute
CoNS	Coagulase negative staphylococci
CLABSI	Central Line Associated Blood Stream Infection
CRBSI	Catheter related blood stream infection
CTA	Cystine trypticase agar
DD	Double disk
DS	Double strength
EDS	EDTA disk synergy
ESBL	Extended spectrum β-lactamases
ETA	Endotracheal aspirate
HLAR	High level aminoglycoside resistance
HTM	*Haemophilus* test medium
IAMM	Indian Association of Medical Microbiologists
MAC	McConkey agar
MBC	Minimum bactericidal concentration
MBL	Metallo beta-lactamases
MHA	Müeller Hinton agar
MHB	Mueller Hinton broth
MH-SB	Mueller Hinton sheep blood agar

MHT	Modified Hodge test
MIC	Minimum inhibitory concentration
MRSA	Methicillin resistant *Staphylococcus aureus*
NA	Nutrient agar
NB	Nutrient broth
NS	Non-susceptible
OF	Oxidation-fermentation test
OLB	Open lung biopsy
ONPG	Ortho-nitrophenyl-β-D galactopyranoside
PCN	Percutaneous nephrostomy
PCR	Polymerase chain reaction
PDA	Phenylalanine deaminase
PMNs	Polymorphonuclear cells
PPA	Phenylpyruvic acid
PPE	Personal protective equipment
QC	Quality control
RCM	Robertson's cooked meat
SBP	Spontaneous bacterial peritonitis
SEC	Squamous epithelial cells
SPS	Sodium polyanetholsulfonate
TCBS	Thiosulfate citrate bile salt sucrose agar
TSA	Trypticase soya agar
TSB	Trypticase soya broth
TSBA	Trypticase soya blood agar
UTI	Urinary tract infection
YLO	Yeast-Like Organisms

Chapter 1
General Guidelines

1.1. Introduction

This is a comprehensive standard operating procedure manual for all types of specimens received in a clinical bacteriology diagnostic laboratory serving a tertiary care hospital. The manual has been compiled by referring to international protocols customized to the needs and the infrastructure already available in India or infrastructure that can be achieved by upgradation. Both conventional and automated procedural alternatives are included. It is intended that all participating laboratories, including Nodal Centers and the Regional Center laboratories, will strictly adhere to the procedures. The MANUAL has been organized to place each part of the procedure together, including sample collection, specimen processing, supplies, quality control (QC), and step-by-step testing procedure. This will allow the user to see an overview of the entire procedure together. Guidelines for specimen collection and transport can be separately made available to the collection points and those for processing are to be made available in the processing laboratories. All the laboratories must isolate, identify to species level and carry out susceptibility tests of significant bacterial isolates as per guidelines provided. For isolates, which are difficult to identify, Regional Center laboratories can send the isolates to Nodal Centers for further characterization.

1.2. Role of the laboratory

Microbiologists play a critical role in generating, gathering, and analyzing data both for clinical and public health decision making. Efficient and accurate microbiologic diagnosis of bacterial infections guides the choice of antibiotics and other treatment options for the patient. Similarly, microbiological surveillance is critical to guide appropriate antibiotic therapy through the identification of local resistance profiles. Thus, the role of the microbiology laboratory is essential in preventing infection related morbidity and mortality from bacterial infections.

1.3. Biosafety

Laboratory staff handling infectious agents are at risk of laboratory-acquired infections as a result of accidents or unrecognized incidents. Laboratory-acquired infections occur when microorganisms are inadvertently ingested, inhaled, or introduced into tissues. The degree of hazard depends upon the virulence and dose of the biological agent, route of exposure, mode of transmission, host resistance, proper biosafety training and experience with biohazards.

While laboratory-acquired infections are not as extensively reported, deadly infections with any of the microorganisms are possible if appropriate biosafety procedures are not strictly followed in a properly equipped laboratory. Biosafety Level 2 (BSL-2) practices are required for work involving these agents as they present a potential hazard to personnel and the environment.

The following requirements have been established for laboratorians working in BSL-2 facilities:

- Laboratory personnel must receive specific training in handling pathogenic agents by fully trained and experienced scientists.
- Access to the laboratory must be limited to personnel who have a need to be in the laboratory and have undergone proper training.
- All necessary precautions must be taken with contaminated sharp items and other biomedical wastes (BMWs), and sharps or other BMWs must be disposed of in appropriately labeled hard plastic containers as per the guidelines of the local/central Pollution Control Board (PCB).
- Personal protective equipment (PPE) must be used appropriately, and care must be taken when performing procedures that have the potential to create aerosols.

Table 1.1: Relation of risk groups to biosafety levels, practices and equipment (WHO, 2004)

RISK GROUP	BIOSAFETY LEVEL	LABORATORY TYPE	LABORATORY PRACTICES	SAFETY EQUIPMENT
1	Basic – Biosafety Level 1	Basic teaching, research	GMT	None; open bench work
2	Basic – Biosafety Level 2	Primary health services; diagnostic services, research	GMT plus protective clothing, biohazard sign	Open bench plus BSC for potential aerosols
3	Containment – Biosafety Level 3	Special diagnostic services, research	As Level 2 plus special clothing, controlled access, directional airflow	BSC and/or other primary devices for all activities
4	Maximum containment – Biosafety Level 4	Dangerous pathogen units	As Level 3 plus airlock entry, shower exit, special waste disposal	Class III BSC, or positive pressure suits in conjunction with Class II BSCs, double-ended autoclave (through the wall), filtered air

BSC: biological safety cabinets; GMT: good microbiological techniques

Table 1.2: Summary of biosafety level requirements (WHO, 2004)

	BIOSAFETY LEVEL			
	1	2	3	4
Isolation[a] of laboratory	No	No	Yes	Yes
Room sealable for decontamination	No	No	Yes	Yes
Ventilation:				
— inward airflow	No	Desirable	Yes	Yes
— controlled ventilating system	No	Desirable	Yes	Yes
— HEPA-filtered air exhaust	No	No	Yes/No[b]	Yes
Double-door entry	No	No	Yes	Yes
Airlock	No	No	No	Yes
Airlock with shower	No	No	No	Yes
Anteroom	No	No	Yes	—
Anteroom with shower	No	No	Yes/No[c]	No
Effluent treatment	No	No	Yes/No[c]	Yes
Autoclave:				
— on site	No	Desirable	Yes	Yes
— in laboratory room	No	No	Desirable	Yes
— double-ended	No	No	Desirable	Yes
Biological safety cabinets	No	Desirable	Yes	Yes
Personnel safety monitoring capability[d]	No	No	Desirable	Yes

[a] Environmental and functional isolation from general traffic.
[b] Dependent on location of exhaust (see Chapter 4).
[c] Dependent on agent(s) used in the laboratory.
[d] For example, window, closed-circuit television, two-way communication.

1.3.1. Protective clothing and equipment

1.3.1.1. Laboratory coats

Protective coats, gowns, aprons, or uniforms designated for laboratory use must be worn while working in the laboratory. Laboratory coats should fit properly and should cover arms to the wrist. This protective clothing must be removed and left in the laboratory before leaving for non-laboratory areas, such as offices or eating areas. All protective clothing is either disposed off in the laboratory or laundered by the institution; personnel should never take it home.

1.3.1.2. Gloves

Regardless of the type of infectious material, gloves should be worn when performing potentially hazardous procedures involving infectious materials in which there is a risk of splashing or skin contamination or when the laboratory worker has cuts or broken skin on his or her hands. Gloves should always be worn when handling clinical specimens, body fluids, and tissues from humans and animals. These specimens should be handled as if they are positive for hepatitis B virus, human immunodeficiency virus (HIV), or any other blood borne pathogen. Gloves must be removed when contaminated by splashing or spills or when work with infectious materials is completed. When removing gloves, avoid touching any area of the gloves that may have come in contact with infectious material.

Gloves should not be worn outside the laboratory. Personnel should not use the telephone, computer, or open doors with gloves that have been used in laboratory procedures. All used gloves should be disposed of by discarding them with other disposable materials and autoclaving. Hands should be washed immediately after removing gloves.

1.3.1.3. Barrier precautions

Clinical specimens, body fluids, and tissues from humans and animals should be assumed to be positive for human pathogens. These materials should be handled in a biosafety cabinet (BSC) or using other barrier precautions (*e.g.*, goggles, mask, face shield, or other splatter guards) whenever a procedure is performed that can potentially create an aerosol. Closed-toe comfortable shoes that have low heels should be worn in the laboratory or other areas where chemicals are present. This will reduce injuries that may occur from spills, splashes, falling objects, slipping, and broken glass.

1.3.2. Standard microbiological safety practices

The following safety guidelines apply to all microbiology laboratories, regardless of biosafety level. All procedures requiring handling of infectious materials, potentially infectious materials, or clinical specimens should be performed while wearing appropriate PPE.

1.3.2.1. Limiting access to laboratory

Sometimes non-laboratory staff may attempt to enter the laboratory to obtain test results etc. Although this occurs more frequently in clinical laboratories, access to the laboratory should be limited to trained personnel with a need to work in the laboratory, regardless of the setting. Biohazard warning signs or stickers should be posted near or on all laboratory doors. Children who have not reached the age of adulthood and pets are not allowed in laboratory areas. Laboratories should be locked when not in use. In addition, freezers and refrigerators located in corridors should be locked at all times, especially the ones with infectious organisms or other hazardous materials.

1.3.2.2. Autoclaving

An autoclave must be available for the BSL-2 laboratory and must be operated only by personnel who have been properly trained in its use. Each autoclave load should be monitored with temperature-sensitive tape, thermograph, or by other means (e.g., chemical indicators or biological indicators). Spore strips (such as *Geobacillus stearothermophilus*) or other biological indicators designed to test the efficiency of sterilization should be included in autoclave loads on a regular basis (e.g., monthly). A

logbook should be maintained for each autoclave to record the date, time, and indicator of sterilization of each autoclave run.

1.3.2.3. Disinfection (Georgia Department of Public Health, 2018; University College London, Medical School, 2018)

Different organisms have different susceptibilities to different disinfectants. 70% isopropyl alcohol is though generally effective as a surface disinfectant, is not the disinfectant of choice for decontaminating spills. It should be noted that 100% alcohol is not as effective a disinfectant as 70% alcohol. Phenolic disinfectants, although expensive, are effective against many organisms. Phenolic compounds (e.g. Chloroxylenol) are active against vegetative bacteria and lipid-containing viruses and Mycobacteria, but not against spores and have variable activity against nonlipid viruses. Always refer to manufacturer's label for recommended dilution and exposure times. An effective general disinfectant is a 1:100 dilution of household bleach (sodium hypochlorite, about 5%) in water, which gives roughly 0.05% or 500 ppm of available chlorine. At this dilution, bleach can be used to wipe down benches, hoods, and other equipment. A 1:10 dilution (about 0.5% or 5,000 ppm available chlorine) should be used to clean up spills of cultured or concentrated infectious material where heavy contamination has occurred. However, this stronger solution is more corrosive, will pit stainless steel, and should not be used routinely. After using bleach, wipe the area down with water or 70% alcohol to remove corrosive residue. Diluted bleach solutions lose potency over time, so prepare them fresh from a concentrated stock solution.

1.3.2.4. Disposal of contaminated materials

All discarded plates, tubes, clinical samples, pipettes, gloves, and other contaminated materials should be placed in disposal containers at each bench. Special disposal containers typically constructed of puncture-proof plastic must be used for sharps to minimize the risk of injury. Avoid overfilling disposal containers. The lids should rest flush with the top of the container. Containers of contaminated material should be carefully transported to the autoclave room and autoclaved before disposal. Water should be added to each container to be autoclaved for optimal sterilization. Waste disposal containers in the laboratory should be clearly labeled for disposal of infectious items or non-infectious waste. Waste disposal containers for infectious or potentially infectious items should be lined with a plastic biohazard or otherwise specially marked bag.

1.3.2.5. Decontaminating bench tops and other surfaces

Bench tops and other potentially contaminated surfaces should be wiped with a disinfectant (1% bleach) routinely after working with infectious agents or clinical specimens or after spills, splashes, or contamination by infectious materials. Following disinfection with 1% bleach (10,000 ppm chlorine), the surface must be wiped down with

70% isopropyl or ethyl alcohol to remove the bleach and prevent corrosion of the work surface. Solutions of disinfectants should be maintained at each work station. For regular disinfection (i.e. no spillage) 0.1% (1000 ppm) of bleach solution would suffice.

Table 1.3: Recommended dilutions of chlorine-releasing compounds (WHO, 2004)

	"CLEAN" CONDITIONS[a]	"DIRTY" CONDITIONS[b]
Available chlorine required	0.1% (1 g/l)	0.5% (5 g/l)
Sodium hypochlorite solution (5% available chlorine)	20 ml/l	100 ml/l
Calcium hypochlorite (70% available chlorine)	1.4 g/l	7.0 g/l
Sodium dichloroisocyanurate powder (60% available chlorine)	1.7 g/l	8.5 g/l
Sodium dichloroisocyanurate tablets (1.5 g available chlorine per tablet)	1 tablet per litre	4 tablets per litre
Chloramine (25% available chlorine)[c]	20 g/l	20 g/l

[a] After removal of bulk material.
[b] For flooding, e.g. on blood or before removal of bulk material.
[c] See text.

1.3.2.6. General laboratory cleanliness

All areas of the laboratory must be kept clean and orderly. Dirt, dust, crowding, or clutter is a safety hazard, may lead to contamination of specimens, isolates, and/or biological assays, and is not consistent with acceptable biological research. Floors should be kept clean and free of unnecessary clutter and should be washed with a germicidal solution on a regular basis and after any spill of infectious material.

1.3.2.7. Decontamination of spills

The following procedure is recommended for decontaminating spills:

- Isolate the area to prevent anyone from entering.
- Wear gloves and protective clothing such as a gown or lab coat, shoes, and a mask (if the spill may contain a respiratory agent or if the agent is unknown).
- Absorb or cover the spill with disposable towels, but do not wipe up the spill or remove the towels.
- Saturate the towels and the affected area with an appropriately diluted intermediate or high-level disinfectant (*e.g.*, household bleach- 5,000 ppm to 10,000 ppm of available chlorine) and leave them in place for at least 15 minutes.
- Wipe area using clean disinfectant-soaked towels and allow area to air dry.
- Place all disposable materials used to decontaminate the spill into a biohazard container. If broken glassware is involved, use mechanical means to dispose it.

- Handle the material in the same manner as other infectious waste.

1.3.2.8. Hand washing

All laboratories should contain a sink with running water and soap for hand washing. Frequent hand washing is one of the most effective procedures for avoiding laboratory-acquired infections. Hands should be washed for at least one minute with an appropriate germicidal soap after infectious materials are handled and before exiting the laboratory. If germicidal soap is unavailable, then use 70% isopropyl or ethyl alcohol to cleanse hands. Alcohol based hand cleanser is not a substitute when hands are visibly soiled or contaminated with biological material.

1.3.2.9. Mouth pipetting

Rubber bulbs or mechanical devices must be used for pipetting. Mouth pipetting is strictly prohibited.

1.3.2.10. Sharps

A high degree of precaution must always be taken when handling contaminated sharp items, including needles and syringes, slides, glass pipettes, capillary tubes, broken glassware, and scalpels. Sharps should be disposed off in designated puncture-proof, leak-proof, and sealable sharps containers. To minimize finger sticks, used disposable needles must not be bent, sheared, broken, recapped, removed from disposable syringes, or otherwise manipulated by hand before disposal. Non-disposable sharps should be placed in a labeled discard pan for decontamination before cleaning. Broken glassware should not be handled directly by hand but should be removed by mechanical means (*e.g.*, brush and dustpan, tongs, or forceps).

1.3.2.11. Aerosols

Take all precautions to minimize splashes or aerosolization. When undertaking procedures with a high potential for creating infectious aerosols or splashing or spraying of the face with infectious or other hazardous materials, laboratory work should be conducted in a biosafety cabinet or by laboratorian wearing the appropriate face protection equipment (*e.g.*, goggles, mask, face shield, or other splatter guards). Face protection should also be used when working with high concentrations or large volumes of infectious agents. Procedures that pose such a risk may include:

- Centrifugation, vortexing, and vigorous mixing: these procedures should be performed in closed containers like safety-capped or sealed tubes.
- All body fluids and infectious materials should only be centrifuged in carriers with safety caps.
- Containers of infectious material that may be under pressure or at a different temperature from the room (and so may splash when opened) should be opened inside a BSC. Place gauze around the closure while opening to reduce splashes and aerosols.
- Grinding of tissue specimens should be performed in a biosafety cabinet.

- For sonic disruption, infectious materials should be placed in a sealed container in the sonicator.
- When opening containers of infectious materials, where internal pressure or temperature differences can cause splashing, container should be opened inside a BSC. While opening such containers, gauze may be placed around the closure to minimize the splashes and aerosol generation.
- Loops containing infectious material should be dried in the hot air above a burner before flaming.
- Inoculating wires and loops should be cooled after flame sterilization by holding them still in the air for 5-10 seconds before they touch colonies or clinical material. Disposable loops are preferred if resources are available.

1.3.2.12. Refrigerators and freezers

The temperature of laboratory refrigerators and freezers should be monitored daily to ensure. They should also be regularly inspected for the presence of broken vials or tubes containing infectious agents. When removing and discarding broken material, laboratorians should wear gloves and PPE. If the broken material is suspected of being infectious, disinfectant should be applied to the affected area and kept in place for at least 15 minutes before removal of the broken material. Refrigerators and freezers should be regularly cleaned with a disinfectant and defrosted to prevent possible contamination or temperature failure.

1.3.2.13. Fire prevention

Burners when in use should be placed away from light fixtures and flammable materials. Bulk quantities of flammable material must be stored in a safety cabinet. Small amounts of these flammable materials (*e.g*., ethyl acetate, ethyl alcohol, and methanol) can be stored in safety containers or safety cabinets for chemical storage. Burners must be turned off when not in use. All laboratory staff must know the location of fire extinguishers, fire blankets, alarms, and showers, and fire safety instructions and evacuation routes should be posted in lab areas.

Note: Chemicals should not be stored in alphabetical order. Chemicals must be segregated and stored according to their hazard class and compatibility

1.3.2.14. Eating

Eating, drinking, and smoking are not permitted in laboratory work areas. Food must be stored and eaten outside the laboratory in areas designated for that purpose only. Personal articles (*e.g*., handbags, eyeglasses, or wallets) should not be placed on laboratory workstations.

Table 1.4: General rules for chemical incompatibilities (WHO, 2004)

SUBSTANCE CATEGORY	INCOMPATIBLE SUBSTANCES
Alkali metals, e.g. sodium, potassium, caesium and lithium	Carbon dioxide, chlorinated hydrocarbons, water
Halogens	Ammonia, acetylene, hydrocarbons
Acetic acid, hydrogen sulfide, aniline, hydrocarbons, sulfuric acid	Oxidizing agents, e.g. chromic acid, nitric acid, peroxides, permanganates

1.3.3. Special Practices

1.3.3.1. Accidents

All injuries or unusual incidents should be reported immediately to the supervisor. In event of cuts or puncture wounds from potentially infected needles or glassware occur, the affected area should be promptly washed with disinfectant soap and water for 15 minutes. Needle-stick injury, any other skin puncture, should be immediately reported to the supervisor and appropriate health officials and prophylactic treatment for the personnel, as applicable, performing the procedure may be initiated at the earliest. In the event of a centrifuge accident in which safety carriers have not been used, other personnel in the area should be warned immediately and the area should be sealed to prevent anyone from entering.

1.3.3.2. Laboratory design and equipment

The laboratory should be designed to avoid conditions that pose biosafety risks. Ample space should be provided to allow for safe circulation of staff when working and cleaning. Separate areas for infectious and non-infectious work should be designated. Illumination should be adequate. Walls, ceiling, floors, benches, and chairs must be easy to clean, impermeable to liquids, and resistant to chemicals and disinfectants. Hand-washing basins with running water and soap and disinfectant must be provided in each room. An autoclave or other means of decontamination must be available close to the laboratory. Adequate storage space for specimens, reagents, supplies, or personal items should be provided inside and outside the working area, as appropriate. Safety systems for fire, chemicals, electrical, or radiation emergencies, and an emergency shower and eyewash facilities should be in place and regularly audited. Security measures should also prevent theft, misuse, or deliberate release of the infectious materials.

Table 1.5: Biosafety equipment (WHO, 2004)

EQUIPMENT	HAZARD CORRECTED	SAFETY FEATURES
Biological safety cabinet		
— Class I	Aerosol and spatter	• Minimum inward airflow (face velocity) at work access opening. Adequate filtration of exhaust air. • Does not provide product protection
— Class II	Aerosol and spatter	• Minimum inward airflow (face velocity) at work access opening. Adequate filtration of exhaust air • Provides product protection
— Class III	Aerosol and spatter	• Maximum containment • Provides product protection if laminar flow air is included
Negative pressure flexible-film isolator	Aerosol and spatter	• Maximum containment
Spatter shield	Spatter of chemicals	• Forms screen between operator and work
Pipetting aids	Hazards from pipetting by mouth, e.g. ingestion of pathogens, inhalation of aerosols produced by mouth suction on pipette, blowing out of liquid or dripping from pipette, contamination of suction end of pipette	• Ease of use • Controls contamination of suction end of pipette, protecting pipetting aid, user and vacuum line • Can be sterilized • Controls leakage from pipette tip
Loop microinciner-ators, disposable loops	Spatter from transfer loops	• Shielded in open-ended glass or ceramic tube. Heated by gas or electricity • Disposable, no heating necessary
Leakproof vessels for collection and transport of infectious materials for sterilization within a facility	Aerosols, spillage and leakage	• Leakproof construction with lid or cover • Durable • Autoclavable
Sharps disposal containers	Puncture wounds	• Autoclavable • Robust, puncture-proof
Transport containers between laboratories, institutions	Release of microorganisms	• Robust • Watertight primary and secondary containers to contain spills • Absorbent material to contain spills
Autoclaves, manual or automatic	Infectious material (made safe for disposal or reuse)	• Approved design • Effective heat sterilization
Screw-capped bottles	Aerosols and spillage	• Effective containment
Vacuum line protection	Contamination of laboratory vacuum system with aerosols and overflow fluids	• Cartridge-type filter prevents passage of aerosols (particle size 0.45 μm) • Overflow flask contains appropriate disinfectant. Rubber bulb may be used to close off vacuum auto matically when storage flask is full • Entire unit autoclavable

Table 1.6: Difference between class I, II and III biological safety cabinets (BSCs)(WHO, 2004)

BSC	FACE VELOCITY (m/s)	AIRFLOW (%)		EXHAUST SYSTEM
		RECIRCULATED	EXHAUSTED	
Class I[a]	0.36	0	100	Hard duct
Class IIA1	0.38–0.51	70	30	Exhaust to room or thimble connection
Class IIA2 vented to the outside[a]	0.51	70	30	Exhaust to room or thimble connection
Class IIB1[a]	0.51	30	70	Hard duct
Class IIB2[a]	0.51	0	100	Hard duct
Class III[a]	NA	0	100	Hard duct

1.3.3.3. Medical surveillance of laboratory workers

The employing authority is responsible for providing adequate surveillance and management of occupationally acquired infections. Pre-employment and periodic health checks should be organized and performed. Pre-employment checks should include a history of current/past illness, physical examination, immunization history, ocular examination (visual acuity, color vision), infection screen (HIV (National AIDS Control Organization, 2016), HBV, HCV, tuberculosis) and immunity screen (hepatitis B surface antibody). According to the pollution control board recommendations all health care workers are required to be immunized against HBV and tetanus [Bio-Medical Waste Management (Amendment) Rules, 2018] (Ministry of Environment, Forest and Climate Change, 2018)**.**

- Prophylaxis or other specific protective measures may be applied after a risk assessment of potential exposure and a health check of the individual or individuals. Special attention should be paid to women of childbearing age and pregnant women as some microorganisms present a higher risk for the fetus.
- Immunization of the laboratory workers can also be proposed taking into account the following criteria:
    1) Conclusion of the risk assessment
    2) Verification by serology of the immunization status of the worker (some workers may be already immune from prior vaccination or infection)
    3) The local availability, licensing status, and utility of vaccines (*i.e.*, does it provide protection against the prevalent serogroups or serotypes circulating in the region?)
- The availability of therapeutic drugs (*i.e.*, antibiotics) in case of accident
- The existence of national regulations or recommendations

- A first-aid box containing basic medical supplies should be available along with a written emergency procedure to access a doctor for definitive treatment of the injury. First aid kits should be periodically checked to ensure contents are within the expiration date

1.4. Laboratory safety instructions

- Do not eat or drink in the laboratory.
- Benches should be clear of all non-essential materials including books and notes.
- Wear laboratory coats and gloves while working in the laboratory.
- Wipe working area with a disinfectant at the beginning and end of laboratory session.
- Avoid any activity that introduces objects into the mouth, e.g. mouth pipetting.
- Cover any open cuts on hands and other exposed skin surfaces with a water-resistant dressing.
- Carry out all procedures in a way so as to minimize the risks of spills, splashes and the production of aerosols.
- Always wash your hands before leaving the laboratory.

1.5. Emergency measures: Mishaps with infective material

1.5.1. Spillage or minor spills

Cover the spill with a cloth or tissue soaked in disinfectant, leave for at least 15 minutes and then mop up. If there is a gross spillage or any spillage with a specimen likely to contain a category 3 organism (*e.g*. sputum) outside the safety cabinet, evacuate the room for at least an hour to allow possible aerosols to be dispersed. Then, for group 2 organisms, disinfect and clean up. For group 3 organisms, fumigate the room. Deal with spillages in safety cabinets by disinfecting the affected surfaces in the cabinet, and in group 3 organisms spillage fumigate the cabinet.

1.5.2. Management of Occupational Exposure

Encourage cuts and puncture wounds to bleed and then wash with soap and water. If the eye is splashed, rinse at once with tap water or irrigating solution from the laboratory first aid kit. If skin is soiled with infective material, rinse with 70% alcohol and then with soap and water. Following cut or puncture, if post exposure prophylaxis for HIV is contemplated, use the following schedule.

Dosages of the Drugs for PEP for adults and adolescents- FDC of Tenofovir (TDF) 300 mg plus Lamivudine (3TC) 300 mg plus Efavirenz (EFV) 600 mg once daily for 4 weeks. If the source is already on ART, start the exposed person the above mentioned regimen at the earliest with proper counseling and then refer for an expert opinion.

Chapter 2

Specimen Collection, Transport and Processing

2.1. BLOOD

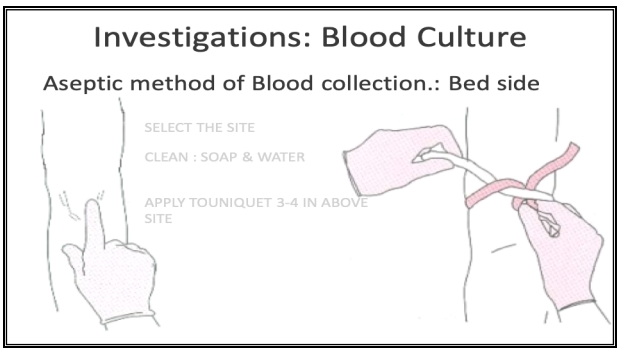
2.1.1. Collection and transport

Purpose: To reduce blood culture contamination rate, collection may be improved by taking the following precautions (Little et al., 1999; Mimoz et al., 1999; Isenberg, 2007; CLSI, 2007).

Note: This is an emergency procedure. The sample should be processed and reported immediately. The results of the smear should be informed to the concerned clinician and documented in the critical alert register.

2.1.1.1. Prepare the site

- Select the site of venipuncture. If the patient is unusually dirty, wash the intended site with soap and water prior to venipuncture.
- Apply a tourniquet, 3-4 inches above the intended site of venipuncture. Alternatively, this can be done after cleaning.
- Put on examination gloves.
- Vigorously cleanse with 70% isopropyl or ethyl alcohol to remove surface dirt and oils. Scrub the venipuncture site gently but firmly with the cotton beginning in the center and continuing in an outward direction circularly for an area of 4 to 5 inches in diameter.
- Allow to dry.

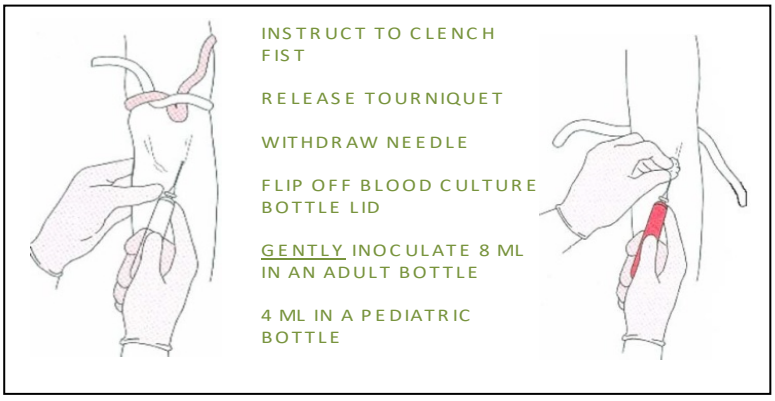


- Swab or wipe concentric circles of 2% w/v chlorhexidine with 70% isopropyl alcohol or 10% w/v povidone iodine/tincture of iodine, in a similar manner as given earlier- beginning in the center and continuing in an outward direction circularly for an area of 4 to 5 inches in diameter.

- Allow the povidone iodine to dry (2 minutes). For chlorhexidine gluconate (2%w/v)/ tincture I_2 (10%w/v), drying period is ~ 30 seconds.
- Do NOT touch the site after cleaning.
- Instruct patient to clench and unclench the fist.



- Perform phlebotomy using the needle and syringe.
- Release the tourniquet and withdraw the needle.
- Apply pressure to the site of venipuncture and place a bandage over the puncture site.



- Skin preparation with either alcohol, alcoholic chlorhexidine (2% w/v), or tincture of iodine (10% w/v) leads to lower blood culture contamination rates than does the use of povidone-iodine (Little et al., 1999; Mimoz et al., 1999).
- **For pediatric patients**

< 2months: Omit the iodine step, and clean two additional times with separate preparation pads saturated with 70% isopropyl alcohol or ethyl alcohol

> 2 months: Chlorhexidine gluconate as a skin antiseptic is approved for use in pediatric patients two months of age and older (CLSI, 2007).

2.1.1.2. Prepare the bottle

Prepare the septum of the blood culture bottle and the rubber stoppers on bottles or tubes. Label the bottles with the patient's name and the date and time of draw.

Site of draw may be listed.

Note: In particular, please mention whether blood is collected from a central line or from peripheral venipuncture.

Collection through an intravenous line

- It is not necessary to discard the initial volume of blood or flush the line with saline to eliminate residual heparin or other anticoagulants (CLSI, 2007).
- Vigorously wipe septa with 70% alcohol and allow drying completely, for 30 to 60 seconds.
- Pediatric bottles should not to be used for adult patients except for those elderly patients in whom it's difficult to obtain larger amounts of blood.

Table 2.1: Recommended total volume and numbers of blood cultures (CLSI, 2007)

Age & body weight	Amount (divided between 2 blood cultures)	Remarks
Neonates to 1 year (<4 kg)	0.5 to 1.5 ml	At least 1 ml Two separate venipunctures are generally not possible
Children (< 40 kg)	10 to 20 ml	Blood culture volumes should be limited to <1% of total blood volume (usually about 0.7 ml/kg). e.g. total sample limit would be 7 ml for a 10 kg patient and 28 ml for a 40 kg patient.
Adults & children (>40 kg)	30 to 40 ml	At least 10-20 ml of blood

- **Adult patient (50 kg):** 10-20 ml, divided between **two** blood cultures from **separate**, peripheral venipuncture **sites**.
- Anerobic blood cultures should be taken only if there are adequate resources (James & al-Shafi, 2000).
- **Pediatric patient:** 6-10 ml, divided between two blood cultures.
- Initially obtain **three** blood culture sets within a 30 minute period **before administration of empiric antimicrobial agents** from patients presenting with possible **infective endocarditis**. If those sets are negative **at 24 hours**, obtain two more sets of cultures, for a total of five sets overall (CLSI, 2007).

2.1.1.3. Timing of blood cultures

Note: Although drawing blood cultures before or during the fever spike is optimal for recovery**,** volume is more important **than** timing in the detection of agents of septicemia.

Thoroughly mix bottles to avoid clotting.

Don't forget: After phlebotomy, remove residual tincture of iodine from the patient's skin by cleansing with alcohol to avoid skin irritation.

Manual blood culture inoculation

For conventional blood culture method, blood culture for bacterial infections should be carried out in two bottles containing 50 ml each of tryptone soya broth and bile broth. After removing the kraft paper, inoculate the blood culture bottles. Incubate at 37ºC and examine daily for 7 days for evidence of growth, indicated by turbidity, hemolysis, gas production, discrete colonies, or a combination of these. DO NOT USE CULTURE BOTTLE IF OLDER THAN 7 DAYS. If beyond, replace and issue a fresh bottle. Clean the surface with alcohol wipe after removing date tag and let it dry before inoculating the blood sample to broth.

Automated blood culture inoculation

Remove the plastic caps and disinfect the rubber septa on the bottles with a 70%alcohol. Let the bottles dry. Ensure barcode is not placed over the barcode already present on the automated culture bottle.

Inoculation of blood to the culture bottles: Change the used needle for sampling with a new sterile needle to insert 8–10 mL of blood into each bottle.

1. Start with the aerobic bottle, then fill the anaerobic bottle.
2. For a conventional bottle insert needle through the hole in the metallic cap.

2.1.1.4. Transport of blood culture bottles

In case of delay between collection and processing, **never refrigerate the bottle**. Preferably keep the bottle in a 35ºC incubator, if available. Otherwise, leave the bottle at room temperature.

2.1.2. Processing of blood cultures

a. Safety

   i. Keep the culture bottles within a biosafety cabinet or behind a shield, or wear a face mask.

   ii. Always wear gloves, because blood cultures contain material from patients who may harbor blood-borne pathogens.

   iii. Use needleless transfer devices or safety needles, and never recap them.

   iv. Dispose off needles and syringes in puncture-proof container.

b. Incubate blood cultures for the predetermined period at 35°C (usually 5 days, unless quality monitors indicate less time).

c. Maintain incubation conditions to allow recovery of microorganisms (follow manufacturer's instructions) and maintain rotation or agitation of the media if possible.

d. Examine the cultures at least daily, whether detection of positives is by visual inspection or by an automated system. For visual inspection, observe for hemolysis, turbidity, gas production, pellicle formation, "puffballs," and clotting, which are indicative of microbial growth.

Table 2.2: Visible signs of growth caused by organisms commonly encountered in blood cultures

Microscopic observation	Associated microorganisms
Hemolysis	Streptococci, staphylococci, *Listeria* spp., *Clostridium* spp., *Bacillus* spp.
Turbidity	Aerobic Gram-negative bacilli, staphylococci, *Bacteroides* spp.
Gas formation	Aerobic Gram-negative bacilli, anaerobes
Pellicle formation	*Pseudomonas* spp., *Bacillus* spp., yeast cells
Clotting	*Staphylococcus aureus*
Visible colonies, "puffballs"	Staphylococci, Streptococci

e. For manual broth systems, perform at least one blind subculture to solid agar from visually negative bottles. Perform blind subculture after overnight incubation, and on 5th (final) day post-incubation on sheep blood agar and McConkey agar (MAC). In between, examine the bottles daily and subculture on solid media whenever there is a visible sign of growth in the bottle.

Note: Subculture of automated systems has little clinical utility.

f. In special circumstances when cultures appear to be negative, perform a Gram stain, wet mount, or acridine orange stain from the culture or its sediment to determine the presence of organisms.

g. Discard positive and negative bottles safely after autoclaving at 121ºC for 30 minutes. After autoclaving, open the automated system bottles with an opener and discard the inoculated medium in a designated shank for biomedical waste. For manual systems, open the screw-capped bottles, and discard the cultured media.

2.1.3. Further processing of positive blood cultures

a. Gram-stain a thin smear from the broth or agar immediately when suggestive of growth, for optimal patient care.

b. Subculture to agar media and put up biochemical tests based on the Gram stain results.

c. Follow-up workup of positive blood culture isolates.

2.1.4. Reporting results

a. For "No growth" cultures, indicate the length of incubation: "No growth after x days of incubation" for both preliminary and, final reports (automated systems: 5 days, manual systems: 5-7 days).

b. Positive cultures

i. Immediately report Gram stain results of all positive cultures to the physician in-charge, with as much interpretive information as possible.

ii. Follow immediately with a written or computer-generated final report including the following:

- AST result.
- Date and time of collection and receipt.
- Date and time positive result is reported and whether it was from a catheter draw or a peripheral draw.

Note: Such information is useful in the diagnosis of catheter-related bloodstream infections (CRBSI).

2.1.5. Interpretation

a. The report of a positive culture generally means that the patient is bacteremic. However, skin microbiota may contaminate the culture, causing a false-positive result, or pseudo-bacteremia; the latter has many other causes too.

For criteria to consider the labeling of isolates as pathogenic, including list of important pathogens, please refer to the CDC list of common commensals, that of mucosal barrier injury related organisms causing bacteremia

(https://www.cdc.gov/nhsn/xls/2018-NHSN-Organisms-List-Validation.xlsx).

- Examples of common commensals: Coagulase Negative *Staphylococcus, Bacillus* species, *Corynebacterium* species (Diphtheroids), *Micrococcus, Propionibacterium* species
- Examples of organisms recognized as pathogens: *Staphylococcus aureus, Streptococcus pyogenes, Streptococcus pneumoniae, Enterococcus faecalis, Enterococcus faecium, E. coli, Klebsiella pneumoniae, Salmonella, Pseudomonas aeruginosa, Acinetobacter* species*, Haemophilus influenzae, Neisseria meningitidis, Candida* species

b. Mixed cultures can be present and account for a small but significant number of bacteremias. Be aware of this when examining smears and plates.

c. For diagnosing CRBSI, differential time to positivity (DTP) should be noted that includes growth of microbes from a blood sample drawn from a catheter hub at least 2 hours before microbial growth is detected in a blood sample obtained from a peripheral vein, which best defines CRBSI (Mermel et al., 2009).

2.1.6. Limitations

- Low levels of organisms may not be detected in the incubation interval of the culture.
- The media used may not support the growth of all organisms. Use of multiple formulations increases the yield.
- Sodium polyanethol sulphonate (SPS) may inhibit the growth and viability of some organisms.
- Other diseases can present similarly to bacteremia, since there are many causes of fever of unknown origin.
- Bacterial metabolism may not produce sufficient CO_2 for detection in automated systems.
- There are a number of fastidious microorganisms that infect the blood but cannot be grown in routine blood culture.

2.2. CSF

2.2.1. Collection and transport

Purpose: To identify the organisms causing pyogenic meningitis.

Note: This is an emergency procedure. The samples should be processed and reported immediately. The results of the smear should be informed to the concerned clinician and documented in the critical alert register.

2.2.1.1. Specimen collection

2.2.1.1.1. *Lumbar puncture*

- Cap, face mask, gown and gloves for physician drawing CSF are useful adjuncts to infection prevention. Disinfect the puncture site with antiseptic solution and alcohol in a manner identical to phlebotomy skin preparation for blood culture to prevent specimen contamination and introduction of infection.
- Insert a needle with stylet at the L3-L4, L4-L5, or L5-S1 interspace. When the subarachnoid space is reached, remove the stylet; spinal fluid will appear in the needle hub.
- Measure the hydrostatic pressure with a manometer.

- **Note:** Lumbar puncture opening pressure should not be considered a reliable measure of intracranial pressure in children (Cartwright & Igbaseimokumo, 2015) .
- Collect the CSF into three calibrated sterile labeled tubes.
- Physicians should be instructed to sequentially collect 2.0 ml of CSF each into three sterile calibrated tubes if only routine chemistry (total protein and glucose), bacteriology (culture & susceptibility), and hematology (cell count) are required.

2.2.1.1.2. *Ventricular shunt fluid*

- Clean the reservoir site with antiseptic solution and alcohol prior to removal of fluid to prevent introduction of infection.
- Remove fluid by aspiration of CSF from the Ommaya reservoir or by collection from the ventricular drain or shunt.
- Collect CSF into a minimum of three sterile calibrated tubes if only routine chemistry (total protein and glucose, tube no. 1), bacteriology (culture & susceptibility, tube no. 2), and hematology (cell count, tube no. 3) are required.
- An initial CSF sample should be collected prior to antimicrobial therapy for highest diagnostic sensitivity.

2.2.1.2. Specimen transport

- Submit to laboratory as soon as possible and alert laboratory that specimen is in transit.
- Do not refrigerate.
- Each sterile calibrated tube containing CSF must be properly labeled with the patient's name, unique identification number, and the date and time of collection.
- Requisition must be complete with demographic and specimen collection information. Record the patient diagnosis for proper processing of specimen.

2.2.1.3. Rejection criteria

- Call physician to prioritize requests if there is insufficient volume.
- Specimens in leaky containers must be processed, but alert the physician of the possibility of contamination.

2.2.2. CSF Processing

Day 1

2.2.2.1. CSF gross appearance

- Clear, slightly turbid, cloudy, purulent
- Contains blood

- Contains clots
- Xanthochromic

2.2.2.2. Centrifuge CSF

- If <1 ml volume, vortex 30 seconds.
- If >1 ml volume, centrifuge at 1500 × g for 15 min/cytocentrifuge (1000 rpm for 10 minutes)

2.2.2.3. Wet mount

Look for pus cells, RBCs, bacteria, yeast cells.

2.2.2.4. India ink preparation

Look for capsulated organisms like *Cryptococcus neoformans*, *S. pneumoniae*.

2.2.2.5. Gram's smear

- Place 5-6 drops of sample or if the specimen is cloudy prepare the smear by placing 1-2 drops of CSF.
- Allow the drop(s) to form one large drop. Do not spread the fluid.
- Air-dry the slide.
- Fix smear with methanol or heat.
- Perform Gram's stain. Interpret CSF microscopy immediately.
- Any bacteria seen are considered significant.
- Report: Number of WBCs, and describe the morphology for bacteria

2.2.2.6. Culture (Yoo et al., 2016)

- Chocolate agar (CHOC), blood agar, McConkey agar or RCM broth (or a blood culture bottle).
- Incubate at 37ºC for 48 hours; examine daily.
- If no growth in plates but RCM is turbid, subculture from RCM on blood agar, McConkey agar and chocolate agar.

Day 2

2.2.2.7. Culture examination

- Examine all plates and broth media for macroscopic evidence of growth at 24 hours.
- If no visible growth is observed on the culture media, reincubate.
- Cultures with growth:
  - Notify physician of positive culture findings.

- Identify all organisms, using the rapid tests.
- Perform rapid bile solubility spot test on all alpha hemolytic streptococci to identify *S. pneumoniae*. If positive, report it as *S. pneumoniae.*
- Perform catalase and Gram stain of organisms growing on BAP and/or CHOC.
- Antigen test for *S. pneumoniae, N. meningitidis, H. influenzae* is also performed directly on CSF, on request of clinician.

2.2.3 Reporting of results and interpretation (Public Health England, 2018a)

- Appearance: report the appearance of CSF and the presence of a clot, if applicable
- Microscopy
  - Cell count: report numbers of RBCs x 10^6 per litre and report numbers of PMNs and lymphocytes x 10^6 /L or Report PMNs and lymphocytes as percentages of the total WBC (which is reported as x 10^6).
  - In certain cases, referral to cytology for identification of abnormal (e.g. malignant) cells may be indicated. Gram stain: report on organisms detected and presence or absence of pus cells.
  - Supplementary tests: India ink or nigrosin.
  - Report on encapsulated yeasts detected.
  - Microscopy for *Mycobacterium* species and parasites
- Culture reports the organisms isolated or report absence of growth
- Molecular testing results (if applicable).

Interpretation

- Abnormalities associated with bacterial meningitis
- Reduced glucose concentration: educed, with a CSF:serum glucose ratio below about 0.4 (<40%). Take the serum glucose at about the same time as the lumbar puncture. A CSF glucose below roughly 2.2 mmol/L is also commonly used.
- Elevated protein concentration
- Raised white blood cell (WBC) count: typically above about 1,000 × 10^6/L (1,000 cells/μL), often several thousand. It is predominantly polymorphonuclear (neutrophils), usually more than 80%.
- Elevated intracranial pressure

Table 2.3: Commonly isolated pathogens of clinical significance

Patient group (age / condition)	Commonly isolated pathogens

Neonates <28 days	*E.coli*, Group B streptococci, *Listeria monocytogenes*
<2 months	Group B streptococci and *Haemophilus influenzae*
<10 years	*H. influenzae*, *Streptococcus pneumoniae*
Adult	*S. pneumoniae, Neisseria meningitidis*
Immunocompromised	Common organisms plus *Cryptococcus neoformans, Listeria monocytogenes*
CNS shunt infection	Coagulase negative staphylococci (CoNS), *S. aureus*

2.3. BODY FLUIDS FROM STERILE SITES

2.3.1. Specimen collection

- Body fluids from sterile sites should be collected by percutaneous aspiration for pleural, pericardial, peritoneal, amniotic, and synovial fluids.
- Use care to avoid contamination with commensal microbiota.
- Clean the needle puncture site with alcohol, and disinfect it with an iodine solution [1-2% tincture of iodine or a 10% solution of povidone iodine (1% free iodine)] to prevent specimen contamination or infection of patient (if tincture of iodine is used, remove with 70% ethanol after the procedure to avoid burn).
- Aseptically perform percutaneous aspiration with syringe and needle to obtain pleural, pericardial, peritoneal, or synovial fluid. Use safety devices to protect from needle exposure.
- Immediately place a portion of the joint fluid or peritoneal fluid collected from patients with CAPD or SBP into aerobic and anaerobic blood culture bottles, retaining some (0.5 ml) in sterile tubes for Gram stain and direct plating.
- Use the minimum and maximum volumes recommended by the bottle manufacturer (generally up to 10 ml is the maximum for each bottle).
- Alternatively, inoculate the blood culture bottles after receipt in the laboratory.
- Submit other fluids and the remainder of specimens after inoculation of blood culture bottles in one of the following: a sterile, gassed-out tube or a sterile blood collection tube without preservative; however, fluids in such tubes may clot during transport.

2.3.2 Specimen transport

- Submit to laboratory as soon as possible and, if from a normally sterile site, alert laboratory that specimen has been submitted.
- Do not refrigerate.

- Label specimens with patient demographics and date, time, and site of collection *e.g*. left knee joint fluid.
- Record the patient diagnosis for improved processing of specimen.

Note:

- If specimens inoculated into blood culture bottles are received, Gram stain cannot be performed.
- Collect specimen prior to antimicrobial therapy for greatest diagnostic sensitivity.
- Do not submit specimens from drains after they have been infused with antimicrobial agents.
- Call physician when fluid specimens are received on a swab.
- Contact physician if specimen is insufficient for the number of tests requested.
- Swabs constitute the least desirable sample for culture of body fluids and should be discouraged, since the quantity of sample may not be sufficient to ensure recovery of a small number of organisms.
- Routine bacterial culture is sufficient for culture for *Candida* species, if blood culture bottles are used or specimen is centrifuged.

Important considerations

- Specimens received by the laboratory in a syringe with the needle still attached should be rejected because of the risk of a needle-stick/sharp exposure by laboratory staff. The physician should be immediately contacted to recollect the sample and send it in proper container.

  Note: Establish a policy for the proper collection and transport of clinical specimens not collected on swabs. Educate the physicians that needles must be removed from the syringe and the syringe cap secured prior to transport to avoid leakage.

- Syringes that have been capped with a Luer-Lock (with needle removed) prior to transport may be accepted for culture provided the specimen has not clotted inside the syringe and there is no leakage during transport which could result in contamination of the culture. The laboratory may reject specimens that have clotted in a capped syringe because they cannot be processed for culture without inadvertently contaminating the specimen.

2.3.3. Processing

Day 1

a. Gross appearance

- Clear, slightly turbid, cloudy, purulent

- Contains blood
- Contains clots

b. Gram smear

- Place 5 to 6 drops of sample, or if the specimen is cloudy, prepare the smear by placing 1 or 2 drops of fluid.
- Allow the drop(s) to form one large drop. Do not spread the fluid.
- Air-dry the slide.
- Fix smear with methanol.
- Perform Gram's stain and interpret immediately.
- Report: Number of WBCs and describe the morphology for bacteria.

c. Culture

- Inoculate blood agar, MAC and RCM.
- Incubate at 37ºC overnight.
- Body fluid samples should also be incubated anaerobically, examined after 48 hours.
- Body fluids can also be inoculated in relevant blood culture bottles..

Day 2

d. Culture examination

- Examine all plates and broth media for macroscopic evidence of growth after 24 hours.
- If no visible growth is observed on the culture media, re-incubate.
- Cultures with growth:
- Notify physician of positive culture findings.
- Correlate culture results with those of the direct Gram stain microscopy.
- Identify all organisms, using the rapid tests.
- Do not perform complete identification if the physician indicates that the organism is a probable contaminant or that the isolate is one or two colonies of a coagulase-negative staphylococcus on one medium with no growth in the broth.

2.3.4 Reporting of results and interpretation (Public Health England, 2018b)

Microscopy

- Gram stain: report on WBCs and organisms detected. Cell count (if requested), report numbers of WBCs x 10^6 per litre. Also report PMNs and mononuclear leucocytes as percentage of the total WBCs,
- Microscopy for *Mycobacterium* species

Interpretaion

- High WBC count is likely to indicate a bacterial infection
- Organisms detected in sterile body fluids are indicative of infection

2.4. OCULAR SPECIMENS (Isenberg, 2007; Rajput et al., 2026)

2.4.1. Specimen collection and transport

Note: Most eye specimens should be collected by an ophthalmologist. These specimens should be inoculated onto culture media at the bedside, in the clinic or the physician's office. A variety of techniques are used to collect material from different parts of the eye. The conjunctiva is constantly contaminated by various bacteria from the environment and ocular adnexa. Therefore, specimens from the conjunctiva serve as a control when compared with specimens collected by more aggressive or invasive techniques.

Considerations

- Provide fresh media to the clinical areas routinely collecting ocular cultures, and instruct physicians to immediately transport inoculated media and slides to the laboratory.
- Obtain viral and chlamydial samples before topical anesthetics are instilled.
- Obtain samples for chlamydial cultures with calcium alginate swabs.
- **Note:** Calcium alginate swabs may be toxic for *Neisseria gonorrhoeae* (for which rayon or cotton swabs could be used) (Lauer & Masters, 1988).
- For viral cultures, use Dacron or cotton swabs with non-wood shafts.

2.4.1.1. Collection by anatomic site (Rajput et al., 2026)

2.4.1.1.1. *Conjunctiva (bacterial conjunctivitis) and lid margin (blepharonconjunctivitis)*

- Obtain the specimen with a sterile, pre-moistened cotton or calcium alginate swab.
- Roll the calcium alginate or cotton swab over the conjunctiva before topical medications are applied.
- Culture both eyes with separate swabs.
- Immediately inoculate the material at the bedside onto BAP and CHOC.

- Inoculate the swab from the right conjunctiva in horizontal streaks, and inoculate the swab from the left conjunctiva in vertical streaks, each on one half of the same agar plate.
- Inoculate specimens from the right and left lid margins, if collected, by making an R and an L to represent the respective sites on another agar plate.
- Obtain conjunctival scrapings for a smear preparation as follows:
- Instill 1 or 2 drops of proparacaine hydrochloride.
- Using a Kimura spatula, gently scrape across the lower right tarsal conjunctiva.
- Smear the material in a circular area 1 cm in diameter on a clean glass slide.
- Prepare at least two slides.
- Immerse the slides in 95% methyl alcohol or 95% methanol for 5 minutes.
- Repeat steps for the left conjunctiva.

2.4.1.1.2. ***Cornea (bacterial keratitis)*** (Schaefer et al., 2001)

- Instill 1 or 2 drops of proparacaine hydrochloride (local anesthetic for ophthalmic instillation).
- Obtain conjunctival samples as described above, and then obtain corneal scrapings from the advancing edge of the ulcer by scraping multiple areas of ulceration and suppuration with a sterile Kimura spatula, using short, firm strokes in one direction (keep the eyelid open, and be careful not to touch the eyelashes).
- Obtain approximately three to five scrapings per cornea.
- Inoculate each set of scrapings onto BAP and CHOC, using a 'C' formation for each scraping.
- Prepare smears by applying the scrapings in a gentle circular motion over a clean glass slide or by compressing material between two clean glass slides and pulling the slides apart.

2.4.1.1.3. ***Bacterial endophthalmitis***

- Collect an aspirate of the vitreous fluid or perform a paracentesis of the anterior chamber using a needle aspiration technique to collect intraocular fluid.
- Collect specimens for conjunctival cultures along with the fluid to determine the significance of indigenous microbiota.
- If a small volume of fluid is collected, inoculate cultures at the bedside by inoculating 1 or 2 drops of fluid onto culture media.

2.4.2. Specimen processing (Rajput et al., 2026)

Direct smears

Gram stain

- Prepare two or three smears from the clinical material if glass slides do not accompany the specimen. If cheesy tissue bits are present, crush them onto a slide for Gram stain.
- Place slides in 95% methanol for 5 -10 minutes to fix material.
- Perform a Gram stain.
- Examine the stained smear for the presence of somatic cells and extra- and intracellular organisms.
- The presence of PMNs suggests a bacterial infection.
- The presence of mononuclear cells may indicate viral conjunctivitis.

Note: Pigment granules that resemble Gram-positive cocci may be present on the Gram-stained smear. They can be differentiated from cocci because they are large, oval, and brown.

2.4.3. Culture inoculation, examination, and interpretation

- Inoculate culture media – blood agar and McConkey agar
- If a scanty specimen of intraocular fluid is submitted in a syringe, wash out the syringe by drawing up a small amount of broth.
- Use the broth to inoculate plate media with 2 drops per plate.
- Place the remainder of the broth and specimen in broth culture tube. Avoid creating an aerosol.
- Incubate cultures at 35ºC in 5-7% CO_2 for 24 hours.
- Examine daily for the presence of microorganisms.

  Note: minimum culture duration (or incubation time) for ocular specimens is often 10 days (Rajput et al., 2026)
- Estimate and report the number of each organism on each plate. The presence of moderate numbers of colonies or many colonies on one or more culture plates should indicate the bacterial etiology of the infection.

Quantitation of C streaks (Rajput et al., 2026)

- Less than half of the C streaks are positive per plate
- More than half of the streaks, but not all, are positive
- All streaks are positive for bacteria

2.4.4. Post-analytical considerations

Reporting results

- Convey positive reports from invasively collected specimens to the physician as soon as possible.
- Report the relative number and morphology of all microorganisms seen, the presence and numbers of somatic cells (especially PMNs), and whether the organisms were observed intracellular as well as extracellular.
- Report the quantity and organism identity for each morphological type observed on culture media.

Interpretation

- All organisms grown in any amount from critical eye specimens (i.e., aqueous and vitreous fluid) should be identified and AST results reported.
- All organisms present in the direct smear that grow on primary culture plates are considered clinically significant and should be worked up.
- The following criteria may assist the laboratory to determine the clinical significance of bacterial isolates from critical eye specimens that may otherwise be considered indigenous microbiota.
- Coagulase-negative staphylococci, diphtheroids, *P. acnes* (now *Cutibacterium acnes*), or viridans group streptococci growing on more than one medium are generally considered significant.
- Indigenous commensal isolates may also be clinically significant when growth occurs only on one medium or in broth. In such cases a comment may be added to the final report indicating that the clinical significance of organisms that are part of the commensal microbiota must be determined by clinical correlation.

2.5. RESPIRATORY SPECIMENS (CLSI, 2007)

Purpose: To isolate and identify the potentially pathogenic organisms from upper and lower respiratory tracts (URT and LRT) aiding in the diagnosis of infections.

Sputum cultures are done primarily to identify the pathogens that cause pneumonia or bronchopneumonia: community-acquired or hospital-acquired.

This procedure is for the isolation and identification of the common respiratory pathogens such as *S. pneumoniae*, *H. influenzae*, *S. aureus*, Gram-negative bacilli, including *P. aeruginosa* and other non-fermenters, *Moraxella catarrhalis*, *Streptococcus pyogenes*, etc.

Table 2.4: Appropriate specimens for diagnosis of bacterial infection in upper and lower respiratory diseases.

Clinical condition	Primary pathogen(s)	Specimen(s)
Chronic bronchitis	*S. pneumoniae* *S. aureus,* including MRSA *H. influenzae* Gram-negative bacilli	Lower respiratory
Pneumonia	**Community-acquired pneumonia** *S. pneumoniae* *S. aureus,* including MRSA *H. influenzae* *K.pneumoniae* Anaerobes (if aspiration) Agents of bioterrorism, including *Bacillus anthracis, Brucella* spp., *Francisella tularensis*, *Yersinia pestis*, and *Burkholderia pseudomallei* **Hospital-acquired pneumonia, including ventilator associated pneumonia (VAP)** Gram-negative bacilli, including *P. aeruginosa* and other non-fermenters Anaerobes (if aspiration) *S. aureus*	Lower respiratory
Lung abscess	*S. aureus* *S. pyogenes* *K. pneumoniae* *P. aeruginosa* Anaerobes	Lung aspirate or biopsy sample
Sinusitis	**Acute:** *S. aureus, S. pneumoniae, H. influenzae, M. catarrhalis* **Chronic:** Agents of acute sinusitis Gram-negative bacilli, including *P. aeruginosa,* Anaerobes	Sinus aspirate
Staphylococcal carriage	*S. aureus*, including MRSA	Nasal swab
Cystic fibrosis	*Pseudomonas aeruginosa* *S. aureus* *Burkholderia cepacia* complex *Aspergillus* spp.	Deep throat (young children) Lower respiratory

2.5.1. Specimen collection and transport

2.5.1.1. Sputum

- Spontaneous: Early morning specimen generated after a bout of cough.
- Having the patient brush his or her teeth and gargle with water immediately before obtaining the sputum specimen reduces the number of contaminating oropharyngeal bacteria.

- Collect specimen resulting from deep cough in a sterile screw-cap cup or other suitable sterile collection assembly of about 100 ml capacity.
- To prevent contamination of the outside of the container, the patient should be instructed to press the rim of the container under the lower lip to catch the entire expectorated cough sample.
- Tightly screw on the cap of the container. Wipe off any spilled material on its outside with a tissue moistened with disinfectant, but take care not to let any disinfectant enter the container. In addition, patients should remove dentures during the specimen collection.
- Early-morning sputum samples should be obtained because they contain pooled overnight secretions in which pathogenic bacteria are more likely to be concentrated. Twenty four hour collections should be discouraged (Miller et al., 2024)
- Deliver the specimen to the laboratory as quickly as possible, preferably within 2 hours, because delicate bacterial, viral and mycoplasma pathogens may die out during longer delay.

2.5.1.2. Endotracheal aspirate (ETA) (Wu et al., 2002)

- Endotracheal aspiration should be done with a sterile technique using a 22 inch, 12F suction catheter. The catheter should be introduced through the endotracheal tube for at least 30 cm. Gentle aspiration is then performed without instilling saline solution. The first aspirate is discarded.
- The second aspirate should be collected after tracheal instillation of 5 ml saline in a mucus collection tube. [If very little secretion is produced by the patient, chest vibration or percussion for 10 minutes should be used to increase the retrieved volume ($\geq$ 1 ml)].
- The specimens should be sent to laboratory and cultured within 1 hour of collection.

2.5.1.3. Bronchoalveolar lavage (BAL) (Sampsonas et al., 2011)

In this procedure 120 ml of saline should be infused into a lung segment through the bronchoscope to obtain cells and protein of the pulmonary interstitium and alveolar spaces. Send a portion of it to the laboratory.

2.5.1.4. Sinus aspirate

Collection of specimens from patients with sinusitis should be performed by otolaryngologists who perform nasal endoscopy or sinus puncture and aspiration.

2.5.2. Type of container

Collect in a sterile leak proof screw-cap container.

2.5.3. Rejection criteria

2.5.3.1. For sputum and endotracheal aspirate specimens

- Reject duplicate specimens received on the same day unless the initial sample was inappropriate for culture according to microscopic evaluation.
- Do not accept repeat cultures at intervals of less than every 48 hours.
- Reject the following specimens for diagnosis of lower respiratory tract disease:
  - 24 hour sputum collection
  - Contaminated sputum and endotracheal specimens as per Gram stain rejection criteria (see below)
  - Specimens that are visually saliva only
  - Specimens that are visibly contaminated with toothpaste or other substances
  - Nasal washes or swabs of nares to diagnose sinusitis
- Sputum samples are highly contaminated with normal anaerobic flora of the upper respiratory tract. Therefore, anaerobic culture should not be done.

2.5.3.2. For BAL, lung aspirates and OLB specimens

- BAL specimens, lung aspirates, and OLB specimens should never be rejected by the laboratory, since the patient has undergone an invasive procedure for their collection.
- For specimens delayed in transit more than 2 hours without refrigeration, indicate on the report that the delay in transit may compromise the culture results.
- Anaerobic culture should be performed on lung aspirates, pleural fluid, and OLB specimens by request or when the original specimen Gram stain demonstrates morphotypes suggestive of anaerobic infection.

2.5.4. Processing

2.5.4.1. Media and Reagents

- CHOC
- Blood agar and MAC (optional); add when indicated by the consultant.
- Gram's stain

2.5.4.2. Sputum and ETA, note the following:

Features of the specimen: purulent, mucopurulent, mucoid, blood tinged saliva.

2.5.4.3. Microscopic examination

Gram stain (G/S) Sputum (Wong et al., 1982; Public Health England, 2019)

Examine 20 to 40 fields from sputum smears under low power and endotracheal smears under both low power and oil immersion. Average the number of cells in representative fields that contain cells. Reject the following for culture (Sputum or ETA from adults: ≥25 SECs/LPF), as poorly collected or not consistent with a bacterial infectious process.

Note: Salivary specimens may be rejected before homogenization or on the basis of a ratio <2:1 of WBC: SECs determined by a Gram stain at low power magnification (x100)

BAL (Rea-Neto et al., 2008)

Microscopic examination of cells from lavage has also been used to diagnose pneumonia (Bennett et al.,2015). If more than 5% of cells from BAL contain intra-cellular bacteria, diagnosis can be interpreted as pneumonia with sensitivities of up to 90% and specificities of 89% to 100%.

2.5.5. Limitations in studying the sputum specimens by Gram staining

- Not all patients can provide an adequate sample. This may be either due to an inability to produce a sample, or because the sample is of poor quality.
- Interpretation is observer dependent.
- Atypical pathogens, common either singly or as co-infecting agents, cannot be seen.
- The definition of "positive" varies from study to study, and a positive result for pneumococcus is poorly predictive of the ability to recover that organism from a sputum or blood culture (Mandell et al., 2007).

2.5.6. Culture

- CA, BA with *Staphylococcus* streaking, MAC
- Incubate in candle jar at 37ºC aerobically.

Suction tips (ST) (Friedland et al., 2001)

These are received in sterile test tubes. Wash the tips (including the bore) using approximately 0.5 ml sterile normal saline. This fluid should be inoculated onto BA & MAC with a 4 mm diameter nichrome wire loop. Subsequent procedure is same as for sputum.

Inoculation (ETA, BAL and lung aspirates)

ETA and BAL received are processed for a semi-quantitative estimation; a standardised wire loop is therefore a must for inoculation. A wire loop of 1.2 mm diameter (1µl

volume) should be used for the purpose. The inoculation should be done on the blood agar and MAC. The plates should be incubated aerobically overnight at 37°C.

2.5.7. Reporting of results and interpretation (on Day 2)

2.5.7.1. Sputum

Report the growth of following:

- *Streptococcus pneumoniae*
- *H. influenzae*
- *S. aureus*
- Gram-negative bacilli, including *P. aeruginosa* and other non-fermenters
- *Moraxella catarrhalis*
- *Streptococcus pyogenes*

2.5.7.2. ETA & BAL

- A CFU count of 10^5/ml (*i.e*. 100 colonies) or 10^4/ml (*i.e*. 10 colonies) respectively, is considered significant.
- Antibiotic sensitivity testing: As per the procedure described for the clinically significant isolates**.**

2.5.8. Limitations

This procedure is not suitable for the isolation of all respiratory pathogens *e.g*. *M. tuberculosis*, *Legionella*, *Chlamydiae*, *Mycoplasma*, which can also cause pneumonia.

2.6. PUS

Purpose: To isolate and identify bacterial etiological agent(s) in deep-seated pus/wound specimens.

2.6.1 Specimen collection

- Preferably collect specimen prior to initiation of therapy and only from wounds that are clinically infected or deteriorating or that fail to heal over a long period.
- Cleanse surrounding skin or mucosal surfaces.
- For closed wounds and aspirates, disinfect with 2% chlorhexidine or 70% alcohol followed by an iodine solution [1 to 2% tincture of iodine or a 10% solution of povidone-iodine (1% free iodine)]. Remove iodine with alcohol prior to specimen collection.

- For open wounds, debride, if appropriate, and thoroughly rinse with sterile saline prior to collection. Sample viable infected tissue, rather than superficial debris.

2.6.1.1 Wound or abscess aspirates

- Samples collected by using a syringe and needle should be placed in a sterile container or blood collection tube without anticoagulant for submission to the laboratory.
- A portion of the sample should also be placed in a sterile tube containing anaerobic medium like RCM if an anaerobic culture is required.

2.6.1.2. Open wounds

- Cleanse the superficial area thoroughly with sterile saline, changing sponges with each application. Remove all superficial exudates.
- Remove overlying debris with scalpel and swabs or sponges.
- Collect biopsy or curette sample from base or advancing margin of lesion.

2.6.1.3. Pus

- Aspirate the deepest portion of the lesion or exudate with a syringe and needle.
- Collect a biopsy sample of the advancing margin or base of the infected lesion after excision and drainage.
- For bite wounds, aspirate pus from the wound, or obtain it at the time of incision, drainage, or debridement of infected wound.

2.6.1.4. Tissues and biopsy samples

- Tissue biopsy samples should be collected from areas within and adjacent to the area of infection. Large enough tissue samples should be collected to perform all of the tests required (i.e., 3 to 4 mm biopsy samples).
- If anaerobic culture is required, a separate piece of tissue should be submitted in a sterile tube containing anaerobic medium like RCM.
- Collect swabs only when tissue or aspirate cannot be obtained.
- Limit swab sampling to wounds that are clinically infected or those that are chronic and non-healing.
- Remove superficial debris by thorough irrigation and cleansing with non-bacteriostatic sterile saline. If wound is relatively dry, collect with two cotton-tipped swabs moistened with sterile saline.
- Gently roll swab over the surface of the wound approximately five times, focusing on area where there is evidence of pus or inflamed tissue.

Note: Organisms may not be distributed evenly in a burn wound, so sampling different areas of the burn is recommended. Blood cultures should be used to monitor patient status.

2.6.2. Standard precautions to be followed while handling the specimen

Note: Syringes with the needle attached should not be accepted due to the sharps and biohazard risk to staff.

- Grossly contaminated specimen or leaky containers and collection containers of doubtful sterility must be noted and mentioned.
- Deliver aspirates and tissues to the laboratory within 30 minutes for best recovery.
- Keep tissues moist to preserve organism viability.
- Do not refrigerate or incubate before or during transport. If there is a delay, keep sample at room temperature, because at lower temperature there is likely to be more dissolved oxygen, which could be detrimental to anaerobes.

2.6.3. Rejection criteria

- For anaerobic culture, avoid swab collection if aspirates or biopsy samples can be obtained**.**
- Do not accept specimens for microbiological analysis in container with formalin.

2.6.4. Specimen processing

Day 1

Aspirate, pus and swab

a. Mix the specimen thoroughly. Place a drop of the specimen onto each medium *i.e*. RCM, blood agar and MAC.

b. Prepare smear for Gram stain by placing a drop of specimen on a slide and spreading it to make a thin preparation.

c. Perform a Gram stain on all specimens and use in the evaluation of culture. Record the relative numbers of WBCs and bacterial and fungal morphotypes. If clinically important organisms are recognized or suspected (*e.g*., from a normally sterile site) based on the Gram stain interpretation, telephone or report results to the appropriate caregiver immediately. Report any bacteria seen in a surgically collected specimen from a normally sterile site.

d. Aerobic incubation conditions:

i. Incubate RCM, blood agar and MAC in an incubator at 37°C. Incubate for a minimum of 24 hours for open wound cultures. Incubation may be extended to 2-3 days for invasive specimens (*i.e.*, aspirated fluids and tissues) that remain culture negative after 24-48 hours of aerobic incubation.

ii. Critical deep-wounds, abscesses, and tissue samples should have anaerobic cultures requested in order to recover all the primary pathogen(s) causing infection in specific clinical conditions (*e.g.*, tissue or pus from brain, lung, liver tissue, deep wounds, abscesses, etc.).

Reporting results

Report Gram stain results as soon as possible, generally within 1 hour for specimens from critical sites.

Day 2

Culture interpretation

- Report growth on blood agar and McConkey agar.
- Correlation with results on Gram stain is to be done.
- In case of no growth on both plates and RCM sterile, report as 'No growth after x days'.
- In case of just a film of growth on the plates and turbid RCM, plates should be further incubated and subculture from RCM and looked for growth on the next day.
- If there is growth of 3 or more organisms on culture plates, report as mixed flora of doubtful significance with suggestion of repeat sample.

Table 2.5: Common organisms isolated from various abscesses (Public Health England, 2016; Public Health England, 2015)

Brain abscess	Anaerobic streptococci, anaerobic Gram-negative bacilli, "*Streptococcus anginosus*" group, Enterobacteriaceae, *Streptococcus pneumoniae*, β-haemolytic streptococci, *Staphylococcus aureus*
Breast abscess	Anaerobic streptococci, β-haemolytic streptococci, *Pseudomonas aeruginosa*, *Proteus* species, *S. aureus*
Dental abscess	α-haemolytic streptococci, anaerobic Gram negative bacilli, anaerobic streptococci, "*S. anginosus*" group, *Aggregatibacter actinomycetemcomitans, Actinomyces* species
Liver abscess	Enterobacteriaceae, *Bacteroides* species*, Clostridium* species, anaerobic streptococci, "*S. anginosus*" group, enterococci , *Pseudomonas aeruginosa*, *Burkholderia pseudomallei* (in endemic areas), *Candida* species

Lung abscess	*S.aureus, Streptococcus pneumoniae, Klebsiella* spp., *Fusobacterium necrophorum, Burkholderia pseudomallei, Nocardia* spp., *Actinomyces* spp, *Aspergillus* spp.
Pancreatic abscess	Enterobacteriaceae, Enterococci, Anaerobes, *Candida* spp.
Peri-rectal abscess	Anaerobes, Enterobacteriaceae, streptococci, *S. aureus*
Pilonidal abscess	Anaerobes, Enterobacteriaceae, *S. aureus*, β-haemolytic streptococci
Prostatic abscess	*E. coli* and other Enterobacteriaceae, Enterococci, Anaerobes, *Neisseria gonorrhoeae, S. aureus, Cryptococcus neoformans*
Psoas abscess	Enterobacteriaceae, *Bacteroides* species, *S. aureus*, streptococci, *Mycobacterium tuberculosis*
Renal abscess	Gram-negative bacilli, *E. coli* and other Enterobacteriaceae, *S. aureus, Candida*
Salivary gland abscess	*S. aureus*, Anaerobes
Spinal Epidural abscess	*S. aureus, S. epidermidis*, Beta- hemolytic Streptococci, Alpha hemolytic Streptococci, *Streptococcus pneumoniae*, Enterobacteriaceae, Pseudomonads
Sub-phrenic abscess	Mixed infections from gastro-intestinal flora
Throat/Neck abscess	β-haemolytic streptococci, anaerobes
Skin and Soft Tissue infection	β-haemolytic streptococci (including *Streptococcus pyogenes*), *S. aureus, Bacteroides* species, *Bacillus cereus* (especially after trauma or orthopaedic surgery), Enterobacteriaceae, *Pseudomonas aeruginosa, Clostridium* species, *Actinomyces* species, *Mycobacterium* species, Dermatophytes, *Candida, Mucor, Fusarium*
Bone and Joint infection	*S. aureus*, Coagulase Negative *Staphylococcus* (especially prosthetic joint infection), Beta-haemolytic Streptococci, *Mycobacterium* species (especially *Mycobacterium tuberculosis), Enterococcus* species, *Haemophilus* species, Enterobacteriaceae, *Pseudomonas aeruginosa, Actinomyces*

2.7. URINE

The most common urine specimen received is the per-urethral voided urine. Healthy urethra is unsterile and it is extremely critical that urine specimens be collected carefully to minimise urethral contamination. There are several types of urine specimens and the results of each type are determined by different guidelines. Therefore, it is essential that

each urine specimen received by the laboratory is clearly labelled as to the type of collection of urine specimen.

2.7.1. Collection of urine

2.7.1.1. Midstream clean catch urine

- The midstream clean catch urine is the most common type of urine specimen.
- The technique involved in collection is based on discarding the first portion of urine, which is most likely to be contaminated by urethral commensals.
- It is recommended that the first voided morning specimen be collected, as bacteria would have multiplied to high levels after overnight incubation in the bladder.
- If not possible, the urine can be collected during the day, preferably 4 hours after the last void, keeping in mind that the counts may be lower, yet significant.
- Midstream clean catch urine should be collected in a sterile, wide mouth, screw capped bottle after very thorough preliminary cleaning of external genitalia with soap and water. Antiseptics should not be used for this purpose.

2.7.1.2. Indwelling catheter

- Hospitalized patients with indwelling catheter are especially at risk of developing UTI.
- To avoid contamination, the specimen should be collected by disinfecting a portion of the catheter tubing with alcohol & puncturing the tubing directly with a sterile syringe with needle and aspirating the urine.
- The urine MUST NOT be collected from the drainage bag.

2.7.1.3. Suprapubic collection

- The suprapubic collection avoids urethral contamination but is invasive.
- This procedure is usually reserved for infants and adults, from whom it is difficult to obtain a midstream clean catch urine specimen.
- Disinfect the skin above the bladder and plunge a sterile needle with syringe into the bladder; aspirate the urine and transfer to a sterile container.

2.7.1.4. Percutaneous nephrostomy (PCN) aspirate

- Percutaneous nephrostomy aspirate is urine collected directly from renal pelvis.
- If the sample is a PCN catheter sample, collection must be done as explained for indwelling catheters and not from the drainage bag.

2.7.1.5. Cystoscopy specimens

- Cystoscopy specimen is urine collected from the bladder during cystoscopy.

2.7.1.6. Ileal conduit specimen

- Ileal conduit specimen is collected after cleaning stoma site.
- A fresh drain of urine is collected. It must not be collected from the urine drainage bag.

2.7.1.7. Intermittent catheter specimen

- A red rubber catheter should be introduced into the urethra periodically to drain urine from the bladder.
- It should be collected directly into a specimen container.

2.7.2. Specimen Transport

- Urine must be transported to the lab as soon as possible.
- It should be cultured as early as possible after collection, preferably within 2 hours.
- In case of delay, it may be refrigerated up to a maximum of 24 hours before plating.

2.7.3. Processing of specimen

2.7.3.1. Smear

- Transfer approximately 2 ml of well mixed, un-centrifuged urine specimen using a sterile Pasteur pipette into a labelled tube, and place one-drop of urine on a clean glass slide using the same pipette.
- Do not spread.
- Allow to dry (air dry or on a dryer), heat fix and stain by Gram stain.
- Keep the specimen tubes in the refrigerator till plating and thereafter store the specimen tubes at 2-8ºC until the final report is sent.

2.7.3.2. Examination of wet smear of uncentrifuged urine

- Look for pus cells and microorganisms.
- Quantify the presence of pus cells and microorganisms, most commonly Gram-negative bacilli and also Gram-positive cocci, into many, moderate, few or occasional.
- Also make a note of presence of epithelial cells and other microorganisms, *viz*. yeast like organisms, Gram-positive bacilli.

2.7.3.3. Culture

2.7.3.3.1. *Choice of media and dilution*

Results of direct smear examination should be used as a guide for choice of media and dilution of specimen as indicated in the Table 2.6.

Table 2.6: Recommended media according to microscopic findings

Pus cell	Epithelial Cell	Bacteria	Media Recommended	
			BA*	MA**
Nil	Nil	Nil	½ plate, 0.01 ml undiluted (10 µl)	½ plate, 0.01 ml undiluted (10 µl)
Variable (occasional – many)	Nil	Occasional to few GNB and/ GPC	Full plate 0.01 ml undiluted	Full plate 0.01 ml undiluted
Variable (occasional – many)	Nil	Moderate GNB	Full plate 0.01 ml of 1/10 diluted urine	Full plate 0.01 ml of 1/10 diluted urine
Variable (occasional – many)	Nil	Moderate to many GNB	Full plate 0.01 ml of 1/100 diluted urine	Full plate 0.01 ml of 1/100 diluted urine
Variable (occasional – many)	Nil	Occasional to many GPC or YLO or GPB	Full plate 0.01 ml undiluted urine	Full plate 0.01 ml undiluted urine
Variable (occasional – many)	Nil	Only YLO	Full plate 0.01 ml undiluted	Full plate 0.01 ml undiluted add SAB**
Many	Nil	No bacteria	Full plate 0.01 ml undiluted	Full plate 0.01 ml undiluted
Variable (occasional – many)	Few to many	Many GNB with or without diptheroides, YLO etc.	Full plate 0.01ml of 1/100 diluted urine + SAB undiluted urine 0.01ml	Full plate 0.01 ml of 1/100 diluted urine

*** 4-area streaking without flaming in between for isolation**

****Criss-cross streaking for colony count**

CLED media (Cystine-Lactose-Electrolyte-Deficient Agar) may be used as an alternative to Blood Agar and MacConkey for urine culture.

2.7.3.3.2. *Special situations*

- Suprapubic collection– use full plate BA and MA

- PCN aspirate-use full plate BA, MA, CA and thioglycolate broth, irrespective of the smear finding.
- Make 1:10 dilution when moderate GNB and 1:100 dilution when many GNB are present in the smear.

2.7.3.3.3. *Inoculation*

- To evaluate the clinical significance of a growth in urine culture, estimation of the number of organisms present per ml of urine is essential. If needed dilute the urine sample 1:10 or 1:100 using sterile normal saline.
- For 1:10 dilution, mix 0.5 ml urine with 4.5 ml sterile normal saline.
- For a 1:100 dilution, mix 0.1 ml (100 µl) urine with 9.9 ml sterile normal saline.
- Inoculate well-mixed, un-centrifuged, undiluted or diluted urine on to BA and MA using a pipette that delivers 0.01 ml.
- As shown in the figure 2.1, streak on BA as guide for 4 area streaking. Be sure to progress from one area to next and DO NOT go over to the previously streaked areas. Do not flame the streaking loop between streak areas. This gives adequate isolation of colonies in the fourth area of streaking.

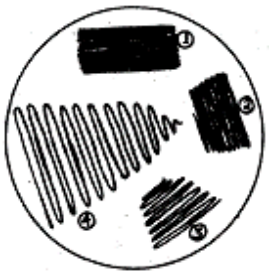


Figure 2.1: Representation of four areas streaking on BA

- Use a triangular loop on MA to achieve even distribution of the inoculum by rotating the plate (CRISS-CROSS STREAKING). Do NOT flame the loop during this streaking maneuver. If half plate is used on MA, use streaking with streaking loop and spread the inoculum evenly by close streaking, once, over half the plate.
- If chocolate agar is used, follow the same streaking pattern as for blood agar.
- Incubate all the inoculated plates aerobically at 37ºC.

2.7.4. Results and interpretation

2.7.4.1. Colony Counts

After overnight incubation count the number of colonies manually on each plate and multiply the number of colonies counted by

- 100 for undiluted urine

- 1000 for a 1:10 dilution of urine
- 10000 for a 1:100 dilution of urine. This gives total number of viable bacteria present in 1.0 ml undiluted urine and express as CFU/ml of urine.

Note: For disposable plastic calibrated loop of 1 microliter, multiply above mentioned number of colonies by a further factor of 10.

2.7.4.2. Interpretation of counts

The significance of a positive urine culture is most reliably assessed in terms of the number of colony forming units (viable bacteria) present in the urine. The following is offered as a guide for midstream clean catch urine.

<1000 CFU/ml	INSIGNIFICANT bacteriuria; UTI-unlikely
1000- 100,000 CFU/ml	PROBABLY SIGNIFICANT bacteriuria; UTI Probable
> 100,000 CFU /ml	SIGNIFICANT bacteriuria; UTI certain

For SPC, PCN and cystoscopic specimens, any CFU is significant irrespective of number.

2.7.4.3. Identification of isolates

- Identify all coliform bacteria that are considered probably significant.
- The preliminary screening media should be mannitol motility medium, triple sugar iron agar medium, peptone water, citrate and if needed include Christensen's urea agar and lysine iron agar
- Identify all non-lactose fermenting organisms even if they are in insignificant range in order to rule out *Salmonella* spp. (carrier state).
- Identify beta haemolytic streptococci.
- Perform grouping for beta hemolytic streptococci in pregnant women, even if they are few in numbers and along with skin contaminants in order to rule out presence of group B *Streptococcus* infection.

Note: Pseudomonads, *Candida* and *Staphylococcus* spp. May cause UTI.

2.7.4.4. Antimicrobial susceptibility testing

- For the panel and detailed methodology of AST please refer to procedure on antimicrobial susceptibility testing.
- When requested, follow guidelines below for testing organism's susceptibility to antibiotics.

$<10^3$ CFU/ml	AST not done, except for cystoscopic, PCN or SPCspecimens
10^3-10^5 CFU/ml	AST done on two organisms depending on their probable significance and relative numbers
$>10^5$CFU/ml	AST done on 1-2 organisms and rarely three organisms depending on their significance and relative numbers

2.7.5. Reporting (Magliano et al., 2012)

- When there is no growth after 24 hours of incubation, send a preliminary report as "No growth".
- When growth shows 1-2 types of organisms with >100,000 CFU/ml (in presence of pus cells), report as "Significant, >100,000 CFU/ml, with organism or organisms".
- When growth suggests gross contamination, *e.g.* mixture of diphtheroids, coagulase negative staphylococci, micrococci, YLO and >2 types of GNB and the smear shows pus cells,report as "Mixture of organisms along with contaminants" and suggest repeat "mid-stream clean-catch" urine sample for culture to confirm significance.

2.8. FECAL SPECIMENS

2.8.1. Specimen collection and transport

- A small quantity of solid/semisolid stool or one third of the container in case of watery stool is collected in a sterile screw-capped disposable 40 ml container.
- A rectal swab is not recommended as the material obtained is never adequate for all the tests or for inoculating all the media used for culture.
- The sample should be collected preferably prior to initiation of antibiotics in the container directly, taking care not to soil the outside of the container. Samples should not be collected from bedpan.
- The sample should be immediately transported to the laboratory on collection.
- If there is a delay in transporting faecal specimens or if samples need to be sent by post, one of the following transport media may be employed:
  - Phosphate buffered glycerol saline solution
  - Stuart's transport medium
  - Cary and Blair transport medium

Note**:** Wasfy et al., study confirms that Cary-Blair medium (CB) is suitable for the preservation of *Salmonella* and *Shigella* isolates for more than 2 weeks at 25ºC, 4ºC, or -70ºC (Wasfy et al., 1995). *Campylobacter jejuni* was not recovered after 2 days of storage in CB at 25ºC when an inoculum of 12 x 10^8 cells per ml was used.

2.8.2. Microscopy

- For all watery faeces samples, whether the doctor orders or not, examine a hanging drop (HD) immediately or wet preparation by darkfield (DF) microscopy.
- If dark-field microscopy is positive, proceed with immobilisation test with *V. cholerae* polyvalent O1 antiserum and *V. cholerae* O139 specific antiserum and examine again under darkfield microscope.

2.8.3. Culture and Isolation

- Commonly encountered enteric pathogens and potential pathogens include *Salmonella*, *Shigella*, *V. cholerae*, *Campylobacter*, *Edwardsiella*, *Aeromonas*, *Plesiomonas*, diarrhoegenic *E. coli* and *V. parahaemolyticus.*
- Routine media to be included are BA, MA, XLD or DCA, and Selenite F broth.

  Note: Many laboratories also include a blood agar plate (BA), in order to aid with the recovery of *Aeromonas* spp., *Plesiomonas* spp., and *Vibrio* spp., whereas other add this on request only (Humphries & Linscott, 2015).
- BA is included for all stool samples as primary plating.
  - Use a swab/pasteur pipette.
  - Place a loopful of the specimen over a small area of each plate, then flame the loop, and streak from the inoculated area over the entire plate.
  - Inoculation on DCA: 1 in 10 dilution of specimen in saline is streaked with the help of triangular loop to get maximum isolation of colonies.
- If *V. cholerae* is suspected, a TCBS and alkaline peptone water (APW) medium with a pH 8.4 to 8.6 are also inoculated. After 4 hours of incubation at 37°C, a drop is taken from the surface of the APW is examined under DFM or phase contrast microscopy. A subculture is also made on BA and TCBS.
- Place 1-2 ml or 1 g of faecal suspension into a tube containing selenite F broth, or pick up an amount approximately the size of a pea and emulsify it in the selenite F broth.
- For all samples, media incubated are examined after 18 hours and a subculture is done immediately from the selenite F broth onto a DCA plate.

2.8.4. Colony characteristics on different media

2.8.4.1. Deoxycholate citrate agar

1. DCA is examined at the end of 24 hours and again after 48 hours.
2. Colonies of all non-lactose fermenters (NLF) may look similar: reliance should not be placed on colony characteristics for differentiation. Commonly isolated microorganisms are *Shigella, Salmonella and Vibrio*. Occasionally 48 hours incubation may be needed for the NLFs to appear.
3. *E.coli, Klebsiella* and *Enterobacter* will look pink being lactose fermenters. Generally suppressed on DCA.

2.8.4.2. Xylose lysine deoxycholate medium

- *Shigella*, *Salmonella* and NFGNB: Small pink colonies with or without black centers (*Salmonella)*.

- Coliforms: yellow colonies, or yellow with black centres (*Citrobacter freundii* and *Proteus vulgaris*)

2.8.4.3. Thiosulphate citrate bile salts sucrose (TCBS) agar

- *V. cholerae* O1 and *V. cholerae* O139 produce flat yellow disk-like colonies due to the fermentation of sucrose in the medium.
- *V. parahaemolyticus* produces green colonies.

2.8.5. Biochemical tests for screening

Inoculation

- Choose a single colony of each type of isolate from each plate (pink colony from XLD plate) and inoculate the media listed below in that order using a straight needle touching the colony once.
- Recharge of the needle should not be necessary.
  - Mannitol motility medium: Stab down the centre of the medium, reaching the bottom, but not touching the sides.
  - TSI: Stab into the butt and streak along the surface.
  - Peptone water: Dip the needle into the medium.
  - Citrate utilization test: Inoculate lightly from a young culture over the entire surface of the slant of Simmon's citrate agar using a straight wire. Incubate at 37ºC for 2-7 days. Blue medium with a streak of growth is positive, e.g. *Klebsiella* spp. Original green colour and no growth indicate a negative reaction, e.g. *E. coli*
  - LIA: For black centre colonies on XLD & DCA, stab the butt and streak the slope. Examine after overnight incubation.

2.8.6. Reading

- Examine mannitol motility medium for evidence of motility and mannitol fermentation.
- Examine TSI for glucose, lactose and sucrose fermentation; presence of gas and H_2S.
- Examine LIA for decarboxylation of lysine and presence of H_2S shown as a black precipitation. Note the amount of H_2S produced & deamination.
- Test peptone water culture for the presence of indole after overnight incubation. Indole test after extraction may be done with a lipid solvent like xylol when reactions are doubtful.

2.9. TISSUE SAMPLE PROCESSING (Public Health England, 2025)

Grind or homogenise specimen with appropriate instruments or equipments, using a sterile tissue grinder (beads), a sterile scalpel or sterile scissors and petri dish.

- A small volume (approximately 0.5mL) of sterile, saline/water, peptone or broth should be added to aid the homogenisation process.
- Ideally, all grinding or homogenisation should be performed in a biosafety cabinet.

Note: Surgically obtained specimens for fungal culture should be cut (finely sliced) rather than homogenized

- Select media, incubation times, temperature and other features following SOP like pus/aspirates.

Chapter 3

Identification of isolates to species level

ICMR took a considered decision to include following bacteria in its AMRSN programme with overall supervision and higher molecular studies assigned to following Nodal centers.

A. Enterobacterales:

Citrobacter freundii, C. koseri, Citrobacter spp., *Enterobacter cloacae, Enterobacter* spp., *Escherichia coli, Klebsiella aerogenes (*formerly *Enterobacter aerogenes)*, *K. pneumoniae, K. oxytoca, Klebsiella* spp., *Proteus mirabilis, P. vulgaris, Morganella morganii, Providencia rettgeri, Providencia stuartii, Serratia marcescens*

B. Typhoidal *Salmonella*:

Salmonella Typhi, *S*. Paratyphi A, S. Typhimurium, *Salmonella* spp.

C. Nonfermenter Gram-negative Bacilli:

Pseudomonas aeruginosa, Acinetobacter baumannii, A. lwoffii, Acinetobacter spp., *Burkholderia cepacia* complex*, Stenotrophomonas maltophilia*

D. Staphylococci, Enterococci:

Staphylococcus aureus, MRSA, MSSA, Coagulase Negative Staphylococci (SOSA)*, S. epidermidis, S. haemolyticus, S. hominis, S. lugdunensis, S. pseudointermedius, S. saprophyticus, Staphylococus* spp. *Enterococcus faecalis, E. faecium, Enterococcus* spp.

E. Faecal bacterial pathogens:

E. coli Diarrhoeagenic (EPEC, EHEC, STEC), *Campylobacter jejuni, Aeromonas* spp., *Salmonella* Enteritidis, *S.* Heidelberg, *S.* Newport, *Salmonella* spp. faecal*, S.* Typhimurium faecal, *Shigella dysenteriae, S. boydii, S. flexneri, S. sonnei, Shigella* spp*., Plesiomonas shigelloides, Vibrio cholerae, V. parahaemolyticus, Vibrio* spp., *Clostridioides difficle, Yersinia enterocolitica*

F. Streptococci:

Streptococcus pneumoniae, S. pyogenes, S. agalactiae, S. viridans

3.1. Identification of isolates of family Enterobacterales

Table 3.1: Identification of commonly isolated members of family Enterobacterales

	Lactose	Motility	Gas	Indole	VP	Citrate	PDA	Urease	Lysine	H_2S	Inositol	ONPG	MR	Ornithine	Arginine	TSI
Escherichia coli	+	+	+	+	-	-	-	-	+	-	-	+	+	-	±	A/A
Shigella groups A, B, C	-	-	-	±	-	-	-	-	-	-	-	-	+	-	-	K/A
Shigella sonnei	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	K/A
Salmonella (most serotypes)	-	+	+	-	-	+	-	-	+	+	±	-	+	-	±	K/A
Salmonella Typhi	-	+	-	-	-	-	-	-	+	+	-	-	+	-	-	K/A
Salmonella Paratyphi A	-	+	+	-	-	-	-	-	-	-	-	-	+	-	±	K/A
Citrobacter freundii	±	+	+	-	-	+	-	±	-	±	-	+	+	-	±	A/A
Citrobacter koseri	±	+	+	+	-	+	-	±	-	-	-	+	+	-	±	A/A
Klebsiella pneumoniae	+	-	+	-	+	+	-	+	+	-	+	+	-	+	-	A/A
Klebsiella oxytoca	+	-	+	+	+	+	-	+	+	-	+	+	±	+	-	A/A
Klebsiella aerogenes	+	+	+	-	+	+	-	-	+	-	+	+	-	+	-	A/A
Enterobacter cloacae	+	+	+	-	+	+	-	±	-	-	-	+	-	+	+	A/A
Hafnia alvei	-	+	+	-	+	-	-	-	+	-	-	+	±	±	-	K/A
Serratia marcescens	-	+	±	-	+	+	-	-	+	-	±	+	±	+	-	A/A
Proteus mirabilis	-	+	+	-	±	±	+	+	-	+	-	-	+	±	-	A/A
Proteus vulgaris	-	+	+	+	-	-	+	+	-	+	-	-	+	-	-	A/A
Morganella morganii	-	+	+	+	-	-	+	+	-	±	-	-	+	-	-	K/A
Providencia rettgeri	-	+	-	+	-	+	+	+	-	-	+	-	+	-	-	K/A
Providencia stuartii	-	+	-	+	-	+	+	±	-	-	+	-	+	-	-	K/A
Providencia alcalifaciens	-	+	+	+	-	+	+	-	-	-	-	-	+	-	-	K/A
Yersinia enterocolitica	-	-	-	±	-	-	-	±	-	-	±	+	+	-	-	A/A
Yersinia pestis	-	-	-	-	-	-	-	-	-	-	-	±	±	-	-	K/A
Yersinia pseudotuberculosis	-	-	-	-	-	-	-	+	-	-	-	±	+	-	-	K/A

+ positive reaction in ≥ 90% isolates; - negative reaction in ≥ 90% isolates; ± variable reaction; A/A acid slant and acid butt; K/A alkaline slant and acid butt

Distinguishing reactions of common and pathogenic Enterobacterales: Lactose and inositol indicate fermentation of lactose and inositol; gas indicates gas from glucose; citrate indicates citrate utilization (Simmons'); PDA indicates phenylalanine deaminase; lysine indicates lysine decarboxylase, H_2S indicates H_2S production in TSI agar. ONPG

indicates metabolism of o-nitrophenyl-β-D-galactopyranoside. + indicates ≥ 90% of strains positive; - indicates ≥ 90% strains negative; V indicates 11%-89% of strains positive (Reference: Konemans Diagnostic microbiology 7th Edition)

3.1.1. Identification of *Salmonella enterica*

Isolate identification

Based on colony morphology and non-lactose fermentation, the isolates are identified using standard biochemical tests. The enterobacterales with biochemicals indicating *Salmonella* Typhi or Paratyphi A, B or C should be further confirmed by slide agglutination test using the *Salmonella* antisera.

Method for slide agglutination test for serological identification (based on Kauffmann -White scheme) (Guibourdenche et al., 2010)

Take a drop of saline on a clean slide. Make a smooth suspension of the growth and check for absence of auto-agglutination. Add a drop of the required antisera and mix thoroughly. Look for agglutination within 30 seconds.

Algorithm to be followed

Note: To save reagent, all the isolates need not be tested with all the antisera.

a. If gram negative, NLF, motile, acid without gas in glucose, TSI with alkaline slant, acid butt, H_2S +ve, no gas, urea negative, citrate negative, indole negative- test with O**9** and **d**H antisera for *Salmonella* Typhi.
b. If Gram-negative, NLF, motile, acid and gas in glucose, urea negative and citrate positive, TSI with alkaline slant, acid butt, no H_2S with gas, indole negative - test with O**2** and **a**H antisera for *Salmonella* Paratyphi A.
c. If Gram-negative, NLF, motile, acid and gas in glucose, TSI with alkaline slant, acid butt, H_2S +ve with gas, urea negative, citrate positive, indole negative- test with poly O and poly H first. If positive, then tests for other groups starting from A-G groups *
   (* This will also be applied for the non-typhoidal *Salmonella* isolated from stool or any other specimen)
d. For nonmotile variants *S. gallinarum* and *S. pullorum* again specific antisera only can be used.

3.1.2. Identification of *Pseudomonas spp.*

Table 3.2: Criteria for biochemical characterization of *Pseudomonas spp.*

***Pseudomonas* groups**	**Species**	**Oxidase**	**Motility**	**Pyoverdin**	**Yellow pigment**	**Glucose**	**Maltose**	**Lactose**	**Mannitol**	**Arginine**	**Lysine**	**NO_3 - NO_2**	**NO_3 - N_2**	**Urea**	**DNAse**	**Esculin**	**H_2S**	**Acetamide**
Fluorescent group	*P. aeruginosa*	+	+	+	-	+	V	-	V	+	-	+	V	V	-	-	-	+
	P. fluorescens	+	+	+	-	+	V	-	V	+	-	V	-	V	-	-	-	-
	P. putida	+	+	+	-	+	V	-	V	+	-	-	-	V	-	-	-	-
Stutzeri group	*P. stutzeri*	+	+	-	-	+	+	-	V	-	-	+	+	V	-	-	-	-
	P. mendocina	+	+	-	-	+	-	-	-	+	-	+	+	V	-	-	-	-
	CDC group Vb-3	+	+	-	-	+	+	-	+	+	-	+	+	V	-	-	-	-
Alcaligenes group	*P. alcaligenes*	+	+	-	-	-	-	-	-	V	-	V	V	-	-	-	-	-
	P. pseudoalcaligenes	+	+	-	-	-	-	-	-	V	-	+	V	-	-	-	-	-
	Pseudomonas. spp. Group-1	+	+	-	-	-	-	-	-	V	-	+	+	-	-	NA	-	-
Yellow pigment group	*P. luteola*	-	+	-	+	+	+	-	+	+	-	V	V	V	-	+	-	-
	P. oryzihabitans	-	+	-	+	+	+	-	+	-	-	-	-	V	-	-	-	-

+,≥ 90% strains positive;- ≥90% strains negative; V, 11%-89% of strains positive; NA, Not available; NO_3 - Nitrate, NO_2 - Nitrite, N_2 -Nitrogen gas

3.1.3. Identification of *Acinetobacter spp.*

Table 3.3: Criteria for biochemical characterization of *Acinetobacter spp.* (Winn et al., 2005)

***Acinetobacter* spp. (genomospecies no.)**	**Temp 41°C**	**Motility**	**Oxidase**	**Growth at 37°C**	**Growth at 44°C**	**Haemolysis on BA**	**Gelatin test**	**OF dextrose**	**Arginine**	**Malonate**	**Nitrate reduction**
A.johnsonii (7)	NG	-	-	NG	NG	NH	-	-	V	V	-
A.baumannii (2)	G	-	-	G	G	NH	-	+	+	+	V
A.haemolyticus	NG	-	-	G	NG	H	+	V	+	-	-
Acinetobacter spp. *(6)*	NG	-	-	G	NG	H	+	V	+	-	-
Acinetobacter spp. *(10)*	NG	-	-	G	NG	NH	-	+	-	-	-
A.calcoaceticus(1)	G	-	-	G	NG	NH	-	+	+	+	-
Acinetobacter spp. *(3)*	NG	-	-	G	NG	NH	-	+	+	V	-
Acinetobacter spp. *(12)*	NG	-	-	G	NG	NH	-	-	+	+	-
Acinetobacter spp. *(5)*	G	-	-	G	NG	NH	-	-	+	-	-
A.lwoffii (8/9)	NG	-	-	G	NG	NH	-	-	-	-	-
Acinetobacter spp. *(11)*	NG	-	-	G	NG	NH	-	-	-	-	-

OF: Oxidative-Fermentative , G- Growth, NG- No growth,+ Positive, - Negative, H-haemolytic, NH-Non-haemolytic,V- Variable,

3.1.4. Identification of *Stenotrophomonas maltophilia* and *Burkholderia cepacia* complex

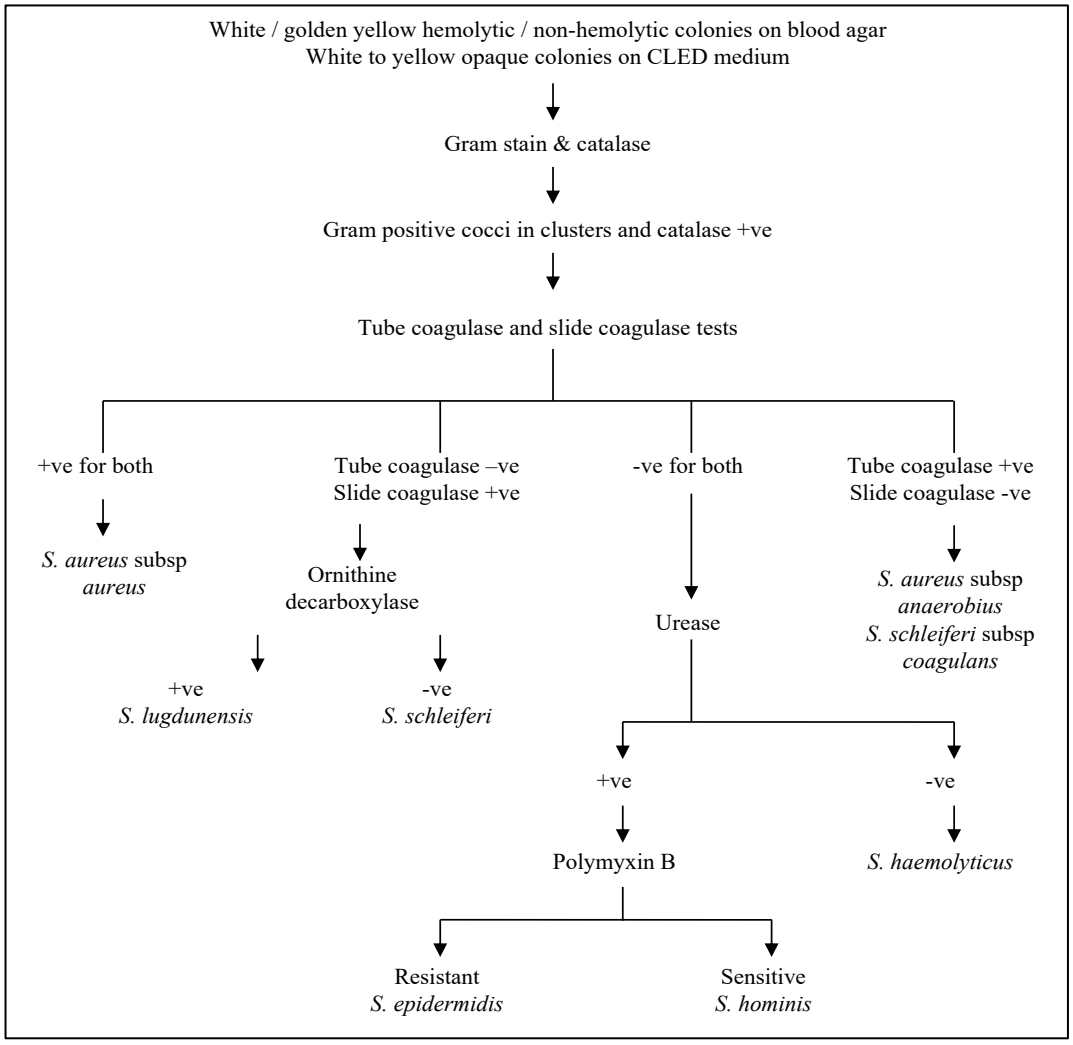
Table 3.4: Criteria for biochemical characterization of *Stenotrophomonas maltophilia* and *Burkholderia cepacia* complex

Test	***S. maltophilia***	***B. cepacia* complex**
MMTP	**- + = -**	**- + = -**
Oxidase	Negative	Positive
Catalase	Positive	Negative
OF Maltose	Positive	-
OF Mannitol	Negative	-
OF Glucose	-	Positive
OF Lactose	-	Positive
OF Salicin	Positive	-
OF Adonitol	Positive	-
Esculin	Positive	-
Gelatin liquefaction	Negative	Positive
Lysine decarboxylation	Positive	Positive
DNAse	Positive	Negative

PB 300	Sensitive	Resistant
Gentamicin (10 µg)	-	Resistant
MR	Negative	Negative
VP	Negative	Negative
Indole	Negative	Negative
Citrate	Variable	Positive
Urea	Negative	Negative
Lysine	Positive	Positive
Ornithine	Positive	Negative
Arginine	Negative	Negative

3.1.6. Identification of Staphylococci and Enterococci

MALDI-TOF MS may be used as the primary method for species-level identification of *Staphylococcus* and *Enterococcus* isolates from pure cultures. In instances where MALDI-TOF MS is unavailable, yields no identification, or provides a low-confidence or inconclusive result, conventional identification methods based on morphological, cultural and biochemical characteristics may be employed. The following flowchart describe the conventional identification pathways for Staphylococcus species.



3.1.6.1 Identification of Enterococci

Morphology and cultural characteristics (Arias & Murray, 2012)

Members of the genus *Enterococcus* are catalase-negative, Gram-positive cocci that can occur either as pairs or in the form of short chains. After growth on blood agar for 2 4 hours, most of the isolates of *Enterococcus* show α-hemolysis or γ-hemolysis on sheep blood agar, although some strains may exhibit β-hemolysis. About a third of the isolates of *Enterococcus* show β-hemolysis, if inoculated onto blood agar containing human blood or rabbit blood.

Identification

Preliminary identification of genus *Enterococcus* should be made on the basis of tests like hydrolysis of bile-esculin, growth in 6.5% NaCl broth and heat tolerance. However, some other genera of catalase negative bacteria like *Leuconostoc*, *Pediococcus* and *Vagococcus* resemble enterococci in some phenotypic characteristics. Using tests like the PYR (L-pyrollidonyl β-naphthylamide) test and detection of Lancefield's group D antigen can help to distinguish *Enterococcus* from other genera.

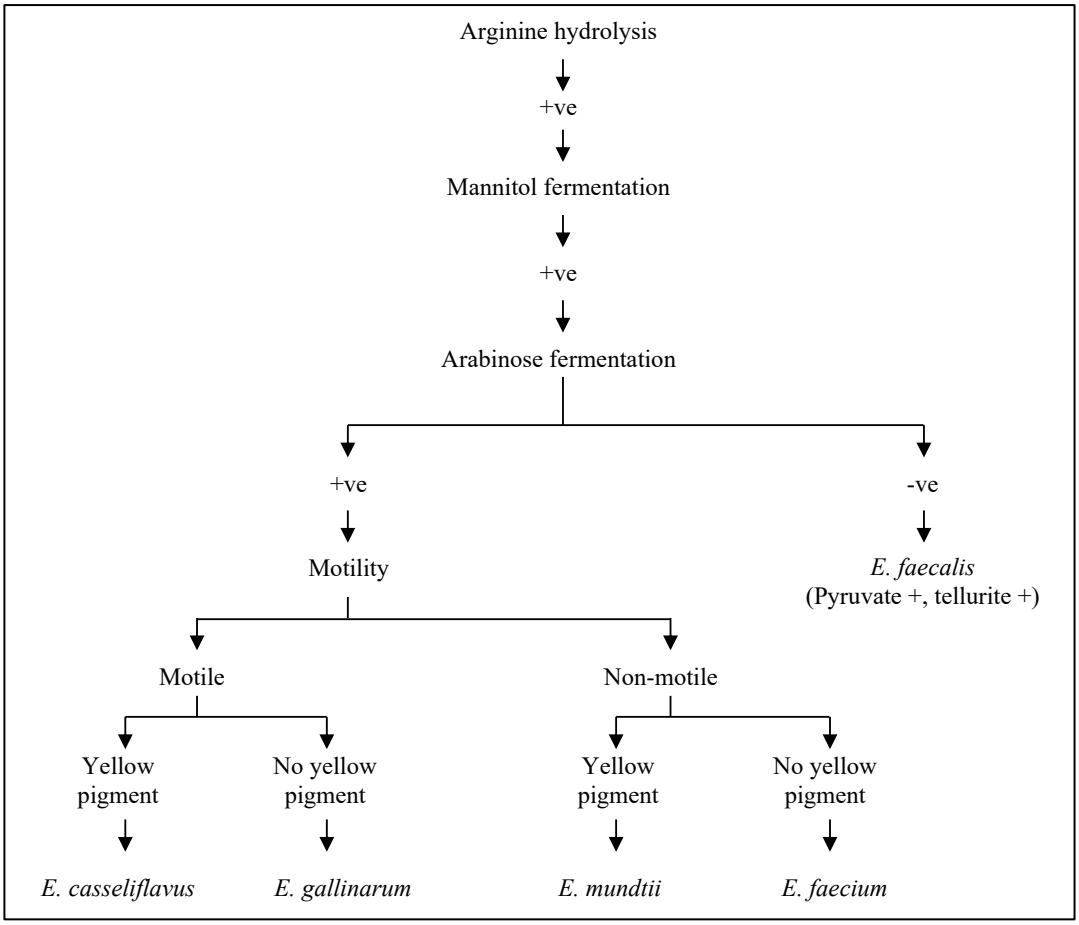
Speciation of Enterococci

The most widely used identification and characterization scheme for *Enterococcus* species is the Facklam and Collins identification scheme. However, most of the frequently isolated species of *Enterococcus* can be identified by using simpler identification schemes using lesser number of biochemical tests.

Enterococci have been put into 5 groups for easier characterization and identification on the basis of some biochemical tests like acid production from mannitol and sorbose and hydrolysis of arginine.

Group I	*E.avium, E.raffinosus, E.pallens, E.malodoratus*
Group II	*E.faecalis, E.faecium, E.casseliflavus, E.gallinarum, E.mundtii*
Group III	*E.dispar, E.hirae, E.durans*
Group IV	*E.cecorum, E.asini, E.phoeniculicola*
GroupV	*E.canis, E.columbae*

Algorithm for phenotypic speciation of *Enterococcus* isolates



3.1.7. Identification, characterization, and serotyping of *Streptococcus pneumoniae*

The major clinical syndromes of pneumococcal disease are pneumonia, bacteremia, and meningitis. The main age groups affected are young children and the elderly. In children, it also causes sinusitis and otitis media.

3.1.7.1. Identification of *S. pneumoniae*

Specimen received: CSF, Blood and sterile body fluids

Note: Microscopic examination of the specimen by smear helps in preliminary identification of the organism and thereby early diagnosis.

Gram staining and culture: *S. pneumoniae* are Gram-positive cocci (lanceolate shape) typically arranged in pairs. The specimen should be inoculated into blood agar, chocolate agar and incubated overnight at 37ºC in 5-7% CO_2.

Colony morphology: Alpha-hemolytic colonies with central pitting, smooth colonies on BAP (Figure 3.1). Greenish discoloration on blood agar is due to partial hemolysis of red blood cells.

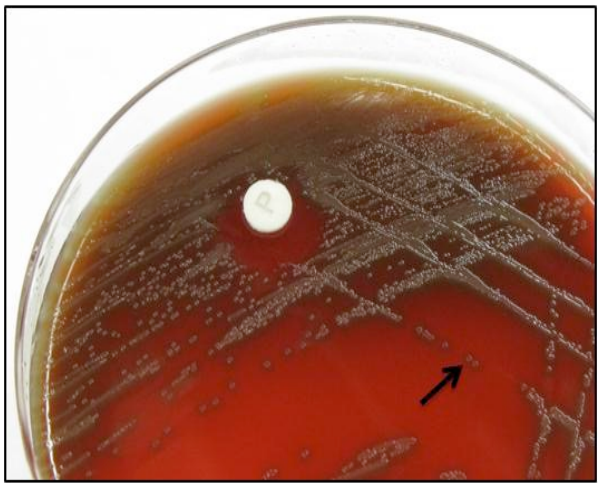


Figure 3.1: *S. pneumoniae* colonies with a surrounding green zone of alpha hemolysis (black arrow) on a BAP

If the colony morphology suggests of *Streptococcus pneumoniae*, further biochemical tests like catalase, and optochin tests simultaneously, with bile solubility as a confirmatory test should be done for the identification. If these tests confirm the isolate as *S. pneumoniae*, serological tests to identify the serotype can be performed.

3.1.7.2. Characterization of *S. pneumoniae*

3.1.7.2.1. Catalase test (MacFaddin, 1976)

Purpose: To differentiate between Gram-positive cocci. Members of the genus *Staphylococcus* are catalase positive, and members of the genera *Streptococcus* and *Enterococcus* are catalase negative.

Principle: To test for the presence of the enzyme catalase. The enzyme catalase present in bacteria breaks down hydrogen peroxide (H_2O_2) into H_2O and O_2. The oxygen is given off as bubbles seen as effervescence.

Performing catalase test

i. Grow the isolate(s) to be tested for 18-24 hours on a Chocolate agar plate at 35-37°C with ~5% CO_2 (or in a candle jar).

ii. From overnight growth on the Chocolate agar plate, use a disposable loop to carefully remove a colony and place it on a glass slide.

iii. Growth on Chocolate agar can be tested for Catalase (Do not use growth on blood agar as erythrocytes in the blood agar will cause a false positive reaction)

iv. Add 20-30 µl of 3% H_2O_2 to the slide and mix with the bacteria. H_2O_2 can be obtained from a commercial drug store.
Note: Commercially, H_2O_2 is sold as 100 volumes (30%), which must be diluted to 10 volumes (3%) in distilled water before use.

v. After initial opening, store H_2O_2 at 4°C in a tightly closed bottle as it will slowly lose potency once opened.

vi. Observe the bacterial suspension on the slide immediately for vigorous bubbling.

vii. It is essential to use a known positive and negative quality control (QC) strain. A *Staphylococcus* spp. strain can be used for a positive control and a known *S. pneumoniae* strain or any other streptococcal spp., i.e., *S. pyogenes* can be used for a negative control.

Interpretation: The absence of bubbling from a transferred colony indicates a negative test. Any bubbling from a transferred colony indicates a positive test.

Troubleshooting: False positives will result from transfer of red blood cells so take care when picking colonies from the BAP for this test.

Quality control: It is essential to use a known positive and negative QC strain as described in the procedure. Opened bottles should be checked against a known catalase positive organism every 6 months.

3.1.7.2.2. Optochin test (Arias & Murray, 2012)

Purpose: To differentiate between *Streptococcus pneumoniae* (Optochin-sensitive) and other alpha hemolytic *Streptococcus* species (Optochin-resistant)

Principle: To test an organism's susceptibility to the chemical, optochin (ethyl hydrocupreine hydrochloride).

Procedure: Optochin (P) disks (6 mm, 5 µg) can be obtained from a commercial vendor. Optochin disks are often called "P disks" and many commercial versions are labeled with a capital "P". If a commercial source of P disks is not available, a 1:4000 solution of ethyl hydrocupreine hydrochloride can be applied to sterile 6 mm filter paper disks.

i. Grow the strain(s) to be tested for 18-24 hours on a BAP at 35-37°C with ~5% CO_2 (or in a candle jar).

ii. Use a disposable loop to remove an isolated colony from the overnight culture on the BAP and streak onto one half of a BAP.

iii. Two different isolates can be tested on the same plate, but care must be taken to ensure that the cultures do not overlap.

iv. Place a P disk within the streaked area of the plate and incubate the BAP overnight at 35-37°C with ~5% CO_2 (or in a candle jar).

v. Observe the growth on the BAP near the P disk and measure the diameter of the zone of inhibition, if applicable.

Interpretation

Sensitive: A zone of inhibition of 14 mm or greater indicates sensitivity.

Resistant: A zone of inhibition less than 14 mm indicates resistance. In the case of an isolate completely resistant to optochin, the diameter of the disk (6 mm) should be recorded.

Troubleshooting: A smaller zone of inhibition (< 14 mm) or no zone of inhibition indicates that the bile solubility test is required. It is important to remember that pneumococci are sometimes optochin resistant.

Quality control: Each new lot of optochin disks should be tested with positive and negative controls. The growth of *S. pneumoniae* strain ATCC 49619 is inhibited by optochin and growth of *S. mitis* strain ATCC 49456 is not inhibited by optochin.

3.1.7.2.3. Bile solubility test (Mac Faddin, 1976; WHO, 2011)

Purpose: To differentiate between bile soluble *Streptococcus pneumoniae* and other bile insoluble alpha hemolytic *Streptococcus* spp.

Principle: To test the ability of bacterial cells to lyse in the presence of bile salts within a specific time and temperature

Reagents required: 2% sodium deoxycholate (bile salt) solution (dissolve 2 g of sodium deoxycholate into 100 ml sterile distilled water) and 0.85% normal saline

Procedure

i. Grow the isolate(s) to be tested for 18-24 hours on a BAP at 35-37°C with ~5% CO_2 (or in a candle-jar).
ii. Add bacterial growth from the overnight BAP to 1.0 ml of 0.85% saline to achieve turbidity in the range of a 0.5-1.0 McFarland standard.
iii. Divide the cell suspension equally into 2 tubes (0.5 ml per tube).
iv. Add 0.5 ml of 2% sodium deoxycholate (bile salts) to one tube. Add 0.5 ml of 0.85% saline to the other tube. Mix each tube well.
v. Incubate the tubes at 35-37°C in CO_2.
vi. Vortex the tubes.
vii. Observe the tubes for any clearing of turbidity after 10 minutes. Continue to incubate the tubes for up to 2 hours at 35-37^{0}C in CO_2 if negative after 10 minutes. Observe again for clearing.

Interpretation: A clearing of the turbidity in the bile tube but not in the saline control tube indicates a positive test (Figure 3.2).

Troubleshooting: Partial clearing (partial solubility) should not be considered positive for pneumococcal identification. Partially soluble strains that have optochin zones of inhibition of less than 14 mm should not be considered pneumococci.

Quality control: Each new lot of sodium deoxycholate should be tested with positive and negative QC strains. *S. pneumoniae* strain ATCC 49619 can be used as a positive control and *S. mitis* strain ATCC 49456 can be used as a negative control.

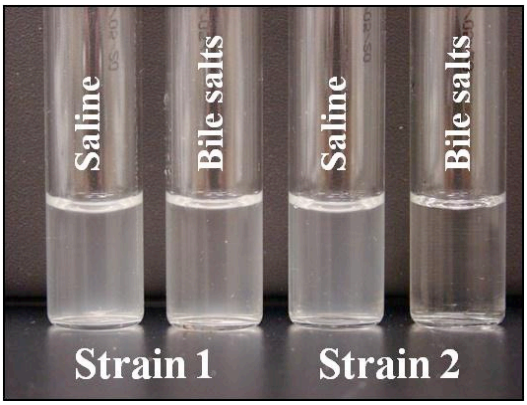


Figure 3.2: Bile solubility test for two different strains of bacteria. For strain 1, a slight decrease in turbidity is observed in the tube containing the bile salts (2nd from left), but the contents are almost as turbid as the control tube (far left); therefore, strain 1 is not *S. pneumoniae*. For strain 2, all turbidity in the tube containing the bile salts (far right) has cleared, indicating that the cells have lysed, in contrast to the control tube (2nd from right), which remains turbid; therefore, strain 2 is *S. pneumoniae*. Figure adapted from WHO manual (WHO, 2011)

3.1.7.3. Serotyping of *Streptococcus pneumoniae* by Quellung (WHO, 2011; Forbes et al., 2002)

Purpose: To determine capsular serotype of the *Streptococcus pneumoniae* isolates.

Principle: A positive Quellung or Neufeld reaction is the result of the binding of the capsular polysaccharide of pneumococci with type specific antibody contained in the typing antiserum. An antigen-antibody reaction causes a change in the refractive index of the capsule so that it appears "swollen" and more visible. After the addition of a counter stain (methylene blue), the pneumococcal cells stain dark blue and are surrounded by a sharply demarcated halo which represents the outer edge of the capsule. The light transmitted through the capsule appears brighter than either the pneumococcal cell or the background. Single cells, pairs, chains, and even clumps of cells may have positive Quellung reactions.

Performance specification: Gold standard test

Primary sample: *S. pneumoniae* fresh culture suspension

Reagents required

- 0.85 % saline
- Pneumococcal typing antisera (pool, group, or serotype-specific)
- Loeffler's Methylene blue (0.2 %)

Procedure

i. Grow the isolate(s) to be tested for 18-24 hours on a blood agar plate (BAP) at 35-37°C with ~5% CO_2 (or in a candle jar).

ii. From overnight growth on the BAP, use a sterile loop to prepare a light to moderate cell suspension (approximately equal to a 0.5 McFarland density standard) in 0.5

ml of 0.85% saline. Optimum Quellung reactions can be observed when there are 25-50 cells visible in a microscopic field at 1000X magnification.

iii. Dispense equal amounts of antiserum (5 µl) and methylene blue (5 µl) onto a microscope slide. Add approximately 0.2-1.0 µl of the diluted cell suspension and mix all three with a pipette tip.

iv. Cover the suspension with a 22 mm square cover-slip and incubate at room temperature (25°C) for 10-15 minutes.

v. Do not allow the fluid on the slide to dry.

vi. Examine the slide at 1000X using an oil immersion lens.

vii. Begin testing with pooled antisera. Once a positive reaction is obtained, proceed with individual group and serotype-specific antisera included in the pooled antisera that gave the positive reaction to determine the serogroup and serotype.

Reading the Quellung reaction results

- A positive Quellung reaction is observed when the capsule appears as a sharply demarcated halo around the dark blue-stained cell
- A negative Quellung reaction is observed when there is no appearance of a clear, enlarged halo surrounding the stained cell.

Troubleshooting

- In some instances, observing a positive reaction can be difficult. Prepare and read all Quellung reactions on the same day that the cell suspension is prepared.
- When reading the reactions, look for free floating single or paired cells.
- Agglutination (cells clumping together) is NOT a positive Quellung reaction.
- If a Quellung reaction is not observed in any of the antisera pools, the strain may be non-typeable, but identification of the strain as *S. pneumoniae* should be confirmed by optochin susceptibility and bile solubility testing.

Quality control: Each new lot of antisera received should be tested for positive Quellung reactions using *S. pneumoniae* reference strains with known capsular serotypes.

3.1.8. Identification and characterization of β-haemolytic Streptococci (βHS) (Betty et al., 2002; *Lancefield, 1933; Harvey & McIllmurray, 1984; CDC, 2010; CDC,2019; Facklam, 1980)*

Group A streptococcus (GAS) mainly causes sore throat, scarlet fever and post streptococcal glomerulonephritis. Group B *Streptococcus* (GBS) causes illness in people of all ages. GBS disease can be especially severe in newborns, most commonly causing sepsis, pneumonia, and sometimes meningitis. The most common problems caused by group B streptococci in adults are bloodstream infections, pneumonia, skin and soft-tissue infections, and bone and joint infections.

On blood agar, colonies are usually small, 1-2 mm in size, smooth and glistening, with well-defined zones of β-haemolysis. Colonies of both bacteria (Figure 3.3) group A streptococci (GAS) (on the right) and group B streptococci (GBS) (on the left) appear about the same color and size, but the degree of hemolytic activity is very different. The group A *Streptococcus* has more hemolytic activity than the group B *Streptococcus.*

On Gram staining, beta hemolytic streptococci are Gram positive cocci in pairs and short chains.

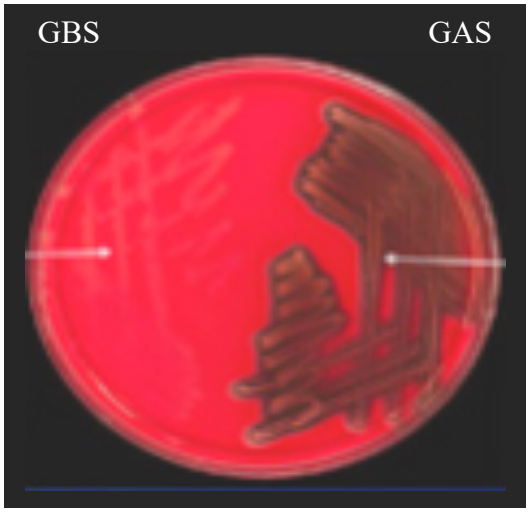


Figure 3.3: Beta haemolytic colonies with varying zones of hemolysis of GAS and GBS on blood agar

GBS are gray to whitish-gray surrounded by a weak zone of beta hemolysis of the red blood cells in the culture medium. Non-hemolytic strains can also be encountered (left hand side) and if whitish-gray colonies are identified and suspected of being GBS, then these should be further tested for catalase and confirmed by CAMP test. Since Group A streptococci (GAS) often lead to post-streptococcal sequelae, it is important to identify the antigenic groups of βHS to institute penicillin prophylaxis at the earliest.

If colonies suspecting of group A beta hemolytic colonies are grown, further confirmation is done by catalase and grouping. Catalase test has been described under *Streptococcus pneumoniae* section.

3.1.8.1. Identification of group A *Streptococcus* (GAS)

Specimens: Throat swab

Processing of swabs

- No smear examination should be done on throat swabs unless diphtheria or Vincent's angina are suspected.
- Inoculate the throat swabs directly on to blood agar plate.
- For detection of βHS, cut BA at the area of the first streaking, since more clear-cut hemolysis may be obtained from deeper colonies at the cut area due to the activity

of both oxygen stable and oxygen labile hemolysins. It is important that sheep blood be used in BA for recognizing β-hemolytic streptococci.

- If colony morphology suggests of beta hemolytic group A streptococci, grouping should be performed.

3.1.8.2. Identification of group B *Streptococcus* (GBS)

Specimens: Genital swabs, urine specimens, throat swabs and skin swabs from neonates may also be sent. These will be taken from the ear, umbilical stump and skin.

Processing of swabs

- All collected swabs (vaginal/rectal/skin surface/throat) should be processed by direct plating onto CHROMagar Strep B (primary isolation) and also by using selective broth followed by plate culture onto a CHROMagar Strep B.
- Inoculate swabs into LIM BROTH (DO NOT REMOVE SWABS) and incubate at 37°C in 5% CO_2 for 24-48 hours.
- After 24 hours, examine BA + CHROMagar StrepB
- Check for presence of beta hemolytic fine colonies / non-hemolytic fine colonies on BA and on CHROMagar StrepB for mauve colonies (Figure 3.4).
- Perform grouping for beta hemolytic streptococcal by the micro-nitrous acid extraction method and using either latex agglutination method or using the in-house preparation of antisera
- If grouping is positive for group B *Streptococcus*, perform the CAMP test for confirmation.



Figure 3.4: GBS (mauve) and *Enterococcus* (blue) colonies on CHROM agar

CAMP Test (Christie, Atkins, Munch-Peterson) for GBS (Darling, 1975)

The **CAMP test** identifies GBS on the basis on the formation of the CAMP factor that enlarges the area of haemolysis formed by β-haemolysin which is produced by *S. aureus*. The haemolytic activity of staphylococcal β-lysin on red blood cells is enhanced by the extracellular CAMP factor. Therefore, wherever the two reactants overlap on sheep blood

agar, an accentuation of a ß-haemolytic reaction occurs. This can be seen as an "arrow-head like" zone of haemolysis between the line of growth of the GBS and that of the *S. aureus*. The presence of CAMP factor is one of the major biochemical identification tests.

i. Make a single streak of a known *S. aureus* ATCC 25923 down the middle of a pre-warmed 5% sheep blood plate.
ii. Streak out a positive and negative control on both side of, and perpendicular to the *S. aureus* streak using growth scraped from the applicable control culture STGG cryovials prepared as per SOP.
iii. The streaks must be close but not touch one another.
iv. Add the test subject numbers onto the plate below that of the controls. Make similar streaks of the test organisms parallel to the control strains, 1cm apart.
v. Incubate the plate at 35-37˚C in 5% CO_2 for 18-24 hours.
vi. Examine the plate for a zone of complete lysis in the shape of an arrow-head at the junction of the streak lines, indicative of GBS (Figure 3.5). Some strains of Staphylococci can produce small arrow heads of lysis. Re-check the catalase reaction to be sure it is negative indicating a *Streptococcus*.

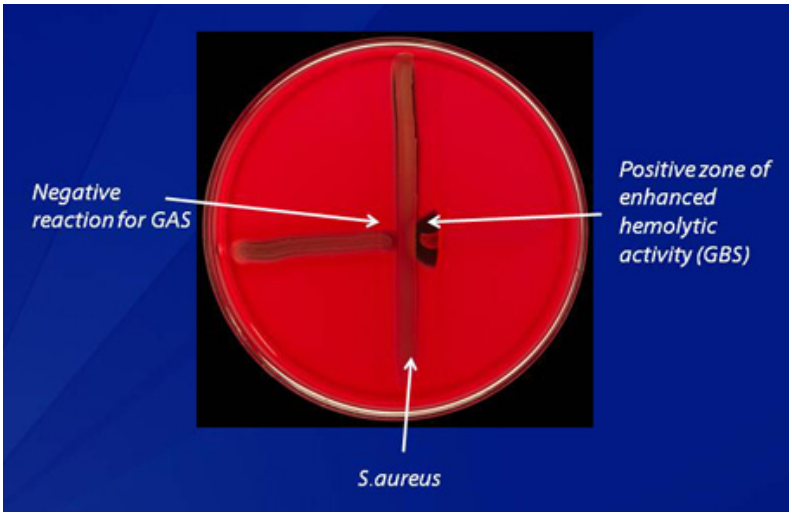


Figure 3.5: CAMP test, negative for *Enterococcus faecalis* (and other *Streptococcus* spp.); positive for *Streptococcus agalactiae* (GBS)

3.1.8.3. Grouping of beta haemolytic Streptococci

3.1.8.3.1. Antigen extraction methods

i. Micro nitrous acid extraction

- Take 20 µl of 2M sodium nitrite solution in a test tube.
- Inoculate 3 to 4 colonies of β- haemolytic streptococci into the test tube.
- Add 3 µl of glacial acetic acid to the suspension. Leave it at room temperature for 15 minutes.

- Add 16 to 24 µg of sodium bicarbonate ($NaHCO_3$) for neutralization. Add 100 µl of distilled water.
- Extract is now ready for CoA.

ii. Broth suspension

Inoculate multiple colonies of β-hemolytic streptococci into 1.0 ml Todd Hewitt Broth. Incubate for 4-6 hours at 37°C. If enough growth had occurred to give a visible turbidity, proceed for co-agglutination grouping; otherwise continue incubation overnight.

3.1.8.3.2. Grouping Method

Streptococcal grouping test kit

Intended Use: The kit is used for the qualitative detection and identification of the Lancefield group of Streptococci (A, B, C, D, F, and G) by agglutination of specific antibody-coated latex particles in the presence of enzymatically extracted antigen.

Clinical significance: Lancefield groups have quite different clinical significance and, in many cases, different biochemical and haemolytic differences within the same group. The majority of *Streptococcus* species possess group specific antigens of carbohydrate components in the cell wall. Lancefield demonstrated that these antigens could be isolated and identified by precipitation reactions with homologous antisera. There are several reported methods for the extraction of the antigen. This test utilizes an enzyme based rapid latex test system for providing a simple and rapid extraction process.

Health and Safety warnings

- All patient samples and isolates derived from patient samples and reagents should be treated as potentially infectious and the user should wear protective gloves, eye protection and laboratory coats when performing the test.
- Non-disposable apparatus must be sterilized after use by an appropriate method
- Disposable apparatus must be treated as bio-hazardous waste and autoclaved or incinerated.
- Spillages of potentially infectious material should be absorbed and disposed of as above. The site of spillage must be sterilized with disinfectant or 70% alcohol
- Do not pipette by mouth
- These reagents contain micro-fine latex suspensions coated with rabbit serum. The product also contains aqueous buffer salts including less than 0.1% sodium azide as a preservative – see material safety data sheet (MSDS) available on request.
- The product may contain dry natural rubber.

Analytical precautions

- Do not modify the test procedure.
- Resuspend latex reagent preparation gently but thoroughly

- Discard the reagent if the suspension becomes rough (i.e. shows signs of auto-agglutination).

Reagent preparation

- Do not dilute or modify the reagent in any way.
- Allow all reagents and samples to reach room temperature (18-30ºC) before use.

Composition

i. 6x50T (2.5 ml) latex determinations for the grouping of streptococci A: B: C: D: F: G
ii. Polyvalent positive control 2 ml
iii. Freeze dried extraction enzyme 2 vials. Reconstitute each with 10 ml of distilled RO water
iv. Disposable test cards x 50
v. Mixing sticks 300 and kit insert

Storage and shelf life

- Store latex reagents and controls upright at 2-8ºC.
- DO NOT FREEZE LATEX REAGENTS
- Do not use reagents after the stated expiry date.
- Once opened latex reagents may be used until the expiry date provided, they have been stored correctly and have not been contaminated.
- The freeze-dried extraction enzyme should be stored at 2-8ºC. Once reconstituted with 10 ml of sterile distilled water, it will retain its activity for at least 3 months or until the date shown on the bottle label, whichever is sooner. Alternatively, the enzyme may be stored in aliquots of 0.4 ml frozen at -20ºC, when it will remain active for at least 6 months or until the date shown on original bottle.
- Do not freeze and thaw the enzyme more than once.

Specimen and sample preparation

Cultures: The media normally used include blood agar base. In such cases, note colonial characteristics, haemolysis, and cell morphology before starting the test. Ensure that the organisms to be tested are Gram-positive and catalase-negative. Any blood agar plate culture yielding 2-6 well-separated colonies may be used, they should have been inoculated from a pure culture of the organism. If a clear and conclusive result of cultures that appear to contain streptococci is not obtained further subculture of suspect colonies is recommended. Organisms of groups A, B, C, D, F and G are normally beta-haemolytic, any alpha or non haemolytic organisms showing positive results should be confirmed by further biochemical test. (Some B & D strains can be either alpha or non haemolytic).

Principle: Streptococci carry group specific carbohydrate antigens in their cell walls. After extraction by a specially developed enzyme preparation these antigens will agglutinate latex particles coated with the corresponding antibody. The latex remains in smooth suspension in the absence of group specific antigen.

Method

i. Using a sterile bacteriological loop, pick 2-6 colonies of streptococci (avoiding other types of colony on the plate) and emulsify them in 0.4 ml extraction enzyme. (If a broth culture is to be grouped, pipette 0.1 ml of an overnight culture into 0.4 ml extraction enzyme).
ii. Incubate the mixture in a water bath at 37ºC for 10 minutes. Shake the tubes vigorously after 5 minutes incubation.
iii. Re-suspend the latex reagents by gentle agitation. Dispense 1 drop of each latex onto a circle on the test slide.
iv. Add one drop of the extract from a Pasteur pipette (or another device delivering approximately 50 microliters) to each drop of latex reagents, and mix the contents of each circle with a separate mixing stick.
v. Rock the slide for not longer than 1 minute, then observe for agglutination.

Note: The positive control is supplied so that the reactivity of all the latex reagents can be checked with each batch of test. It requires no extraction or dilution before use, and should be used as in steps iii to iv above. All latex reagents should show strong agglutination within 1 minute.

Interpretation of results

- A positive result is indicated by the visible agglutination of the latex particles. This should normally occur within a few seconds of mixing, depending on the strength of the antigen extract.
- A negative result is indicated by a milky appearance without any visible agglutination of the latex particles.
- Strong rapid agglutination with the FIRST latex reagent indicates a positive identification of that group, subsequent weak or delayed reactions with the same extract should be ignored. Only strong agglutination is significant; occasional strains of streptococci may give weak reactions with more than one group. If agglutination occurs in all groups, either the enzyme has been over-inoculated in which case repeat the test using a lighter inoculum, or a mixed culture was tested, in which case subculture and retest.

Performance characteristics

		Test reagent	
		+	-
Reference method	+	607	55
	-	0	24

Limitations of the methods

- False negative results can occur if an insufficient amount of culture is used for the extraction.
- False positive results have been known to occur with organisms from unrelated genera, eg. *Escherichia, Klebsiella or Pseudomonas.* These are lightly to non-specifically agglutinate all latex reagents.
- The group D antigen is common to organisms belonging to groups Q, R and S.
- False positive results can occur if the test is continued for longer than one minute
- Some strains of Group D streptococci have been found which also appear to possess Group G antigen, further biochemical tests are recommended in any case where identification is not conclusive.

Internal quality control

Positive control is provided and should be used to verify that the latex reagents are working satisfactorily under test conditions. Periodically check the following:

- The test reagents agglutinate with a known reference *Streptococcus* strain.
- The test reagents do not auto agglutinate in normal saline solution

3.1.8.4. Storage

Store all GAS and GBS isolates in small RCM broths. Label the tubes. Seal with paraffin wax and store at RT. Alternatively, these can also be stored in **STGG medium**. With a sterile loop, prepare a heavy suspension of GBS in STGG medium. GBS isolated on CAMP test plates can be used for storage purposes directly or if needed can be sub-cultured first on 5% sheep blood agar and stored from that. Label the STGG vial with the participant ID, date of collection of original swab/urine, site of original sample collection/sample type and store at -70˚C. Preparation of the media is given in annexure.

3.1.9. Identification of faecal isolates

Table 3.5: Biochemical tests for screening of fecal isolates

TSI Agar			Oxidase	Mannitol	Motility	Indole	Citrate	LIA*	CU Agar/ urea broth	Organism
Reaction		**Interpret-ation**								
1	Yellow slant, yellow butt, gas+	Glucose = acid + gas; lactose &/or sucrose = acid + gas	-	+	+	+	-	V	-	*E. coli*
			-	+	-	-	+	V	W	*Klebsiella pneumoniae*
			+	+	+/-	+/-	V	-	V	*Aeromonas* spp.
2	Yellow slant, yellow butt, gas+ with blackening	Glucose = acid + gas; lactose &/or sucrose = acid + gas with H_2S	-	+	+/-	-/+	+	-	V	*Citrobacter* spp.
			-	+	+	-	+	+	-	*Salmonella enterica* subsp .arizonae
			-	-	+	+/-	V	R	+	*Proteus* spp.
3	Red slant, yellow butt with gas	Glucose = acid + gas; lactose & sucrose negative	-	+	+	-	-	-	-	*Salmonella* Paratyphi A
			-	+	+	-	V	+	-	*Salmonella* spp. (H_2S-)
			-	-	+	+/-	-	R	+	*Morganella* spp.
			-	+	+/-	+/-	+	-	-	*Citrobacter* spp.
4	Red slant, yellow butt with gas with blackening	Glucose = acid + gas; lactose & sucrose negative with H_2S	-	+	+	-	V	+	-	*Salmonella* spp. (H_2S+) & *Arizona* spp.
			-	+	+	-	+	-	W	*Citrobacter* spp.
			-	-	+	+/-	-	R	+	*Proteus* spp.
			-	-	+	+	+	+	-	*Edwardsiella tarda*
5	Red slant, yellow butt, no gas	Glucose = acid no gas; lactose & sucrose negative	-	+	+	-		+	-	*S.* Typhi**
			-	+/-	-	+/-	-	-	-	*Shigella* spp., Alkalescens Dispar group
			-	+/-	+	+	+	-	V	*Providencia* group
			-	+/-	+	+	-	R	+	*Morganella morganii*
			+	-	+	+	-	+	-	*Plesiomonas shigelloides*
6	Red slant, yellow butt, no gas, with slight blackening	Glucose = acid no gas; lactose & sucrose negative with little H_2S	-	+	+	-	-	+	-	*S.* Typhi
7	Red slant, red butt	Glucose, lactose & sucrose negative	+	-	+	-	+		V	*Pseudomonas* spp.***
			V	-	-	-	+		V	Other non-fermenting Gram- negative bacilli

8	Yellow slant; usually no change in butt in 24 hours*(Incubation for >24hours can change to acid butt	Lactose negative, glucose & sucrose = slight acid	-	+	+ at 22°C; - at 37°C	-	-	+	+	*Yersinia enterocolitica*
9	Yellow slant, yellow butt	Glucose = acid; lactose &/or sucrose = acid	+	+	+	+	+	+	-	*V. cholerae* and other vibrios
			-	+	+/-	+	-	V	-	Anaerogenic *E. coli*
			-	+	+/-	+	V	-	-	*Aeromonas* spp.

*** Incubation for >24 hours can give acid butt, for *Yersinia enterocolitica***

Note: *Plesiomonas* is an oxidase positive organism which is an exception for Enterobacteriaceae

3.1.9.1. Further biochemical tests for identification

3.1.9.1.1. Proteus group (*O'Hara et al., 2000)*

- When the TSI and LIA reactions are suggestive of *Proteus or Morganella* species, transfer a heavy inoculum from the TSI to CU agar or urea broth and incubate in a water bath at 37°C.
- If the organism belongs to the *Proteus* group, rapid and abundant urease production is detected, for most strains, within 30 minutes to three hours.
- However, if urease production occurs within 24 hours, the culture should be considered as *Proteus*.
- If negative at 24 hours, incubate Christensen's medium for an additional 24 hours since some strains of *Citrobacter* and some *Klebsiella* strains split urea only after longer incubation. Urea broth may also be inoculated for confirmation.

3.1.9.1.2. Salmonella and salmonella-like organisms (Mooijman et al., 2019)

- When TSI and LIA reactions are suggestive of non-H_2S-producing *Salmonella*, use additional biochemical reactions, which correlate with the *Salmonella* 'O'groups for which they are characteristic.
- Inoculate LIA, malonate, dulcitol, urease and citrate (with a known *Pseudomonas aeruginosa* control).
- Serological confirmation: Rapid slide agglutination test
  - When the TSI and LIA reactions on a motile culture are suggestive of H_2S-producing *Salmonella*, proceed to rapid slide agglutination tests with the fresh TSI culture and specific diagnostic serum for identification. Subcultures on nutrient agar (NA) may be tested alternately.

- Proceed also to serologic identification of non-H_2S-producing *Salmonella* species.

Procedure

- Place 2 separate drops of saline above each other on a slide and mix a heavy inoculum of TSI agar growth in each across the slide in a linear fashion and not circular.
- To one, add the required antiserum, to the other, more saline to serve as the control.
- Rock the slide back and forth for 1-3 minutes, watching closely for appearance of agglutination.
- Read results with the naked eye, being certain that no spontaneous agglutination (auto agglutination) has occurred in the saline control.

Note:

- Four tests may be done on one slide without any waste.
- In heterologous mixtures, cross agglutination may occur, but it usually takes longer and is less marked than a specific reaction. It is not uncommon to find cross-reactions between *Citrobacter* and *Proteus* spp and polyvalent *Salmonella* antiserum; or between coliforms and *Shigella*.
- Some freshly isolated strains of *S.* Typhi are not agglutinated by polyvalent or specific somatic and flagellar antisera because of the presence of Vi-antigen.
- Slide agglutination tests with Vi antiserum must be done, remembering that some strains of the *Citrobacter* group as well as *S*. Paratyphi C possess the same Vi-antigen. So, in slide agglutination test with salmonella O polyvalent and Vi antisera, only if both are negative, it is considered negative.

Additional biochemical tests for serologically unidentifiable isolates

- An organism which is salmonella like, but which cannot be serologically typed, must be studied further biochemically for the following reasons:
  - First, it may actually be a *Salmonella* strain belonging to the groups which are not usually looked for in our laboratory (*i.e*., other than a member of Groups A-E)
  - Second, it may be an *Arizona* strain, and if so, must be identified and reported since the arizona group is similar to *Salmonella* species in pathogenicity.
  - Third, it may belong to the citrobacter group or be a strain of *E.tarda*.
- Hold the CU agar and inoculate the following additional media. LIA, citrate, malonate broth, dulcitol, lactose broth, gelatin.

- See Table below for correlation of reactions in these media with the organisms thus characterized.

Table 3.6: Special biochemical test for differentiating *Salmonella*, *Arizona*, *Edwardsiella tarda* and *Citrobacter* isolates

LIA	Malonate	Dulcitol	Lactose	CU Agar	Gelatin liquefaction	Organism
+/H_2S	V*	+	-	-	-	*Salmonella* spp.
+/H_2S	+	-	RLF or LFD	-	+	*Arizona* spp.
-/H_2S	-V	+	RLF orLFD	Delayed + or -	-	*Citrobacter* spp.
+/H_2S	-	-	-	-	-	*E. tarda*

***Salmonella enterica* subspecies *arizonae* is malonate +**

- When an organism with typical *Salmonella*-like reactions in LIA is malonate negative, dulcitol variable, and lactose negative, consider it to be organism "biochemically resembling *Salmonella*".
- If the organism with *Salmonella*-like reactions in TSI gives a *Salmonella*, *Arizona*-like reaction in LIA, positive malonate test within 24-48 hours, does not ferment dulcitol (though lactose may be fermented rapidly, or after some delay), liquefies gelatin and does not show urease production on Christensen's urea medium, consider it to be "organism biochemically resembling *Arizona*".
- If an organism with salmonella-like reactions in TSI gives a *citrobacter* -like reaction in LIA, with a negative/positive malonate test and rapid dulcitol fermentation, it is definitely not *Salmonella* or *Arizona* strain. Consider it to be possibly a *citrobacter* strain.
- CU agar may show a weakly positive reaction on prolonged incubation.
- If an organism with salmonella-like reactions in TSI and LIA gives negative reaction in the supplementary tests, consider it to be a strain of *E.tarda*.

3.1.9.1.3. Shigella and shigella-like organisms

Inoculate suspected NLF colonies on MA, DCA or colorless (red) colonies on XLD in MM, TSI, peptone water and LIA. Include urease and phenyl pyruvic acid (PPA) to rule out *Morganella* species.

Serologic identification: rapid slide agglutination test

- If the test for urease is negative on non-motile cultures with TSI reactions suggestive of *Shigella* species, do rapid slide agglutination tests with the appropriate sera, according to indole test and mannitol fermentation test results.

- *Shigella* is divided based on mannitol fermentation
  Mannitol negative - *Shigella dysenteriae* (Group A)
  Mannitol positive - *Shigella flexneri* (Group B)
  Shigella boydii (Group C)
  Shigella sonnei (Group D)
- Group A (10 serotypes) is divided on the basis of indole production into following:
  Indole negative serotypes 1, 3, 4, 5, 6, 9, 10
  Indole positive serotypes 2, 7, and 8
- Groups B, C, D are based on lactose fermentation and indole production
- Group D is the only late lactose fermenter; it doesn't produce indole and has only one serotype
- Between Group B (6 serotypes) and Group C (15 Serotypes); only serotypes 6 of Group B is indole negative along with serotypes 1, 2, 3, 4, 6, 8, 10, 12, 14 of group C and serotypes 1-5 of group B with serotypes 5, 7, 9, 11, 13, 15 of group C are indole positive.
- Suspend growth from cultures, which appear to be *Shigella*, but which do not react with shigella antisera in saline, heat at 100°C for 30 minutes, and retest.
  Note: Such heating will destroy a labile antigen, which, if present, may have inhibited agglutination of the bacilli by the antiserum. Check the heated suspension for auto-agglutination before proceeding further.

Additional tests for biochemically and serologically unidentifiable isolates

- An indole-positive shigella-like organism, which in the living state is not agglutinated by any of the various shigella antisera, must be studied further, since it may belong to the Alkalescens-Dispar (A-D) group.
- Use a heated suspension for slide agglutination tests. Unheated Alkalescens-Dispar (A-D) group strains are inagglutinable.
- If agglutination is negative, proceed as follows with cultures giving shigella-like TSI reactions.
  - Smell the TSI slant to detect a fruity odour, if present.
  - Inoculate the following media: LIA, Simmon's citrate slope, PPA, lactose, sucrose, mannitol, glucose and salicin broths.
- When a shigella-like organism is not identifiable either as *Providencia* strain or an anaerogenic strain of *E.coli*, hold carbohydrate broth cultures for 3 weeks, and send subcultures to a reference laboratory for identification.
  Note: A preliminary report may be sent to indicate isolation of 'an organism biochemically resembling *Shigella* species, but serologically untypable with available facilities.

3.1.9.1.4. *V. cholerae* 01 and *V. cholerae* 0139

i. Direct microscopic examination

- Prepare a hanging drop and look for highly motile, darting bacillary forms as presumptive evidence.
- Test motility by dark-field examination if specimen is received. Dark-field (DF) examination is more dependable than HD.
  - Take a few loopfuls of the liquid faeces on a slide, place a cover slip and examine under DF for actively darting organisms.
  - If typically motile forms are present, test for immobilization (DFI) by mixing a loopful of specimen with a loopful of *V. cholerae* 01 antiserum, put a cover slip on and examine again by dark-field microscopy. If now the motility is absent then, it is confirmed that the motile organisms were *V.cholerae*, if negative, perform DFI with *V. cholerae* 0139 antiserum.

ii. Isolation

- Streak directly on to thiosulfate citrate bile salt sucrose agar (TCBS), MA, DCA and XLD plates. Incubate overnight at 35+/- 1°C. BA is included if the patient is a child < 2 years.
- Inoculate alkaline peptone water (APW) and selenite F broth; incubate at 35+/- 1°C. Make a hanging drop/DF examination from the APW culture after 4 to 6 hours as given above.
- After 4-6 hours incubation of the APW culture, inoculate TCBS and BA plates and incubate at 35+/- 1°C overnight.
- After 16-18 hours inoculate DCA from selenite F broth.
- Examine plates for presence of colonies suggestive of *V. cholerae*, or of any other enteric pathogens.

Note: TCBS agar usually suppresses growth of *E. coli* and enteric pathogens other than the vibrios. All vibrios show similar colony characteristics. They are large, flat and yellow.

iii. However, *Vibrio parahaemolyticus* shows exception in that it is sucrose non-fermenter and produces blue-green colonies.

iv. Subculture from suspicious colonies according to directions given earlier.

v. Subculture from the APW after over-night incubation onto TCBS and BA if not already subcultured after 4-6 hours (in the case of specimens handled in the night).

Further biochemical tests for identification

i. If TSI reactions indicate possible sucrose fermentation without gas formation, indole positive, motile cultures, suspect the organisms to be *V. cholerae*. Set up a Greig Test for determination of test tube haemolysis.

- Inoculate a tube of glycerolated heart infusion broth and incubate overnight to obtain a suitable culture.
- Add 1.0 ml of 1% saline suspension of washed sheep red blood cells to 1.0 ml of the 24-hour culture.

- Incubate at 37°C for 2 hours, and then refrigerate overnight.
- Look for presence or absence of hemolysis.

Note: Classical *V. cholerae* does not produce test tube haemolysis, though haemolytic colonies may be seen on BA as a result of haemodigestion. El Tor vibrios, which are also agglutinated by *V. cholerae* antiserum, produce test tube haemolysis. With the gradual disappearance of classical vibrios and emergence and endemicity of El Tor vibrios, it is desirable that agglutinable strains be examined by additional tests.

ii. The panel of tests for identification of classical *V. cholerae* from E1 Tor biotype is

- Growth on BA demonstrating haemolysis
- Greig test
- Polymyxin B (50 units) reaction
- Voges Proskauer reaction

Table 3.7: Differential characteristics of *V. cholerae* types

Test	**Classical *V. cholerae***	**El Tor biotype**
Growth on blood agar	Hemodigestion	Hemolysis and/or hemodigestion
Test tube hemolysis (Greig test)	Negative	Positive
Polymyxin B (50 units)	Susceptible	Resistant
Voges Proskauer reaction	Negative	Positive

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Table 3.8: Biochemical reactions of *V. parahaemolyticus*, *V. cholerae* O1 & O139

Test	***V. parahaemolyticus***	***V. cholerae* O1 & O139**
Oxidase	Positive	+
Hemolysis on 5% SBA	Non – hemolytic	Beta
TCBS	Bluish green colonies	Yellow
Growth at 0% NaCl	Negative	Positive
Growth at 7% NaCl	Positive	Negative
TSI – slope/butt	Alk./acid	acid/acid
Nitrate reduction	Positive	+
Gelatin liquefaction	Positive	+

Indole	Positive	+
Citrate	Variable	+
MR	Positive	Variable
VP	Negative	Variable
Arginine dihydrolase	Negative	-
Lysine decarboxylase	Positive	+
Ornithine decarboxylase	Positive	+
String test	Negative	+
Glucose	Positive – acid	+
Lactose	Negative	-
Mannitol	Positive – acid	+
Sucrose	Negative	+
Arabinose	Positive – acid	-
Mannose	Positive – acid	+

Serological confirmation

- Test the cultures for slide agglutination by specific diagnostic serum, *V. cholerae* sero group O1 polyvalent.
- If positive, proceed with ogawa and inaba serotypes. If agglutination is negative with *V. cholerae* serogroup O1 try agglutination with *V. cholerae* serogroup O139 antiserum. If this also is negative, proceed as for identification of non-O1 *V.cholerae*.
- These are organisms which are morphologically, culturally and biochemically identical to *Vibrio cholerae* but serologically not serogroup O1 or O139.
- They could be any of serogroups O2 to O138. They cause an acute diarrhoeic illness -paracholera. Hence, even if slide agglutination tests with *V. cholerae* anti-serum is negative, all suspicious colonies should be biochemically characterised fully.
- *V. parahaemolyticus* is a marine halophilic vibrio, which can tolerate upto 7% NaCl.
- If clinical history suggests consumption of seafood prior to onset of gastroenteritis, a search for this organism should be included.

Isolation

In addition to the media mentioned, the faeces sample may be inoculated on TCBS. Also inoculate a small amount of the sample into enrichment medium, peptone water with 3% NaCl. After 6 to 8 hours incubation, subculture onto TCBS agar.

Identification

From the TCBS plates, pick out green umbonate colonies with a deep green centre and transparent periphery and proceed with inoculation of TSI, MM medium, peptone water, nitrate broth, gelatin, indole, citrate, methyl red, Voges Proskauer, arginine dihydrolase, lysine dihydrolase, ornithine decarboxylase, individual glucose, mannitol, sucrose, arabinose and mannose. *V.parahaemolyticus* grows better if the NaCl content of the media is enhanced. This would help differentiate between *V. parahaemolyticus* and *V. cholerae* O1/O139.

3.1.9.1.5. Campylobacter jejuni

They are Gram-negative comma or 'S' shaped organisms, actively motile, and microaerophilic. Ideal atmosphere for its growth should contain 5-6% O_2, 10% CO_2 atmosphere and the rest N_2; growth is best at a temperature of 42°C.

Isolation: Besides the media recommended commonly for faeces, streak directly on campylobacter agar base supplemented with campylobacter selective supplement (BLASER WANG).

***Campylobacter* agar base** constitutes following:

Lab-Lemco powder	10 g/L
Peptone	10 g/L
Sodium chloride	5 g/L
Agar	12 g/L
pH	7.5 ± 0.2 at 25ºC

***Campylobacter* selective supplement (BLASER WANG)**

Vancomycin	5 mg
Polymyxin B	1,250 IU
Trimethoprim	2.5 mg
Amphotericin B	1 mg
Cephalothin	7.5 mg

Each 500 ml of the campylobacter agar base is mixed with one vial of the campylobacter selective supplement and 10% defibrinated sheep blood or horse blood or 5-6% laked horse blood mixed gently and poured into sterile petri dishes.

Biochemical characterization

Generally they are inert organisms. The morphology on Gram's stain should be considered confirmatory. Reactions are as follows for *Campylobacter jejuni*.

- Oxidase-positive,
- Nitrate reduction positive,
- Indole negative,
- Urease negative,
- Methyl red negative,
- Voges-Proskauer negative,

Report

- Send provisional report based on DF test/ hanging drop in cases where cholera is suspected or smear examinations in cases of pseudomembranous colitis.
- Send final report as soon as isolated organisms are identified (usually on day three).

Chapter 4
Identification by Automation

Automated platforms shorten the time to identification of bacterial and fungal pathogens and improve the reproducibility of results. This chapter describes such platform used in the bacteriology laboratory. Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) for the identification of bacterial isolates grown in culture. Instrument-specific operating steps should always be read together with the manufacturer's instructions for use.

4.1 Identification by MALDI-TOF Mass Spectrometry

4.1.1 Purpose

To identify bacterial isolates obtained from specimens sent for bacterial culture to the genus and/or species level, rapidly and from a pure culture.

4.1.2 Principle

Mass spectrometry is an analytical technique in which sample molecules are converted into charged ions and separated according to their mass-to-charge ratio (*m/z*). In MALDI-TOF MS, the ions are produced by matrix-assisted laser desorption/ionisation (MALDI) and are separated by a time-of-flight (TOF) mass analyser. Whole microbial cells are analysed directly, without prior extraction of nucleic acids or biochemical testing. The instrument records the profile of the most abundant cellular proteins, chiefly ribosomal proteins, in the range of approximately 2,000-20,000 Daltons. This profile is highly reproducible and characteristic of a genus, species or in some groups, a species complex. (Clark et al., 2013; Singhal et al., 2015).

The working mechanism comprises the following sequential steps (Figure 4.1):

1. **Sample-matrix co-crystallisation:**

   A small amount of a fresh colony is smeared as a thin film on a target plate and overlaid with a matrix solution. The matrix is a small organic acid dissolved in an organic solvent containing trifluoroacetic acid (commonly α-cyano-4-hydroxycinnamic acid in 50% acetonitrile with 2.5% trifluoroacetic acid). The solvent lyses the cells and extracts proteins; on drying, the matrix and the proteins co-crystallise.

2. **Laser desorption and ionisation:**

   The target is placed in the vacuum chamber and short pulses of an ultraviolet laser (commonly a nitrogen laser, 337 nm) are fired at the crystals. The matrix absorbs the laser energy, vaporises and carries intact protein molecules into the gas phase, where proton transfer from the matrix produces mainly single charged ions (z = 1), so that *m/z* is effectively equal to the mass.

3. **Acceleration:**

   A high-voltage electric field accelerates all ions to the same kinetic energy.

4. **Time-of-flight separation:**

   The ions drift through a field-free, evacuated flight tube. Because their kinetic energy is the same, lighter ions travel faster and reach the detector earlier than heavier ones; the time of flight is proportional to the square root of *m/z*.

5. **Detection:**

   The detector records the arrival time of each ion packet. After calibration, arrival times are converted into *m/z* values, and a mass spectrum (intensity plotted against *m/z*) is generated.

6. **Spectral matching and identification:**

   After baseline correction and peak picking, the spectrum is compared by the software with a database of reference spectra of well-characterised organisms. A similarity score or confidence value is generated and the organism is reported at the level of species (or genus/complex) for which the match is reliable.

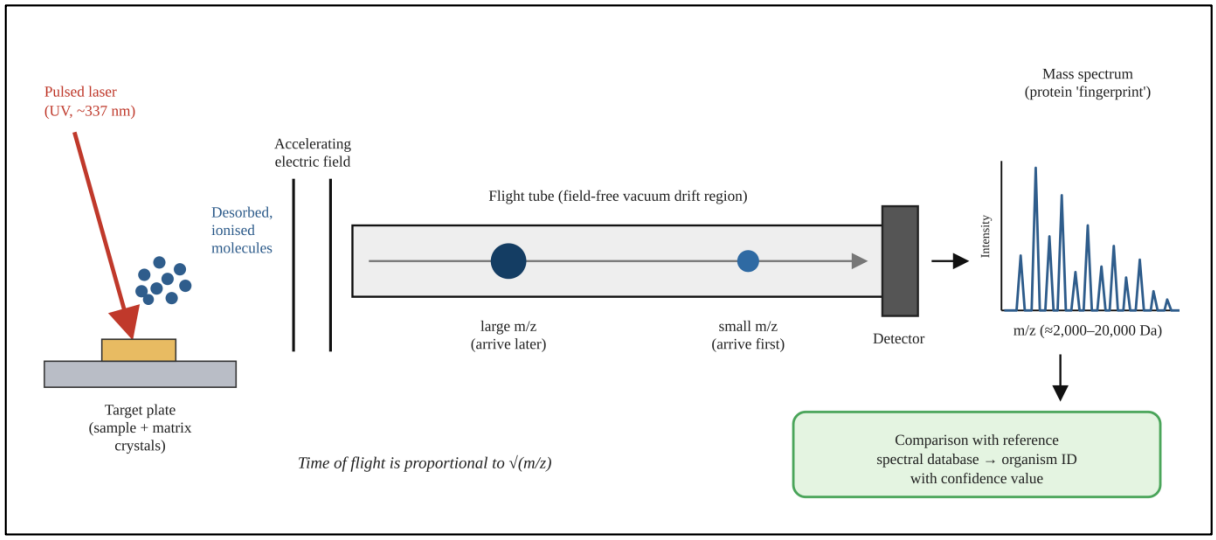


Figure 4.1. Schematic representation of the working principle of MALDI-TOF MS: from sample-matrix crystals on the target plate to spectrum generation and database matching.

Advantages and limitations:

Identification is obtained within minutes of colony picking, at a low consumable cost per isolate, and the method is applicable to bacteria, yeasts and (after extraction) mycobacteria and filamentous fungi. However, the result depends on the quality and accuracy of the reference database and on the purity and age of the culture. Closely related organisms (for example, *Streptococcus pneumoniae* and the *S. mitis* group, *Escherichia coli* and *Shigella* species, and the serovars of *Salmonella*) may not be reliably separated and need supplementary testing (Section 4.1.5).

4.1.3 Sample preparation

- Use a fresh (18-24 hours), pure culture. Remove any mucoid layer with a sterile swab before sampling.
- Label the target plate with the date and plate number. Pick a single colony with a loop or a wooden stick and smear it as a thin, even, confluent film on the designated spot. Record the specimen/laboratory identification number against the spot position.
- Overlay the dried smear with 1 µL of matrix and allow it to dry completely before loading the plate into the instrument.
- Include a control spot of the reference strain (*E. coli* ATCC 8739) in every acquisition group (Section 4.1.6).

4.1.4 Analysis and review of results

Load the dried target plate into the instrument as per the manufacturer's instructions, with the plate identification entered or scanned so that the spot positions are linked to the specimen numbers, and start the run. The results are reviewed by a microbiologist in the instrument software on the same day and handled as shown in Table 4.1.

Table 4.1 Review of MALDI-TOF MS results

Result category	Action
High confidence (single identification, ≥ 98%)	Accept, subject to the reporting criteria in Section 4.1.5. Results below 98% are re-fired (the same spot is re-run).
Medium confidence / low discrimination (a list of closely related organisms)	Review the list in detail. Select the most appropriate identification if acceptable, otherwise discard and re-test.
Low confidence or no identification	Discard and re-test.
Poor result persisting after re-analysis	Evaluate spectral quality. A good-quality spectrum indicates that an alternative identification method is needed; a poor-quality spectrum indicates that the preparation must be repeated.

After the review, validated results are confirmed and cleared from the review list, the plate is discarded or cleaned as per the manufacturer's instructions, and the matrix and formic acid are returned to the refrigerator.

4.1.5 Acceptance and reporting of identification

- **Common isolates** with a confidence of >98% are reported as identified, provided that the colony morphology and Gram-stain appearance match the identification.
- **Uncommon isolates** with a confidence of >98% are reported as presumptive, pending additional in-house testing, provided that the colony morphology and Gram-stain appearance match.

- **Low discrimination** results that list a few organisms of the same genus are reported as the species complex or group, according to the full list of organism identifications in the instrument database.
- **Beta-haemolytic streptococci:** any isolate identified as Group A, C or G *Streptococcus* requires confirmation by Lancefield group agglutination before reporting.
- ***Streptococcus pneumoniae:*** any identification of >98% requires confirmation. An identification of 99.9% in an isolate that is bile soluble and/or optochin sensitive is confirmed and needs no further work. An identification of 98-99.8% in an isolate that is bile insoluble and optochin resistant is reported as presumptive viridans streptococci and referred for whole-genome sequencing.
- **Anaerobes and atypical mycobacteria** with >99% agreement are considered confirmed; otherwise they are confirmed by 16S rRNA gene sequencing.
- **Enteric pathogens** identified by MALDI-TOF MS need the additional testing shown in Table 4.2.

Table 4.2 Additional testing for enteric pathogens identified by MALDI-TOF MS

MALDI-TOF MS identification	Additional testing
Salmonella species (including *S.* Typhi and *S.* Paratyphi A)	Serotyping from a blood agar subculture (on the same day, if possible).
Non-lactose-fermenting *E. coli* (possible *Shigella* species)	Multiplex PCR for the identification of *Shigella* species.

4.1.6 Quality control

Positive and negative quality control (QC) is performed on receipt of every new lot of reagents and once daily for each type of microorganism tested. One bacterial and one yeast reference isolate are used for positive QC; a spot of matrix alone serves as the negative control. The strains and expected results are given in Table 4.3.

Table 4.3 Daily quality control

QC organism	Expected result
E. coli ATCC 8739	*E. coli*
No organism (matrix only)	No identification

- If the negative control gives an identification, check that the surface of the target plate is clean and repeat the negative control.
- Repeated low-confidence identifications, despite correct procedure and technique, may indicate that the instrument needs calibration.

Chapter 5
Antimicrobial Susceptibility Testing

5.1 Scope

This document outlines the principles, methodologies, and quality assurance practices involved in antimicrobial susceptibility testing (AST) within diagnostic laboratories. It covers:

- The clinical significance of AST in guiding antimicrobial therapy.
- Standardized testing methods.
- Selection and maintenance of reference strains and quality control procedures.
- Interpretation of susceptibility results according to internationally recognized guidelines (e.g., CLSI, EUCAST) (Clinical and Laboratory Standards Institute [CLSI], 2026; European Committee on Antimicrobial Susceptibility Testing [EUCAST], 2026a)

5.2 Introduction & Definitions

Antimicrobial resistance (AMR) has emerged as one of the most pressing global public health challenges, threatening the effective treatment of a growing number of infections caused by bacteria, parasites, viruses, and fungi. Accurate and timely AST is critical in combating AMR, guiding clinicians in selecting the most effective therapy, and supporting surveillance of resistance trends.

Diagnostic laboratories play a pivotal role in this effort, providing essential data on the susceptibility patterns of clinical isolates. AST not only informs patient care but also contributes to infection control strategies and antibiotic stewardship programs. Various standardized methods, such as disk diffusion, broth micro dilution, and automated systems, are utilized in laboratories to determine the susceptibility of pathogens to antimicrobial agents. Given the rise in multidrug-resistant organisms and the evolving landscape of infectious diseases, ensuring the quality, consistency, and accuracy of AST procedures in diagnostic laboratories has become more important.

Definitions:

- **Susceptible (S)** isolates are inhibited by the usually achievable concentrations of anti-microbial agent and infection is expected to respond when the recommended dosage is used for the site of infection (CLSI, 2026).
- **Intermediate (I)** indicates that an antimicrobial agent may still have clinical efficacy when its concentration is higher than standard levels at the site of infection or when alternative/variable dosing applies, while serving as a buffer zone to prevent minor technical variations from causing major interpretation errors.

- **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.
- **Non-susceptible (NS)** is a reporting category for isolates tested against an agent for which only a susceptible breakpoint has been established, because resistant strains are absent or rare. An isolate whose MIC is above the susceptible breakpoint, or whose zone diameter is below it, is reported as non-susceptible. It is not reported as intermediate or resistant, because no such categories exist for that drug–organism combination. A susceptible only interpretive category may be applied to new antimicrobial agents for whom no resistant isolates have been encountered at the time initial interpretive criteria are determined.
- **Susceptible-Dose Dependent (SDD)** organisms are not reliably inhibited by standard dosing, but can be effectively treated if higher exposure is achieved through optimized dosing strategies, such as increased dose, increased dosing frequency, or both. Thus, clinical efficacy is expected when appropriate, evidence-based high-dose regimens are used to attain sufficient drug concentrations at the site of infection.

5.3 Media recommended for AST

1. Mueller-Hinton agar (MHA)
2. MHA with 5% sheep blood for Streptococcus sp.
3. Haemophilus test medium (HTM) for *Haemophilus* sp.
4. GC agar with 1% supplements for *Neisseria* sp.
5. Mueller-Hinton broth (MHB)
6. MH-F broth (supplemented with 5% mechanically defibrinated horse blood and 20 mg/L β-NAD)

5.3.1 Mueller-Hinton Agar (MHA)

MHA preparation includes the following steps (CLSI, 2024a):

1. Prepare MHA from a commercially available dehydrated base according to the manufacturer's instructions.
2. Immediately after autoclaving, allow the agar to cool at 45 to 50°C water bath.
3. Pour the freshly prepared and cooled medium into glass or plastic, flat-bottomed Petri dishes on a level, horizontal surface to give a uniform depth of approximately 4 mm. This corresponds to 60 to 70mL of medium for plates with a diameter of 150 mm and 25 to 30 mL for plates with a diameter of 100 mm.
4. Allow the agar plates to cool further to room temperature and, unless the plates are used the same day, store in a refrigerator (2 - 8°C).
5. Use the plates within seven days after preparation unless adequate precautions, such as wrapping in plastic, are taken to minimize drying of the agar.

6. A representative sample of each batch of plates should be examined for sterility by incubating at 35 ± 2°C for 24 hours.
7. Check the pH of each batch of MHA when the medium is prepared. The exact method used depends largely on the type of equipment available in the laboratory. The agar medium should have a pH between 7.2 and 7.4 at room temperature, and must therefore be checked after gelling.
   Note: If the pH is less than 7.2 certain drugs will appear to lose potency (eg: Aminoglycosides, Macrolides), whereas other agents may appear to have excessive activity (eg: Tetracyclines). If the pH is greater than 7.4 the opposite effects can be expected (CLSI, 2024a).

Check the pH by one of the following means:

- ✓ Macerate enough agar to submerge the tip of a pH electrode.
- ✓ Allow a small amount of agar to solidify around the tip of a pH electrode in a beaker or cup.
- ✓ Use a surface electrode.
- ✓ Do not add supplemental calcium or magnesium cations to MHA.

5.3.2 Mueller-Hinton Agar + 5% Sheep Blood:

- Prepare MHA as described above. When it has cooled to 45 to 50°C, add 50mL of defib-rinated sheep blood to 1 L of MHA. Continue as described in above protocol.
- Check the pH after aseptic addition of the blood to the autoclaved and cooled medium. The final pH should be the same as unsupplemented MHA (pH 7.2 to 7.4).

5.3.3. Haemophilus Test Medium:

Haemophilus Test Medium (HTM) consists of the following ingredients (CLSI, 2024a, 2026):

- Mueller-Hinton medium base
- 15 µg/mL β-NAD
- 15 µg/mL bovine or porcine hematin
- 5 g/L yeast extract

HTM preparation includes the following steps:

1. Prepare a fresh hematin stock solution by dissolving 50 mg hematin powder in 100 mL of 0.01 mol/L NaOH (heat gently) and stir until completely dissolved.
2. Prepare an NAD stock solution by dissolving 50 mg of NAD in 10 mL of distilled water; filter sterilize.

3. Prepare MHA from a commercially available dehydrated base according to the manufacturer's directions, adding 5 g of yeast extract and 30 mL of hematin stock solution to 1 L of MHA.
4. After autoclaving, cool to 45 to 50°C.
5. Aseptically add 3 mL of the NAD stock solution.
6. The pH should be 7.2 to 7.4.

5.3.4 GC Agar +1% Defined Growth Supplement

1. Use a 1% defined growth supplement that contains the following ingredients per litre (CLSI, 2024a):

⇒ L-cystine - 1.1 g
⇒ Guanine HCl - 0.03 g
⇒ Thiamine HCl - 0.003 g
⇒ P-aminobenzoic acid - 0.013 g
⇒ Vitamin B12 - 0.01 g
⇒ Thiamine pyrophosphate (cocarboxylase) - 0.1 g
⇒ Nicotinamide adenine dinucleotide (NAD) - 0.25 g
⇒ Adenine - 1 g
⇒ L-glutamine - 10 g
⇒ Glucose - 100 g
⇒ Ferric nitrate - 0.02 g
⇒ L-cysteine HCl- 25.9 g

2. Prepare 1 L of single strength GC agar base from a commercially available dehydrated base according to the manufacturer's instructions. After autoclaving, cool to 45 to 50°C in a 45 to 50°C water bath. Add 10 mL of 1% defined growth supplement.

5.3.5 Mueller-Hinton broth (MHB)

MH broth, un-supplemented cation-adjusted Mueller-Hinton broth, is used for testing of non-fastidious organisms according to the ISO standard 20776-1, 2019 (International Organization for Standardization [ISO], 2019).

MH-F broth, cation-adjusted MH broth supplemented with 5% lysed ((mechanically defibrinated)) horse blood and 20 mg/L β-NAD, is used for testing *Streptococcus* spp. (including *S. pneumoniae*), *Haemophilus influenzae, Moraxella catarrhalis, Listeria monocytogenes, Campylobacter jejuni and coli, Pasteurella multocida, Corynebacterium spp., Aerococcus sanguinicol*a and *urinae, Kingella kingae* and several other fastidious organisms.

Un-supplemented MH broth may be purchased from commercial sources or prepared locally according to the manufacturers' instructions. MH broth should meet the requirements in the ISO Technical specification, ISO/TS 16782, 2016 and the quality control criteria published by EUCAST (ISO, 2016; EUCAST, 2026a).

5.3.6 MH-F broth (MHF)

MH-F broth is prepared as follows:

Reagents

- Cation-adjusted MHB from commercial source.
- 50% lysed horse blood.
- β-Nicotinamide adenine dinucleotide (β-NAD), purity ≥98%.

5.3.6.1 Preparation of 50% lysed horse blood stock solution:

- Aseptically dilute mechanically defibrinated horse blood with an equal amount of sterile deionized water.
- Freeze the blood at -20°C overnight and thaw. Repeat the cycle until the cells are completely lysed (Repeat the freeze-thaw cycle until the cells are completely lysed (usually three cycles; ISO 20776-1 notes that up to seven may be required).
- Clarify the 50% lysed horse blood by centrifugation and discard the pellet. A clear solution is essential for reading. Failure to clarify the solution may be due to inadequate lysis or centrifugation. Repeating the centrifugation may improve the clarity of the solution.
- The stock solution may be stored at -20°C in aliquots and defrosted as required. Do not refreeze unused solution.

5.3.6.2 Preparation of β-NAD stock solution

- Dissolve β-NAD in sterile deionized water to a concentration of 20 mg/mL.
- Sterilize the solution through a 0.2 µm membrane filter.
- The stock solution may be stored at -20°C in aliquots and defrosted as required. Do not refreeze unused solution.

5.3.6.3 Preparation MH-F broth

- Prepare and autoclave cation-adjusted MHB according to the manufacturer's instructions, but with 100 mL less deionized water per litre to allow for the addition of lysed horse blood. Cool medium to 42-45°C.
- Aseptically add 100 mL 50% lysed horse blood and 1 mL β-NAD stock solution per litre medium and mix well.

- Dispense MH-F broth in sterile containers with screw caps (EUCAST, 2026b).

5.3.6.4 Storage of MH-F broth

- Store MH-F broth at 4-8°C.
- Storage conditions and shelf life should be determined as part of the laboratory quality assurance programme. A shelf life of 3 months can be expected

5.3.6.5 Sterility check:

- Check the sterility of the media by incubating for 24-48hrs at 37°C in ambient air

5.3.6.6 Quality control

- Check that the pH is within the range 7.2-7.4.
- Check that the medium supports good growth of control strain(s) of the intended test organisms.
- Check that MICs are within control limits for all bacteria-antimicrobial agent combinations used (EUCAST QC tables).

5.3.7 Quality control of Media:

The media should contain sufficient concentrations of cations to provide adequate growth and to permit the user to determine zone diameters for quality control strains.

MHA should have following cation concentrations (ISO, 2016; CLSI, 2024a),

- ✓ Calcium ≤ 25mg/L (20-25mg/L)
- ✓ Magnesium ≤ 12.5mg/L (10-12.5mg/L)
- ✓ Manganese ≤ 8mg/L
- ✓ Zinc ≤ 3mg/L (2-3 mg/L)
- ✓ Thymidine ≤ 0.03mg/L

1. Calcium and magnesium can be validated by testing (DD/MIC) aminoglycosides + quinolone to avoid false resistance with these drugs.
2. Manganese concentration to avoid false resistance can be validated by testing glycylcy-clines by performing (DD/MIC) with tigecycline using WDCM 00013 E. coli/ E. coli ATCC 25922.
3. Zinc to avoid false resistance with imipenem or other carbapenem can be validated by performing *Pseudomonas aeruginosa* WDCM 00025/ *Pseudomonas aeruginosa* ATCC 27853 with imipenem.
4. Thymidine content can be determined by using *Enterococcus faecalis* WDCM 00087/ *Enterococcus faecalis* ATCC 29212 against SXT

5. All abovementioned cation can be determined by inductively coupled plasma mass spectrometry (ICP-MS) or flame atomic absorption spectroscopy (FAAS)
6. When testing daptomycin MHB+50mg/L of Calcium to be added
7. 2% w/V NaCl is for oxacillin MIC testing (broth/agar dilution) of staphylococci; the oxacillin screen agar uses 4% NaCl + 6 µg/mL oxacillin (Table 5.18 notes); cefoxitin disk diffusion needs no added NaCl.
8. For Tigecycline testing medium should be fresh on the day or use not more than 12 hours old prepared media.

5.4 Reference control strain

Purpose and Responsibilities

Quality control (QC) in antimicrobial susceptibility testing (AST) monitors the precision, accuracy, reagent performance, and personnel competence of a test system, mainly by testing reference strains with known susceptibility. Quality assurance (QA) is broader, covering monitoring, corrective action, recordkeeping, equipment calibration, proficiency testing, and training. Responsibility is shared: manufacturers ensure the product is made correctly (stability, labelling, potency, traceability), while laboratories ensure proper storage, personnel proficiency, and adherence to current CLSI standards or manufacturer instructions.

Selection and Maintenance of QC Strains

QC strains must come from a recognized source (e.g., ATCC®), with expected results established per CLSI M23. Ideal strains have MICs near the middle of the tested range, and procedures must be modified when three or fewer adjacent dilutions are tested. Supplemental QC strains (e.g., *H. influenzae* ATCC® 10211) assess specific test characteristics and are used for new tests or training, not routine QC. Stocks are stored at -70°C to - 80°C in a suitable stabilizer, sub-cultured twice before use, and refreshed with new working cultures daily, new subcultures weekly, and new primary subcultures at least monthly.

Batch or Lot Quality Control

Each new lot of tubes, trays, or plates must be tested with appropriate QC strains and rejected if MICs fall outside expected ranges. At least one uninoculated unit per lot is incubated to verify sterility. Cation content of Mueller-Hinton broth should be checked (e.g., gentamicin with *P. aeruginosa* ATCC® 27853), with calcium at 50 mg/L for daptomycin, and lot numbers of all materials must be recorded.

Frequency of QC Testing

QC is performed daily, or weekly once satisfactory performance is documented. Daily testing is satisfactory when no more than 3 of 30 consecutive results are out of range. To

convert to weekly testing, no more than 1 of 20 or 3 of 30 results may be out of range. Weekly QC is also required whenever a reagent component changes and does not apply to tests run less than weekly. Screening tests (e.g., vancomycin and high-level aminoglycoside plates) may be tested weekly if used routinely, but labile or infrequently used tests (e.g., oxacillin agar screen) need daily QC.

Corrective Action

When an out-of-control result has an identifiable cause (QC strain, supplies, process, or equipment), the error is corrected, documented, and the strain retested the same day. If no cause is found, the combination is tested and monitored for five consecutive days, and daily QC continues until resolved. If problems persist, a system error is likely, requiring new strains, new lots of materials, contact with manufacturers, or exchange of materials with another laboratory. Returning to weekly QC requires five satisfactory days if a problem was corrected, or 20 or 30 consecutive satisfactory days if no cause was identified.

Reporting Patient Results and Verification

When QC fails, reporting decisions are made case by case, considering the size and direction of the error, proximity to the breakpoint, and results with other organisms or drugs. Options include suppressing results, retrospective review, or using an alternative method or reference laboratory. Acceptable QC does not guarantee accurate patient results, so all results should be checked for consistency with the organism's identification and expected drug-class activity hierarchies. Unusual results should be investigated and repeated if needed, under a laboratory-specific verification policy.

Table 5.1a: Reference control strains

Escherichia coli	ATCC	25922
Escherichia coli	ATCC	35218
Escherichia coli	NCTC	13353
Klebsiella pneumoniae	ATCC	700603
Pseudomonas aeruginosa	ATCC	27853
Enterococcus faecalis	ATCC	29212
Staphylococcus aureus	ATCC	25923
Staphylococcus aureus	ATCC	29213
Neisseria gonorrhoeae	ATCC	49226
Streptococcus pneumoniae	ATCC	49619
H.influenzae	ATCC	49247

Table 5.1b: Performance quality control of AST media using reference control strains

Media	QC organism	Observation	Interpretation
MHA	*S.aureus* ATCC 25923	Good growth.	Satisfactory

	E.faecalis ATCC 29212 with SXT	Observation of ≥20 mm zone diameter.	Satisfactory
MHA with 5% sheep blood for *Streptococcus sp*.	*Streptococcus pneumoniae* ATCC 49619	Good growth with alpha haemolytic pitting colonies	Satisfactory
GC agar with 1% supplements for *Neisseria sp*.	*Neisseria gonorrhoeae* ATCC 49226	Good growth with translucent colonies	Satisfactory
Mueller-Hinton broth	*S.aureus* ATCC 25923 *E.coli* ATCC 25922	Good growth with uniform turbidity	Satisfactory
MH-F broth (supplemented with 5% mechanically defibrinated horse blood and 20 mg/L β-NAD)	*Streptococcus pneumoniae* ATCC 49619 *H.influenzae* ATCC 49247	Good growth with uniform turbidity	Satisfactory

Other Control Procedures

Four additional controls support test validity:

- **Growth control:** a drug-free medium confirming organism viability and serving as a turbidity reference.
- **Purity control:** a streak of each inoculum to detect mixed cultures.
- **Inoculum control:** plate counts at least quarterly to confirm that the standardization procedure remains in control.
- **End-point interpretation control:** periodic comparison of readers so all agree within ±1 twofold dilution.

5.5 Antimicrobial susceptibility testing methods

AST methods are of three types, which differ in the result they give and in when each is best used.

- Diffusion method
- Dilution method
- Diffusion+ Dilution

5.6 Diffusion method

5.6.1 Disk diffusion method

5.6.1.1 Selection of the isolate

- Select isolated colonies of each organism type that may play a pathogenic role from the primary agar plate. Only pure cultures are tested.

- Do not test mixtures of different organism types on the same plate. For mixed cultures, obtain isolated colonies by overnight subculture with proper streaking before testing.
- Where testing is carried out directly on clinical material (urine, normally sterile body fluids) in a clinical emergency, and the direct Gram stain shows a single pathogen, the report is issued as preliminary and susceptibility must be repeated by the standard method.

5.6.1.2 Inoculum Preparation:

Turbidity standard for inoculum preparation:

Antimicrobial susceptibility testing inoculum is prepared using BaSO4 turbidity as standard equivalent to a 0.5 McFarland. Alternatively photometric (Nephelometer) device can be used (CLSI, 2024a).

Direct Colony Suspension Method:

- Direct colony suspension is the preferable method for fastidious organisms such as *Streptococci*, *Haemophilus* spp.; *N. gonorrhoeae*, *N. meningitidis*, and also for testing Staphylococci for potential methicillin or Oxacillin resistance (CLSI,2024).
- Prepare the inoculum by making a direct broth or saline suspension of isolated colonies selected from an 18- to 24-hour agar plate (use a non-selective medium, such as blood agar).
- Adjust the suspension to achieve a turbidity equivalent to a 0.5 McFarland standard. This results in a suspension containing approximately 1-2 × 10^8 colony-forming units (CFU)/mL for Escherichia coli ATCC25922.
- For visual turbidity adjustment, use adequate light to compare the inoculum tube and 0.5 McFarland standards against a card with a white background and contrasting black lines.
- *Streptococcus pneumoniae* colonies from a blood agar plates for inoculum preparation 0.5 McFarland is the standard equivalent of density. When S. pneumoniae is suspended from colonies on a chocolate agar plate, the inoculum must be equivalent to a McFarland 1.0 standard in order to contain a sufficient number of viable cells.
- Alternatively a well calibrated photometric (nephlometer) device can be used for the accurate inoculum preparation.

Growth Method

- The growth method is preferable when colony growth is difficult to suspend directly and a smooth suspension cannot be made (CLSI, 2024).
- It can also be used for non-fastidious organisms (except staphylococci) when fresh (24-hour) colonies, as required for the direct colony suspension method, are not available.
- Using loop or sterile swab three to five well-isolated colonies of the same morphological type has to be taken and mix it in a tube containing 4-5 ml of enriched medium like nutrient broth or tryptic soy broth.

- Incubate the broth culture at 35 ± 2°C for 2 to 6 hours. Adjust the turbidity of the actively growing broth culture with sterile saline or broth to achieve a turbidity equivalent to that of a 0.5 McFarland standard.
- This results in a suspension containing approximately 1-2 × 108 CFU/mL for E. coli ATCC25922.
- Use adequate light to compare the inoculum tube and 0.5 McFarland standards against a card with a white background and contrasting black lines. Alternatively a well calibrated photometric (nephlometer) device can be used for the accuracy.

5.6.1.3 Antibiotic solution preparation:

CLSI recommended concentration of antimicrobial agent added to 6mm filter paper disks (Whatman filter paper no.1) (CLSI, 2026).

Antibiotic pure substance from manufacturer's with ≥95% to ≥98% purity

Purity of the substance will varies based on the manufacturers; always check the Certificate of Analysis (COA) of substrate for potency, solvents and storage conditions

Required antibiotic stock concentration=Needed concentration of antibiotic on disk (in ug)/ Final volume to be added on disk (in µL)

For example,

As per CLSI, the required meropenem disk content is 10µg, the standard volume of antibiotic solution to be placed on the disk is 5µl

Step 1: Convert microgram (µg) to milligram (mg), 10µg/1000 = 0.010 mg Step 2: Convert microliter (µL) to milliliter (mL), 5µl/1000 = 0.005 mL. Converting concentration to same units will reduce the uncertainty of antibiotic addition.

Required stock antibiotic concentration = 0.010 mg/0.005ml

Therefore, required antibiotic stock concentration = 2 mg/mL

Table 5.2: Solvents and diluents for preparation of stock solutions of antimicrobial agents (CLSI M100 Ed36)

Antimicrobial Agents[a]	**Solvent**	**Diluent**
Amoxicillin, clavulanic acid	Phosphate buffer, pH 6.0, 0.1mol/L	Phosphate buffer, pH 6.0, 0.1mol/L
Ampicillin	Phosphate buffer, pH 8.0, 0.1mol/L	Phosphate buffer, pH 6.0, 0.1mol/L
Azithromycin	95% ethanol or glacial acetic acid[a,c]	Broth media
Aztreonam	Saturated solution sodium bicarbonate	Water
Amikacin	Water	Water

Avibactam	Water	Water
Cefepime	Phosphate buffer, pH 6.0, 0.1mol/L	Phosphate buffer, pH 6.0, 0.1mol/L
Cefpodoxime	(0.10 %; 11.9 mmol/L) aqueous sodium bicarbonate	Water
Cefazolin	Phosphate buffer, pH 6.0, 0.1mol/L	Phosphate buffer, pH 6.0, 0.1mol/L
Cefotaxime	Water	Water
Ceftriaxone	Water	Water
Cefoperazone	Water	Water
Colistin	Water	Water
Cefoxitin	Water	Water
Ceftazidime	Sodium carbonate	Water
Chloramphenicol	95 % ethanol	Water
Ciprofloxacin	Water	Water
Clindamycin	Water	Water
Clarithromycin	Methanol[b] or glacial acetic acid[a,b]	Phosphate buffer, pH 6.5, 0.1mol/L
Daptomycin	Water	Water
Enmetazobactam	Water	Water
Erythromycin	95% ethanol or glacial acetic acid[a,b]	Water
Ertapenem	Phosphate buffer, pH 7.2, 0.01mol/L	Phosphate buffer, pH 7.2, 0.01mol/L
Fosfomycin	Water	Water
Gentamicin	Water	Water
Imipenem	Phosphate buffer, pH 7.2, 0.01mol/L	Phosphate buffer, pH 7.2, 0.01mol/L
Linezolid	Water	Water
Levonadifloxacin	27.5µg/ml or 27.5 mg/L solution of L-arginine in water	Water
Minocycline	Water	Water
Meropenem	Water	Water
Nalidixic acid	½ volume of water, then add NaOH 1 mol/L, dropwise to dissolve	-
Norfloxacin, Ofloxacin and levofloxacin	½ volume of water, then 0.1 mol/L NaOH dropwise to dissolve	Water
Nitrofurantoin[c]	Phosphate buffer,	Phosphate buffer,

	pH 8.0, 0.1mol/L	pH 8.0, 0.1mol/L
Oxacillin	Water	Water
Piperacillin	Water	Water
Penicillin	Water	Water
Plazomicin	Water	Water
Polymixin B	Water	Water
Rifampin	Methanol (maximum concentration = 640 µg/mL)	Water (with stirring)
Sulbactam	Water	Water
Sulfonamides	½ volume hot water & minimal amount of 2.5 mol/L NaOH to dissolve	Water
Tazobactam	Water	Water
Tigecycline	Water	Water
Ticarcillin	Phosphate buffer, pH 6.0, 0.1mol/L	Phosphate buffer, pH 6.0, 0.1mol/L
Trimethoprim	0.05 mol/L lactic or hydrochloric acid, 10 % of final volume	Water (may require heat)
Tetracycline	Water	Water
Teicoplanin	Water	Water
Tobramycin	Water	Water
Vancomycin	Water	Water

Notes:

a. Consult the safety data sheets before working with any antimicrobial reference standard powder, solvent, or diluent. Some of the compounds (eg, solvents such as DMSO, methanol) are more toxic than others and may necessitate handling in a chemical fume hood.

b. For glacial acetic acid, use 1/2 volume of water, then add glacial acetic acid dropwise until dissolved, not to exceed 2.5 µl/ml.

c. Alternatively, Nitrofurantoin is dissolved in DMSO

5.6.1.4 Inoculating AST plates:

MHA plate should be inoculated within 15 minutes after the inoculum has been adjusted. A sterile cotton swab is dipped into the suspension, rotated several times, and gently pressed onto the inside wall of the tube above the fluid level to remove excess inoculum from the swab. The swab should be then streaked over the entire surface to the agar plate three times, with the plate rotated approximately 60^{o} each time to ensure even distribution of the inoculum. A final sweep of the swab should be made around the agar rim. The lid may be left ajar for 3 to 5 minutes but no longer than 15 minutes to allow any excess surface moisture to be absorbed before the drug-impregnated disks are applied (CLSI, 2024a).

5.6.1.5 Application of antimicrobial disks on plate:

Ideally, this should be done within 15 minutes of inoculation of plates. The selected antimicrobial disks will be dispensed evenly onto the agar plate with the help of a forceps/sterile needle/surgical blade. Flame the tips of the applicator intermittently. Each disk must be pressed down to ensure complete contact with the agar surface (CLSI, 2024a).

- Ordinarily no more than 12 disks are applied on a 150 mm plate or 5 disks on a 100 mm plate, keeping at least a distance of 24 mm (center to center) between disks. Dispensing too near to the edge of the plate should be avoided. Because some of the drugs diffuse instantaneously, a disk should not be relocated once it has come in contact with the agar surface.
- It is advisable to place disks that give predictably small zones like aminoglycosides, next to those disks that give larger zones like cephalosporins.
- Disk containers should be removed from the refrigerator or freezer one to two hours before use, so they may equilibrate to room temperature before opening. This procedure minimizes the amount of condensation that occurs when warm air contacts cold disks.
- Note: Sealed packages of the disks that contain drugs from the β-lactam class should be stored frozen, except for a small working supply, which may be refrigerated for one week at most. Some labile agents (e.g. imipenem, cefaclor, and clavulanic acid combinations) may retain greater stability if stored frozen until the day of use.
- Only those disks that have not reached the manufacturer's expiration date stated on the label will be used. Unused disks should be discarded on the expiration date.

5.6.1.5.1 Incubation:

No longer than 15 minutes after disks are applied, the plates should be inverted and incubated at 35º± 2ºC for 16-18 h in ambient air for routine disk diffusion; 20-24 h in 5% CO2 for the fastidious organisms; and the 24h read for staphylococcal oxacillin/vancomycin and enterococcal vancomycin (CLSI, 2024a).

5.6.1.5.2 Interpretation and reporting results:

- Each plate should be examined after overnight incubation (16-18 hour), for confluent growth and circular zones of inhibition. The diameters of the zones of complete inhibition, including the diameter of the disk, should be measured to the nearest whole millimeter with calipers or a ruler. With unsupplemented MHA, the measuring device should be held on the back of the inverted petri dish, which is illuminated with reflected light located a few inches above a black, nonreflecting background (CLSI, 2024a, 2026).
- Zone margin should be considered the area showing no obvious visible growth detectable with the unaided eye. Faint growth of tiny colonies visible only by lens should be ignored.
- Zone sizes should be measured from the upper inoculated surface of opaque media like MHA with added blood, illuminated with reflected light, with the cover removed. In

case of presence of discrete colonies within clear zone of inhibition, repeat test with a subculture of a single colony/pure culture from the primary culture plate. If discrete colonies still appear, inner colony free zone size should be measured. For Proteus spp., swarming should be ignored.

- With trimethoprim, the sulfonamides, and combinations of the two agents, antagonists in the medium may allow some minimal growth; therefore, the zone diameter should be measured at the obvious margin, and slight growth (20% or less of the lawn of growth) should be disregarded.

5.6.1.6 Storage of antimicrobial disks (CLSI, 2024a)

Cartridges containing commercially prepared paper disks specifically for susceptibility testing are generally packaged to ensure appropriate anhydrous conditions. Disks should be stored at 8°C or below (working supply), or frozen at -14°C or below, in a non-frost-free freezer until needed. Sealed packages of disks that contain drugs from the ß-lactam class should be stored frozen (-20ºC), except for a small working supply, which may be refrigerated for at most 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 (CLSI, 2024a).

Antimicrobial disks are supplied in cartridges packaged to maintain anhydrous conditions, and must be stored at −20 °C ± 8 °C and never in a self-defrosting freezer. Sealed packages containing disks of the β-lactam class should be kept frozen, except for a small working supply, which may be refrigerated for not more than one week. Some labile agents, such as imipenem, cefaclor and clavulanate combinations, retain greater stability if kept frozen until the day of use. Only disks that have not reached the manufacturer's expiration date stated on the label are used, and disks are discarded on that date.

5.7 Dilution method

Disc diffusion is the routine method, but it has limits where the result is ambiguous or where the infection is severe and prolonged, such as endocarditis. MIC testing is indicated to:

- establish the level of resistance of a strain rather than only its category;
- guide therapy where the S/I/R category alone gives insufficient clinical information;
- test agents for which disc diffusion is not a valid method, in particular colistin and polymyxin B;
- confirm results that fall in the intermediate range or that are unusual for the organism;
- monitor MIC creep in surveillance (vancomycin, teicoplanin and linezolid in MRSA; linezolid in enterococci; ciprofloxacin, ceftriaxone and azithromycin in *S.* Typhi).

Dilution methods are of three kinds: broth macrodilution, broth microdilution and agar dilution. Gradient diffusion combines diffusion and dilution.

5.7.1. Macro broth dilution

5.7.1.1 Aim:

The macro broth dilution method is a standardized procedure used to determine the Minimum Inhibitory Concentration (MIC) of antimicrobial agents against bacterial isolates. It is particularly suited for testing a small number of isolates or even a single iso-late. This method also allows for subsequent minimum bactericidal concentration (MBC) testing using the same tubes (CLSI, 2024b).

- For fastidious organisms such as *Streptococcus pneumoniae*, use Mueller-Hinton Broth (MHB) supplemented with 2.5-5% lysed horse blood.
- For *Haemophilus influenzae*, use Haemophilus Test Medium (HTM).

5.7.1.2 Requirements:

- Sterile capped 75 x 12 mm tubes or small screw-capped bottles
- Sterile Pasteur pipettes
- Overnight culture of test and control organisms
- Required antibiotic powder (with certified potency; clinical preparations not allowed)
- Required solvent for antibiotic dissolution
- Sterile distilled water (500 ml)
- Cation-adjusted Mueller-Hinton Broth (CAMHB)
- Test tube rack (holds 22 tubes: 11 in each row)

5.7.1.3 Preparation of Antimicrobial Stock Solutions:

Use one of the following formulas:

Weight (mg) = Volume (ml) • Concentration (µg/ml) / Potency (µg/mg)

Or

Volume (mL) = Weight (mg) • Potency (µg/mg) / Concentration (µg/ml)

- Prepare stock solution at ≥ 1000 µg/ml or 10× of the highest final test concentration.
- If using non-aqueous solvents, minimize solvent volume, and complete dilution with water or appropriate diluent.
- Aliquot and store stock solutions at ≤-60°C. Can be stored at ≤ -20 °C only for some agents/short periods
- Use thawed aliquots on the same day and discard after use.

5.7.1.4 Procedure:

1. Prepare serial two-fold dilutions of the antimicrobial agent in broth.

2. Prepare 1 ml of each dilution per test (minimum)
3. Prepare a 0.5 McFarland suspension using either:
    a. Growth method, or
    b. Direct colony suspension method.
4. Dilute the suspension in broth to achieve a density of ~1 × 10^6 CFU/ml.
5. Within 15 minutes of inoculum preparation, add 1 ml of the diluted inoculum to each tube containing 1 ml of antibiotic dilution.
    a. Final inoculum concentration: ~5 × 10^5 CFU/ml.
6. Include control tubes:
    a. Growth Control: Inoculated tube with no antibiotic.
    b. Sterility Control: Broth only, no inoculum or antibiotic.
7. Incubate tubes at 35 ± 2°C for 16 to 20 hours in ambient air.

5.7.1.5 Inoculum Verification and Purity Check:

Subculture the inoculum on non-selective media to check the purity. Verify inoc-ulum:

- Take 0.01 ml from the growth control and dilute into 10 ml sterile saline.
- Plate 0.1 ml onto non-selective agar.
- Incubate overnight and count colonies.
- ~50 colonies indicate correct inoculum (~5 × 10^5 CFU/ml).

5.7.1.6 Interpretation:

1. Check purity of inoculum
2. Check sterility control tube. It should be clear/non-turbid.
3. Check growth control tube for adequate turbidity.
4. MIC is expressed as the lowest dilution, which inhibited growth judged by lack of turbidity in the tube
5. Standard strain of known MIC/ATCC strain should be run along with the test strain.
6. It is used to check the reagents and conditions and techniques.
7. For trimethoprim and sulfonamides, read the end point at the concentration in which there is ≥ 80% reduction in growth as compared to the control.

5.7.2 Broth microdilution (reference method for colistin and polymyxin B)

5.7.2.1 Principle and scope

Broth microdilution (BMD) tests a standardized bacterial suspension against two-fold dilutions of the agent in a liquid medium in a 96-well tray. It is the only method recommended by the joint CLSI-EUCAST Polymyxin Breakpoints Working Group for colistin and polymyxin B. Disc diffusion, agar dilution and gradient diffusion are not acceptable for these agents. BMD is performed with cation-adjusted Mueller-Hinton broth

(CAMHB), without added polysorbate-80, in untreated polystyrene trays (ISO 20776-1), with a final inoculum of 5×10^5 CFU/mL per well (EUCAST, 2016; ISO, 2019).

For Enterobacterales and *Pseudomonas aeruginosa*, colistin broth disc elution (CBDE) and the colistin agar test (CAT) are acceptable alternatives were stated in CLSI M100. They are not acceptable for *Acinetobacter baumannii* complex (CLSI, 2026).

5.7.2.2 Materials

- Untreated 96-well U-bottom polystyrene microwell plates (not tissue-culture treated)
- Colistin sulfate or polymyxin B sulfate reference powder with a stated potency on the certificate of analysis
- Cation-adjusted Mueller-Hinton broth
- Sterile water, normal saline, low-retention tips, multichannel pipettes, reagent reservoir
- Densitometer or 0.5 McFarland standard; class II biosafety cabinet; incubator at 35 ± 2 °C

5.7.2.3 Antibiotic stock

Correct for potency. The CLSI reference potency for colistin sulfate is 30,000 U/mg; commercial powder is commonly ≥ 19,000 U/mg, so 1.57 mg of such powder in 1 mL of distilled water gives a 1 mg/mL stock. Alternatively calculate the volume of a prepared stock using $V_1 = N_2 \times V_2 \div N_1$; from a 4 mg/mL stock, 320 µL in 10 mL of medium gives 128 µg/mL (CLSI, 2026).

Solutions prepared aseptically but not filter-sterilized are acceptable; they may be membrane-filtered if preferred. Dispense in small volumes into sterile vials, seal, and store preferably at −60 °C or below, never warmer than −20 °C and never in a self-defrosting freezer. Thaw as needed and use the same day.

5.7.2.4 Inoculum

Prepare a 0.5 McFarland suspension (1.5×10^8 CFU/mL) in 1 mL sterile saline from a 24-hour non-selective plate. Dilute so that each well contains approximately 5×10^5 CFU/mL after inoculation (acceptable range $2–8 \times 10^5$). Where 50 µL of inoculum is added to a 100 µL well, a 150-fold dilution of the 0.5 McFarland suspension (25 µL into 3725 µL CAMHB) gives the required density. Inoculate within 15 minutes of preparing the suspension.

5.7.2.5 Plate layout

- Distribute 100 µL of CAMHB containing colistin at 128 µg/mL into each well of column 1, and 50 µL of CAMHB into each well of columns 2 to 12.
- Serially transfer and mix 50 µL from column 1 through to column 11, discarding 50 µL from column 11. This gives a colistin range of 64 to 0.12 µg/mL after inoculation.
- Add 50 µL of inoculum to columns 1 to 10 and to column 12.
- Column 11 is the sterility (drug) control and column 12 the growth control.

- Rows A to H carry the test isolates and the QC strains, one per row, so that every isolate has its own growth control in column 12. The same convention must be applied to the plate maps in 5.9.2.c and 5.9.4.c, which at present define only a single growth-control well (A12).

5.7.2.6 Incubation

Incubate at 35 ± 2 °C for 16 to 20 hours in ambient air, within 15 minutes of adding the inoculum. Do not stack trays more than four high.

5.7.2.7 Purity check and count verification

- Streak 50 µL of the final inoculum onto nutrient agar for a purity check.
- Transfer 10 µL from the growth-control well into 10 mL saline, plate 100 µL onto nutrient agar and streak; 30 to 50 colonies indicate an adequate inoculum.
- Incubate purity and count plates with the sealed tray at 35 ± 2 °C for 16 to 18 hours.

5.7.2.8 Reading and interpretation

- Confirm the purity of the inoculum.
- The sterility control must show no growth.
- The growth control must show acceptable growth (a button ≥ 2 mm or definite turbidity); otherwise, the test is invalid.
- The QC strains must fall within the ranges below; otherwise, no test result is reported.
- The MIC is the lowest concentration completely inhibiting growth as seen by the unaided eye. Reading aids may be used provided they do not compromise the ability to discern growth.
- Where a single well is skipped, read the highest MIC. Where more than one well is skipped, do not report the result.

Interpretive criteria (CLSI, current edition): colistin and polymyxin B, S ≤ 2 µg/mL and R ≥ 4 µg/mL for Enterobacterales, *Pseudomonas aeruginosa* and *Acinetobacter* spp. There is no susceptible category for polymyxins in some editions; confirm against the edition named in 5.0.

5.7.2.9 Quality control strains and ranges (CLSI, 2026; EUCAST, 2016)

- *Escherichia coli* ATCC 25922 - colistin MIC 0.25–2 µg/mL
- *Pseudomonas aeruginosa* ATCC 27853 - colistin MIC 0.5–2 µg/mL
- MCR-1 positive *Escherichia coli* (resistant control) - colistin MIC 4–8 µg/mL

5.7.2.10 Organisms intrinsically resistant to colistin (CLSI, 2026)

Colistin must not be tested or reported for:

- *Morganella morganii*
- *Proteus* spp. (*mirabilis*, *penneri*, *vulgaris*)
- *Providencia* spp. (*rettgeri*, *stuartii*)
- *Serratia marcescens*
- *Burkholderia cepacia* complex

5.7.2.11 Safety

- Open plates of drug-resistant bacteria only inside a biosafety cabinet.
- Staff performing the test wear an impermeable laboratory coat and gloves throughout.
- Dispose of waste as per the local pollution control board guideline.

5.7.3 Agar dilution method

5.7.3.1 Aim

Agar dilution method for determining antimicrobial susceptibility is a well-established technique. The antimicrobial agent is incorporated into the agar medium, with each plate containing a different concentration of the agent. The inoculum can be applied rapidly and simultaneously to the agar surfaces using an inoculum replicating apparatus capable of transferring to 32-36 inocula to each plate (CLSI, 2024b).

Agar dilutions are most often prepared in Petri-dishes and have advantage that it is possible to test several organisms on each plate. The dilutions are made in a small volume of water and added to agar which has been melted and cooled to not more than 45-50°C. Add blood to agar cooled to 45-50 °C; for chocolate agar, heat the blood agar first, then cool before adding the antibiotic. It would be convenient to use 90 mm diameter petri dishes and add 2 ml of desired drug dilutions to 18 ml of melted agar.

5.7.3.2 Calculation:

Example: To prepare agar containing a maximum antibiotic concentration of 64 µg/mL in a final volume of 20 mL:

- Final volume of agar medium = 20 mL (19 mL agar + 1 mL antibiotic solution).
- Total antibiotic required = 64 × 20 = 1280 µg.
- Therefore, prepare an antibiotic solution of 1280 µg/mL.
- For preparing 2 mL of the starting solution, 2560 µg of antibiotic is required.
- Perform serial two-fold dilutions of the antibiotic solution.
- Add 1 mL of each dilution to 19 mL of molten agar to obtain the required final antibiotic concentrations.

5.7.3.3 Requirements

1. 90 mm diameter petridishes
2. Antimicrobial pure substance
3. Required solvent for the antibiotic
4. Sterile graduated pipettes – 10 ml, 5 ml, 2 ml and 1 ml.
5. Sterile capped 75 x 12 mm tubes / small screw-capped bottles
6. Sterile Pasteur pipettes
7. Sterile Distilled Water - 500 ml

8. Pre-sterilized melted Muller Hinton agar
9. Supplements like blood for testing fastidious organism

5.7.3.4 Procedure

1. Prepare antimicrobial stock solutions using sterile distilled water or an appropriate solvent, as recommended for antimicrobial agent. and serial two-fold dilution of the antimicrobial range. Prepare serial two-fold dilutions using a suitable diluent. The antimicrobial solutions should be 10 times the intended final concentration in agar (e.g., for a final concentration of 4 µg/mL, prepare a 40 µg/mL solution. Label petridishes with the antimicrobial concentration to be poured
2. Add 2ml of the antimicrobial solution to18 ml of melted MHA at 45-50 °C and mix thoroughly
3. Pour the mixture onto the petridish with appropriate label and allow to set
4. Include one plate without antimicrobial agents to serve as growth control
5. Dry the plates at 37ºC with their lids tipped for 20 to 30 minutes in an incuba-tor
6. Inoculum preparation:
7. Prepare a 0.5 McFarland matched suspension of the organism either by growth method or direct colony suspension
8. Dilute 1:10 in either sterile broth or saline
9. Inoculate either with a multiple inoculator as spots or with a wire loop or a platinum loop calibrated to deliver 0.001ml and spread over a 5mm to 8 mm spot on the MHA containing antimicrobial agents
10. Allow the plates to stand at room temperature until moisture in inoculum spots has been absorbed in the agar
11. Incubate at 35± 2°C for 16 to 20 hours in ambient atmosphere. Incubate in CO_2(5%) for *N. gonorrhoea, N. meningitidis* and *Streptococcus* sp.

5.7.3.5 Reading and Interpretation:

Place the plates on a dark, non-reflecting surface and record MIC as the lowest concentration of antimicrobial agent that completely inhibits growth

Disregard a single colony or faint haze caused by inoculum

With trimethoprim and sulfonamides read the end point at the concentration showing 80% or more reduction in growth compared to the control.

If two or more colonies persist in concentrations beyond an obvious end point, or there is no growth at lower concentration and growth at higher concentration, check purity and repeat if required.

5.7.4 Minimum bactericidal concentration (MBC)

The MBC is the lowest concentration giving a predetermined reduction, usually 99.9%, in CFU/mL relative to the starting inoculum. The MIC dilution and at least two more concentrated dilutions are subcultured and enumerated (Collee et al., 2003; CLSI, 2024b).

- Prepare dilutions and inoculations as for MIC determination by macrobroth dilution (5.7.1).
- Before incubation, subculture the antibiotic-free control tube by spreading a loopful evenly over a quarter of a suitable agar plate and incubate overnight at 35 ± 2 °C. This represents the original inoculum.
- Incubate the tubes overnight at 35 ± 2 °C and read the MIC of the control organism to confirm that the drug concentrations are correct.
- Record the lowest concentration inhibiting growth as the MIC.
- Subculture all tubes showing no visible growth in the same way and incubate overnight.
- Compare the growth obtained with that from the pre-incubation control subculture.
- Include a second set of the same dilutions inoculated with an organism of known susceptibility; these are not subcultured, as their only purpose is to confirm by MIC that the drug levels are correct.

Interpretation: a similar number of colonies indicates bacteriostatic activity; a reduced number indicates partial or slow bactericidal activity; no growth indicates the whole inoculum has been killed. The lowest dilution giving the predetermined reduction is the MBC.

5.8 Diffusion+ Dilution

5.8.1 E-test method

5.8.1.1 Introduction:

- The E-test (bioMérieux) which is a quantitative method for antimicrobial susceptibility testing applies both the dilution of antibiotic and diffusion of antibiotic into the medium. A predefined stable antimicrobial gradient is present on a thin inert carrier strip (CLSI, 2024b).
- E-test can be used to determine MIC for fastidious organisms like S. pneumoniae, ß-hemolytic Streptococci, N. gonorrhoeae, Haemophilus spp. and anaerobes.
- It can also be used for Non-fermenting Gram Negative bacilli (NFGNB) for e.g. Pseudomonas spp and Burkholderia pseudomallei.
- Used to detect the resistance of major consequence, for e.g. the test is very useful in detecting glycopeptide resistant Enterococci (GRE) and glycopeptide intermedi-ate S.aureus (GISA).
- When this E-test strip is applied onto an inoculated agar plate, there is an immedi-ate release of the drug. Following incubation, a symmetrical inhibition ellipse is produced.

- The intersection of the inhibitory zone edge and the calibrated carrier strip indi-cates the MIC value over a wide concentration range (>10 dilutions) with inherent precision and accuracy.

5.8.1.2 Requirements

1. E-test strips
2. Appropriate media (MHA, HTM, GC)
3. 0.5 McFarland turbidity standard
4. Cotton swabs
5. Forceps/ e-test applicator
6. Overnight culture of QC and test organisms

5.8.1.3 Procedure:

1. Remove e-test strips from freezer and allow to reach room temperature
2. 0.5 McFarland standard matched suspension of the organism is prepared by growth method or direct colony suspension
3. Streak a lawn culture of the standardized inoculum as in disk diffusion testing within 15 minutes of inoculum preparation
4. Allow excess moisture to be absorbed into the media
5. Apply strips onto agar surface with the forceps making sure the graduated surface is facing up.
6. Place only one strip for 90 mm petridish and 4 to 6 strips on 150 mm plate.
7. When using more than one strips, the E-end of the strip (highest concentration) should be towards the edge of the plate.
8. Do not change position of the strip once placed on the plate
9. Incubate at 35± 2°C for 16 to 20 hours in ambient atmosphere. Incubate in CO2 for N. gonorrhoea, N. meningitidis and Streptococcus sp.

5.8.1.4 Reading and Interpretation:

1. Read MIC as the point where the inhibition ellipse intersects the scale.
2. MIC value should be read where the ellipse intersects the strip, round up to the next two-fold dilution, and for bacteriostatic agents (e.g. linezolid, tetracyclines) read at ~80% inhibition ignoring haze. At present the 80% rule is stated only for trimethoprim/sulfonamide

5.9 Other bacterial AST and specific antimicrobial resistance tests (CLSI, 2023)

5.9.1 Determination of Cefepime/enmetazobactam (30/20µg) by Disk diffusion

Antibiotic pure substance from various manufacturers, ≥95% to ≥98% purity

Purity of the substance will varies based on the manufacturers; always check the

Certificate of Analysis (COA) of substrate for potency, solvents and storage conditions

Required antibiotic stock concentration= Needed concentration of antibiotic on disk/ Final volume to be added on disk

For a Cefepime/enmetazobactam disk containing 30/20µg, with an application volume of 5 µL

(A) Cefepime-30µg

Step 1: Convert microgram (µg) to milligram (mg), 30µg/1000 = 0.030 mg Step 2: Convert microliter (µL) to milliliter (mL), 5µl/1000 = 0.005 mL

Step 3: Converting concentration to same units will reduce the uncertainty of antibiotic addition.

As per CLSI, the required Cefepime disk content is 30 µg, the standard volume of antibiotic solution to be placed on the disk is 5µl (U.S. Food and Drug Administration [FDA], n.d.).

Concentration will be reduced while adding the two different antibiotics together, due to this reason the combinations antibiotics calculated with doubling of required concentration.

Required stock antibiotic concentration = 0.060 mg/0.005ml

Therefore, Required antibiotic stock concentration = 12 mg/mL

Note:

Cefepime to be dissolved in Phosphate buffer, pH 6, 0.1 mol/L.

(B) Enmetazobactam -20µg

As per CLSI, the required enmetazobactam disk content is 20 µg, the standard vol-ume of antibiotic solution to be placed on the disk is 5µl

Step 1: Convert microgram (µg) to milligram (mg), 20µg/1000 = 0.020 mg Step 2: Con-vert microliter (µL) to milliliter (mL), 5µl/1000 = 0.005 mL

Step 3: Converting concentration to same units will reduce the uncertainty of antibiotic addition.

- Concentration will be reduced while adding two different antibiotics together, due to this reason the combinations antibiotics calculated with doubling of required concentration.

Required stock antibiotic concentration = 0.040 mg/ 0.005ml

Therefore, Required antibiotic stock concentration = 8 mg/mL

Note:

- Enmetazobactam to be dissolved in water
- Mix equal volumes (1:1) of the cefepime stock (12 mg/mL) and enmetazobactam stock (8 mg/mL). Apply 5 µL of the resulting mixture to each sterile disk to achieve the required disk content of 30 µg cefepime and 20 µg enmetazobactam.

5.9.2 Determination of Cefepime/enmetazobactam Minimum inhibitory concentration (MIC) using the reference broth-micro dilution method

5.9.2.1 Introduction

Extended-spectrum beta-lactamases (ESBLs) and ampC-producing Enterobacterales limit treatment options, and carbapenems remain the usual choice for serious infections, but heavy carbapenem use drives resistance, so β-lactam/β-lactamase inhibitor (BL/BLI) combinations that spare carbapenems are of interest. This procedure describes determination of the MIC of cefepime/enmetazobactam by reference broth microdilution (BMD), so that isolates can be interpreted against the breakpoints in 5.9.2.d. It applies to pure, freshly isolated cultures of Enterobacterales from clinical specimens, including ESBL- and AmpC-producing isolates for which carbapenem-sparing options are being considered. It is not intended for carbapenemase-producing isolates, particularly metallo-β-lactamase producers, because enmetazobactam is not expected to restore cefepime activity against them, and no interpretive criteria are provided for other organisms, including P. aeruginosa. Cefepime is relatively stable to AmpC hydrolysis, and enmetazobactam, a tazobactam-derived β-lactamase inhibitor, protects it from ESBL activity; cefepime is therefore tested in two-fold serial dilutions with enmetazobactam at a fixed final concentration of 8 µg/mL in each well. The MIC is the lowest cefepime concentration, tested with 8 µg/mL enmetazobactam, that completely inhibits visible growth, and it is reported as cefepime/enmetazobactam (e.g. 0.5/8 µg/mL).

5.9.2.2 Antibiotic preparation

Cefepime antibiotic calculation:

V1=N2*V2/N1

N1: Antibiotic stock concentration (eg 8mg/ml).

V1: Volume of stock to be added in media.

N2: Final required concentration of antibiotic in media.

V2: Volume of media

V1= 256µg/ml x 10 ml/8mg/ml = 320µl

320µl from 8mg/ml stock to be added in 10ml media to get final concentration of 256µg/ml

Enmetazobactam antibiotic calculation:

V1= N2 x V2/ N1

N1: Antibiotic stock concentration (eg 1mg/ml).

V1: Volume of stock to be added in media.

N2: Final required concentration of antibiotic in media.

V2: Volume of media.

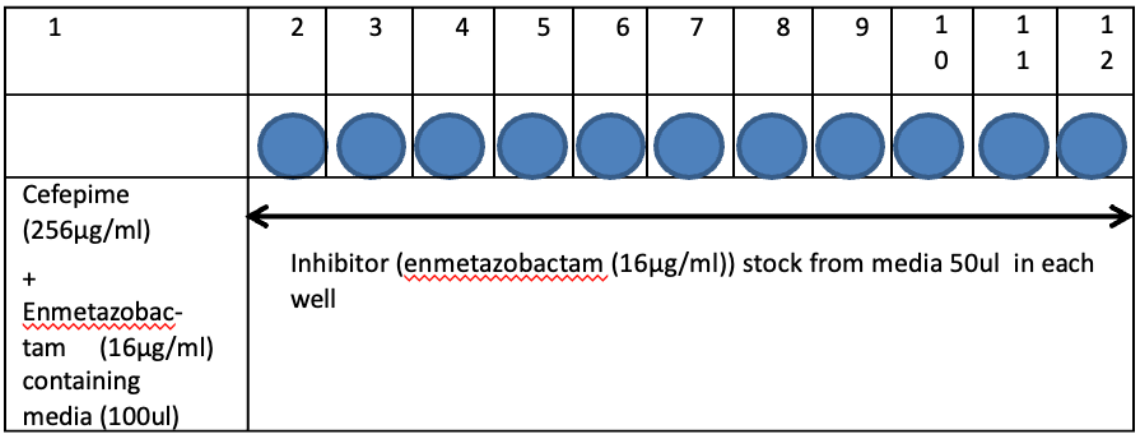
V1= 16µg/ml x 10 ml/ 1mg/ml= 160µl

*160µl of 1mg/ml main stock solution added in 10 ml of CaMHB broth (Consider as working antibiotic solution)

5.9.2.3 Antibiotic preparation on 96 well U- bottoms Plate:

For Cefepime/enmetazobactam combinations.

- Fixed 8µg/ml of inhibitor is combined with respective Cefepime antibiotic dilutions.
- For uniform distribution of fixed concentrations of an enmetazobactam, prepare separate stock of 16µg/ml in media (16µg/ml is due to the inhibitor concentraion remain 8µg/ml even after the cefepime antibiotic dilution) and add 50 µl of enmetazobactam stock into the well.
- From 16µg/ml enmetazobactam stock, add appropriate concentration of antibiotic (Cefepime- 256µg/ml).
- Serially dilute antibiotic stock from 1st well till 11th well. With this antibiotic gets serially diluted while inhibitor concentration will not change.



5.9.2.4 Cefepime/enmetazobactam

Table 5.3: QC strains recommended for Routine QC of ß- lactam combination agents as per CLSI 2025 guidelines (CLSI M100 Ed36-2026)**.**

Quality Control Strains for (Cefepime-enmetazobactam)	CLSI Reference Zone Diameter (mm)	CLSI Reference MIC range (mg/L)
E.coli **ATCC 35218**	32-38	0.008/8-0.06/8
K.pneumoniae **ATCC 700603**	26-32	0.12/8-0.5/8
E.coli **NCTC 13353**	27-33	0.03/8-0.12/8

Table 5.4: Cefepime/enmetazobactam -FDA breakpoints

Pathogen	MIC (mg/L)		DD(mm)	
	≤S	≥R	≥S	≥R
Enterobacterales	≤8/8	≥16/8	≥21	<20

5.9.2.5 Inoculum preparation:

- Inoculate 2-3 morphologically similar colonies of 24 hours pure growth from nu-trient agar plate into 1.5ml nutrient broth. Incubate at 37ºc for 2 hours and then adjust to 0.5 McFarland standards.
- 100 µl of 0.5 McFarland adjusted inoculum, is transferred to 10 ml (target final inoculum 5×10^5 CFU/mL and the acceptable colony-count range ($2\text{-}8 \times 10^5$) in the purity check of Muller-hinton broth and used as final inoculum for performance of MIC testing.

5.9.2.6 Procedure:

1. Label the wells from higher to lower concentration, Add 50µl of Cation adjusted Mueller-Hinton broth (CAMHB) containing 'enmetazobactam' each of these labeled wells from A2 to A11 & wells 2-11 of each row.
2. Add 100µl of CAMHB containing enmetazobactam(16µg/ml) along with 256µg/ml concentration of cefepime, added from the A1 to H1 respective wells of the 96-wells microtiter plate.
3. Serially dilute 50µl of the antibiotic from A1 to A11 discard 50 µl of the antibiotic from A11.
4. Add 50 µl of the final inoculum to the wells of the micro titer plate.
5. Add 100µl of CAMHB into Sterility control well.
6. Add 50µl of CAMHB and 50µl of final inoculum into growth control well which is A12 like each row's last well and H1 to H12 will be the sterility control (atleast 2 to 3 wells).

5.9.2.7 Purity check and count verification:

1. Add 50µl of final inoculum to nutrient agar plates and do tertiary streaking for purity check.
2. Add 10µl of inoculum from growth control (GC) well and transfer to 10mlof saline for count verification.
3. From the count verification tube, add 100µl onto nutrient agar plate and per-form criss cross streaking (should show at least 30 – 50 colonies).
4. Nutrient agar plates and sealed microtiter plates are incubated at 37°C for 16-18 hours.

5.9.3 Determination of Aztreonam/avibactam (30/20µg) by Disk diffusion

5.9.3.1 Antibiotic stock solution preparation for Aztreonam/avibactam

Antibiotic pure substance from various manufacturers, ≥95% to ≥98% purity

Purity of the substance will varies based on the manufacturers; always check the Certificate of Analysis (COA) of substrate for potency, solvents and storage conditions

Required antibiotic stock= Needed concentration of antibiotic on disk/ Final volume to be added on disk

For example, Aztreonam-30µg

As per CLSI, the required Aztreonam disk content is 30 µg, the standard volume of antibiotic solution to be placed on the disk is 5µl

Step 1: Convert microgram (µg) to milligram (mg), 30µg/1000 = 0.030 mg Step 2: Convert microliter (µL) to milliliter (mL), 5µl/1000 = 0.005 mL

Step 3: Converting concentration to same units will reduce the uncertainty of antibi-otic addition.

Concentration will be reduced while adding two different antibiotics together, due to this reason the combinations antibiotics calculated with doubling of required concentration

Required stock antibiotic concentration =0.060 mg/0.005ml

There, Required antibiotic stock concentration = 12 mg/mL

Note:

Aztreonam to be dissolved in saturated solution sodium bicarbonate

For example, Avibactam-20µg

As per CLSI, the required Avibactam disk content is 20 µg, the standard volume of antibiotic solution to be placed on the disk is 5µl

Step 1: Convert microgram (µg) to milligram (mg), 20µg/1000 = 0.020 mg

Step 2: Convert microliter (µL) to milliliter (mL), 5µl/1000 = 0.005 mL

Step 3: Converting concentration to same units will reduce the uncertainty of antibiotic addition.

Concentration will be reduced while adding two different antibiotics together, due to this reason the combinations antibiotics calculated with doubling of required concentration.

Required stock antibiotic concentration =0.040 mg/0.005ml

Therefore, Required antibiotic stock concentration = 8 mg/mL

Note:

- Avibactam to be dissolved in water
- Mix both Aztreonam (30µg) and Avibactam (20µg)

Note: For inoculum preparation and interpretation refer disk diffusion method procedure.

5.9.4 Determination of Aztreonam/avibactam minimum inhibitory concentration (MIC) using the reference broth-micro dilution

5.9.4.1 Introduction:

To determine the minimum inhibitory concentration (MIC) of the aztreonam/avibactam combination against aerobic Gram-negative bacteria using the reference broth micro dilution (BMD) method. Results support clinical management of infections due to

organisms producing, metallo-β-lactamases (MBLs) and co-producing oxacillinases (OXA-48 like), ESBLs/AmpC that hydrolyse aztreonam alone (Bakthavatchalam et al., 2022; CLSI, 2026).

Applies to clinical microbiology laboratories performing quantitative susceptibility testing on Enterobacterales and selected non-fermenters from clinically significant specimens (e.g., blood, sterile sites, and lower respiratory tract). Not intended for Stenotrophomonas malto-philia, Acinetobacter spp., anaerobes, or organisms without established interpretive criteria. Use only on pure, freshly isolated cultures.

5.9.4.2 Antibiotic preparation:

1. Aztreonam antibiotic calculation:

V1= N2*V2/N1

N1: Antibiotic stock concentration (eg 8mg/ml).

V1: Volume of stock to be added in media.

N2: Final required concentration of antibiotic in media.

V2: Volume of media.

V1= 256µg/ml x 10 ml/ 8mg/ml= 320µl

320µl from 8mg/ml stock to be added in 10ml media to get final concentration of 256µg/ml

2. Avibactam antibiotic calculation:

V1=N2 x V2 /N1

N1: Antibiotic stock concentration (eg 1mg/ml).

V1: Volume of stock to be added in media.

N2: Final required concentration of antibiotic in media.

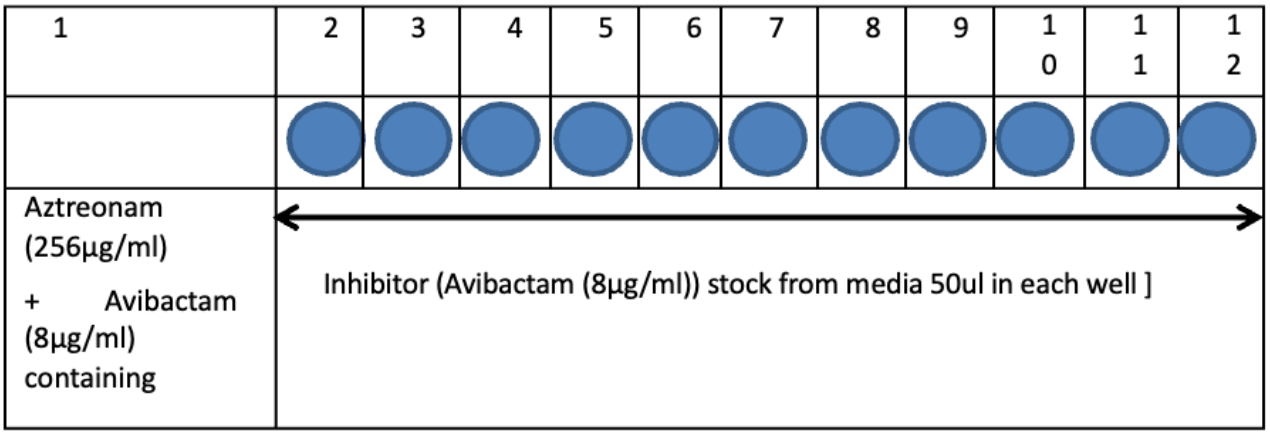
V2: Volume of media.

V1= 8µg/ml x 10 ml/1mg/ml = 80µl

*80µl of 1mg/ml main stock solution added in 10 ml of CaMHB broth (Consider as working antibiotic solution)

5.9.4.3 Antibiotic preparation on 96 well U- bottoms Plate:

- For Aztreonam and avibactam combinations.
- Fixed 4µg/ml of inhibitor is combined with respective Aztreonam antibi-otic dilutions.
- For uniform distribution of fixed concentrations of an avibactam, prepare separate stock of 8µg/ml in media and add 50 µl of avibactam stock into the well.
- From 8µg/ml Avibactam stock, add appropriate concentration of antibi-otic (Aztreonam- 256µg/ml).
- Serially dilute antibiotic stock from 1st well till 11th well. With this antibiotic gets serially diluted while inhibitor concentration will not change.



Antibiotic pure substance:

- Aztreonam
- Avibactam

Table 5.5 Quality control (QC) strains & QC Range of the antibiotic

Aztreonam/avibactam		
QC Organism	**QC Range (MM) (DD)**	**QC Range mg/L (MIC)**
E. coli ATCC 25922	32-38	0.03/4-0.12/4
P. aeruginosa ATCC 27853	24-30	2/4-8/4
E. coli ATCC 35218	31-38	0.016/4-0.06/4
K.pneumoniae ATCC 700603	26-32	0.06/4-0.5/4

5.9.4.4 Inoculum preparation

- Inoculate 2-3 morphologically similar colonies of 24 hours pure growth from nutri-ent agar plate into 1.5ml nutrient broth. Incubate at 37ºc for 2 hours and then adjust to 0.5 McFarland standards.
- 100 µl of 0.5 McFarland adjusted inoculum, is transferred to 10 ml of Muller-hinton broth and used as final inoculum for performance of MIC testing.

5.9.4.5 Procedure:

1. Label the wells from higher to lower concentration, Add 50µl of Cation adjusted Mueller-Hinton broth (CAMHB) containing Avibactam each of these labeled wells from A2 to A11.
2. Add 100µl of CAMHB containing avibactam (8µg/ml) along with 256µg/ml concentration of Aztreonam, added from the A1 to H1 respective wells of the 96-wells microtiter plate.
3. Serially dilute 50µl of the antibiotic from A1 to A11 discard 50 µl of the antibiotic from A11.
4. Add 50 µl of the final inoculum to the wells of the microtiter plate.

5. Add 100µl of CAMHB into Sterility control well.
6. Add 50µl of CAMHB and 50µl of final inoculum into growth control well which is A12

5.9.4.6 Purity Check and Count Verification:

- Add 50µl of final inoculum to nutrient agar plates and do tertiary streaking for puri-ty check.
- Add 10µl of inoculum from GC well and transfer to 10mlof saline for count verifi-cation.
- From the count verification tube, add 100µl onto nutrient agar plate and perform criss cross streaking (should show at least 30 – 50 colonies).
- Nutrient agar plates and sealed microtiter plates are incubated at 37°C for 16- 18 hours.

Table 5.6: Interpretation

Aztreonam/ avibactam	Disk content (µg)	DD Breakpoints (mm)			MIC Breakpoints (mg/L)		
		S	I	R	S	I	R
CLSI 2026	30/20	≥25	22-24	≤21	≤4/4	8/4	≥16/4

CLSI 2026 Comments:

Confirmatory MIC testing is indicated for isolates with zones of 22-24 mm to avoid overcalling susceptible results as intermediate

5.9.5 Determination of Levonadifloxacin by Disk diffusion

5.9.5.1 Disk diffusion:

Antibiotic stock solution preparation for Levonadifloxacin:

Antibiotic pure substance from various manufacturers, ≥95% to ≥98% purity

Purity of the substance will varies based on the manufacturers; always check the Certifi-cate of Analysis (COA) of substrate for potency, solvents and storage conditions

Required antibiotic stock concentration= Needed concentration of antibiotic on disk/ Final volume to be added on disk

For example,

As per CLSI, the required Levonadifloxacin disk content is 10 µg, the standard volume of antibiotic solution to be placed on the disk is 5µl

Step 1: Convert microgram (µg) to milligram (mg), 10µg/1000 = 0.010 mg

Step 2: Convert microliter (µL) to milliliter (mL), 5µl/1000 = 0.005 mL

Step 3: Converting concentration to same units will reduce the uncertainty of antibi-otic addition.

Required stock antibiotic concentration = 0.010 mg/0.005ml

Therefore, Required antibiotic stock concentration = 2 mg/mL

Note:

Levonadifloxacin antibiotic to be dissolved in L-arginine (27.5 µg/ml) solution. L-arginine solution has to be prepared in distilled water.

5.9.5.2 Methodology:

Inoculation of test Plates:

1. Optimally, within 15 minutes after adjusting the 0.5 Mcfarland of the inoculum, dip a sterile cotton swab into the adjusted suspension.
2. Rotate the swab several times and press firmly on the inside wall of the tube above the fluid level. This removes excess fluid from the swab.
3. Inoculate the dried surface of an MHA plate by streaking the swab over the entire sterile agar surface. Repeat this procedure by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, swab the rim of the agar. Alternatively, an automated plate rotator can be used for lawn preparation.
4. Leave the lid ajar for three to five minutes, but no more than 15 minutes, to allow for any excess surface moisture to be absorbed before applying the drug-impregnated disks.

NOTE: Avoid extremes in inoculum density. Never use undiluted overnight broth cul-tures or other unstandardized inoculum for streaking plates.

Application of Disk

1. Dispense the predetermined battery of antimicrobial disks onto the surface of the in-oculated agar plate. Each disk must be pressed down to ensure complete contact with the agar surface.
2. Disks must be distributed evenly so they are no closer than 24 mm from centre to centre. Ordinarily, not more than five disks on a 100-mm plate.
3. If disks are placed too close to the edge of the plate, the zones may not be fully round with some drugs.
4. For in-house prepared antimicrobial solution,Use a calibrated 2-10 µL micropipette 2 mm calibrated loop to de-liver 5µl of the solution into 6 mm disk prepared from Whatman No.1 filter pa-per, and placed on the surface of the plate and dry it for 10-15 minutes.
5. Some of the drug diffuses almost instantaneously; due to that disk should not be re-located once it has contact with the agar surface. Instead, place a new disk in another location on the agar.
6. Invert the plates and place in an incubator set to 35±2°C within 15 minutes after the disks are applied

5.9.5.3 Interpretation:

Table 5.7: Quality Control strains & Quality Control ranges – CLSI 2026

Levonadifloxacin	CLSI Reference Zone Diameter (mm)	CLSI Reference MIC range(mg/L)
***E.coli* ATCC 25922**	27 – 33	0.03 – 0.25
***P.aeruginosa* ATCC 27853**	17 – 23	0.5 – 4
S.aureus* ATCC 25923	32 - 39	NA
S.aureus* ATCC 29213	NA	0.008 - 0.03
***H.influenzae* ATCC 49247**	31 - 41	0.008 - 0.06
***S.pneumoniae* ATCC 49619**	24 - 31	0.12 - 0.25

*NA - Not available;: *S. aureus ATCC 29213 - Quality control strain only for MIC determination.m*S. aureus ATCC 25923 - Quality control strain only for disk diffusion assay.

5.9.5.4 Interpretive criteria for Levonadifloxacin (IV & oral regimens):

Table 5.8: CDSCO Approved drug breakpoints based on PK/PD and clinical efficacy in phase III trial (CTRI/2017/06/008843)

Pathogens	MIC Breakpoints (in mg/L)			Disk diffusion breakpoints (in mm)		
	S	I	R	S	I	R
***Staphylococcus aureus* (MRSA)**	≤2	4	≥8	≥17	14-16	≤13
Streptococcus pyogenes*	≤1	2	≥4	≥20	-	≤19
Enterococcus faecalis*	≤8	-	≥16	≥10	-	≤9
Streptococcus dysgalactiae* ssp. *dysgalactiae*	≤0.25	-	≥0.5	≥20	-	≤19
Streptococcus agalactiae*	≤0.5	-	≥1	≥20	-	≤19

*Susceptibility breakpoints for non-Staphylococcus pathogens are based on Levonadifloxacin's clinical efficacy in phase 3 study patients infected with these organisms

5.9.6 Determination of Nafithromycin by Disk diffusion

5.9.6.1 Disk diffusion:

Antibiotic stock solution preparation for Nafithromycin:

Antibiotic pure substance from various manufacturers, ≥95% to ≥98% purity

Purity of the substance will varies based on the manufacturers; always check the Certificate of Analysis (COA) of substrate for potency, solvents and storage conditions

Required antibiotic stock concentration= Needed concentration of antibiotic on disk/ Final volume to be added on disk

For example,

As per CLSI, the required Nafithromycin disk content is 15 µg, the standard volume of antibi-otic solution to be placed on the disk is 5µl

Step 1: Convert microgram (µg) to milligram (mg), 15µg/1000 = 0.015 mg

Step 2: Convert microliter (µL) to milliliter (mL), 5µl/1000 = 0.005 mL

Step 3: Converting concentration to same units will reduce the uncertainty of antibiotic addition.

Required stock antibiotic concentration = 0.015 mg/ 0.005ml

Therefore, Required antibiotic stock concentration = 3 mg/mL

Note:

Nafithromycin antibiotic to be dissolved in ½ volume of water, then glacial acetic acid dropwise to dissolve (acetic acid not to exceed2.5µl/ml)

5.9.6.2 Methodology:

5.9.6.2.1 Inoculation of Test Plates

1. Optimally, within 15 minutes after adjusting the 0.5Mcfarland of the inoculum, dip a sterile cotton swab into the adjusted suspension.
2. Rotate the swab several times and press firmly on the inside wall of the tube above the fluid level. This removes excess fluid from the swab.
3. Inoculate the dried surface of an MHA plate by streaking the swab over the entire sterile agar surface. Repeat this procedure by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, swab the rim of the agar. Alternatively, an automated plate rotator can be used for lawn preparation.
4. Leave the lid ajar for three to five minutes, but no more than 15 minutes, to allow for any excess surface moisture to be absorbed before applying the drug-impregnated disks.

NOTE: Avoid extremes in inoculum density. Never use undiluted overnight broth cultures or other unstandardized inoculum for streaking plates.

5.9.6.2.1 Application of Disk

1. Dispense the predetermined battery of antimicrobial disks onto the surface of the inoculated agar plate. Each disk must be pressed down to ensure complete contact with the agar sur-face.
2. Disks must be distributed evenly so they are no closer than 24 mm from centre to centre. Ordinarily, not more than five disks on a 100-mm plate.
3. If disks are placed too close to the edge of the plate, the zones may not be fully round with some drugs.
4. For in-house prepared antimicrobial solution, a 2 mm calibrated loop is used to deliver 5µl of the solution into 6 mm disk prepared from Whatman No.1 filter paper, and placed on the surface of the plate.
5. Some of the drug diffuses almost instantaneously; due to that disk should not be relocated once it has contact with the agar surface. Instead, place a new disk in another location on the agar.
6. Invert the plates and place in an incubator set to 35±2°C within 15 minutes after the disks are applied.

5.9.6.3 Interpretation:

Quality Control strains & Quality Control ranges CLSI M100 Ed36

Table 5.9: QC range is available for Broth micro dilution & DD

Quality Control Strains for Nafithromycin	**CLSI Reference Zone Diameter (mm)**	**CLSI Reference MIC range (mg/L)**
S.aureus ATCC 25923*	25 - 31	**NA**
S.aureus ATCC 29213*	**NA**	0.06 - 0.25
E.faecalis ATCC 29212	**NA**	0.016 - 0.12
H.influenzae ATCC 49247	16 - 20	2 - 8
S.pneumoniae ATCC 49619	25 - 31	0.008 - 0.03

NA : Not available. * S. aureus ATCC 29213 & E.faecalis ATCC 29212 - Quality control strain only for MIC determination. * S. aureus ATCC 25923 - Quality control strain only for disk diffusion assay

5.10 Zone diameters and MIC breakpoints (CLSI M100 Ed36)

Antimicrobial susceptibility testing results for bacterial pathogens included in the ICMR AMR Surveillance Network are interpreted using the Clinical and Laboratory Standards Institute (CLSI) M100, 36th Edition (2026) guidelines. The corresponding zone diameter and MIC breakpoints for susceptible, intermediate (or susceptible-dose dependent, where applicable), and resistant categories are provided in the following section.

Table 5.10: List of antimicrobial agents, zone diameters and MIC interpretive criteria for Order Enterobacterales except *Salmonella and Shigella*.

Antimicrobial agent	Disk Content (µg)	Zone diameter (mm)			MIC (µg/ml)		
		S	I	R	S	I	R
Piperacillin-tazobactam (PTZ)	100/10	≥ 25	21-24 (SDD)	≤ 20	≤8/4	16/4 (SDD)	≥32/4
Cefotaxime (TAX) OR	30	≥ 26	23-25	≤ 22	≤ 1	2	≥ 4
Ceftriaxone	30	≥ 23	20-22	≤ 19	≤ 1	2	≥ 4
Ceftazidime (CAZ)	30	≥ 21	18-20	≤ 17	≤ 4	8	≥ 16
Ceftazidime-avibactam***	30/20	≥ 21	-	≤ 20	≤8/4	-	≥16/4
Cefoperazone-sulbactam^	75/30	≥21	16-20	≤15	≤ 16	32	≥ 64
Aztreonam/avibactam @	30/20	≥ 25	22-24	≤ 21	≤4/4	8/4	≥16/4
Ertapenem (ETP)	10	≥ 22	19-21	≤ 18	≤ 0.5	1	≥ 2
Imipenem (IMI)	10	≥ 23	20-22	≤ 19	≤ 1	2	≥ 4
Meropenem (MEM)	10	≥ 23	20-22	≤ 19	≤ 1	2	≥ 4
Colistin (CST) #***		-	-	-	-	≤ 2	≥ 4
Amikacin (AMK)	30	≥20	17-19	≤16	≤4	8	≥16
Plazomicin(PLZ) ##	30	≥18	15-17	≤14	≤2	4	≥8
Minocycline	30	≥ 16	13-15	≤ 12	≤ 4	8	≥ 16
Ciprofloxacin (CIP)	5	≥26	22-25	≤21	≤0.25	0.5	≥1
Levofloxacin	5	≥21	17-20	≤16	≤ 0.5	1	≥2
Tigecycline&***	15	≥19	15-18	≤14	≤ 2	4	≥ 8
Cefepime/enmetazobactam*	30/20	≥21	-	≤20	≤8/8		≥16/8

1. #: For colistin, broth microdilution, colistin broth disk elution (CBDE) and colistin agar test (CAT MIC) methods are acceptable. For polymyxin B, broth microdilution is the only approved method. Disk diffusion and gradient diffusion method should not be performed. .
2. &: CLSI breakpoints are not available, FDA-approved breakpoints are included. For Tigecycline Broth microdilution MIC determination, the medium must be prepared fresh on the day of use. Zone diameter breakpoints validated for *E.coli* only. For C.koseri use an MIC method
3. ^: No CLSI and EUCAST or FDA breakpoints are available. Use company breakpoints (Pfizer)
4. ##: Plazomicin(PLZ) breakpoints do not apply to the family Morganellaceae which includes but is not limited to the genera *Morganella , Proteus* and *Providencia.*
5. *FDA breakpoints
6. *** If the isolate is resistant to carbapenems (ertapenem, imipenem, or meropenem), then test for Ceftazidime-avibactam, Aztreonam/avibactam, Tigecycline and colistin.
7. @ Mandatory antibiotics

Table 5.11: List of antimicrobial agents, zone diameters and MIC interpretive criteria for Order Enterobacterales except *Salmonella and Shigella*. (for urine samples only)

Antimicrobial agent	Disk content(µg)	Zone diameter (mm)			MIC (µg/ml)		
		S	I	R	S	I	R
Piperacillin-tazobactam (PTZ)	100/10	≥ 25	21-24 (SDD)	≤ 20	≤8/4	16/4 (SDD)	≥32/4
Cefazolin*@	30	≥ 15		≤14	≤16		≥ 32
Cefotaxime (TAX) OR	30	≥ 26	23-25	≤ 22	≤ 1	2	≥ 4
Ceftriaxone	30	≥ 23	20-22	≤ 19	≤ 1	2	≥ 4
Ceftazidime (CAZ)	30	≥ 21	18-20	≤ 17	≤ 4	8	≥ 16
Cefoperazone-sulbactam^	75/30	≥21	16-20	≤15	≤ 16	32	≥ 64
Ertapenem (ETP)	10	≥ 22	19-21	≤ 18	≤ 0.5	1	≥ 2
Imipenem (IMI)	10	≥ 23	20-22	≤ 19	≤ 1	2	≥ 4
Meropenem (MEM)***	10	≥ 23	20-22	≤ 19	≤ 1	2	≥ 4
Colistin (CST) #					-	≤ 2	≥ 4
Amikacin (AMK)	30	≥20	17-19	≤16	≤4	8	≥16
Minocycline	30	≥ 16	13-15	≤ 12	≤ 4	8	≥ 16
Ciprofloxacin (CIP)	5	≥26	22-25	≤21	≤0.25	0.5	≥1
Levofloxacin	5	≥21	17-20	≤16	≤ 0.5	1	≥2
Norfloxacin *	10	≥ 17	13-16	≤ 12	≤4	8	≥16
Trimethoprim-sulfamethoxazole(SXT) *	1.25/23.75	≥ 16	11-15	≤ 10	≤ 2/38	-	≥ 4/76
Fosfomycin*$	200	≥ 16	13-15	≤ 12	≤ 64	128	≥ 256
Nitrofurantoin (NIT) *	300	≥ 17	15-16	≤ 14	≤ 32	64	≥ 128

1. *: To be tested only for isolates from urine.
2. @: Cefazolin: uncomplicated UTI due to *E. coli, K. pneumoniae, P. mirabilis*.
3. $: Fosfomycin- DD & MIC breakpoints apply only to *E.coli* Urinary tract isolates and should not be extrapolated to other spp of Enterobacterales. Disc should have 50 µg glucose-6-phosphate. MIC can be tested only by agar dilution (not broth dilution) method with agar media supplemented with 25 µg/ ml glucose-6-phosphate.
4. ***If the isolate is resistant to ertapenem, perform testing for meropenem.
5. #: For colistin, broth microdilution, colistin broth disk elution (CBDE) and colistin agar test (CAT MIC) methods are acceptable. For polymyxin B, broth microdilution is the only approved method. Disk diffusion and gradient diffusion method should not be performed.

Table 5.12: List of antimicrobial agents, zone diameters and MIC interpretive criteria for *Salmonella* Typhi and *Salmonella*Paratyphi A, B and C and *Shigella*.

Antimicrobial Agent	Disk content (µg)	Zone Diameter (mm)			MIC (µg/ml)		
		S	I	R	S	I	R
Ampicillin (AMP)	10	≥ 17	14-16	≤ 13	≤ 8	16	≥ 32
Ceftriaxone (CTR) OR	30	≥ 23	20-22	≤ 19	≤ 1	2	≥ 4
Cefotaxime (TAX)	30	≥ 26	23-25	≤ 22	≤ 1	2	≥ 4
Ertapenem (ETP)	10	≥22	19-21	≤18	≤0.5	1	≥2
Imipenem (IMI)	10	≥23	20-22	≤19	≤1	2	≥4
Meropenem (MEM)	10	≥23	20-22	≤19	≤1	2	≥4
Azithromycin* *Salmonella*	15	≥ 13		≤ 12	≤ 16		≥ 32
Azithromycin *Shigella spp*	15	≥ 16	11-15	≤ 10	≤ 8	16	≥ 32
Ciprofloxacin (CIP) for *Salmonella*	5	≥ 31	21-30	≤ 20	≤ 0.06	0.12-0.5	≥ 1
Ciprofloxacin (CIP) for *Shigella*	5	≥26	22-25	≤21	≤0.25	0.5	≥1
Levofloxacin** for *Salmonella*	-	-	-	-	≤ 0.12	0.25-1	≥ 2
Levofloxacin for *Shigella*	5	≥21	17-20	≤16	≤0.5	1	≥2
Ofloxacin (OFX) ** for *Salmonella*	-	-	-	-	≤ 0.12	0.25-1	≥ 2
Ofloxacin (OFX) for *Shigella*	5	≥16	13-15	≤12	≤2	4	≥8
Trimethoprim-sulfamethoxazole (SXT)	1.25/23.75	≥ 16	11-15	≤ 10	≤ 2/38	-	≥ 4/76
Chloramphenicol (CHL)	30	≥ 18	13-17	≤ 12	≤ 8	16	≥ 32

1. *Azithromycin for *S. enterica ser.*Typhi only
2. **: Only by MIC: Ofloxacin, Levofloxacin.
3. Carbapenems to be reported only when Ceftriaxone/ Cefotaxime resistant.

Table 5.13: List of antimicrobial agents, zone diameters and MIC interpretive criteria for *Pseudomonas aeruginosa*

Antimicrobial agent	Disk content(µg)	Zone diameter(mm)			MIC (µg/mL)		
		S	I	R	S	I	R
Piperacillin-tazobactam (PTZ)	100/10	≥22	18-21	≤17	≤ 16/4	32/4	≥64/4
Cefepime (FEP)	30	≥ 18	15-17	≤ 14	≤ 8	16	≥ 32
Ceftazidime (CAZ)	30	≥ 18	15-17	≤ 14	≤ 8	16	≥ 32
Ceftazidime-avibactam***	30/20	≥ 21	-	≤ 20	≤8/4	-	≥16/4
Cefoperazone-sulbactam^	75/30	≥21	16-20	≤15	≤ 16	32	≥ 64
Imipenem (IMI)	10	≥ 19	16-18	≤ 15	≤ 2	4	≥ 8
Meropenem (MEM)	10	≥ 19	16-18	≤ 15	≤ 2	4	≥ 8
Colistin (CST) ***	-	-	-	-	-	≤ 2	≥ 4
Polymyxin B	-	-	-	-	-	≤ 2	≥ 4
Amikacin (AMK)*	30	≥ 17	15-16	≤ 14	≤ 16	32	≥ 64
Tobramycin	10	≥19	13-18	≤ 12	≤1	2	≥4
Ciprofloxacin (CIP) OR	5	≥ 25	19-24	≤18	≤ 0.5	1	≥ 2
Levofloxacin (LEV)	5	≥ 22	15-21	≤14	≤ 1	2	≥ 4

1. First line antimicrobial agents for isolates from blood, urine, sputum, ETA, suction tip, throat, nasal and ear swabs, BAL, pus, genital tract specimen: Ceftazidime, Levofloxacin, Tobramycin, Piperacillin-tazobactam
2. Colistin: Broth microdilution, Colistin broth disc elution and Colistin agar tests are recommended.Colistin MIC can be used to predict MIC of Polymyxin B.
3. Polymyxin B: MIC by microbroth dilution only.
4. Amikacin: Report only for Urinary isolates.
5. ^: No CLSI and EUCAST or FDA breakpoints are available. Use company breakpoints
6. *** If the isolate is resistant to carbapenems (imipenem, or meropenem), then test for Ceftazidime-avibactam and colistin.

Table 5.14: List of antimicrobial agents, zone diameters and MIC interpretive criteria for *Acinetobacter* spp.

Antimicrobial agent	Disk Content (µg)	Zone diameter(mm)			MIC (µg/mL)		
		S	I	R	S	I	R
Piperacillin-tazobactam (PTZ)	100/10	≥ 21	18-20	≤ 17	≤ 16/4	32/4-64/4	≥ 128/4
Ampicillin-sulbactam	10/10	≥22	17-21	≤16	≤8/4	16/8	≥32/16
Cefoperazone-sulbactam^	75/30	≥21	16-20	≤15	≤ 16	32	≥ 64
Cefepime (FEP)	30	≥ 18	15-17	≤ 14	≤ 8	16	≥ 32
Ceftazidime (CAZ)	30	≥ 18	15-17	≤ 14	≤ 8	16	≥ 32
Imipenem (IMI)	10	≥ 22	19-21	≤ 18	≤ 2	4	≥ 8
Meropenem (MEM)	10	≥ 18	15-17	≤ 14	≤ 2	4	≥ 8
Colistin***	-	-	-	-	-	≤ 2	≥ 4
Polymyxin B	-	-	-	-	-	≤ 2	≥ 4
Amikacin (AMK)$	30	≥ 20	17-19	≤ 16	≤ 8	16	≥ 32
Minocycline	30	≥22	18-21	≤17	≤1	2	≥4
Levofloxacin (LEV)	5	≥ 17	14-16	≤ 13	≤ 2	4	≥ 8
Co-trimoxazole	1.25/23.75	≥16	11-15	≤10	≤2/38	-	≥4/76
Tigecycline&***	15	≥19	15-18	≤14	≤ 2	4	≥ 8

1. First line antimicrobial agents for isolates from blood , urine, sputum, ETA, suction tip, BAL, pus, genital tract specimen : Ceftazidime, Levofloxacin, Amikacin, Piperacillin-tazobactam.
2. First line antimicrobial agents for isolates from throat, nasal and ear swabs: Ceftazidime, Levofloxacin, Amikacin, Piperacillin-tazobactam.
3. Colistin: Only for *Acinetobacter baumannii* complex MIC by microbroth dilution, CBDE, CAT, DD or gradient diffusion methods are not recommended. Colistin MIC predict for Polymyxin B only for *Acinetobacter baumannii complex*.
4. ^: No CLSI and EUCAST or FDA breakpoints are available. Use company breakpoints
5. **&:**Since CLSI breakpoints are not available, FDA-approved breakpoints are included.
6. *** If the isolate is resistant to carbapenems (ertapenem, imipenem, or meropenem), then test for tigecycline and colistin.
7. $Amikacin breakpoint revised-CLSI 2026

Table 5.15: List of antimicrobial agents, zone diameters and MIC interpretive criteria for *Stenotrophomonas maltophilia*

Antimicrobial agent	Disk content	Zone diameter(mm)			MIC (µg/mL)		
		S	I	R	S	I	R
Ticarcillin-clavulanic acid	-	-	-	-	≤ 16/2	32/2-64/2	≥ 128/2
Minocycline (MIN)	30 µg	≥26	21-25	≤20	≤1	2	≥4
Levofloxacin (LEV)	5 µg	≥ 17	14-16	≤ 13	≤ 2	4	≥ 8
Trimethoprim-sulfamethoxazole (SXT)	1.25/ 23.75 µg	≥ 16	11-15	≤ 10	≤ 2/38		≥ 4/76
Chloramphenicol (CHL)*	-	-	-	-	≤ 8	16	≥ 32

Note: *: Chloramphenicol not for UTI;

Table 5.16: List of Antimicrobial Agents, zone diameters and MIC break points for faecal bacterial pathogens (*Arizona* spp, *Vibrio* spp., *Aeromonas*spp., *Plesiomonas shigelloides*)

Antimicrobial agent	Disk content	Zone diameter (mm)			MIC (µg/mL)		
		S	I	R	S	I	R
Ampicillin (AMP)	10 µg	≥ 17	14-16	≤ 13	≤ 8	16	≥ 32
Ceftriaxone*	30	≥23	20-22	≤19	≤1	2	≥4
Imipenem	10 µg	≥ 23	20-22	≤ 19	≤ 1	2	≥ 4
Meropenem	10 µg	≥ 23	20-22	≤ 19	≤ 1	2	≥ 4
Nalidixic acid (NAL)#	30 µg	≥ 19	14-18	≤ 13	≤ 16	-	≥ 32
Azithromycin $		-	-	-	≤ 2	-	-
Ciprofloxacin (CIP)$	5 µg	≥ 21	16-20	≤ 15	≤ 1	2	≥ 4
Tetracycline (TET) for Vibrio	30 µg	≥ 15	12-14	≤ 11	≤ 4	8	≥ 16
Trimethoprim-sulfamethoxazole (SXT)	1.25/23 75 µg	≥ 16	11-15	≤ 10	≤ 2/38		≥ 4/76
Chloramphenicol	30 µg	≥ 18	13-17	≤ 12	≤ 8	16	≥ 32

1. *For *Aeromonas* only
2. #Nalidixic acid –Enterobacterales interpretation CLSI 2026
3. $Ciprofloxacin & Azithromycin –Interpreted as per M45, 3 rd edition CLSI 2016

Table 5.17: List of Antimicrobial Agents, zone diameters and MIC break points for *Campylobacter* spp.

Antimicrobial agent	Disk content	Zone diameter (mm)			MIC (µg/mL)		
		S	I	R	S	I	R
Erythromycin* (Azithromycin can be predicted by testing Erythromycin)	15	≥16	13-15	≤ 12	≤ 8	16	≥ 32
Tetracycline (TET)*	30 µg	≥ 26	23-25	≤ 22	≤ 4	8	≥ 16
Ciprofloxacin (CIP)*	5 µg	≥ 24	21-23	≤ 20	≤ 1	2	≥ 4
Norfloxacin (NOR)$	10 µg	≥ 17	13-16	≤ 12	≤ 4	8	≥ 16
Clindamycin (CLI) *	-	-	-	-	≤ 4	-	≥ 8

* Erythromycin - (Azithromycin can be predicted by testing Erythromycin), Tetracycline, Ciprofloxacin- **Interpreted as per M45, 3 rd edition CLSI 2016**

Table 5.18: List of antimicrobial agents, zone diameters and MIC interpretive criteria for *Staphylococcus* species

Antimicrobial agent	Disk content	Zone diameter (mm)			MIC(µg/mL)		
		S	I	R	S	I	R
Cefoxitin (FOX)	30 µg						
*		≥ 22	-	≤ 21	≤ 4	-	≥ 8
**		≥ 25	-	≤ 24	-	-	-
****		≥ 25	-	≤ 24	-	-	-
Oxacillin							
*	-	-	-	-	≤ 2	-	≥ 4
**	1 µg	≥ 18	-	≤ 17	≤ 0.5	-	≥1
***	1 µg	≥ 18	-	≤ 17	≤ 0.5	-	≥1
****	-	-	-	-	≤ 0.5	-	≥1
Ceftaroline$	30 µg	≥ 25	20-24 (SDD)	≤ 19	≤ 1	2-4 (SDD)	≥ 8
Vancomycin (VAN)	-	-	-	-			
*S. aureus*including MRSA					≤ 2	4-8	≥ 16
All CoNS (SOSA)					≤ 4	8-16	≥ 32
Teicoplanin (TEC)					≤ 8	16	≥ 32
Daptomycin@@	-	-	-	-	≤1	-	-
Erythromycin @	15 µg	≥ 23	14-22	≤ 13	≤ 0.5	1-4	≥ 8
Tetracycline (TET)	30 µg	≥ 19	15-18	≤ 14	≤ 4	8	≥ 16

Tigecycline (TIG) #	15 µg	≥ 19	-	≤18	≤ 0.5	-	≥ 1
Ciprofloxacin (CIP)	5 µg	≥ 21	16-20	≤ 15	≤ 1	2	≥ 4
Clindamycin (CLI)	2 µg	≥ 21	15-20	≤ 14	≤ 0.5	1-2	≥ 4
Trimethoprim-sulfamethoxazole	1.25/23.75 µg	≥ 16	11-15	≤ 10	≤**2**/38	-	≥**4**/76
Linezolid (LNZ)	30 µg	≥26	23-25	≤22	≤ 4	-	≥ 8
Nitrofurantoin (for urinary isolates)	300 µg	≥17	15-16	≤14	≤32	64	≥128
Levonadifloxacin[&]	10 µg	≥17	14-16	≤13	≤ 2	4	≥ 8

Notes:

1. **S. aureus*, *S. lugdunensis*
2. ** *S. epidermidis*
3. *** *S. pseudointermedius, S. schleiferi*
4. ****CoNS (non- *S. epidermidis, S. lugdunensis, S. pseudointermedius, S. schleiferi)*
5. Oxacillin screen agar (6 µg/ml): Only for *S. aureus*. MHA with 4% NaCl& 6 µg/mL of Oxacillin: > 1 colony or light film of growth denotes resistance.
6. Cefoxitin is used as surrogate marker for *mecA* mediated oxacillin resistance. If *mecA* positive, report as methicillin (oxacillin) (not cefoxitin) resistant. Rare isolates *mecA*negative with oxacillin MIC ≥ 4 µg/mL, should be reported as methicillin (oxacillin) (not cefoxitin) resistant
7. For non-*S. epidermidis*CoNS, with oxacillin MIC 0.5-2 µg/mL, test for *mecA*or PBP2a. If negative, report as methicillin (oxacillin) Susceptible.
8. If any discrepancy is observed between the results of cefoxitin disc diffusion and oxacillin screen agar in detection of MRSA, latex agglutination test for PBP2a or *mec*A PCR may be performed for confirmation if facilities are available.
9. Vancomycin screen agar (6 µg/mL) (VSA): Only for *S. aureus*. Examine carefully with transmitted light for >1 colony or light film of growth. More than 1 colony - presumptive reduced susceptibility to vancomycin. For the isolates which grow on VSA, perform vancomycin MIC using a validated MIC method (E-test or broth micro dilution) to determine vancomycin MICs. VISA with MIC of 4 µg/mL will not grow on VSA.
10. @: Erythromycin: Not to be tested for isolates from urine.
11. #: Tigecycline: Only for MRSA isolates: S :≤ 0.5, R : ≥ 1 (EUCAST).
12. Creeping MIC: MRSA: Vancomycin, Teicoplanin, Linezolid.
13. $: Ceftaroline Only by NC.
14. @@ Daptomycin (Not for lower respiratory tract)

Table 5.19: List of antimicrobial agents, zone size and MIC interpretive criteria for Enterococci

Antibiotics	**Disc potency**	**Zone diameter (mm)**			**MIC (µg/ml)**		
		S	**I**	**R**	**S**	**I**	**R**
Penicillin OR	10 units	≥ 15	-	≤ 14	≤ 8	-	≥ 16
Ampicillin (AMP)	10 µg	≥ 17	-	≤ 16	≤ 8	-	≥ 16
Vancomycin (VAN)	30 µg	≥ 17	15-16	≤ 14	≤ 4	8-16	≥ 32
Teicoplanin	30 µg	≥ 14	11-13	≤ 10	≤ 8	16	≥ 32
Daptomycin$	-	-	-	-	-	≤ 4 (SDD)	≥ 8

Daptomycin$$	-	-	-	-	≤ 2	4	≥ 8
Gentamicin (HLG)	120 µg	≥ 10	7-9	≤ 6	≤ 500	-	>500
Ciprofloxacin (CIP) *	5 µg	≥ 21	16-20	≤ 15	≤ 1	2	≥ 4
Nitrofurantoin (NIT)*	300 µg	≥ 17	15-16	≤ 14	≤ 32	64	≥ 128
Fosfomycin #	200 µg	≥ 16	13-15	≤ 12	≤ 64	128	≥ 256
Linezolid (LNZ)	30 µg	≥ 23	21-22	≤ 20	≤ 2	4	≥ 8

Notes:

1. Vancomycin readings should be taken after 24 hours incubation. Perform MIC test for enterococci falling in intermediate zone for Vancomycin.
2. Vancomycin screen agar (6 µg/mL): For the isolates which grow on VSA, perform vancomycin MIC and to rule out *E. gallinarum*and *E. casseliflavus*(having intermediate vancomycin resistance due to *vanC*), perform motility and pigment production tests.
3. *: Ciprofloxacin, Nitrofurantoin tested only for urinary isolates.
4. HLG: DD : Intermediate results : Verify with agar /broth dilution method.
5. MIC Creep: Linezolid.
6. #: Fosfomycin: Urinary *E. faecalis* only. Disk contains glucose-6-phosphate 50µg. MIC only by agar dilution supplemented with glucose-6-phosphate 25 µg/ml.
7. $ Daptomycin. *E. faecium* only. Not for lower respiratory isolates.
8. $$ Daptomycin. *Enterococcus*spp other than *E. faecium*. Not for lower respiratory isolates.

Table 5.20: List of antimicrobial agents, zone size and MIC interpretive criteria for *Streptococcus pneumoniae*

Antimicrobial agent	Disk content	Zone diameter (mm)			MIC (µg/ml)		
		S	I	R	S	I	R
Penicillin*	Oxacillin 1µg	≥20	-	-	-	-	-
Penicillin parenteral (non-meningitis)@	-	-	-	-	≤2	4	≥8
Penicillin parenteral (meningitis)#&	-	-	-	-	≤0.06	-	≥0.12
Penicillin (oral penicillinV)$	-	-	-	-	≤0.06	0.12-1	≥2
Cefotaxime (meningitis) #& OR	-	-	-	-	≤0.5	1	≥2
Ceftriaxone (meningitis) #&	-	-	-	-	≤0.5	1	≥2
Cefotaxime (non-meningitis)@ OR	-	-	-	-	≤1	2	≥4
Ceftriaxone (non-meningitis)@	-	-	-	-	≤1	2	≥4
Meropenem&					≤0.25	0.5	≥1
Vancomycin	30µg	≥17	-	-	≤1	-	-

Erythromycin	15µg	≥21	16-20	≤15	≤0.25	0.5	≥1
Levofloxacin	5µg	≥17	14-16	≤13	≤2	4	≥8
Chloramphenicol	30µg	≥21	-	≤20	≤4	-	≥8
Linezolid	30µg	≥21	-	-	≤2	-	-

Notes:

1. *: Isolates of pneumococci with oxacillin zone size of ≥20mm are susceptible (MIC ≤0.06 µg/mL) to penicillin. Penicillin and cefotaxime, ceftriaxone or meropenem MICs should be determined for those isolates with oxacillin zone diameters of ≤19mm, because zones of ≤19mm occurs with penicillin–resistant, -intermediate, or certain – susceptible strains. For isolates with oxacillin zones ≤19mm, do not report penicillin as resistant without performing a penicillin MIC test.

2. @: For all isolates other than those from CSF, report interpretation for both meningitis and non-meningitis.

3. #: For CSF isolates, report only meningitis interpretation

4. $: Interpretations for oral penicillin may be reported for isolates other than those from CSF

5. &:For*S. pneumoniae* isolates from CSF, penicillin and cefotaxime or meropenem should be tested by a reliable MIC method and reported routinely. Such isolates can also be tested against vancomycin using the MIC or disk diffusion methods

Table 5.21: List of antimicrobial agents, zone size and MIC interpretive criteria for *Streptococcus* β-Haemolytic group

Antimicrobial Agent	Disk content	Zone diameter (mm)			MIC (µg/ml)		
		S	I	R	S	I	R
Penicillin	10 units	≥24	-	-	≤0.12	-	-
Ampicillin	10µg	≥24	-	-	≤0.25	-	-
Ceftriaxone	30µg	≥24	-	-	≤0.5	-	-
Erythromycin	15µg	≥21	16-20	≤15	≤0.25	0.5	≥1
Levofloxacin	5µg	≥17	14-16	≤13	≤2	4	≥8
% Levonadifloxacin	10µg	≥20	-	≤19	≤1	2	≥4

Table 5.22: List of antimicrobial agents, zone size and MIC interpretive criteria for *Streptococcus* viridans group

Antimicrobial agent	Disk content	Zone diameter (mm)			MIC(µg/mL)		
		S	I	R	S	I	R
Cefotaxime	30µg	≥28	26-27	≤25	≤1	2	≥4
Vancomycin	30µg	≥17	-	-	≤1	-	-

Notes: Small colony forming beta hemolytic strains with group A, C, F or G antigens (*S. anginosus* group, previously termed as "*Streptococcus milleri*") are considered part of the viridans group, and break points for the viridans group should be used.

Chapter 6
Special Phenotypic Tests

This chapter describes phenotypic methods that can be used to detect and characterise antimicrobial resistance in clinically important bacterial pathogens. It covers selected tests for the detection of specific resistance mechanisms and determination of antimicrobial susceptibility, including methods for carbapenemase detection, colistin susceptibility testing, assessment of antimicrobial combinations, inducible clindamycin resistance, vancomycin susceptibility and investigation of efflux pump activity. These procedures are intended to support the standardised performance and interpretation of phenotypic tests across participating laboratories.

6.1 Carba-NP test (For Enterobacterales and *Pseudomonas spp.*) (CLSI, 2026; CLSI, 2019)

Principle: Based on a technique designed to identify the hydrolysis of the β-lactam ring of a carbapenem, as indicated by a colour change in the pH indicator phenol red.

6.1.1 Requirements

1. 1.5 mL microcentrifuge tubes
2. Imipenem sodium salt
3. B-PERII, Bacterial Protein Extraction Reagent
4. $ZnSO_4.7H_2O$
5. Negative (wild-type *E. coli*) and positive (*K. pneumoniae* OXA-48 or *K. pneumoniae* KPC-2) controls. As per CLSI, *K. pneumoniae* ATCC BAA-1705 is a carbapenemase-positive control, and *K. pneumoniae* ATCC BAA-1706 is a carbapenemase-negative control.

6.1.2 Preparation and storage of Solution A

1. Prepare a concentrated solution of red phenol (0.5% w/v).
2. Add 2 mL of the concentrated red phenol solution (vortex thoroughly before pipetting to resuspend the solution) to 16.6 mL of distilled water.
3. Adjust the pH to 7.8 by adding 1N NaOH solution dropwise.
4. Add 180 µL of 10 mM $ZnSO_4$ to obtain a final concentration of 0.1 mM.

Solution A is stable at room temperature for 1 week and may be stored at -20°C for several months. Solution A containing imipenem (6 mg/mL) should be prepared immediately before use. However, aliquots of imipenem powder may be weighed and prepared in advance and stored at 4°C for up to 2 weeks, provided Solution A has not been added. A total of 600 µL of Solution A is required per test, including the Solution A-only control, the Solution A+imipenem mixture, and the positive and negative controls.

6.1.3 Procedure

1. Add 100 µL of 20 mM Tris-HCl lysis buffer in each of two 1.5 mL microcentrifuge tubes.
2. Resuspend a 1/4 to 1/3 calibrated loopful of bacterial colonies (approximately 10 µL) in each tube containing 100 µL of lysis buffer. Bacterial colonies may be recovered directly from the area around a carbapenem disk on an antibiogram performed using the disk diffusion method.
3. Ensure that the bacterial colonies are completely resuspended. If necessary, mix by pipetting up and down.
4. Add 100 µL of Solution A to the first microcentrifuge tube and 100 µL of Solution A containing imipenem (6 mg/mL) to the second tube.
5. Incubate at 37°C for a maximum of 2 hours.
6. Visually assess the colour of each tube and interpret the results as follows **(Figure 6.1)**.

Interpretation	**Without imipenem**	**With Imipenem**
No carbapenemase	Red	Red
Carbapenemase producer	Red	Orange/Yellow
Not interpretable	Yellow	Yellow

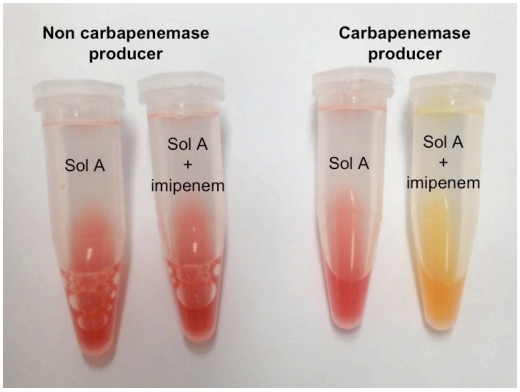


Figure 6.1: Carba NP test

The time required to obtain positive results generally varies as follows:

1. KPC producers: 30 minutes to 2 hours
2. OXA-48-like producers: 20 minutes to 1 hour
3. Metallo-β-lactamases (NDM, VIM, IMP): 15 minutes to 1 hour

6.2 Modified Carbapenem Inactivation Method (mCIM and eCIM) for Enterobacterales and *Pseudomonas spp.* (CLSI, 2026; CLSI, 2019)

- mCIM is used for detecting carbapenemases in *Enterobacterales* and *P. aeruginosa,* whereas eCIM is used together with mCIM to differentiate metallo-β-lactamases from serine carbapenemases in *Enterobacterales*

- mCIM can be performed alone; however, eCIM must be performed together with mCIM
- eCIM result is valid only if mCIM result is positive.

6.2.1 mCIM (Figure 6.2)

i. Grow the test organism on Mueller-Hinton agar plates
ii. For each isolate to be tested, emulsify 1 µl loopful of bacteria *Enterobacterales* or 10 µl of *P. aeruginosa* colonies from an overnight-grown plate in 2 mL of trypticase soy broth (TSB).
iii. Vortex the suspension for 10-15 seconds.
iv. Using sterile forceps, add a 10 µg meropenem disk to the suspension, ensuring that the disk is fully immersed.
v. Incubate at 37°C for 4 hours
vi. Prepare a lawn culture of *E. coli* ATCC 25922, adjusted to 0.5 McFarland turbidity, on Mueller-Hinton agar (MHA).
vii. Using a sterile 10 µL loop, remove the meropenem disk from the suspension and place it on the lawn culture of *E. coli* ATCC 25922.
viii. Invert the plate and incubate at 37°C for 18–24 hours.
ix. After incubation, measure the zone of inhibition.

6.2.2 eCIM (Figure 6.2)

i. For each isolate, label a second tube containing 2 mL of TSB for the eCIM test.
ii. Add 20 µL of 0.5 M EDTA to the 2 mL TSB tube to obtain a final EDTA concentration of 5 mM.
iii. Follow steps i to ix as above for the mCIM procedure. Process the mCIM and eCIM tubes in parallel.
iv. Place the meropenem disks from the mCIM and eCIM tubes on the same MHA plate inoculated with the meropenem-susceptible *E. coli* ATCC® 25922 indicator strain.

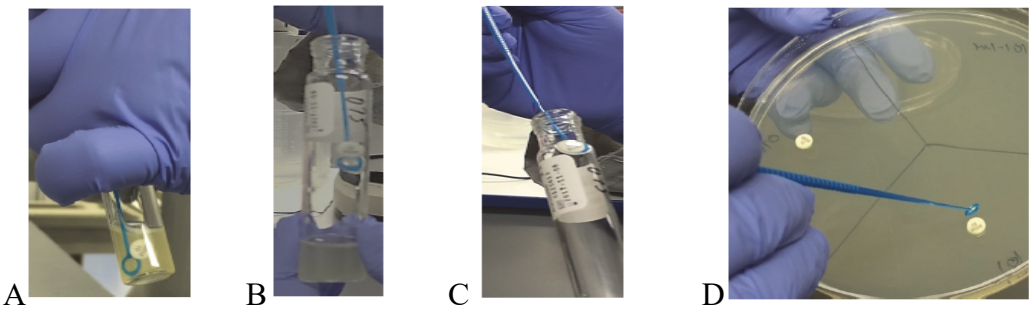


Figure 6.2: Method for performing the mCIM/eCIM test. (A) Remove the meropenem disk using a 10 µL loop. (B) Drag the loop against the inside edge of the tube to remove excess liquid. (C) Use the same loop to remove the disk from the tube. (D) Place the disk on a MHA plate previously inoculated with the meropenem-susceptible indicator strain *E. coli* (ATCC®25922) (CLSI, 2019).

6.2.3 Interpreting and reporting results (Figure 6.3)

6.2.3.1 mCIM

- Carbapenemase positive**:** Zone of inhibition of 6–15 mm, or the presence of colonies within a zone of inhibition measuring 16–18 mm.
- Carbapenemase negative**:** Zone of inhibition ≥ 19 mm.
- Carbapenemase indeterminate**:** Zone of inhibition of 16–18 mm, or a zone of inhibition ≥ 19 mm with pinpoint colonies present within the zone.

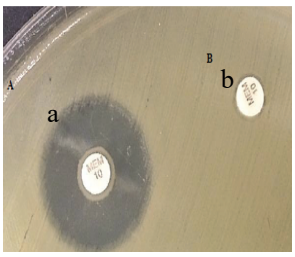
6.2.3.2 eCIM

Metallo-β-lactamase positive

- An increase of ≥ 5 mm in the zone diameter for eCIM compared with mCIM (e.g., mCIM = 6 mm; eCIM = 15 mm; difference in zone diameters = 9 mm). For eCIM interpretation, ignore pinpoint colonies within any zone of inhibition.
- If the test isolate produces a metallo-β-lactamase, its activity will be inhibited in the presence of EDTA, such that meropenem in the disk will not be hydrolysed as efficiently as in the tube without EDTA. Consequently, the zone of inhibition of the meropenem-susceptible *E. coli* indicator strain will increase in the eCIM compared with the mCIM.

Metallo-β-lactamase negative

- An increase of ≤ 4 mm in the zone diameter for eCIM compared with mCIM (e.g., mCIM = 6 mm; eCIM = 8 mm; difference in zone diameters = 2 mm). For eCIM interpretation, ignore pinpoint colonies within any zone of inhibition.
- If the test isolate produces a serine carbapenemase, its activity will not be affected by the presence of EDTA. Therefore, there will be no increase or only a marginal increase (≤ 4 mm) in the zone diameter in the presence of EDTA compared with the mCIM zone diameter.

(A) 

(B) 

Figure 6.3: mCIM result interpretation. (A) Quality control (QC) strains: (a) negative control, *K. pneumoniae* ATCC BAA-1706; and (b) positive control, *K. pneumoniae* ATCC BAA-1705.

Note: A narrow ring of growth around the meropenem disk, as observed with the negative control (a), results from carryover of the test organism in the TSB broth and should be ignored. (B) Positive mCIM result (CLSI, 2019).

6.3 Tests for Colistin resistance for *Enterobacterales* and *P. aeruginosa*

The Polymyxins (colistin & Polymyxin B) are antimicrobial agents of last resort for treating multidrug-resistant infections. Clinical and pharmacokinetic/pharmacodynamic (PK/PD) data suggest that these agents have limited clinical efficacy. Alternative agents are strongly preferred. If alternative agents are unavailable, knowledge of the colistin MIC may help inform treatment decisions (CLSI, 2026; CLSI, 2019).

For colistin, broth microdilution, broth disk elution, and agar dilution MIC methods are acceptable. Broth microdilution is the only approved method for testing polymyxin B. Disk diffusion and gradient diffusion methods should not be performed.

Colistin and polymyxin B are considered equivalent agents; therefore, MICs obtained by testing colistin are expected to predict MICs for polymyxin B, and vice versa. At present, CLSI has not evaluated polymyxin B testing methods, and the procedures described below should not be adapted for polymyxin B. These methods were evaluated by CLSI for *Acinetobacter* spp. and were found to yield inaccurate results.

These methods were established with limited disk and /or media manufacturers and are considered provisional until additional data are evaluated by CLSI and shown to meet CLSI M23 guidance (Goodwin and Wikler, 2023, CLSI Guideline M23).

6.3.1 Colistin broth disk elution method

Principle:

The colistin broth disk elution method is a tube dilution method in which colistin disks serve as the source of colistin. The method is intended for testing *Enterobacterales* and *P. aeruginosa*, primarily to determine the colistin susceptibility of multidrug-resistant isolates for clinical or infection prevention purposes (CLSI, 2026).

6.3.2 Requirements

1. Cation-adjusted Mueller-Hinton broth (CA-MHB), in 10 mL volumes
2. Colistin sulfate disks (10 µg)
3. Colistin concentrations of 0 µg/mL (growth control), 1 µg/mL, 2 µg/mL, and 4 µg/mL
4. Sterile swab
5. Sterile normal saline
6. 0.5 McFarland turbidity standard
7. Fresh culture (24 hours)

6.3.3 Procedure (Figure 6.4)

1. Dispense 10 mL of CA-MHB into sterile test tubes. Allow the colistin disks to equilibrate to room temperature.

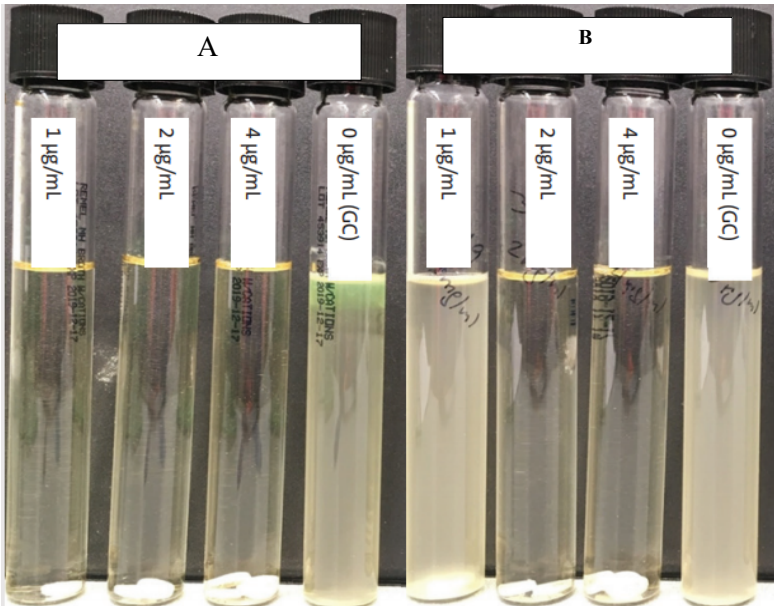


Figure 6.4: Colistin broth elution method. (A) *E. coli* ATCC BAA-3170 (formerly AR bank # 0349mcr-1) (≤1-4µg/mL). (B) *P. aeruginosa* ATCC 27853 (≤1-4µg/mL)

2. For each isolate, label four tubes containing CA-MHB for the final colistin concentrations of 1, 2, and 4 µg/mL and the growth control (0 µg/mL).
3. Using aseptic technique, add one colistin disk to the 1 µg/mL tube, two colistin disks to the 2 µg/mL tube, and four colistin disks to the 4 µg/mL tube. Do not add colistin disks to the growth control tube.
4. Gently vortex the tubes containing the disks and allow the colistin to elute from the disks for at least 30 minutes, but no longer than 60 minutes, at room temperature.
5. Add 50 µL of the standardised inoculum to each of the growth control and colistin-containing tubes to obtain a final inoculum concentration of approximately 7.5×10^5 CFU/mL.
6. Using a 10 µL loop, subculture the original inoculum onto a blood agar plate to check culture purity.
7. Close the screw-cap tubes tightly and vortex each inoculated tube at low speed to mix. Loosen the caps slightly before incubation. Incubate the tubes at 33–35°C in ambient air for 16–20 hours.

6.3.4 QC recommendations

- *E. coli* ATCC BAA-3170 (formerly AR bank # 0349mcr-1): ≤1-4µg/mL
- *P. aeruginosa* ATCC 27853: ≤1-4µg/mL

6.3.5 Interpretation

1. Check the purity plate to ensure the inoculum was pure, and also check the growth control (GC) tube, which must be turbid for the test to be valid.
2. Read the MIC as the lowest concentration that completely inhibits the growth of the test isolates.
3. For *Enterobacterales* and *P. aeruginosa,* an MIC of ≤2µg/mL is classified as Intermediate and an MIC of ≥ 4µg/mL is classified as Resistant.

6.4 Colistin Agar method

6.4.1 Principle

This is an agar dilution method using colistin as the antimicrobial agent. It is a slight modification of the agar dilution method described in CLSI M07, involving a different inoculum and a different approach to interpreting results. The colistin agar method is intended for *Enterobacterales* and *P. aeruginosa*, primarily for testing multidrug-resistant isolates for clinical or infection prevention purposes (CLSI, 2026).

6.4.2 Requirements

1. Mueller-Hinton agar (20 mL in a 100-mm Petri dish)
2. Colistin sulfate disks
3. Colistin concentrations of 0 µg/mL (growth control, GC), 1 µg/mL, 2 µg/mL, and 4 µg/mL
4. Sterile swab
5. Sterile normal saline
6. 0.5 McFarland turbidity standard
7. Fresh culture (24 hours)

6.4.3 Procedure

1. Using a marker, divide each colistin agar plate into up to 10 sections to test up to 10 isolates per plate, with colistin concentrations increasing in twofold increments (Figure 6.5).

2. Label each section with the appropriate isolate number.
3. Using a pipette or a 10 µL loop, inoculate 10 µL of the 1:10 dilution onto the appropriate section of each colistin agar plate.
4. Using a 10 µL loop, subculture from the original inoculum onto a blood agar plate as a purity check.
5. Incubate the colistin agar plates and the purity plate at 33-35ºC in ambient air for 16-20 hours.

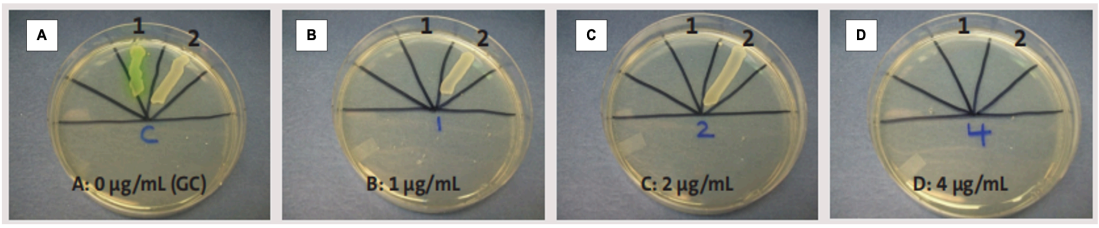


Figure 6.5: Colistin agar Test. (A) Growth control (0 µg/mL colistin). (B) Colistin at 1 µg/mL. (C) Colistin at 2 µg/mL. (D) Colistin at 4 µg/mL. Bacterial growth is assessed at each colistin concentration to determine the minimum inhibitory concentration (MIC).

6.4.4 QC recommendations

E. coli ATCC BAA-3170 (formerly AR bank # 0349mcr-1): ≤1-4µg/mL

P. aeruginosa ATCC 27853: ≤1-4µg/mL

6.4.5 Interpretation

1. Check the purity plate to ensure the inoculum was pure, and also check the growth control (GC) tube, which must be confluent growth for the test to be valid.
2. Read the colistin plates carefully with transmitted light for colonies or a light film of growth.
3. MIC is the lowest colistin agar plate concentration that completely inhibits the growth of the test isolate (e.g. even 1 colony would be considered growth).
4. For *Enterobacterales* and *P. aeruginosa,* an MIC of ≤2µg/mL is classified as Intermediate and an MIC of ≥ 4µg/mL is classified as Resistant.

6.5 Tests for Aztreonam plus ceftazidime-Avibactam Broth Disk Elution Method

6.5.1 Introduction

Due to limited therapeutic options, there may be a clinical need to assess the in vitro activity of the combination of aztreonam and ceftazidime-avibactam to guide the treatment of

multidrug-resistant Gram-negative bacterial infections, particularly those caused by metallo-β-lactamase (MBL) producers.

The aztreonam plus ceftazidime-avibactam broth disk elution method was established with limited disk and/or media manufacturers and is considered provisional until additional data are evaluated by CLSI and shown to meet CLSI M23 guidance (CLSI, 2026).

6.5.2 Purpose

Aztreonam plus ceftazidime-avibactam broth disk elution method is intended for testing *Enterobacterales* and *S. maltophilia,* primarily multidrug-resistant isolates, particularly those producing MBLs. This is a tube dilution method in which aztreonam and ceftazidime-avibactam disks serve as the sources of the antimicrobial agents (CLSI, 2026).

6.5.3 Requirements

1. CA-MHB (5 mL tubes)
2. Aztreonam disks (30 µg)
3. Ceftazidime-avibactam disks (30 µg/20 µg)
4. Final concentration: aztreonam 6 µg/mL, ceftazidime 6 µg/mL, and avibactam 4 µg/mL
5. Sterile swab
6. Sterile normal saline
7. 0.5 McFarland turbidity standard
8. Fresh culture (24 hours)

6.5.4 Procedure

1. Dispense 5 mL of CA-MHB into sterile test tubes. Allow the antimicrobial disks to equilibrate to room temperature.
2. For each isolate, label four tubes containing CA-MHB as follows: aztreonam (ATM), ceftazidime-avibactam (CZA), aztreonam plus ceftazidime-avibactam (ATM + CZA), and growth control.
3. From a fresh, non-selective agar plate (18–24 hours old), select 3–5 colonies, suspend them in sterile normal saline, and adjust the suspension to 0.5 McFarland turbidity.
4. Using aseptic technique, add one aztreonam disk to the tube labelled ATM, one ceftazidime-avibactam disk to the tube labelled CZA, and one aztreonam disk plus one ceftazidime-avibactam disk to the tube labelled ATM + CZA. Do not add antimicrobial disks to the growth control tube.

5. Gently vortex the tubes containing the disks and allow the antimicrobial agents to elute from the disks for at least 30 minutes, but no longer than 60 minutes, at room temperature.
6. Add 25 µL of the standardised inoculum to each of the ATM, CZA, ATM + CZA, and growth control tubes to obtain a final inoculum concentration of approximately 7.5 × 10^5 CFU/mL.
7. Using a 10 µL loop, subculture the original inoculum onto a blood agar plate as a purity check.
8. During mixing, avoid allowing the disks to adhere to the tube caps or glass surfaces.
9. Close the screw-cap tubes tightly and vortex each inoculated tube at low speed to mix. Loosen the caps slightly before incubation. Incubate the tubes at 33–35°C in ambient air for 16–20 hours.

6.5.5 QC recommendation and Result Interpretation

QC Strain	Organism characteristics	Expected Results
E.coli ATCC 25922	Susceptible to all antimicrobial agents **(Figure 6.6)**	**ATM:** No growth = Susceptible **CZA:** No growth = Susceptible **ATM + CZA:** No growth = Susceptible
K. pneumoniae BAA1705	Not susceptible to Aztreonam. Susceptible to ceftazidime/avibactam and ceftazidime/avibactam + Aztreonam **(Figure 6.7)**	**ATM:** Growth = Not susceptible **CZA:** No growth = Susceptible **ATM + CZA:** No growth = Susceptible
K. pneumoniae BAA2146	Not susceptible to Aztreonam or ceftazidime/avibactam. Susceptible to ceftazidime/avibactam + Aztreonam **(Figure 6.8)**	**ATM:** Growth = Not susceptible **CZA**: Growth = Not susceptible **ATM + CZA:** No growth = Susceptible
E.coli AR Bank #0348 (Alternative strains: *E.coli* AR Bank #0434 or *E.coli* AR Bank #0450)	Not susceptible to any antimicrobial agents evaluated **(Figure 6.9)**	**ATM:** Growth = Not susceptible **CZA:** Growth = Not susceptible **ATM + CZA:** Growth = Not susceptible

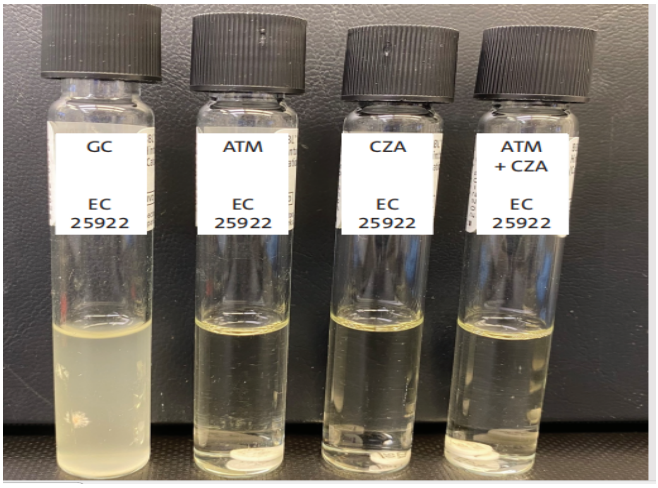


Figure 6.6 Disc elution test with QC strain of *E. coli* ATCC 25922 using Aztreonam (ATM), Ceftazidime-Avibactam (CZA), and their combination (ATM + CZA)

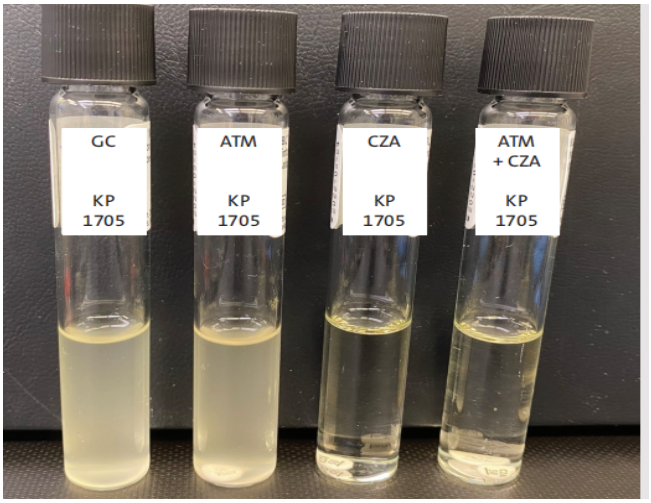


Figure 6.7 Disc elution test with QC strain of *K. pneumoniae* BAA1705 using Aztreonam (ATM),

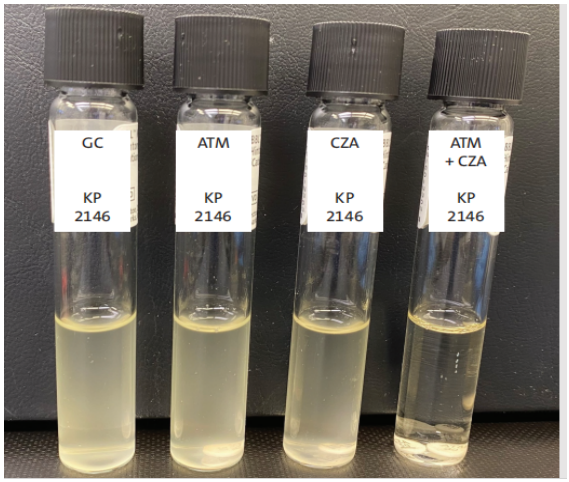


Figure 6.8 Disc elution test with QC strain of *K. pneumoniae* BAA2146 using Aztreonam (ATM), Ceftazidime-Avibactam (CZA), and their combination (ATM + CZA)

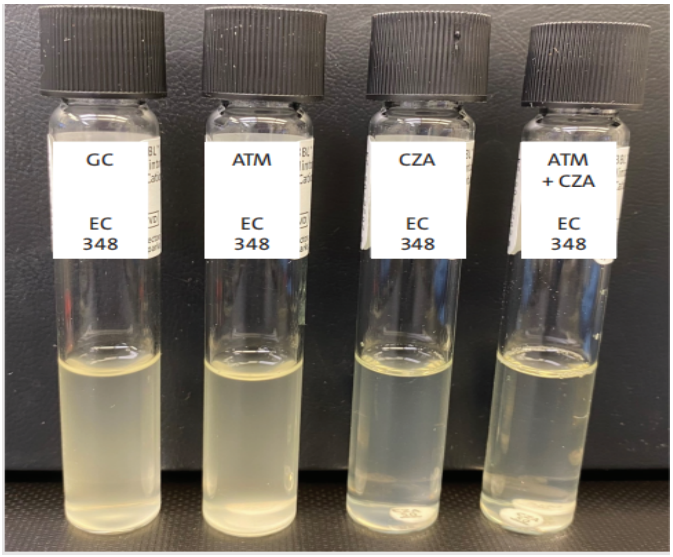


Figure 6.9: Disc elution test with QC strain of *E. coli* AR Bank #0348 using Aztreonam (ATM), Ceftazidime-Avibactam (CZA), and their combination (ATM + CZA) (Alternative strains: *E. coli* AR Bank #0434 or *E. coli* AR Bank #0450)

6.5.6 Interpretation

1. Check the purity plate to ensure the inoculum was pure, and also check the growth control (GC) tube, which must show visible growth for the test to be valid.
2. Examine each of the ATM, CZA, and ATM + CZA tubes for growth or no growth. Any visible turbidity should be interpreted as growth.
3. For *Enterobacterales* and *S. maltophilia*, interpret the results as follows:
    - No growth = Susceptible
    - Growth = Not susceptible

6.6 D-test for inducible clindamycin resistance (CLSI, 2026)

To detect inducible clindamycin resistance in staphylococci resistant to erythromycin and susceptible or intermediate to clindamycin, perform the D-test.

1. Place the clindamycin disk (2 µg) at a distance of 15-26 mm, edge to edge, from the erythromycin disk (15 µg) on Mueller-Hinton agar (MHA).
2. Incubate the plate at 35ºC for 18 hours.
3. Examine the clindamycin inhibition zone for D-shaped blunting adjacent to the erythromycin disk or for hazy growth within the zone of inhibition around the clindamycin disk. Either finding indicates inducible clindamycin resistance.
4. If the inducible clindamycin resistance test is positive, the clindamycin zone size should be entered as 14 mm or MIC as 4 µg/mL.

6.7 Vancomycin screen agar for *S. aureus* and *Enterococcus* spp. (CLSI, 2026)

Brain heart infusion (BHI) agar supplemented with vancomycin hydrochloride at a concentration of 6 µg/mL is used for vancomycin screening.

1. Prepare a suspension of the test strain equivalent to a 0.5 McFarland standard.
2. Using a 10 µL loop, spot-inoculate the suspension onto the surface of vancomycin screen agar containing 6 µg/mL vancomycin. Include the control strains *E. faecalis* ATCC 51299 (resistant) and *E. faecalis* ATCC 29212 (susceptible) on each plate.
3. Incubate at 35ºC for 24 hours.
4. Examine the plates using transmitted light.
5. The presence of more than one colony of the strain or a light film of growth indicates reduced susceptibility to vancomycin. Confirm vancomycin resistance by determining the MIC.

6.8 MIC for vancomycin by broth microdilution method (CLSI 2026; CLSI 2024)

6.8.1 Principle

This test uses double-strength Müeller-Hinton broth, 4x-strength antibiotic solutions prepared as serial two-fold dilutions and the test organism at a concentration of 2 x 10^6/mL. In a 96-well plate, 100 µl of double-strength MHB, 50 µl each of the antibiotic dilutions and the organism suspension are mixed and incubated at 35°C for 18-24 hours. The lowest concentration showing inhibition of growth will be considered the MIC of the organism.

6.8.2 Procedure

1. Prepare a standardised inoculum using either direct colony suspension or growth method.
2. Optimally, within 15 minutes of preparation, dilute the adjusted inoculum suspension in water, saline, or broth so that, after inoculation, each well contains approximately 5 x 10^5CFU/mL.
3. The 0.5 McFarland suspension (1 x 10^8 CFU/mL) should be diluted 1:10 to yield 10^7 CFU/mL. When 0.005 mL of this suspension is inoculated into the broth, the final test concentration of bacteria will be approximately 5 x 10^5 CFU/mL (or 5 x 10^4 CFU/well in the microdilution method).

4. Within 15 minutes after the inoculum has been standardised as described above, inoculate each well of a microdilution tray using an inoculator device that delivers a volume that does not exceed 10% of the volume in the well.
5. It is advisable to perform a purity check of the inoculum suspension by subculturing an aliquot onto a nonselective agar plate for simultaneous incubation.
6. To prevent drying, seal each tray in a plastic bag, with plastic tape, or with a tight-fitting plastic cover before incubating.

6.8.3 Interpretations of results

The MIC is the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in the tubes or microdilution wells as detected by the unaided eye. The lowest concentration of vancomycin on which the strain showed no growth is taken as the minimum inhibitory concentration of vancomycin for that strain.

6.9 MIC Determination by E-test (CLSI 2026)

6.9.1 Preparation of inoculum and E-test procedure:

1. Remove the E-test package from the freezer (-20ºC) at least 30 minutes before use.
2. Using a sterile loop, touch the top of 3-4 individual colonies and transfer them into a tube containing sterile saline.
3. Emulsify the inoculum against the inner wall of the tube to avoid clumping.
4. Adjust the turbidity of the suspension to match a 0.5 McFarland standard.
5. Inoculate the agar plate and ensure the agar surface is dry, but not excessively dry.
6. Swab the plate within 15 minutes of preparing the adjusted inoculum.
7. Dip a sterile cotton swab into the inoculum and, before removal, rotate it several times against the inner wall of the tube above the fluid level to remove excess liquid.
8. Streak the swab evenly across the entire agar surface. Rotate the plate approximately 60º and then repeat streaking. Rotate another 60º and streak again. Finally, run the swab around the rim of the agar to complete inoculation.
9. Leave the plate lid slightly ajar for 5-15 minutes to allow excess surface moisture to absorb before applying strips.
10. Apply E-test strips onto the agar surface using sterile forceps (or E-test applicator if available). Place the strip with the ‘E end’ at the centre of the plate, ensuring the scale is clearly visible.
11. Do not remove a strip once it has touched the agar.
12. Incubate plates at 37 °C for 18 hours in ambient air.

6.9.2 Interpretation and reporting of results

1. Read the MIC at the point where the ellipse of inhibition intersects the scale on the strip.
2. If the MIC value falls between two two-fold dilutions, round up to the higher value.
3. Always read the MIC value at the point of complete inhibition of all visible growth, including isolated colonies.
4. If the intersection differs on either side of the strip, record the higher MIC value.
5. For sulfonamide and trimethoprim, interpret the MIC at 80% inhibition of growth.
6. Ignore swarming of *Proteus* species.
7. Ignore any faint growth directly along the edge of the strip.
8. Breakpoint values are the MIC thresholds used to categorise isolates as **S** (susceptible), **I** (intermediate), or **R** (resistant).

6.10 MIC Determination by Automated System

Note: The example of VITEK2 is being used as an illustrative example

6.10.1 Reagents

The reagent cards contain 64 wells, each holding an individual test substrate for identification and antibiotics at different concentrations for susceptibility testing. The substrates measure various metabolic activities such as acidification, alkalinization, enzyme hydrolysis, and growth in the presence of inhibitory substances. An optically clear film on both sides of the card allows appropriate oxygen transmission while maintaining a sealed system that prevents contact with the organism-substrate mixtures. Each card is equipped with a pre-inserted transfer tube for inoculation. Barcodes on the cards provide information on product type, lot number, expiration date, and a unique identifier that links the card to the sample after loading onto the system.

For the identification of different organism classes, four reagent cards are currently available:

1. GN (21341) - Gram-negative fermenting and non-fermenting bacilli
2. GP (21342) - Gram-positive cocci and non-spore-forming bacilli
3. YST (21343) - Yeasts and yeast-like organisms
4. NH (21346) - Fastidious microorganisms

For the sensitivity of different organism classes, currently five reagent cards are available:

1. AST N281 (414532) - Sensitivity of Gram-negative bacilli
2. AST P628 (414534) - Sensitivity of Gram-positive cocci
3. AST YS08 (420739) - Sensitivity for Yeast

4. AST ST03 (421040) - Sensitivity for streptococci
5. AST GN 69 (413400) - Sensitivity for urinary Gram-negative bacilli.

6.10.2 Suspension preparation

Using a sterile swab or applicator stick, transfer an adequate number of colonies from a pure culture and suspend the microorganism in 3.0 mL of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) in a 12 x 75 mm clear plastic (polystyrene) test tube. Adjust the suspension to the required turbidity and measure using a turbidity meter.

The acceptable McFarland turbidity range is as follows:

- GN and GP cards: 0.50–0.63 McFarland
- YST cards: 1.80–2.20 McFarland

6.10.3 Inoculation

Reagent cards should be inoculated with microorganism suspensions. The test tube containing the suspension is placed in a special rack (cassette), and the identification card is positioned in the adjacent slot with the transfer tube into the corresponding suspension tube. Each cassette can accommodate up to 10 tests; the filled cassette should be placed manually into the incubation chamber.

6.10.4 Card sealing and incubation

Inoculated cards are processed by a mechanism that cuts off the transfer tube and seals the card before loading it into the carousel incubator. The carousel incubator can accommodate up to 60 cards, and all card types are incubated on-line at 35.5 + 1.0ºC. Each card is removed from the carousel incubator every 15 minutes, transferred to the optical system for reaction readings, and then returned to the incubator. Results will be interpreted using software**.**

6.11 Detection of Efflux Pump Overexpression by MIC Determination

Antibiotic efflux is an important mechanism of antimicrobial resistance in bacteria. In Gram-negative bacteria, multidrug efflux pumps with broad substrate specificity can act synergistically with the outer membrane permeability barrier, contributing to intrinsic and acquired multidrug resistance (MDR). Overexpression of these pumps can confer resistance to multiple antibiotics by actively exporting structurally unrelated compounds.

6.11.1 Levofloxacin with and without efflux pump inhibitor phenyl-arginine-beta-napthylamide (EPI-PAβN)

Checkerboard assay for screening the efflux pump overexpression (EPO) phenotype

1. This test is done using levofloxacin with and without efflux pump inhibitor to rule out the efflux pump overexpression (EPO) phenotype by MIC determination according to CLSI protocol.
2. EPI – PAβN of 20µg/mL should be used as a standard with levofloxacin ranging from 1.25 to 80 µg/mL.
3. Incubate the plates for 16-18 hours at 37^0C.

6.11.2 Results and interpretation

- A > 4-fold difference between levofloxacin and the efflux pump inhibitor than levofloxacin alone is considered to be positive for EPO.
- MIC ranges should be compared with the standard strains PAO1 and ATCC 27853.

Chapter 7

Genotypic Tests for Mechanisms of Resistance

This chapter describes molecular methods used for the detection and characterisation of antimicrobial resistance determinants in selected bacterial pathogens. It outlines the procedures for DNA extraction, PCR-based detection and sequencing-based approaches used in the assessment of resistance mechanisms.

7.1 Enterobacterales

To determine the prevalence of antimicrobial resistance genes among members of the order *Enterobacterales*, molecular testing must be performed for the detection of the following β-lactamase and carbapenemase genes: *bla*TEM, *bla*SHV, *bla*OXA, *bla*CTX-M, AmpC β-lactamase genes, *bla*KPC (KPC1 and KPC2), *bla*VIM, *bla*IMP, and *bla*NDM. In addition, polymerase chain reaction (PCR) assays should be performed for the detection and characterisation of Class A (metallo-beta-lactamase), Class C (AmpC) and Class D carbapenemase genes, as applicable **(Figure 7.1)**.

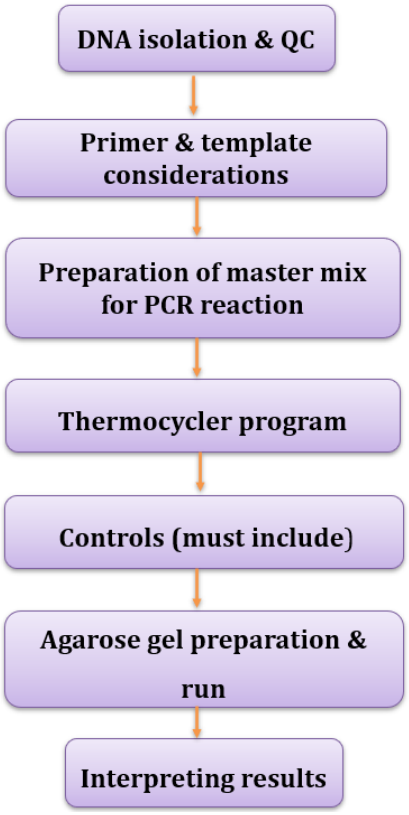


Figure 7.1: Practical workflow to be followed from DNA isolation through running an agarose gel electrophoresis after PCR

7.1.1 DNA isolation and QC

The total genomic DNA from bacterial isolates should be extracted using either a commercially available DNA extraction kit or a validated manual extraction protocol (e.g. DNA extraction by boiling method) appropriate for the sample type and downstream application.

A) DNA extraction by the boiling method

Principle: The boiling method is based on heat-induced lysis of bacterial cells. Exposure to freezing and high temperatures disrupts the bacterial cell wall and membrane, resulting in the release of genomic DNA into the solution.

Procedure:

1. Transfer 3–4 well-isolated bacterial colonies (or one loopful of bacterial growth) into 100 µL of sterile nuclease-free water or Tris-EDTA (TE) buffer.
2. Vortex thoroughly to obtain a uniform suspension.
3. Freeze the suspension at -20°C for 20 minutes.
4. Subject the suspension to heat shock by incubating at 95°C, followed by boiling for 20 minutes.
5. Centrifuge the lysate at 8,000 rpm for 10 minutes.
6. Carefully transfer the supernatant containing the DNA to a sterile nuclease-free microcentrifuge tube without disturbing the pellet.
7. Store the extracted DNA at −20°C until further use.

Advantages: Rapid, cost-effective, requires minimal reagents, suitable for routine PCR-based applications.

Limitations: Produces crude DNA preparations; not suitable for long-term DNA storage or applications requiring highly purified DNA, such as whole-genome sequencing or other sensitive molecular analyses.

B) QC of extracted DNA

1. Measure DNA concentration using a NanoDrop spectrophotometer by recording the absorbance at 260 nm (A260).
2. Check DNA purity by determining the A260/A280 ratio. A ratio of approximately 1.8 is considered indicative of acceptable DNA purity.
3. Where required (when high-quality DNA is required for applications such as sequencing or other sensitive molecular analyses), DNA integrity may be assessed by electrophoresis of 1.0 µL of the extracted DNA on a 0.8% agarose gel. Intact DNA appears as a high-molecular-weight band with minimal or no smearing.

7.1.2 Polymerase chain reaction (PCR)

For the detection of the 16 target resistance genes, simplex PCR assays should be standardized initially to optimize amplification conditions and ensure assay performance. Subsequently, the multiplex PCR assays described by Dallenne et al. (Dallenne et al., 2010, JAC) must be evaluated and implemented, as appropriate, for simultaneous detection of multiple target genes. Due to its specific assay requirements, detection of the *bla*NDM gene is performed using a simplex PCR assay rather than as part of the multiplex PCR panel (Zarfel et al., 2011, Clin Microbiol Infect).

The target genes and corresponding primer sequences to be used for PCR are listed in **Table 7.1**.

Table 7.1:PCR gene targets and primers for mechanisms of resistance in Enterobacterales

PCR name	β-lactamase targeted	Primers	Amplicon (bp)
Multiplex I TEM, SHV and OXA-1	TEM1 and TEM2	F: CATTTCCGTGTCGCCCTTATTC R: CGTTCATCCATAGTTGCCTGAC	800
	SHV	F: AGCCGCTTGAGCAATTAAAC R: ATCCCGCAGATAAATCACCAC	713
	OXA1, 4 and 30	F: GGCACCAGATTCAACTTTCAAG R: GACCCCAAGTTTCCTGTAAGTG	564
Multiplex II CTX-M1, 2 and 9	Variants of CTX-M group 1, M3 and 15	F: TTAGGAARTGTGCCGCTGYA R: CGATATCGTTGGTGGTRCCCAT	688
	Variants of CTX-M group 2	F: CGTTAACGGCACGATGAC R: CGATATCGTTGGTGGTRCCAT	404
	Variants of CTX-M group 9 and CTX-M14	F: TCAAGCCTGCCGATCTGGT R: TGATTCTCGCCGCTGAAG	561
Multiplex III ACC, FOX, MOX, DHA, CIT and EBC	AmpC beta lactamases ACC1 and 2	F: CACCTCCAGCGACTTGTTAC R: GTTAGCCAGCATCACGATCC	346
	FOX1 to 5	F: CTACAGTGCGGGTGGTTT R: CTATTTGCGGCCAGGTGA	162
	MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11 and CMY-19	F: GCAACAACGACAATCCATCCT R: GGGATAGGCGTAACTCTCCCAA	895
	DHA-1 and DHA-2	F: TGATGGCACAGCAGGATATTC R: GCTTTGACTCTTTCGGGTATTCG	997
	LAT-1 to LAT-3, BIL-1, CMY-2 to CMY-7, CMY-12 to CMY-18 & CMY-21 to CMY-23	F: CGAAGAGGCAATGACCAGAC R: ACGGACAGGGTTAGGTTAGGA TAGY	538

Multiplex IV Metallo beta lactamases and carbapenemases	IMP	F: TTGACACTCCATTTACDG R: GATYGAGAATTAAGCCACYCT	139
	VIM	F: GATGGTGTTTGGTCGCATA R: CGAATGCGCAGCACCAG	390
	KPC	F: CATTCAAGGGCTTTCTTGCTGC R: ACGACGGCATAGTCATTTGC	538
Simplex	NDM-1	F: ACCGCCTGGACCGATGACCA R: GCCAAAGTTGGGCGCGGTTG	264
Simplex	OXA-48	F: TATATTGCATTAAGCAAGGG R: CACACAAATACGCGCTAACC	800

bp, base pair product size

I) Preparation of master mixture and PCR conditions:

A) <u>Multiplex PCR for TEM, SHV, OXA1:</u>

i) PCR for Multiplex I:

A 25 µL PCR reaction mix must be prepared, containing 200 ng of DNA, 1x PCR buffer, 1U Taq polymerase, 1.5 mM $MgCl_2$, 0.2 mM each of the four dNTPs, 10 pm/µL of primers (Table 7.1), and dH_2O to make the volume to 25 µL.

PCR reaction mixture (1 PCR reaction) for multiplex I:

PCR reaction mixture	Volume (µL)
PCR buffer	2.5
dNTP	0.5
TEM-F	1
TEM-R	1
SHV-F	1
SHV-R	1
OXA-F	1
OXA-R	1
Taq Polymerase	0.2
H_2O	13.8
DNA	2

Thermal cycler program used for Multiplex I:

94°C for 10 min
94°C for 40 sec
60°C for 40 sec
72°C for 1 min
} 30 cycles
72°C for 7 min
4°C ∞

ii) PCR for Multiplex II:

25 µL PCR reaction mix must be prepared, containing 200 ng of DNA, 1x PCR buffer, 1U Taq polymerase, 1.5 mM $MgCl_2$, 0.2 mM each of four dNTPs, 10 pm/µL of primers (Table 7.1) and dH_2O to make the volume to 25 µL.

PCR reaction mixture for multiplex II:

PCR reaction mixture	Volume (µL)
PCR buffer	2.5
dNTP	0.5
CTXM-1-F	0.5
CTXM-1-R	0.5
CTXM-2-F	0.5
CTXM-2-R	0.5
CTXM-9-F	1
CTXM-9-R	1
CTXM-8/25-F	1
CTXM-8/25-R	1
Taq DNA polymerase	0.2
H_2O	13.8
DNA	2

Detailed thermal cycler program used for multiplex II:

94°C for 10 min
94°C for 40 sec
60°C for 40 sec
72°C for 1 min } 30 cycles
72°C for 7 min
4°C ∞

iii) PCR for Multiplex III:

25 µL PCR reaction mix must be prepared, containing 200 ng of DNA, 1x PCR buffer, 1U Taq polymerase, 1.5 mM $MgCl_2$, 0.2 mM of each of four dNTPs, 10 pm/µL of primers (Table 7.1) and dH_2O to make the volume to 25 µL.

PCR reaction mixture for multiplex III:

PCR reaction mixture	Volume (µL)
PCR buffer	2.5

dNTP	0.5
ACC-F	0.5
ACC-R	0.5
FOX-F	1.25
FOX-R	1.25
MOX-F	0.5
MOX-R	0.5
DHA-F	1.25
DHA-R	1.25
CIT-F	0.5
CIT-R	0.5
EBC-F	0.5
EBC-R	0.5
Taq DNA polymerase	0.2
H_2O	10.8

Detailed thermal cycler program used for multiplex III:

94°C for 10 min
94°C for 40 sec
60°C for 40 sec
72°C for 1 min } 30 cycles
72°C for 7 min
4°C ∞

iv) PCR for Multiplex IV:

25 µL PCR reaction mix must be prepared, containing 200 ng of DNA, 1x PCR buffer, 1U Taq polymerase, 1.5 mM $MgCl_2$, 0.2 mM of each of four dNTPs, 10 pm/µL of primers (Table 7.1) and dH_2O to make the volume to 25 µL.

PCR reaction mixture for multiplex IV:

PCR reaction mixture	**Volume (µL)**
PCR buffer	2.5
dNTP	0.5
IMP-F	1.25
IMP-R	1.25
VIM-F	1.25
VIM-R	1.25
KPC-F	0.5

KPC-R	0.5
Taq DNA polymerase	0.2
H_2O	13.8
DNA	2

Detailed thermal cycler program used for multiplex IV:

94°C for 10 min
94°C for 40 sec
55°C for 40 sec
72°C for 1 min } 35 cycles
72°C for 7 min
4°C ∞

B) <u>Monoplex PCR for *bla*NDM gene:</u>

25 µL PCR reaction mix should be prepared, containing 200 ng of DNA, 1x PCR buffer, 1U Taq polymerase, 1.5 mM $MgCl_2$, 0.2mM of four dNTPs, 10 pm/µL of primers (Table 7.1) and dH_2O to make the volume to 25 µL.

PCR reaction mixture for NDM:

PCR reaction mixture	**Volume (µL)**
PCR buffer	2.5
dNTP	0.5
NDM-F	1
NDM-R	1
Taq DNA polymerase	0.2
H_2O	17.8
DNA	2

Detailed thermal cycler program used for NDM:

95°C for 5 min
95°C for 1 min
60°C for 30 sec } 35 cycles
72°C for 1 min
72°C for 10 min
4°C ∞

C) <u>Monoplex for *bla*OXA-48 like gene:</u>

25 µL PCR reaction mix will be prepared, containing 200 ng of DNA, 1x PCR buffer, 1U Taq polymerase, 1.5 mM $MgCl_2$, 0.2 mM of each of four dNTPs, 10 pm/µL of primers (Table 7.1) and dH_2O to make the volume to 25µL.

PCR reaction mixture for OXA-48:

PCR reaction mixture	Volume (µL)
PCR buffer	2.5
dNTP	0.5
OXA48-F	1
OXA48-R	1
Taq DNA polymerase	0.2
H_2O	17.8
DNA	2

Detailed thermal cycler program used for OXA-48:

94°C for 10 min
95°C for 30 sec
51°C for 1 min
72°C for 30 sec
} 30 cycles
72°C for 7 min
4°C ∞

II) Agarose Gel Electrophoresis

Materials required:

- Agarose powder
- Buffer: 1× TAE *or* 1× TBE
- DNA stain: EtBr (0.5 µg/mL)
- 6× loading dye
- DNA ladder (100 bp or mixed range)
- Gel tray, comb, casting tape
- Electrophoresis tank & power supply
- Microwave or hot plate, heat-resistant flask
- UV or blue-light transilluminator

Preparation of Gel:

1. Calculate agarose for 1.5% for 100 mL
2. Add agarose to 100 mL 1× buffer (TAE/TBE) in a flask.
3. Heat until fully clear. Swirl carefully.
4. Cool to ~60°C (flask comfortably hot to hold).
5. Add EtBr stain to a final concentration of 0.5 µg/mL

6. Seal tray ends, insert comb. Pour gel (3–5 mm thick).
7. Let solidify for 20–30 min, then place gel in tank and cover with 1× buffer.

Gel run conditions:

1. Load 5–10 µL PCR sample product and 5 µL ladder.
2. Voltage: ~5–8 V/cm (distance between electrodes). Typical mini-gel (10 cm) (80–100 V).
3. Run 30–45 min (or until dye front is ~70–80% of gel).
4. Visualize bands on UV or blue light; photograph.

Interpreting results:

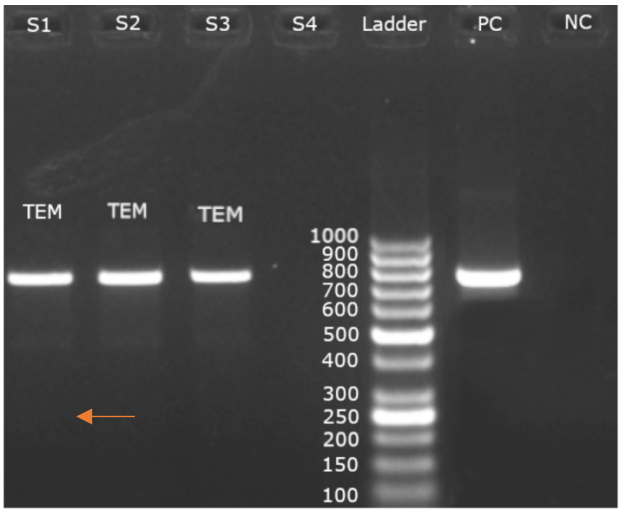


Figure 7.2: Agarose gel electrophoresis showing PCR amplification of the TEM gene in clinical isolates. Lanes S1–S4 represent clinical samples; Ladder represents the DNA molecular weight marker (100–1,000 bp); PC, positive control; and NC, negative control (no-template control). Distinct bands of approximately 800 bp observed in the clinical samples and positive control indicate successful amplification of the target TEM gene, while no amplification is observed in the negative control

7.2. Typhoidal *Salmonella: Salmonella* Typhi

7.2.1. Mechanism of resistance test panel

Table 7.2: Genes, primers, product sizes and characteristics for mechanisms of resistances of *Salmonella* Typhi (Sushila et al., 2014, IJMR; Sharma et al., 2013; IJMM; Lunn AD et al., 2010, Int Microbiol)

Gene	Primer (5'-3')	Amplicon (bp)	Purpose/ Interpretation
gyrA	F: ATGAGCGACCTTGCGAGAGAAATTACACCG R: TTCCATCAGCCCTTCAATGCTGATGTCTTC	630	Fluoroquinolones resistance; sequence QRDR region and interpret relevant substitutions

gyrB	F: GCGCTGTCCGAACTGTACCT R: T GATCAGCGTCGCCACTTCC	181	Fluoroquinolones resistance; sequence QRDR region and interpret relevant substitutions
parC	F: ATGAGCGATATGGCAGAGCG R: TGACCGAGTTCGCTTAACAG	412	Fluoroquinolones resistance; sequence QRDR region and interpret relevant substitutions
parE	F: TCTCTTCCGATGAAGTGCTG R: ATACGGTATAGCGGCGGTAG	240	Fluoroquinolones resistance; sequence QRDR region and interpret relevant substitutions
qnrA	F: ATTTCTCACGCCAGGATTTG R: GATCGGCAAAGGTTAGGTCA	516	Plasmid-mediated resistamce
qnrB	F: GATCGTGAAAGCCAGAAAGG R: ACGATGCCTGGTAGTTGTCC	469	Plasmid-mediated resistamce
qnrS	F: ACGACATTCGTCAACTGCAA R: TAAATTGGCACCCTGTAGGC	417	Plasmid-mediated resistamce
acrR	F: GGTCCTTAAACCCATTGCTG R: ACAGAATAGCGACACAGAAA	816	Regulator of the AcrAB efflux system to identify regulatory variants

Additional azithromycin and efflux-associated analysis

- Include acrB in the molecular analysis and analyse relevant amino-acid substitutions, including R717Q and R717L.
- Interpret acrB variants cautiously: a sequence variant should not automatically be considered a resistance determinant without phenotype–genotype assessment.
- For fluoroquinolones, examine relevant quinolone-resistance-determining regions (QRDRs) in gyrA, gyrB, parC and parE, together with acquired qnr genes.
- For beta-lactam resistance, identify the specific beta-lactamase allele.

7.2.2. Whole Genome Sequencing (WGS) Methodology (Kidgell et al., 2002, Smith et al., 2025, WHO, 2023)

Day 1: Revival and Purity Check of Bacterial Isolates (Smith et al., 2025, WHO, 2023)

- Revive preserved *Salmonella* isolates from glycerol stocks, cryovials or lyophilised cultures.
- Streak onto Blood Agar (BA) and/or MacConkey Agar (MAC) plates using aseptic technique.
- Incubate aerobically at 37°C for 18–24 hours.

- Examine colony morphology and check for contamination.
- Select well-isolated colonies from a pure culture for DNA extraction.

Day 2: Genomic DNA Extraction and Quality Assessment (Smith et al., 2025, WHO, 2023, Katiyar et al., 2020)

- Select 2–3 well-isolated colonies from a fresh culture.
- Extract genomic DNA using a validated bacterial DNA extraction kit, following the manufacturer's instructions.
- Elute DNA in the recommended volume of nuclease-free water or elution buffer.
- Determine DNA concentration and purity using Qubit fluorometer with the dsDNA High Sensitivity (HS) assay.
- Record DNA concentration and A260/A280 ratio (acceptable target: 1.8–1.9).
- Store DNA at 2–8°C for short-term or −20°C for long-term use.

Day 3: Library Preparation

- Quantify and normalize genomic DNA according to the DNA Prep kit requirements.
- Perform DNA fragmentation, adapter addition and indexing according to the manufacturer's instructions.
- Amplify and purify libraries according to the manufacturer's protocol.
- Assess library concentration using Qubit dsDNA HS assay.
- Evaluate fragment size distribution using Bioanalyzer.
- Ensure libraries meet the manufacturer's recommended quality and concentration requirements.

Day 4: Library Pooling and Sequencing

- Normalize and pool libraries in equimolar concentrations.
- Quantify and prepare the pooled library according to manufacturer guidelines.
- Load the prepared library, reagent cartridge and flow cell into the Illumina MiSeq instrument.
- Set up paired-end sequencing (2 × 150 bp or 2 × 250 bp).
- Initiate sequencing and generate raw FASTQ files.

Day 5: Bioinformatics Analysis of *Salmonella* Typhi Whole Genome Sequencing Data (Smith et al., 2025, WHO, 2023, Katiyar et al., 2020, Chen et al., 2018, Bolger et al., 2014, Prjibelski et al., 2020, Gurevich et al., 2013, Bortolaia et al., 2020, Alcock et al., 2023, Carattoli et al., 2014, Argimón et al., 2021, CLSI, 2024)

- After completion of sequencing on the Illumina MiSeq platform, analyse raw sequence data (FASTQ files) using the following workflow.

1: Quality Control of Raw Reads (Chen et al., 2018)

- Analyse quality control for Per-base sequence quality, GC content distribution, Adapter contamination, Overrepresented sequences and Sequence duplication levels.
- Majority of bases should have Phred score ≥ Q30 and minimal adapter contamination.

2: Read Trimming and Filtering (Bolger et al., 2014, Prjibelski et al., 2020)

- Perform trimming and Filtering by using fastp/or Trimmomatic remove Illumina adapter sequences and low-quality bases from read ends.
- Filter reads should have Quality score < Q20–Q30

3: Genome Assembly (Andrews, 2010, Gurevich et al., 2013)

- To reconstruct the bacterial genome from short sequencing reads into longer sequences (contigs), perform genome assembly using SPAdes.
- Use quality-filtered and trimmed paired-end sequencing reads as input for genome assembly.
- Generate assembled genome contigs in FASTA format for downstream analyses, including genome annotation, antimicrobial resistance gene detection, serotype prediction, and phylogenetic analysis.
- Keep number of contigs as low as possible, N50 higher value and coverage depth ≥30×

4: Antimicrobial Resistance and Virulence Analysis

- Upload assembled contigs or FASTQ files on ResFinder / CARD/
- Pathogenwatch (Alcock et al., 2023, Carattoli et al., 2014, CLSI, 2024)
- Screen for acquired resistance genes. Detect chromosomal mutations.

7.2.3 Molecular Characterisation of Antimicrobial Resistance-Associated Genetic Elements in Typhoidal

7.2.3.1. Detection of Class 1 Salmonella Integrons and Mobile Genetic Elements (Katiyar et al., 2020, Alcock et al., 2023, Carattoli et al., 2014, Argimón et al., 2021)

Whole-genome sequencing (WGS) data shall be analysed to identify integrons and mobile genetic elements associated with the acquisition and dissemination of antimicrobial resistance determinants.

1. Screen assembled genome sequences for integron-associated genes, particularly intI1, using IntegronFinder.
2. Identify integron-associated conserved regions and gene cassette arrays.

3. Identify mobile genetic elements, including insertion sequences and transposons.

7.2.3.2. Plasmid Replicon Typing (Argimón et al., 2021)

The following analyses shall be performed using Plasmid Finder to identify whether AMR genes are chromosomal or plasmid-mediated-

1. Identify plasmid replicon types using PlasmidFinder e.g. IncY, IncHI1, IncFIB, IncQ.
2. Screen for plasmid-associated antimicrobial resistance genes, including blaCTX-M, blaSHV, blaTEM, blaNDM, qnr and dfrA.
3. Assess the association between identified plasmid replicons and antimicrobial resistance determinants.

7.2.3.3. Pathogenicity Island Analysis

Use SPI Finder to identify virulence-associated pathogenicity islands e.g. SPI-1, SPI-2, SPI-3, SPI-4, SPI-5, SPI-7.

7.2.4. Molecular Clonality Analysis of Typhoidal Salmonella

Sequence typing, lineage assignment and phylogenetic analysis shall be performed to characterise the genomic diversity and population structure of the isolates (Kidgell et al., 2002, Smith et al., 2025, WHO, 2023, Katiyar et al., 2020, CLSI, 2024).

1. Multilocus Sequence Typing (MLST): Perform in-silico MLST using the seven housekeeping genes aroC, dnaN, hemD, hisD, purE, sucA and thrA. Assign sequence types using the Salmonella enterica MLST database (Kidgell et al., 2002, Smith et al., 2025, CLSI, 2024).

2. Lineage assignment: Perform S. Typhi lineage and sub-lineage assignment using GenoTyphi or an equivalent validated tool (Katiyar et al., 2020, CLSI, 2024).

3. **Core-genome SNP analysis:** Identify core-genome single nucleotide polymorphisms (SNPs) to assess genetic differences and relatedness among isolates (Smith et al., 2025, CLSI, 2024)

4. Phylogenetic analysis: Construct phylogenetic trees -

- To determine genetic relatedness between isolates and compare them with national or international strains use Snippy for SNP calling. Select a reference genome (e.g., *Salmonella* Typhi CT18).
- For Whole Genome Comparison of multiple *Salmonella* Typhi genomes simultaneously use Parsnp. Use Interactive Tree of Life (iTOL) to visualize the tree.
- Use Proksee for Genome Visualization and Annotation**,** Circular genome maps, Linear genome maps, GC content visualization, GC skew analysis, AMR gene

locations, Virulence gene locations and Plasmid visualization (Smith et al., 2025, WHO, 2023, Katiyar et al., 2020, CLSI, 2024)

5. Comparative genomic analysis: Compare sequence types, lineage distribution and antimicrobial resistance profiles to investigate the genomic epidemiology and clonal distribution of typhoidal Salmonella isolates.

Table 7.3a: Common Resistance Markers in *S.* Typhi (Dahiya et al., 2014, Sharma et al., 2013, Lunn et al., 2010, Katiyar et al., 2020, Alcock et al., 2023, Carattoli et al., 2014, CLSI, 2024)

Antibiotic class	Resistance genes / molecular targets	Resistance mechanism
Fluoroquinolones	gyrA, gyrB, parC, parE mutations; qnrB, qnrS	QRDR mutations and plasmid-mediated quinolone resistance
Azithromycin	acrB mutations (R717Q, R717L)	Alteration of AcrB efflux pump
ESBL	blaCTX-M-15, blaCTX-M-14, blaSHV-12; confirmed ESBL-associated variants of blaTEM	Extended-spectrum β-lactamase production
Ampicillin / Penicillins	blaTEM-1, blaTEM-1B, blaTEM-1D, blaTEM-116	Narrow-spectrum β-lactamase production
Carbapenems	blaNDM-5	Carbapenemase production
Aminoglycosides	aph(3″)-Ib and aph(6)-Id, aph(3′)-Ia, aac(6′)-Ib	Aminoglycoside-modifying enzymes
Chloramphenicol / Phenicols	catA1, floR	Enzymatic inactivation and efflux
Sulphonamides	sul1, sul2	Altered dihydropteroate synthase
Trimethoprim	dfrA7, dfrA15, other acquired dfrA variants	Altered dihydrofolate reductase
Tetracyclines	tetA, tetB	Efflux-mediated resistance

Table 7.3b: XDR-associated resistance profile (Dahiya et al., 2014, Katiyar et al., 2020, Alcock et al., 2023, Carattoli et al., 2014, CLSI, 2024)

Antibiotic class	Resistance genes / molecular targets
Fluoroquinolones	gyrA, gyrB, parC, parE mutations; qnr genes
Third-generation cephalosporins	blaCTX-M-15, blaSHV-12, other ESBL genes
Ampicillin	blaTEM-1 and other acquired β-lactamase genes
Chloramphenicol	catA1, floR
Trimethoprim-sulfamethoxazole	dfrA7, sul1, sul2

5: Pan-Genome Analysis

- Perform pan genome analysis by using BPGA to compare multiple *Salmonella* Typhi genomes for Core genome identification, Accessory genome analysis, Unique genes, Functional annotation, Gene presence/absence matrix

Pathogenicity Island Analysis

- Use SPI Finder to identify virulence-associated pathogenicity islands. E.g. SPI-1, SPI-2, SPI-3, SPI-4, SPI-5, SPI-7

7.3 Non-Fermenting Gram-negative bacteria (NFGNB) and Diarrheal pathogens

7.3.1 Molecular Detection of Antimicrobial Resistance Genes

Targets include:

- Carbapenemases: *bla*$_{NDM}$, *bla*$_{KPC}$, *bla*$_{IMP,}$ *bla*$_{VIM}$ (*P. aeruginosa*), *bla*$_{OXA-23,}$ *bla*$_{OXA-24,}$ *blaOXA-51*(*A. baumannii*)
- ESBLs: *bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV,}$ *bla*$_{VEB}$, *bla*$_{GES}$ (*P. aeruginosa*), *bla*$_{PER}$ (*A. baumannii*)
- AmpC: *bla*$_{CMY}$, *bla*$_{DHA}$, *bla*$_{FOX}$, *bla*$_{CIT}$
- Other: *sul*, *dfrA*, *bla*$_{OXA-1/10}$, *qnrS*/*qnrB*, *mphA*, *ermB*, *tetO,* etc.

Biosafety

- Follow BSL-2 practices
- Use PPE (lab coat, gloves, eye protection)
- Perform DNA extraction in a biosafety cabinet if required
- Separate areas for:
  - Pre-PCR
  - PCR setup
  - Post-PCR analysis

Sample Requirements

- Pure bacterial isolates from culture
- Confirmed identification (biochemical/MALDI-TOF)

Materials Required

Equipment

- Thermal cycler

- Gel electrophoresis unit
- UV transilluminator/gel documentation system
- Micropipettes with aerosol-resistant tips

Reagents

- DNA extraction kit/boiling method reagents
- PCR master mix
- Primers (validated for AMR targets)
- Agarose, TAE buffer
- DNA ladder
- Ethidium bromide / safe dye

I) Procedure

a) DNA Extraction

Principle

Genomic DNA is extracted from pure bacterial cultures using the kit based on silica membrane spin-column technology. Bacterial cells are lysed enzymatically and chemically, and DNA binds selectively to the membrane, followed by washing and elution of purified DNA. The standard protocol for extraction of genomic DNA from both Gram-negative and Gram-positive bacteria

Reagents and Consumables

Buffer AL, Buffer ATL, Proteinase K, Buffer AW1, Buffer AW2, Buffer AE (elution buffer), Proteinase K, Lysozyme (for enhanced lysis), Absolute ethanol (96–100%), Collection tubes, Spin columns, Sterile 1.5 mL microcentrifuge tubes , Filtered pipette tips: (10 µL, 20 µL, 200 µL, 1000 µL)), 200 µL 1000 µL

Equipment

- Incubator (35–37°C)
- Vortex mixer
- Water bath or thermal block (compatible with 1.5 mL tubes)
- Microcentrifuge (capable of 13,000–14,000 rpm)
- Micropipettes (single-channel): 10 µL, 20 µL, 100 µL, 200 µL, and 1000 µL

Procedure

1. Emulsify a loopful of similar bacterial colonies in 200 µL of 0.85% normal saline and mix thoroughly by vortexing.
2. Add 100 µL of lysozyme and vortex briefly.

3. Incubate at 37°C for 1 hour in a water bath or heat block.
4. Add 20 µL of Proteinase K and mix thoroughly by vortexing.
5. Incubate at 56°C for 30 minutes until complete cell lysis is achieved.
6. Add 200 µL of Buffer AL (cell lysis buffer) and mix well.
7. Incubate at room temperature for 10 minutes.
8. Add 250 µL of chilled absolute ethanol (99.9%) and vortex thoroughly.
9. Transfer the mixture to a DNeasy Mini spin column placed in a 2 mL collection tube.
10. Centrifuge at 8000 rpm for 1 minute. Discard the flow-through and collection tube.
11. Place the spin column into a new collection tube.
12. Add 500 µL of Buffer AW1.
13. Centrifuge at 8000 rpm for 1 minute. Discard the flow-through and collection tube.
14. Add 500 µL of Buffer AW2.
15. Centrifuge at 14,000 rpm for 3 minutes. Discard the flow-through and collection tube.
16. Place the spin column in a new 2 mL collection tube and centrifuge at 20,000 × g for 1 minute to remove residual ethanol.
17. Transfer the spin column to a clean 1.5 mL microcentrifuge tube. Add 100 µL of Buffer AE directly onto the membrane.
18. Incubate at room temperature for 5 minutes.
19. Centrifuge at 8000 rpm for 1 minute to elute the DNA.
20. Transfer the eluted DNA to a fresh, sterile microcentrifuge tube.
21. Store the DNA at -20°C. DNA concentration and purity may be assessed using spectrophotometry.

Notes:

- Ensure complete lysis for Gram-positive organisms (extend lysozyme incubation if needed).
- Avoid ethanol carryover (critical for PCR/WGS).
- Use aerosol-resistant tips to prevent contamination.

b) DNA Quality Control

Principle

DNA quality and quantity are assessed using spectrophotometric analysis based on the Beer–Lambert Law, measuring absorbance at 230 nm, 260 nm, and 280 nm.

Equipment

- NanoDrop One Spectrophotometer

Determination of DNA quality using the NanoDrop One Spectrophotometer

1. Before taking measurements with NanoDrop One, lift the instrument arm and clean the upper and lower pedestals with nuclease-free water.
2. Wipe the pedestals dry with a new laboratory wipe.
3. To measure the nucleic acid, select the Nucleic acid tab from the home screen and tap dsDNA.
4. Pipette 1-2 µL blanking solution (Elution buffer in this case) onto the lower pedestal and lower the arm.
5. Tap Blank and wait for the measurement to complete.
6. Lift the arm and clean both the arm and pedestal with a fresh laboratory wipe.
7. Pipette 1-2 µL DNA sample onto the pedestal and lower the arm.
8. Sample measurement will start if Auto-measure is on; if Auto-measure is off, tap Measure.
9. When sample measurement is completed, the spectrum and reported values will be displayed.
10. Tap End experiment, lift the arm, and clean both pedestals with a clean wipe.
11. For each DNA sample, the UV absorbance spectrum and summary of the results will be available on the screen as shown below **(Figure 7.3)**.

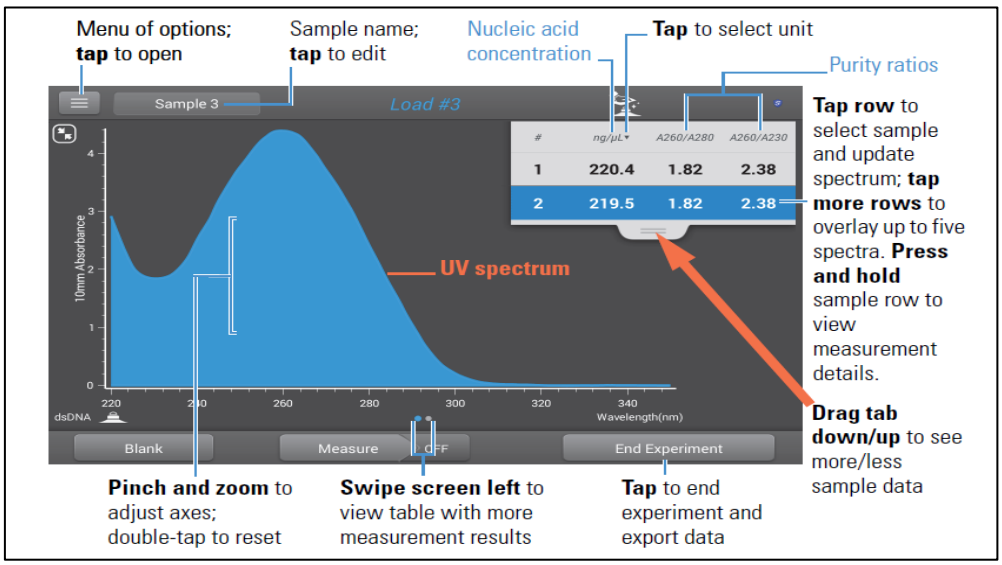


Figure 7.3: NanoDrop spectrophotometric analysis of extracted DNA. Representative absorbance spectrum obtained using a NanoDrop spectrophotometer, showing nucleic acid quantification and purity assessment. The absorbance peak at 260 nm indicates nucleic acid concentration, while the A260/A280 and A260/A230 ratios are used to evaluate protein and organic contaminant levels, respectively. Acceptable purity is indicated by an A260/A280 ratio of ~1.8 and an A260/A230 ratio between 1.8–2.2.

Interpretation Criteria

Parameter	Acceptable Range	Interpretation
DNA concentration	≥30 ng/µL	Suitable for PCR/WGS
A260/280 ratio	~1.8 (1.7–2.0)	Pure DNA
A260/230 ratio	1.8–2.2	Low contamination

Interpretation Guidelines

- Low A260/280 (<1.6) - Protein contamination
- Low A260/230 (<1.5) - Salt/organic contamination
- High ratios (>2.2) - Possible RNA contamination

Troubleshooting

Issue	Possible Cause	Corrective Action
Low DNA yield	Incomplete lysis	Increase lysozyme/proteinase K incubation
Poor purity (low 260/280)	Protein contamination	Repeat extraction
Low 260/230	Salt carryover	Additional wash or ethanol removal
No amplification	Inhibitors present	Dilute DNA or re-extract

c) Storage of DNA samples (pre-PCR handling)

Purpose

To ensure integrity, stability, and contamination-free handling of extracted DNA prior to PCR and downstream molecular applications.

Principle

DNA stability is influenced by temperature, enzymatic degradation (DNases), and repeated freeze–thaw cycles. Proper storage minimises degradation and preserves template quality for reliable amplification.

Storage Conditions

Storage Type	Temperature	Duration	Remarks
Short-term	2–8°C	≤24–48 hours	For immediate PCR use
Medium-term	−20°C	Up to 6 months	Routine storage
Long-term	−80°C	>6 months	For archival/reference samples

Procedure

- Transfer eluted DNA into sterile, DNase-free microcentrifuge tubes.
- Label clearly with:
  - Sample ID
  - Organism
  - Date of extraction
  - Operator initials
- Aliquot DNA into multiple tubes (20–50 µL each) to avoid repeated freeze–thaw cycles.
- Store at −20°C for routine PCR use.
- For long-term storage or sequencing applications, store at −80°C.

Handling Guidelines

- Avoid more than 2–3 freeze–thaw cycles.
- Thaw DNA on ice before use.
- Mix gently (avoid vortexing high molecular weight DNA).
- Use aerosol-resistant tips to prevent contamination.

d) Positive Control Management

Purpose

To ensure validity, reliability, and reproducibility of PCR assays by including appropriate positive controls for each target gene.

Principle

Positive controls contain known AMR gene targets and confirm PCR reagents are functional, primers are working, and thermal cycling conditions are optimal

Types of Positive Controls

- Reference Strain Controls: Previously characterised bacterial isolates carrying specific AMR genes
- Previously WGS-confirmed clinical isolates: Sequencing-validated strains

Table 7.4:Recommended Gene-wise Controls

S. No	Target	Organism
1	*bla*$_{NDM}$	*K. pneumoniae*
2	*bla*$_{OXA-51}$	*A. baumannii*
3	*bla*$_{OXA-23}$	*A. baumannii*

4	*bla*$_{\text{OXA-24}}$	*A. baumannii*
5	*bla*$_{\text{OXA-58}}$	*A. baumannii*
6	*bla*$_{\text{KPC}}$	*K. pneumoniae*
7	*bla*$_{\text{VIM}}$	*P. aeruginosa*
8	*bla*$_{\text{IMP}}$	*P. aeruginosa*
9	*bla*$_{\text{GES}}$	*P. aeruginosa*
10	*bla*$_{\text{VEB}}$	*A. baumannii*
11	*bla*$_{\text{PER}}$	*A. baumannii*
12	*bla*$_{\text{CTX-M}}$	*E. coli*
13	*bla*$_{\text{SHV}}$	*K. pneumoniae*
14	*bla*$_{\text{TEM}}$	*E. coli*
15	*bla*$_{\text{CMY}}$	*E. coli*
16	*bla*$_{\text{DHA}}$	*E. coli*
17	*bla*$_{\text{OXA-1}}$	*E. coli*
18	*sul*	*Salmonella sp*
19	*dfrA*	*Salmonella sp*
20	*qnrS*	*Salmonella sp*
21	*qnrB*	*Salmonella sp*
22	*mphA*	*Shigella sp*
23	*ermB*	*Shigella sp*

Preparation of Positive Control DNA

- Extract DNA using standardised protocol (same as test samples)
- Quantify DNA concentration
- Aliquot into small volumes (10–20 µL)
- Label with:
    - Gene target
    - Strain ID
    - Date

Storage of Positive Controls

Type	Storage Condition
DNA aliquots	−20°C
Long-term archival	−80°C

Working aliquot	−20°C (separate from stock)

Usage in PCR

- Include at least one positive control per gene target in each PCR run.
- Add control DNA in a separate area (template addition zone).
- Use minimal volume (1–2 µL) to reduce contamination risk.

Acceptance Criteria

- Positive control must show:
  - Band at expected size
- Negative control must show:
  - No amplification
- If criteria are not met, invalidate run and repeat.

Troubleshooting

Issue	Possible Cause	Action
No amplification in control	PCR failure	Check reagents
Weak band	DNA degradation	Use fresh aliquot
Contamination in NTC	Carryover contamination	Decontaminate workspace

e) Primer Reconstitution, Stock Preparation and Dilution

Purpose

To standardise preparation, handling, and storage of oligonucleotide primers to ensure:

- Consistent PCR performance
- Reproducibility across laboratories
- Minimal degradation and contamination

Principle

Primers are supplied in lyophilised form and must be reconstituted to a defined stock concentration (typically 100 µM), followed by preparation of working solutions (2 µM) for routine PCR use.

Materials

- Lyophilised primers (forward and reverse)
- Nuclease-free water or TE buffer (pH 8.0)

- Sterile DNase/RNase-free microcentrifuge tubes
- Low-retention pipette tips

Stock Solution Preparation (100 µM)

Formula: Volume (µL) = nmol of primer × 10

Example:

- If primer supplied = 25 nmol
- Add 250 µL nuclease-free water = gives 100 µM stock

Procedure

1. Briefly centrifuge primer vial.
2. Add calculated volume of nuclease-free water.
3. Mix gently (pipette up/down; avoid vigorous vortexing).
4. Incubate at room temperature for 10 minutes to ensure complete dissolution.
5. Label clearly:
    - Primer name
    - Concentration (100 µM)
    - Date

Working Solution Preparation (10 µM)

Dilution Formula

$$C1 \text{ x } V1 = C2 \text{ x } V2$$

Example

From 100 µM → 2 µM

Take 2 µL stock + 98 µL water

Procedure

- Prepare in a sterile tube:
    - 2 µL stock primer
    - 98 µL nuclease-free water
- Mix gently
- Label as Working Primer (2 µM)

Recommended Use in PCR

Application	Final Primer Concentration
Singleplex PCR	0.2–0.5 µM

Multiplex PCR	0.1–0.3 µM (optimize per primer)

Storage Conditions

Solution Type	Temperature	Duration
Stock (100 µM)	-20°C	Long-term (>1 year)
Working (10 µM)	-20°C	3–6 months
Short-term use	4°C	≤1 week

Troubleshooting

Issue	Possible Cause	Action
No amplification	Incorrect dilution	Recalculate and prepare fresh
Primer dimers	High concentration	Reduce primer concentration
Non-specific bands	Low annealing temp	Optimise PCR conditions
Weak bands	Degraded primer	Use fresh aliquot

7.3.2 Detection of Antimicrobial resistancegenes by multiplex conventional PCR

Purpose

To detect major AMR genes in clinically relevant NFGNB and diarrheal pathogens using multiplex conventional PCR for AMR surveillance, clinical correlation and epidemiological tracking.

Scope

Applicable to:

- NFGNB (*P. aeruginosa*, *A. baumannii* spp. etc.)
- Diarrhoeal pathogens (*E. coli, Shigella, Salmonella, Vibrio cholerae,* etc.)

Principle

Simultaneous amplification of multiple resistance genes in a single PCR reaction using gene-specific primers, followed by agarose gel electrophoresis for detection based on expected amplicon sizes.

A) Target gene panel for molecular AMR detection

This panel includes clinically relevant AMR determinants covering

- Carbapenemases
- ESBLs
- AmpC β-lactamases
- Non-β-lactam resistance genes

Table 7.5: Standardised Target Gene Table for Carbapenemases, ESBL and AmpC

S. No	Target Gene	Gene Family / Variant	Resistance Mechanism	Target Organism
1	*bla*$_{NDM}$	Metallo-β-lactamase	Carbapenem resistance	***P. aeruginosa* & *A. baumannii***
2	*bla*$_{OXA-51-like}$	Class D (intrinsic)	Carbapenem (Acinetobacter marker)	***A. baumannii***
3	*bla*$_{OXA-23-like}$	Class D	Carbapenem resistance	***A. baumannii***
4	*bla*$_{OXA-24like}$	Class D	Carbapenem resistance	***A. baumannii***
5	*bla*$_{OXA-58-like}$	Class D	Carbapenem resistance	***A. baumannii***
6	*bla*$_{KPC}$	Class A carbapenemase	Carbapenem resistance	***P. aeruginosa***
7	*bla*$_{VIM}$	Metallo-β-lactamase	Carbapenem resistance	***P. aeruginosa***
8	*bla*$_{IMP}$	Metallo-β-lactamase	Carbapenem resistance	***P. aeruginosa***
9	*bla*$_{GES}$	ESBL / Carbapenemase variants	Broad β-lactam resistance	***P. aeruginosa***
10	*bla*$_{VEB}$	ESBL	β-lactam resistance	***P. aeruginosa***
11	*bla*$_{PER}$	ESBL	β-lactam resistance	***A. baumannii***
12	*bla*$_{CTX-M}$ (Group 1,2,9)	ESBL	Cephalosporin resistance	**Diarrhoeal**
13	*bla*$_{SHV}$	ESBL	β-lactam resistance	**Diarrhoeal**
14	*bla*$_{TEM}$	ESBL	β-lactam resistance	**All**
15	*bla*$_{OXA-1-like}$	Narrow/ESBL variant	β-lactam resistance	**Diarrhoeal**
16	*bla*$_{CMY}$	Plasmid AmpC	Cephalosporin resistance	**Diarrhoeal**
17	*bla*$_{DHA}$	Plasmid AmpC	Cephalosporin resistance	**All**

Table 7.6: Standardised Target Gene Table for Non-β-lactam Resistance Genes

S. No	Target Gene	Gene Family / Variant	Resistance Mechanism	Target Organism
19	*sul1* / *sul2*	Sulfonamide resistance	Folate pathway inhibition	**Diarrhoeal**
20	*dfrA* (variants)	Trimethoprim resistance	DHFR enzyme alteration	**Diarrhoeal**

21	*qnrS*	PMQR	Fluoroquinolone resistance	**Diarrhoeal**
22	*qnrB*	PMQR	Fluoroquinolone resistance	**Diarrhoeal**
23	*mphA*	Macrolide phosphotransferase	Macrolide resistance	**Diarrhoeal**
24	*ermB*	rRNA methylase	Macrolide resistance	**Diarrhoeal**

B) Organism-wise multiplex PCR panels

To improve standardisation and efficiency, multiplex PCR panels are designed based on organism groups:

- ***A. baumannii***
- ***P. aeruginosa***
- **Diarrhoeal pathogens**

PANEL 1: *Acinetobacter* spp./ *A. baumannii*

Panel 1a: Carbapenemases genes

Purpose: Detection of major carbapenem resistance determinants in *Acinetobacter*

Target Gene	Primer Sequence (5'→3')	Amplicon Size (bp)	Reference
bla$_{\text{OXA-51-like}}$	F – TAATGCTTTGATCGGCCTTG R- TGGATTGCACTTCATCTTGG	353	Woodford et al., 2004, JAC
bla$_{\text{OXA-23-like}}$	F- GATCGGATTGGAGAACCAGA R- ATTTCTGACCGCATTTCCAT	501	Woodford et al., 2004, JAC
bla$_{\text{OXA-24like}}$	F- GGTTAGTTGGCCCCCTTAAA R- AGTTGAGCGAAAAGGGGATT	246	Woodford et al., 2004, JAC
bla$_{\text{OXA-58-like}}$	F- AAGTATTGGGGCTTGTGCTG R- CCCCTCTGCGCTCTACATAC	599	Woodford et al., 2004, JAC
bla$_{\text{NDM}}$	F- GGTTTGGCGATCTGGTTTTC R- CGGAATGGCTCATCACGATC	621	Poirel et al., 2011, Diagnostic microbiology and infectious disease

Panel 1b: ESBL

Purpose: Detection of extended-spectrum β-lactam resistance

Target Gene	Primer Sequence (5'→3')	Amplicon Size (bp)	Reference
bla$_{\text{TEM}}$	F- TCGCCGCATACACTATTCTCAGAATGAC R- ACGCTYACYGGYTCCAGATTTAT	445	Ogutu et al., 2015
bla$_{\text{PER}}$	F- GCTCCGATAATGAAAGCGT	520	Poirel et al., 2011, Diagnostic

	R- TTCGGCTTGACTCGGCTGA		microbiology and infectious disease

PANEL 2: *P. aeruginosa*/ *Pseudomonas* spp.

Panel 2a: Carbapenemases Genes

Purpose: Detection of major carbapenemases in *P. aeruginosa*

Target Gene	Primer Sequence (5'→3')	Amplicon Size (bp)	Reference
*bla*IMP	F- GGAATAGAGTGGCTTAAYTCTC R- GGTTTAAYAAAACAACCACC	232	Poirel et al., 2011, Diagnostic microbiology and infectious disease
*bla*VIM	F- GATGGTGTTTGGTCGCATA R- CGAATGCGCAGCACCAG	390	Poirel et al., 2011, Diagnostic microbiology and infectious disease
*bla*KPC	F- CGTCTAGTTCTGCTGTCTTG R- CTTGTCATCCTTGTTAGGCG	798	Poirel et al., 2011, Diagnostic microbiology and infectious disease
*bla*NDM	F- GGTTTGGCGATCTGGTTTTC R- CGGAATGGCTCATCACGATC	621	Poirel et al., 2011, Diagnostic microbiology and infectious disease

Panel 2b: ESBL

Target Gene	Primer Sequence (5'→3')	Amplicon Size (bp)	Reference
*bla*TEM	F- CATTTCCGTGTCGCCCTTATTC R- CGTTCATCCATAGTTGCCTGAC	800	Ogutu et al., 2015
*bla*VEB	F- GATTAATAACGACTTCCATTWCC R- CATCGCTGTTGGGGTTGCCCAATTTT	391	Poirel et al., 2011, Diagnostic microbiology and infectious disease
*bla*GES	F- AGTCGGCTAGACCGGAAAG R- TTTGTCCGTGCTCAGGAT	399	Poirel et al., 2011, Diagnostic microbiology and infectious disease

PANEL 3: Diarrhoeal Pathogens

Panel 3a: ESBL

Target Gene	Primer Sequence (5'→3')	Amplicon Size (bp)	Reference
*bla*TEM	F- CATTTCCGTGTCGCCCTTATTC R- CGTTCATCCATAGTTGCCTGAC	800	Ogutu et al., 2015
*bla*SHV	F- AGCCGCTTGAGCAAATTAAAC R- ATCCCGCAGATAAATCACCAC	713	Ogutu et al., 2015

bla$_{CTX-M}$ (Groups 1,2,9)	F- SCNATGTGCAGYACCAGTAARG R- CCGCRATATGRTTGGTGGTG	415	Kulkarni et al., 2025
bla$_{OXA-1-like}$	F- ATGAAAAACACAATACATATCAACTTCGC R- GTGTGTTTAGAATGGTGATCGCATT	820	Dallene et al., 2010, JAC
bla$_{CMY}$	F- ATTGATGAAAAAATCGWTATGCT R- TTAYTTCAGYTTTTCAAGAATGCG	1146	Dallene et al., 2010, JAC
bla$_{DHA}$	F- AACTTTCACAGGTGTGCTGGGT R- CCGTACGCATACTGGCTTTGC	405	Pérez-Pérez et al., 2002

Panel 3b: Other

Target Gene	Primer Sequence (5'→3')	Amplicon Size (bp)	Reference
sul1	F- CGGCGTGGGCTACCTGAACG R- GCCGATCGCGTGAAGTTCCG	432	Arabi et al., 2015
sul2	F – GCGCTCAAGGCAGATGGCATT R - GCGTTTGATACCGGCACCCGT	293	Arabi et al., 2015
dfrA1	F – TGGTAGCTATATCGAAGAATGGAGT R- TATGTTAGAGGCGAAGTCTTGGGTA	425	Grape et al., 2007
dfrA5	F- AGCTACTCTTTAAAGCCTTGACGTA R- GTGTTGCTCAAAAACAACTTCG	341	Grape et al., 2007
dfrA7/17	F- ACATTTGACTCTATGGGTGTTCTTC R - AAAACTGTTCAAAAACCAAATTGAA	280	Grape et al., 2007
qnrS	F- ACGACATTCGTCAACTGCAA R- TAAATTGGCACCCTGTAGGC	423	Cattoir et al., 2007
qnrB	F- GATCGTGAAAGCCAGAAAGG R- ACGATGCCTGGTAGTTGTCC	469	Cattoir et al., 2007
mphA	F: GTGAGGAGGAGCTTCGCGAG R: TGCCGCAGGACTCGGAGGTC	403	Brenciani et al., 2007
ermB	F: GAAAAGGTACTCAACCAAATA R: AGTAAGGGTACTTAAATTGTTTAG	639	Brenciani et al., 2007

7.3.2.1 PCR Reaction Setup

Purpose

To prepare a standardised reaction mixture for amplification of AMR genes using multiplex PCR.

Principle

Multiplex PCR involves simultaneous amplification of multiple target genes within a single reaction using gene-specific primer sets. Optimal primer concentration and reagent balance are critical to ensure uniform amplification efficiency and minimise non-specific products.

Procedure

1. Thaw all reagents on ice and mix gently by pipetting.
2. Prepare the master mix in a designated pre-PCR area to minimise contamination.
3. Aliquot the required volume of master mix into sterile PCR tubes.
4. Add template DNA separately in a designated template addition area.
5. Include appropriate positive and negative controls (NTC) in each run.

Notes:

- Primer concentrations may require optimisation depending on multiplex panel complexity.
- Avoid repeated freeze–thaw cycles of reagents.
- Use aerosol-resistant filter tips to prevent contamination.

Master Mix preparation and Thermal Cycling Profile

A) Panel 1: *A. baumannii*/Acinetobacter spp.

i) Panel 1a: Carbapenemases

Master mix preparation

Reagents	Volume per Reaction (µL)	Volume for 5 Reactions (µL)	Volume for 10 Reactions (µL)
2X Multiplex Master mix	10.0	50.0	100.0
Working Stock Primer	2.0	10.0	20.0
Molecular-grade water	6.0	30.0	60.0
Total Master Mix Volume	18.0	90.0	180.0

Multiplex Primer Working Stock (*bla*$_{OXA\text{-}23\text{-}like}$, *bla*$_{OXA\text{-}24\text{-}like}$, *bla*$_{OXA\text{-}58\text{-}like}$):

Primer working stocks for multiplex PCR targeting bla$_{OXA\text{-}23\text{-}like}$, bla$_{OXA\text{-}24\text{-}like}$, and bla$_{OXA\text{-}58\text{-}like}$ genes should be prepared as follows:

- Aliquot 2 µL each of forward and reverse primers corresponding to all three targets (total of six primers).
- This yields a combined primer volume of 12 µL.
- Add 88 µL of nuclease-free water (or molecular-grade water) to achieve a final volume of 100 µL.

Final composition:

- Total primer volume: 12 µL
- Nuclease-free water: 88 µL
- Final volume: 100 µL

Singleplex Primer Working Stock (*bla*$_{\text{NDM}}$ and *bla*$_{\text{OXA-51-like}}$):

Primer working stocks for *bla*$_{NDM}$and *bla*$_{OXA-51-like}$ should be prepared individually (singleplex) as follows:

- Aliquot 2 µL each of forward and reverse primers (total primer volume: 4 µL).
- Add 96 µL of nuclease-free water (molecular grade) to make up the final volume to 100 µL.

Final composition (per target):

- Total primer volume: 4 µL
- Nuclease-free water: 96 µL
- Final volume: 100 µL

Note: If the stock primer concentration is 100 µM, the above dilution results in a final working concentration of 2 µM for each primer in both multiplex and singleplex preparations.

Addition of Template and Controls

- For each reaction, 2 µL of extracted DNA must be added to the respective PCR tubes.
- Template DNA addition must be performed in a designated template addition area to minimise the risk of contamination.
- Each PCR run must include appropriate controls, namely negative extraction control, negative PCR control (no-template control), and positive control.
- For a total of 10 test samples, the master mix should be prepared for 14 reactions, comprising 10 test samples, 3 controls, and 1 additional reaction to account for pipetting variability.

Control Type	Purpose	Template Added
Negative Extraction Control (NEC)	To monitor contamination during the DNA extraction process	2 µL of negative extraction control
Negative PCR Control (NTC)	To check for contamination in PCR reagents (no template control)	2 µL of molecular-grade water (no DNA)
Positive PCR Control	To confirm proper amplification and performance of PCR reagents	2 µL of positive control DNA

Cycling Conditions:

Steps	Temperature (°C)	Duration	No. of Cycles
Denaturation	95°C	15 min	1
Initial denaturation	94°C	30 sec	30 Cycles
Annealing	54°C	90 sec	
Extension	72°C	90 sec	
Final Extension	72°C	10 min	1

ii) Panel 1b: ESBL

Master mix preparation

Reagents	Volume per Reaction (µL)	Volume for 5 Reactions (µL)	Volume for 10 Reactions (µL)
2X Multiplex Master mix	10.0	50.0	100.0
Working Stock Primer	2.0	10.0	20.0
Molecular-grade water	6.0	30.0	60.0
Total Master Mix Volume	18.0	90.0	180.0

Multiplex Primer Working Stock Preparation (*bla*$_{TEM}$ and *bla*$_{PER}$)

The primer working stock for multiplex PCR targeting *bla*$_{TEM}$ and *bla*$_{PER}$ genes should be prepared as follows:

- Aliquot 2 µL each of forward and reverse primers for both targets (total 4 primers).
- This yields a combined primer volume of 8 µL.
- Add 92 µL of nuclease-free water (or molecular-grade water) to make up the final volume to 100 µL.

Final Composition:

- Total primer volume: 8 µL
- Nuclease-free water: 92 µL
- Final volume: 100 µL

Note: If stock primer concentration is 100 µM, the above dilution yields a final working concentration of 2 µM in the multiplex working stock.

Addition of Template and Controls

Addition of template DNA and inclusion of negative extraction control, no-template control (NTC), and positive control should be performed as per the procedure described previously.

Cycling Conditions:

Steps	Temperature (°C)	Duration	No. of Cycles
Denaturation	95°C	15 min	1
Initial denaturation	94°C	30 sec	30 Cycles
Annealing	52°C	90 sec	
Extension	72°C	90 sec	
Final Extension	72°C	10 min	1

B) Panel 2: *P. aeruginosa*/ *Pseudomonas* spp.

i) Panel 2a: Carbapenemases

Master mix preparation

Reagents	Volume per Reaction (µL)	Volume for 5 Reactions (µL)	Volume for 10 Reactions (µL)
2X Multiplex Master mix	10.0	50.0	100.0
Working Stock Primer	2.0	10.0	20.0
Molecular-grade water	6.0	30.0	60.0
Total Master Mix Volume	18.0	90.0	180.0

Multiplex Primer Working Stock Preparation (*bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{KPC}$, *bla*$_{NDM}$)

The primer working stock for multiplex PCR targeting *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{KPC}$, and *bla*$_{NDM}$ genes should be prepared as follows:

- Aliquot 2 µL each of forward and reverse primers for all four targets (total 8 primers).
- This yields a combined primer volume of 16 µL.
- Add 84 µL of nuclease-free water (or molecular-grade water) to make up the final volume to 100 µL.

Final Composition:

- Total primer volume: 16 µL
- Nuclease-free water: 84 µL
- Final volume: 100 µL

Note: If stock primer concentration is 100 µM, this mixture will give each primer a final concentration of 2 µM in the multiplex working stock.

Addition of Template and Controls

Addition of template DNA and inclusion of negative extraction control, no-template control (NTC), and positive control should be performed as per the procedure described previously.

Cycling Conditions:

Steps	Temperature (°C)	Duration	No. of Cycles
Denaturation	95°C	15 min	1
Initial denaturation	94°C	30 sec	30 Cycles
Annealing	54°C	90 sec	
Extension	72°C	90 sec	
Final Extension	72°C	10 min	1

ii) Panel 2b: ESBL

Master mix preparation

Reagents	Volume per Reaction (µL)	Volume for 5 Reactions (µL)	Volume for 10 Reactions (µL)
2X Multiplex Master mix	10.0	50.0	100.0
Working Stock Primer	2.0	10.0	20.0
Molecular-grade water	6.0	30.0	60.0
Total Master Mix Volume	18.0	90.0	180.0

Multiplex Primer Working Stock Preparation (*bla*$_{TEM}$, *bla*$_{VEB}$, *bla*$_{GES}$)

The primer working stock for multiplex PCR targeting *bla*$_{TEM}$, *bla*$_{VEB}$, and *bla*$_{GES}$ genes should be prepared as follows:

- Aliquot 2 µL each of forward and reverse primers for all three targets (total 6 primers).
- This yields a combined primer volume of 12 µL.
- Add 88 µL of nuclease-free water (or molecular-grade water) to make up the final volume to 100 µL.

Final Composition:

- Total primer volume: 12 µL
- Nuclease-free water: 88 µL
- Final volume: 100 µL

Note: If stock primer concentration is 100 µM, the above dilution yields a final working concentration of 2 µM in the multiplex working stock.

Addition of Template and Controls

Addition of template DNA and inclusion of negative extraction control, no-template control (NTC), and positive control should be performed as per the procedure described previously.

Cycling Conditions:

Steps	Temperature (°C)	Duration	No. of Cycles
Denaturation	95°C	15 min	1
Initial denaturation	94°C	30 sec	30 Cycles
Annealing	52°C	90 sec	
Extension	72°C	90 sec	
Final Extension	72°C	10 min	1

C) Panel 3: Diarrhoeal Pathogens

i) Panel 3a: ESBL

Master mix preparation

Reagents	Volume per Reaction (µL)	Volume for 5 Reactions (µL)	Volume for 10 Reactions (µL)
2X Multiplex Master mix	10.0	50.0	100.0
Working Stock Primer	2.0	10.0	20.0
Molecular-grade water	6.0	30.0	60.0
Total Master Mix Volume	18.0	90.0	180.0

Multiplex Primer Working Stock Preparation {*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$ (Groups 1,2,9)}

The primer working stock for multiplex PCR targeting *bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{CTX-M}$ (Groups 1,2,9)genes should be prepared as follows:

- Aliquot 2 µL each of forward and reverse primers for all three targets (total 6 primers).
- This yields a combined primer volume of 12 µL.
- Add 88 µL of nuclease-free water (or molecular-grade water) to make up the final volume to 100 µL.

Final Composition**:**

- Total primer volume: 12 µL
- Nuclease-free water: 88 µL
- Final volume: 100 µL

Multiplex Primer Working Stock Preparation (*bla*$_{CMY}$and *bla*$_{DHA}$)

The primer working stock for multiplex PCR targeting *bla*$_{CMY}$and *bla*$_{DHA}$genes should be prepared as follows:

- Aliquot 2 µL each of forward and reverse primers for both targets (total 4 primers).
- This yields a combined primer volume of 8 µL.

- Add 92 µL of nuclease-free water (or molecular-grade water) to make up the final volume to 100 µL.

Final Composition:

- Total primer volume: 8 µL
- Nuclease-free water: 92 µL
- Final volume: 100 µL

Singleplex Primer Working Stock Preparation (*bla*$_{\text{OXA-1-like}}$)

The primer working stock for singleplex PCR targeting bla$_{\text{OXA-1-like}}$ gene should be prepared as follows:

- Aliquot 2 µL each of forward and reverse primers (total 2 primers).
- This yields a combined primer volume of 4 µL.
- Add 96 µL of nuclease-free water (or molecular-grade water) to make up the final volume to 100 µL.

Final Composition:

- Total primer volume: 4 µL
- Nuclease-free water: 96 µL
- Final volume: 100 µL

Note: If stock primer concentration is 100 µM, the above dilution yields a final working concentration of 2 µM for each primer in the multiplex primer mix.

Addition of Template and Controls

Addition of template DNA and inclusion of negative extraction control, no-template control (NTC), and positive control should be performed as per the procedure described previously.

Cycling Conditions:

***bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{CTX-M}}$ (Group 1,2,9)**

	Steps	Temperature	Duration	No of Cycles
bla$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M\ (Group\ 1,2,9)}$	Denaturation	95°C	15 min	1
	Initial denaturation	94°C	30 sec	30
	Annealing	52°C	90 sec	
	Extension	72°C	90 sec	
	Final Extension	72°C	10 min	1
bla$_{CMY}$and *bla*$_{DHA}$	Denaturation	95°C	15 min	1

	Initial denaturation	94°C	30 sec	30
	Annealing	52°C	90 sec	
	Extension	72°C	90 sec	
	Final Extension	72°C	10 min	1
	Denaturation	95°C	5 min	1
	Initial denaturation	94°C	1 min	30
*bla*OXA-1-like	Annealing	50°C	1 min	
	Extension	72°C	1 min	
	Final Extension	72°C	10 min	1

ii) Panel 3b: Other resistance genes

Singleplex Primer Working Stock Preparation (*dfrA, sul1 / sul2, qnrS, qnrB, mphA, ermB*)

The primer working stocks for PCR targeting *dfrA, sul1 / sul2, qnrS, qnrB, mphA, ermB* genes shall be prepared individually, as all targets are amplified using singleplex PCR assays, as follows:

- Aliquot 2 µL each of forward and reverse primers (total 2 primers).
- This yields a combined primer volume of 4 µL.
- Add 96 µL of nuclease-free water (or molecular-grade water) to make up the final volume to 100 µL.

Final Composition:

- Total primer volume: 4 µL
- Nuclease-free water: 96 µL
- Final volume: 100 µL

Note: If the stock primer concentration is 100 µM, the above dilution yields a final working concentration of 2 µM for each primer in the multiplex primer mix.

Addition of Template and Controls

Addition of template DNA and inclusion of negative extraction control, no-template control (NTC), and positive control should be performed as per the procedure described previously.

Cycling Conditions:

Gene Name	Steps	Temperature	Duration	No. of Cycles
dfrA	Denaturation	95°C	5 min	1
	Initial denaturation	92°C	1 min	30
	Annealing	50°C	1 min	
	Extension	72°C	1 min	
	Final Extension	72°C	8 min	1
sul1 / sul2	Denaturation	94°C	2 min	1
	Initial denaturation	94°C	1 min	35
	Annealing	60°C	1 min	
	Extension	72°C	1 min	
	Final Extension	72°C	10 min	1
qnrS, qnrB	Denaturation	94 °C	5 min	1
	Initial denaturation	94°C	45 sec	35
	Annealing	53°C	45 sec	
	Extension	72°C	1 min	
	Final Extension	72°C	7 min	1
mphA	Denaturation	95°C	5 min	1
	Initial denaturation	95°C	30 sec	30
	Annealing	60°C	1 min	
	Extension	72°C	1 min	
	Final Extension	72°C	10 min	1
ermB	Denaturation	95°C	5 min	1
	Initial denaturation	95°C	45 sec	30
	Annealing	58°C	45 sec	
	Extension	72°C	45 sec	
	Final Extension	72°C	5 min	1

7.3.2.2 Gel Electrophoresis

Purpose

To resolve and visualise PCR amplicons based on molecular size using agarose gel electrophoresis, enabling the detection and confirmation of AMR gene targets following multiplex PCR amplification.

Principle

Agarose gel electrophoresis enables the separation of nucleic acid fragments based on molecular size under the influence of an applied electric field. Negatively charged DNA molecules migrate toward the anode, with smaller fragments exhibiting higher mobility and migrating faster than larger fragments through the agarose matrix. Following electrophoretic separation, amplified DNA products are detected by visualisation under ultraviolet illumination after staining with a nucleic acid intercalating dye.

Materials and Reagents

- Agarose
- 1X TAE / TBE buffer
- DNA staining dye (Ethidium bromide / safe alternative)
- DNA loading dye
- DNA ladder (100 bp)
- PCR products
- Gel casting tray and comb

Equipment

- Gel electrophoresis unit
- Power supply
- Microwave or hot plate
- UV transilluminator/gel documentation system

Reagent Preparation

a) Preparation of 50X TAE Buffer (Stock Solution)

- Dissolve 242 g Tris base in 500 mL distilled water.
- Add 57.1 mL glacial acetic acid.
- Add 100 mL of 0.5 M EDTA (pH 8.0).
- Make up the volume to 1 L with distilled water.
- Mix thoroughly and store at 4-8°C.

b) Preparation of 1X TAE Buffer (Working Solution)

- Dilute 50X TAE buffer in distilled water at a ratio of 1:50.
- Example: Mix 20 mL of 50X TAE with 980 mL of distilled water.
- Mix well prior to use.

c) Preparation of Ethidium Bromide (EtBr) Stock Solution (10 mg/mL)

- Weigh 1 g of Ethidium bromide (EtBr) powder
- Transfer to a suitable light-protected container.
- Add 100 mL of distilled water.
- Mix thoroughly until completely dissolved.
- Store at room temperature or 4°C.

Note: Appropriate personal protective equipment (PPE), including disposable gloves, must be worn at all times during agarose gel electrophoresis and visualisation procedures to prevent contamination and ensure operator safety.

Procedure

a) Gel Casting Setup and Preparation

- Wear appropriate personal protective equipment (PPE), including disposable gloves, prior to handling gels and operating the gel documentation system.
- Place the gel casting tray within the gel caster and securely seal both ends using casting gates to prevent leakage.
- Ensure the casting assembly is placed on a level surface; verify alignment using a level indicator and adjust the clamp screws as required to achieve uniform gel thickness.
- Position the comb appropriately to form wells of the desired size.
- Prepare a 1.5–2% (w/v) agarose gel in 1X TAE buffer. For example, to prepare 1% agarose (100 mL), dissolve 1 g of agarose in 100 mL of 1X TAE buffer.
- Heat the mixture using a microwave oven or hot plate until complete dissolution is achieved, and allow it to cool to approximately 50–60°C.
- Add Ethidium bromide (EtBr), if pre-staining is employed.
- Pour the molten agarose into the casting tray and allow it to solidify at room temperature for 20–30 minutes.

b) Sample Preparation

- Mix 5 µL of PCR product with 1 µL of 6X loading dye, if the dye is not incorporated in the PCR master mix.

c) Electrophoresis

- Wear appropriate personal protective equipment (PPE), including disposable gloves, prior to handling gels and operating the gel documentation system.
- Clean the gel electrophoresis chamber thoroughly and fill the tank to approximately 75% capacity with 1X TAE running buffer.
- Carefully remove the comb and place the solidified gel into the electrophoresis chamber, ensuring it is fully submerged in the running buffer up to the indicated level.
- Load the prepared samples into the wells along with an appropriate 100 bp DNA ladder for size estimation.
- Perform electrophoresis at a constant voltage of 80–120 V for 45–60 minutes, or until the tracking dye migrates approximately 70–80% of the gel length.

d) Visualisation and Documentation

- Wear appropriate personal protective equipment (PPE), including disposable gloves, prior to handling gels and operating the gel documentation system.
- Prior to use, clean the gel documentation (gel doc) platform and UV transilluminator surface with distilled water to remove residual contaminants, stains, or debris. Ensure the surface is dry before placing the gel.
- Carefully transfer the gel from the electrophoresis chamber to the gel documentation system.
- Gently blot excess running buffer from the gel surface using lint-free tissue to prevent interference during imaging.
- Place the gel centrally on the UV transilluminator platform, ensuring proper alignment for uniform illumination.
- Visualise DNA bands under UV illumination using appropriate safety measures (UV shield or protective goggles).
- Adjust exposure settings (e.g., intensity, focus, and exposure time) to obtain clear and well-resolved band images.
- Capture and save the gel image using the gel documentation system software.
- Label the image with relevant details, including date, sample ID, panel/gene targets, and run number, for traceability and record-keeping.

Interpretation of Results

- DNA bands are identified by comparison with an appropriate molecular weight marker (DNA ladder).
- The presence of a distinct band at the expected amplicon size is indicative of positive amplification of the target gene.
- The absence of a corresponding band indicates a negative result for the target gene.

- The presence of non-specific bands or smearing suggests suboptimal amplification conditions, non-specific primer binding, or degraded template DNA and warrants further optimisation or repeat testing.

Quality Control

- A positive control containing a known target gene and a negative control (no-template control, NTC) must be included in each electrophoresis run to validate assay performance.
- The DNA ladder must demonstrate clearly resolved bands to ensure accurate size determination of amplicons.
- The NTC must exhibit no detectable bands, confirming the absence of contamination or non-specific amplification.

Precautions

- Use freshly prepared running buffer and ensure that the electrophoresis apparatus is thoroughly cleaned to minimise the risk of contamination.
- Avoid overloading sample wells, as excessive DNA volume may result in band distortion or smearing.
- Handle Ethidium bromide (EtBr) with caution, as it is a potent mutagen; appropriate personal protective equipment (PPE), including gloves and a lab coat, must be worn at all times.
- Minimise exposure to UV radiation during visualisation to prevent DNA damage and reduce the risk of personal injury; use protective shields or UV safety goggles where applicable.

Troubleshooting

Issue	Possible Cause	Solution
Faint bands	Low DNA concentration or suboptimal PCR yield	Increase template DNA concentration or optimise PCR conditions
Smear	Degraded DNA or excessive template loading	Use high-quality DNA and reduce the amount of loaded sample
No bands	PCR failure or reagent malfunction	Check reagents and cycling conditions
Extra bands	Non-specific amplification or primer mismatch	Optimise annealing temperature and primer concentrations

7.4 *Staphylococcus* species

7.4.1 Genotypic confirmation of antibiotic resistance

A) DNA Extraction

DNA extraction from bacterial isolates should be performed using the kit according to the manufacturer's instructions.

Procedure:

1. Obtain a freshly subcultured plate with isolated colonies.
2. Take 2 mL of sterile normal saline in a 2 mL microcentrifuge tube.
3. Pick 3–4 isolated colonies and emulsify them in the saline.
4. Homogenise and centrifuge at 13,000 rpm for 10 minutes at 4°C**.**
5. Carefully discard the supernatant without disturbing the pellet.
6. Add 400 µL of Fast Lysis Buffer to the pellet and resuspend by vigorous vortexing.
7. Transfer the suspension to a Pathogen Lysis Tube (with beads) provided in the kit.
8. Vortex at low speed for 10 minutes**.**
9. Centrifuge the Pathogen Lysis Tube at 13,000 rpm for 10 minutes**.**
10. Transfer 100 µL of the supernatant into a fresh 1.5 mL microcentrifuge tube.
11. Use the eluate directly for PCR reactions, or store it at –80°C for long-term preservation.

B) Polymerase Chain Reaction (PCR)

Singleplex and multiplex PCR will be carried out on all isolates (both susceptible and resistant) to detect antibiotic resistance determinants using previously published primers and protocols. For *Staphylococcus* species, the following genes will be targeted in different reaction mixtures: methicillin resistance (*mec*A), erythromycin (*erm*A, *erm*B, and *erm*C), and linezolid resistance (*cfr*A). The reaction mixtures will contain 2x Taq Multiplex/Singleplex PCR Smart Mix, 10 pmol of each primer, and 10 ng of bacterial DNA. Visualise amplicons in a 1.5% agarose gel containing ethidium bromide. The primer sequences, PCR cycling conditions, and expected amplicon sizes are provided in **Table 7.7**.

Table 7.7: PCR for the detection of antibiotic resistance genes in *Staphylococci*

Genes	Primer sequence (5' - 3')	PCR conditions	Amplicon size (bp)	Reference
*mec*A	F-GTAGAAATGACTGAACGTCCGATA R-CCAATTCCACATTGTTTCGGTCTAA	94°C for 4 min; 30 cycles of 94°C for 15 s; 58°C for 15 s; 72°C for 30 s final extension at 72°C for 10 min	310	(Strommenger B. et al., 2003, J. Clin. Microbiol)
*cfr*A	F-TGAAGTATAAAGCAGGTTGGGAGTCA R-ACCATATAATTGACCACAAGCAGC	94°C for 5 min; 30 cycles of 94°C for 30 s; 55°C for 30 s; 72°C for 30 s final extension at 72°C for 5 min	746	(Manoharan M et al., 2021, Microb Drug Resist)
*erm*A	F-GTTCAAGAACAATCAATACAGAG R-GGATCAGGAAAAGGACATTTTAC	95°C for 3 min; 30 cycles of 94°C for 30 s; 55°C for 30 s; 72°C for 1 min final extension at 72°C for 10 min	421	(Manoharan M et al., 2021, Microb Drug Resist)
*erm*B	F-CCGTTTACGAAATTGGAACAGGTAAA GGGC R-GAATCGAGACTTGAGTGTGC		359	
*erm*C	F-GCTAATATTGTTTAAATCGTCAATTCC R-GGATCAGGAAAAGGACATTTTAC		572	

7.5 *Enterococcus* spp.

For *Enterococcus* species, the targeted genes will include vancomycin resistance (*van*A and *van*B), and linezolid resistance (*cfr*A, *cfr*B, *cfr*D, *optr*A and *poxt*A) genes. The reaction mixtures will contain 2xTaq Multiplex/Singleplex PCR Smart Mix, 10 pmol of each primer, and 10 ng of bacterial DNA. Visualise amplicons in a 1 or 1.5% agarose gel containing

ethidium bromide. The primer sequences, PCR cycling conditions, and expected amplicon sizes are provided in **Table 7.8**.

Table 7.8: Genotypic confirmation of antibiotic resistance in *Enterococci*

Target genes	Primer sequence(5'-3')	PCR conditions	Amplicon size (bp)	References
*van*A	F-GGGAAAACGACAATTGC R-GTACAATGCGGCCGTTA	94°C for 2min 30 cycles of 94°C for 1 min 54°C for 1min 72°C for 1 min final extension at 72°C for 10 min	732	(Dutka-Malen S et al., 1995, J Clin Microbiol.)
*van*B	F-ATGGGAAGCCGATAGTC R-GATTTCGTTCCTCGACC	94°C for 2 min 30 cycles of 94°C for 1 min 54°C for 1min 72°C for 1 min final extension at 72°C for 10 min	635	(Dutka-Malen S et al., 1995, J Clin Microbiol.)
cfrA	F-TGAAGTATAAAGCAGGTTGGGAG TCA R-CCATATAATTGACCACAAGCAGC	94°C for 2min 30 cycles of 94°C for 1min 54°C for 1 min 72°C for 1 min final extension at 72°C for 10 min	746	(Kehrenberg C et al., 2006, AAC)
*cfr*B	F-TGAGCATATACGAGTAACCTCA AGA R-CGCAAGCAGCGTCTATATCA	94°C for 5min 35 cycles of 94°C for 30 sec 58°C for 30 sec 72°C for 1 min final extension at 72°C for 5 min	293	(Abdullahi IN et al., 2023, Eur J Clin Microbiol Infect Dis.)
*cfr*D	F-AGAAGTCGCAACAAGTGAGGA R-GCAACTGCATGAGTCAAAGAA	94°C for 7min 30 cycles of 94°C for 1min 60°C for 1 min 72°C for 1 min final extension at 72°C for 10 min	595	(Abdullahi IN et al., 2023, Eur J Clin Microbiol Infect Dis.)
*optr*A	F-AGGTGGTCAGCGAACTAA R-ATCAACTGTTCCCATTCA	94°C for 2min 30 cycles of 94°C for 1min 54°C for 1 min	1395	(Wang et al., 2015, JAC)

Target genes	Primer sequence(5'-3')	PCR conditions	Amplicon size (bp)	References
		72°C for 1 min final extension at 72°C for 10 min		
poxtA	F: TCAATGCAGAGCAGGAAGCA R: GGTGGATTTACCGACACCGT	94°C for 7min 35 cycles of 94°C for 1min 60°C for 1 min 72°C for 1 min final extension at 72°C for 10 min	791	(Abdullahi IN et al., 2023, Eur J Clin Microbiol Infect Dis.)

Whole-Genome Sequencing of Linezolid-Resistant *Enterococcus* (LRE) isolates

Whole-genome shotgun sequencing will be performed using the Ion TorrentPGM™ System (Life Technologies, Waltham, MA) with 400-bp chemistry. The generated data will be assembled *de novo* using SPAdes Genome Assembler v.5.0.0.0 embedded in Torrent Suite Server v.5.0.4. Genome assembly and annotation will be carried out on the Galaxy webserver (https://usegalaxy.org) and the PATRIC webserver (https://www.patricbrc.org) using default parameters. Identification of antibiotic resistance genes will be performed using ABRicate,a command-line tool for mass screening of contigs for antibiotic resistance (ARGs) and virulence genes (Galaxy Version 1.0.1). It comes with several databases such as Resfinder, NCBI (Bacterial Antimicrobial Resistance Reference Gene Database), CARD (Comprehensive Antibiotic Resistance Database), ARG-ANNOT (Antibiotic resistance gene-annotation), megares, PlasmidFinder, Ecoli VF and VFDB (Virulence Finder Database). Downstream analysis and MLST will be performed using the Center for Genomic Epidemiology(CGE) server (http://www.cbs.dtu.dk/services) (Babraham Bioinformatics - FastQC ; Seemann T et al., 2014; Davis JJ et al., 2020, NAR; Seemann T, 2017, Github).

Chapter 8
Quality control (QC)

8.1 Reference strains for QC (CLSI M100, 2019)

- *Escherichia coli ATCC 25922 (beta-lactamase negative)*
- *Escherichia coli ATCC 35218 (beta-lactamase positive)*
- *Enterococcus faecalis ATCC 29212 (for checking of thymidine or thymine level of MHA)*
- *Enterococcus faecalis ATCC 51299 (resistant to Vancomycin)*
- *Haemophilus influenzae ATCC 49766 (for cephalosporins/ampicillin susceptible)*
- *Haemophilus influenzae ATCC 10211 (for HTM control)*
- *Haemophilus influenzae ATCC 49247 (BLNAR)*
- *Klebsiella pneumoniae ATCC 700603 (for cefpodoxime/ESBL positive)*
- *Pseudomonas aeruginosa ATCC 27853 (for aminoglycosides)*
- *Staphylococccus aureus ATCC 25923 (beta-lactmase negative, oxacillin susceptible, mecA negative)*
- *Staphylococccus aureus ATCC 43300 (Cefoxitin resistant – MRSA mecA positive)*
- *Staphylococccus aureus ATCC 29213 (beta-lactmase positive)*
- *Streptococcus pneumoniae ATCC 49619 (oxacillin resistant/ Penicillin Inter médiate resistant by altered binding proteins)*
- *Streptococcus pneumoniae ATCC 33400 (oxacillin susceptible)*

8.2 Storing and testing QC strains (CLSI M100, 2019)

These strains will be tested by the standard procedures mentioned. Zone sizes will be compared with that mentioned in the table for control strains. For prolonged storage, strains will be preserved in BHI broth with 10% glycerol at -20ºC / -70ºC / or liquid nitrogen, else the strains will be lyophilized. Working cultures will be stored on trypticase soya agar or CHOC at 2 to 8ºC. Before testing, strains will be subcultured to obtain isolated colonies. Freeze dried or frozen cultures will be subcultured twice prior to testing. If an unexplained result suggests a change in the organism's inherent susceptibility, a new culture of the control strain will be obtained.

Caution: Careful attention to organism maintenance (e.g., minimal subcultures) and storage (e.g., -60°C or below) is especially important for these QC strains because

spontaneous loss of the plasmid encoding the β-lactamase has been documented. If stored at temperatures above -60°C or if repeatedly subcultured, these strains may lose their resistance characteristics and QC results may be outside the acceptable ranges.

Frequency of testing

It applies only to antimicrobial agents for which satisfactory results have been obtained with either the 15-replicate (3- × 5-day) plan or 20 or 30 consecutive test day plan. Otherwise QC is required each test day.

Quality control of media

Mueller Hinton agar or the various other broth and agar media not containing antimicrobials should always be kept overnight at 37ºC for sterility checking prior to inoculation. Each batch or new lot of media should be checked with reference ATCC strains. Zone sizes and MICs obtained must be within acceptable CLSI limits. Else the batch should be rejected and any patient results obtained not reported.

Chapter 9

External Quality Assurance Scheme (EQAS)

9.1. Introduction:

Advanced AMR EQAS is a national EQAS program intended for tertiary level hospitals across India, in collaboration with the Indian Council of Medical Research (ICMR). This program is specifically developed for microbiology laboratories participating in Antimicrobial Resistance Surveillance Network (AMRSN), who are the primary source of resistance data to represent the AMR burden in India. The purpose of this EQAS exercise is to determine the accuracy of AST results reported by individual laboratories.

Currently, the National EQAS scheme being conducted by the Indian Association of Medical Microbiologist (IAMM) in a tier level approach is for 1200 laboratories. This is for primary health care centers (Tier 1), secondary level (Tier 2) and tertiary level hospitals (Tier 3). IAMM-EQAS tests the ability of the participant to perform bacterial identification and antimicrobial susceptibility testing by disc diffusion. Detailed protocol on methodology and list of antimicrobials to be tested are provided. No skills other than identification and AST profiling by disc diffusion are assessed.

9.2 How is the advanced-AMR-EQAS different from other National EQAS scheme in place?

With rising antimicrobial resistance in India, microbiology laboratories need to scale up their testing skills and knowledge to meet the requirements.

EQAS panel consisting of Gram negative and Gram positive lyophilized cultures isolated from different syndromes with a brief case history will be given. Participants are expected to do the following:

- Determine the clinical significance of the isolate provided by analyzing the clinical syndrome/scenario described
- Identify the organism up to the species level
- Carry out the appropriate susceptibility testing method (DD/MIC-BMD, AD, E-test: Where automation is not suitable)
- Include the appropriate antimicrobials for the identified organisms
- Interpret the AST results accurately based on CLSI and/or EUCAST guidelines
- Carry out clinical reporting, based on the AST profile as Cascade reporting, as recommended by the antimicrobial stewardship program

9.3 EQAS Program Schedule

This EQAS program will be conducted three times a year that every four months once with 10 exercises/cultures per each cycle, isolated from various clinical specimens. The most common organisms will be included and rare organisms will be avoided.

9.4 EQAS Panel preparation

A panel of 10 lyophilized cultures containing gram negative and gram positive bacterial cultures will be included in the package.

i) These cultures once chosen will be subjected to the following characterization:

a) Bacterial identification

- Standard culture and biochemical tests
- Automated system
- MALDI-TOF
- 16 S rRNA sequencing/ WGS

b) Antimicrobial susceptibility profile (as per CLSI/EUCAST guidelines)

- Disk diffusion
- MIC determination by broth micro dilution (BMD), agar dilution, E-test
- MIC determination by automated susceptibility testing

c) Molecular Detection of Antimicrobial Resistance

- Conventional PCR for antimicrobial resistant determinants
- Whole genome sequencing by Ion Torrent (short read) and MinION (long read)

ii) Once characterized, the cultures will be freeze-dried by lyophilization followed by viability and purity check

iii) Homogeneity and stability of the lyophilized cultures will be checked by exposing the cultures to various temperatures

iv) A total 30% of the lyophilized cultures will be checked once a week starting from the date of preparation to till the date of dispatch of results by the EQAS coordinator along with expert comments and suggestions

9.5 EQAS process

✓ A panel of 10 well-characterized cultures with a brief clinical case history of the clinical specimen source from which it is isolated will be given

✓ Participants are expected to identify the organisms up to species level according to their standard operative procedures by biochemical/any automated methods, choose right

antimicrobials for respective pathogens and clinical specimen source, use right methodology to determine antimicrobial susceptibility testing and clinical reporting as cascade reporting, preferably

✓ Once the reports are received by the EQAS coordinator, the evaluation will be performed as per international standards and be circulated to all the advisory board members for their inputs and suggestions

✓ On receiving the board member's feedback, reasoning out and justification will be elaborately detailed and shared with the participating centers

Clinical case history examples:

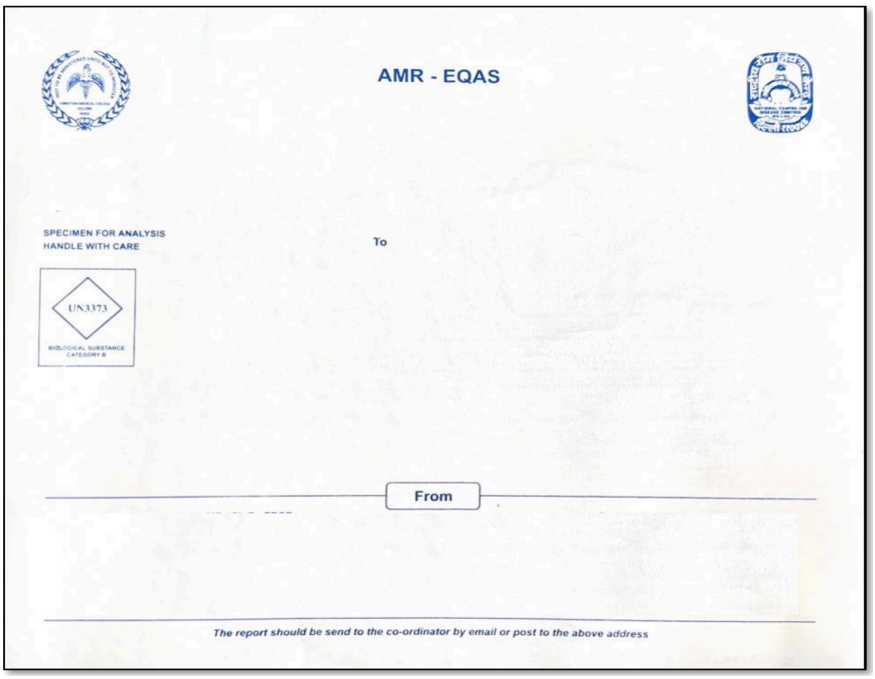
Culture 1A	
Clinical Presentation	A 56-year-old man with history of previous successful treatments of MRSA bacteremia with vancomycin. He was later hospitalized for catheter-related MRSA bacteremia that persisted despite vancomycin treatment. Catheter was removed. He had a regular heart rate and rhythm, and normal first and second heart sounds without murmurs. His blood culture, grew gram positive cocci in clusters and were identified later as methicillin resistant *Staphylococcus aureus* (MRSA). Therapy was changed from vancomycin to daptomycin, and the patient's blood cultures were sterilized.
Given Challenges	Antimicrobial susceptibility testing for syndrome-specific antibiotic panels.
Received result	Susceptibility to cefoxitin gentamicin, erythromycin, clindamycin, trimethoprim-sulfamethoxazole, rifampicin, vancomcyin, and linezolid were reported using disc diffusion testing. The given MRSA culture was reported to be susceptible to vancomycin. Daptomycin susceptibility was not reported.
Expected results	The given MRSA culture was identified as vancomycin intermediate *S. aureus* (VISA) with the vancomycin MIC of 4 or 8 µg/ml. Vancomycin MIC value should be reported. Daptomycin susceptibility testing should be performed.

Post evaluation (This will be shared)	MRSA-infected patients who fail or have a history of vancomycin therapy so that additional susceptibility testing and appropriate antibiotic therapy can be promptly commenced to reduce the morbidity associated with VISA infection. The patient was previously treated with vancomycin for MRSA infection. Therefore, the probability of MRSA to develop VISA can be considered. For treating VISA infection, daptomycin is considered as a viable option. Therefore, testing of daptomycin MIC is mandatory. BMD is considered as the reference method for vancomycin susceptibility. Vancomycin is a large molecule and diffuse poorly in the agar medium. The DD method, did not reliably differentiate vancomycin-susceptible from vancomycin non-susceptible isolates. Results of DD test are qualitative, derived from the test, rather than an MIC. Vancomycin DD for *S. aureus* was reported with an unacceptable very major error rate. If the patient was treated with vancomycin based on the reported result (vancomycin susceptible based on DD), it would be end up with poor outcome.

Culture 1B	
Clinical Presentation	A 25-year-old patient with 2-day history of chest tightness and shortness of breath was admitted to the intensive care unit (ICU) due to worsening dyspnea over 6 h. Two days before admission, the patient started having sore throat and cough after flu-like symptoms that were self-treated with unknown medications without improvement. Patient was treated empirically with meropenem but did not improve significantly. Respiratory secretions were sent for cultures and gram negative bacilli was grown
Given Challenges	*Pseudomonas aeruginosa* with discrepant carbapenem susceptibility profile was given
Received result	Susceptibility to ceftazidime, cefepime, piperacillin/tazobactam, aztreonam, imipenem, levofloxacin, amikacin and tobramycin. Susceptibility to meropenem was not tested and reported.
Expected results	The given *P.aeruginosa* cultures was meropenem resistant, but imipenem susceptible. Susceptibility testing to both the carbapenems (imipenem and meropenem) is expected to be performed and reported critically.

Post evaluation (This will be shared)	*P.aeruginosa* was the infected organism identified in respiratory specimen. As only imipenem was found susceptible by disk diffusion, the same susceptibility was extrapolated to meropenem and the patient was continued on meropenem. However, the infected strain had a profile of discrepant carbapenem susceptibility with imipenem being susceptible, but meropenem resistant. This is due to the interplay of complex chromosomal mediated resistance mechanisms, which is commonly seen in *P.aeruginosa.* Imipenem resistance meropenem susceptible (IRMS) is due to the loss of porin – OprD, while meropenem resistance imipenem susceptible (MRIS) is due to overexpression of efflux pump mexAB. Due to the difference in the resistance mechanisms, resistance to individual agents (imipenem/meropenem) within the carbapenem group differs. Therefore it is mandatory to perform susceptibility testing to both carbapenems (imipenem and meropenem) and not to extrapolate one agent's susceptibility to other agents. In this case scenario, testing of only imipenem and extrapolating its susceptibility to meropenem led to the poor clinical outcome, which could have been avoided if meropenem was tested and reported resistant.

9.6 EQAS Package and dispatch PT items





9.7 Evaluation format

a) Scoring system for identification of pathogens

Level of identification reported	Points awarded
Correct species	**2**
Correct genus, incorrect species	**1**
Correct genus, lack of discrimination to species level	**1**
Incorrect genus	**-1**
Negative result or no growth	**0**

b) Scoring system for AST

Reference results	Participant's result		
	Susceptible	**Intermediate**	**Resistant**
Susceptible	2	1	0
Intermediate	1	2	1
Resistant	-1	1	2

*Automated susceptibility testing/ E-test MIC will be awarded complete marks if the interpretation is consistent with the expected report

Susceptibility Interpretation errors and validation

- Minor error (mE): Susceptible / Resistant isolate reported as intermediate susceptible OR intermediate isolate reported as susceptible or resistant (1 mark)
- Major error (ME): Susceptible isolate reported as resistant (0 mark)
- Very major error (VME): Resistant isolate reported as susceptible (NEGATIVE marking -1 mark)
- No error: 2 marks

ANNEXURE – I
Biochemical reactions

1. Amino acid decarboxylase tests

Use: To determine the ability of bacteria to decarboxylase an amino acid to the corresponding amine with the liberation of carbon dioxide.

Ingredients and preparation

Peptone	5.0 g
Meat extract	5.0 g
Glucose	0.5 g
Pyridoxal	5.0 mg
Bromocresol purple	5.0 ml
Cresol red	2.5 ml
Distilled water	1.0 litre

Dissolve the solids in water and adjust the pH to 6.0 before the addition of the indicators. This is the basal medium and to it is added the amino acid whose decarboxylation is to be tested. Divide the basal medium into four portions and treat as follows:

Add 1% L-lysine hydrochloride.

Add 1% L- ornithine hydrochloride.

Add 1% L- arginine hydrochloride.

No additions (control)

Readjust the pH to 6.0 if necessary. Distribute 1 ml quantities in small tubes containing sterile liquid paraffin to provide a layer about 5 mm thick above the medium.

Procedure: The isolated colony from the test isolate is inoculated in two tubes of each amino acid media, one containing the amino acid and the other to be used as control tube devoid of amino acid. Overlay the tubes with sterile mineral oil to cover about 1cm above the surface and incubate at 35ºC for 18-24 hours.

Principle and interpretation: This test is based on the ability of some bacteria to decarboxylase an amino acid to the corresponding amine with the liberation of carbon dioxide. The production of these decarboxylases is induced by a low pH and, as a result of their action; the pH rises to neutrality or above.

The medium first becomes yellow due to acid production during glucose fermentation; later if decarboxylation occurs, the medium becomes violet. Control should remain yellow.

Result

Amino acid	Positive	Negative
Lysine	*E.coli* *Edwardsiella* *Salmonella*	*Shigella spp* *Citrobacter spp*
Ornithine	*E.coli*	*Klebsiella*
Arginine	*Pseudomonas*	*E.coli*

2. β-galactosidase (ONPG) test

Use: To rapidly differentiate delayed lactose-fermenting organisms from lactose-negative organisms.

Ingredients and preparation

Sodium phosphate buffer 0.01M, pH 7.0

O-nitrophenyl-β-D-galactopyranoside

(Add 80 mg ONPG to 15.0 ml distilled water and warm to dissolve crystals. Add 5.0 ml phosphate buffer and adjust pH to 7.0. Store in a dark bottle.

Principle: O-nitrophenyl-β-D-galactopyranoside (ONPG) is structurally similar to lactose, except that orthonitrophenyl has been substituted for glucose. On hydrolysis, through the action of the enzyme β-galactosidase, ONPG cleaves into two residues, galactose and o-nitrophenyl. ONPG is a colourless compound; o-nitrophenyl is yellow, providing visual evidence of hydrolysis.

Lactose-fermenting bacteria possess both lactose permease and β-galactosidase, two enzymes required for the production of acid in the lactose fermentation test. The permease is required for the lactose molecule to penetrate the bacterial cell where the β-galactosidase can cleave the galactoside bond, producing glucose and galactose. Non-lactose fermenting bacteria are devoid of both enzymes and are incapable of producing acid from lactose. Some bacterial species appear to be non-lactose fermenters because they lack permease, but do possess β-galactosidase and give a positive ONPG test. So called late lactose fermenters may be delayed in their production of acid from lactose because of sluggish permease activity. In these instances, a positive ONPG test may provide a rapid identification of delayed lactose fermentation.

Procedure: A loopful of bacterial growth is emulsified in 0.5 ml of saline to produce a heavy suspension. One drop of toluene is added to the suspension and vigorously mixed

for a few seconds to release the enzyme from the bacterial cells. An equal quantity of buffered ONPG solution is added to the suspension and the mixture is placed in a 37ºC water bath. When using ONPG tablets, a loopful of bacterial suspension is added directly to the ONPG substrate resulting from adding 1 ml of distilled water to a tablet in a test tube. This suspension is also placed in a 37ºC water bath.

ONPG positive	**ONPG negative**
Escherichia coli	*Proteus mirabilis*
Salmonella choleraesuis subsp. *arizonae*	*Neisseria meningitidis*
Neisseria lactamica	

3. Glucose phosphate (GP) broth

Use: It is employed to conduct methyl red test and Voges-Proskauer test

Ingredients and preparation

Peptone	5.0 g
Glucose, 10% solution	50 ml
KH_2PO_4	5.0 g
Distilled water	1.0 litre
pH	7.6

Dissolve the peptone and phosphate. Adjust the pH to 7.6, filter, then dispense in 5 ml amounts and sterilize at 121ºC for 15 minutes. Sterilize the glucose solution by filtration and add 0.25 ml to each tube (final concentration 0.5%).

4. Gelatin liquefaction test

Use: It is used to determine the ability of an organism to produce proteolytic type enzymes (gelatinase) that liquefy gelatin.

Ingredients and preparation

Nutrient broth	1 litre
Gelatin	120 g

Add the gelatin to the broth and allow it to stand at 4ºC overnight. Warm to 45ºC to dissolve the gelatin. Adjust to pH 8.4 and steam for 10 minutes. Cool quickly to 45ºC and slowly add the beaten whites of 2 eggs, or 10 g egg albumin dissolved in 50 ml water (this helps to clear colloidal particles from the medium). Steam for 30 minutes, stirring occasionally, filter through hardened filter paper. Adjust the pH to 7.6 and dispense in 12 ml amounts. Autoclave for 10 minutes at 115ºC; remove from autoclave as quickly as possible and keep at low temperature (< 22ºC). (The resulting medium is perfectly transparent when solid and should be of firm consistency.)

Principle and interpretation: Gelatin is a protein derivative of animal collagen which is hydrolysed by gelatinase into its constituent amino acids with a loss of its gelling characteristics.

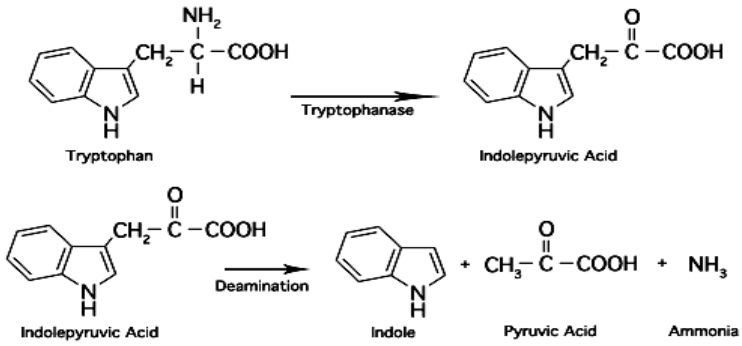
Positive	**Negative**
S.aureus	*Listeria monocytogenes*
P.mirabilis, P.vulgaris	

5. Indole

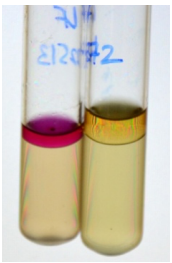
Use: To determine the ability of an organism to split indole from tryptophan.

Ingredients and preparation: The medium is peptone water.

Principle: Indole is generated by reductive deamination from tryptophan via the intermediate molecule indolepyruvic acid. Tryptophanase catalyzes the deamination reaction, during which the amine (NH_2) group of the tryptophan molecule is removed. Final products of the reaction are indole, pyruvic acid, ammonia (NH_3) and energy. Pyridoxal phosphate is required as a coenzyme.



Procedure: Inoculate peptone broth with the test organism and incubate at 35ºC for 18 to 24 hours. At the end of this time, add 15 drops of Kovac's reagent down the inner wall of the tube.



Result: Reddening of ring formed---positive test

No red colour---negative test

Note: 1) Kovac's reagent is recommended in preference to Ehrlich's reagent.

2) Tryptone can also be used instead of peptone as an ingredient.

Positive	**Negative**
E.coli	*Klebsiella*
P.vulgaris	*Citrobacter freundii*
P. rettgeri	

6. Methyl red

Use: The medium most commonly used is methyl red-Voges Proskauer broth, as formulated by Clark and Lubs. This medium also serves for the performance of the VP test.

Ingredients and preparation

Polypeptone	7.0 g
Glucose	5.0 g
Dipotassium phosphate	5.0 g
Water	1.0 litre
pH	6.9

Methyl red pH indicator; Methyl red, 0.1g in 300 ml of 95% ethyl alcohol

Distilled water, 200 ml

Principle and interpretation: Methyl red is a pH indicator with a range between 6.0 (yellow) and 4.4(red). The pH at which methyl red detects acid is considerably lower than the pH for other indicators used in bacteriologic culture media. Thus, to produce a color change, the test organism must produce large quantities of acid from the carbohydrate substrate being used.

The methyl red test is a quantitative test for acid production, requiring positive organisms to produce strong acids from glucose through the mixed acid fermentation pathway. Because many species of the Enterobacteriaceae may produce sufficient quantities of strong acids that can be detected by MR indicator during the initial phases of incubation, only organisms that can maintain this low pH after prolonged incubation, overcoming the pH –buffering system of the medium, can be called MR—positive.

The development of stable red color in the surface of the medium indicates sufficient acid production to lower the pH to 4.4 and constitutes positive test.

Procedure and interpretation: Inoculate the glucose phosphate broth with a pure culture of the test organism. Incubate the broth at 35ºC for 48 to 72 hours. At the end of this time, add 5 drops of methyl red reagent directly to the broth.

The development of stable red color in the surface of the medium indicates sufficient acid production to lower the pH to 4.4 and constitutes positive test.

Results

Positive	**Negative**
E.coli	*Enterobacter aerogenes*
Shigella spp.	

7. Motility test medium (Edwards and Ewing)

Use: This medium is used for checking the motility of organisms. Low agar concentration allows free movement of bacteria.

Ingredients and preparation:

Peptic digest of animal tissue (peptone)	10.0 g
Beef extract	3.0 g
Sodium chloride	5.0 g
Agar	4.0 g
Final pH at 25°C	7.4±0.2

Dissolve the ingredients by boiling in 1 litre of distilled water. Pour in tubes. Autoclave the medium at 121°C for 20 minutes. Cool the tubed medium in an upright position.

Principle and interpretation: Bacteria are motile by means of flagella. This test is done to determine whether an organism is motile or non-motile.

Procedure: The test isolate is inoculated by stabbing in the center of media in the tube with straight wire.

Result

1) Non motile—growth restricted to stab line

2) Motile—Diffused growth ‘‘swarm” extends as a zone of turbidity from the stab line.

Positive	**Negative**
Enterobacter aerogenes	*Staphylococcus aureus*
Escherichia coli	*Shigella sonnei*
Proteus mirabilis	*Klebsiella pneumoniae*

8. Nitrate reduction

Use: To determine the ability of an organism to reduce nitrate to nitrites or free nitrogen gas.

Ingredients and preparation:

Beef extract	3.0 g
Peptone	5.0 g
Potassium nitrate	1.0 g
Agar (nitrate free)	12.0 g
Distilled water	1.0 litre
Reagent A	
α---Naphthylamine	5.0 g
Acetic acid (5N), 30%	1.0 litre
Reagent B	
Sulfanilic acid	8.0 g
Acetic acid (5N), 30%	1.0 litre

Principle and interpretation: The capability of an organism to reduce nitrates to nitrites is an important characteristics used in the identification and species differentiation of many groups of microorganisms.

Organisms demonstrating nitrate reduction have the capability of extracting oxygen from nitrates to nitrites and other reduction products. The presence of nitrites in the test medium is detected by the addition of α- naphthylamine and sulfanilic acid, with the formation of a red diazolinum dye, p-sulfobenzeneazo-α – naphthylamine.

The development of a red color within 30 seconds after adding the test reagents indicates the presence of nitrites and represents a positive reaction for nitrate reduction to nitrite. If no color develops after adding the test reagents this may indicate either that nitrate has not been reduced (a true negative reaction) or nitrate has been converted to nitrite and further converted into nitrogen gas. Addition of zinc powder will reduce unbroken nitrate to nitrite producing red color indicating a true negative reaction. Failure of zinc powder to produce red color indicates that nitrate has been converted beyond nitrite to nitrogen gas.

Procedure: Inoculate the nitrate medium with a loopful of the test organism isolated in pure culure on agar medium and incubate at 35ºC for 18 to 24 hours. At the end of incubation, add 1 ml each of reagents A and B to the test medium in that order.

Positive	Negative
Enterobacteriaceae	*Neisseria gonorrhoeae*
M.tuberculosis	

9. Oxidation-fermentation test

Use: To distinguish between aerobic and anaerobic breakdown of carbohydrate.

Ingredients and preparation

Peptone	2.0 g
Sodium chloride	5.0 g
Dipotassium hydrogen phosphate	0.3 g
Bromothymol blue 1%	3.0 ml
Agar	3.0 g
DW	1.0 litre

The pH is adjusted to 7.1 before adding the bromothymol blue and the medium is autoclaved in a flask at 121ºC for 15 minutes. The carbohydrate to be added is sterilized separately and added to give a final concentration of 1%. Medium is then tubed to a depth of about 4 cm.

Principle and interpretation: This method depends upon the use of a semisolid tubed medium containing the carbohydrate together with a pH indicator. If acid is produced only at the surface of the medium, where condition is aerobic, the attack on the sugar is oxidative. If acid is found throughout the tube including the lower layers where conditions are anaerobic, the breakdown is fermentative.

Fermenting organisms (*e.g*. Enterobacteriaceae, *Aeromonas*, and *Vibrio*) produce an acid reaction throughout the medium the covered (anaerobic) as well as the open (aerobic) tube. Oxidizing organisms produce an acid reaction only in the open tube. This begins in the surface and gradually extends downwards; and may appear only after an alkaline reaction has been present for several days.

Procedure: The isolated colony from the test isolate is inoculated in two tubes using straight wire, stabbing the media halfway to the bottom of tube. One tube is covered with 1 cm layer of sterile mineral oil, leaving the other tube open to air. Incubate at 35ºc for 18-24 hours.

Acid production is detected in medium by appearence of yellow colour.

Oxidative	Fermentative
Alkaligenes spp.	*Aeromonas* spp
Pseudomonas spp.	*Staphylococcus* spp (except *S. saprophyticus*)

10. Peptone water

Use: This medium is used as a growth medium for the detection of indole. Testing of indole is important in the identification of enterobacteria.

It is also used for the basal medium for sugar fermentation.

Ingredients and preparation

Peptone (peptic digest of animal tissue)	10.0 g
Sodium chloride	5.0 g
Distilled water	1.0 litre
Final pH at 25°C	7.5 ±0.2

Dissolve by warming. Pour in tubes and autoclave the medium at 121°C for 20 minutes.

11. Phenylpyruvic acid medium (PPA)

Use: It is used to determine the ability of an organism to deaminate phenyl alanine to phenyl pyruvic acid enzymatically with resulting acidity.

Ingredients and preparation

Yeast extract	3.0 g
DL-phenylalanine	2.0 g
Or L-phenylalanine	1.0 g
Disodium hydrogen phosphate	1.0 g
Sodium chloride	5.0 g
Agar	12.0 g
Distilled water	1.0 litre

Adjust the pH to 7.4, distribute and sterilize by autoclaving at 121°C for 15 minutes. Allow to solidify in tubes as long slopes.

Principle and interpretation: This test indicates the ability of an organism to deaminate phenylalanine with the production of phenylpyruvic acid, which will react with ferric salts to give a green color. Deamination of phenylalanine and utilization of malonate can be combined in one test.

Procedure: The agar slant of the medium is inoculated with a single colony of the test organism isolated in pure culture of primary plating agar. After incubation at 35^0C for 18

to 24 hours, 4 to 5 drops of the ferric chloride reagent are added directly to the surface of the agar. As the reagent is added, the tube is rotated to dislodge the surface colonies.

Result:

Positive test: - A green color will develop in fluid and in the slope within one minute.

Negative test: - No color change

Positive	**Negative**
Proteus vulgaris	*E.coli*
Providencia spp	

12. Simmon's citrate medium

Use: This medium is used for the ability of the bacteria to utilize citrate as a source of carbon and energy.

Ingredients and preparation

NaCl	5.0 g
$MgSO_4$	0.2 g
$NH_4H_2PO_4$	1.0 g
$KH_2\ PO_4$	1.0 g
Sodium Citrate	5.0 g
Distilled water	1.0 litre
Bromothymol blue (0.2% aq solution)	10 ml
Agar (2%)	20 ml

Adjust the pH to 6.8 by adding 1N NaOH (8 ml/L)

Steam for 1 hour and distribute 2.5 ml in each tube and autoclave at 121ºC for 15 minutes. Finally make slopes.

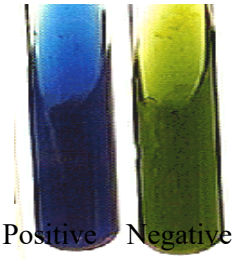
Principle and interpretation: The medium contains citrate, ammonium ions, and other inorganic ions needed for growth. It also contains bromothymol blue, a pH indicator. Bromothymol blue turns blue at a pH of 7.6 or greater. When an organism catabolizes citrate, it produces alkaline waste products, causing the medium to turn blue. Furthermore, only an organism that can utilize citrate will produce visible growth on the citrate slant.

Result

Positive: Blue colored growth

Negative: No growth/ no color change

Positive	Negative
Klebsiella	*E.coli*
Salmonella (except *S.* Typhi *and S.* Paratyphi A)	*Shigella*
Proteus rettgeri	*Edwardsiella*


Positive Negative

13. Sugar fermentation test

Use: It is used to determine the ability of an organism to ferment a specific carbohydrate incorporated in a basal medium and produce acid or acid with visible gas.

Ingredients and preparation

Peptone (peptic digest of animal tissue)	10.0 g
Sodium chloride	5.0 g
Distilled water	1.0 litre
Final pH at 25°C	7.4 ±0.2
Used as basal medium for sugar fermentation	
Andrade's indicator	1%

To it add different sugar at a concentration of 1% (dextrose, lactose, maltose, sucrose) sterilized by seitz filtration method or autoclave at 10 lbs pressure for 15 minutes.

Principle and interpretation: Organism ferments the sugar leading to production of acid with or without gas. Acid production is indicated by dark pink colour and gas produced will collect in Durham's tube.

14. Triple sugar iron agar

Use: Triple sugar iron agar is used for the differentiation of enteric pathogens by ability to determine carbohydrate fermentation and hydrogen sulphide production.

Ingredients and preparation

Beef extract	3.0 g
Yeast extract	3.0 g
Peptone	20.0 g
Glucose	1.0 g
Lactose	10.0 g
Sucrose	10.0 g

Ferric citrate	0.3 g
Sodium chloride	5.0 g
Sodium thiosulphate	0.3 g
Agar	12.0 g
Phenol red, 0.2% solution	12.0 ml
Distilled water	1.0 litre
Final pH	7.4

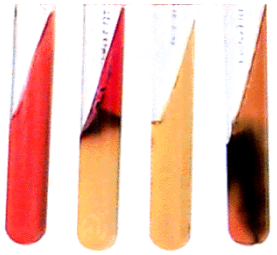
Heat to boiling to dissolve the medium completely. Mix well and pour in the tubes. Sterilize at 121ºC for 15 minutes and cool to form slopes with deep butts (3 cm).

Results

Butt colour	**Slant colour**	**Interpretation**
Yellow	Red	Glucose only fermented
Yellow	Yellow	Glucose fermented, also lactose and/or sucrose
Red	Red	No action on glucose, lactose or sucrose

Bubbles or cracks present: gas production

Black precipitates present: hydrogen sulphide gas production



Butt:	Red	Yellow	Yellow	Red
Slant:	Red	Red	Yellow	Red

15. Urease test

Use: To determine the ability of an organism to split urea into two molecules of ammonia by the action of the enzyme, urease, with resulting alkalinity

Ingredients and preparation

Peptone	1.0 g
Sodium chloride	5.0 g
Di potassium hydrogen phosphate	2.0 g
Phenol red	6.0 ml
Agar	20.0 g
Distilled water	900 ml

Adjust pH at 6.8—6.9 with 1N NaOH

Then add sterile glucose solution 1gm/l. Autoclave at 10 lbs pressure for 20 minutes. Sterilize urea 20 g in 100 ml. by filtration. Distribute 2.5 ml in each sterile tube and slopes are made.

Principle and interpretation: Bacteria, particularly those growing naturally in an environment exposed to urine may decompose urea by enzyme urease. The occurrence of this enzyme can be tested for by growing the organism in the presence of urea and testing the alkali production by means of a suitable pH indicator.

Heavy inoculum of growth is inoculated on the surface of the slants. When urea is utilized ammonia is formed during incubation which makes the medium alkaline showing a pink red color by the change in the phenol red indicator.

Positive	**Negative**
Proteus	*E.coli*
K.pneumoniae (weakly)	

16. VOGES-PROSKAUER TEST

Use: To determine the ability of some organisms to produce a neutral end product, acetylmethyl carbinol (AMC, acetoin) from glucose fermentation.

Ingredients and preparation: Medium is Glucose phosphate (GP) Broth

Reagents:

- α-naphthol 5% color intensifier

α-naphthol	5.0 g
Absolute alcohol	100 ml

- Potassium hydroxide 40% oxidizing agent

KOH	40.0 g
Distilled water	100 ml

Principle and interpretation: Pyruvic acid the pivotal compound formed in the fermentative degradation of glucose is further metabolized through various metabolic pathways, depending on the enzyme systems possessed by different bacteria. One such pathway results in the production of acetoin (acetyl methyl carbinol) a neutral reacting end product in the presence of atmospheric oxygen and 40% potassium hydroxide, acetoin is converted to diacetyl, and α-naphthol serves as a catalyst to bring out a red complex.

Procedure: Inoculate glucose phosphate broth with a pure culture of test organism. Incubate for 24 hours at 35^0C. At the end of this time, aliquot 1 ml of broth to a clean test tube. Add 0.6 ml of 5% α-naphthol followed by 0.2 ml of 40% KOH. It is essential that the reagents be added in this order. Shake the tube gently to expose the medium to atmospheric oxygen and allow the tube to remain undisturbed for 10 to 15 minutes.

Positive	**Negative**
Enterobacter aerogenes	*E.coli*

A positive test is represented by the development of a red color 15 minutes or more after the addition of the reagents.

ANNEXURE II

Preparation of Bacteriological Media and Reagents

1. Biphasic McConkey's medium (BPMM)

Agar slant

Peptone	20.0 g
Lactose	10.0 g
Bile salts	5.0 g
Sodium Chloride	5.0 g
Agar	30.0 g
Neutral red (1%)	4.0 ml
Distilled water	1.0 litre

Broth

Peptone	30.0 g
Lactose	7.5 g
Bile salts	7.5 g
Neutral red (1%)	7.5 ml
Distilled water	1.0 litre

Dissolve ingredients under 'agar' in distilled water by boiling and adjust pH to 7.6. Filter through gauze and dispense in 25 ml amounts in screw – capped prescription bottles. Sterilize at 121°C for 15 minutes. Remove the bottles from autoclave while hot and place them in a horizontal position, so as to form slants on the broader side. Allow solidifying.

Dissolve ingredients under 'broth' in distilled water and sterilize by autoclaving at 121ºC for 15 minutes. Add 30 ml to each bottle under strict aseptic conditions. Incubate all bottles at 37ºC for 48 hours to check sterility.

Use: This medium is used for direct inoculation of blood for culture.

2. Blood agar

Sterile defibrinated sheep blood	7 ml
Nutrient agar (melted)	100.0 ml

Pour about 7 ml of melted nutrient agar, as a base, into sterile petri dishes and allow setting. This forms a thin base for pouring in the blood agar. Add sterile defibrinated sheep blood (5 – 7%) to nutrient agar, the latter should be cooled to about 45 – 50°C before blood is added. Mix well and pour about 15 ml of blood agar over the base in each petri dish. **Human blood is not recommended for the preparation of blood agar** as it may contain certain antibacterial substances and hence unsuitable for use in media preparation.

Alternately blood agar may be made with no agar base.

Use: It serves as an enriched medium and a differential medium for haemolytic organisms. Most common pathogens grow on it.

3. Brain heart infusion broth (BHIB)

Sodium citrate	1.0 g
Sodium chloride	4.0 g
Sodium Phosphate	5.0 g
Dextrose	10.0 g
Peptone	10.0 g
Brain Heart infusion	
Brain Infusion broth	250.0 ml
Heart infusion broth	750.0 ml
Sodium polyanethol sulphonate	0.25 g

Obtain ox brain and heart. Remove all the fat from the heart. Cut into small pieces and grind. Add distilled water three times (v/w). Keep at 4ºC overnight.

From the brain, remove meninges fully and then, weigh. Add distilled water, (3 times v/w) and mash by using hand. Keep in the cooler overnight.

Next morning, boil the brain and heart separately, for 30 minutes. Then filter through cotton gauze layer. Measure each broth separately and then mix 1:3 (brain: heart). Mix both infusions and the remaining ingredients. Dissolve well and adjust pH of the entire amount to 7.4 to 7.6. Autoclave at 121ºC for 15 minutes. Filter through filter paper and distribute in screw capped prescription bottles in 50 to 100 ml amounts. Autoclave once more at 115ºC for 10 minutes.

To prepare media from commercially available dehydrated medium dissolve the recommended amount in distilled water, heat to dissolve, and autoclave at 15 psi at 121ºC for 20 minutes.

Brain heart infusion (Medium)	Difco		Hi-Media	
	Grams per Litre	Code	Grams per Litre	Code
Agar	52	241830	52	M211
Broth	37	237500	37	M210

Use: This is used for direct inoculation of whole blood, bone marrow and body fluids for culture.

4. Chocolate agar (CHOC)

CHOC is heated blood agar

Sterile defibrinated sheep blood	10.0 ml
Nutrient agar (melted)	100.0 ml

Melt the Nutrient agar. When the temperature is about 45 to 50ºC add the blood and mix well. After the addition of blood, heat in a water bath. Slowly bringing up the temperature to 75ºC with constant agitation. Special care should be taken to avoid fluctuation in the temperature. Heating is continued till the blood changes to chocolate color. This color is very critical. Remove from the water bath. Cool to about 50ºC and pour about 20 ml into plates with sterile precautions. Special care must be taken to avoid air bubbles.

Use: This is an enriched medium used for the cultivation of pathogenic neisseriae and *H.influenzae.*

5. Cystine trypticase agar (CTA) sugar for *Neisseria* spp:

CTA is made from commercially available dehydrated medium. Catalogue No: M159 (Himedia, Mumbai, India).

Suspend 28.5 g in 1000 ml distilled water. Adjust pH to 7.3 $\pm$ 0.2. Boil to dissolve the medium completely. Dispense in tubes 8 – 10 ml and sterilize by autoclave at 121°C for 15 minutes. Cool to 50°C and add appropriate carbohydrate. Mix well and allow the tubed medium to cool in upright position.

Casein enzymic hydrolysate	20.0 g
L-cystine	0.50 g
Sodium chloride	5.0 g
Sodium sulphite	0.50 g
Phenol red	0.017 g
Agar	2.50 g
Distilled water	1000 ml
Carbohydrates	0.5 g

Note: Add extra agar of 1.25 g/100 ml of the dehydrated medium

6. Haemophilus test medium (HTM)

Hemin stock solution

Hematin	50.0 mg
NaOH, 0.01 mol/l	100.0 ml

Dissolve the hemin with heat, stirring until the powder is completely dissolved.

NAD stock solution (Sterilize by filtration)

NAD	50.0 mg
Dist. Water	10.0 ml

Preparation

MHA	1.0 l
Hematin stock solution	30.0 ml
Yeast extract	5.0 gm.

Sterilize by autoclaving, cool to 50°C, add 3.0 ml NAD stock solution aseptically.

pH 7.2 to 7.4

7. MacConkey agar (MAC)

Peptone Hi-Media RM015	20.0 g
Sodium chloride	5.0 g
Bile salt HI Media RM 008	10.0 g
Difco Bile salt	1.125 g
Lactose	15.0 g
Agar	15.0 g
Distilled water	1000 ml

Dissolve the ingredients except lactose in distilled water by heating. Adjust pH to 7.6. Add 0.4 ml of 1% neutral red solution to every 100.0 ml of medium with lactose. Sterilize by autoclaving at 121ºC for 15 minutes.

To prepare media from commercially available dehydrated medium dissolve the recommended amount in distilled water, heat to dissolve, and autoclave at 15 psi at 121ºC for 20 minutes.

Medium	Difco		Hi-Media	
	Grams per Litre	Code	Grams per Litre	Code
McConkey agar	47.3	294584	55	M082

Use: This is a partially selective and a differential medium, used for the differentiation of lactose fermenting and non–lactose fermenting enteric bacteria.

Note: MAC, for exclusive use in faeces cultures, may be prepared with bile salts No.3 (Difco) a purified product at a concentration of 0.15%. Incorporation of this purified bile salt will suppress the growth of enterococci and to some extent the commensal coliform bacteria as well.

8. Mueller–Hinton agar (MHA)

MHA is made from commercially available dehydrated medium Difco™ MHA. Catalogue No: 225250, (BD, Sparks, MD, USA). 38 g of the powder provided is dissolved in 1 litre of distilled water mixed thoroughly, boiled for 1 minute to completely dissolve. Adjust pH to 7.3±0.2. Autoclave at 121°C at 15 minutes. Do not over heat.

Approximate formula per Litre is as given below:

Beef extract	2.0 g
Acid digest of casein	17.5 g
Starch	1.5 g
Agar	17.0 g
Distilled water	1000 ml

To prepare media from commercially available dehydrated medium dissolve the recommended amount in distilled water, heat to dissolve, and autoclave at 15 psi at 121ºC for 20 minutes.

Medium	Difco		Hi-Media	
	Grams per Litre	Code	Grams per Litre	Code
Mueller Hinton agar	38	225250	38	M173

Use: Standard medium for antimicrobial susceptibility testing.

9. Nutrient agar (NA)

Agar powder	1.5 to 1.8 g
KH_2PO_4	0.05 g
NaH_2PO_4	0.05 g
Nutrient broth	100 ml

Mix the agar in nutrient broth and heat to dissolve. When cool adjust the pH to 7.5-7.6. Sterilize by autoclaving. Pour as plates or slopes. To make deeps, reduce agar concentration to 0.5%.

Use: This is used as a base for many media. Only nonfastidious organisms will grow on this.

10. Nutrient Broth (NB)

Peptone	1.0 g
Beef extract (Lab Lemco)	0.4 g
Sodium chloride	0.5 g
Distilled water	100 ml

Weigh out all the ingredients as above, peptone should be taken last, because it sticks to the paper on exposure. Mix the ingredients and dissolve them by heating. When cool, adjust the pH to 7.4 – 7.6. Distribute in tubes, bottles or flasks and sterilize by autoclaving.

To prepare media from commercially available dehydrated medium dissolve the recommended amount in distilled water, heat to dissolve, and autoclave at 15 psi at 121ºC for 20 minutes.

Medium	Difco		Hi-Media	
	Grams per Litre	Code	Grams per Litre	Code
Nutrient agar	23	213000	28	M001
Nutrient broth	8	234000	13	M002

Use: This is a basal medium and is also used to grow non fastidious organisms for various purpose.

11. Thioglycollate broth:

Thioglycollate broth is made from commercially available dehydrated medium. Catalogue No: M009 (Hi-Media, Mumbai, India)

Suspend 29.75 g in 1000 ml of distilled water. Adjust pH 7.1 $\pm$ 0.2. Heat with frequent agitation and boil until solution is complete. Dispense in 15 x 150 mm test tubes to fill about 2 / 3 of its length. *i.e* approximately 10 ml. Sterilize by autoclaving at 118ºC for 15 minutes. Store at room temperature. Do not use the medium if more than the upper third of the medium is pink in color.

Pancreatic digest of casein	15.0 g
Yeast extract	5.0 g
Dextrose (glucose)	5.50 g
Sodium chloride	2.50 g
L- cystine	0.50 g
Sodium thioglycollate	0.50 g
Resazurin sodium	0.001 g
Agar	0.75 g
Distilled water	1000 ml

To prepare media from commercially available dehydrated medium dissolve the recommended amount in distilled water, heat to dissolve, and autoclave at 15 psi at 121ºC for 20 minutes.

Medium	Difco		Hi-Media	
	Grams per Litre	Code	Grams per Litre	Code
Thioglycollate broth	29.8	225650	29.75	M009

Use: For the cultivation of anaerobic and microaerophilic bacteria. Heat in a boiling water bath and cool before use. Such restoration of anaerobic condition may be done once only.

12. Trypticase soya agar (TSA)

TSA is made from commercially available dehydrated medium, Difco ™ TSBA. Catalogue No: 212305 (BD, Sparks, MD, USA).

Suspend 40 g of the powder in 1 litre of distilled water. Mix the ingredients and dissolve by heating in a water bath. Sterilize by autoclaving at 121°C for 15 minutes. Repeated heating should not be done.

Pancreatic digest of casein	15.0 gm
Papaic digest of soyabean meal	5.0 gm
Sodium chloride	5.0 gm
Agar	15.0 gm
Distilled water	1000 ml

Use: As a base for blood agar especially for the cultivation of *S. pneumoniae* and other streptococci.

13. Trypticase soya broth (TSB)

TSB is made from commercially available dehydrated medium, Difco TM TSBA. Catalogue No: 211825 (BD, Sparks, MD, USA).

Suspend 30 g of powder in 1 litre of distilled water. Adjust pH 7.3±0.2. Warm gently to dissolve. Dispense and autoclave at 121°C for 15 minutes.

Pancreatic digest of casein	17.0 g
Papaic digest of soyabean meal	3.0 g
Sodium chloride	5.0 g
Dipotassium phosphate	2.5 g
Dextrose	2.5 g
Distilled water	1.0 litre

To prepare media from commercially available dehydrated medium dissolve the recommended amount in distilled water, heat to dissolve, and autoclave at 15 psi at 121ºC for 20 minutes.

Medium	Difco		Hi-Media	
	Grams per Litre	Code	Grams per Litre	Code
Tryptone soya agar	40	236950	40	M290
Tryptone soya broth	30	211825	27.5	M322

Use: This is an all-purpose medium.Is also suitable for blood cultures, in place of BHI.

14. Trypticase soya blood agar (TSBA)

TSBA is made from commercially available dehydrated medium, Difco ™ TSBA. Catalogue No: 227300 (BD, Sparks, MD, USA).

40 g of the powder provided is dissolved in 1 litre of distilled water. Adjust pH 7.3±0.2. Autoclave at 121°C for 15 minutes.

Approximate formula per Litre is as given below:

Tryptone H	15.0 g
Soyatone	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Distilled water	1000.0 ml

To 100.0 ml of Trypticase Soya Agar cooled to 45–50ºC add 5 to 7 ml of defibrinated sheep blood. Mix and pour plates.

Use: For the better growth and isolation of streptococci including *S. pneumoniae.*

15. Skim milk (10%) and glycerol (15%) solution

10% skim milk and 15% glycerol solution is used for long term preservation of isolates by freezing at -70°C.

Media preparation

i. Place 10 g dehydrated skim milk and 85 ml distilled water into flask A. Swirl to mix.

ii. Place 15 ml of glycerol into flask B.

iii. Autoclave both flasks at 115°C for 10 minutes, and exhaust the pressure carefully.

iv. While still hot, pour the contents of flask A into flask B in a safety cabinet.

v. Store at 4°C when not in use.

Quality control: Streak 10 µl onto a BAP or a CAP and incubate for 48 hours at 35-37°C with ~5% CO_2 (or in a candle-jar) to verify the sterility of the solution. Passing result will show no growth.

16. Mueller -Hinton sheep blood agar (MH-SB)

Prepare Mueller-Hinton agar according to the instructions on the label on the dehydrated medium. Autoclave at 121^{o}C for 15 minutes and cool to 50^{o}C in a water bath .Add 5% defibrinated sheep blood (5 ml per 100 ml media) and dispense into 15 x 100 mm petri dishes. Allow to solidify, place in plastic bags and store at +4^{o}C.This medium should appear bright red in color. Before inoculation plates should be dried with lids ajar so that there are no droplets of moisture on the agar surface. The time taken to achieve this depends on the drying conditions.

17. STGG media (Skim milk-Tryptone-Glucose-Glycerol)

Ingredients

- Skim milk powder (from Difco) 2 g
- Tryptone soya broth (TSB, from Oxoid) 3 g
- Glucose 0.5 g
- Glycerol 10 ml
- Distilled water 100 ml

Preparation

- Mix to dissolve all ingredients.
- Dispense 1.0 ml of STGG medium into 1.5 ml screw-capped vials.
- Loosen the screw-cap tops and autoclave for 10 minutes (at 15 pounds) at 121 °C.
- Tighten caps after autoclaving.
- STGG vials can be stored frozen at −20 °C (or colder) or refrigerated until use.

- A standard volume of 1.0 ml is preferred to allow for comparisons across studies in quantification of pneumococci.
- Allow tubes of STGG medium to reach room temperature before use.
- Usually the milk solids pellet in the bottom of the tube is resuspended by vortexing for 10–20 seconds

Note: STGG Medium should be used within 6 months of preparation whether stored frozen or refrigerated

Quality control

- A quality control test for sterility of the STGG medium must be performed on each batch- Streak 10 µl onto a blood agar plate (BAP) or a chocolate agar plate (CAP) and incubate for 48 hours at 35-37°C with ~5% CO_2 (or in a candle-jar) to verify the sterility of the solution
- The ability of STGG medium to support recovery of viable pneumococci should also be checked- From a batch of newly prepared medium, inoculate *Streptococcus pneumoniae* into two to three vials, incubate at 37°C with ~5% CO_2 (or in a candle-jar) for 18- 24 hours, subsequently subculture onto BAP or CAP, incubate the plates at 37°C with ~5% CO_2 (or in a candle-jar) for 18- 24 hours, look for the presence of *Streptococcus pneumoniae* colonies
- Growth and recovery of ATCC49619 *S.peumoniae* after freezing in STGG medium at -80^{0}C for 48 hours

18. Quality control (QC) of media

For QC check for all the above media, see chart for quality control in this section.

Media	Organisms	Expected Results
Blood agar	*S. pyogenes*	Small colonies with beta haemolysis
	Viridans streptococcus	Small colonies with alpha haemolysis
	S. pneumoniae	Alpha haemolytic smooth/mucoid colonies
Chocolate agar	*H. influenzae*	Translucent colonies
McConkey agar	*E. coli*	Non-mucoid lactose fermenting colonies
	P. mirabilis	Non-lactose fermenting colonies; no swarming
	E. faecalis	Magenta pink colonies
	V. cholerae	Non lactose fermenting colonies with dew drop appearance.
Mueller-Hinton Agar	*S. aureus*	Good growth/ATCC 29212 E. *faecalis* – sxt >20mm satisfactory
Mueller-Hinton blood agar/TSBA	*S. pneumoniae*	Large alpha haemolytic colonies with pitting
Nutrient agar	*S. aureus*	Pigmented colonies
Nutrient broth	*E. coli*	Growth
	S. aureus	Growth
	P. aeruginosa	Growth with pellicle

Thioglycollate broth	*B. fragilis* *C. perfringens*	Look for growth and smear morphology
BPMM	*E. coli* *S.* Typhi	Non-mucoid lactose fermenting colonies Non Lactose fermenting colonies
HTM	*H. influenzae*	Small translucent colonies

19. Quality control strains

- **For fastidious organisms**

  S. pneumoniae ATCC 49619; OXA resistant; penicillin intermediate resistant

  S. pneumoniae ATCC 33400; OXA – susceptible

  H. influenzae ATCC 10211: type b; hemophilus test medium (HTM) medium control

  H. influenzae ATCC 49247; ampicillin résistant non type b strain

  H. influenzae ATCC 49766; ampicillin susceptible

  H. influenzae CCUG 23946; type b strain (Culture Collection University of Gothenburg, Sweden)

- **For non-fastidious organisms**

  Escherichia coli ATCC 25922 (beta-lactamase negative)

  Escherichia coli ATCC 35218 (beta-lactamase positive)

  Enterococcus faecalis ATCC 29212 (to check thymidine or thymine level of MHA)

  Enterococcus faecalis ATCC 51299 (vancomycin resistant, *vanB* and HLAR)

  Klebsiella pneumoniae ATCC 700603 (for cefpodoxime)

  Pseudomonas aeruginosa ATCC 27853 (for aminoglycosides)

  Staphylococccus aureus ATCC 38591 (beta-lactamase positive)

  Staphylococccus aureus ATCC 43300 (oxacillin resistant)

  Staphylococccus aureus ATCC 29213 (beta-lactamase positive)

Stock cultures should be kept at -70ºC in brucella broth with 10% glycerol for up to 3 years. Before use as a QC strain, the strain should be subcultured at least twice and retested for characteristic features. Working cultures are maintained on TSA slants at 2-8°C for up to 2 weeks.

20. McFarland standard

A barium chloride 0.5 McFarland density standard solution is prepared by adding 0.5 ml of a 1.175% (v/v) barium chloride dihydrate ($BaCl_2.2H2O$) to 99.5 ml of 1% sulfuric acid. The resulting mixture is placed in a tube identical to that used for preparing the dilution for the antimicrobial susceptibility tests. The same size tube (screw capped) and volume of liquid must be used. Store in the dark, at room temperature when not in use. Prepare a fresh standard solution every 6 months. Mark tube to indicate level of liquid, check before use to be sure that evaporation has not occurred. If evaporation has occurred, prepare a fresh standard.

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