Standard Operating Procedures
Bacteriology

Antimicrobial Resistance
Surveillance and Research Network

2nd Edition, 2019

Indian Council of Medical Research
New Delhi, India

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Foreword

This Standard Operating Procedure (SOP) manual is intended to assist as a reference material for routine laboratory activities of ICMR's 'Antimicrobial Resistance Surveillance and Research Network' (AMRSN). This edition of SOP embodies the continued efforts of ICMR to provide clinically relevant and practical document for the standardized in vitro susceptibility testing (AST) of bacterial pathogens. The manual guide the expected and required procedures needs to be followed by laboratory personnel while handling bacterial pathogens and performing AST to enhance the operational performance. I am glad that ICMR's national network on anti-microbial drug resistance is continuously thriving for containment of AMR and providing in-depth understanding of clonality of drug resistant pathogens and their transmission dynamics.

The manual describes the well accepted methods of specimen collection, transport, culture, identification and anti-microbial susceptibility testing on clinically important gram positive and gram negative bacteria. The major revisions include procedures for tissue samples collection, identification of Streptococcus spp. (beta and alpha hemolytic) and Streptococcus pneumoniae, automated methods of Minimum Inhibitory Concentration (MIC) testing and updated zone diameters and MIC breakpoints. This edition has been updated for phenotypic and genotypic methods (molecular mechanism of resistance) of AST and External Quality Assurance Scheme (EQAS) in a concise and easy manner. The manual has been improved and updated to be in sync with CLSI standard documents. Reference to any commercial method or equipment does not mean endorsement of ICMR, this is only for the purpose of this research study.

I applaud the diligent efforts of editorial board members and ICMR team. I am hopeful that users will be benefited immensely from this manual and it will meet its intended objectives. I am optimistic that it will further evolve for the clinical as well as research purposes through periodic revisions and updates.

I convey my best wishes to all.

Prof. Balram Bhargava
Secretary, DHR & Director General, ICMR

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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

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AMENDMENT SHEET

S. No.	Page No	Clause No	Date of amendment	Amendment made	Reason	Signature SR	Signature Lab Director

General Guidelines

CHAPTER 1

General Guidelines

1. Introduction

This is a comprehensive standard operative 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.

2. Role of the laboratory

Microbiologists play a critical role in gathering 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 to preventing morbidity and mortality from bacterial infections.

3. Biosafety

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

While laboratory-acquired infections are not as extensively reported, deadly infections with any of the organisms 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 and be directed 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 when work is being conducted.
- Extreme precautions must be taken with contaminated sharp items and other biomedical wastes (BMWs), and sharps or other BMWs must be disposed off in appropriately labeled hardened plastic containers as per the guidelines of the local/central Pollution Control Board (PCB).
- Personal protective equipment (PPE) must be used appropriately, and particular 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[1]

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[1]

	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	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
[c] Dependent on agent(s) used in the laboratory.
[d] For example, window, closed-circuit television, two-way communication.

3.1. Protective clothing and equipment

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.

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 off by discarding them with other disposable materials and autoclaving. Hands should be washed immediately after removing gloves.

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.

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.

3.2.1. Limiting access to laboratory

Sometimes non-laboratorians attempt to enter the laboratory to obtain test results. 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 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. All laboratories should be locked when not in use. In addition, all freezers and refrigerators located in corridors should be locked, especially those that contain infectious organisms or other hazardous materials.

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. To verify that each autoclave is working properly, spore strips (such as *Geobacillus stearothermophilus*) or other biological indicators designed to test for efficiency of sterilization should be included in autoclave loads on a regular basis (i.e., monthly). Each autoclave load should be monitored with temperature-sensitive tape, thermograph, or by other means (i.e., chemical indicators or biological indicators). A logbook should be maintained for each autoclave to record the date, times, and indicator of sterilization of each autoclave run.

3.2.3. Disinfection[2,3]

Organisms may have different susceptibilities to various disinfectants. As a surface disinfectant, 70% isopropyl alcohol is generally effective. However, 70% alcohol 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. They are not active against spores and their activity against nonlipid viruses is variable. Always read disinfectant labels for manufacturers' recommendations for dilution and for exposure times for efficacy. An effective general disinfectant is a 1:100 (1% or 10,000 ppm) dilution of household bleach (sodium hypochlorite) in water; at this dilution, bleach can be used for wiping surfaces of benches, hoods, and other equipment. A 1:100 dilution of bleach (10,000 ppm of available chlorine) should be used to clean up spills of cultured or concentrated infectious material where heavy contamination has occurred; however, it is more corrosive, will pit stainless steel, and should not be used routinely. If bleach is used, wipe down the area with 70% alcohol to inactivate the bleach. If bleach is used as a disinfectant, the diluted solutions should be made weekly from a concentrated stock solution.

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 items. Waste disposal containers for infectious or potentially infectious items should be lined with a plastic biohazard or otherwise specially marked bag.

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 inactivate 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[1]

	"Clean" Conditions[a]'	**"Dirty" Conditions[b]**
Available chlorine required	0.1% (1 g/I)	0.5% (5 g/1)
Sodium hypochlorite solution (5% available chlorine)	20 m1/1	100 m1/I
Calcium hypochlorite (70% available chlorine)	1.4 g/I	7.0 g/I
Sodium dichloroisocyanurate powder (60% available chlorine)	1.7 g/I	8.5 g/I
Sodium dichloroisocyanurate tablets (1.5 g available chlorine per tablet)	1 tablet per litre	4 tablets per litre
Chloramine (25% available chlorine)	20 g/I	20 g/I

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

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.

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- 1% dilution or 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.

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.

3.2.9. Mouth pipetting

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

3.2.10. Sharps

A high degree of precaution must always be taken with any 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).

3.2.11. Aerosols

All procedures must be carefully performed to minimize splashes or aerosolization. When procedures with a high potential for creating infectious aerosols are conducted or when a procedure that can result in splashing or spraying of the face with infectious or other hazardous materials is used, 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. If safety-capped tubes are not available, sealed tubes should be used.
- All body fluids and infectious materials should only be centrifuged in carriers with safety caps.

- Handling tissue specimens or body fluids: gauze should be used to remove the tops on blood specimens and should be placed around the top of blood culture bottles to minimize aerosol production during removal of the needle. Grinding of tissue specimens should be performed in a biosafety cabinet.
- Sonic disruption: infectious materials that undergo sonic disruption should be placed in a sealed container within the sonicator.
- Opening containers of infectious materials whose internal pressures or temperatures may be different from ambient pressures or temperatures.
- 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.

3.2.12. Refrigerators and freezers

The temperature of laboratory refrigerators and freezers should be monitored daily to ensure that they are functioning properly. 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.

3.2.13. Fire prevention

Burners should be used away from light fixtures and flammable materials. Bulk 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 such safety cabinets for chemical storage. Burners must be turned off when not in use. All laboratorians must know the location of fire extinguishers, fire blankets, alarms, and showers, and fire safety instructions and evacuation routes should be posted.

Note: Chemicals should not be stored in alphabetical order.

Table 1.4: General rules for chemical incompatibilities[1]

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

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.

3.3. Special Practices

3.3.1. Accidents

All injuries or unusual incidents should be reported immediately to the supervisor. When 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. Report a needle-stick injury, any other skin puncture, to the supervisor and appropriate health officials immediately as prophylactic treatment of the personnel performing the procedure may be indicated. 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 isolated to prevent anyone from entering.

3.3.2. Laboratory design and equipment

The laboratory should be designed to avoid conditions that pose biosafety problems. Ample space should be provided to allow for safe circulation of staff when working and cleaning. There should be clear separation of areas for infectious and non-infectious work. 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. Security measures should also prevent theft, misuse, or deliberate release of the infectious materials.

Table 1.5: Biosafety equipment[1]

Equipment	Hazard Corrected	Safety Feature
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 pathogents, 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 bube. 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 um) • 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)[1]

BSC	Face Velocity (m/s)	Airflow (%)		Exhaust System
		Recirculated	Exhausted	
Class I"	0.36	0	100	Hard duct
Class I1A1	0.38-0.51	70	30	Exhaust to room or thimble connection
Class IIA2 vented to the outside'	0.51	70	30	Exhaust to room or thimble connection
Class I1B1'	0.51	30	70	Hard duct
Class 11823	0.51	0	100	Hard duct
Class 111'	NA	0	100	Hard duct

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[4], HBV, HCV, tuberculosis) and immunity screen (hepatitis B surface antibody). According to the latest pollution control board recommendations all health care workers are required to be immunized against HBV and tetanus [Bio-Medical Waste Management (Amendment) Rules, 2018][5]**.**

- Prophylaxis or other specific protective measures may be applied after a risk assessment of possible 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:
- Conclusion of the risk assessment
- Verification by serology of the immunization status of the worker (some workers may be already immune from prior vaccination or infection)
- The local availability, licensing state, 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

4. Laboratory safety instructions

- Do not eat or drink in the laboratory.
- 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 minimise the risks of spills, splashes and the production of aerosols.
- Always wash your hands before leaving the laboratory.
- Benches should be clear of all non-essential materials including books and notes.

5. Emergency measures: Mishaps with infective material

5.1. Spillage or minor spills

Put on gloves, cover the spill with a cloth or tissue soaked in disinfectant, leave for at least 10 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.

5.2 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. If following cut or puncture, post exposure prophylaxis for HIV is contemplated, use 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.

Specimen Collection, Transport & Processing

CHAPTER 2

Specimen Collection, Transport and Processing

1. BLOOD

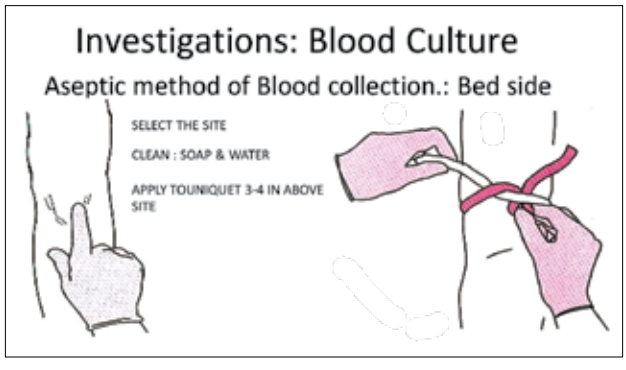
1.1. Collection and transport

Purpose: To reduce blood culture contamination rate, collection may be improved by taking the following precautions[6,7,8,9].

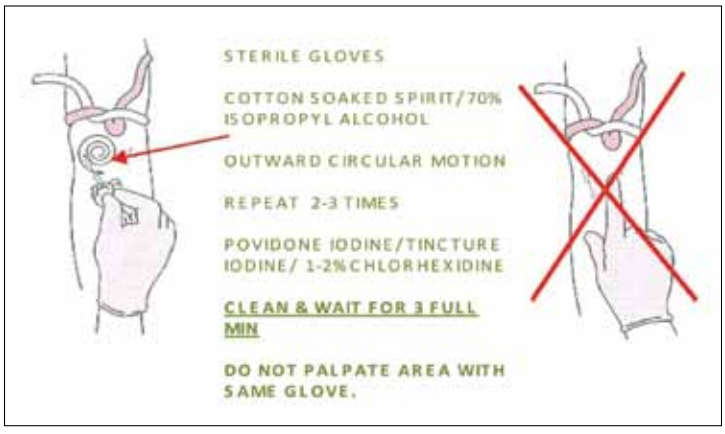
Note: This is an emergency procedure. The sample should be processed and reported immediately. The results of the smear should be informed to the concerned clinician and documented in the critical alert register.

1.1.1. Prepare the site

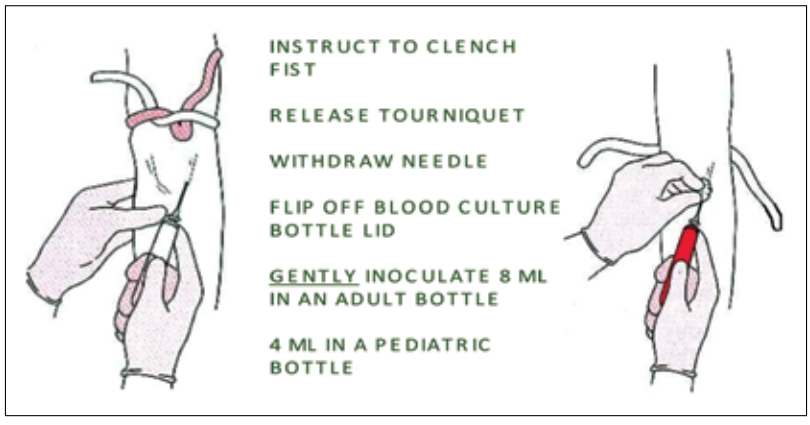
- Select the site of venipuncture. If the patient is unusually dirty, wash the intended site with soap and water prior to venipuncture.
- Apply a tourniquet, 3-4 inches above the intended site of venipuncture. Alternatively this can be done after cleaning.
- Put on examination gloves.
- Vigorously cleanse 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 (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[6,7].

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[9].

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[9].
- 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[9]

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[10].
- **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[9].

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 37°C and examine daily for 7 days for evidence of growth, indicated by turbidity, hemolysis, gas production, discrete colonies, or a combination of these.

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.

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.

1.3. Further processing of positive blood cultures

a. Gram-sain 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.

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).

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[11].

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. CSF

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.

2.1.1. Specimen collection

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[12].
- Collect the CSF into five 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.

2.1.1.2. 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.

2.1.2. 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.

2.1.3. 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. CSF Processing

Day 1

2.2.1. CSF gross appearance

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

2.2.2. Centrifuge CSF

- If <1 ml volume, vortex 30 seconds.
- If >1 ml volume, centrifuge at 3000 g for 20 minutes /cytocentrifuge (1000 rpm for 10 minutes)

2.2.3. Wet mount

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

2.2.4. India ink preparation

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

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

2.2.6. Culture[13]

- Chocolate agar (CHOC), blood agar, McConkey agar and 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

2.2.7. 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.
  - Triple antigen test for *S. pneumoniae, N. meningitidis, H. influenzae* is also performed directly on CSF, on request of clinician.

2.3. Reporting of results and interpretation[14]

- 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 report the organisms isolated or report absence of growth
- Molecular testing results (if applicable).

Interpretaion

- Abnormalities associated with bacterial meningitis
- Reduced glucose concentration: <60% serum glucose
- Elevated protein concentration
- Raised white blood cell (WBC) count: 10^1 - 10^4 predominantly polymorphs
- Elevated intracranial pressure

Table 2.3: Commonly isolated pathogens of clinical significance

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*

3. BODY FLUIDS FROM STERILE SITES

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 syringe 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.

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 needless 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.

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 BACTEC® bottles like CSF.

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.

3.4 Reporting of results and interpretation[15]

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

4. OCULAR SPECIMENS[8]

Note: For detailed procedures on ocular microbiology, please refer to http://www.ijmm.org/documents/ocular.pdf [16].

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) [17].
- For viral cultures, use Dacron or cotton swabs with non-wood shafts[18].

4.1.1. Collection by anatomic site[16]

4.1.1.1. Conjunctiva (bacterial conjunctivitis) and lid margin (blepharoconjunctivitis)

- 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.

4.1.1.2. Cornea (bacterial keratitis)

- 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.

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.

4.2. Specimen processing[16]

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.

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[16]
- 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[16]

- 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

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,* 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.

5. RESPIRATORY SPECIMENS[8]

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

5.1. Specimen collection and transport

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. Such communication with patients can be rewarding. 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[6,7,19].
- Deliver the specimen to the laboratory as quickly as possible, preferably within 2 hours, for delicate bacterial, viral and mycoplasma pathogens may die out during longer delay.

5.1.2. Endotracheal aspirate (ETA) [17]

- 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.

5.1.3. Bronchoalveolar lavage (BAL) [21]

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.

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.

5.2. Type of container

Collect in a sterile leak proof screw-cap container.

5.3. Rejection criteria

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 hours 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.

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.

5.4. Processing

5.4.1. Media and Reagents

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

5.4.2. Sputum and ETA, note the following:

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

5.4.3. Microscopic examination

Gram stain (G/S) Sputum[22,23]

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 homogenisation or on the basis of a ratio <2:1 of WBC: SECs determined by a Gram stain at low power magnification (x100)

BAL[24]

Microscopic examination of cells from lavage has also been used to diagnose pneumonia[25]. 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%.

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[26].

5.6. Culture

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

Suction tips (ST) [27]

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.

5.7. Reporting of results and interpretation (on Day 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*

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**.**

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.

6. PUS

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

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.

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 (*e.g*., Vacutainer® or similar type) 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.

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.

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.

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.

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.

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.

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, bood 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 sterile.
- 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[28,29]

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*,
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- hemoplytic 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*

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.

7.1. Collection of urine

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 voiding 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.

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.

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.

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.

7.1.5. Cystoscopy specimens

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

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.

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.

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.

7.3. Processing of specimen

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.

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.

7.3.3. Culture

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 GNBand/ 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.

7.3.3.2 Special situations

- Suprapubic collection– use full plate BA and MA
- PCN aspirate-use full plate BA, MA, CA and thioglycollate 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.

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.

7.4. Results and interpretation

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.

7.4.2. Interpretation of counts

The significance of a positive urine culture is most reliably assessed in terms of the number atedaof 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.

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 *Staphylocioccus* spp. may cause UTI.

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

7.5. Reporting[30]

- 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.

Table 2.7: Distribution of urine pathogens according to age groups and gender, data are reported as percentages of total patients in each age group

Organism	Gender	≤14 years	15–29 years	30–59 years	≥60 years
E. coli	All	58.9	71.0	71.0	66.3
	Female	64.1	71.8	71.7	70.8
	Male	51.3	60.3	66.7	52.2
K. pneumoniae	All	6.1	9.2	8.6	8.8
	Female	6.3	9.1	9.0	9.3
	Male	5.9	10.3	8.0	7.2
E. faecalis	All	2.6	5.4	6.2	6.7
	Female	3.4	5.4	6.1	5.2
	Male	1.4	5.2	6.5	11.6
P. mirabilis	All	15.6	3.7	3.8	5.2
	Female	11.8	3.1	3.5	4.3
	Male	21.2	12.1	5.8	8.0
P. aeruginosa	All	5.7	1.2	0.9	3.2
	Female	5.1	0.8	0.5	1.7
	Male	6.6	6.9	3.3	7.8
S. agalactiae	All	0.3	3.7	3.8	1.5
	Female	0.2	4.0	4.1	1.4
	Male	0.3	0	1.3	1.8

Table 2.8: Distribution of the six more frequently isolated species according to patients' gender[31]

Organism	All	Females	Males
E. coli	67.6	71.0	55.0
K. pneumoniae	8.8	9.1	7.3
E. faecalis	6.3	5.4	9.5
P. mirabilis	5.2	4.2	9.0
P. aeruginosa	2.5	1.4	6.8
S. agalactiae	2.3	2.5	1.5
All other G−	6.8	5.9	9.8
All other G+	0.7	0.5	1.1

8. FECAL SPECIMENS

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[32]. *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.

8.2 Microscopy

- For all watery faeces samples, whether the doctor orders or not, examines 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* O1 non-differential and *V. cholerae* O139 specific antiserum and examine again under darkfield microscope.

8.3 Culture and Isolation

- Commonly encountered enteric pathogens and potential pathogens include *Salmonella*, *Shigella*, *V. cholerae*, *Arizona*, *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[33].
- 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.

8.4 Colony characteristics on different media

8.4.1. Desoxycholate citrate agar and MacConkey's 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.

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*)

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.

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.

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.

9. TISSUE SAMPLE PROCESSING[34]

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 Class II exhaust protective 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.

Identification of Isolates to Species Level

CHAPTER 3

Identification of Isolates to Species Level

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

1. Enterobacteriaceae : Dr Pallab Ray : PGIMER, Chandigarh

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

2. Typhoidal *Salmonella* : Dr Arti Kapil, AIIMS, New Delhi

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

3. Nonfermenter Gram Negative Bacilli : Dr V Balaji, CMC, Vellore

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

4. Staphylococci, Enterococci : Dr Sujatha Sistala, JIPMER, Puducherry

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

5. Faecal bacterial pathogens : Dr V Balaji, CMC, Vellore

E. coli Diarrhoeagenic (EPEC, EHEC, STEC), *Campylobacter jejuni, Aeromonas* spp., *Arizona* 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., *Clostridium difficile, Yersinia enterocolitica*

6. Streptococci : Dr V Balaji, CMC, Vellore

Streptococcus pneumoniae, S. pyogenes, S. agalactiae, S. viridians

1. Identification of isolates of family Enterobacteriaceae

Table 3.1: Identification of commonly isolated members of family Enterobacteriaceae

	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
Enterobacter 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 Enterobacteriaceae: 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 ≥ 85% of strains positive; - indicates ≥ 85% strains negative; ± indicates 16-84% of strains positive.

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 enterobacteriaceae with biochemicals indicating *Salmonella* Typhi or Paratyphi A, B or C should be further confirmed by slide agglutination test using the *Salmonella* antisera (Murex or Denka Seiken).

Method for slide agglutination test for serological identification (based on Kauffman -White scheme)

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.

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	-	-	-	-

1.3. Identification of *Acinetobacter spp.*

Table 3.3: Criteria for biochemical characterization of *Acinetobacter spp.*[35]

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
A. johnsonii (7)	NG	-	-	NEG	NEG	NH	NEG	NEG	V	V
A. baumannii (2)	G	-	-	+	+	NH	NEG	+	+	+
A. haemolyticus	NG	-	-	+	NEG	+	+	V	+	NEG
Acinetobacter spp. (6)	NG	-	-	+	NEG	+	+	V	+	NEG
Acinetobacter spp (10)	NG	-	-	+	NEG	NH	NEG	+	NEG	NEG
A. calcoaceticus(1)	G	-	-	+	NEG	NH	NEG	+	+	+
Acinetobacter spp (3)	NG	-	-	+	NEG	NH	NEG	+	+	V
Acinetobacter spp. (12)	NG	-	-	+	NEG	NH	NEG	NEG	+	+
Acinetobacter spp. (5)	G	-	-	+	NEG	NH	NEG	NEG	+	NEG
A. lwoffii (8/9)	NG	-	-	+	NEG	NH	NEG	NEG	NEG	NEG
Acinetobacter spp. (11)	NG	-	-	+	NEG	NH	NEG	NEG	NEG	NEG

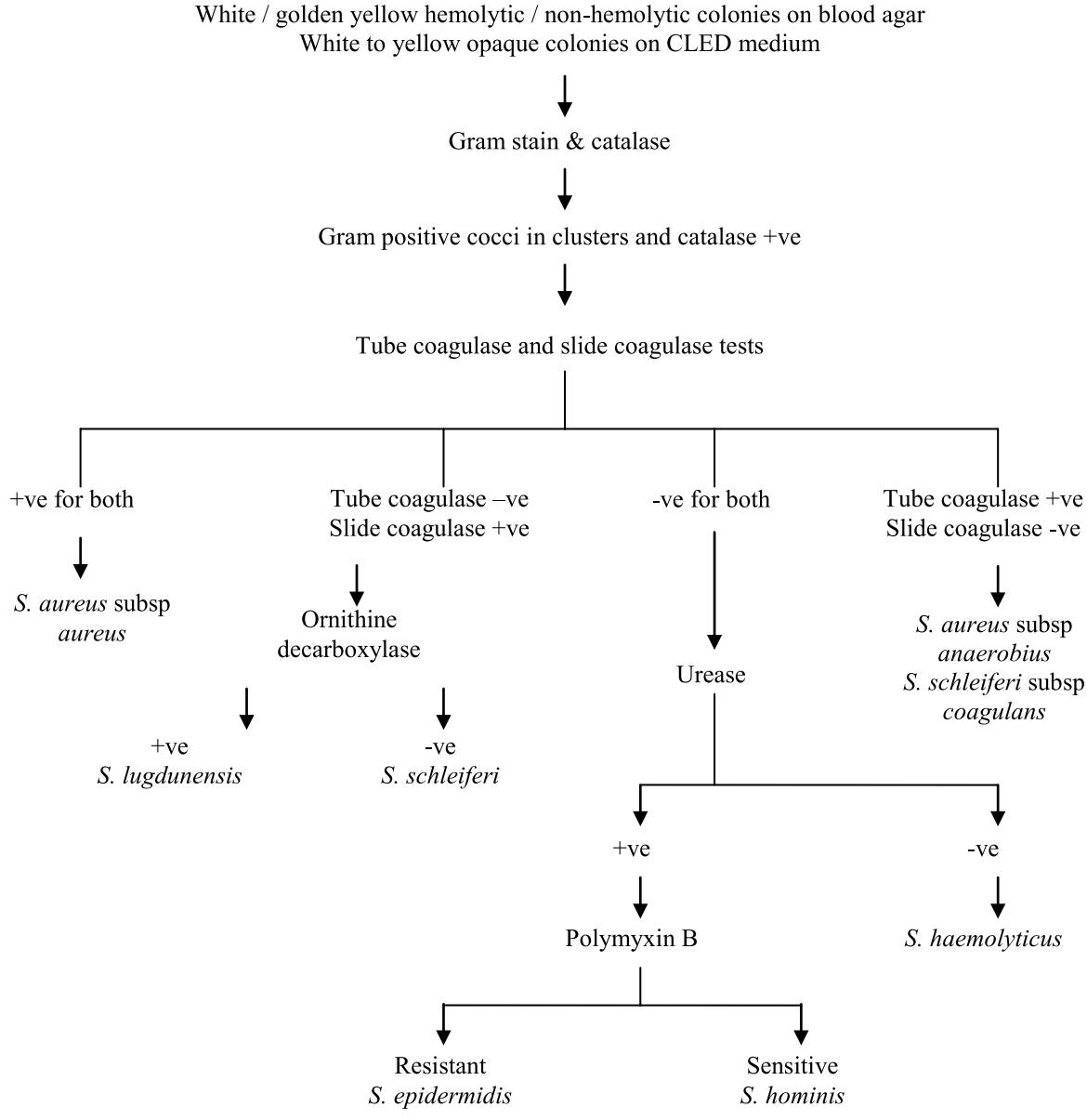
OF: Oxidative-Fermentative

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	V	Positive
Urea	Negative	Negative
Lysine	Positive	Positive
Ornithine	Positive	Negative
Arginine	Negative	Negative

1.5. Identification of staphylococci



1.5. Identification of Enterococci

Morphology and cultural characteristics

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 be β-hemolytic. 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

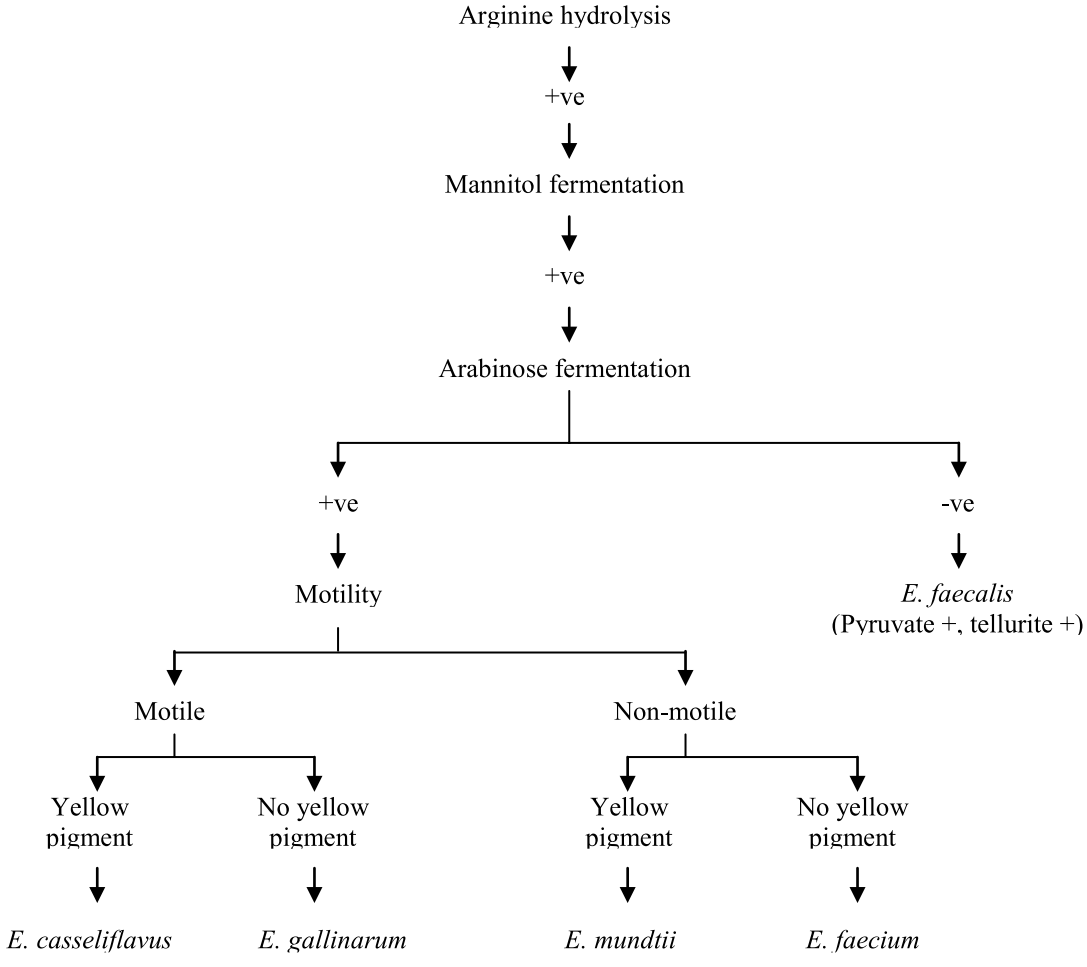
of bile-esculin, growth in 6.5% NaCl broth and heat tolerance. However, some other genera of catalase negative bacteria like *Leuconostoc*, *Pediococcus* and *Vagococcus* resemble enterococci in some phenotypic characteristics.Using tests like the PYR (L-pyrollidonyl β-naphthylamide) test and detection 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 dihydrolysis 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*
Group V	*E. canis, E. columbae*

Algorithm for phenotypic speciation of *Enterococcus* isolates



1.6. 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.

1.6.1. 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 stain: *S. pneumoniae* are Gram positive cocci (lanceolate shape) 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) on chocolate agar, fine colonies with greenish discoloration.

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

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.

1.6.2. Characterization of *S. pneumoniae*

1.6.2.1. Catalase test[36]

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 BAP at 35-37°C with ~5% CO_2 (or in a candle jar).
ii. From overnight growth on the CAP, use a disposable loop to carefully remove a colony and place it on a glass slide.
iii. Do not use growth on blood agar as erythrocytes in the blood agar will cause a false positive reaction.
iv. Add 2-3 loopfull (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 (https://www.microrao.com/micronotes/pg/catalase.pdf).
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.

1.6.2.2. Optochin test[36]

Purpose: To differentiate between *Streptococcus pneumoniae* (sensitive) and other alpha hemolytic *Streptococcus* species (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 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, 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.

1.6.2.3. Bile solubility test[36,37]

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

Reagent 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.

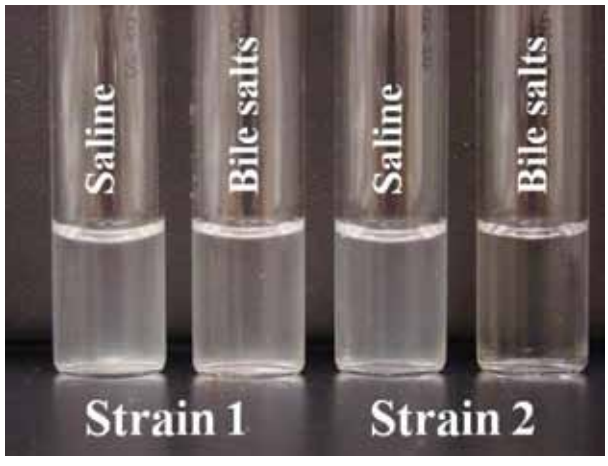


Figure 3.2: Bile solubility test for two different strains of bacteria. For strain 1, a slight decrease in turbidity is observed in the tube containing the bile salts (2nd from left), but the contents are almost as turbid as the control tube (far left); therefore, strain 1 is not *S. pneumoniae*. For 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 is adapted from CDC WHO manual[37].

1.6.3. Serotyping of *Streptococcus pneumoniae* by Quellung[37,38]

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 sera (pool, group, or serotype-specific) from Statens Serum Institute, Denmark
- 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 nontypeable, but identification of the strain as *S. pneumoniae* should be confirmed by optochin susceptibility and bile solubility testing.

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

1.7. Identification and characterization of β-haemolytic Streptococci (βHS)[38,39,40,41,42, 43]

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 Gram stain, beta hemolytic streptococci are Gram positive cocci in pairs and short chains

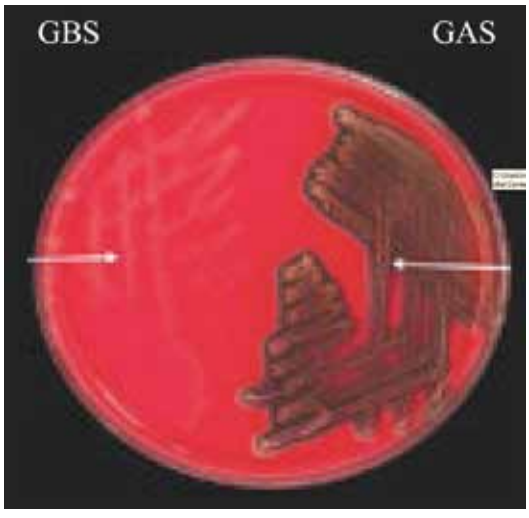


Figure 3.3: Beta haemolytic colonies with varying zones of hemolysis of GAS and GBS on blood agar.

On blood agar colonies are usually small, 1-2 mm in size, smooth and glistening, with well-defined zones of β-haemolysis. They are the commonest and are glossy colonies. 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.*
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 1.6.2.1.

1.7.1. 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 is suspected.
- Inoculate the 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.

1.7.2. 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 + CHROMagarStrepB
- Check for presence of beta hemolytic fine colonies / non-hemolytic fine colonies on BA and on CHROMagarStrepB for mauve colonies (Figure 3.4).
- Perform grouping for beta hemolytic streptococcus by the micro-nitrous acid extraction method and using either latex agglutination method or using the in- house preparation of anti-sera
- If grouping is positive for group B streptococcus, perform the CAMP test for confirmation.

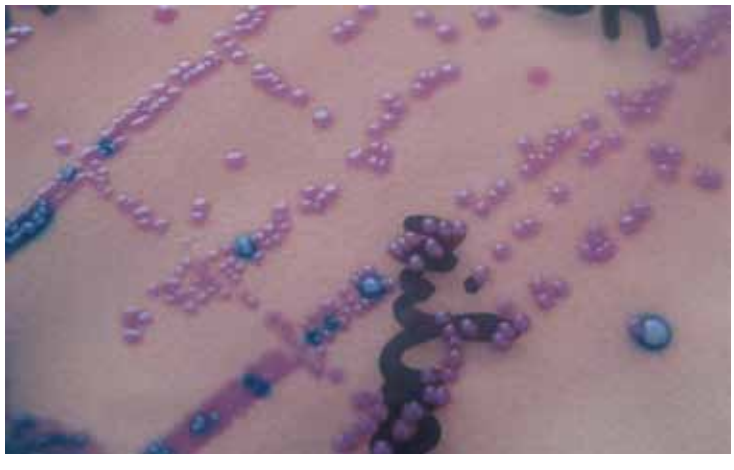


Figure 3.4: GBS (mauve) and *Enterococcus* (blue) colonies on CHROM agar.

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

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*.

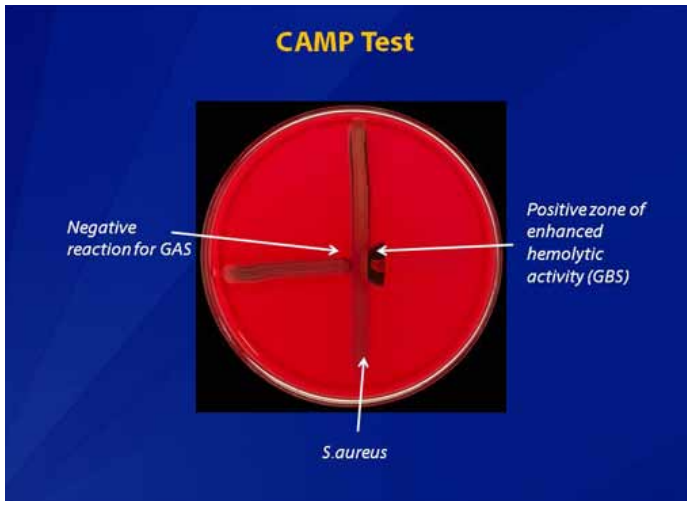


Figure 3.5: CAMP test, negative for *Enterococcus faecalis* (and other *Streptococcus* spp.); positive for *Streptococcus agalactiae* (GBS); Source:CDC

1.7.3. Grouping of beta haemolytic Streptococci

1.7.3.1. 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.

1.7.3.2. Grouping Method

Streptococcal grouping test kit

Company Name: PLASMATEC

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 2-8^{o}C.
- **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 2-8^{o}C. 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 -20^{o}C, 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 37°C 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 inoculums, 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

1.7.4. 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.

1.8. Identification of faecal isolates

Table 3.5: Biochemical tests for screening of fecal isolates

	TSI Agar		Mannitol	Motility	Indole	Citrate	LIA*	CU Agar/ urea broth	Organism
	Reaction	Interpretation							
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	Same as 1, with blackening	Same as 1 with H_2S	+	+/-	-/+	+	-	V	*Citrobacter* spp.
			+	+	-	+	+	-	*Arizona* spp.
			-	+	+/-	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	Same as 3, with blackening	Same as 3 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	Same as 5, with slight blackening	Same as 5 with little H_2S	+	+	-	-	+	-	*S.* Typhi
7	Red slant, red butt	Glucose, lactose & sucrose negative	-	+	-	+		V	*Pseudomonas* spp.***
			-	-	-	+		V	Other non-fermenting Gram- negative bacilli
8	Yellow slant; usually no change in butt in 24 hours*	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;

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

1.8.1. Further biochemical tests for identification

1.8.1.1. *Proteus* group

- 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.

1.8.1.2. *Salmonella* and salmonella-like organisms

- 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*.

1.8.1.3. *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'.

1.8.1.4. *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 parahaemolytic*us 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

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.

1.8.1.5. *Campylobacter jejuni*

They are Gram-negative comma or 'S' shaped organisms, actively motile, and microaerophilic. Ideal atmosphere for its growth should contain 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) [OXOID].

***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,
- Nitrate reduction 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).

Antimicrobial Susceptibility Testing

CHAPTER 4

Antimicrobial Susceptibility Testing

All the participating centers have to follow CLSI methodology, for antimicrobial susceptibility testing[44]

1. Definitions

- **Susceptible (S)** isolates are inhibited by the usually achievable concentrations of antimicrobial agent and infection is expected to respond when the recommended dosage is used for the site of infection.

- **Intermediate (I)** isolates have antimicrobial agent MICs that approach usually attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates with normal recommended doses. It implies
  - ♦ clinical efficacy in body sites where the drugs are physiologically concentrated (*e.g.* quinolones in urine) or
  - ♦ at sites where the drug is not specifically concentrated, clinical efficacy at higher than normal dosage of a drug (*e.g.* β-lactams).

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

- **Non-susceptible (NS)** organisms have only a susceptible interpretive category, but not intermediate or resistant interpretive categories. 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. Isolates that test with an MIC above the susceptible interpretive breakpoint are designated as non-susceptible.

2. Disc diffusion testing

2.1. Inoculum

Isolated colonies of each type of organism that may play a pathogenic role should be selected from primary agar plates. Mixtures of different types of organisms should not be tested on the same test plate. In case of mixed cultures, isolated colonies may be obtained after overnight subculture with proper streaking.

When testing has been carried out directly with the clinical material (*e.g.*, urine and normally sterile body fluids) in clinical emergencies when the direct Gram stain shows a single pathogen, the report is to be dispatched as preliminary, and the susceptibility must be repeated by the standard methodology.

2.2. Preparation of inoculum

- **Direct colony suspension method** – Prepare a saline suspension of the isolate from an overnight incubated agar plate (use a nonselective medium, such as blood agar) to obtain 0.5 McFarland turbidity (1.5 x 10^8 cfu/ ml of *E. coli* ATCC®25922).

- **Growth method** – With a sterile straight wire touch the top of each of four to five colonies of the same morphological type, and inoculate tryptic soya or any suitable broth. Incubate tube at 35ºC till turbidity of 0.5 McFarland tube or more is achieved. Then with sterile normal saline adjust turbidity to exactly 0.5 McFarland.

Use of the spectrophotometric method is preferable over visual determination with Brown's tubes. For visual comparison, look through the broths in transmitted light against a white background with contrasting black stripes.

2.3. Inoculating test 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 discs are applied.

2.4. Application of antimicrobial discs to an agar plate

Ideally, this should be done within 15 minutes of inoculation of plates. The selected antimicrobial discs 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 disc must be pressed down to ensure complete contact with the agar surface.

- Ordinarily no more than 12 discs are applied on a 150 mm plate or 5 discs on a 100 mm plate, keeping at least a distance of 24 mm between discs. Dispensing too near to the edge of the plate should be avoided. Because some of the drugs diffuse instantaneously, a disc should not be relocated once it has come in contact with the agar surface.
- It is advisable to place discs that give predictably small zones like aminoglycosides, next to those discs that give larger zones like cephalosporins.
- Disc 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 discs that have not reached the manufacturer's expiration date stated on the label will be used. Unused discs should be discarded on the expiration date.

2.5. Incubation

No longer than 15 minutes after discs are applied, the plates should be inverted and incubated at 35º ± 2ºC in ambient air.

2.6. Interpretation and reporting of 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 disc, should be measured to the nearest whole millimeter with callipers 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.

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.

2.7 Recommended Media: Müeller Hinton agar (MHA)

Fresh plates should be used the same day or stored in a refrigerator (2-8 ºC); if refrigerated, they should be wrapped in plastic to minimize evaporation. Just before use, if excess moisture is visible on the surface, plates should be placed in an incubator (35ºC) or, with lids ajar, in a laminar-flow hood at room temperature until the moisture evaporates (usually 10 to 30 minutes).
Organisms susceptible to tetracycline should also be considered susceptible to doxycycline and minocycline. However, some organisms intermediate or resistant to tetracycline may be susceptible to doxycycline or minocycline or both.

3. ATCC control strains

Each center has to procure its own control strains or Nodal Center can purchase the strain and distribute the strains.

Table 4.1: ATCC control strains

Escherichia coli	ATCC	25922
Klebsiella pneumoniae	ATCC	700603
Pseudomonas aeruginosa	ATCC	27853
Enterococcus faecalis	ATCC	29212
Staphylococcus aureus	ATCC	25923

4. Preparing antibiotic discs in-house

4.1. Method

- From the table 4.2, find the appropriate solvent for desired antimicrobial agent. In most cases it is sterile distilled water or phosphate buffered saline, pH 7.2.
- On the vial of the antibiotic powder, read the potency of the antibiotic, amount, its value in international units, if any.
- From the CLSI tables, find out how much of the antibiotic is to be added to a single disc. If mentioned in units, and the powder is assayed in milligrams, apply proper conversion.
- The amount mentioned per disc is to be dissolved in 10 µl of the solvent. Adding correction for the potency, calculate how much is to be dissolved in say, 4 ml.
- Now take 4 ml of the sterile solvent in a test tube.
- Measure the exact quantity of antimicrobial to be added to 4 ml of the solvent. Use the milligram balance, and aluminium foils (sterile, if possible) to weigh the antimicrobial.
- Now dissolve antimicrobial in the solvent (depending on the solubility, it may take seconds to an hour or more). If it does not dissolve fast, keep the test tube in the incubator and shake intermittently.
- Now dispense sterile discs (400 for 4 ml of antimicrobial solution) onto pre-sterilized Petri plates. Work in a biosafety hood from this step. Keep the discs apart, do not touch the discs; use a forceps to separate the discs. Flame the tip of the forceps intermittently.
- Add exactly 10 µl of the antibiotic solution to each disc. When finished, leave Petri-dish slightly open in the incubator for 0.5-1 hour. This allows for the drying of discs.
- When dry, use the forceps to transfer the discs to an appropriate container. You may add a small pack of silica gel to keep moisture away.
- Store discs at 4ºC. For degradable antimicrobials like clavulanic acid and imipenem, store at -20ºC and take these out only just before use.

Table 4.2: Solvents and diluents for preparation of stock solutions of antimicrobial agents

Antimicrobial Agents[a]	Solvent	Diluent
Amoxicillin, clavulanic acid and ticarcillin	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[b,c]	Broth media
Aztreonam	Saturated solution sodium bicarbonate	Water
Cefepime	Phosphate buffer, pH 6.0, 0.1mol/L	Phosphate buffer, pH 6.0, 0.1mol/L
Cefotetan	Dimethyl sulfoxide(DMSO)	Water
Cefpodoxime	(0.10 %; 11.9 mmol/L) aqueous sodium bicarbonate	Water
Ceftazidime	Sodium carbonate	Water
Cephalothin	Phosphate buffer, pH 6.0, 0.1mol/L	Water
Chloramphenicol	95 % ethanol	Water

Antimicrobial Agents[a]	Solvent	Diluent
Cinoxacin and nalidixic acid	½ volume of water, then add NaOH 1 mol/L, dropwise to dissolve	Water
Clarithromycin	Methanol[c] or glacial acetic acid[b,c]	Phosphate buffer, pH 6.5, 0.1mol/L
Enoxacin, fleroxacin, norfloxacin, ofloxacin and levofloxacin	½ volume of water, then 0.1 mol/L NaOH dropwise to dissolve	Water
Erythromycin	95% ethanol or glacial acetic acid[b,c]	Water
Imipenem	Phosphate buffer, pH 7.2, 0.01mol/L	Phosphate buffer, pH 7.2, 0.01mol/L
Moxalactam (diammonium salt)	0.04 mol/L HCl (let sit for1.5 to 2 hour)	Phosphate buffer, pH 6.0, 0.1mol/L
Nitrofurantoin[c]	Phosphate buffer, pH 8.0, 0.1mol/L	Phosphate buffer, pH 8.0, 0.1mol/L
Rifampin	Methanol (maximum concentration = 640 µg/mL)	Water (with stirring)
Sulfonamides	½ volume hot water & minimal amount of 2.5 mol/L NaOH to dissolve	Water
Telithromycin	½ volume of water, then add glacial acetic acid dropwise until dissolved; not to exceed 2.5 µL/ml	Water
Trimethoprim	0.05 mol/L lactic or hydrochloric acid, 10 % of final volume	Water (may require heat)

Notes:

a. The following antimicrobials will be soluble in sterile distilled water: amikacin, azlocillin, carbenicillin, cefaclor, cefamandole, cefonicid, cefotaxime, cefoperazone, cefoxitin, ceftizoxime, ceftriaxone, ciprofloxacin, clindamycin, gatifloxacin (with stirring), gemifloxacin, gentamicin, kanamycin, linezolid, mecillinam, meropenem, methicillin, mezlocillin, minocycline, moxifloxacin, nafcillin, netilmicin, oxacillin, penicillin, piperacillin, quinupristin-dalfopristin, sparfloxacin, sulbactam, tazobactam, teicoplanin, tetracycline, tobramycin, trimethoprim (if lactate), trospectomycin and vancomycin.
b. 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.
c. 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.

4.2. Storage of antimicrobial discs[44]

Cartridges containing commercially prepared paper disks specifically for susceptibility testing are generally packaged to ensure appropriate anhydrous conditions. Discs should be stored at 8°C or below, 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.

5. Minimum Inhibitory Concentration (MIC) testing

Methods of MIC testing

- Agar dilution
- Broth dilution methods
  - ♦ Broth macrodilution
  - ♦ Micro broth dilution
  - ♦ Broth dilution automated method
  - ♦ E-test

5.1. MIC agar dilution method

Media

Prepare appropriate agar plates containing the correct amount of the antibiotic. For this use sterile McCartney bottles (or appropriate tubes) with 20 ml of sterile agar media cooled to 48ºC-50ºC in a water bath. Add 256 µl, 128 µl, 56 µl and 28 µl of antibiotic stock 1 (10,000 µg / ml), 160 µl, 80 µl, and 40 µl of stock 2 (1,000 µg / ml), and 200 µl, 100 µl, and 50 µl of stock 3 (100 µg/ml) to get agar media containing 128 µg/ml to 0.25 µg/ml of media. Pour plates (90 mm); allow solidifying and drying. Depth of media should be 3-4 mm. Use without sterility check as antibiotics degrade if plate is incubated. Make a checkerboard on the back of the plates to identify spots for various strains.

Note: MHA should contain 20-25 mg/litre of Ca^{++} ions and 10-12.5 mg/litre of Mg^{++} ions.

Inoculum

To obtain pure culture of target organism, either suspend 3-5 colonies in normal saline or use the growth method as described previously to get an inoculum of 0.5 McFarland (1.5×10^8 cfu /ml). Make a one in ten dilution of this suspension (10^7cfu/ml).Carefully spot 1µl on to the agar plate to get the final inoculum of 10^4 cfu. For sulphonamides spot 10^3 cfu.

Incubation: 35-37ºC.

Controls: Use one negative control and positive control spot.

Period of incubation: overnight, except for oxacillin for staphylococci and vancomycin for enterococci - 24 hours.

Reading: MIC is defined as lowest concentration of antibiotic at which there is no visible growth. Ignore film or one or two colonies.

5.2. MIC broth dilution methods

5.2.1. Broth macrodilution MIC testing[45]

Several sterile tubes should be arranged and labeled in the range of MICs to the particular antimicrobial under consideration (for example, to test between 0.25µg/ml and 128µg/ml).

Inoculum

- For pure culture of target organism, either suspend 3-5 colonies in normal saline or use the growth method as described previously to get an inoculum of 0.5 McFarland. (1.5 x 108 cfu/ml).
- Make a one in hundred dilution of the suspension by adding 0.1 ml growth into 9.9 ml normal saline (~106 cfu/ml).
- Final inoculum should be 3-5 X 10^5 cfu/ml. For this, add 0.25 ml of the above suspension into each tube.

Media

Use double strength Müeller Hinton broth (DS MHB). Add 0.5 ml of DS MHB into each of the several tubes. For oxacillin MIC of staphylococci, use DS MHB with 2-4% NaCl. For *S. pneumoniae,* use DS MHB with 5% lysed horse blood, and for *H. influenzae* use DS *Haemophilus* test medium (broth).
Note: MHB should contain 20-25 mg/litre of Ca^{++} ions and 10-12.5 mg/litre of Mg^{++} ions.

Antibiotic dilutions

- Add 0.25 ml of the 4x antibiotic solution to each of the specified tubes.
- To prepare the 4x solutions prepare the stock solutions of the antibiotic as follows:
  - Dissolve 10 mg of the desired antibiotic in the appropriate diluent (mostly DW). Also take into consideration the potency of the antibiotic as specified by the manufacturer and obtain a stock solution of 10 mg active salt (10,000 µg/ml). (label as stock 1)
  - Next prepare 2 further stock solutions of 1 mg/ml (1000 µg/ml) (stock 2) and 100 µg/ml (stock 3) by serial 10 fold dilutions (1 ml in 9 ml DW twice).
  - Add 2 ml of sterile diluent to each of test tubes labeled as 4x antibiotic solution (tubes labeled 512 µg/ml to 0.25 µg/ml for final concentrations of 128 µg/ml to 0.0625 µg/ml).
  - Add 102.4 µl, 51.2 µl, 25.6 µl and 12.8 µl of stock 1 (10,000 µg/ml) to get antimicrobial solutions containing 512 µg/ml, 256 µg/ml, 128 µg/ml, and 64 µg/ml antibiotic. Similarly add 64 µl, 32 µl, and 16 µl of stock 2 (1000 µg/ml) and 80 µl, 40 µl, and 20 µl of stock 3 (100 µg/ml) to get solutions 32 µg/ml, 16 µg/ml, 8 µg/ml, 4 µg/ml, 2 µg/ml, 1.0 µg/ml. These are the 4x stock solutions[46]. Final tube volume: 1 ml.

Incubation: 35-37ºC

Controls: Use one growth control tube with no antibiotic, use one sterility control tube (negative control) with no inoculum. Also test one standard strain together as test control.
Period of incubation: overnight, except for oxacillin for staphylococci and vancomycin for enterococci where it will be 24 hours.

Reading: MIC is defined as lowest concentration of antibiotic at which there is no visible growth.

5.2.2. MIC Micro broth dilution method44, 47, 48, 49

Note: The method described is for colistin susceptibility testing

Limited therapeutic options have forced clinicians and microbiologists to reappraise the clinical application of colistin for treating infections caused by multidrug-resistant (MDR), especially carbapenem-resistant (CR) Gram-negative bacilli (GNB). In 2016, CLSI and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) jointly recommended broth microdilution (BMD) as the reference method for minimum inhibitory concentration (MIC) susceptibility testing of colistin, and abandoned the use of other methods including agar dilution (AD), disk diffusion (DD) and gradient diffusion[47].

5.2.2.1. Purpose

To circumvent the crisis, both false susceptible and false resistant of colistin susceptibility testing should be equally weighed. Colistin susceptibility testing by BMD guides clinicians to facilitate optimal patient management.

5.2.2.2. Principle

CLSI-EUCAST working group has recommended the ISO-20776 standard BMD method for MIC testing of colistin using the sulfate salt of colistin and CAMHB without adding P-80 in non-treated polystyrene trays[47]. Bacterial isolate is subjected to various dilutions of antibiotics. The highest dilution of antibiotic that inhibit the growth of bacteria should be considered as MIC. A polystyrene tray containing 96 wells should be filled with small volumes of serial two-fold dilutions of different antibiotics. The inoculum suspension and standardization should be done according to the McFarland standard. The bacterial inoculum should be then inoculated into the wells and incubated at 37□C overnight. The lowest concentration of antibiotic that will prevent macroscopically visible growth of microorganism in a 96-well plate should be taken as the endpoint or MIC.

5.2.2.3. Materials

- Untreated 96-well polystyrene microwell-plates (Greiner, Cat no. 650161)
- Colistin sulfate salt (Sigma Aldrich, Cat no. C2700000)
- Cation-adjusted Mueller-Hinton broth (CAMHB) (BD, Cat no. 212322)
- Pure Bacterial culture
- Injection water/ Sterile water
- Densi check
- Multi-channel pipettes
- Low retention tips
- Biosafety cabinet level 2
- Incubator ($37^{O}C$)
- Reagent reservoir

5.2.2.4. Methods

5.2.2.4.1. Preparation of colistin stock solution

- Colistin stock solution should be prepared in sterile water (5mg/ml) and stored at -20°C.

- For working stock, 256µl of colistin solution (5mg/ml) should be mixed with 744µl of sterile water to prepare a concentration 1280µg/ml.
- For adding directly to plate, 100µl of colistin solution (1280µg/ml) should be mixed with 900µl of sterile CAMHB to prepare a concentration 128µg/ml.

5.2.2.4.2. Preparation of inoculum

- A 0.5 McFarland turbidity standard (1.5x108 CFU/ml) inoculum should be prepared for test organism in sterile saline (1ml) from 24 hours old non-selective plate (use a non-selective medium, such as nutrient agar or blood agar).
- Optimally within 15 minutes of preparation, 150-fold dilution (106 CFU/ml) should be prepared by adding 25µl of 0.5McFarland scale organism in 3725µl of CAMHB.

5.2.2.4.3. Preparation of 96-well plate

- Distribute 100µl of CAMHB-colistin (128µg/ml) into each well of column 1 and 50µl of CAMHB into each well of column 2 to 12.
- Mix 50µl CAMHB-colistin (128µg/ml) from column 1 sequentially in serial 2-fold dilution with column 2 to 11 (colistin conc. range, 64-0.25µg/ml).
- Mix 50µl of bacterial inoculum in column 1 to 10 (colistin conc. range, 64-0.12µg/ml) and 12.
- Column 11 and 12 serve as drug control and growth control, respectively.

5.2.2.5. Incubation

Incubate the inoculated microdilution trays at 35±2°C for 16 to 20 hours in an ambient air incubator within 15 minutes of adding the inoculum. To maintain the same incubation temperature for all cultures, do not stack microdilution trays more than four high[48].

5.2.2.6. Interpretation

Compare the amount of growth in the wells or tubes containing the antimicrobial agent with the amount of growth in the growth-control wells or tubes (no antimicrobial agent) used in each set of tests when determining the growth end points. Isolates having MIC of ≤2µg/ml should be considered as susceptible and >2µg/ml is resistant[49].

For a test to be considered valid, acceptable growth (≥ 2 mm button or definite turbidity) must occur in the growth-control well.

The lowest concentration at which the isolate is completely inhibited (as evidenced by the absence of visible bacterial growth) is recorded as the minimal inhibitory concentration or MIC.
When a single skipped well occurs in a microdilution test, read the highest MIC. Do not report results for drugs for which there is more than one skipped well[44].

5.2.2.7. Quality control

E.coli ATCC 25922 and *P. aeruginosa* ATCC 27853 strains should be included with each test. A set of growth control (without drug) and drug control (without microorganism) should be tested to ensure the quality, sensitivity and specificity of the test.

5.2.2.8. Safety prcautions

- Prevention of antimicrobial resistance depends on appropriate clinical practices that should be incorporated into all routine patient care.
- Always open drug resistant bacterial plate inside a biosafety cabinet.
- Each technologist performing the testing should wear an impermeable laboratory coat and gloves during all procedures.
- Waste should be disposed as per local pollution control board guideline.

5.2.3. MIC broth dilution automated method

Identification and antibiotic sensitivity by Vitek

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

5.2.3.1. Purpose

VITEK 2 Compact Instrument (V2C) is an automated identification system for identification and sensitivity testing of microorganisms.

5.2.3.2. Principle

VITEK 2 is an automated microbiology system utilizes growth-based technology which accommodates the colorimetric reagent cards for organism identification and colometry based antimicrobial susceptibility using MIC test cards that are incubated and interpreted automatically.

5.2.3.3. Type of primary sample

For cultural requirements, follow manuacturer's instructions given in table. These parameters include acceptable culture media, culture age, incubation conditions, and inoculum turbidity.

5.2.3.4 Equipment

VITEK 2 Compact Densicheck Plus (bioMérieux Inc. Vitek 2 Compact Hardware User Manual)

5.2.3.5. Reagents

The **reagent cards** have 64 wells that can each contain an individual test substrate for identification and antibiotics with different concentration for sensitivity. Substrates measure various metabolic activities such as acidification, alkalinization, enzyme hydrolysis, growth in the presence of inhibitory substances. An optically clear film present on both sides of the card allows for the appropriate level of

oxygen transmission while maintaining a sealed vessel that prevents contact with the organism-substrate admixtures. Each card has a preinserted transfer tube used for inoculation. Cards have bar codes that contain information on product type, lot number, expiration date, and a unique identifier that can be linked to the sample after loading the card onto the system.

For the identification of different organism classes, currently four reagent cards 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 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.

5.2.3.6. Procedure (bioMérieux Inc. Vitek 2 Compact Online Software User Manual)

5.2.3.6.1. Suspension preparation

Use a sterile swab or applicator stick to transfer a sufficient number of colonies of a pure culture and to 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 turbidity accordingly and measure using a turbidity meter called the DensiChekTM.

Product McFarland turbidity range follows as:

GN	0.50- 0.63
GP	0.50- 0.63
YST	1.80- 2.20

Microorganism suspensions from each test tube should be sub-cultured on CLED agar media for checking the purity of corresponding suspension tube.

5.2.3.6.2. Inoculation

Reagent cards should be inoculated with microorganism suspensions. A test tube containing the microorganism suspension should be placed into a special rack (cassette) and the identification card should be placed in the neighbouring slot while inserting the transfer tube into the corresponding suspension tube. The cassette can accommodate up to 10 tests, the filled cassette should be placed manually in to incubation chamber.

5.2.3.6.3. Card sealing and incubation

Inoculated cards are passed by a mechanism, which cuts off the transfer tube and seals the card prior to loading into the carousel incubator. The carousel incubator can accommodate up to 60 cards. All card types are incubated on-line at 35.5 + 1.0ºC. Each card is removed from the carousel incubator once every 15 minutes, transported to the optical system for reaction readings, and then returned to the incubator

until the next read time. Data are collected at 15-minute intervals during the entire incubation period. The VITEK system analyses the data results and determines the identification and sensitivity of the test organism. Result data is automatically recorded and generated by the computer in the form of a printout. The printout for the identification and sensitivity of the test organism will be filed in the VITEK 2 Compact Software.

5.2.3.6.4. Quality control

Quality control organisms and their expected results are listed for each product and are tested according to the procedures outlined in the on-line product information (Ref chapter on Quality control, section B). Frequency of testing should be once every week using all the cards in use. For AST- YST, QC should be done once every month and /or along with each test run. This is because of the low frequency of yeast isolates.

For antimicrobial susceptibility testing by Vitek 2 compact, the antibiotic panels provided in the cards are fixed and need to be edited in the final result to suit organism and or site of infection in the following manner.

i. **Gram Positive Cocci (*Staphylococcus, Enterococcus*):** Exclude Nitrofurantoin in all isolates except those from urine.

ii. **Gram Negative Bacilli (*E. coli, Pseudomonas* etc)**
- Do not report Tigecycline for Pseudomonas or for any isolate from urine and blood.
- Do not report Nitrofurantoin in any isolate except those from urine.
- Do not report Ertapenem in Non-Fermenters
- Do not report Colistin in Burkhloderia.
- Do not report Ampicillin/sulbactum, cefriaxone, tetracycline, Co-trimoxazole for Pseudomonas

iii. **Following antimicrobial agents should not be routinely reported for bacteria isolated from CSF**
- Agents administrated by oral route only
- 1st and 2nd generation Cephalosporins.
- Clindamycin.
- Macrolides
- Tetracyclines
- Fluoroquinolones

For fastidious bacteria, antibiotic panels are chosen as per CLSI Guideline[44].

5.2.3.6.7. Standardization of DensiCHEKTM Plus using DensiCHEKTM Plus standard kit (bioMérieux Inc. DensiChek User's manual, as per manufacturer's instructions)

DensiCHEKTM plus standards are used to verify the DensiCHEKTM plus instrument measurement performance. Use of three standards enables the accuracy of the instrument to be monitored and therefore confirm accuracy of the organism suspension.

Principle: The true value expressed in McFarland (McF) is printed on the label of the standard tube. Place the standard tube into the DensiCHEKTM plus, the value displayed on the instrument can be compared to the value printed on the label.

Kit content: Set of four standards 0.0 (blank), 0.5 McF, 2.0 McF, and 3.0 McF
Precautions: Do not open the tubes and do not shake any of the standard tubes, since air bubbles can affect readings.

Storage conditions: Do not freeze or over-heat. The kit can be used till the expiration date indicated on the pack.

Temperature: Storage: 5ºC to 30ºC (41ºF to 86ºF); Operating: 15ºC to 30ºC (59ºF to 86ºF)

Relative humidity: Storage: 20% to 85% non-condensing; Operating: 20% to 80% non-condensing

Test frequency: It is recommended to use the standard kit to test accuracy of the standard tube into the DensiCHEKTM Plus prior to first use and on a monthly basis.

Important instructions for use

- Confirm that the DensiCHEKTM Plus instrument is set to the GLASS tube setting.
- Select the 0.0 McF standards and clean the outside surface with a lens tissue.
- Gently invert the 0.0 McF standard five to six times to ensure it is homogenous.
- Ensure the instrument is on and insert the 0.0 McF standards into the instrument and press the Zero/Scroll key.
- Slowly rotate the standard one full rotation. The instrument will display a series of dashes followed by 0.00.
- Select the desired standard (0.5, 2.0, and 3.0) and clean outside surface with lens tissue.
- Gently invert the standard five to six times to ensure it is homogenous.
- Insert the standard into the instrument and slowly rotate the standard one full rotation until a numerical value is displayed.
- Check the displayed McFarland value is within the acceptable range.
- If any standards are outside the acceptable range repeat above mentioned steps. If it is still out of the range contact with vendors.

Reference range

Standard	Acceptable Range
0.5 McF	0.44 - 0.56
2.0 McF	1.85 – 2.15
3.0 McF	2.79 – 3.21

Safety precautions

- For in vitro diagnostic use only.
- Suspension not within the appropriate range on VITEK 2 Densichek may compromise
- card performance
- Do not use card after the expiration date shown on the package liner

- Allow the card to come to room temperature before use.
- Only pure isolates are used for sensitivity testing. Mixed cultures used for testing give erroneous results. In case, inoculum has a mixed growth as identified by 'unidentified' flag by Vitek, the isolate should be subcultured and the test should be repeated.

Unusual results (Recheck result and isolate purity before reporting)

- *Klebsiella* sensitive to ampicillin.
- *Staphylococcus* resistant to vancomycin.
- *Proteus mirabilis* resistant to ampicillin.
- MRSA should not be reported as sensitive to any beta lactam antibiotics.

5.2.4. MIC broth dilution method E-test

Day 1

Prepare inoculums by method given below:

- Remove the E-test package from the freezer (-20ºC) at least 30 minutes before required.
- With a loop, touch the top of 3 or 4 individual colonies and transfer to a tube of saline.
- Emulsify the inoculum on the inside of the tube to avoid lumps.
- Compare turbidity to that in the 0.5 McFarland standard. Adjust turbidity of inoculum to match that standard.
- Inoculate agar plate
- Ensure the agar surface is dry, but not overly dry.
- Swab plate within 15 minutes of preparing the adjusted inoculum.
- Dip a sterile cotton swab into the inoculum and pulling out slightly, rotate the swab several times against the inside of tube above the fluid level to remove excess liquid.
- Streak the swab over the entire surface of the agar plate. Rotate the plate approximately 60º then repeat streaking motion. Rotate 60º again and repeat streaking. Complete inoculation by running the swab around the rim of the agar.
- Leave the lid of the plate ajar for 5 minutes (no more than 15 minutes) to allow any excess moisture to be absorbed before applying strips.
- Apply E-test strips.
- Apply strips to agar surface using forceps (or E-test applicator if available). Place the strip with the'E end' at the center of the plate and with the scale visible.
- Position one (seldom two) strip(s) onto a 90 mm plate or 4 to 6 strips onto a 150 mm plate. Do not remove a strip once it has touched the agar.
- Incubate plates at 37ºC for 18 hours in ambient air.

Day 2

Results

- Read MIC at the point where ellipse intersects the scale. If a MIC value between two two-fold dilutions is seen, always round up to the highest value.
- Remember to read the MIC value at **complete inhibition** of all growth including isolated colonies.

- If the intersect differs on either side of the strip, read the MIC as the greater value.
- However, remember that sulfonamide and trimethoprim should be read at **80% of growth** and that swarming of proteus should be ignored.
- Ignore any growth at the edge of the strip.
- Breakpoint values are MIC values at which the bacteria should be interpreted as S (susceptible), I (intermediate) or R (resistant).

6. Zone diameters and MIC breakpoints

Table 4.1: List of antimicrobial agents, zone diameters and MIC interpretive criteria for Enterobacteriaceae

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

Notes:

1. *: Not to be tested for isolates from urine.
2. **: To be tested only for isolates from urine.
3. #: Colistin to be tested only by MIC test. EUCAST guidelines, 2015 give cut-offs only for colistin.
4. @: Cefazolin: as surrogate marker for oral agents for Urine (uncomplicated UTI due to *E. coli, K. pneumoniae, P. mirabilis*).
5. $: Only NC: Fosfomycin for Urinary *Escherichia coli.* 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.
6. $$: Only NC (i): Ceftazidime-avibactam; DD zone size 18-20 mm: confirm resistance by MIC (ii) Meropenem-vaborbactam

Table 4.2: List of antimicrobial agents, zone diameters and MIC interpretive criteria for *Salmonella* Typhi and *Salmonella* Paratyphi A, B and C

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
Azithromycin	15	≥ 13		≤ 12	≤ 16		≥ 32
Cefixime (FIX)	5	≥ 19	16-18	≤ 15	≤ 1	2	≥ 4
Ceftriaxone (CTR)	30	≥ 23	20-22	≤ 19	≤ 1	2	≥ 4
Chloramphenicol (CHL)	30	≥ 18	13-17	≤ 12	≤ 8	16	≥ 32
Ciprofloxacin (CIP)	5	≥ 31	21-30	≤ 20	≤ 0.06	0.12-0.5	≥ 1
Levofloxacin*	-	-	-	-	≤ 0.12	0.25-1	≥ 2
Ofloxacin (OFX) *	-	-	-	-	≤ 0.12	0.25-1	≥ 2
Pefloxacin	5	≥ 24	-	≤ 23	-	-	-
Trimethoprim-sulfamethoxazole (SXT)	1.25/23.75	≥ 16	11-15	≤ 10	≤ 2/38	-	≥ 4/76

Notes:

1. Creeping MIC (MIC 50): Ciprofloxacin, Ceftriaxone, Azithromycin.
2. Azithromycin for *S.* Typhi
3. *: Only by NC: Ofloxacin, Levofloxacin.

Table 4.3: 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
Amikacin (AMK)	30	≥ 17	15-16	≤ 14	≤ 16	32	≥ 64
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
Ciprofloxacin (CIP)	5	25	19-24	18	0.5	1	2
Colistin (CST)	-	-	-	-	≤ 2	-	≥ 4
Gentamicin (GEN)	10	≥ 15	13-14	≤ 12	≤ 4	8	≥ 16
Imipenem (IMI)	10	≥ 19	16-18	≤ 15	≤ 2	4	≥ 8
Levofloxacin (LEV)	5	22	15-21	14	1	2	4
Meropenem (MEM)	10	≥ 19	16-18	≤ 15	≤ 2	4	≥ 8
Piperacillin-tazobactam (PTZ)	100/10	≥ 21	15-20	≤ 14	≤ 16/4	32/4-64/4	≥ 128/4
Polymyxin B	-	-	-	-	≤ 2	4	≥ 8
Tobramycin	10	≥ 15	13-14	≤ 12	≤ 4	8	≥ 16

Notes:

1. First line antimicrobial agents for isolates from blood, urine, sputum, ETA, suction tip, BAL, pus, genital tract specimen: Ceftazidime, Levofloxacin, Tobramycin, Amikacin, Piperacillin-tazobactam,
2. First line antimicrobial agents for isolates from throat, nasal and ear swabs: Ceftazidime, Levofloxacin, Tobramycin, Amikacin, Piperacillin-tazobactam.
3. For isolates from eye: Test only Ciprofloxacin, Gentamicin, Tobramycin and Ceftazidime.
4. Colistin: Only by MIC by broth microdilution. Not by DD or gradient diffusion methods. Colistin MIC predict for Polymyxin B.
5. * : Only NC : Ceftazidime-avibactam

Table 4.4: List of antimicrobial agents, zone diameters and MIC interpretive criteria for *Acinetobacter baumannii*

Antimicrobial agent	Disk content (µg)	Zone diameter (mm)			MIC (µg/mL)		
		S	I	R	S	I	R
Amikacin (AMK)	30	≥ 17	15-16	≤ 14	≤ 16	32	≥ 64
Cefepime (FEP)	30	≥ 18	15-17	≤ 14	≤ 8	16	≥ 32
Ceftazidime (CAZ)	30	≥ 18	15-17	≤ 14	≤ 8	16	≥ 32
Colistin	-	-	-	-	≤ 2	-	≥ 4
Imipenem (IMI)	10	≥ 22	19-21	≤ 18	≤ 2	4	≥ 8
Levofloxacin (LEV)	5	≥ 17	14-16	≤ 13	≤ 2	4	≥ 8
Meropenem (MEM)	10	≥ 18	15-17	≤ 14	≤ 2	4	≥ 8
Minocycline	30	≥ 16	13-15	≤ 12	≤ 4	8	≥ 16
Piperacillin-tazobactam (PTZ)	100/10	≥ 21	18-20	≤ 17	≤ 16/4	32/4-64/4	≥ 128/4
Polymyxin B	-	-	-	-	≤ 2	=	≥ 4

Notes:

1. First line antimicrobial agents for isolates from blood , urine, sputum, ETA, suction tip, BAL, pus, genital tract specimen : Ceftazidime, Levofloxacin, Amikacin, Piperacillin-tazobactam, Cefoperazone-sulbactam.
2. First line antimicrobial agents for isolates from throat, nasal and ear swabs: Ceftazidime, Levofloxacin, Amikacin, Piperacillin-tazobactam, Netilmicin.
3. Colistin: Only for *Acinetobacter baumannii* complex and only by MIC by microbroth dilution. Not by DD or gradient diffusion methods. Colistin MIC predict for Polymyxin B only for *Acinetobacter baumannii complex*.

Table 4.5: List of antimicrobial agents, zone diameters and MIC interpretive criteria for *Burkholderia cepacia* complex

Antimicrobial agent	Disk content (µg)	Zone diameter (mm)			MIC (µg/mL)		
		S	I	R	S	I	R
Ceftazidime (CAZ)	30	≥ 21	18-20	≤ 17	≤ 8	16	≥ 32
Chloramphenicol (CHL)*	-	-	-	-	≤ 8	16	≥ 32
Levofloxacin (LEV)	-	-	-	-	≤ 2	4	≥ 8
Meropenem (MEM)	10	≥ 20	16-19	≤ 15	≤ 4	8	≥ 16
Minocycline (MIN)	30	≥ 19	15-18	≤ 14	≤ 4	8	≥ 16
Ticarcillin-clavulanic acid **	-	-	-	-	≤ 16/2	32/2-64/2	≥ 128/2
Trimethoprim-sulfamethoxazole (SXT)	1.25/23.75	≥ 16	11-15	≤ 10	≤ 2/38		≥ 4/76

Notes: *: Chloramphenicol not for UTI; **: Ticarcillin-clavulanic acid only by MIC and only by NC

Table 4.6: List of antimicrobial agents, zone diameters and MIC interpretive criteria for *Stenotrophomonas maltophilia*

Antimicrobial agent	Disk content (µg)	Zone diameter (mm)			MIC (µg/mL)		
		S	I	R	S	I	R
Ceftazidime (CAZ)	-	-	-	-	≤ 8	16	≥ 32
Chloramphenicol (CHL)*	-	-	-	-	≤ 8	16	≥ 32
Levofloxacin (LEV)	5	≥ 17	14-16	≤ 13	≤ 2	4	≥ 8
Minocycline (MIN)	30	≥ 19	15-18	≤ 14	≤ 4	8	≥16
Ticarcillin-clavulanic acid **	-	-	-	-	≤ 16/2	32/2-64/2	≥ 128/2
Trimethoprim-sulfamethoxazole (SXT)	1.25/23.75	≥ 16	11-15	≤ 10	≤ 2/38		≥ 4/76

Notes: *: Chloramphenicol not for UTI; **: Ticarcillin-clavulanic acid only by MIC and Only by NC

Table 4.7: List of Antimicrobial Agents, zone diameters and MIC break points for fecal bacterial pathogens (*Shigella* spp., Diarrhoeagenic *E. coli, Salmonella* spp., *Arizona* spp, *Vibrio* spp., *Aeromonas* spp., *Plesiomonas shigelloides*)

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
Cefixime (FIX)	5	≥ 19	16-18	≤ 15	≤ 1	2	≥ 4
Chloramphenicol	30	≥ 18	13-17	≤ 12	≤ 8	16	≥ 32
Ciprofloxacin (CIP)	5	≥ 31	21-30	≤ 20	≤ 0.06	0.12-0.5	≥ 1
Imipenem	10	≥ 23	20-22	≤ 19	≤ 1	2	≥ 4
Meropenem	10	≥ 23	20-22	≤ 19	≤ 1	2	≥ 4
Nalidixic acid (NAL)	30	≥ 19	14-18	≤ 13	≤ 16	-	≥ 32
Tetracycline (TET)	30	≥ 15	12-14	≤ 11	≤ 4	8	≥ 16
Trimethoprim-sulfamethoxazole (SXT)	1.25/23.75	≥ 16	11-15	≤ 10	≤ 2/38		≥ 4/76

Notes:

1. For *Shigella* spp., Diarrhoeagenic *E. coli*: Test Ampicillin, Cefixime, Nalidixic acid, Trimethoprim-sulfamethoxazole.
2. For *Salmonella* spp., *Arizona* spp.: Test Ampicillin, Chloramphenicol, Ciprofloxacin, Trimethoprim-sulfamethoxazole.
3. For *Vibrio* spp.: Test Ampicillin, Nalidixic acid, Tetracycline, Trimethoprim-sulfamethoxazole.
4. For *Aeromonas* spp., *Plesiomonas shigelloides*: Test Cefixime, Ciprofloxacin, Imipenem, Meropenem, Tetracycline.

Table 4.8: List of Antimicrobial Agents, zone diameters and MIC break points for *Campylobacter* spp.

Antimicrobial agent	Disk content (µg)	Zone diameter (mm)			MIC (µg/mL)		
		S	I	R	S	I	R
Azithromycin (AZM)	-	-	-	-	≤ 8	16	≥ 32
Ciprofloxacin (CIP)	5	≥ 31	21-30	≤ 20	≤ 0.06	0.12-0.5	≥ 1
Clindamycin (CLI) *	-	-	-	-	≤ 4	-	≥ 8
Norfloxacin (NOR)	10	≥ 17	13-16	≤ 12	≤ 4	8	≥ 16
Tetracycline (TET)	30	≥ 15	12-14	≤ 11	≤ 4	8	≥ 16

Notes: *Clindamycin data based on EUCAST 2014

Table 4.9: List of antimicrobial agents, zone diameters and MIC interpretive criteria for *Staphylococcus* species

Antimicrobial agent	Disk content (µg)	Zone diameter (mm)			MIC(µg/mL)		
		S	I	R	S	I	R
Cefoxitin (FOX)	30						
*		≥ 22	-	≤ 21	≤ 4	-	≥ 8
**		≥ 25	-	≤ 24	-	-	-
****		≥ 25	-	≤ 24	-	-	-
Ciprofloxacin (CIP)	5	≥ 21	16-20	≤ 15	≤ 1	2	≥ 4
Clindamycin (CLI)	2	≥ 21	15-20	≤ 14	≤ 0.5	1-2	≥ 4
Dalbavancin $	-	-	-	-	≤ 0.25	-	-
Erythromycin @	15	≥ 23	14-22	≤ 13	≤ 0.5	1-4	≥ 8
Linezolid (LNZ)	30	≥ 21	-	≤ 20	≤ 4	-	≥ 8
Oritavancin $	-	-	-	-	≤ 0.12	-	
Oxacillin							
*	-	-	-	-	≤ 2	-	≥ 4
**	1	≥ 18	-	≤ 17	≤ 0.25	-	≥ 0.5
***	1	≥ 18	-	≤ 17	≤ 0.25	-	≥ 0.5
****	-	-	-	-	≤ 0.25	-	≥ 0.5
Tedizolid $	-	-	-	-	≤ 0.5	1	≥ 2
Teicoplanin (TEC)					≤ 8	16	≥ 32
Telavancin $	-	-	-	-	≤ 0.12	-	
Tetracycline (TET)	30	≥ 19	15-18	≤ 14	≤ 4	8	≥ 16
Tigecycline (TIG) #	-	-	-	-	≤ 0.5	-	≥ 1
Trimethoprim-sulfamethoxazole	1.25/23.75	≥ 16	11-15	≤ 10	≤ 2/38	-	≥ 4/76
Vancomycin (VAN)	-						
S. aureus					≤ 2	4-8	≥ 16
All CoNS					≤ 4	8-16	≥ 32

Notes:

1. **S. aureus*, *S. lugdunensis*
   ** *S. epidermidis*
   *** *S. pseudointermedius, S. schleiferi*
   ****CoNS (non- *S. epidermidis, S. lugdunensis, S. pseudointermedius, S. schleiferi)*
2. 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.
3. Cefoxitin is used as surrogate marker for *mecA* mediated oxacillin resistance. If *mecA* positive, report as oxacillin (not cefoxitin) resistant. Rare isolates *mecA* negative with oxacillin MIC ≥ 4 µg/mL, should be reported as oxacillin resistant[44].
4. For non-*S. epidermidis* CoNS, with oxacillin MIC 0.5-2 µg/mL, test for *mecA* or PBP2a. If negative, report as S[44].
5. 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.

6. 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-strip or broth micro dilution) to determine vancomycin MICs. VISA with MIC of 4 µg/mL will not grow on VSA.
7. @: Erythromycin: Not to be tested for isolates from urine.
8. #: Tigecycline: Only for MRSA isolates: S : ≤ 0.5, R : ≥ 1 (EUCAST). Only by NC.
9. Creeping MIC: MRSA: Vancomycin, Teicoplanin, Linezolid.
10. $: Oritavancin, telavancin, tedizolid, dalbavancin for *S. aureus* only. Only by NC.

Table 4.10: List of antimicrobial agents, zone size and MIC interpretive criteria for enterococci

Antibiotics	Disc potency (µg)	Zone diameter (mm)			MIC (µg/ml)		
		S	I	R	S	I	R
Ampicillin (AMP)	10	≥ 17	-	≤ 16	≤ 8	-	≥ 16
Ciprofloxacin (CIP) *	5	≥ 21	16-20	≤ 15	≤ 1	2	≥ 4
Dalbavancin @	-	-	-	-	≤ 0.25	-	-
Fosfomycin #	200	≥ 16	13-15	≤ 12	≤ 64	128	≥ 256
Gentamicin (HLG)	120	≥ 10	7-9	≤ 6	≤ 500	-	≥ 1000
Linezolid (LNZ)	30	≥ 23	21-22	≤ 20	≤ 2	4	≥ 8
Nitrofurantoin (NIT)*	300	≥ 17	15-16	≤ 14	≤ 32	64	≥ 128
Oritavancin @	-	-	-	-	≤ 0.12	-	
Tedizolid @					≤ 0.5	-	
Teicoplanin	30	≥ 14	11-13	≤ 10	≤ 8	16	≥ 32
Telavancin @	-	-	-	-	≤ 0.25	-	
Vancomycin (VAN)	30	≥ 17	15-16	≤ 14	≤ 4	8-16	≥ 32

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 (6µg/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. @: Oritavancin, telavancin, tedizolid, dalbavancin : for vancomycin susceptible *E. faecalis*:. Only by NC
7. #: Fosfomycin: Urinary *E. faecalis* only. Only by NC. Disk contains glucose-6-phosphate 50 µg. MIC only by agar dilution supplemented with glucose-6-phosphate 25 µg/ml.

Table 4.11: List of antimicrobial agents, zone size and MIC interpretive criteria for *Streptococcus pneumoniae*

Antimicrobial agent	Disk content (µg)	Zone diameter (mm)			MIC (µg/ml)		
		S	I	R	S	I	R
Penicillin *	Oxacillin 1	≥20	-	-	-	-	-
Penicillin parenteral (non-meningitis) @	-	-	-	-	≤2	4	≥8
Penicillin parenteral (meningitis) #&	-	-	-	-	≤0.06	-	≥0.12
Penicillin (oral penicillin) $	-	-	-	-	≤0.06	0.12-1	≥2
Cefotaxime (meningitis) #&	-	-	-	-	≤0.5	1	≥2
Cefotaxime (non-meningitis) @	-	-	-	-	≤1	2	≥4
Erythromycin	15	≥21	16-20	≤15	≤0.25	0.5	≥1
Levofloxacin	5	≥17	14-16	≤13	≤2	4	≥8
Vancomycin	30	≥17	-	-	≤1	-	-
Linezolid	30	≥21	-	-	≤2	-	-
Chloramphenicol	30	≥21	-	≤20	≤4	-	≥8
Meropenem&					≤0.25	0.5	≥1

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

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

Notes:

1. *: Penicillin and ampicillin is drug of choice for treatment of beta hemolytic streptococcal infections. Susceptibility testing of beta lactams does not need to be performed routinely because nonsusceptiblre isolates are extremely rare in any beta hemolytic streptococcus. Resistant isolates has to be re-identified, retested, and, if confirmed, submitted to a public health laboratory.
2. @: Recommendations for intrapartum prophylaxis for group B streptococci are penicillin or ampicillin. Athough cefazolin is recommended for penicillin allergic women at low risk for anaphylaxis, those at high risk for anaphylaxis may receive clindamycin. Group B streptococci are susceptible to ampicilin, penicillin, and cefazolin but may be resistant to erythromycin and clindamycin. When a group B streptococcus is isolated from a pregnant woman with severe penicillin allergy (high risk for anaphylaxis) erythromycin and clindamycin (including inducible clindamycin resistance) should be tested and only clindamycin should be reported.

For this table, the beta hemolytic group includes the large colony forming pyogenic strains of streptococci with group A (*Streptococcus pyogenes*), C, or G antigens and strains with group B (*S. agalactiae* antigen).

Table 4.13: List of antimicrobial agents, zone size and MIC interpretive criteria for *Streptococcus* viridans group

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

Special Tests (Phenotypic)

CHAPTER 5

Special Tests (Phenotypic)

1. Carba-NP test (For Enterobacteriaceae and *Pseudomonas spp.*) [44]

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

Requirements

1. 1.5 ml Eppendorf tubes
2. Imipenem sodium salt (Sigma-Aldrich)
3. B-PERII, Bacterial Protein Extraction Reagent (Thermo Scientific, Pierce)Cat:78260
4. ZnSO4, 7H2O (Sigma-Aldrich, Cat: 221376)
5. Negative (wild-type *E. coli*) and positive (*K. pneumoniae* OXA-48 or *K. pneumoniae* KPC-2) controls. (CLSI: *K. pneumoniae* ATCC BAA-1705 Carbapenemase positive; *K. pneumoniae* ATCC BAA-1706 Carbapenemase negative)

Preparation and storage of Solution A

1. Prepare a concentrated solution of red phenol 0.5% w/v
2. Mix 2 ml of the concentrated red phenol solution (strongly vortex before pipetting to resuspend the solution) in 16.6 ml of distilled water
3. Adjust the pH at 7.8 by adding drops of a NaOH solution (1 N)
4. Add 180 µl of ZnSO4 (Sigma-Aldrich, Cat: 221376) 10 mM to obtain a final concentration of 0.1 mM

Solution A is stable at room temperature for 1 week and may be kept at -20°C for several months. Solution A + imipenem (6 mg/ml) should be prepared extemporaneously. However, batches of imipenem powders can be weighted and prepared in advance and kept at 4°C for two weeks if solution A is not added. 600 µl of solution A is needed for each test (solution A, solution A + imipenem, positive and negative controls).

Procedure

1. Add 100 µl of 20 mM Tris-HCl lysis buffer (B-PERII, Bacterial Protein Extraction Reagent, Thermo Scientific, Pierce) in each of two 1.5 ml eppendorf tubes
2. Resuspend a 1/4 to 1/3 calibrated dose (10 µl) of bacterial colonies in each of those 100 µl of 20 mM Tris-HCl lysis buffer. (Bacterial colonies may be recovered directly from the antibiogram around disk of carbapenem performed according to the disk diffusion techniques).
3. Check that bacterial colonies have been correctly resuspended. If necessary mix up and down with a pipette.

4. Add (i) 100 µl of Solution A in the first eppendorf tube and (ii) 100 µl Solution A + imipenem 6 mg/ml in the second 1.5 ml eppendorf tube.
5. Incubate at 37°C for a maximum of 2 hours
6. Optical reading of the color of each tube

Interpretation	**No antibiotic**	**Imipenem**
No carbapenemase	Red	Red
Carbapenemase producer	Red	Orange/Yellow
Not interpretable	Yellow	Yellow

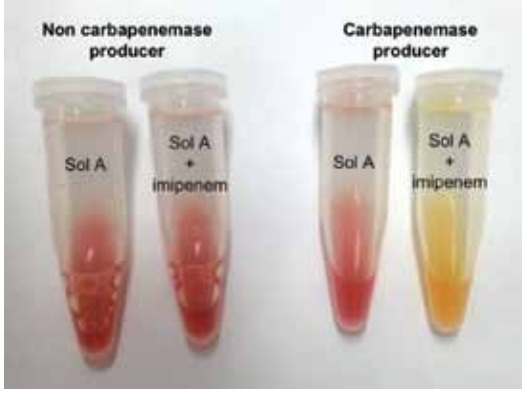


Figure 5.1: Carba NP test

Usually, the time required for obtaining positive results is as follows:

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

2. Modified carbapenem inactivation method (mCIM and eCIM); (For Enterobacteriaceae and *Pseudomonas* spp.) 44

- mCIM is used for detecting carbapenemases in *Enterobacteriaceae* and *P. aeruginosa* whereas eCIM is used together with mCIM to differentiate metallo-β-lactamases from serine carbapenemases in *Enterobacteriaceae*
- mCIM can be performed alone; however, eCIM must be performed together with mCIM
- eCIM is only valid if mCIM is positive.

2.1. mCIM

i. Grow the organism to be tested in the Mueller Hinton agar plates.
ii. For each isolate to be tested, emulsify 1 µl loopful of bacteria Enterobacteriaceae or 10 µl of *P. aeruginosa* from the overnight grown plates in 2 ml of trypticase soy broth (TSB)
iii. Vortex it for 10-15 seconds
iv. Add a 10 µg meropenem disk into the suspension using a sterile forceps (Ensure the disk is immersed in the suspension)
v. Incubate it at 37°C for 4 hours

vi. Make a lawn culture of 0.5 McFarland adjusted culture of *E.coli* ATCC 25922 into Mueller Hinton agar
vii. Remove the meropenem disk from the suspension using 10 µl loop and place it on the lawn culture of *E. coli* ATCC®25922
viii. Invert and incubate the plates at 37°C for 18 - 24 hours
ix. Following the incubation, measure the zone of inhibition

2.1.2. eCIM

i. For each isolate, label a second 2-mL TSB tube for the eCIM test.
ii. Add 20 µL of the 0.5 M EDTA to the 2-mL TSB tube to obtain a final concentration of 5 mM EDTA.
iii. Follow steps i through ix above as for 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.

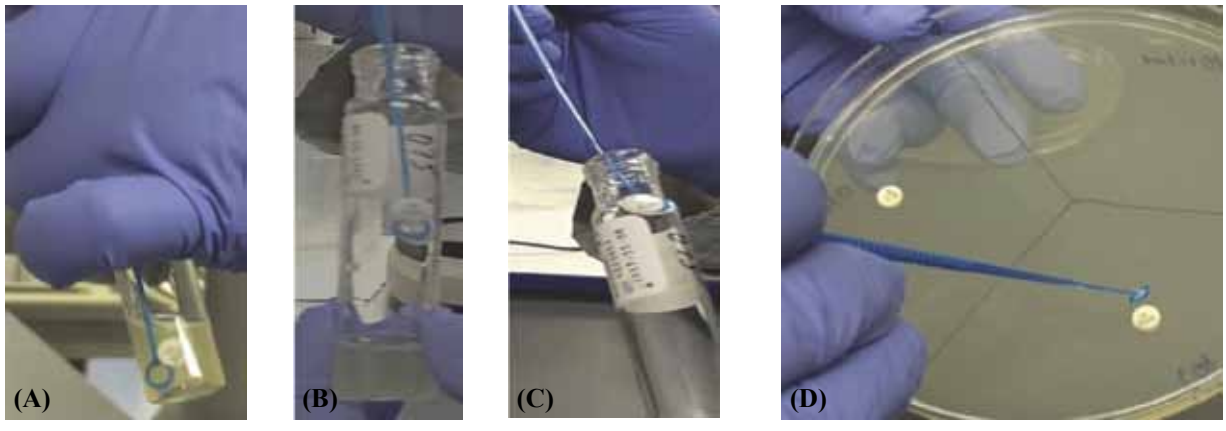


Figure 5.2: Method of mCIM/eCIM suspetibility (A) Remove the meropenem disk with a 10ul loop, (B) drag the loop against the inside edge of the tube to remove excess liquid, (C) use same loop to remove the disk from the tube, (D) place disk on MHA plate previously inoculated with the meropenem-susceptible *E. coli* (ATCC®25922)[44].

2.1.3. Test Results Interpretation

2.1.3.1. mCIM

- Carbapenemase positive : if zone of inhibition is 6-15 mm (or) presence of colonies if zone of inhibition is 16-18 mm zone
- Carbapenemase negative : zone ≥ 19mm
- Carbapenemase indeterminate : zone 16-18mm (or) ≥ 19 mm and the presence of pinpoint colonies within the zone

2.1.3.2. eCIM

Metallo-β-lactamase positive

- A ≥ 5 mm increase in zone diameter for eCIM vs zone diameter for mCIM (eg, mCIM = 6 mm; eCIM = 15 mm; zone diameter difference = 9 mm). For only the eCIM test, ignore pinpoint colonies within any zone of inhibition
- If the test isolate produces a metallo-β-lactamase, the activity of the carbapenemase will be inhibited in the presence of EDTA such that the meropenem in the disk will not be hydrolyzed as efficiently as in the tube without EDTA. The result is inhibition of the meropenem-susceptible *E. coli* and an increase in the zone diameter for the eCIM zone diameter when compared to the mCIM zone diameter.

Metallo-β-lactamase negative

- A ≤ 4 mm increase in zone diameter for the eCIM vs zone diameter of mCIM (eg, mCIM = 6 mm; eCIM = 8 mm; zone diameter difference = 2 mm). For only the eCIM test, ignore pinpoint colonies within any zone of inhibition
- If the test isolate produces a serine carbapenemase, the activity of the carbapenemase will not be affected by the presence of EDTA and there will be no or marginal (≤ 4 mm) increase in zone diameter in the presence of EDTA compared to the mCIM zone diameter.

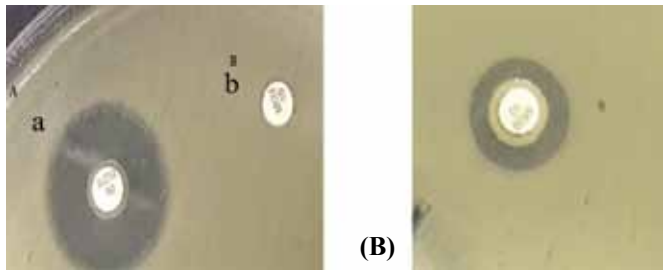


Figure 5.3: mCIm results interpretation; (A) for QC strains: (a) negative control BAA1706 and (b) positive control BAA1705. Note: A narrow ring of growth around the meropenem disk as seen with the negative control (a) results from carryover of the test organism in the TSB broth and should be ignored; (B) Positive mCIM result[44].

3. D-test for inducible clindamycin resistance

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

i. Place the clindamycin disk (2 µg) at a distance of 15 to 26 mm from the erythromycin disk (15 µg), edge to edge, on Mueller Hinton agar medium.
ii. Incubate the plate at 35ºC for 18 hours.
iii. Check for a **D shaped** blunting in the clindamycin inhibition zone adjacent to the erythromycin disc: or hazy growth within the zone of inhibition around clindamycin which indicates inducible clindamycin resistance[44].
iv. If inducible clindamycin resistance test is positive, clindamycin zone size should entered as 14 mm or MIC as 4 µg/ml.

4. Vancomycin screen agar for *S. aureus* and *Enterococcus* spp.

BHI agar supplemented with vancomycin hydrochloride at a concentration of 6 µg/ml.

i. Prepare a suspension of strain to be tested, equivalent to 0.5 McFarland standard.
ii. Inoculate the suspension using a 10 µl loop to make a spot on the surface of the vancomycin screen agar (6 µg/ml). Include the control strains (*E. faecalis* ATCC 51299: R; *E. faecalis* ATCC 29212: S) on every plate.
iii. Incubate at 35ºC for 24 hours.
iv. Read the plates in transmitted light.
v. Presence of more than one colony of the strain or light film of growth is interpreted as reduced susceptibility to vancomycin. Confirm the vancomycin resistance after performing MIC[44].

5. MIC for vancomycin by broth micro dilution method

Micro-broth dilution test

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 $2x10^6$/ml. In a 96 well plate, 100 ml of double-strength MHB, 50 ml 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.

Procedure

i. Prepare a standardized inoculum using either direct colony suspension or growth method.
ii. 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.
iii. 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).
iv. Within 15 minutes after the inoculum has been standardized 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.
v. It is advisable to perform a purity check of the inoculum suspension by subculturing an aliquot onto a nonselective agar plate for simultaneous incubation.
vi. To prevent drying, seal each tray in a plastic bag, with plastic tape, or with a tight-fitting plastic cover before incubating.

Reading of result

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. Detection of heteroresistant vancomycin intermediate *Staphylococcus aureus* (hVISA) population analysis profile/area under curve (PAP/AUC) analysis[50]

Inoculate test isolate and Mu3 strain (*S.aureus* ATCC 700698) in trypticase soya broth (TSB) and incubate at 37°C for 24 hours.

i. Dilute the culture in saline to 10^{-3}& 10^{-6} and inoculate on to brain heart infusion agar (BHIA) plates containing 0.5, 1, 2, 2.5, 4 mg/L vancomycin and incubate at 37°C for 48 hours.
ii. Count the colonies after 48 hours of incubation.
iii. Plot the graph of viable colony count against vancomycin concentration using Graph Pad Prism and calculate ratio of area under curve (AUC) of test divided by corresponding AUC for Mu3 strain.
iv. AUC ratio of ≥ 0.9 is considered as hVISA.

7. Combination antimicrobial testing to evaluate the best combination of drugs for MRSA

i. To evaluatethe best combination of drugs for MRSA, perform checkerboard assay**.** Determine the MIC of test drug –A, the MIC of test drug –B and MIC of the combination (A + B) by agar dilution technique as given above).
ii. Interpret the interaction between drug A and B using the total fractional inhibitory concentration (ΣFIC). This is calculated by the formula, Σ FIC = FIC of drug A + FIC of drug B, where
FIC of drug A = MIC of drug A / MIC of (A+B) combination
FIC of drug B = MIC of drug B / MIC of (A+B) combination.
iii. Interpretation of Σ FIC value:

≤ 0.5	Synergy
0.5 – 4	Indifference
> 4	Antagonism

8. Detection of over-expression of efflux pumps MIC

Antibiotic extrusion by efflux pumps is a major determinant of antimicrobial resistance in bacteria. Particularly, in gram negative organisms, multidrug efflux pumps with broad specificities function synergistically with the outer membrane barrier to provide intrinsic and acquired multidrug resistance (MDR). Over-expression of efflux pumps contributes to multiple antibiotic resistances due to their ability to extrude or pump out structurally unrelated compounds and thus involving in multi drug resistance.

Levofloxacin with and without efflux pump inhibitor phenyl-arginine-beta-napthylamide (EPI-PAβN)

Checker Board Assay (to screen EPO phenotype)

i. This test is done using levofloxacin with and without efflux pump inhibitor to rule out the efflux pump overexpressing (EPO) phenotype by MIC determination according to CLSI protocol
ii. EPI – PAβN of 20µg/ml should be used as a standard with levofloxacin ranging from 1.25 to 80 µg/ml
iii. Incubate the plates for 16-18 hours at 37^0C

Results and interpretation

- > 4 fold difference in the levofloxacin and efflux pump inhibitor than levofloxacin alone is considered to be positive for EPO.
- MIC ranges should be compared with the standard strain of PAO1 and ATCC 27853 *P. aeruginosa.*

Quality Control (QC)

CHAPTER 6

Quality control (QC)

Reference strains for QC[44]

- *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)*

Storing and testing QC strains[44]

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[44]

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[44]

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.

Genotypic Tests for Mechanisms of Resistance

CHAPTER 7

Genotypic Tests for Mechanisms of Resistance

1. Enterobacteriaceae

1.1. Polymerase chain reaction (PCR)

For 16 genes, initially simplex assays should be standardized and multiplex PCR assays described by Dallenne et al.[51] should be tried later. Table 9.1 shows the list of genes and primers to be used for PCR. Simplex PCR would be used for NDM-1[52].

Table 7.1: PCR gene targets and primers for mechanisms of resistance in Enterobacteriaceae

PCR name	β-lactamase targeted	Primers	Amplicon (bp)
Multiplex I TEM,SHV and OXA-1	TEM1 and TEM 2	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 to5,	F: CTACAGTGCGGGTGGTTT R: CTATTTGCGGCCAGGTGA	162
	MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11and CMY19	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:ACGGACAGGGTTAG-GTTAGGATAGY	538

PCR name	β-lactamase targeted	Primers	Amplicon (bp)
Multiplex IV Metallo beta lactamases and carbapene-mases	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

bp; base pair

1.2. Molecular typing of isolates by PFGE

The strains should be evaluated for genetic relatedness using pulsed-field gel electrophoresis (PFGE) with *Xba*I digestion of the genomic DNA separated by electrophoresis in a 1.2% agarose gel, in accordance with the standard protocol established by the Centers for Disease Control and Prevention (PulseNet; www.cdc.gov/pulsenet/pathogens/index.html).

- Grow the bacterial strain overnight on tryptic soya agar (TSA) plates at 37°C.
- Suspend bacterial colonies in cell suspension buffer to an optical density (OD) of 1.3–1.4 using a spectrophotometer set at 590 nm.
- Mix 400 µl adjusted cell suspension with 20 µl of proteinase K and an equal volume (400 µl) of melted 1% agarose containing 1% sodium dodecyl sulfate.
- Carefully dispense the mixture into appropriate wells of a reusable plug mould. After solidification, transfer the plugs individually to round bottom tubes containing 1.5 mL of cell lysis buffer (50 mmol/l Tris–HCl 50 mmol/l EDTA, pH 8·0; 1% sarcosine) and 0.5 mg/ml of proteinase K.
- Lyse the cells in a 54°C water bath for 2 hours with constant and vigorous agitation at 175–200 rev/minute.
- After lysis, wash the plugs twice with preheated water and four times with preheated TE buffer for 10–15 minutes per wash at 50°C, with agitation as above. Store the plugs in 2 ml of TE buffer at 4°C until digestion with DNA restriction enzyme (RE).
- Digest the DNA in agarose plugs with 50 U of *Xba*I for at least 3 hours at 37°C in a water bath.
- Load the plugs onto wells in a 1% (wt/vol) pulse-field-certified agarose gel.
- Load a DNA size standard ladder as molecular weight standard.
- Separate DNA restriction fragments using a CHEF-DRII (Bio-Rad Laboratories) electrophoresis system with pulse times of 5–50 seconds at 14°C for 14 hours in 0.5x TBE buffer at 6 V/cm.

- Stain the gel with ethidium bromide, and photograph restriction fragment patterns using a gel documentation system (Bio-Rad, United Kingdom). Compare PFGE profiles to identify restriction enzyme digestion pattern clusters with BioNumerics software, version 5.0 (Applied Maths, Austin, Texas, US). Cluster fingerprints by using the Jacard coefficient evaluated by the unweighted-pair group method (UPGMA). Isolates are considered to indistinguishable if they have the same number and size of bands in a PFGE fingerprint pattern. Isolates are considered to be closely related if their PFGE pattern differs by less than three bands.

2. Typhoidal *Salmonella: Salmonella* Typhi

2.1. Mechanism of resistance test panel

Table 7.2: Genes, primers, product sizes and characteristics for mechanisms of resistances of *Salmonella* Typhi [53,54,55]

Gene	Primer (5'-3')	Amplicon (bp)	Characteristic (to study)
gyrA	F: ATGAGCGACCTTGCGAGAGAAATTACACCG R: TTCCATCAGCCCTTCAATGCTGATGTCTTC	630	fluoroquinolones resistance
gyrB	F: GCGCTGTCCGAACTGTACCT R: T GATCAGCGTCGCCACTTCC	181	fluoroquinolones resistance
parC	F: ATGAGCGATATGGCAGAGCG R: TGACCGAGTTCGCTTAACAG	412	fluoroquinolones resistance
parE	F: TCTCTTCCGATGAAGTGCTG R: ATACGGTATAGCGGCGGTAG	240	fluoroquinolones resistance
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	efflux pump mediated drug resistance

2.2. Transmissibility of AMR in *Salmonella* Typhi: Class I integrons[56]

- Plasmid DNA should be extracted by using plasmid DNA extraction Kit according to the manufacturer guidelines.
- Detect integrons by PCR with the degenerate primers hep 35 (5' TGCGGGT CAAAGATCTGGATTT 3') and hep36 (5' CAACACATGCGTATAAAT 3'), which hybridize to conserved regions of integron encoded integrase gene *intl1, intl2* and *intl3.*
- Determine the class of integron by analyzing integrase PCR products through enzyme digestion by *Hinf1*

2.3. Molecular clonality tests panel for *Salmonella* Typhi

- MLST: *thrA, purE, sucA, hisD, aroC, hemD, dnaN* [57]
- PFGE

Table 7.3: Genes, PCR primers and product size of all the seven housekeeping genes used for MLST (http://mlst.ucc.ie/mlst/dbs/Senterica/documents/primersEnterica_html)

Gene	PCR Primers (5'-3')	Amplicon (bp)
AroC	F: CCTGGCACCTCGCGCTATAC R: CCACACACGGATCGTGGCG	826
HemD	F: GAAGCGTTAGTGAGCCGTCTGCG R: ATCAGCGACCTTAATATCTTGCCA	666
HisD	F: GAAACGTTCCATTCCGCGCAGAC R: CTGAACGGTCATCCGTTTCTG	894
PurE	F: ATGTCTTCCCGCAATAATCC R: TCATAGCGTCCCCCGCGGATC	510
SucA	F: AGCACCGAAGAGAAACGCTG R: GGTTGTTGATAACGATACGTAC	643
ThrA	F: GTCACGGTGATCGATCCGGT R: CACGATATTGATATTAGCCCG	852
DnaN	F: ATGAAATTTACCGTTGAACGTGA R: AATTTCTCATTCGAGAGGATTGC	833

2.4. Pulse Field Gel Electophoresis (PFGE)[58]

Methodology

Day 1

- Revive strains in Blood agar plate.

Day 2

- Inoculate single colony in 2 ml BHI (Brain Heart Infusion) broth overnight at 37°C in shaking incubator.

Day 3

- Centrifuge inoculated broth and wash the received pellet thrice in 50 mM EDTA (1ml) by doing vortex at 12000 rpm for 2 minutes each.
- Resuspend final pellet in 500 µl EC buffer and adjust to 4 McFarland with turbidometer.
- To the 100 µl of suspension (in EC buffer) , add 50 µl lysozyme (Lysozyme stock and 500 mg/ml keep at -20°C (Working- 1 mg/ml at 4°C)
- Incubate at 37°C for 30 minutes (Prepare 1% LMA in 0.5X TBE).
- Add equal volume (150 µl) of agarose to 150 µl suspension I.

- Mix this suspension and add immediately to plug mold. Settle for 30 to 45 minutes till the plug appear milky white (make 2 plugs for each isolate).
- Now add (1ml) lysozyme in plugs (both) from (1 mg/ ml working solution) and incubate these plugs for 3 hours at 37°C (keep in some drawer or cupboard).
- Discard lysozyme and 450 µl ESP buffer, aad 50 µl proteinase K (20 mg/ml) to the plugs, and keep overnight at 55°C in water bath.

Day 4

- Take out entire solution by pipette.
- Add 1 ml TE (pH 8.0) and keep at 65°C water bath for 1 hour (1st wash).
- Give 2 simple washes with 1 ml TE (pH 8.0) without incubation but with intermittent shaking or hand inverting.
- Add 50 mM EDTA (1ml) and keep this solution at 37°C for 30 minutes. **Note:** Now, blocks can be kept at 4°C for a week to 10 days.
- Cut blocks in two halves (with cover glass slide). Take both halves in microcentrifuge tube (MCT) containing autoclaved 1 ml MilliQ water.
- Invert MCT several times to remove last traces of EDTA and then pipette out the entire solution.
- Now perform restriction digestion of the blocks by adding 88 µl distilled water, 10 µl buffer and 2 µl restriction enzyme. Incubate the solution at room temperature overnight at 20-25°C.

Day 5

- Run the digested overnight product in 1% agarose in 0.5X TBE.
- Load in CHEF loader at temperature 12°C ± 2°C, at conditions 200 V for 16 hours with pulse time ranging from 10 to 25 s.
- **Running the gel:** Clean the apparatus before pouring the gel.
- Place the comb after ~200 ml (1% LMA) solution poured in gel plate and allow to cool for 30 - 46 minutes.
- Now load the plugs in the wells and overlay the leftover 1% gel to cover the wells (make 3 liter 0.5X TBE using autoclaved water only).
- Cool the loading chamber by adding the buffer and switch on the machine.
- After loading the sample, allow it to run for 18.5 hrs at 200V switch time.

Day 6

- Stain the gel for 30 min and destain for 5 min in 200-300 ml 0.5X TBE buffer or distilled water (add ethidium bromide around 20-50 µl). If Etbr staining is excessive, the gel should be destained otherwise not needed.
- Visualize in gel doc system and perform analysis and interpretation.

3. Resistance gene detection in *Pseudomonas* spp. & *Acinetobacter* spp., using multiplex-PCR

3.1. DNA isolation

Extract whole genomic DNA from overnight colonies grown on blood agar (Remel, Lenexa, KS) using the QIAamp DNA Mini Kit or the QIAsymphony instrument (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Multiplex PCR should be performed as a first screening step on all of the following *bla* genes: TEM, SHV, KPC, NDM, IMP, VIM, OXA-48, VEB, PER, GES and SPM.
For *Acinetobacter*, in addition to the above mentioned genes, uniplex PCR for OXA-51, SIM gene and multiplex PCR for OXA-23, OXA-24 and OXA-58 should be performed.

3.2. Detection of genes by multiplex PCR

A multiplex PCR should be used for the detection of β-lactamase genes using primers presented in Tables 9.4, 9.5, 9.6 and 9.7. Subject total DNA to multiplex-PCR using the Multiplex PCR kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Visualize amplicons in a 2% agarose gel containing ethidium bromide.

Table 7.4: Detection of bla_{SHV} like, bla_{TEM} like, bla_{VEB} like, bla_{PER} like, bla_{GES} like, and bla_{SPM} like genes

Target	Primer pairs	Amplicon (bp)	Reference
TEM	F - CATTTCCGTGTCGCCCTTATTC R - CGTTCATCCATAGTTGCCTGAC	800	51
SHV	F - CCTTTAAAGTAGTGCTCTGC R – TTCGCTGACCGGCGAGTAGT	119	59 (in- house)
GES	F - AGTCGGCTAGACCGGAAAG R - TTTGTCCGTGCTCAGGAT	399	51
SPM	F - AAAATCTGGGTACGCAAACG R - ACATTATCCGCTGGAACAGG	271	60, 61
VEB	F - CATTTCCCGATGCAAAGCGT R - CGAAGTTTCTTTGGACTCTG	648	51
PER	F - GCTCCGATAATGAAAGCGT R - TTCGGCTTGACTCGGCTGA	520	

Reaction volume		Cycling conditions		
2x multiplex master mix (Qiagen)	10 µl	95°C	15 mins	1 cycles
Primer mix (2 µM of each primer)	2 µl	94°C	30 sec	30 cycles
5x Q solution	2 µl	59°C	1.5 mins	
DNA template (< 0.4 µg/ 20 µl reaction)	Variable	72°C	1.5 mins	
Water	Up to 20 µl	72°C	10 mins	1 cycles

Table 7.5: Detection of bla_{IMP}, $bla_{VIM,}$ $bla_{NDM,}$ bla_{KPC} and bla_{OXA-48} genes

Target genes	Primer pairs	Amplicon (bp)	Reference
IMP2	F - GGAATAGAGTGGCTTAAYTCTC R - GGTTTAAYAAAACAACCACC	232	62
VIM	F - GATGGTGTTTGGTCGCATA R - CGAATGCGCAGCACCAG	390	51
KPC	F - TGTCACTGTATCGCCGTC R - CTCAGTGCTCTACAGAAAACC	1011	63

NDM	F - CCGTATGAGTGATTGCGGCG R - GCCCAATATTATGCACCCGG	779	62
OXA-48	F - GCTTGATCGCCCTCGATT R - GATTTGCTCCGTGGCCGAAA	281	62
SIM	F - TACAAGGGATTCGGCATCG R - TAATGGCCTGTTCCCATGTG	570	62

Reaction volume		**Cycling conditions**		
2x multiplex master mix (Qiagen)	10 µl	95°C	15 mins	1 cycle
Primer mix (2 µM of each primer)	2 µl	94°C	30 secs	
5x Q solution	2 µl	59°C	1.5 mins	30 cycles
DNA template (< 0.4 µg/ 20 µl reaction)		72°C	1.5 mins	
Variable Water	up to 20 µl	72°C	10 min	1 cycle

Table 7.6: Detection of bla$_{OXA-51}$ like gene for *Acinetobacter baumannii*

Target genes	**Primer pairs**	**Amplicon (bp)**	**Reference**
OXA-51	F: TAATGCTTTGATCGGCCTTG R: TGGATTGCACTTCATCTTGG	353	64

Reaction volume		**Cycling conditions**		
2x multiplex master mix (Qiagen)	10 µl	94°C	3 mins	1 cycle
Primer mix (2 µM of each primer)	2 µl	94°C	45 secs	
5x Q solution	2 µl	57°C	45 mins	35 cycles
DNA template (< 0.4 µg/ 20 µl reaction)		72°C	1.5 mins	
Variable Water	up to 20 µl	72°C	5 min	1 cycle

Table 7.7: Detection of bla$_{OXA-23,}$ bla$_{OXA-24}$ and bla$_{OXA-58}$ like genes

Genes	**Primer pairs**	**Amplicon (bp)**	**Reference**
OXA-23	F - GATCGGATTGGAGAACCAGA R - ATTTCTGACCGCATTTCCAT	501	65
OXA-24	F - GGTTAGTTGGCCCCCTTAAA R - AGTTGAGCGAAAAGGGGATT	246	
OXA-58	F - AAGTATTGGGGCTTGTGCTG R - CCCCTCTGCGCTCTACATAC	599	

Reaction volume		**Cycling conditions**		
2x multiplex master mix (Qiagen)	10 µl	95°C	15 mins	1 cycle
Primer mix (2 µM of each primer)	2 µl	94°C	30 secs	
5x Q solution	2 µl	52°C	1.30 mins	30 cycles
DNA template (< 0.4 µg/ 20 µl reaction)	Variable	72°C	1.30 mins	
Water	up to 20 µl	72°C	5 min	1 cycle

3.3. Detection of outer membrane porin loss (OMP) genes OprD/CarO by monoplex PCR[66,67]

- OprD is the outer membrane porin channels favors the entry of imipenem inside in *Pseudomonas aeruginosa*. Either the presence of any mutations and or loss of the porin channel as such contributes for imipenem resistance
- CarO is a heat-modifiable carbapenem-associated outer membrane protein which allows the selective uptake of imipenem *A. baumannii*. Gene alterations such as disruption by insertion sequences, changes in the primary structure or decreased expression contribute for imipenem resistance.

Table 7.8: Primers for *OprD*/*CarO* PCR

Primer name	Primer sequence (5'-3')	PCR product size (bp)
OprD	F - 5' CGCCGACAAGAAGAACTAGC -3' R - 5' GTCGATTACAGGATCGACAG -3'	1332
CarO	F – 5' GCTCACCTGATGCTGACATT – 3' R – 5' TTGCTTCTTCAACAGCTTGG -3'	1282

3.3.1. PCR

Set monoplex PCR for the amplification of OMP genes using following master mix and cycling conditions.

Master mix (Single reaction)		Cycling conditions		
Mastermix	12.5 µl	95°C	5 mins	1 cycle
PCR Water	6 µl	94°C	15 secs	
Forward Primer	2 µl	58°C	1.30 mins	35 cycles
Reverse Primer	2 µl	72°C	1.30 mins	
DNA template	2.5 µl	72°C	5 min	1 cycle
Total Reaction Volume	25 µl			

3.3.2. Post amplification DNA detection by gel electrophoresis

i. Mix 7 µL of each amplicon with 1 µL of 6X loading dye bromophenol blue.
ii. Subject the amplified products to electrophoresis in freshly prepared 2% agarose gel containing 0.5 µg/ml ethidium bromide
iii. Load the test samples and negative control in appropriate wells. Use molecular ladder of 100-1000 bp long
iv. Carry out electrophoresis at 100 volts for 60 minutes.
v. Visualize the gel by ultraviolet radiation using Quantity one® (version 4.6.2) software in the gel documentation system.
vi. Check the positive and negative controls of each run for validation. Then take the results of the samples

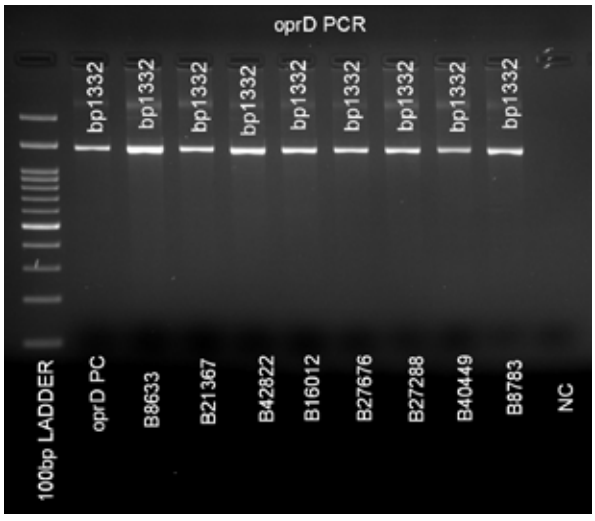


Figure 7.1: OprD profile by agarose gel electrophoresis[66]

3.3.3. *OprD* / *CarO* gene sequencing[66,67]

Use Sanger sequencing method (Dideoxynucleotide chain termination method) for sequencing OprD / CarO gene.

3.3.3.1. Pre-clean up

i. Thaw high preparation PCR reagent for 30 mins and mix well. Add 36µl of HPPCR Reagent to 20 µl PCR product. Make up the volume to 100 µl with sterile water.Mix it thoroughly using pipette.
ii. Incubate for 5mins at room temperature. After appropriate period of incubation, place the tubes on magnetic separation device for 2-3mins until the solution clears.
iii. With the sample tubes still on the magnet, discard the supernatant. Add 200v of 70% ethanol to the pellet in the sample tube. Incubate for 30sec at room temperature
iv. With the tubes still on the magnet, discard the supernatant and repeat the 70% ethanol wash step again.
v. Dry the beads by incubating the tubes at room temperature for 3-5mins with the tubes still on the magnet. Remove the tubes from the magnetic separation device. Add 40µl elution buffer and 5 times.
vi. Place the tubes back on to the magnetic device and wait 1min until the solution clears. Transfer the clear supernatant to new tubes for further processing.

3.3.3.2. Quantitation of DNA (Nanodrop™ check)

Place 1µl of sterile water to set blank and use 1µl of sample for Nanodrop check.

3.3.3.3. Sequencing PCR using following master mix and cycling conditions

Table 7.9: Primers for *OprD / CarO* sequencing

Primer	Primer sequence (5'-3')	Amplicon size (bp)
OprD	F1: CGCCGACAAGAAGAACTAGC F2: GCCGACCACCGTCAAATCG R: GTCGATTACAGGATCGACAG	1332
CarO	F: GCTCACCTGATGCTGACATT R: TTGCTTCTTCAACAGCTTGG	1282

Master mix (Single reaction)		Cycling conditions	
RR mix	0.5 µl	25 cycles of	
PCR Water	4.9 µl		
Primer (Forward or reverse)	1.6 µl	96°C	15 secs
Buffer	1 µl	50°C	20 sec
Product	2 µl	60°C	4 mins
Total Reaction Volume	10 µl		

3.3.3.4. Post clean up

i. Thaw the high prep DTR solution by keeping in room temperature for 10mins.
ii. Mix well and add 10 µl pf HPDTR reagent and add 10 µl of sample (PCR product)
iii. Add 40 µl of 85% ethanol and mix by pipetting 10 times
iv. Place tubes on magnetic separation device for 5 mins. Then discard the supernatant and add 200 µl of 85% ethanol and incubate for 2 mins at room temperature.
v. Discard the supernatant and repeat the 85% ethanol wash step again.
vi. Incubate beads at room temperature for 10 mins with tubes still on magnet for drying.
vii. Remove the tubes from the magnet device and add 40 µl of injection solution (0.1 mM EDTA or Deionized water). Mix 20 times by pipetting.
viii. Incubate at room temperature for 5 mins keeping the tubes on the magnetic device.
ix. After appropriate period of incubation, transfer 30 µl of clear supernatant to fresh tube and load 30 µl of this supernatant in the sequencing plate.

3.4. Multi-locus sequence typing (MLST) for *Pseudomonas aeruginosa*

Primers: MLST scheme for *Pseudomonas aeruginosa* uses internal fragments of the following seven house-keeping genes.

i. *acsA* (Acetyl coenzyme A synthetase)
ii. *aroE* (Shikimate dehydrogenase)
iii. *guaA* (GMP synthase)
iv. *mutL* (DNA mismatch repair protein)
v. *nuoD* (NADH dehydrogenase I chain C, D)
vi. *ppsA* (Phosphoenolpyruvate synthase)
vii. *trpE* (Anthralite synthetase component I)

Table 7.10: Primer pairs for the PCR amplification of internal fragments of genes for MLST for *Pseudomonas aeruginosa*

Gene Name	Primer for PCR	Primer for sequencing
acsA (Acetyl coenzyme A synthetase)	F: ACCTGGTGTACGCCTCGCTGAC R: ACATAGATGCCCTGCCCCTTGAT	F: GCCACACCTACATCGTCTAT R: AGGTTGCCGAGGTTGTCCAC
aroE (Shikimate dehydrogenase)	F: TGGGGCTATGACTGGAAACC R: AACCCGGTTTTGTGATTCCTACA	F: ATGTCACCGTGCCGTTCAAG R: TGAAGGCAGTCGGTTCCTTG
guaA (GMP synthase)	F: CGGCCTCGACGTGTGGATGA R: AACGCCTGGCTGGTCTTGTGGTA	F: AGGTCGGTTCCTCCAAGGTC R: GACGTTGTGGTGCGACTTGA
mutL (DNA mismatch repair protein)	F: CCAGATCGCCGCCGGTGAGGTG R: CAGGGTGCCATAGAGGAAGTC	F: GAAGACCGAGTTCGACCAT R: GTGCCATAGAGGAAGTCAT
nuoD (NADH dehydrogenase I chain C,D)	F: ACCGCCACCCGTACTG R: TCTCGCCCATCTTGACCA	F: CGGCGAGAACGAGGACTAC R: TGGCGGTCGGTGAAGGTGAA
ppsA (Phosphoenol pyruvate synthase)	F: GGTCGCTCGGTCAAGGTAGTGG R: GGTTCTCTTCTTCCGGCTCGTAG	F: GTGACGACGGCAAGCTGTA R: GTATCGCCTTCGGCACAGGA
trpE (Anthralite synthetase component I)	F: GCGGCCCAGGGTCGTGAG R: CCCGGCGCTTGTTGATGGTT	F: TTCAACTTCGGCGACTTCCA R: GGTGTCCATGTTGCCGTTCC

Master mix (Single reaction)		Cycling conditions		
Mastermix	12.5 µl	95°C	5 mins	1 cycle
PCR Water	6 µl	94°C	1 min	
Forward Primer	2 µl	55°C	30 sec	30 cycles
Reverse Primer	2 µl	72°C	1 mins	
DNA template	2.5 µl	72°C	10 min	1 cycle
Total Volume	25 µl			

The genes must be sequenced and analyzed against the reference sequences deposited in the MLST database for *Pseudomonas aeruginosa* https://pubmlst.org/paeruginosa/ .

3.5. Multi-locus sequence typing for *Acinetobacter baumannii*

Oxford scheme of MLST was used for the characterization of clinical isolates of *A. baumannii* and it can be accesed from database available from weblink https://pubmlst.org/abaumannii/. For *A. baumannii*, seven house keeping genes studied for MLST are as follows:

i. *gltA*: citrate synthase
ii. *gyrB*: DNA gyrase subunit B
iii. *gdhB*: glucose dehydrogenase B
iv. *recA*: homologous recombination factor
v. *cpn60*: 60-KDa chaperonin
vi. *gpi*: glucose 6 phosphate isomerase
vii. *rpoD*: RNA polymerase sigma factor *rpoD*

Table 7.11: Primers for MLST of *A. baumannii*

Gene	Primer sequence (5'-3')	Amplicon (bp)
cpn60	GGT GCT CAA CTT GTT CGT GA	479
	CAC CGA AAC CAG GAG CTT TA	
gpi	GAA ATT TCC GGA GCT CAC AA	508
	TCA GGA GCA ATA CCC CAC TC	
gltA	AATTTACAGTGGCACATTAGGTCCC	722
	GCAGAGATACCAGCAGAGATACACG	
gyrB	TGA AGG CGG CTT ATC TGA GT	909
	GCT GGG TCT TTT TCC TGA CA	
recA	CCTGAATCTTCYGGTAAAAC	425
	GTTTCTGGGCTGCCAAACATTAC	
gdhB	GCT ACT TTT ATG CAA CAG AGC C	775
	GTT GAG TTG GCG TAT GTT GTG C	
rpoD	ACC CGT GAA GGT GAA ATC AG	492
	TTC AGC TGG AGC TTT AGC AAT	

Reaction volume		Cycling conditions		
Hot start buffer	12.5 µl	94°C	15 mins	1 cycle
Forward primer (10µM)	1 µl	94°C	30 secs	30 cycles
Reverse primer (10µM)	1 µl	50°C	30 secs	
DNA template (< 0.4 µg/ 20 µl reaction)	Variable	72°C	1.0 min	
Water	up to 25 µl	72°C	5 min	1 cycle

3.6. Class 1 and 2 integron and gene cassette detection for *Acinetobacter* spp. and *Pseudomonas* spp.

Table 7.12: PCR for detection of class 1 and 2 integron and gene cassette (For both *Acinetobacter* spp. and *Pseudomonas* spp.)

Target	Primer sequence (5'-3')	Amplicon (bp)	Reference
intI1F	F: CCTCCCGCACGATGATC	280	68
	R: TCCACGCATCGTCAGGC		
intI2	F: CACGGATATGCGACAAAAAGGT	789	
	R: GTAGCAAACGAGTGACGAAATG		
hep58	F: GGCATCCAAGCAGCAAGC	Variable	
	R: AAGCAGACTTGACCTGAT		
hep74	F: CGGGATCCCGGACGGCATGCACGATTTGTA	Variable	
hep51	R: GATGCCATCGCAAGTACGAG	Variable	

Reaction volume		Cycling conditions			
2x multiplex master mix (Qiagen)	10 µl	94°C	5 min		1 cycle
Primer mix (2 µM of each primer)	2 µl	94°C	1 min	}	
5x Q solution	2 µl	50°C	1 min	}	30 cycles
DNA template (< 0.4 µg/ 20 µl reaction)	Variable	72°C	1 min	}	
Water	up to 20 µl	72°C	7 min		1 cycle

Table 7.13: PCR for detection of insertion sequences for *A. baumannii*

Target	Primer pairs	Amplicon (bp)	Reference
ISAba1	F: CACGAATGCAGAAGTTG R: CGACGAATACTATGACAC	549	69
ISAba1 F with OXA-23 R	F: CACGAATGCAGAAGTTG R: ATTTCTGACCGCATTTCCAT	~1300	70
ISAba1 F with OXA-51 R	F: GATACCAGACCTGGCAACAT R: TGGATTGCACTTCATCTTGG	~1200	70, 71

ISAba1 PCR and ISAba1 F with OXA-23 R PCR		
Reaction volume		**Conditions**
Hot start master mix (Qiagen)	12.5µl	95ºC for 15 min 35 cycles of 95ºC for 45 secs; 56ºC for 45 secs; 72ºC for 2 mins; and 72ºC for 10 mins
Forward primer	1 µl	
Reverse primer	1 µl	
DNA template (<0.4 µg/ 20 µl reaction)	Variable	
Water	up to 20 µl	

ISAba1 F with OXA-51R PCR		
Reaction volume		**Conditions**
Phusion high fidelity PCR master mix (Qiagen)	10 µl	95ºC for 2 min 35 cycles of 95ºC for 10 secs; 57ºC for 30 secs; 72ºC for 30 secs; and 72ºC for 10 mins
Forward primer	1 µl	
Reverse primer	1 µl	
DMSO	0.6 µl	
DNA template (< 0.4 µg/ 20 µl reaction)	Variable	
Water	up to 20 µl	

Table 7.14: PCR for detection of RMTases (for both *Acinetobacter* spp. and *Pseudomonas* spp.)[72]

Genes	Primer pairs	Amplicon (bp)
armA	F - CAAATGGATAAGAATGATGTT R - TTATTTCTGAAATCCACT	777
rmtA	F - ATGAGCTTTGACGATGCCCTA R - TCACTTATTCCTTTTTATCATG	756
rmtB	F - ATGAACATCAACGATGCCCT R - CCTTCTGATTGGCTTATCCA	769
rmtC	F - CGAAGAAGTAACAGCCAAAG R - ATCCCAACATCTCTCCCACT	711
rmtD	F - CGGCACGCGATTGGGAAGC R - CGGAAACGATGCGACGAT	401
rmtE	F - ATGAATATTGATGAAATGGTTGC R - TGATTGATTTCCTCCGTTTTTG	819

rmtF	F - GCGATACAGAAAACCGAAGG R - ACCAGTCGGCATAGTGCTTT	590
rmtG	F - AAATACCGCGATGTGTGTCC R - ACACGGCATCTGTTT CTTCC	250
npmA	F - CTCAAAGGAACAAAGACGG R - GAAACATGGCCAGAAACTC	641

Reaction volume		**Cycling conditions**		
2x multiplex master mix (Qiagen)	10 µl	96°C	5 mins	1 cycle
Primer mix (2 µM of each primer)	2 µl	96°C	1 min	
5x Q solution	2 µl	55°C	30 secs	35 cycles
Dye	2 µl	72°C	1 min	
DNA template (< 0.4 µg/ 20 µl reaction)	Variable	72°C	5 mins	1 cycle
Water	up to 20 µl			

4. Resistance gene detection in *Shigella* spp. by PCR

4.1. DNA isolation

Extract whole genomic DNA from overnight colonies grown on blood agar (Remel, Lenexa, KS) using the QIAamp DNA Mini Kit or the QIAsymphony instrument (Qiagen, Valencia, CA) according to the manufacturer's instructions.

4.2. PCR

As a first screening step, PCR should be performed for the genes: *dhfr, sulI, sulII*, *bla*OXA, *bla*TEM, *bla*CTX-M-1, AmpC, *aac(6)-Ib-cr*, *qnr*A,B,S.

Table 7.15: PCR detection of *sulI, OXA* and *aac(6)-Ib-cr* genes

Gene	**Primer Sequences**	**Amplicon**	**Reference**
sulI	F: CTTCGATGAGAGCCGGCGGC R: GCAAGGCGGAAACCCGCGCC	437bp	73
bla-OXA	F: ATGAAAAACACAATACATATCAACTTCGC R: GTGTGTTTAGAATGGTGATCGCATT	820bp	
aac(6)-Ib-cr	F: GCAACGCAAAAACAAAGTTAGG R: GTGTTTGAACCATGTACA	560bp	74

Note: Master mix preparation is same for all the targets.

Reaction volume		Cycling conditions			
2x multiplex master mix (Qiagen)	10 µl	94°c	5 min		1 cycle
Primer mix (2 µM of each primer)	2 µl	94°c	1 min	}	
5x Q solution	2 µl	50°c	1 min		30 cycles
DNA template (< 0.4 µg/ 20 µl reaction)	Variable	72°c	1 min		
Water	up to 20 µl	72°c	7 min		1 cycle

Table 7.16: PCR for detection of *dhfr1a,* sulII, TEM, CTX-M-1

Gene	Primer Sequences	Amplicon	Cycling conditions	Reference
dhfr1a	F: GGAGTGCCAAAGGTGAACAGC R: GAGGCGAAGTCTTGGGTAAAAAC	367 bp	92ºC for 5 min; 30 cycles of 92 ºC for 1 min, 50 ºC for 1 min, 72ºC for 1 min; and 72ºC for 8 min	75
sulII	F: AGGGGGCAGATGTGATCGAC R: TGTGCGGATGAAGTCAGCTCC	626bp	94ºC for 2 min; 35 cycles of 94ºC for 1 min, 60ºC for 1 min, 72ºC for 1 min; and 72ºC for 10 min	76
bla-TEM	F: CATTTCCGTGTCGCCCTTATTC R: CGTTCATCCATAGTTGCCTGAC	800bp	95ºC for 15 min; 30 cycles of 94ºC for 30 sec, 59ºC for 1 min 30 sec, 72ºC for 1 min 30 sec; and 72ºC for 10 min	51
bla-CTX-M-1	F: AAAAATCACTGCGCCAGTTC R: AGCTTATTCATCGCCACGTT	415bp	95ºC for 15 min; 30 cycles of 94ºC for 30 sec, 58ºC for 1 min 30 sec, 72ºC for 1 min 30 sec; and 72ºC for 10 min	61

Table 7.17: PCR for detection of *qnrA, qnrB* and *qnrS*

Gene	Primer Sequences	Amplicon (bp)	Reference
qnrA	F: ATTTCTCACGCCAGGATTTG R: GATCGGCAAAGGTTAGGTCA	516	77
qnrB	F: GATCGTGAAAGCCAGAAAGG R: ACGATGCCTGGTAGTTGTCC	469	
qnrS	F: ACGACATTCGTCAACTGCAA R: TAAATTGGCACCCTGTAGGC	417	

Cycling conditions: Initial denaturation of 94ºC for 5 min; 32 cycles of 94ºC for 45 sec, 53ºC for 45 sec, 72ºC for 1 min; and final extension of 72ºC for 7 min

Table 7.18: PCR for detection of MOX, CIT, DHA, ACC, EBC and FOX

Gene	Primer Sequences	Amplicon (bp)	Reference
MOX	F- GCTGCTCAAGGAGCACAGGAT R- CACATTGACATAGGTGTGGTGC	520	78
CIT	F –TGGCCAGAACTGACAGGCAAA R- TTTCTCCTGAACGTGGCTGGC	462	
DHA	F – AACTTTCACAGGTGTGCTGGGT R - CCGTACGCATACTGGCTTTGC	405	
ACC	F- AACAGCCTCAGCAGCCGGTTA R- TTCGCCGCAATCATCCCTAGC	346	
EBC	F- TCGGTAAAGCCGATGTTGCGG R - CTTCCACTGCGGCTGCCAGTT	302	
FOX	F- AACATGGGGTATCAGGGAGATG R - CAAAGCGCGTAACCGGATTGG	190	

Cycling conditions: Initial denaturation of 95ºC for 15 min; 30 cycles of 94ºC for 30 sec, 65ºC for 1 min 30 sec, 72ºC for 1 min 30 sec; and final extension of 72ºC for 10 min

Table 7.19: PCR for class 1 and class 2 integron and gene cassette

Gene	Primer sequence (5'-3')	Amplicon	References
intI1F	F: CCTCCCGCACGATGATC	280bp	68
	R: TCCACGCATCGTCAGGC		
intI2F	F: CACGGATATGCGACAAAAAGGT	789bp	
	R: GTAGCAAACGAGTGACGAAATG		
hep58-F *hep59-R*	F: GGCATCCAAGCAGCAAGC AAGCAGACTTGACCTGAT	Variable	
hep74-F	F: CGGGATCCCGGACGGCATGCACGATTTGTA	Variable	
hep51-R	R: GATGCCATCGCAAGTACGAG	Variable	

Cycling conditions: Initial denaturation of 94ºC for 5 min; 30 cycles of 94ºC for 50 sec, 56ºC for 50 sec, 72ºC for 4 min; and final extension of 72ºC for 10 min

5. *Staphylococcus* species

5.1. Genotypic confirmation of antibiotic resistance

Table 7.20: PCR for the detection of antibiotic resistance genes in Staphylococci

Genes	Primer sequence (5' -3')	PCR Conditions	Amplicon (bp)	Reference
mecA	F-GTAGAAATGACTGAACGTCCGATA R-CCAATTCCACATTGTTTCGGTCTAA	94°C for 7 min 30 cycles of 94°C for 45 sec 55°C for 45 sec 72°C for 30 sec	310	79
mupA	F-CCCATGGCTTACCAGTTGA R-CCATGGAGCACTATCCGAA	94°C for 4 min 30 cycles of 94°C for 60 sec 60°C for 45 sec 72°C for 60 sec	1500	80
mupB	F-CTAGAAGTCGATTTTGGAGTAG R-AGTGCTAAAATGATAAGACGATC	94°C for 1 min 35 cycles of 94°C for 30 sec 53°C for 30 sec 68°C for 4 min	674	81
ermA	F-AAGCGGTAAACCCCTCTGA R-TTCGCAAATCCCTTCTCAAC	94°C for 3 min 30 cycles of 94°C for 45 sec 55°C for 30 sec 72°C for 30 sec	190	82
ermB	F-CTATCTGATTGTTGAAGAAGGATT R-GTTTACTCTTGGTTTAGGATGAAA	94°C for 3 min 30 cycles of 94°C for 30 sec 54°C for 30 sec 72°C for 30 sec	142	
ermC	F-AATCGTCAATTCCTGCATGT R-TAATCGTGGAATACGGGTTTG	94°C for 3 min 30 cycles of 94°C for 45 sec 55°C for 30 sec 72°C for 30 sec	299	
vanA	F-CATGAATAGAATAAAAGTTGCAATA R-CCCCTTTAACGCTAATACGACGATCAA	94°C for 10 min 30 cycles of 94°C for 30 sec 50°C for 45 sec 72°C for 30 sec	1030	83

5.2. Pulsed Field Gel Electrophoresis (PFGE) for *Staphylococcus* spp[84]

Methodology

Day 1

Subculture the QC and test strains to obtain overnight culture (16-18 hours) and incubate at 37ºC.

Day 2

- Inoculate 5 to 6 identical colonies of overnight cultures in Todd -Hewitt broth and incubate for 5-6 hours at 37ºC.
- Centrifuge the broth at 8000 rpm for 5 minutes.
- Discard the supernatant and resuspend the pellet with 300 µL sterile TE buffer and 4 µL of lysostaphin at stock concentration of (1 mg/ mL) and adjust to 1.5 McFarland / $5x10^9$ CFU/ mL.
- Equal volumes of cell suspension and 1.8% of low melting temperature agarose with Tris-EDTA buffer (i.e. 100 µL each)
- Mix well and pipette into plug molds and allow solidifying at room temperature for 10 minutes.

In situ digestion of cells

- Place the plug in lysis buffer and incubate at 50ºC- 55ºC for 4-6 hours.
- Discard the lysis buffer and wash the plugs with sterile preheated TE buffer (50^0 C- 55ºC) for 30 minutes. Repeat the step 4 times.
- Slice the plug into half with sterile blade or spatula and store one half in TE-buffer at 4ºC.

Restriction enzyme digestion

- Take 200 mL of NE buffer 2 with 10 mL BSA in a sterile tube and place one half of the plug.
- Add 1.5 mL (30 units) SmaI to the tube and mix to get uniform distribution of enzyme in the buffer.
- Incubate the plugs at 37°C overnight.

Day 3

Electrophoresis

- Soak the plugs in 1 mL of cold 0.5X (TBE) Tris Boric Acid EDTA buffer for 5 minutes at room temperature.
- Discard the buffer and load the restriction-digested plugs along with PFGE lambda marker (New England Biolabs, Maine, USA) into wells in a 1% agarose gel (Pulsed field certified agarose, Bio-Rad, California, USA) and seal the loaded wells with low melting temperature agarose.
- Place the prepared gel in 2 litres of 0.5X Tris Boric Acid EDTA buffer in pulsed field electrophoresis chamber.

- Perform the electrophoresis in the CHEF Mapper pulsed-field gel electrophoresis system (Bio-Rad, California, USA) with the following parameters.
- Running parameters to be followed: 200 V (6 V/ cm); temperature, 14°C; initial switch, 5 seconds; final switch, 40 seconds; and time, 21 hours.
- Following electrophoresis, stain the gel with 60 µL of ethidium bromide at a stock concentration (10 mg/ mL) for 20 minutes with 500 mL fresh distilled water.
- Visualize the bands using gel documentation system.

5.3. Multi-locus sequence typing (MLST) for *Staphylococcus aureus*[85]

Extract genomic DNA using Qiagen bacterial DNA purification kit. Protocol should be followed as per the manufacturer's instructions.

Table 7.21: Sequences of primers for MLST of *Staphylococcus aureus*

S. No	Target Gene	Primer sequence (5' -3')
1	Carbamate kinase (*arcC*)	F: TTGATTCACCAGCGCGTATTGTC R: AGGTATCTGCTTCAATCAGCG
2	Shikimate dehydrogenase (*aroE*)	F: ATCGGAAATCCTATTTCACATTC R: GGTGTTGTATTAATAACGATATC
3	Glycerol kinase (*glpF*)	F: CTAGGAACTGCAATCTTAATCC R: TGGTAAAATCGCATGTCCAATTC
4	Guanylate kinase (*gmk*)	F: ATCGTTTTATCGGGACCATC R: TCATTAACTACAACGTAATCGTA
5	Phosphate acetyltransferase (*pta*)	F: GTTAAAATCGTATTACCTGAAGG R: GACCCTTTTGTTGAAAAGCTTAA
6	Triosephosphate isomerase (*tpi*)	F: TCGTTCATTCTGAACGTCGTGAA R: TTTGCACCTTCTAACAATTGTAC
7	Acetyl coenzyme A acetyltransferase (*yqiL*)	F: CAGCATACAGGACACCTATTGGC R: CGTTGAGGAATCGATACTGGAAC

- Perform PCR with an initial denaturation at 95°C for 5 minutes; 30 cycles at 95°C for 1 minute , 55°C for 1 minute, and 72°C for 1 minute; and final extension at 72°C for 5 minutes. After amplification, stain the gel with ETBR for 20 minutes and visualize the bands using gel documentation system.
- Purify the amplified products using GeneJET purification kit (Thermo Scientific, USA) and send the purified amplicons for custom sequencing (Agrigenome Labs Pvt Ltd, Kochi, Kerala). The gene sequences of each housekeeping gene should be trimmed using codon code aligner software. These trimmed sequneces should be submitted to *S.aureus* MLST database (http://saureus.mlst.net/).
- For each housekeeping gene, different sequences are assigned allelic numbers and each isolate is defined by the allelic profile (a string of 7 allelic numbers). Sequences types (ST) will be assigned based on the allelic profile.
- Clustering of related STs in clonal complexes (CCs) should be determined using eBURST (Based Upon Related Sequence Types) algorithm (http://saureus.mlst.net/eburst/).

6. *Enterococcus* spp.

Table 7.22: Genotypic confirmation of antibiotic resistance in Enterococci

Genes	Primer sequence (5' -3')	PCR Conditions	Amplicon	Reference
vanA	F-GGGAAAACGACAATTGC R-GTACAATGCGGCCGTTA	94°C for 2 min 30 cycles of 94°C for 1 min 54°C for 1 min 72°C for 1 min	732 bp	86
vanB	F-ATGGGAAGCCGATAGTC R-GATTTCGTTCCTCGACC	94°C for 2 min 30 cycles of 94°C for 1 min 54°C for 1 min 72°C for 1 min	635 bp	
vanC1	F-GGTATCAAGGAAACCTC R-CTTCCGCCATCATAGCT	94°C for 2 min 30 cycles of 94°C for 1 min 54°C for 1 min 72°C for 1 min	822 bp	
vanC2/3	F-CTCCTACGATTCTCTTG R-CGAGCAAGACCTTTAAG	94°C for 2 min 30 cycles of 94°C for 1 min 54°C for 1 min 72°C for 1 min	439 bp	

6.1. PFGE Protocol for *Enterococcus*[87]

Day 1

Subculture the QC and test strains to obtain overnight culture (16-18 hours) and incubate at 37ºC.

Day 2

- Inoculate 5 to 6 identical colonies of overnight cultures in Todd-Hewitt broth and incubate for 5-6 hours at 37ºC.
- Centrifuge the broth at 8000 rpm for 5 minutes.
- Discard the supernatant and resuspend the pellet with 300 µL lysis buffer and adjust to 1.5 McFarland / $5x10^9$ CFU/ mL.
- Equal volumes of cell suspension and 1.8% of low melting temperature agarose with Tris-EDTA buffer (i.e. 100 µL each)
- Mix well and pipette into plug molds and allow solidifying at room temperature for 10 minutes.

In situ Digestion of cells

- Place the plug in lysis buffer and incubate at 37ºC for 6-7 hours.
- Replace the lysis buffer with proteolysis buffer and incubate at 50°C for overnight

Day 3

- Discard the proteolysis buffer and wash the plugs with sterile preheated TE buffer (50 - 55ºC) for 30 minutes. Repeat the step 4 times.
- Slice the plug into half with sterile blade or spatula and store one half in TE-buffer at 4ºC.

Restriction enzyme digestion

- Take 200 mL of NE buffer 2 with 10 mL BSA in a sterile tube and place one half of the plug.
- Add 1.5 mL (30 units) SmaI to the tube and mix to get uniform distribution of enzyme in the buffer.
- Incubate the plugs at 37°C for 6-8 hours.

Electrophoresis

- Soak the plugs in 1 mL of cold 0.5X (TBE) Tris Boric Acid EDTA buffer for 5 minutes at room temperature.
- Discard the buffer and load the restriction-digested plugs along with PFGE lambda marker (New England Biolabs, Maine, USA) into wells in a 1% agarose gel (Pulsed field certified agarose, Bio-Rad, California, USA) and seal the loaded wells with Low melting temperature agarose.
- Place the prepared gel in 2 litres of 0.5X Tris Boric Acid EDTA buffer in pulsed field electrophoresis chamber.
- Perform the electrophoresis in the CHEF Mapper pulsed-field gel electrophoresis system (Bio-Rad, California, USA) with the following parameters.
- Running parameters to be followed: 200 V (6 V/ cm); temperature, 14°C; initial switch, 5 seconds; final switch, 40 seconds; and time, 21 hours.
- Following electrophoresis stain the gel with 60 µL of ethidium bromide at a stock concentration of (10 mg/ mL) for 20 minutes with 500 mL fresh distilled water.
- Visualize the bands using gel documentation system.

6.2. MLST for *Enterococcus faecali* [88]

Extract genomic DNA using Qiagen bacterial DNA purification kit. Protocol should be followed as per the manufacturer's instructions.

Table 7.23: Sequences of primers for MLST of *Enterococcus faecalis*

S.No	Target Gene	Primer sequence (5' -3')
1	Glucose-6-phosphate dehydrogenase *(gdh)*	F: GGCGCACTAAAAGATATGGT R: CCAAGATTGGGCAACTTCGTCCCA
2	Glyceraldehyde-3-phosphate dehydrogenase (*gyd*)	F: CAAACTGCTTAGCTCCAATGGC R: CATTTCGTTGTCATACCAAGC
3	Phosphate ATP binding cassette transporter (*pstS*)	F: CGGAACAGGACTTTCGC R: ATTTACATCACGTTCTACTTGC
4	Putative glucokinase (*gki)*	F: GATTTTGTGGGAATTGGTATGG R: ACCATTAAAGCAAAATGATCGC
5	Shikimate 5-dehydrogenase *(aroE)*	F: TGGAAAACTTTACGGAGACAGC R: GTCCTGTCCATTGTTCAAAAGC
6	Shikimate 5-dehydrogenase *(Xpt)*	F: AAAATGATGGCCGTGTATTAGG R: AACGTCACCGTTCCTTCACTTA
7	Acetyl-coenzyme A acetyltransferase (*yiqL)*	F: CAGCTTAAGTCAAGTAAGTGCCG R: GAATATCCCTTCTGCTTGTGCT

- Perform PCR with an initial denaturation at 94°C for 5 minutes ; 30 cycles at 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 1 minute; and final extension at 72°C for 7 minutes. After amplification, stain the gel with ETBR for 20 minutes and visualize the bands using gel documentation system.
- Purify the amplified products using GeneJET purification kit (Thermo Scientific, USA) and send the purified amplicons for custom sequencing (Agrigenome Labs Pvt Ltd, Kochi, Kerala). The gene sequences of each housekeeping gene should be trimmed using codon code aligner software. These trimmed sequences should be submitted to *E.faecalis* MLST database http://efaecalis.mlst.net/).
- For each housekeeping gene, different sequences are assigned allelic numbers and each isolate is defined by the allelic profile (a string of 7 allelic numbers). Sequences types (ST) will be assigned based on the allelic profile.
- Clustering of related STs, clonal complexes (CCs) should be determined using eBURST (Based Upon Related Sequence Types) algorithm (http://efaecalis.mlst.net/eburst).

6.3. MLST for *Enterococcus faecium*[89]

Extract genomic DNA using Qiagen bacterial DNA purification kit. Protocol followed as per the manufacturer's instructions.

Table 7.24: Sequences of primers for MLST of *Enterococcus faecium*

S.No	Target Gene	Primer sequence (5' -3')
1	Adenylate kinase (*adk)*	F: TAT GAA CCT CAT TTT AAT GGG R: GTT GAC TGC CAA ACG ATT TT
2	ATP synthase, alpha subunit (*atpA)*	F: CGG TTC ATA CGGAAT GGC ACA R: AAGTTCACGATA AGC CAC GG
3	D-alanine:D-alanine ligase (*ddl)*	F: GAGACATTGAATATGCCTTATG R: AAAAAGAAATCGCACCG
4	Glyceraldehyde-3-phosphate dehydrogenase (*gyd)*	F: CAA ACTGCT TAG CTCCAAGGC R: CAT TTCGTT GTCATACCAAGC
5	Glucose-6-phosphate dehydrogenase (*gdh)*	F: GGCGCACTA AAA GATATG GT R: CCA AGA TTG GGCAACTTC GTCCCA
6	Phosphoribosylaminoimidazol carboxylase ATPase subunit (*purK)*	F: GCA GATTGGCACATT GAAAGT R: TACATA AAT CCCCCT GTTTY
7	Phosphate ATP-binding cassette transporter *(pstS)*	F: TTG AGCCAAGTCGAAGCTGGA R: CGTGATCACGTT CTACTTCC

- Perform PCR with an initial denaturation at 94°C for 3 minutes; ; 35 cycles at 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30seconds; and final extension at 72°C for 5 minutes. After amplification, stain the gel with ETBR for 20 minutes and visualize the bands using gel documentation system.
- Purify the amplified products using GeneJET purification kit (Thermo Scientific, USA) and send the purified amplicons for custom sequencing (Agrigenome Labs Pvt Ltd, Kochi, Kerala). The gene sequences of each housekeeping gene should be trimmed using codon code aligner software. These trimmed sequences should be submitted to *E.faecium* MLST database (http://efaecium.mlst.net/).
- For each housekeeping gene, different sequences are assigned allelic numbers and each isolate is defined by the allelic profile (a string of 7 allelic numbers). Sequences types (ST) will be assigned based on the allelic profile. .
- Clustering of related STs, clonal complexes (CCs) should be determined using eBURST (Based Upon Related Sequence Types) algorithm (http://efaeciums.mlst.net/eburst).

External Quality Assurance Scheme (EQAS)

CHAPTER 8

External Quality Assurance Scheme (EQAS)

1. Background

ICMR is pleased to introduce bacteriology, molecular biology laboratory supports through conducting external quality assessment program and invites your laboratory to participate in the EQA survey. External quality assessment scheme is an important component in any of the health care surveillance schemes to achieve maximum quality and reliable data. When used in conjunction with daily QC, these external programs will assist laboratories in improving analytical quality, inter-laboratory agreement, identify potential equipment or reagent failures, and identify any training deficiencies.

2. EQA survey objectives

- To assess the capacity of bacteriology/molecular biology laboratories supporting surveillance network
- To assess the quality of microbiological laboratory work to ensure the data collected by participating laboratories meets set targets and standards

3. EQA distribution testing guidelines

The distribution includes lyophilized cultures for bacterial identification, antimicrobial susceptibility testing and genotypic characterization.

- The pathogens included in culture testing should have clinical specimens as mentioned in Table 10.1.
- Antimicrobial susceptibility testing (AST) to be performed for each of the pathogen for the given list of antimicrobials listed in the protocol.

Table 8.1: Clinical specimens for pathogens included in culture testing for EQAS

Bloodstream pathogens	Cerebro spinal fluid pathogens	Respiratory pathogens	Faecal pathogens	Urinary pathogens	Skin and Soft tissue pathogens
E. coli	*E. coli*	*Pseudomonas aeruginosa*	*E. coli (EPEC)*	*E. coli*	*E. coli*
Klebsiella spp.	*Klebsiella spp.*	*Klebsiella spp.*	*Shigella spp.*	*Klebsiella spp.*	*Klebsiella spp.*
Salmonella Typhi	*P. aeruginosa*	*Moraxella catarrhalis*	*Salmonella spp.*	*Proteus spp.*	*Pseudomonas spp.*
Pseudomonas aeruginosa		*Acinetobacter baumannii*	*Vibrio cholerae*	*Enterobacter spp.*	
Acinetobacter baumannii			*Aeromonas spp.*		
Salmonella spp.					

4. Sample distribution

- The sample pack will be sent through courier mode, while packing and shipment of EQA materials will be as per international guidelines as prescribed by international air transport association (IATA) guidelines
- An EQAS pack containing challenge strains will be sent to each participating microbiology laboratory from the reference laboratory, every 4 months with three cycles per year.

Survey Number	Date of sample dispatching	Results Dead line	Responses of Evaluation

5. Test Performance (site specimen processing)

5.1. Lyophilized culture identification

Recovery of Lyophilized Cultures

i. Opening of an ampoule

- Make a file mark on the ampoule about the middle of the cotton wool plug and apply a red hot glass rod at that site to crack the glass.
- Allow time for air, filtered by the plug to seep into the ampoules. Otherwise when the pointed end is snapped off, the plug will be drawn in. Hasty opening may release aerosols.
- The plug should be removed with forceps. The discarded plug and the pointed end of the ampoule should be put into a lotion jar.

ii. Re-hydration and recovery

- Flame the open end of the tube and add about 0.3-0.5ml of nutrient broth / trypticase soy broth / chocolate broth using a sterile Pasteur pipette.
- Mix the contents carefully so as to avoid frothing.
- Subculture a loop-full of broth suspension onto appropriate media.
- Transfer the rest to a tube containing 1ml of appropriate broth.
- Incubate both broth and plate cultures in aerobic / CO_2 tin/incubator at 35-37°C.
- Follow up with the growth in agar media as per individual labs routine procedures

iii. Test media

- Blood Agar
- MacConckey Agar
- Chocolate Agar
- Nutrient agar

5.2. Culture identification

- After overnight incubation the media are to be checked for visible growth and individual colony smears made for Gram staining
- Colony and smear morphology to be recorded as observed
- Biochemical tests (Table 10.2) to be set up as per the standard methods

Table 8.2: Set up of biochemical tests for Gram negative and positive organisms

For Gram negative organisms	For Gram positive organisms
• Oxidase • Catalase • Motility • Indole • Citrate • Tripe Sugar Iron test • Carbohydrate utilization (glucose, lactose, sucrose, mannite, xylose, arabinose, dulcitol) • Enzyme production (gelatinase, • urease and phenyl pyruvic acid) • MR-VP • Amino acid utilization (Nitrate, Lysine, Orinithine, Arginine)	• Oxidase • Catalase • Optochin • Bacitracin • Novobiocin • DNAse • Indole

5.3. Antimicrobial Susceptibility testing methods

Disk diffusion methods

The Kirby-Bauer method is to be used for antimicrobial susceptibility testing as recommended by CLSI guidelines.
Note: *Antimicrobial agents to be tested for each of the clinical specimen will be given along with PT panel.*

5.3.1. Procedure for performing the disk diffusion test

Materials required

- Sterile broth medium in 1.5 ml quantities. (Nutrient broth / Mueller Hinton broth)
- MHA for Non-fastidious organisms
- MHBA for *S.pneumoniae* and other *Streptococci*
- HTM for *Haemophilus* spp.
- GC agar with 1% growth supplements for *Neisseria* spp.
- Calibrated loop of 2 mm diameter
- Antibiotic solution
- Sterile filter paper disks / Commercial disks
- Pasteur pipettes sterile
- Cotton swabs sterile
- Normal saline and / Nutrient broth

- Mc Farland BaSO4 turbidity standard 0.5 and 1.0
- Sterile forceps / needle / disk dispenser
- 12 x 100 mm sterile test tubes
- Measuring scales / sliding calipers
- Table lamp
- Zone diameter interpretation charts
- Quality control reference strains
- Discard jar with disinfectant

Inoculum preparation: Growth method

i. With a sterile needle / loop, touch four or five well isolated colonies of the same morphological type.
ii. Inoculate into 1.5 ml of a sterile suitable broth. Incubate at 35 – 37°C for 2 – 6 hours to produce a bacterial suspension of moderate turbidity.
iii. Adjust the density of the suspension to Mc Farland barium sulphate standard, 0.5 (for gram negative organisms and gram positive organisms) with sterile saline / broth. (Adequate light is needed to visually compare the inoculum tube and the 0.5 Mc Farland standard against a card with a white background and contrasting black lines).

Inoculation of test plates

i. Mark the plates into sections according to the number of antibiotics to be used.
ii. Inoculate the plates within 15 minutes of preparation of bacterial suspension .
iii. Dip a sterile cotton swab into the suspension and remove the excess fluid by rotating the swab against the side of the tube above the fluid level.
iv. Inoculate the dried surface of the appropriate agar plate by streaking the swab over the entire sterile agar surface. This procedure is repeated by streaking two more times, rotating the plate approximately 60º each time to ensure an even distribution of inoculum. As a final step, the rim of the agar has to be swabbed.

Note: Extremes in inoculum density must be avoided. Never use undiluted overnight broth cultures or other unstandardized inocula for streaking plates.

Application of disks to inoculated agar plates

i. Dispense antimicrobial disks with appropriate concentrations onto the surface of the inoculated agar plate.
ii. Each disk must be pressed down to ensure complete contact with the agar surface.
iii. Make sure that they are no closer than 24 mm from center to center. Ordinarily, no more than 6 disks should be placed on a 90 mm plate.
iv. As some of the drug diffuses almost instantaneously, a disk should not be relocated once it has come into contact with the agar surface. Instead, place a new disk in another location on the agar.
v. Incubate the plates in an inverted position in an incubator set to 37ºC at appropriate conditions .

5.3.2. Reading plates and interpreting results

- Examine each plate after 16 - 18 hours of incubation.
- If the plate was satisfactorily streaked, and the inoculum was correct, the resulting zones of inhibition will be uniformly circular and there will be a confluent lawn of growth. If individual colonies are apparent, the inoculum was too light and the test must be repeated.
- The point of abrupt diminution of growth, which in most cases corresponds with the point of complete inhibition of growth, is taken as the zone edge.
- Measure the diameters of the zones of complete inhibition (as judged by the unaided eye), including the diameter of the disk.
- Zones are measured to the nearest whole millimeter, using sliding calipers or a ruler, which is held on the back of the inverted petri plate, with reflected light. Zones can be measured easily by holding the petri plate a few inches above a black, nonreflecting background, illuminated with reflected light.
- If blood was added to the agar base, the zones are measured from the upper surface of the agar illuminated with reflected light, with the cover removed.
- Zone margin should be taken as the area showing no obvious, visible growth that can be detected with the unaided eye. Faint growth of tiny colonies, which can be detected only with a magnifying lens at the edge of zone of inhibited growth should be ignored. However, discrete colonies growing within a clear zone of inhibition should be subcultured, re-identified and retested.Strains of *Proteus* spp. may swarm into areas of inhibited growth around certain antimicrobial agents. With *Proteus* spp, thin veil of swarming growth in an otherwise obvious zone of inhibition should be ignored.
- Refer CLSI guidelines for interpreting the susceptibility pattern.

5.4. Minimum Inhibitory Concentration (MIC)

Methods of determining MIC

i. Broth dilution method
ii. E- test

5.4.1. Broth dilution method

Materials

- Sterile graduated pipettes – 10 ml, 5 ml, 2 ml and 1 ml.
- Sterile capped 75 x 12 mm tubes / small screw-capped bottles
- Sterile Pasteur pipettes
- Overnight broth culture of test and control organisms (same as for disk diffusion)
- Required antibiotic in powder form (either from the manufacturer or standard laboratory accompanied by a statement of its activity in mg/unit or per ml. Clinical preparations should not be used for reference technique)
- Required solvent for the antibiotic
- Sterile distilled water - 500 ml
- Suitable nutrient broth medium (Muller Hinton Broth Medium).
- Test tube rack to hold 22 tubes in two rows i.e. 11 tubes in each row.

Method

i. Prepare stock dilutions of the antibiotic of concentrations as required.
ii. Arrange two rows of 11 sterile 75 x12 mm capped tubes in the rack.
iii. Prepare 8 ml of broth containing the concentration of antibiotic required for the first tube in each row from the appropriate stock solution already made, in a sterile 30 ml (universal) screw capped bottle.
iv. Mix the contents of the universal bottle using a pipette and transfer 2 ml to the first tube in each row.
v. Using a fresh pipette, add 4 ml of broth to the remaining 4 ml in the universal bottle mix and transfer 2ml to the second tube in each row.
vi. Continue preparing dilutions in this way.
vii. Where as many as 10 or more are required the series should be started again half the way down.
viii. Place 2 ml of antibiotic free broth to the last tube in each row.
ix. Inoculate one row with one drop of an overnight broth culture of the test organism diluted approximately to 1 in 1000 in a suitable broth and the second row with the control organism of known sensitivity similarly diluted.
x. Incubate tubes for 18 hours at 37°C.
xi. Inoculate a tube containing 2ml broth with the organism and keep at +4ºC in a refrigerator overnight to be used as standard for the determination of complete inhibition.

Reading of result

- MIC is expressed as the lowest dilution, which inhibited growth judged by lack of turbidity in the tube. Because very faint turbidity may be given by the inoculum itself, the inoculated tube kept in the refrigerator overnight may be used as the standard for the determination of complete inhibition. Standard strain of known MIC value run with the test is used as the control to check the reagents and conditions.
- Check the control tube for the presence of growth by turbidity. Only then the test is read.
- First note the concentration of ciprofloxacin in the last tube with no turbidity as the end point.
- If there is no turbidity in 0.25 µg/ml tube and if there is turbidity in the 0.125 µg/ml tube then 0.25 µg/ml is taken as the MIC.
- The quality control strain should show the MIC within the acceptable range.
- Expected value of MIC range for ATCC *P.aeruginosa* (27853) is **0.25 – 1.0 µg/ml**

Tube No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Control**
MH broth	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Ciprofloxacin in serial dilutions	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	
Discard										0.5	
Culture	Add 0.05 ml or 1 drop of approximately 0.05 ml to each tube Mix gently, incubate 16 – 18 hours at 37°C, read										
Final concentration of Ciprofloxacin µg / ml	2.0	1.0	0.5	0.25	0.125	0.062	0.031	0.015	0.0078	0.0039	

5.4.2. E-test

Materials required

- Sterile broth medium in 1.5 ml quantities. (Nutrient broth / Mueller Hinton broth)
- MHA for Non-fastidious organisms.
- E-test strips
- Pasteur pipettes sterile
- Cotton swabs sterile
- Normal saline and / Nutrient broth
- Mc Farland BaSO4 turbidity standard 0.5 and 1.0
- Sterile forceps / needle / disk dispenser
- 12 x 100 mm sterile test tubes
- Measuring scales / sliding calipers
- Table lamp
- Quality control reference strains
- Zone diameter interpretation charts
- Discard jar with disinfectant

Inoculum preparation: Growth method

i. With a sterile needle / loop, touch four or five well isolated colonies of the same morphological type
ii. Inoculate into 1.5 ml of a sterile suitable broth. Incubate at 35 – 37°C for 2 – 6 hours to produce a bacterial suspension of moderate turbidity
iii. Adjust the density of suspension to Mc Farland barium sulphate standard, 0.5 (for gram negative organisms and gram positive organisms) with sterile saline/broth.
iv. (Adequate light is needed to visually compare the inoculum tube and the 0.5 Mc Farland standard against a card with a white background and contrasting black lines).

Inoculation of test plates

i. Mark the plates into sections according to the number of antibiotics to be used
ii. Inoculate the plates within 15 minutes of preparation of bacterial suspension
iii. Dip a sterile cotton swab into the suspension and remove the excess fluid by rotating the swab against the side of the tube above the fluid level
iv. Inoculate the dried surface of the appropriate agar plate by streaking the swab over the entire sterile agar surface. This procedure is repeated by streaking two more times, rotating the plate approximately 60º each time to ensure an even distribution of inoculum. As a final step, the rim of the agar has to be swabbed.

Note: Extremes in inoculum density must be avoided. Never use undiluted overnight broth cultures or other unstandardized inocula for streaking plates.

Application of E-test strip on to inoculated agar plates

i. Place the strip onto the surface of the inoculated agar plate

ii. Strips must be pressed down to ensure complete contact with the agar surface
iii. As some of the drug diffuses almost instantaneously, a strip should not be relocated once it has come into contact with the agar surface
iv. Incubate plates in inverted position in an incubator at 37ºC at appropriate conditions.

Reading plates and interpreting results

i. Examine each plate after 16 – 18 hours of incubation
ii. If the plate was satisfactorily streaked, and the inoculum was correct, the resulting zones of inhibition will be precise. If individual colonies are apparent, the inoculum was too light and the test must be repeated

Results reading

Refer CLSI guidelines for interpreting the susceptibility pattern[44].

5.5. Commercial Antimicrobial Susceptibility (cAST)

5.5.1. VITEK 2

Antimicrobial susceptibility test based on broth micro dilution with specific cards.

Materials Required

i. For QC organisms, use ATCC lyophilized ampoules or other quality source of ready-to-use microorganism (e.g., REMEL disposable Culti-Loops)
ii. Sterile Inoculating Loops
iii. Supplemental Media: nutrient agar (NA), tryptic soy broth (TSB), trypticase soy agar with 5% Sheep Blood (BAP), and trypticase soy agar (TSA).
iv. VITEK 2 Identification cards (for example, GP, ANC, BCL, and GN): store at 2-8°C in unopened original liner.
v. 75 mm × 12 mm clear polystyrene tubes (single use only)
vi. DensiCHEK Plus Meter with McFarland Standards for calibration (0.0, 0.5, 3.0, and 5.0 McF) (see section 15).
vii. Sterile saline solution (aqueous 0.45% to 0.50% NaCl, pH 4.5-7.0)
viii. Bar-coded 10 well cassette card holders
ix. Internal Carousel for card processing

Preparation of inoculum

Select the appropriate card based on the Gram stain reaction and the organism's microscopic appearance.

- First, bring card to room temperature before use
- Aseptically transfer at least 3 mL of sterile saline (0.45 % Nacl) into a clear polystyrene 12×75 mm test tube
- Prepare a homogenous organism suspension by transferring several isolated colonies from the plates to the saline tube

- Adjust the suspension to McFarland standard required by the ID reagent using a calibrated DensiCHEK Plus Meter according to the standard as given below.

Card	McF Range
GN	0.5-0.63
GP	0.5-0.63
ANC	2.7-3.3
BCL	1.8-2.2

Note: The age of the suspension before loading the instrument for AST testing must be less than 30 minutes.

- A test tube containing the microorganism suspension is placed into a special rack (cassette)
- Identification card is placed in the neighboring slot while inserting the transfer tube into the corresponding suspension tube
- The filled cassette is placed automatically into a vacuum chamber station
- After the vacuum is applied and air is re-introduced into the station, the organism suspension is forced through the transfer tube into micro-channels that fill all the test wells
- Inoculated cards are passed by a mechanism, which cuts off the transfer tube and seals the card prior to loading into the carousel incubator
- All card types are incubated on-line at 35.5 $\pm$ 1.0ºC
- Each card is removed from the carousel incubator once every 15 minutes, transported to the optical system for reaction readings, and then returned to the incubator until the next read time
- Data are collected at 15-minute intervals during the entire incubation period
- When the cards are loaded, remove the cassette and dispose of the tubes and straws in a biohazard container
- When the cards are processed and results obtained, cards will be automatically ejected into the waste collection bin

Results

- The VITEK 2 using the AES software will release results of an organism ID and or antibiotic susceptibility to the laboratory information system (LIS) automatically unless review is needed
- For reporting the results, the acceptable probability level is 99%
- Results are concurrently printed and the data sent to the Results View folder on the left side of the screen also called the Navigation Tree where the information is archived
- A red cassette in the Navigation Tree is indicative of an error. If an error occurs during processing, refer to the Software User Manual
- Review results printout and file appropriately
- The different levels of identification and its associated information are shown in the table below

Limitations

i. Very mucoid organism my not provide acceptable result. Alternative methods should be used for these organisms.
ii. Colonies grown on EMB plates cannot be used due to carry over of the dye present in the medium.

5.5.2. BD Phoenix system

i. Inoculum Preparation

- The isolates to be tested were plated at least once on the sheep blood Columbia agar and incubated at 35-37°C.
- The bacterial suspension for the Phoenix AST test was prepared by adding one drop of AST indicator to 8 ml of Phoenix AST broth.
- The prepared AST suspension was poured into the AST sector of the Phoenix panel.
- Excess suspension was collected by the absorbent pad at the bottom of the panel.
- After sealing with a plastic cover and scanning the panel barcode, the panel was loaded manually into the Phoenix system.

ii. Reading and interpreting results

- Continuous measurements of changes to the indicator as well as bacterial turbidity are used in the bacterial growth determination.
- The instrument takes readings every 20 minutes.
- Organisms growing in the presence of a given antimicrobial agent reduce the indicator, changing it to a pink colour.
- This signals organism growth and resistance to that antimicrobial agent.
- Organisms killed or inhibited by the antimicrobial agent do not cause reduction of the indicator and therefore do not produce a colour change.
- Phoenix instrument reads and records the results of the antimicrobial tests contained in the panel and interpret the reactions to give a minimal inhibitory concentration (MIC) value and category interpretations (susceptible, intermediate, resistant).
- AST results are available within 4 to 16 hours.
- This is an autoread result; no manual readings are possible with this system.

5.5.3. MicroScan WalkAway system

i. Inoculum preparation

- The MicroScan® MIC/Panel contains micro-dilutions of each antimicrobial agent in various concentrations with Mueller Hinton Broth and various nutrients which are dehydrated and dried in panels.
- Each panel contains two control wells: a no-growth control well (contains water only/no nutrients or broth), and a growth control well (contains test medium without antibiotic).
- The panel is rehydrated and inoculated at the same time with 0.1 ml of suspension prepared by the turbidity method (inoculum prepared in water, then 0.1 ml transferred to 25 ml of inoculum water containing pluronic-D/F-a wetting solution).
- The panels are incubated at 35ºC in a non-CO2 for 16- 20 hours.

ii. Reading and interpreting results

- The panel is read by visual observation for growth.
- Panels may also be read automatically with the WalkAway® and autoSCAN®-4 Systems, which uses an optics system with growth algorithms to directly measure organism growth.
- The results are reported as minimum inhibitory concentration (MIC) and as categorical interpretation (susceptible, intermediate and resistant).
- MICs for the test organism are read by determining the lowest antimicrobial concentration showing inhibition of growth.

5.6. Reporting cAST

Below mentioned criteria should not be reported with cAST results due to 1) Difference in the card and the performance, 2) Major error is common among antimicrobials with narrow breakpoints either in mm or µg/ml.

- 3rd gen cephalosporin (SPICE organisms)
- Cefepime – GNB
- Pip/tazo – GNB
- Colistin – GNB
- SXT – *S. maltophilia, S. aureus*
- Clindamycin susceptibility for *S. aureus* should not be reported (Erythromycin resistant and clindamycin susceptible isolates may exhibit inducible clindamycin resistance)
- Meropenem *(A. baumannii* or other than *E. coli, K.pneumoniae, N. meningitidis, P. aeruginosa, and P. mirabilis*

Caution: cAST approved by FDA before 2007, thereafter AMR has increased but revisions of break points were not done to re-evaluate the performance.

Quality control

i. The instrument should be serviced annually under a preventative maintenance agreement
ii. Ensure proper functioning of optical, movements and temperature control with monthly maintenance
iii. Quality controls run should be done once in a month with the new batch of reagents and with known susceptible and resistant organisms.
iv. Test minimum of 30 each of susceptible and resistance phenotype to validate the antibiotics used routinely

Calibration

- Verify the DensiCHEK Plus using the calibration standards on a monthly basis when in use. DensiCHEK Plus instrument verification results should be within the established range of standards used for the verification.
- To use the DensiCHEK Plus meter with the calibration standards:

i. Ensure the instrument is ON and set to the GLASS tube setting (the default setting is plastic).
Note: To change the tube type, press the Menu key. SEL and a flashing triangle will display under the current tube type setting. Press the Read/Enter key to move the triangle. When the triangle is pointing to the correct setting, press the Menu key to exit configuration.
ii. Clean the outside of the 0.0 McF standard (blank) with lens tissue and gently invert (do not shake) the blank 5-6 times.
iii. Insert the blank and press the "0" key.
iv. Slowly rotate the blank one full rotation. The instrument will display a series of dashes followed by a numerical value (0.00 will be displayed for the blank).
v. Repeat steps b-d for the remaining standards (0.5 McF, 2.0 McF, and 3.0 McF).
vi. Check that the displayed value is within the acceptable range and record on the DensiCHEK Plus Calibration Log.
vii. If the reading is outside the acceptable range, repeat steps a-e. If still out of range, contact Technical Support.

5.7. Colistin Susceptibility Testing

Broth-micro dilution (BMD) is currently the only method recommended by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for polymyxin antimicrobial susceptibility testing. According to CLSI recommendations, BMD is performed with cation-adjusted Mueller-Hinton broth (CA-MHB), a range of 2-fold dilutions of polymyxins (ranging from 0.12 to 512 g/ml), and a final bacterial inoculum of $5X\ 10^5$ CFU/ml in each well. BMD is considered to be the optimal method and is currently recommended for susceptibility testing in the recent document proposed by the joint CLSI-EUCAST Polymyxin Breakpoints Working Group (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/Recommendations_for_MIC_determination_of_colistin_March_2016.pdf).

The joint CLSI-EUCAST Polymyxin Breakpoints Working Group doesn't recommend disc diffusion, agar dilution and E-test for colistin/polymyxin B susceptibility testing.

Antibiotic preparation in distilled water

$$\text{Volume in ml} = \frac{\text{Potency X weight of antibiotic}}{\text{Needed concentration (Higher concentration)}}$$

From the prepared stock antibiotic solution (needed concentration of antibiotic is prepared in distilled water), antibiotic is serially diluted in 1 ml distilled water from higher concentration to the lower concentration.

Example

Antibiotic pure substance
Colistin sulfate (Sigma Aldrich) – Potency 15,000 IU/mg

Stock solution

1 µg = 15 IU (X 1024 µg)
1024 µg = 15,360 IU

Preparation of 1024 µg/ml stock solution

$$\frac{15000 \times 5 \text{ mg of colistin}}{15{,}360 \text{ IU}} = 4.88 \text{ ml sterile distilled water (diluent)}$$

Weigh 5 mg of colistin (15,000 IU) and dissolve in 4.88 ml sterile distilled water to make 1024 µg/ml.

Quality control (QC) strains

- *E. coli* ATCC 25922 (QC range, 0.25 - 2 µg/ml)
- *P. aeruginosa* ATCC 27853 QC range, 0.5 - 4 µg/ml)
- MCR-1 positive *E. coli* (MIC, 4-8 µg/ml)

Inoculum preparation

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

Procedure

- Label the tubes from higher to lower concentration, add 1 ml of cation adjusted Mueller-Hinton broth (CAMHB) to each of these labeled tubes
- Add 1ml of stock solution containing 1024 µg of colistin to the tube labeled as 512 µg/ml and perform serial dilution from higher to lower concentration (labeled as, 512 µg/ml to 0.12 µg/ml)
- Add 100 µl of CAMHB containing different concentration of colistin from the tubes to respective wells of the labelled 96-wells microtiter plate
- Add 10 µl of final inoculum to the wells of microtiter plate (as mentioned previously)
- Add 100 µl of CAMHB into Sterility control well
- Add 100 µl of CAMHB (without antibiotic) and 10 µl of final inoculum into growth control well
- Take 10 µl of final inoculum and plate on to nutrient agar (tertiary streaking for purity check)
- Transfer 10 µl of inoculum from GC well and add to 10 ml of saline for count verification
- From the count verification tube, plate 100 µl onto nutrient agar and perform criss cross streaking (30 – 50 CFU/ml – satisfactory, less/more CFU/ml than mentioned indicates insufficient or more inoculum)
- Incubate the microtiter plates and nutrient agar plates at 37°C for 16-18 hours

Reading and Interpretation

- Check purity of inoculum
- Check sterility control well. It should be clear/non-turbid
- Check growth control for adequate growth of at least 2mm button formation
- The MIC is the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in the wells as detected by the unaided eye
- When single skipped well is seen, read the highest MIC
- Do not report results if more than one skipped well is present
- Take readings for the quality control organisms tested
  - ♦ *E. coli* ATCC 25922 (QC range, 0.25 - 2 µg/ml)
  - ♦ *P. aeruginosa* ATCC 27853 (QC range, 0.5 - 4 µg/ml)
  - ♦ MCR-1 positive *E. coli* (MIC, 4-8 µg/ml)

Note: *Consider taking reading for the test isolates only if the quality control is satisfactory and found within the ranges mentioned*

6. Returned Data (Result Submission)

Culture Characteristics Culture ID/Specimen ID:	
Testing not performed on this specimen/isolate ϒ Comments / Reasons:	
Media	**Morphology**
Blood Agar	
MacConkey Agar	
Chocolate Agar	
Nutrient Agar	
Others (if any)	

Identification: Gram Negative organisms Culture ID/Specimen ID: _____ (use separate sheet for each of the specimen type)		
Testing not performed on this specimen/isolate ϒ		
Basic Biochemical Identification		
	Positive	Negative
Motility	ϒ	ϒ
Oxidase	ϒ	ϒ
Catalase	ϒ	ϒ
Indole	ϒ	ϒ
Citrate	ϒ	ϒ

Hemolysis		α ϒ	β ϒ	None ϒ
TSI		Slope:	Butt:	Gas:	H_2S:
CHO Reaction	Glucose	ϒ	ϒ
	Lactose	ϒ	ϒ
	Sucrose	ϒ	ϒ
	Mannite	ϒ	ϒ
	Xylose	ϒ	ϒ
	Arabinose	ϒ	ϒ
	Dulcitol	ϒ	ϒ
Protein Reactions	Gelatinase	ϒ	ϒ
	Urease	ϒ	ϒ
	PPA	ϒ	ϒ
OF Glucose	Oxidative	ϒ	ϒ
	Fermentative	ϒ	ϒ
Methyl Red		ϒ	ϒ
Voges Proskaeur		ϒ	ϒ
Nitrate		ϒ	ϒ
Lysine		ϒ	ϒ
Ornithine		ϒ	ϒ
Arginine		ϒ	ϒ
LIA		ϒ	ϒ

Species Specific Identification Culture ID/Specimen ID: _____

	1st Identification	2nd Identification	3rd Identification
Conventional Biochemical Methods	ϒ	ϒ	ϒ
API	ϒ	ϒ	ϒ
MICROSCAN	ϒ	ϒ	ϒ
VITEK 2	ϒ	ϒ	ϒ
Other Commercial Kit	ϒ	ϒ	ϒ
PCR	ϒ	ϒ	ϒ
MALDI TOF	ϒ	ϒ	ϒ
16S rRNA	ϒ	ϒ	ϒ

Antimicrobial Susceptibility Testing (Disc Diffusion) Culture ID/Specimen ID: _____							
S. No	**Antibiotic Code (DISC)**	**Disc Content (µg)**	**Disc zone diameter (mm)**	**S**	**I**	**R**	**Comments**
1				ϒ	ϒ	ϒ	
2				ϒ	ϒ	ϒ	
3				ϒ	ϒ	ϒ	
4				ϒ	ϒ	ϒ	
5				ϒ	ϒ	ϒ	
6				ϒ	ϒ	ϒ	
7				ϒ	ϒ	ϒ	
8				ϒ	ϒ	ϒ	
9				ϒ	ϒ	ϒ	
10				ϒ	ϒ	ϒ	

Antimicrobial Susceptibility Testing (MIC) Culture ID/Specimen ID: _____							
S. No	**Antibiotic Code (MIC)**	**Tested range (µg/ml)**	**MIC value**	**S**	**I**	**R**	**Comments**
1				ϒ	ϒ	ϒ	
2				ϒ	ϒ	ϒ	
3				ϒ	ϒ	ϒ	
4				ϒ	ϒ	ϒ	
5				ϒ	ϒ	ϒ	
6				ϒ	ϒ	ϒ	
7				ϒ	ϒ	ϒ	
8				ϒ	ϒ	ϒ	
9				ϒ	ϒ	ϒ	
10				ϒ	ϒ	ϒ	

7. Assessment of data (Scoring system)

7.1. Scoring

Gram's stain reaction	Points awarded
Correct result	1
Incorrect result	0

Identification results (viable and non viable specimens)	
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

7.2. Rationale

Participants were expected to identify these important pathogens to species level. Discrimination of the isolates to genus level only, or reporting an incorrect species within the species is unhelpful in the clinical management of the patients and was therefore awarded only one point. Reporting an incorrect genus could lead to inappropriate clinical management and therefore carries a higher penalty score than reporting an incorrect species, hence a score of minus one.

7.3. Antimicrobial susceptibility testing (AST) results

Although an identical panel of antibiotic agents was listed for all the EQA specimens, participants were expected to select appropriate agents for antimicrobial susceptibility testing depending on the organism identified. Some participants tested all the agents in the panel regardless of the type of organism, whilst others noted where an agent was not tested because it was inappropriate for the organism identified. In the list of AST results on pages 1, 2 and 3 only agents that were appropriate for the organism are listed. All the agents are listed in the tables on pages 21 to 23 with associated results. Results for appropriate agents are scored unless the participant consensus fell below 80%, in which case the agent is excluded from scoring. Lack of concordance was more commonly associated with agents where only a small number of results were returned and therefore further analysis was considered inappropriate.
Note: For beta lactamase reporting, a negative result is denoted by sensitive and positive result is denoted by resistant.

7.4. Summary of scoring criteria for the antimicrobial susceptibilities

Assigned scores			
Reference results	**Participant's result**		
	S	**I**	**R**
S	2	1	0
I	1	2	1
R	-1	1	2

8. Complete report of participants (Result announcement and feedback)

From the closure of results deadline an intented report will be sent to the participating centers in two weeks time. Final report will be sent to the participating centers soon after the data compilation and analysis when available

Surveillance of Carriage of MDR Enterobacteriaceae in General Population

Subjects and sites of collection of samples

Three groups of subjects will be included for this part of the study:

i. One hundred patients being admitted to Nehru Hospital, PGIMER, Chandigarh on admission (day zero of hospital stay)
ii. One hundred patients attending the outpatient department of PGIMER for unrelated complaints (hypertension, gynaecology and obstetrical conditions and dermatology, etc)
iii. Three hundred volunteers from the general population in the community. The survey is intended to be stratified, multistage, cross-sectional, sample survey.

The following sites will be chosen:

- Primary health care settings include private practioners based in Panchkula
- Secondary care setting includes patients presenting at ESI hospital, Ram Darbar, Chandigarh
- Field setting (Aanganwadi and school children). There are total 420 Anganwadis in Chandigarh under three ICDS Projects. Each ICDS project area, will contribute to 10 Anganwadis with a probability of being selected proportional to the number of centers in three area types– urban, rural and urban slum. Thus, a total of 30 *anganwadi* centers will be selected. 10 children will be selected from each Anganwadi from the list available from each Anganwadi worker for the collection of stool samples (total of 300 healthy school children included from Aanganwadis).

Exclusion criteria

All subjects with any condition with the potential to affect the endogenous flora such as the following will be excluded from the study

i. Diabetes mellitus
ii. Pregnancy
iii. Any immunosuppressive disorder or medication history
iv. History of recent (within 3 months) consumption of antibiotics
v. History of hospitalization in the past one year

Details of patients and consent form

A small proforma will be required to be filled up. Proper written consent will be taken. The details will include relevant demographic details of the subjects.

Processing of stool samples

Media used for isolation of enterobacteriaceae will be McConkey agar containing break point concentrations of cefotaxime (1 µg/ml), ceftazidime (4 µg/ml), cefepime (8 µg/ml), amikacin (16 µg/ml), gentamicin (4 µg/ml), imipenem (1 µg/ml), meropenem (1 µg/ml), ciprofloxacin (1 µg/ml) and piperacillin-tazobactam (16 µg/ml). All samples will be processed in the laboratory within 6 hours of collection. *E. coli* and *Klebsiella pneumoniae* colonies growing on this media will be selected for further processing by Gram staining and standard biochemical tests[90].

Surveillance of Antimicrobial Resistance in Environmental Isolates

Surveillance of antimicrobial resistance in environmental isolates will be optional for Regional Centers.

Sites from which environmental samples to be collected

Chandigarh is divided into 62 sectors (numbered 1-63 excluding 13). Sewage samples will be collected from major drainage points of sector 12 (drains PGIMER, Chandigarh), sector 4, sector 17, sector 22 (drains a major bus terminal of Chandigarh), sector 32 (drains Government Medical College and Hospital, Chandigarh), sector 43 (drains another major bus terminal) and sector 61 (adjoins Punjab). Help will be taken from staff of Municipal Corporation of Chandigarh in determining which manholes to be targeted (Map of Chandigarh with proposed sites of sampling attached as annexure).

Collection of samples

Two methods will be used to collect sewage/waste water samples from each of these sites. In the first method, sewage (50 ml) will be directly collected in autoclaved sampling bottles. In the second method, the method described by Moore will be used. Briefly, a piece of gauze 1200 mm×150 mm will be folded into eight thicknesses and attached to a length of wire or string. The swab will be suspended in flowing waste water or sewage for 48 hours, transferred into a sterile wide-mouthed, screw-capped jar. The sources and time of collection of all samples will be recorded on maps and photographs of all sites will be taken. Sampling from each site will be repeated every six months in order to assess trends in seasonal prevalence[46]. Samples will be collected in duplicate from a given site and immediately transported and then stored at 4°C till processing.

Moore Swab

Moore swabs will be made by cutting pieces of cotton gauze 2 to 4 feet long by 6 inches wide (60 to 120 cm x 15 cm), folding the gauze lengthwise several times to form a tight cylindrical roll, and tying the center with a strong wire. The swabs will be wrapped in heavypaper and sterilized by autoclaving. The ends of the wires holding the swabs will be tied to nylon fish line, and the swabs will be suspended by the lines in the water or sewage to be tested and left inplace for 24 to 48 (usually 24) hours. The swabs will then be removed, the wires holding the swabs cut aseptically, and the swabs will be submerged in buffered glycerol saline media in a conical flask. The flasks will be transported to the laboratory in an ice chest. At the laboratory the flask contents will be subcultured to appropriate media as detailed below.

Culture, isolation and identification of Enterobacteriaceae

Media used for isolation of enterobacteriaceae will be Mueller Hinton agar and McConkey agar containing break point concentrations of cefotaxime (1 µg/ml), ceftazidime (4 µg/ml), cefepime (8 µg/ml), amikacin (16 µg/ml), gentamicin (4 µg/ml), imipenem (1 µg/ml), meropenem (1 µg/ml), ciprofloxacin (1 µg/ml) and piperacillin-tazobactam (16/1.6 µg/ml).

All samples will be processed in the laboratory within 6 hours of collection. Serial 10-fold dilutions of the sewage samples will be prepared in 0.85% NaCl. Each dilution will be plated onto the prepared agar plates. Plates will be incubated at 37°C for 16-18 hours. All *E. coli* and *Klebsiella pneumoniae* colonies growing on this media will be selected for further processing by Gram staining and standard biochemical tests[90].

For the Moore swab, resuscitation of the trapped bacteria will be done in non-selective buffered peptone water for 24 hours at 37°C. Then subculture will be done on non-selective agar and representative colonies of each species will be identified and processed further.

Storage of isolates

Isolates will be stored in nutrient agar deeps at room temperature for short term storage and -80º C in trypticase soya broth with glycerol for long term storage.

Annexures

ANNEXURE I

Map of Chandigarh with proposed sites for surveillance

ANNEXURE II

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*	*Shigella spp*
	Edwardsiella	*Citrobacter spp*
	Salmonella	
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 lactamica*
Neisseria meningitidis	

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.

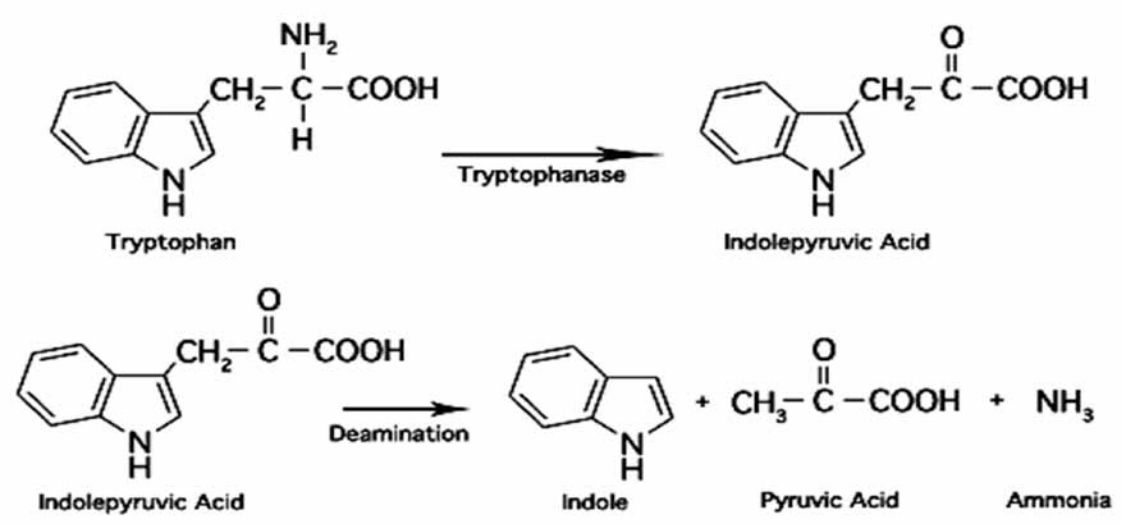
Ingredients and preparation: The medium is peptone water.

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


+ve -ve

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 35°c 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 *K.pneumoniae* (weakly)	*E. coli*

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 III

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 ± 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 121°C 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 118^{o}C 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 45–50°C 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°C for 15 minutes and cool to 50°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°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*	Look for growth and smear morphology
	C. perfringens	
BPMM	*E. coli*	Non-mucoid lactose fermenting colonies
	S. Typhi	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)

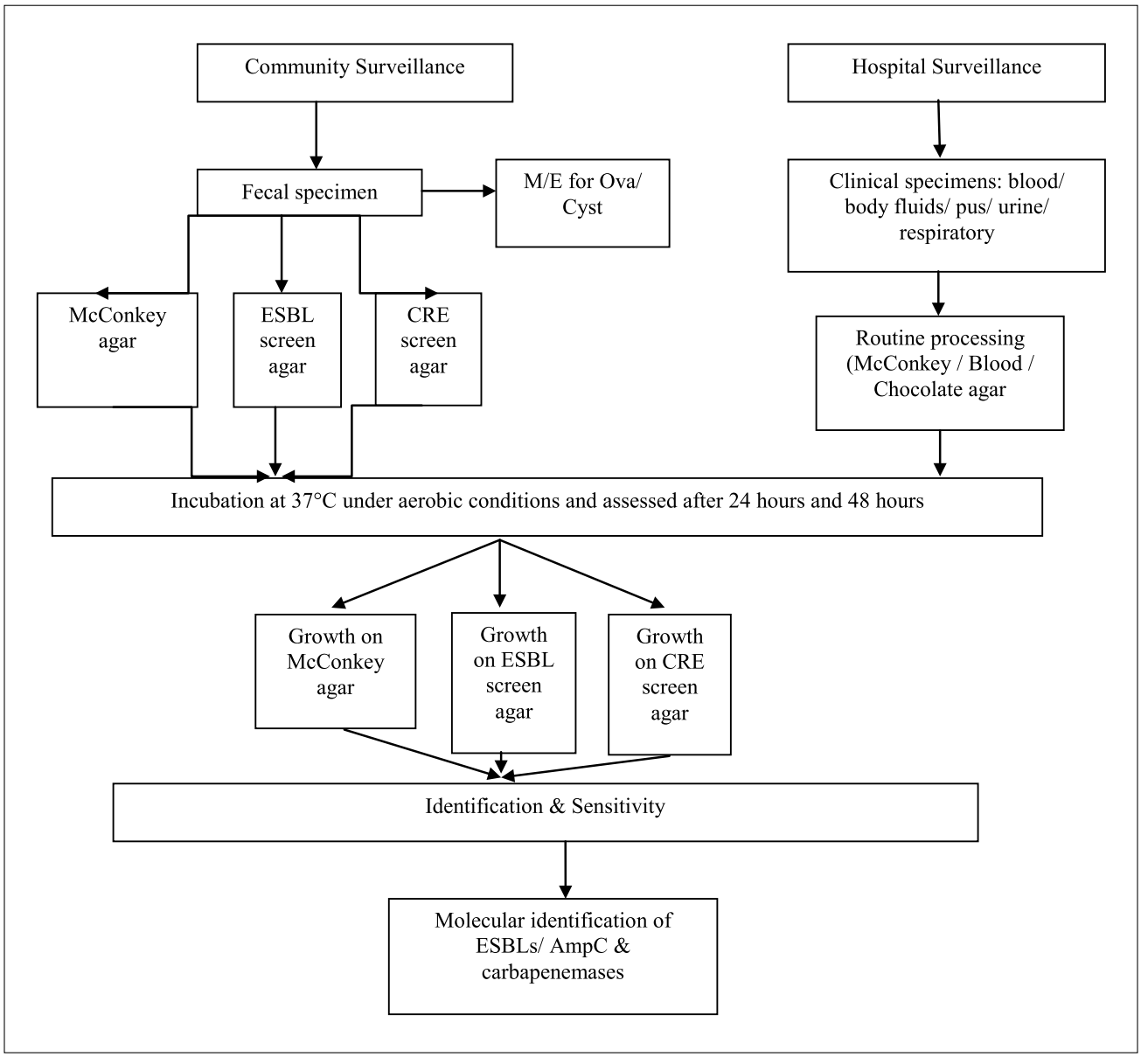
Stock cultures should be kept at -70ºC in brucella broth with 10% glycerol for up to 3 years. Before use as a QC strain, the strain should be subcultured at least twice and retested for characteristic features. Working cultures are maintained on TSA slants at 2-8°C for up to 2 weeks.

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.

ANNEXURE IV

Flowchart of Workflow for Enterobacteriaceae



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Standard Operating Procedures Bacteriology

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