

Standard Operating Procedures

Bacteriology

Antimicrobial Resistance
Surveillance and Research Network



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Foreword

It is my privilege to write this foreword for the manual on Standard Operating Procedure for ICMR Antimicrobial Resistance Surveillance and Research Network. During the last one decade there is a strong global movement building up to tackle the problem of anti-microbial resistance. All will agree that any results of any research can be extrapolated only when the clinical, epidemiological and laboratory methods are standardized and common. Development of this academic/ research network by ICMR on anti-microbial drug resistance is an important landmark. I am happy to have played catalytic role in this movement in India. However, credit goes to ICMR team, chairperson and members of expert committee and finally the participants. Synthesizing this capability (human resource as well as infrastructure) into a coherent targeted action will be remembered as significant contribution of ICMR. Currently more than 400 medical colleges and several specialized state of the art laboratories/ centres are generating data on drug resistance which is of very limited use because of problems of development/ adaptation and use of standardized methods and quality assurance. This manual describes well accepted methods to carry out drug susceptibility testing on important gram positive and gram negative clinically relevant bacteria. Methods of specimen collection, transport, culture, anti-microbial drug susceptibility testing (common, special phenotypic and molecular techniques) as well as quality control and quality assurance have been described in a concise manner. Reference to any commercial method or equipment does not mean endorsement of ICMR, this is only for the purpose of this research study.

I am optimistic that over the years this manual will become a base document – it will evolve not only for the use of ICMR Research network but others will use it for clinical as well as research purpose and will modify according to their needs. I compliment the contributors and ICMR team for this effort. I am hopeful that users/ readers will also have same opinion. I convey my best wishes to all.

Dr V.M. Katoch

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Abbreviations

AST	Antimicrobial susceptibility testing
ATCC	American Type Culture Collection
BAL	Bronchoalveolar lavage
BCC	<i>Burkholderia cepacia</i> 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
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	<i>Haemophilus</i> test medium
IAMM	Indian Association of Medical Microbiologists
MAC	McConkey agar
MBC	Minimum bactericidal concentration
MBL	Metallo beta-lactamases
MHA	Müller Hinton agar
MHB	Mueller Hinton broth
MH-SB	Mueller Hinton sheep blood agar
MHT	Modified Hodge test
MIC	Minimum inhibitory concentration
MRSA	Methicillin resistant <i>Staphylococcus aureus</i>
NA	Nutrient agar
NB	Nutrient broth
NS	Non-susceptible
OF	Oxidation-fermentation test
OLB	Open lung biopsy
ONPG	Ortho-nitrophenyl- β -D galactopyranoside

ORSA	Oxacillin resistant <i>Staphylococcus aureus</i>
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

Distribution List (Controlled Copies)

S. No.	Institutes
1.	ICMR, New Delhi
2.	PGIMER, Chandigarh
3.	CMC, Vellore
4.	AIIMS, New Delhi
5.	JIPMER, Puducherry

Amendment Sheet

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

General guidelines

General Guidelines

A. 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 collection, specimen processing, supplies, 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.

B. 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.

C. 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 sharps must be disposed off in appropriately labeled hardened plastic containers.

Personal protective equipment (PPE) must be worn at all times, and particular care must be taken when performing procedures that have the potential to create aerosols.

1. Protective clothing and equipment

a. Laboratory coats

Protective coats, gowns, smocks, 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.

b. 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.

c. 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.

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.

a. 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 and on all equipment used for laboratory work (e.g., incubators, hoods, microwaves, ice machines, refrigerators, and freezers). 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.

b. 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., biological indicators). A logbook should be maintained for each autoclave to record the date, times, and indicator of sterilization of each autoclave run.

c. Disinfection

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. Always read disinfectant labels for manufacturers' recommendations for dilution and for exposure times for efficacy. An effective general disinfectant is a 1:100 (1%) 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 1:10 (10%) dilution of bleach 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.

d. 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.

e. Decontaminating bench tops and other surfaces

Bench tops and other potentially contaminated surfaces should be wiped with a disinfectant (10% bleach) routinely after working with infectious agents or clinical

specimens or after spills, splashes, or contamination by infectious materials. Following disinfection with 10% bleach, 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.

f. 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.

g. 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.*, a phenolic formulation or household bleach) 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.

h. 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.

i. Mouth pipetting

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

j. 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).

k. 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.

l. 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.

m. 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 as a safety bench can or dispenser can. 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.

n. 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. Special Practices

a. 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.

b. 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.

c. 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. 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 the working area with a disinfectant at the beginning and end of the 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

- a. Spillage or minor spills: Put on gloves, cover the spill with a cloth or tissue soaked in disinfectant, leave for 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.
- b. 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 the skin is soiled with infective material, rinse with 70% alcohol or dilute hypochlorite solution, and then with soap and water.

Specimen Collection, Transport & Processing

Specimen Collection, Transport and Processing

A. Blood

1. Collection and transport

Purpose: To reduce blood culture contamination rate, collection may be improved by taking the following precautions¹⁻⁴:

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

a. 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.

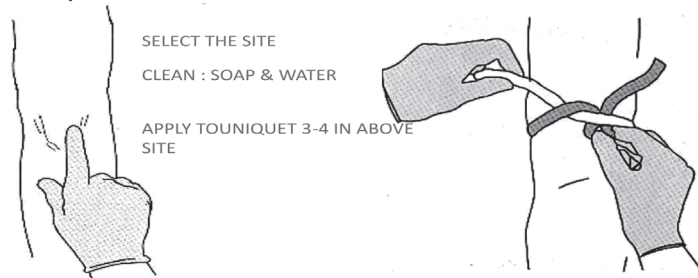
Put on examination gloves.

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.

Investigations: Blood Culture

Aseptic method of Blood collection.: Bed side



Swab or wipe concentric circles of povidone/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.

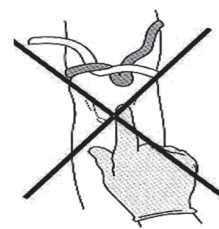
Allow the povidone iodine to dry (2 minutes).

Do NOT touch the site after cleaning.

Instruct patient to clench and unclench the fist.



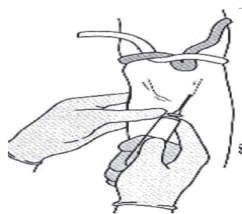
STERILE GLOVES
 COTTON SOAKED SPIRIT/ 70% ISOPROPYL ALCOHOL
 OUTWARD CIRCULAR MOTION
 REPEAT 2 -3 TIMES
 POVIDONE IODINE/ TINCTURE IODINE/ 1 -2%CHLORHEXIDINE
CLEAN & WAIT FOR 3 FULL MIN
 DO NOT PALPATE AREA WITH SAME GLOVE.



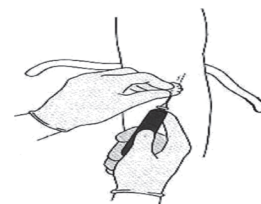
Perform phlebotomy using the needle and syringe.

Release the tourniquet and withdraw the needle.

Apply pressure to the site of venipuncture and place a bandage over the puncture site.



INSTRUCT TO CLENCH FIST
 RELEASE TOURNIQUET
 WITHDRAW NEEDLE
 FLIP OFF BLOOD CULTURE BOTTLE LID
 GENTLY INOCULATE 8 ML IN AN ADULT BOTTLE
 4 ML IN A PEDIATRIC BOTTLE



	Povidone I ₂	Chlorhexidine gluconate (2%)/ tincture I ₂ (10%)
Drying period	2 minutes	~ 30 seconds

Skin preparation with either alcohol, alcoholic chlorhexidine (2%), or tincture of iodine (10%) leads to lower blood culture contamination rates than does the use of povidone-iodine^{1,2}.

For pediatric patients

< 2months

Omit the iodine step

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 older⁴

b. 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 ⁴.

Vigorously wipe septa with 70% alcohol and allow drying completely, for 30 to 60 seconds.

Pediatric bottles are not to be used for adult patients except for those elderly patients in whom it's difficult to obtain larger amounts of blood ⁵.

c. Recommended total volume and numbers of blood cultures

Age & body weight	Amount (divided between 2 blood cultures)	Remarks
Neonates to 1 year (<4kg)	0.5 to 1.5 ml	At least 1 ml Two separate venipunctures are generally not possible
Children ⁶ (< 40 kg)	10 to 20 ml	Blood culture volumes should be limited to <1% of total blood volume (usually about 70 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 and 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**.

Pediatric patient: 6-10 ml, divided between two blood cultures.

*Usually, a properly collected paired sample of blood culture **need not be repeated up to 5 days**.*

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 ⁴.

d. 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 to be carried out in two bottles containing 50 ml each of tryptone soya broth and bile broth (Hi-Media Labs, India). 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.

e. Transport of blood culture bottles

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

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 1. Visible signs of growth caused by organisms commonly encountered in blood cultures

Microscopic observation	Associated microorganisms
Hemolysis	Streptococci, staphylococci, <i>Listeria</i> spp., <i>Clostridium</i> spp., <i>Bacillus</i> spp.
Turbidity	Aerobic gram-negative bacilli, staphylococci, <i>Bacteroides</i> spp.
Gas formation	Aerobic gram-negative bacilli, anaerobes
Pellicle formation	<i>Pseudomonas</i> spp., <i>Bacillus</i> spp., yeast cells
Clotting	<i>Staphylococcus aureus</i>
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 121°C 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.

3. Further processing of positive blood cultures

- a. Gram-stain a thin smear from the broth or agar immediately when suggestive of growth, for optimal patient care.
- b. Subculture to agar media and put up biochemical tests based on the Gram stain results.
- c. Follow-up workup of positive blood culture isolates.

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).

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.
- 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) is 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.⁷

6. Limitations

- a. Low levels of organisms may not be detected in the incubation interval of the culture.
- b. The media used may not support the growth of all organisms. Use of multiple formulations increases the yield.
- c. Sodium polyanethol sulphonate (SPS) may inhibit the growth and viability of some organisms.

- d. Other diseases can present similarly to bacteremia, since there are many causes of fever of unknown origin.
- e. Bacterial metabolism may not produce sufficient CO₂ for detection in automated systems.
- f. There are a number of fastidious microorganisms that infect the blood but cannot be grown in routine blood culture.

B. CSF³

1. Collection and transport

Purpose: To identify the organisms causing pyogenic meningitis.

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

a. Specimen collection

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.

Collect the CSF into five calibrated sterile labeled tubes.

Physicians should be instructed to sequentially collect 2.0 ml of CSF into three sterile calibrated tubes if only routine chemistry (total protein and glucose), bacteriology (culture & susceptibility), and hematology (cell count) are required.

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. Subsequent CSF samples are then collected every 2 to 3 days once antimicrobial therapy is started to monitor for resolution of the infection.

b. 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.

c. 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. CSF Processing

Performance specifications:

The procedure described below is suited to identify common aerobic bacteria/fungi listed below:

Neonates <28 days	<i>E.coli</i> , Group B streptococci
<2 months	Group B streptococci and <i>Haemophilus influenzae</i>
<10 years	<i>H. influenzae</i> , <i>Streptococcus pneumoniae</i>
Adult	<i>S. pneumoniae</i> , <i>Neisseria meningitidis</i>
Immunocompromised	<i>Cryptococcus neoformans</i>
CNS shunt infection	Coagulase negative staphylococci (CoNS)

Day 1

a. CSF gross appearance

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

b. 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)

c. Wet mount

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

d. India ink preparation

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

e. 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

Bacteria - describe the morphology

f. Culture

Chocolate agar (CHOC), blood agar, McConkey agar and RCM broth.

Incubate at 37°C 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

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.

C. Body fluids from sterile sites³

1. Collection and transport

Specimen collection

- Body fluids from sterile sites are 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.
 - Anaerobic transport vial.
 - Specimens received by the laboratory in a syringe with the needle still attached should be rejected because of the risk of a needlesharp 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.

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.

Rejection criteria

- If specimens inoculated into blood culture bottles are received, Gram stain cannot be performed.
- Collect 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.
- NOTE: 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.

2. 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

Bacteria- describe the morphology

c. Culture

Inoculate blood agar, MAC and RCM.

Incubate at 37°C 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.

NOTE: Empiric antimicrobial therapies are selected for the treatment of gastrointestinal tract microbiota, including anaerobes, enteric gram negative bacilli, and enterococci. To attempt to isolate and report each of these agents is labor-intensive and does not add to the requirement to treat the patient with agents that are effective against all the usual microbiota.

Hold positive culture plates or tubes for at least 7 days or, preferably, freeze isolates for long-term retrieval.

D. Ocular Specimens³

1. Specimen collection and transport

NOTE: Most eye specimens are collected by an ophthalmologist. These specimens are 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.

Specimen collection

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.

For viral cultures, use Dacron or cotton swabs with non-wood shafts.

Collection by anatomic site

Conjunctiva (bacterial conjunctivitis) and lid margin (blepharoconjunctivitis suspected)

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 100% methanol for 5 minutes.

Repeat steps for the left conjunctiva.

Cornea (bacterial keratitis)

Instill 1 or 2 drops of proparacaine hydrochloride.

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.

Bacterial endophthalmitis

Collect an aspirate of the vitreous fluid or perform a paracentesis of the anterior chamber using a needle aspiration technique to collect intraocular fluid.

Collect specimens for conjunctival cultures along with the fluid to determine the significance of indigenous microbiota.

If a small volume of fluid is collected, inoculate cultures at the bedside by inoculating 1 or 2 drops of fluid onto culture media.

2. Specimen processing

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 100% 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.

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 35°C in 5-7% CO₂ for 24 hours.

Examine daily for the presence of microorganisms.

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:

1: Less than half of the C streaks are positive per plate

2: More than half of the streaks, but not all, are positive

3: All streaks are positive for bacteria

3. 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.

E. Respiratory specimens³

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

Clinical condition	Primary pathogen(s)	Specimen(s)
Chronic bronchitis	<i>S. pneumoniae</i> <i>S. aureus</i> , including MRSA <i>H. influenzae</i> Gram-negative bacilli	Lower respiratory
Pneumonia	Community-acquired pneumonia <i>S. pneumoniae</i> <i>S. aureus</i> , including MRSA <i>H. influenzae</i> <i>K. pneumoniae</i> Anaerobes (if aspiration) Agents of bioterrorism, including <i>Bacillus anthracis</i> , <i>Brucella</i> spp., <i>Francisella tularensis</i> , <i>Yersinia pestis</i> , and <i>Burkholderia pseudomallei</i> Hospital-acquired pneumonia, including ventilator associated pneumonia (VAP) Gram-negative bacilli, including <i>P. aeruginosa</i> and other non-fermenters Anaerobes (if aspiration) <i>S. aureus</i>	Lower respiratory

Lung abscess	<i>S. aureus</i> <i>S. pyogenes</i> <i>K. pneumoniae</i> <i>P. aeruginosa</i> Anaerobes	Lung aspirate or biopsy sample
Sinusitis	Acute <i>S. aureus</i> , <i>S. pneumoniae</i> , <i>H. influenzae</i> , <i>M. catarrhalis</i> Chronic Agents of acute sinusitis Gram-negative bacilli, including <i>P. aeruginosa</i> Anaerobes	Sinus aspirate
Staphylococcal carriage	<i>S. aureus</i> , including MRSA	Nasal swab
Cystic fibrosis	<i>Pseudomonas aeruginosa</i> <i>S. aureus</i> <i>Burkholderia cepacia</i> complex <i>Aspergillus</i> spp.	Deep throat (young children) Lower respiratory

Specimen collection and transport

Primary specimen

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.^{1,2,5}

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.

Sinus aspirate:

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

Endotracheal aspirate (ETA):

Endotracheal aspiration is done with a sterile technique using a 22 inch, 12F suction catheter. The catheter is 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 is 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 is used to increase the retrieved volume (≥ 1 ml)].

The specimens are sent to the laboratory and cultured within 1 hour of collection.

Bronchoalveolar lavage (BAL) collection:

In this procedure 100-300 ml of saline is infused into a lung segment through the bronchoscope to obtain cells and protein of the pulmonary interstitium and alveolar spaces. A portion of it is sent to the laboratory.

Type of Container

Collect in a sterile leak proof screw-cap container.

Rejection Criteria

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 under Day 1)

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.

For BAL specimens, lung aspirates and open-lung biopsy (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.

Processing

Media and Reagents

CHOC

Blood agar and MAC (optional); add when indicated by the consultant.

Gram's stain

Sputum and ETA, note the following:

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

Microscopic examination

Gram staining (G/S): 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, as poorly collected or not consistent with a bacterial infectious process.

Sputum: ≥ 10 SECs/LPF

NOTE: If the number of WBCs is 10 times the number of squamous epithelial cells (SECs) and there is 3+ to 4+ of a single morphotype of bacteria, accept the specimen for culture.

ETA from adults: If ≥ 10 SECs/LPF or no organisms seen; from pediatric patients: if no organisms seen.

BAL: Microscopic examination of cells from lavage has also been used to diagnose pneumonia. When more than 5% of cells from BAL contain bacteria, pneumonia has been diagnosed with sensitivities of up to 90% and specificities of 89% to 100% (Principles and Practice of Infectious Diseases, 2005).

Limitations in studying the sputum specimens by Gram staining include:

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 (Guidelines, ATS, 2001).

Culture

CA, BA with *Staphylococcus* streaking, MAC

Incubate in candle jar at 37°C aerobically.

Suction tips (ST)

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

Endotracheal aspirate (ETA), bronchoalveolar lavage (BAL) and lung aspirates inoculation

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 (1 μ l volume) is used for the purpose. The inoculation is done on the blood agar and MAC. The plates are incubated aerobically overnight at 37°C.

Day 2

Examination and reporting

Sputum

Report the growth of:

Streptococcus pneumoniae

H. influenzae

S. aureus

Gram-negative bacilli, including *P. aeruginosa* and other non-fermenters

Moraxella catarrhalis

Streptococcus pyogenes

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.

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.

Assay time 1 hour

Turnaround time (TAT) 48 hours

Safety Precautions: Specimen should be processed in the biosafety cabinet IIA.

F. Pus

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

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.

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.

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.

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.

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.

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.

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.

Specimen Processing:

Day 1:

Aspirate, pus and swab:

- a. Mix the specimen thoroughly. Place a drop of the specimen onto each medium *i.e.* RCM, blood agar and MAC.
- b. Prepare smear for Gram stain by placing a drop of specimen on a slide and spreading it to make a thin preparation.
- c. Perform a Gram stain on all specimens and use in the evaluation of culture.

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

d. Aerobic incubation conditions:

- i. Incubate RCM, blood agar and MAC in an incubator at 37°C. Incubate for a minimum of 24 hours for open wound cultures. Incubation may be extended to 2-3 days for invasive specimens (*i.e.*, aspirated fluids and tissues) that remain culture negative after 24-48 hours of aerobic incubation.
- ii. Critical deep-wounds, abscesses, and tissue samples should have anaerobic cultures requested in order to recover all the primary pathogen(s) causing infection in specific clinical conditions (*e.g.*, tissue or pus from brain, lung, liver tissue, deep wounds, abscesses, etc.).

Reporting results

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

DAY 2:

Culture interpretation:

- Report growth on blood agar and McConkey agar.
- Correlation with results on Gram stain is to be done.
- In case of no growth on both plates and RCM sterile, report as sterile.
- In case of just a film of growth on the plates and turbid RCM, plates should be further incubated and subculture from RCM done 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.

G. 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.

1. Collection of urine:

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.

Indwelling catheter:

- Hospitalized patients with indwelling catheter are especially at risk of developing UTI.
- To avoid contamination, the specimen is 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.

Suprapubic collection:

- The suprapubic collection is IDEAL and it 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.

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.

Cystoscopy specimens:

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

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.

Intermittent catheter specimen:

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

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.

3. Processing of specimen:**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 – 8°C until the final report is sent.

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.

Culture:

Choice of media and dilution:

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

Table 3. Recommended media according to microscopic findings.

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

Special situations:

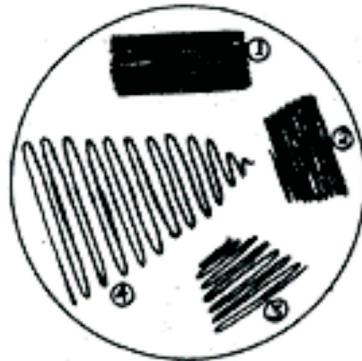
- Suprapubic collection– use full plate BA and MA
- PCN aspirate-use full plate BA, MA, CA and thioglycollate broth, irrespective of the smear finding.
- Make 1:10 dilution when moderate GNB and 1:100 dilution when many GNB are present in the smear.

* 4-area streaking without flaming in between for isolation

**Criss-cross streaking for colony count

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 diagram below, 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.



- 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 37°C.

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.

Interpretation of Counts:

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

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

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

Identification of isolates:

- Identify all coliform bacteria that are considered probably significant.
- The preliminary screening media:
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.

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.

- <10³ CFU/ml AST not done, except for cystoscopic, PCN or SPCspecimens
- 10³-10⁵ CFU/ml AST done on two organisms depending on their probable significance and relative numbers
- >10⁵CFU/ml AST done on 1-2 organisms and rarely three organisms depending on their significance and relative numbers

Reporting:

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.

H. Fecal specimens

Purpose

To describe the collection of faecal samples for microbiological examination, and processing in the laboratory for microbiological examination.

Procedure

Specimen Collection and Transport

1. 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.
2. 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.
3. The sample has to 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.
4. The sample has to be immediately transported to the laboratory on collection.
5. If there is a delay in transporting faecal specimens or if samples have 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

Microscopy

1. For all watery faeces samples, whether the doctor orders or not, examine a hanging drop (HD) immediately, or wet preparation by darkfield (DF) microscopy.
2. 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.

Culture and Isolation

1. Commonly encountered enteric pathogens and potential pathogens include *Salmonella*, *Shigella*, *V.cholerae*, *Arizona*, *Edwardsiella*, *Aeromonas*, *Plesiomonas*, diarrhoeogenic *E. coli* and *V. parahaemolyticus*.
2. Routine media to be included are BA, MA, XLD or DCA, and Selenite F broth.
3. 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.
4. 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.

5. 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.
6. 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.

Colony characteristics on different media:

Desoxycholate citrate agar

- *Shigella* species: Opaque ground-glass NLF colonies, with even margins. *Salmonella* Typhi and *S. Paratyphi*: Translucent, colourless NLF colonies. Other *Salmonella* species: Large NLF opaque colonies, may have a brownish/blackish centre.
- *Vibrio cholerae*: Small, colourless, translucent colonies, which may appear after 48 hours.
- *Pseudomonas aeruginosa*: Large or small NLF colonies, transversely elongated, often with a detectable pigment.
- Proteus group: Colourless NLF, raised, opaque colonies.
- *E.coli*: Raised, opaque, pink colonies surrounded by a pink halo of precipitated bile salt.
- *Klebsiella* and *Enterobacter*: Mucoid, pink colonies.
- DCA is examined at the end of 24 hours and again after 48 hours.
- **NOTE:** Colonies of all non-lactose fermenters (NLF) may look similar: reliance should not be placed on colony characteristics for differentiation. Occasionally 48 hours incubation may be needed for the NLFs to appear.
- DCA is found to be highly satisfactory for the isolation of non-lactose fermenting faecal pathogens, e.g. *Salmonella* and *Shigella*.
- The growth of coliform bacteria is inhibited or greatly suppressed. Gram-positive bacteria are generally inhibited.
- Occasionally, however, coliforms do grow on this medium and such isolates produce acid from lactose, precipitate bile salt, and cause a pink opacity in the medium, which makes it difficult to differentiate the pathogens that may also be present.

MacConkey Agar

- *Shigella* species: Colourless NLF colonies varying from small to large and from translucent to moderately opaque.
- *Salmonella* Typhi: Colourless NLF colonies, varying from small to large and from translucent to slightly opaque, smooth with even or slightly, irregular leaf-like edges.
- Other *Salmonella* species: Colourless NLF colonies, usually more opaque than the above.

- *Vibrio cholerae*: Medium sized colourless, transparent colonies.
- *Pseudomonas aeruginosa*: Large, glistening NLFs, moist colonies, often elongated, with detectable greenish pigment.
- Proteus group: Large, colourless NLF colonies.
- *E.coli*: Large non-mucoid colonies, with even pink colour.
- *Klebsiella* and *Enterobacter*: Large mucoid colonies, with pink centers and colourless peripheries or even pink.
- **NOTE:** Colonies of all non-lactose fermenters (NLF) may look very similar: Reliance should not be placed on colony characteristics for differentiation.

Xylose lysine deoxycholate medium

- *Shigella* species: Small pink colonies.
- *Salmonella* species: Small pink colonies with or without black centers.
- Coliforms: yellow colonies, or yellow with black centres (*Citrobacter freundii* and *Proteus vulgaris*)
- *Pseudomonas*: pink colonies.
- NFGNB: pink colonies

Black centre colonies grown on XLD without isolation need to be subcultured on another XLD.

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.

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 37°C for 2 - 7 days.

Reading: 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.

Reading

- Examine mannitol motility medium for evidence of motility and mannitol fermentation.
- Examine the TSI for fermentation of glucose, lactose, and sucrose, presence of gas and H₂S.
- Examine LIA for decarboxylation of lysine and presence of H₂S shown as a black precipitation. Note the amount of H₂S 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.

Identification of Isolates to Species Level

Identification of Isolates

Table 4. Identification of commonly isolated members of family Enterobacteriaceae Distinguishing reactions of common and pathogenic Enterobacteriaceae

Bacteria	Lactose	Motility	Gas	Indole	VP	Citrate	PDA	Urease	Lysine	H ₂ S	Inositol	ONPG
<i>Escherichia coli</i>	+	+	+	+	-	-	-	-	+	-	-	+
<i>Shigella</i> groups A, B, C	-	-	-	±	-	-	-	-	-	-	-	-
<i>Shigella sonnei</i>	-	-	-	-	-	-	-	-	-	-	-	+
<i>Salmonella</i> (most serotypes)	-	+	+	-	-	+	-	-	+	+	±	-
<i>Salmonella</i> Typhi	-	+	-	-	-	-	-	-	+	+	-	-
<i>Salmonella</i> Paratyphi A	-	+	+	-	-	-	-	-	-	-	-	-
<i>Citrobacter freundii</i>	±	+	+	-	-	+	-	±	-	±	-	+
<i>Citrobacter koseri</i>	±	+	+	+	-	+	-	±	-	-	-	+
<i>Klebsiella pneumoniae</i>	+	-	++	-	+	+	-	+	+	-	+	+
<i>Klebsiella oxytoca</i>	+	-	++	+	+	+	-	+	+	-	+	+
<i>Enterobacter aerogenes</i>	+	+	++	-	+	+	-	-	+	-	+	+
<i>Enterobacter cloacae</i>	+	+	+	-	+	+	-	±	-	-	-	+
<i>Hafnia alvei</i>	-	+	+	-	+	-	-	-	+	-	-	+
<i>Serratia marcescens</i>	-	+	±	-	+	+	-	-	+	-	±	+
<i>Proteus mirabilis</i>	-	+	+	-	±	±	+	++	-	+	-	-
<i>Proteus vulgaris</i>	-	+	+	+	-	-	+	++	-	+	-	-
<i>Morganella morganii</i>	-	+	+	+	-	-	+	++	-	±	-	-
<i>Providencia rettgeri</i>	-	+	-	+	-	+	+	++	-	-	+	-
<i>Providencia stuartii</i>	-	+	-	+	-	+	+	±	-	-	+	-
<i>Providencia alcalifaciens</i>	-	+	+	+	-	+	+	-	-	-	-	-
<i>Yersinia enterocolitica</i>	-	-	-	±	-	-	-	±	-	-	±	+
<i>Yersinia pestis</i>	-	-	-	-	-	-	-	-	-	-	-	±
<i>Yersinia pseudotuberculosis</i>	-	-	-	-	-	-	-	+	-	-	-	±

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, H₂S indicates H₂S production in TSI agar. ONPG indicates metabolism of o-nitrophenyl-b-D-galactopyranoside.

+ indicates ≥ 85% of strains positive; - indicates ≥ 85% strains negative; ± indicates 16-84% of strains positive.

Scheme for identification of *Salmonella enterica*

1. 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 will 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-

(to save reagent, all the isolates need not be tested for all the antisera)

- If gram negative, NLF, motile, acid without gas in glucose, TSI with alkaline slant, acid butt, H₂S +ve, no gas, urea negative, citrate negative, indole negative- test with O₉ and dH antisera for *Salmonella* Typhi.
 - If gram negative, NLF, motile, acid and gas in glucose, urea negative and citrate positive, TSI with alkaline slant, acid butt, no H₂S with gas, indole negative - test with O₂ and aH antisera for *Salmonella* Paratyphi A.
 - If gram negative, NLF, motile, acid and gas in glucose, TSI with alkaline slant, acid butt, H₂S +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)
- For nonmotile variants *S. gallinarum* and *S. pullorum* again specific antisera only can be used.

Table 5. Criteria for biochemical characterization of *Stenotrophomonas maltophilia* and *Burkholderia cepacia* complex

Test	<i>S. maltophilia</i>	<i>B. cepacia</i> 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

Table 6. Criteria for biochemical characterization of *Pseudomonas* spp

<i>Pseudomonas</i> groups	Species	Oxidase	Motility	Pyoverdinin	Yellow pigment	Glucose	Maltose	Lactose	Mannitol	Arginine	Lysine	NO ₃ - NO ₂	NO ₃ - N ₂	Urea	DNase	Esculin	H ₂ S	Acetamide
		Fluorescent group	<i>P. aeruginosa</i>	+	+	+	-	+	V	-	V	+	-	+	V	V	-	-
	<i>P. fluorescens</i>	+	+	+	-	+	V	-	+	+	-	V	-	V	-	-	-	-
	<i>P. putida</i>	+	+	+	-	+	V	-	V	+	-	-	-	V	-	-	-	-
Stutzeri group	<i>P. stutzeri</i>	+	+	-	-	+	+	-	V	-	-	+	+	V	-	-	-	-
	<i>P. mendocina</i>	+	+	-	-	+	-	-	-	+	-	+	+	V	-	-	-	-
	CDC group Vb-3	+	+	-	-	+	+	-	+	+	-	+	+	V	-	-	-	-
Alcaligenes group	<i>P. alcaligenes</i>	+	+	-	-	-	-	-	-	-	-	V	-	V	-	NA	-	-
	<i>P. pseudoalcaligenes</i>	+	+	-	-	-	-	-	-	V	-	+	-	-	-	-	-	-
	<i>Pseudomonas. spp. Group-1</i>	+	+	-	-	-	-	-	-	V	-	+	+	-	-	NA	-	-
Yellow pigment group	<i>P. luteola</i>	-	+	-	+	+	+	-	+	V	-	V	-	V	-	+	-	-
	<i>P. oryzihabitans</i>	-	+	-	+	+	+	-	+	-	-	-	-	V	-	-	-	-

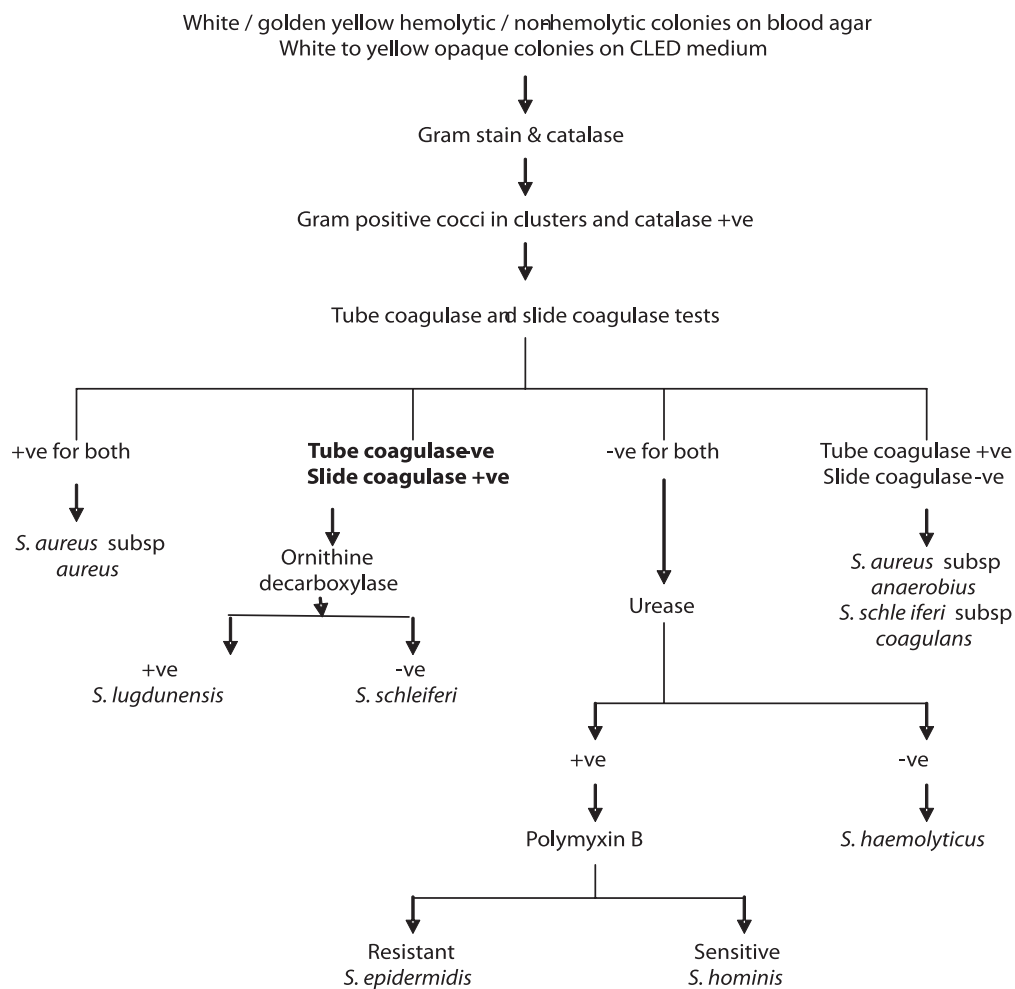
Table 7. Criteria for biochemical characterization of *Acinetobacter* spp

<i>Acinetobacter</i> spp (genomospecies no.)	Motility	Oxidase	Growth at 37°C	Growth at 44°C	Haemolysis on BA	Gelatin test	OF dextrose	Arginine	Malonate
<i>A. johnsonii</i> (7)	-	-	NEG	NEG	NH	NEG	NEG	V	V
<i>A. baumannii</i> (2)	-	-	+	+	NH	NEG	+	+	+
<i>A. haemolyticus</i>	-	-	+	NEG	+	+	NEG	+	NEG
<i>Acinetobacter spp. (6)</i>	-	-	+	NEG	+	+	NEG	+	NEG
<i>Acinetobacter spp (10)</i>	-	-	+	NEG	NH	NEG	+	NEG	NEG
<i>A. calcoaceticus</i> (1)	-	-	+	NEG	NH	NEG	+	+	+
<i>Acinetobacter spp (3)</i>	-	-	+	NEG	NH	NEG	+	+	V
<i>Acinetobacter spp. (12)</i>	-	-	+	NEG	NH	NEG	NEG	+	+
<i>Acinetobacter spp. (5)</i>	-	-	+	NEG	NH	NEG	NEG	+	NEG
<i>A. lwoffii</i> (8/9)	-	-	+	NEG	NH	NEG	NEG	NEG	NEG
<i>Acinetobacter spp. (11)</i>	-	-	+	NEG	NH	NEG	NEG	NEG	NEG

OF – Oxidative-Fermentative

(Ref: Winn W, Allen S, Janda W. Koneman's colour atlas and textbook of diagnostic microbiology. 6th edn. Lippincott Williams & Wilkins; 2005)

Identification of staphylococci



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 24 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* is 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-pyrrolidonyl 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	<i>E. avium</i> , <i>E. raffinosus</i> , <i>E. pallens</i> , <i>E. malodoratus</i>
Group II	<i>E. faecalis</i> , <i>E. faecium</i> , <i>E. casseliflavus</i> , <i>E. gallinarum</i> , <i>E. mundtii</i>
Group III	<i>E. dispar</i> , <i>E. hirae</i> , <i>E. durans</i>
Group IV	<i>E. cecorum</i> , <i>E. asini</i> , <i>E. phoeniculicola</i>
Group V	<i>E. canis</i> , <i>E. columbae</i>

Algorithm for phenotypic speciation of *Enterococcus* isolates (simplified)

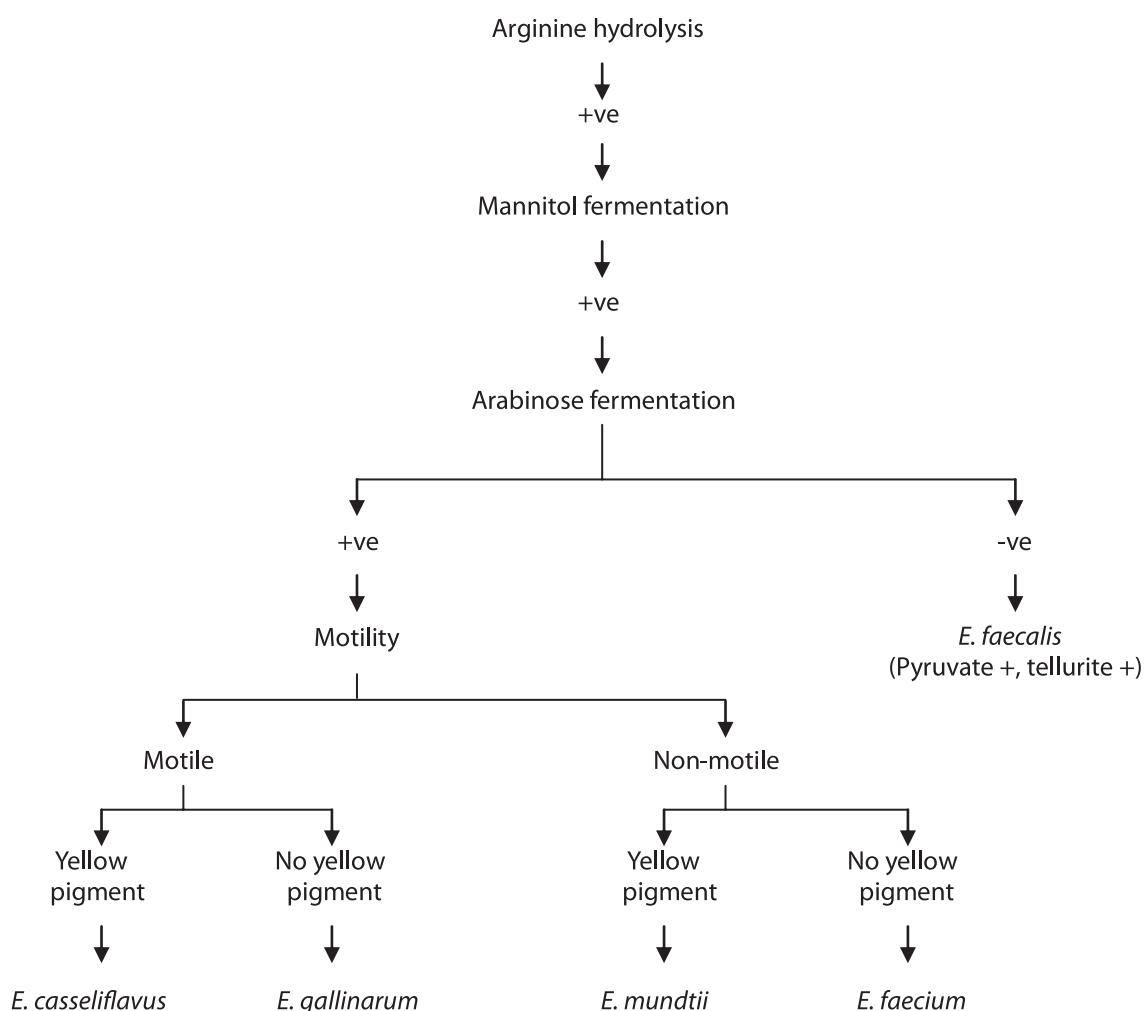


Table 8. Biochemical tests for screening of fecal isolates

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	-	<i>E. coli</i>
		+	-	-	+	V	W	<i>Klebsiella pneumoniae</i>
		+	+/-	+/-	V	-	V	<i>Aeromonas</i> spp
2. Same as 1, with blackening	Same as 1 with H ₂ S	+	+/-	-/+	+	-	V	<i>Citrobacter</i> spp
		+	+	-	+	+	-	<i>Arizona</i> spp
		-	+	+/-	V	R	+	<i>Proteus</i> spp
3. Red slant, yellow butt with gas	Glucose = acid + gas; lactose & sucrose negative	+	+	-	-	-	-	<i>Salmonella</i> Paratyphi A
		+	+	-	V	+	-	<i>Salmonella</i> spp. (H ₂ S-)
		-	+	+/-	-	R	+	<i>Morganella</i> spp
		+	+	+	+	-	-	<i>Citrobacter</i> spp
4. Same as 3, with blackening	Same as 3 with H ₂ S	+	+	-	V	+	-	<i>Salmonella</i> spp (H ₂ S+) & <i>Arizona</i> spp
		+	+	-	+	-	W	<i>Citrobacter</i> spp
		-	+	+/-	-	R	+	<i>Proteus</i> spp
		-	+	+	+	+	-	<i>Edwardsiella tarda</i>
5. Red slant, yellow butt, no gas	Glucose = acid no gas; lactose & sucrose negative	+	+	-	-	+	-	<i>S. Typhi</i> **
		+/-	-	+/-	-	-	-	<i>Shigella</i> spp., <i>Alkalescens</i> <i>Dispar</i> group
		+/-	+	+	+	-	v	<i>Providencia</i> group
		+/-	+	+	-	R	+	<i>Morganella morganii</i>
		-	+	+	-	+	-	<i>Plesiomonas shigelloides</i>
6. Same as 5, with slight blackening	Same as 5 with little H ₂ S	+	+	-	-	+	-	<i>S. Typhi</i>
7. Red slant, red butt	Glucose, lactose & sucrose negative	-	+	-	+	-	V	<i>Pseudomonas</i> 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	-	-	+	-	<i>Yersinia enterocolitica</i>
9. Yellow slant, yellow butt	Glucose = acid; lactose &/or sucrose = acid	+	+	+	+	+	-	<i>V. cholerae</i> and other vibrios
		+	+/-	+	-	V	-	Anaerogenic <i>E. coli</i>
		+	+/-	+	v	-	-	<i>Aeromonas</i> spp

*Incubation for >24 hours can give acid butt

Further Biochemical Tests for Identification

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 is still considered to be *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.

Salmonella and salmonella-like organisms

- When TSI and LIA reactions are suggestive of non-H₂S-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 H₂S-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-H₂S-producing *Salmonella* species.

Procedure

1. 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.
2. To one, add the required antiserum, to the other, more saline to serve as the control.
3. Rock the slide back and forth for 1-3 minutes, watching closely for appearance of agglutination.
4. 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.
5. 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

1. 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*.
2. Hold the CU agar and inoculate the following additional media. LIA, citrate, malonate broth, dulcitol, lactose broth, gelatin.
3. See Table below for correlation of reactions in these media with the organisms thus characterized.

Table 9. Special biochemical test for differentiating *Salmonella*, *Arizona*, *Edwardsiella tarda* and *Citrobacter* isolates

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

4. 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*".
5. 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*".
6. 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.
7. CU agar may show a weakly positive reaction on prolonged incubation.
8. 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*.

Shigella and shigella-like organisms

1. 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

1. 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.
2. *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)
3. Group A (10 serotypes) is divided on the indole production into
 - Indole negative serotypes 1, 3, 4, 5, 6, 9, 10
 - Indole positive serotypes 2, 7, and 8
4. Groups B, C, D are based on lactose fermentation and indole production
5. Group D is the only late lactose fermenter; it doesn't produce indole and has only one serotype
6. 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.
7. 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.
8. 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

1. 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 Alkaescens-Dispar (A-D) group.
2. Use a heated suspension for slide agglutination tests. Unheated Alkaescens-Dispar (A-D) group strains are inagglutinable.
3. 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.

4. 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.
5. **NOTE:** A preliminary report may be sent to indicate isolation of 'an organism biochemically resembling *Shigella* species, but serologically untypable with available facilities'.

V. cholerae 01 and V. cholerae 0139

1. 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.
2. 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 APW culture.
 - Examine plates for presence of colonies suggestive of *V. cholerae*, or of any other enteric pathogens.
3. 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.
4. However, *Vibrio parahaemolyticus* shows exception in that it is sucrose non-fermenter and produces blue-green colonies.
5. Subculture from suspicious colonies according to directions given earlier.
6. 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

1. 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.

2. The panel of tests for the 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 10. Differential characteristics of vibrios.

Test	Classical <i>V. cholerae</i>	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 11. Biochemical reactions of *V. parahaemolyticus*, *V. cholerae* O1 & O139.

Test	<i>V. parahaemolyticus</i>	<i>V cholerae</i> 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	Positive	+

MR	Positive	+
VP	Negative	-
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

1. Test the cultures for slide agglutination by specific diagnostic serum, *V. cholerae* sero group O1 polyvalent.
2. 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*.
3. These are organisms which are morphologically, culturally and biochemically identical to *Vibrio cholerae* but serologically not serogroup O1 or O139.
4. 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.
5. *V. parahaemolyticus* is a marine halophilic vibrio, which can tolerate upto 7% NaCl.
6. If clinical history suggests consumption of seafood prior to onset of gastroenteritis, a search for this organism should be included.

Isolation

1. 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 in this, subculture onto TCBS agar.
2. 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.

Campylobacter jejuni

1. They are Gram-negative comma or 'S' shaped organisms, actively motile, and microaerophilic. Ideal atmosphere for its growth should contain 6% O₂, 10% CO₂ atmosphere and the rest N₂; 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:

Ingredients	Gm/l
Lab-Lemco powder	10
Peptone	10
Sodium chloride	5
Agar	12

pH 7.5 ± 0.2

Campylobacter Selective supplement (BLASER WANG):

Ingredients	per vial
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.

- Biochemical characterization:

- Generally they are inert organisms. The morphology on Gram's stain is confirmatory. Reactions are as follows.
- Oxidase-positive, nitrate reduction positive, indole negative, urease negative, methyl red negative, Voges-Proskauer negative.

Report

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

Name of the antiserum

Salmonella polyvalent (A-I and Vi)

Salmonella Group A (1, 2, 12)

Salmonella Group AO (2)

Salmonella Group B (1, 4, 5, 12)

Salmonella Group C (7)

Salmonella Group C2 (8)

Salmonella Group D (9)

Salmonella Vi antiserum

Company Name¹⁹

- Gemini

- Gemini

- Gemini

- Gemini

- Gemini

- Gemini

- Gemini

- Gemini

<i>Salmonella</i> Group E (poly valent) (1, 3, 10, 15, 19, 34)	- Gemini
<i>Salmonella</i> Paratyphi aH	- Gemini
<i>Salmonella typhi</i> d H (STH)	- Gemini
<i>Salmonella</i> i H	- Gemini
<i>Shigella</i>	- Denka Seiken
<i>Shigella dysenteriae</i> poly A	-Gemini
<i>Shigella dysenteriae</i> poly A1	-Gemini
<i>Shigella dysenteriae</i> 1	-Denka Seiken
<i>Shigella dysenteriae</i> 2	-Denka Seiken
<i>Shigella dysenteriae</i> 3	-Denka Seiken
<i>Shigella dysenteriae</i> 4	-Denka Seiken
<i>Shigella dysenteriae</i> 5	-Denka Seiken
<i>Shigella dysenteriae</i> 6	-Denka Seiken
<i>Shigella dysenteriae</i> 7	-Denka Seiken
<i>Shigella dysenteriae</i> 8	-Denka Seiken
<i>Shigella dysenteriae</i> 9	-Denka Seiken
<i>Shigella dysenteriae</i> 10	-Denka Seiken
<i>Shigella flexneri</i> Poly B	- Gemini
<i>Shigella flexneri</i> 1	- Denka Seiken
<i>Shigella flexneri</i> 3	- Denka Seiken
<i>Shigella flexneri</i> 4	- Denka Seiken
<i>Shigella flexneri</i> 5	- Denka Seiken
<i>Shigella flexneri</i> 6	- Denka Seiken
<i>Shigella boydii</i> (1-7)	- Gemini
<i>Shigella boydii</i> poly C1 (8 – 13)	- Gemini
<i>Shigella boydii</i> poly C2 (14 – 18)	- Gemini
<i>Shigella Sonnei</i> Group D	- Gemini
Vibrio	
<i>V. cholerae</i> (polyvalent)	- King institute, Chennai
<i>V. cholerae</i> - Ogawa	- King institute, Chennai
<i>V. cholerae</i> - Inaba	- King institute, Chennai

EPEC

EPEC 0157 - Denka Seiken

In house (CMC)- Raised in rabbits

Shigella flexneri 2

Shigella flexneri sero 2

V. cholerae 0139

EPEC 0127B8

EPEC 0111B4

EPEC 055B5

EPEC 026B6

EPEC 086B7

EPEC 0125B15

EPEC 0126B16

EPEC 0128B12

EPEC 0124B17

EPEC 0119B14

Antimicrobial Susceptibility Testing

Antimicrobial Susceptibility Testing

All the participating centers have to follow CLSI methodology, 2015 for antimicrobial susceptibility testing.⁸

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
 - a) clinical efficacy in body sites where the drugs are physiologically concentrated (*e.g.* quinolones in urine) or
 - b) at sites where the drug is not 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.

Disc diffusion testing: Basic Procedure

- **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.

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 (1.5×10^8 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 35°C 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.

- **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 will then be streaked over the entire surface to the agar plate three times, with the plate rotated approximately 60° each time to ensure even distribution of the inoculum. A final sweep of the swab will 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.
- **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.
 1. 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.
 2. It is advisable to place discs that give predictably small zones like aminoglycosides, next to those discs that give larger zones like cephalosporins.
 3. 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.

N.B. 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.
- **Incubation**– No longer than 15 minutes after discs are applied, the plates will be inverted and incubated at $35^{\circ} \pm 2^{\circ}\text{C}$ in ambient air.
- **Interpretation and reporting of results** – Each plate will 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, will be measured to the nearest whole millimetre with callipers or a ruler. With unsupplemented MHA, the measuring device is 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 will 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.

- **Recommended Media:**

Müller Hinton agar (MHA): Fresh plates should be used the same day or stored in a refrigerator (2-8°C); 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 (35°C) 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.

ATCC control strains

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

	ATCC	25922
<i>Klebsiella pneumoniae</i>	ATCC	700603
<i>Pseudomonas aeruginosa</i>	ATCC	27853
<i>Enterococcus faecalis</i>	ATCC	29212
<i>Staphylococcus aureus</i>	ATCC	25923

Preparing antibiotic discs in-house

Method

- 1) From the table below find the appropriate solvent for your antimicrobial agent. In most cases it is sterile distilled water or phosphate buffered saline, pH 7.2.
- 2) On the vial of the antibiotic powder, read the potency of the antibiotic, amount, its value in international units, if any.
- 3) 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.
- 4) 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.
- 5) Now take 4 ml of the sterile solvent in a test tube.
- 6) 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.
- 7) 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.

- 8) 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.
- 9) Add exactly 10 µl of the antibiotic solution to each disc. When finished, leave the Petri-dish slightly open in the incubator for 0.5-1 hour. This allows for the drying of the discs.
- 10) 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.
- 11) Store discs at 4°C. For degradable antimicrobials like clavulanic acid and imipenem, store at -20°C and take these out only just before use.

Table 12. 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, 0.1 mol/L	Phosphate buffer, pH 6.0, 0.1 mol/L
Ampicillin	Phosphate buffer, pH 8.0, 0.1 mol/L	Phosphate buffer, pH 6.0, 0.1 mol/L
Azithromycin	95% ethanol or glacial acetic acid ^{b,c}	Broth media
Aztreonam	Saturated solution sodium bicarbonate	Water
Cefepime	Phosphate buffer, pH 6.0, 0.1 mol/L	Phosphate buffer, pH 6.0, 0.1 mol/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, 0.1 mol/L	Water
Chloramphenicol	95 % ethanol	Water
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, 0.1 mol/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, 0.01 mol/L	Phosphate buffer, pH 7.2, 0.01 mol/L
Moxalactam (diammonium salt)	0.04 mol/L HCl (let sit for 1.5 to 2 hour)	Phosphate buffer, pH 6.0, 0.1 mol/L
Nitrofurantoin ^c	Phosphate buffer, pH 8.0, 0.1 mol/L	Phosphate buffer, pH 8.0, 0.1 mol/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)

Footnotes

- The following antimicrobials will be soluble in sterile distilled water: amikacin, azlocillin, carbenicillin, cefaclor, cefamandole, cefonicid, cefotaxime, cefoperazone, ceftoxitin, 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.
- 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.
- 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.

Storage of antimicrobial discs²

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 β-lactam class should be stored frozen (-20°C), except for a small working supply, which may be refrigerated for at most one week. Some labile agents (eg, imipenem, cefaclor, and clavulanic acid combinations) may retain greater stability if stored frozen until the day of use.

Table 13. List of antimicrobial agents, zone diameters and MIC interpretive criteria for Enterobacteriaceae.

Antimicrobial agent	Disk content	Zone diameter interpretive criteria (mm)			MIC interpretive criteria (µg/ml)		
		S	I	R	S	I	R
Amikacin (AMK)	30 µg	≥17	15-16	≤14	≤16	32	≥64
Cefepime (FEP)	30 µg	≥25	19-24	≤18	≤2	4-8	≥16
Cefotaxime (TAX)	30 µg	≥26	23-25	≤22	≤1	2	≥4
Cefoperazone-sulbactam (CSL)	75/30 µg	≥21	16-20	≤15	≤16	32	≥64
Ceftazidime*(CAZ)	30 µg	≥21	18-20	≤17	≤4	8	≥16
Ciprofloxacin (CIP)	5 µg	≥21	16-20	≤15	≤1	2	≥4
Doxycycline*	30 µg	≥14	11-13	≤10	≤4	8	≥16
Ertapenem (ETP)	10 µg	≥22	19-21	≤18	≤0.5	1	≥2
Imipenem (IMI)	10 µg	≥23	20-22	≤19	≤1	2	≥4
Meropenem (MEM)	10 µg	≥23	20-22	≤19	≤1	2	≥4
Nitrofurantoin** (NIT)	300 µg	≥17	15-16	≤14	≤32	64	≥128
Norfloxacin** (NOR)	10 µg	≥17	13-16	≤12	≤4	8	≥16
Piperacillin-tazobactam (PTZ)	100/10 µg	≥21	18-20	≤17	≤16/4	32/4-64/4	≥128/4
Colistin (CST) #					≤2 ⁴	-	≥2 ⁴
Trimethoprim-sulfamethoxazole** (SXT)	1.25/23.75 µg	≥16	11-15	≤10	≤2/38	-	≥4/76
Tigecycline (TIG) §	5 µg	≥19	15-18	≤14	≤2	4	≥8

Notes:

- * Not to be tested for isolates from urine.
- **To be tested only for isolates from urine.
- #Colistin to be tested only by MIC test. EUCAST guidelines, 2015 give cut-offs only for colistin.
- Creeping MIC: *E. coli*, *K. pneumoniae*, *Proteus* spp.: cefotaxime, imipenem.
- ⁵Tigecycline – breakpoints as per FDA (accessed on March, 2015).

Table 14. 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 Interpretive criteria (mm)			MIC Interpretive Criteria (µg/ml)		
		S	I	R	S	I	R
Ampicillin (AMP)	10	≥17	14-16	≤13	≤8	16	≥32
Trimethoprim-sulfamethoxazole (SXT)	1.25/23.75	≥16	11-15	≤10	≤2/38	-	≥4/76
Ciprofloxacin (CIP)	5	≥31	21-30	≤20	≤0.06	0.12-0.5	≥1
Ofloxacin (OFX)	-	-	-	-	≤0.12	0.25-1	≥2
Chloramphenicol (CHL)	30	≥18	13-17	≤12	≤8	16	≥32
Ceftriaxone (CTR)	30	≥23	20-22	≤19	≤1	2	≥4
Cefixime (FIX)	5	≥19	16-18	≤15	≤1	2	≥4

Notes: 1. Creeping MIC: ciprofloxacin, ofloxacin, ceftriaxone.

Table 15. List of antimicrobial agents, zone diameters and MIC interpretive criteria for *Pseudomonas aeruginosa*.

S. No.	Antimicrobial agent	Disk content (µg)	Zone diameter Interpretive criteria (nearest whole mm)			MIC Interpretive criteria (µg/mL)		
			S	I	R	S	I	R
1.	Ceftazidime (CAZ)	30	≥18	15-17	≤14	≤8	16	≥32
2.	Levofloxacin (LEV)	5	≥17	14-16	≤13	≤2	4	≥8
3.	Tobramycin (TOB)	10	≥15	13-14	≤12	≤4	8	≥16
4.	Amikacin (AMK)	30	≥17	15-16	≤14	≤16	32	≥64
5.	Colistin (CST)	10	≥11	-	≤10	≤2	4	≥8
6.	Cefepime (FEP)	30	≥18	15-17	≤14	≤8	16	≥32
7.	Netilmicin (NET)	30	≥15	13-14	≤12	≤8	16	≥32
8.	Piperacillin-tazobactam (PTZ)	100/10	≥21	15-20	≤14	≤16/4	32/4-64/4	≥128/4
9.	Aztreonam (AZT)	30	≥22	16-21	≤15	≤8	16	≥32
10.	Imipenem (IMI)	10	≥19	16-18	≤15	≤2	4	≥8
11.	Meropenem (MEM)	10	≥19	16-18	≤15	≤2	4	≥8
12.	Ciprofloxacin (CIP)	5	≥21	16-20	≤15	≤1	2	≥4
13.	Gentamicin (GEN)	10	≥15	13-14	≤12	≤4	8	≥16

Notes:

1. First line antimicrobial agents for isolates from 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, netilmicin.
3. For isolates from eye: Test only ciprofloxacin, gentamicin, tobramycin and ceftazidime.

Table 16. List of antimicrobial agents, zone diameters and MIC interpretive criteria for *Acinetobacter baumannii*

S. No.	Antimicrobial agent	Disk content	Zone diameter Interpretative criteria (nearest whole mm)			MIC Interpretative criteria ($\mu\text{g/mL}$)		
			S	I	R	S	I	R
1.	Ceftazidime (CAZ)	30 μg	≥ 18	15-17	≤ 14	≤ 8	16	≥ 32
2.	Levofloxacin (LEV)	5 μg	≥ 17	14-16	≤ 13	≤ 2	4	≥ 8
3.	Amikacin (AMK)	30 μg	≥ 17	15-16	≤ 14	≤ 16	32	≥ 64
4.	Piperacillin-tazobactam (PTZ)	100/10 μg	≥ 21	18-20	≤ 17	$\leq 16/4$	32/4-64/4	$\geq 128/4$
5.	Tetracycline (TET)	30 μg	≥ 15	12-14	≤ 11	≤ 4	8	≥ 16
6.	Cefepime (FEP)	30 μg	≥ 18	15-17	≤ 14	≤ 8	16	≥ 32
7.	Netilmicin (NET)	-	-	-	-	≤ 8	16	≥ 32
8.	Imipenem (IPM)	10 μg	≥ 22	19-21	≤ 18	≤ 2	4	≥ 8
9.	Meropenem (MEM)	10 μg	≥ 18	15-17	≤ 14	≤ 2	4	≥ 8
10.	Cefoperazone-sulbactam (CSL)	75/30 μg	≥ 21	16-20	≤ 15	-	-	-

Notes:

1. First line antimicrobial agents for isolates from urine, sputum, ETA, suction tip, BAL, pus, genital tract specimens: 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.

Table 17. List of antimicrobial agents, zone diameters and MIC interpretive criteria for *Burkholderia cepacia*.

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

Table 18. List of antimicrobial agents, zone diameters and MIC interpretive criteria for *Stenotrophomonas maltophilia*.

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

Table 19. List of antimicrobial agents, zone diameters and MIC interpretive criteria for fecal bacterial pathogens (*Shigella* spp., diarrhoeagenic *E. coli*, *Salmonella* spp. *Vibrio* spp.)

Antimicrobial agent	Disk content	Zone diameter Interpretative criteria (nearest whole mm)			MIC Interpretative criteria (µg/mL)		
		S	I	R	S	I	R
Ampicillin (AMP)	10 µg	≥17	14-16	≤13	≤8	16	≥32
Tetracycline (TET)	30 µg	≥15	12-14	≤11	≤4	8	≥16
Trimethoprim-sulfamethoxazole (SXT)	1.25/23.75 µg	≥16	11-15	≤10	≤2/38		≥4/76
Nalidixic acid (NAL)	30 µg	≥19	14-18	≤13	≤16	-	≥32
Norfloxacin (NOR)	10 µg	≥17	13-16	≤12	≤4	8	≥16
Cefixime (FIX)	5 µg	≥19	16-18	≤15	≤1	2	≥4
Ciprofloxacin (CIP)	5 µg	≥31	21-30	≤20	≤0.06	0.12-0.5	≥1
Azithromycin (AZM)	-	-	-	-	≤8	16	≥32
Clindamycin* (CLI)	-	-	-	-	≤4	-	>4

Notes:

1. For *Shigella* spp., diarrhoeagenic *E. coli*: test cefixime, ampicillin, trimethoprim-sulfamethoxazole, nalidixic acid, norfloxacin.
2. For *Salmonella* spp., *Arizona* spp.: test ampicillin, nalidixic acid, ciprofloxacin, and trimethoprim-sulfamethoxazole.
3. For *Vibrio* spp.: test ampicillin, tetracycline, trimethoprim-sulfamethoxazole, norfloxacin, nalidixic acid.
4. For *Campylobacter* spp.: test azithromycin, ciprofloxacin, norfloxacin, clindamycin, and tetracycline.
5. For *Aeromonas* spp., *Plesiomonas shigelloides*: test tetracycline, ciprofloxacin, norfloxacin, cefixime.
6. *Clindamycin data based on EUCAST 2014.

Table 20. List of antimicrobial agents, zone diameters and MIC interpretive criteria for *Staphylococcus* species.

Antimicrobial agent	Disk content	Zone diameter interpretive criteria (mm)			MIC interpretive criteria (µg/mL)		
		S	I	R	S	I	R
Cefoxitin (FOX) * **	30 µg	≥ 22 ≥ 25	- -	≤ 21 ≤ 24	≤ 4 -	- -	≥ 8 -
Ciprofloxacin (CIP)	5 µg	≥ 21	16-20	≤ 15	≤ 1	2	≥ 4
Clindamycin (CLI)	2 µg	≥ 21	15-20	≤ 14	≤ 0.5	1-2	≥ 4
Co-trimoxazole (SXT)	1.25/23.75 µg	≥ 16	11-15	≤ 10	≤ 2/38	-	≥ 4/76
Erythromycin (ERY)	15 µg	≥ 23	14-22	≤ 13	≤ 0.5	1-4	≥ 8
Linezolid (LNZ)	30 µg	≥ 21	-	≤ 20	≤ 4	-	≥ 8
Mupirocin High Level	200 µg	≥ 8	-	≤ 7	≤ 256	-	> 256
Penicillin (PEN)	10 units	≥ 29	-	≤ 28	≤ 0.12	-	≥ 0.25
Teicoplanin (TEC)	30 µg	≥ 14	11-13	≤ 10	≤ 8	16	≥ 32
Tetracycline (TET)	30 µg	≥ 19	15-18	≤ 14	≤ 4	8	≥ 16
Tigecycline (TIG)	-	-	-	-	≤ 0.5	-	≥ 0.5
Vancomycin (VAN) <i>S. aureus</i> All CoNS	-				≤ 2 ≤ 4	4-8 8-16	≥ 16 ≥ 32

Notes:

1. Mupirocin high level: Only for *S. aureus*.
2. Detection of MRSA: (a) DD: Cefoxitin (FOX, 30 µg):
* *S. aureus*, *S. lugdunensis*
** CoNS (non- *S. lugdunensis*)

(b) Oxacillin screen agar (6 µg/ml): MHA with 4% NaCl & 6 µg/ml of Oxacillin: > 1 colony or light film of growth denotes resistance. 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 *mecA* PCR may be performed for confirmation if facilities are available.

3. Vancomycin screen agar (6 µg/mL): 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 (Using E-strip or broth micro dilution) to determine vancomycin MICs.
4. D-Test for inducible Clindamycin resistance: To be done for *S. aureus*, *S. lugdunensis* resistant to erythromycin and susceptible /intermediate to clindamycin.
5. Erythromycin: Not to be tested for isolates from urine.
6. Tigecycline: Only for MRSA isolates: $S \leq 0.5$, $R \geq 0.5$ (EUCAST)
7. Creeping MIC: MRSA-vancomycin, teicoplanin, linezolid, daptomycin, tigecycline.

Special AMST for staphylococci

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.

1. 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.
2. Incubate the plate at 35°C for 18 hours.
3. 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.(CLSI Guidelines, 2015)⁸

Vancomycin screen agar:

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

1. Prepare a suspension of strain to be tested, equivalent to 0.5 McFarland standard.
2. 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) on every plate.
3. Incubate at 35°C for 24 hours.
4. Read the plates in transmitted light.

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 MIC⁸.

MIC for vancomycin by broth micro dilution method:

Micro-broth dilution test

This test uses double-strength Müller-Hinton broth, 4x strength antibiotic solutions prepared as serial two-fold dilutions and the test organism at a concentration of 2×10^6 /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:

1. Prepare a standardized inoculum using either the direct colony suspension or growth method.
2. Optimally within 15 minutes of preparation, dilute the adjusted inoculum suspension in water, saline, or broth so that, after inoculation, each well contains approximately 5×10^5 CFU/mL.
3. The 0.5 McFarland suspension (1×10^8 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 5×10^5 CFU/mL (or 5×10^4 CFU/well in the microdilution method).
4. 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

It is advisable to perform a purity check of the inoculum suspension by subculturing an aliquot onto a nonselective agar plate for simultaneous incubation.

5. 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.

MIC for other antibiotics by agar dilution method:

1. Prepare the stock solutions at ten times the concentrations to be tested and make twofold dilutions of these stock solutions.
2. Add the antimicrobial solutions to molten MHA agar at 50°C.
3. Allow the agar to solidify.
4. Inoculate the strains to be tested in sterile normal saline to make a suspension with turbidity equivalent to 0.5 MacFarland standard.
5. Using 10 µl loop, inoculate a spot of the suspension on plates of different concentrations of antibiotics starting from low concentration.
6. Always include a drug free control medium to check the viability of the strains.

7. Incubate the plates at 35°C for 24 hours.
8. Examine the plates both in reflected and transmitted light.
9. *S. aureus* ATCC 29213 is used as QC for determining MIC values.
10. The lowest concentration on which the strain showed no growth is taken as the minimum inhibitory concentration for that strain.

Detection of heteroresistant vancomycin intermediate *Staphylococcus aureus* (hVISA) population analysis profile/area under curve (PAP/AUC) analysis (Wooten. M *et al.*, 2001)

Procedure:

1. Inoculate test isolate and Mu3 strain (*S.aureus* ATCC 700698) in trypticase soya broth (TSB) and incubate at 37°C for 24 hours.
2. 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.
3. Count the colonies after 48 hours of incubation.
4. 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.
5. AUC ratio of ≥ 0.9 is considered as hVISA.

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

To evaluate the best combination of drugs for MRSA, perform checkerboard assay.

Procedure:

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). 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.

Interpretation of Σ FIC value:

FIC value	Interpretation
≤ 0.5	Synergy
0.5 – 4	Indifference
> 4	Antagonism

Table 21. List of antimicrobial agents, zone size and MIC interpretive criteria for enterococci.

Antibiotics	Disc potency	Zone diameter interpretive criteria (mm)			MIC interpretive criteria (µg/ml)		
		S	I	R	S	I	R
Ampicillin (AMP)	10 µg	≥ 17	-	≤ 16	≤ 8	-	≥ 16
Gentamicin (HLG)	120 µg	≥ 10	7-9	6	≤ 500	-	> 500
Nitrofurantoin (NIT)	300 µg	≥ 17	15-16	≤ 14	≤ 32	64	≥128
Ciprofloxacin (CIP)	5 µg	≥ 21	16-20	≤ 15	≤ 1	2	≥ 4
Teicoplanin (TEC)	30 µg	≥ 14	11-13	≤ 10	≤ 8	16	≥ 32
Vancomycin (VAN)	30 µg	≥ 17	15-16	≤ 14	≤ 4	8-16	≥ 32
Linezolid (LNZ)	30 µg	≥ 23	21-22	≤ 20	≤ 2	4	≥ 8

Notes:

1. Perform MIC test for enterococci falling in intermediate zone for vancomycin.
2. Ciprofloxacin, nitrofurantoin tested only for urinary isolates.
3. HLG: Intermediate results: verify with agar dilution method.
4. Vancomycin readings should be taken after 24 hours incubation.
5. MIC creep: linezolid MIC-to be tested only against *Enterococcus* spp.

Special AMST for enterococci**Identification of vancomycin resistant enterococcus (VRE)**

Vancomycin screening agar (with 6 µg/ml of vancomycin) is used for identification of VRE isolates.

Procedure

1. Prepare 180 ml of BHI agar by dissolving 9.3 gram dehydrated media in 180 ml water.
2. Prepare stock solution of vancomycin containing 60 mg/10 ml of vancomycin stock solution- 1(S1).
3. Add 1 ml of S1 to 9 ml of sterile distilled water to make stock solution- 2 (S2).
4. Add 18 ml of sterile distilled water to 2 ml of S2,to make the working solution.
5. Add 20 ml of the working solution to 180 ml of the molten BHI agar and pour 20 ml agar into petridishes.
6. Suspend the test strain of *Enterococcus* in nutrient broth with a turbidity corresponding to 0.5 MacFarland standard. Spot inoculate on to the agar using a 10 µl loop. Include positive control (*Enterococcus faecalis* 51299) and negative control (*Enterococcus faecalis* 29212) on each plate.
7. Incubate for 24 hours at 37°C.
8. Appearance of even a single colony or film of growth indicates vancomycin resistance.

MIC for vancomycin by agar dilution method:

Perform MIC using brain heart infusion agar incorporated with vancomycin in concentrations ranging from 1 µg/ml to 32 µg/ml.

1. Prepare the vancomycin stock solutions at ten times the concentrations to be tested and make twofold dilutions of these stock solutions as per CLSI guidelines.
2. Add 2 ml of the antimicrobial solution to 18 ml of molten BHI agar at 50°C.
3. Allow the agar to solidify.
4. Inoculate the strains to be tested, in sterile normal saline to make a suspension with turbidity equivalent to 0.5 MacFarland standard.
5. Using 10 µl loop, inoculate a spot of the suspension on plates of different concentrations of vancomycin.
6. Incubate the plates at 35°C for 24 hours.
7. Always include a drug free control medium to check the viability of the strains.
8. Examine the plates both in reflected and transmitted light.
9. ATCC *Enterococcus faecalis* 29212 is used as QC for determining MIC values.

The lowest concentration of vancomycin on which the strain shows no growth is taken as the minimum inhibitory concentration of vancomycin for that strain.

MIC for other antibiotics by agar dilution method:

1. Prepare the stock solutions at ten times the concentrations to be tested and make twofold dilutions of these stock solutions.
2. Add the antimicrobial solutions to molten MH agar at 50°C.
3. Allow the agar to solidify.
4. Inoculate the strains to be tested in sterile normal saline to make a suspension with turbidity equivalent to 0.5 MacFarland standard.
5. Using 10 µl loop, inoculate a spot of the suspension on plates of different concentrations of antibiotics starting from low concentration.
6. Always include a drug free control medium to check the viability of the strains.
7. Incubate the plates at 35°C for 24 hours.
8. Examine the plates both in reflected and transmitted light.
9. ATCC *Enterococcus faecalis* 29212 is used as QC for determining MIC values.

The lowest concentration on which the strain shows no growth is taken as the minimum inhibitory concentration for that strain.

Mechanism of resistance: Phenotypic and genotypic tests and clonality tests

A. Enterobacteriaceae

1. Phenotypic tests for mechanism of resistance

- a. ESBL detection: ceftazidime and ceftazidime + clavulanic acid.
- b. AmpC- β lactamase detection: AmpC discs.
- c. Metallo-β lactamase detection: Imipenem 10 µg + 10 µl of 0.5 M EDTA.
- d. Modified Hodge test for carbapenemase detection: meropenem.

2. Genotypic tests for mechanism of resistance

- a. *TEM-1* and 2.
- b. *SHV* variants including *SHV-1*
- c. *OXA-1, OXA-4, OXA-38, OXA-48*.
- d. *CTX-M* Group 1, Group 2 and Group 9.
- e. *CTX-M* Group 8/25.
- f. ESBL: *ACC, MOX, DHA, CIT* and *EBC*.
- g. AmpC- β lactamases: *VEB, GES, PER*.
- h. Carbapenemases: *VIM, IMP, and KPC-1 to KPC-5*.
- i. *NDM-1*.

3. Molecular Clonality Tests

- a. PFGE
- b. MLST

B. Enteric fever *Salmonella*

1. Phenotypic tests for mechanism of resistance

ESBL detection in ceftriaxone resistant isolates.

2. Genotypic tests for mechanism of resistance

- a. *gyrA* gene
- b. *qnr* gene
- c. Efflux genes

3. Molecular clonality tests

- a. PFGE
- b. MLST

C. *Pseudomonas* spp

1. Phenotypic tests for mechanism of resistance

- a. ESBL detection: ceftazidime and ceftazidime + clavulanic acid.
- b. AmpC detection: ceftazidime/tazobactam and ceftazidime/tazobactam + cloxacillin.
- c. Carbapenemase detection
 - Preliminary screening Carba NP.
 - Combination disc test
 - Imipenem with aminophenylboronic acid
 - Imipenem with phenyl boronic acid
 - Imipenem with cloxacillin

Imipenem with EDTA

- d. Outer membrane porin loss detection: SDS PAGE- OprD.
- e. Detection of over-expression of efflux pumps: MIC- Levofloxacin with and without efflux pump inhibitor (EPI-PA β N)

2. Genotypic tests for mechanism of resistance

- a. AmpC genes: *CMY/CIT, DHA, ACC, ACT/MIR, FOX*
- b. ESBL genes: *TEM, SHV, CTX-M, PER, VEB, GES*
- c. Carbapenemase genes: *SPM, IMP, VIM, NDM, KPC, Oxa 48 like*

3. Molecular clonality tests

MLST: *acsA, aroE, guaA, mutL, nuoD, ppsA, trpE*

D. *Acinetobacter* spp

1. Phenotypic tests for mechanism of resistance

- a. ESBL detection: ceftazidime and ceftazidime + clavulanic acid.
- b. AmpC detection: ceftazidime/tazobactam and ceftazidime/tazobactam + cloxacillin.
- c. Carbapenemase detection
 - Preliminary screening CarbAcineto NP.
 - Combination disc test

Imipenem with aminophenylboronic acid

Imipenem with phenyl boronic acid

Imipenem with cloxacillin

Imipenem with EDTA

- d. Outer membrane porin loss detection: SDS Page- CarO.
- e. Detection of over-expression of efflux pumps: MIC: Levofloxacin with and without efflux pump inhibitor (EPI-PA β N)

2. Genotypic tests for mechanism of resistance

- a. AmpC genes: *CMY/CIT, DHA, ACC, ACT/MIR, FOX*
- b. ESBL genes: *TEM, SHV, CTX-M, PER, VEB, GES*
- c. Carbapenemase genes: *SPM, IMP, VIM, NDM, KPC, Oxa 48 like, Oxa 51, Oxa 23, Oxa 24, Oxa 58 like.*

3. Molecular clonality tests

MLST: *gltA, gyrB, gdhB, recA, cpn60, Gpi, rpoD.*

E. Diarrhoeagenic bacteria

1. Phenotypic tests for mechanism of resistance

Nil

2. Genotypic tests for mechanism of resistance

- a. Diarrhoeagenic *E. coli* and *Shigella*: *bla-OXA*, *dhfrIa*, *Sul 1*, *aac(6')-Ib-cr*
- b. *Vibrio cholerae*: *dfrA1*, *Sul 2*, *tetA*.
- c. *Clostridium difficile*: *nim*, *ermB*, *tet*
- d. *Campylobacter* spp.: *tetO*.

3. Molecular clonality tests

- a. Diarrhoeagenic *E. coli* and *Shigella*: *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, *recA*.
- b. *Vibrio cholerae*: *adk*, *gyrB*, *metE*, *mdh*, *pntA*, *purM*, *pyrC*.
- c. *Clostridium difficile*: *adk*, *atpA*, *dxr*, *glyA*, *recA*, *soda*, *tpi*, *tcdA*, *tcdB*, *tcdC*, *cdd1*, *cdv1*.
- d. *Campylobacter* spp.: *aspA*, *glnA*, *gltA*, *glyA*, *tkt*, *pgm*, *unca*.

F. *Staphylococcus* spp.

1. Phenotypic tests for mechanism of resistance

- a. Betalactamase detection: Penicillin zone edge test (cliff and beach pattern).
- b. MRSA latex agglutination test.
- c. D-test for inducible clindamycin resistance.

2. Genotypic tests for mechanism of resistance

- a. Cefoxitin resistant isolates: PCR for *mecA*.
- b. Mupirocin high level resistance: *mupA*, *mupB* genes.
- c. Inducible Clindamycin resistance: *ermA*, *ermB*, *ermC* genes.
- d. VRSA isolates: PCR for *vanA* genes.

3. Molecular clonality tests

- a. PFGE.
- b. MLST: *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, *yquiL*.

G. *Enterococcus* spp.

1. Phenotypic tests for mechanism of resistance

None.

2. Genotypic tests for mechanism of resistance

- a. VRE: PCR for *vanA*, *vanB*, *vanC*, *vanHAX* genes.
- b. Gentamicin high level resistance: PCR for *aac(6')-Ie*, *aph(2'')-Ia*, *aph(2'')-Ib*, *aph(2'')-Ic*, *aph(2'')-Id*, *aph(3')-IIIa*, *ant(4')-Ia* genes.

3. Molecular clonality tests

- a. PFGE.
- b. MLST: *Enterococcus faecalis*- *gdh*, *gyd*, *pstS*, *gki*, *aroE*, *xpt*, *yiqL*.
Enterococcus faecium: *adk*, *atpA*, *ddl*, *gdh*, *gyd*, *purK*, *pstS*.

Special Tests

Special Tests

Detection of ESBL: (CLSI, 2015)⁸

Table 22. CLSI recommended screening and confirmatory tests for ESBLs in *Klebsiella pneumoniae*, *K. oxytoca*, *Escherichia coli*, and *Proteus mirabilis* (only when clinically relevant, e.g. a bacteremic isolate):

Method	Initial screen test	Phenotypic confirmatory test
Medium	Mueller-Hinton agar	Mueller-Hinton agar
Antimicrobial disk content	<p>For <i>K. pneumoniae</i>, <i>K. oxytoca</i>, and <i>Escherichia coli</i>:</p> <p>Cefpodoxime 10 µg or ceftazidime 30 µg or aztreonam 30 µg or cefotaxime 30 µg or ceftriaxone 30 µg</p> <p>For <i>P. mirabilis</i>:</p> <p>Cefpodoxime 10 µg or ceftazidime 30 µg or cefotaxime 30 µg</p> <p>(The use of more than one antimicrobial agent for screening improves the sensitivity of detection).</p>	<p>Ceftazidime 30 µg Ceftazidime-clavulanic acid 30/10 µg and Cefotaxime 30 µg Cefotaxime-clavulanic acid 30/10 µg</p> <p>(Confirmatory testing requires use of both cefotaxime and ceftazidime, alone and in combination with clavulanic acid).</p>
Inoculum	Standard disk diffusion recommendations as described above	Standard disk diffusion recommendations as described above
Incubation conditions		
Incubation length		
Results	<p>For <i>K. pneumoniae</i>, <i>K. oxytoca</i>, and <i>E. coli</i>:</p> <p>Cefpodoxime zone ≤ 17 mm Ceftazidime zone ≤ 22 mm Aztreonam zone ≤ 27 mm Cefotaxime zone ≤ 27 mm Ceftriaxone zone ≤ 25 mm</p> <p>For <i>P. mirabilis</i>:</p> <p>Cefpodoxime ≤ 22 mm Ceftazidime ≤ 22 mm Cefotaxime ≤ 27 mm</p> <p>Zones above may indicate ESBL production</p>	<p>A ≥ 5-mm increase in a zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone = ESBL (e.g., ceftazidime zone = 16; ceftazidime-clavulanic acid zone=21).</p>

<p>QC recommendation</p>	<p>When testing ESBL-screening antimicrobial agents, <i>K. pneumoniae</i> ATCC® 700603 should be used for quality assessment (e.g., training, competency, or test evaluation). Either strain, <i>K. pneumoniae</i> ATCC® 700603 or <i>E. coli</i> ATCC® 25922, should be used for routine QC (e.g., weekly or daily).</p> <p><i>E. coli</i> ATCC® 25922:</p> <p>Cefpodoxime zone 23-28 mm Ceftazidime zone 25-32 mm Aztreonam zone 28-36 mm Cefotaxime zone 29-35 mm Ceftriaxone zone 29-35 mm</p> <p><i>K. pneumoniae</i> ATCC® 700603:</p> <p>Cefpodoxime zone 9-16 mm Ceftazidime zone 10-18 mm Aztreonam zone 9-17 mm Cefotaxime zone 17-25 mm Ceftriaxone zone 16-24 mm</p>	<p>When performing the ESBL confirmatory tests, <i>K. pneumoniae</i> ATCC® 700603 and <i>E. coli</i> ATCC® 25922 should be tested routinely (e.g., weekly or daily).</p> <p><i>E. coli</i> ATCC® 25922: ≤ 2-mm increase in zone diameter for antimicrobial agent tested alone versus its zone when test in combination with clavulanic acid.</p> <p><i>K. pneumoniae</i> ATCC® 700603: 5-mm increase in ceftazidime-clavulanic acid zone diameter; 3-mm increase in ceftriaxone-clavulanic acid zone diameter.</p>
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ESBL confirmatory tests

Phenotypic confirmatory testing depends on demonstrating a synergy between clavulanic acid and an indicator cephalosporin. These tests distinguish AmpC β-lactamases (not inhibited by β-lactamase inhibitors) from ESBLs. Numerous variations of confirmatory testing have been described but few are convenient for routine use: combined disk method, double disk (DD) approximation test, minimum inhibitory concentration (MIC) methods, and E-test ESBL strips. The CLSI recommends the combined disk method and the MIC method for ESBL confirmation and the same will be followed.

Combined disk test

The combined disk method depends on comparing the inhibition zones around disks containing an indicator cephalosporin with and without clavulanic acid. As per CLSI guidelines, 10 µg of clavulanic acid is added to each of a cefotaxime (30 µg) and a ceftazidime (30 µg) disk. If ESBL is produced, the zone diameters given by the disks with clavulanate are >5 mm larger than those without the inhibitor. A variation of the combination disk method is based on comparing the zone given by cefpodoxime (10 µg) and cefpodoxime/clavulanate (10 mg + 1 µg). ESBL production is inferred if the zones given by the disks with clavulanate are >5 mm larger than those without the inhibitor.

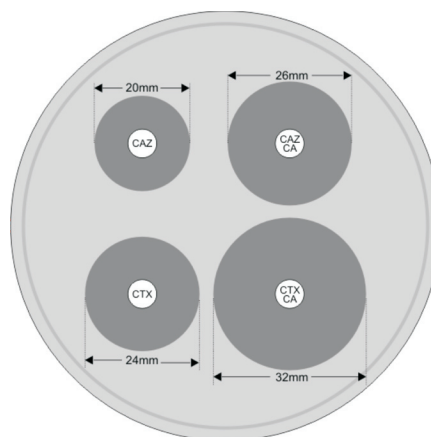


Fig. 1. Combined disk test

Double disk approximation test (DD)⁹

The ESBL-producing strains will be tested by the clavulanate double-disk approximation procedure.

- A plate is inoculated as described for a standard disk diffusion test.
- Disks containing aztreonam and expanded-spectrum cephalosporins are placed 30 mm (center to center) from an amoxicillin-clavulanate or clavulanic acid (10 mg) disk.
- After overnight incubation at 35°C, the production of an ESBL by the test organism is inferred by the presence of characteristic distortions/expansions of the inhibition zones towards the clavulanate disk indicative of clavulanate potentiation of the activity of the test drug.
- Negative double disk tests are repeated with a disk spacing of 20 mm (center to center).

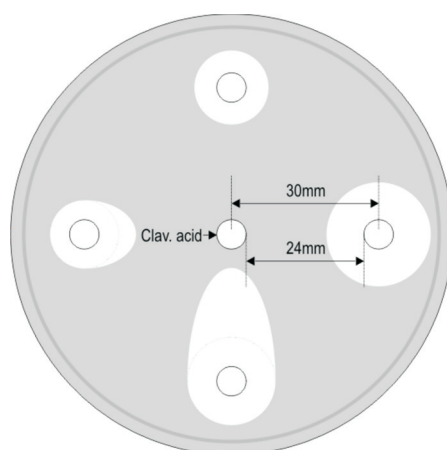


Fig. 2. Double disk approximation test

Detection of AmpC- β -lactamases

AmpC disk test¹⁰

The test is based on use of tris-EDTA to permeabilize a bacterial cell and release β -lactamases into the external environment.

AmpC disks (filter paper disks containing tris-EDTA) are prepared in-house by applying 20 μ l of a 1:1 mixture of normal saline and 100x tris-EDTA (1.0 M tris-HCl, pH approximately 8.0, containing 0.1 M EDTA, filter sterilized) to sterile filter paper disks, allowing the disks to dry, and storing them at 2 to 8°C. The surface of a Mueller-Hinton agar plate should be inoculated with a lawn of *E. coli* ATCC 25922 according to standard disk diffusion method. Immediately prior to use, AmpC disks will be rehydrated with 20 μ l of distilled water and several colonies of each test organism will be applied to the disk.

A 30 μ g cefoxitin disk is placed on the inoculated surface of the Mueller-Hinton agar. The inoculated AmpC disk will be placed almost touching the antibiotic disk with the inoculated disk face in contact with the agar surface. The plate will be inverted and incubated overnight at 35°C in ambient air. Plates will be examined for either an indentation or a flattening of the zone of inhibition, indicating enzymatic inactivation of cefoxitin (positive result), or the absence of a distortion, indicating no significant inactivation of cefoxitin (negative result).

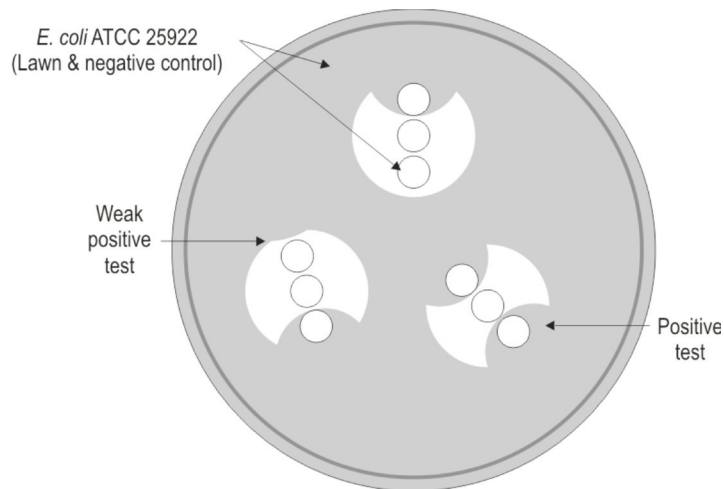


Fig. 3. AmpC disk test

Detection of Metallo- β -lactamases

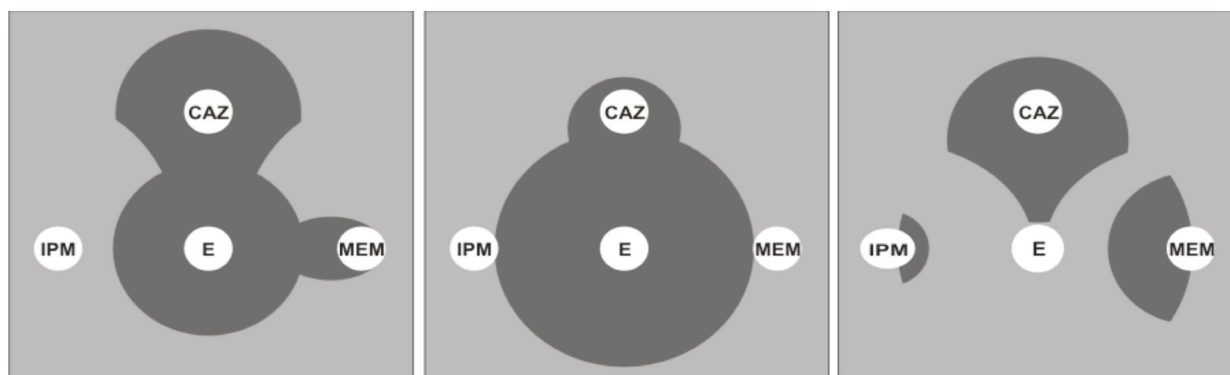


Fig. 4. EDTA- disk synergy test

EDTA-disk synergy (EDS) test

1. An overnight Luria broth liquid culture of the tested isolate will be diluted to a turbidity of a 0.5 McFarland standard and spread on the surface of a Mueller-Hinton agar plate.
2. A disk containing 10 μ g of imipenem (IPM) will be placed on the surface, and a second disk containing 10 μ l of 0.5 M EDTA will be placed 15 mm (edge-to-edge) from the first disk.
3. After incubating overnight at 37°C, the presence of an expanded growth inhibition zone between the two disks will be interpreted as positive for synergy.

Modifications introduced to increase the sensitivity of the EDS include the following:

- (i) The incorporation of MEM and ceftazidime (CAZ) to the original assay and
- (ii) Parallel test employing a disk with a lower EDTA concentration (10 ml of 0.1 M EDTA) placed 10 mm, edge to edge, from the other disk.

The EDS that includes both modifications is named the extended EDS (eEDS).

The use of various β -lactam and EDTA concentrations increases EDS sensitivity.

- (a) The use of only IPM in the EDS may fail to detect MBL producers, as shown for this *P. fluorescens* strain; clear synergistic zones are observed when MEM and CAZ are incorporated to the assay.

- (b) The use of 10 µl of 0.5 M EDTA may result in some cases in undesirable large growth inhibition zones, making MBL detection difficult.
- (c) The problem can be avoided by reducing the EDTA amount; the use of 10 µl of 0.1 M EDTA results in clear synergy zones, unequivocally revealing MBL production by this strain.

Double-disk synergy test (DDST) using mercaptopropionic acid (MPA)

A 0.5 McFarland bacterial suspension will be inoculated on a Mueller- Hinton (MH) agar plate. Imipenem disks will be aligned around blank filter disks containing 5 µl of the chosen mercaptopropionic acid (MPA) inhibitor solution added directly on the disk already placed on the MH agar plate. The distance between the inhibitor and substrates will be tested at 2.0 cm (from center to center). The appearance of either an enhanced or a phantom zone between the antimicrobial agents and the inhibitor disk will be considered a positive result and indicative of MBL production.

Combined disk method (CD)

To identify MBL production in the isolate IPM-EDTA disk synergy test developed by Yong D *et al*⁸ will be used. A 0.5 M EDTA solution containing 186.1 gm of disodium EDTA will be dissolved in 1000 ml of distilled water and pH adjusted to 8.0 by using NaOH. The mixture will be then sterilized by autoclaving. Imipenem disks will be prepared by adding EDTA solution to 10 µg imipenem discs to obtain an EDTA content of 750 µg. The disk will be dried immediately in an incubator and stored at 4°C.

The test strains will be adjusted to McFarland 0.5 standard and inoculated to Mueller Hinton agar. A 10 µg imipenem disk and an imipenem plus 750 µg EDTA disc will be placed on Mueller Hinton agar. Another disk containing only 750 µg EDTA will be also placed as a control. After overnight incubation, the established zone diameter difference of ≥7 mm between imipenem disk and imipenem plus EDTA will be interpreted as EDTA synergy positive.

Carbapenemase detection

Only when using the new interpretive criteria for carbapenems first published in June 2010 (M100-S20-U):

1. The initial screen test (described in Supplemental Table 2A-S3) and the confirmatory Modified Hodge test (MHT) are no longer necessary for routine patient testing.
2. The MHT may be useful for testing isolates for epidemiological or infection control purposes.
3. No change in the interpretation of carbapenem susceptibility test results is required for MHT-positive isolates.

Modified Hodge test (MHT)

Carbapenemase production is detected by the MHT when the test isolate produces the enzyme and allows growth of a carbapenem susceptible strain (*E.coli* ATCC 25922) towards a carbapenem disk. The result is a characteristic cloverleaf-like indentation. A positive MHT indicates that this isolate is producing a carbapenemase. A negative MHT indicates that this isolate is not producing a carbapenemase. The class of carbapenemase cannot be determined by the results of the MHT.

Briefly, a 0.5 McFarland dilution of the *E. coli* ATCC 25922 will be prepared in 5 ml of broth or saline, 0.5 ml of which will be added to 4.5 ml of MHB or saline to make a working dilution of

1:10. A lawn culture of the above will be made on a Mueller Hinton agar plate and allowed to dry for 3–5 minutes. A meropenem (10 µg) or ertapenem (10 µg) susceptibility disk will be placed in the centre of plate. The test organism will be streaked from the edge of the disk to the edge of the plate in a straight line. Up to four organisms can be tested on the same plate with one drug. Then plates will be incubated overnight at $35 \pm 2^\circ\text{C}$ in ambient air for 16–24 hours of incubation. After incubation the plates will be examined for a clover-leaf-type indentation at the intersection of the test organism and the *E. coli* 25922, within the zone of inhibition of the carbapenem susceptibility disk. The test organism having a clover-leaf-like indentation of the *E. coli* 25922 growing along the test organism growth streak within the disk diffusion zone will be considered as MHT positive. Isolates showing a slight indentation will not be considered to produce carbapenemase. With each test, positive and negative controls will be put viz. MHT positive *Klebsiella pneumoniae* ATCC BAA-1705 and MHT negative *Klebsiella pneumoniae* ATCC BAA-1706.

Quality Control (QC) in CLSI Method

Quality Control (Qc) in CLSI Method (2015)⁸

Reference strains for QC:

- *Escherichia coli* ATCC® 25922
- *Escherichia coli* ATCC® 35218 – ESBL detection
- *Klebsiella pneumoniae* ATCC® 700603 – ESBL detection
- *Pseudomonas aeruginosa* ATCC® 27853

Storing and testing QC strains (CLSI, 2015)

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 -20°C / -70°C / or liquid nitrogen, else the strains will be lyophilized. Working cultures will be stored on trypticase soya agar or CHOC at 2 to 8°C. 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.

Frequency of testing (CLSI, 2015)

Daily quality control testing will be advised at the beginning for at least 20-30 days. When no more than 1 out of 20 or 3 out of 30 consecutive tests turn out to be outside acceptable limit, performance will be considered satisfactory to proceed to weekly testing. If not, corrective action will be taken. Daily testing will also be required when manufacturer of agar media are changed or when converting from manual to automated system of reading results.

Along with weekly testing, QC will be performed whenever reagent component of the test is changed. If any weekly test result is out of range, corrective action will be required.

Quality control of media (CLSI, 2015)

Mueller Hinton agar or the various other broth and agar media not containing antimicrobials will always be kept overnight at 37°C for sterility checking prior to inoculation. Each batch or new lot of media will be checked with reference ATCC strains. Zone sizes and MICs obtained must be within acceptable CLSI limits. Else the batch will be rejected and any patient results obtained not reported.

Minimum Inhibitory Concentration (MIC) Testing

Minimum Inhibitory Concentration (MIC) Testing

Broth dilution MIC

The broth macrodilution MIC method will be used to measure (semiquantitatively) the *in vitro* activity of an antimicrobial agent against a bacterial isolate.

Broth macrodilution MIC testing¹¹

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

Inoculum

- 1) 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).
- 2) Make a one in hundred dilution of the suspension by adding 0.1 ml growth into 9.9 ml normal saline ($\sim 10^6$ cfu/ml).
- 3) Final inoculum will be $3-5 \times 10^5$ cfu/ml. For this we will add 0.25 ml of the above suspension into each tube.

Media

- 1) Double strength Mueller 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

- 1) Add 0.25 ml of the 4x antibiotic solution to each of the specified tubes.
- 2) To prepare the 4x solutions prepare the stock solutions of the antibiotic as follows:
 - a. 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)
 - b. 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).
 - c. 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).
 - d. 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 solutions¹⁵.

Final tube volume: 1 ml.

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.

Incubation: 35-37°C 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.

Agar dilution MIC testing¹¹

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 48°C-50°C 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 100 µg/ml stock 3 to get agar media containing 128 µg/ml to 0.25 µg/ml of media. Pour plates (90 mm); allow to solidify and dry. 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

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 ten dilution of this suspension (10⁷ cfu/ml). Carefully spot 1µl on to the agar plate to get the final inoculum of 10⁴ cfu. For sulphonamides spot 10³ cfu.

Controls: Use one negative control and positive control spot.

Incubation: 35-37°C 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.

MIC testing by E Test

Day 1

Prepare inoculum

Remove the E-test package from the freezer (-20°C) 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 overtly 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 the tube above the fluid level to remove excess liquid.

Streak the swab over the entire surface of the agar plate. Rotate the plate approximately 60° then repeat streaking motion. Rotate 60° 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 37°C 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).

**Molecular Mechanisms of Antimicrobial
Resistance in Clinical Isolates
(Nodal Center and Optional for
Regional Centers)**

Molecular Mechanisms of Antimicrobial Resistance in Clinical Isolates (Nodal Center and Optional for Regional Centers)

Molecular basis of resistance in representative indicator organisms (every six months 30 consecutive isolates each of *E. coli* and *Klebsiella* spp., *Proteus* spp., *Enterobacter* spp., *Citrobacter* spp., and other species of *Enterobacteriaceae*):

Salmonella

1. PCR for *gyrA* gene containing the quinolone resistance-determining region (QRDR)

Primers: 5' ATGAGCGACCTTGCGAGAGAAATTACACCG 3'

5' TTCCATCAGCCCTTCAATGCTGATGTCTTC 3'

Cycling profile:

94°C	2 minutes
94°C	30 seconds
70°C	60 seconds x 30 cycles
72°C	90 seconds
72°C	5 minutes

2. Molecular typing of representative isolates by PFGE and MLST.

Enterobacteriaceae

Polymerase chain reaction (PCR)

For 16 genes initially simplex assays will be standardised. The multiplex PCR assays described by Dallenne et al.¹² will be tried later. Table below shows the list of genes and primers to be used for PCR. Simplex PCR would be used for NDM-1.¹³

Table 23. PCR gene targets and primers to be used.

PCR name	β-lactamase targeted	Primers	Bp
Multiplex I TEM,SHV and OXA-1	TEM1 and TEM 2	F:CATTTCCGTGTCGCCCTTATTC R:CGTTCATCCATAGTTGCCTGAC	800
	SHV	F:AGCCGCTTGAGCAATTAAC 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:CGATATCGTTGGTGGTRCCAT	688
	Variants of CTX-M group 2	F:CGTTAACGGCAGATGAC 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: GCTTTGACTCTTTTCGGGTATTTCG	997
	LAT-1 to LAT-3, BIL-1, CMY-2 to CMY-7,CMY-12 to CMY-18 and CMY-21 toCMY-23	F: CGAAGAGGCAATGACCAGAC R:ACGGACAGGGTTAGGTTAGGATAGY	538
Multiplex IV Metallo beta lactamases and carbapene-mases	IMP	F: TTGACACTCCATTTACDG R: GATYGAGAATTAAGCCACYCT	139
	VIM	F: GATGGTGTGGTTCGCATA R: CGAATGCGCAGCACCCAG	390
	KPC	F: CATTCAAGGGCTTTCTTGCTGC R: ACGACGGCATAGTCATTTGC	538
Simplex	NDM-1	F: 5'-ACC GCC TGG ACC GAT GAC CA-3' R: 5'-GCC AAA GTT GGG CGC GGT TG-3'	264

bp, base pair product size

Molecular typing of isolates by PFGE

The strains will 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; Centers for Disease Control and Prevention, Atlanta, GA).

- 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-DR11 (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 indistinguishable if they have the same number and size of bands in a PFGE fingerprint pattern. Isolates are reconsidered to be closely related if their PFGE pattern differs by less than three bands.

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

DNA isolation

Whole genomic DNA will be extracted from overnight colonies grown on blood agar (Remel, Lenexa, KS) using the QIAamp DNA Mini Kit and the QIAcube instrument (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Multiplex PCR needs to be performed as a first screening step on all of the following *bla* genes: TEM, SHV, CTX-M, KPC, NDM, IMP, VIM, OXA-48, VEB, PER, GES, SPM and AmpC-types.

Detection of genes by multiplex PCR

A multiplex PCR will be used for the detection of β -lactamase genes: using primers presented in Tables 24, 25, 26 and 27. Total DNA will be subjected to multiplex-PCR using the Multiplex PCR kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Amplicons will be visualized in a 2% agarose gel containing ethidium bromide.

Table 24. Detection of bla_{AmpC} genes (Reaction 1, annealing temperature: 65°C)

Target genes	Primer pairs	Expected Amplicon size (bp)	Reference
MOX	F - GCTGCTCAAGGAGCACAGGAT R - CACATTGACATAGGTGTGGTGC	520 bp	Perez-Perez, Hanson, 2002
CIT	F - TGGCCAGAACTGACAGGCAAA R - TTTCTCCTGAACGTGGCTGGC	462 bp	
DHA	F - AACTTTCACAGGTGTGCTGGGT R - CCGTACGCATACTGGCTTTGC	405 bp	
ACC	F - AACAGCCTCAGCAGCCGGTTA R - TTCGCCGCAATCATCCCTAGC	346 bp	
EBC	F - TCGGTAAGCCGATGTTGCGG	302 bp	
	R - CTTCCACTGCGGCTGCCAGTT		
FOX	F - AACATGGGGTATCAGGGAGATG	190 bp	
	R - CAAAGCGCGTAACCGGATTGG		

Table 25. Detection of bla_{SHV}, bla_{TEM}, bla_{VEB}, bla_{PER}, bla_{GES}, and bla_{SPM} genes (Reaction 2, annealing temperature: 60°C)

Target genes	Primer pairs	Expected Amplicon size (bp)	Reference
TEM	F - CATTTCGGTGCGCCCTTATTC R - CGTTCATCCATAGTTGCCTGAC	800 bp	Dallenne <i>et al.</i> , 2010
SHV	F - CCTTTAAAGTAGTGCTCTGC R - TTCGCTGACCGGCGAGTAGT	119 bp	C. Lascols (in- house)
KPCy	F - TGTCACTGTATCGCCGTC R - CTCAGTGCTCTACAGAAAACC	1011 bp	Yigit <i>et al.</i> , 2001
NDM	F - CCGTATGAGTGATTGCGGCG R - GCCCAATATTATGCACCCGG	779 bp	Poirel <i>et al.</i> , 2011
GES	F - AGTCGGCTAGACCGGAAAG R - TTTGTCCGTGCTCAGGAT	399 bp	Dallenne <i>et al.</i> , 2010
SPM	F - AAAATCTGGGTACGCAAACG R - ACATTATCCGCTGGAACAGG	271 bp	Ellington, Woodford <i>et al.</i> , 2007
VEB	F - CATTTCGGATGCAAAGCGT R - CGAAGTTTCTTTGGACTCTG	648 bp	Dallenne <i>et al.</i> , 2010
PER	F - GCTCCGATAATGAAAGCGT R - TTCGGCTTGACTCGGCTGA	520 bp	

Table 26. Detection of bla_{CTX-M} group genes (Reaction 3, annealing temperature: 52°C)

Target genes	Primer pairs	Expected Amplicon size (bp)	Reference
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CTX-M1	F - AAAAATCACTGCGCCAGTTC R - AGCTTATTCATCGCCACGTT	415 bp	Woodford, 2003
CTX-M2	F - CGACGCTACCCCTGCTATT R - CCAGCGTCAGATTTT TCA GG	552 bp	
CTX-M9	F - CAAAGAGAGTGCAACGGATG R - ATTGGAAAGCGTTCATCACC	205 bp	
CTX-M8	F - TCGCGTTAAGCGGATGATGC	688bp	
CTX-M25	F - GCACGATGACATTCGGG	347 bp	
CTX-M8-M25	R - AACCCACGATGTGGGTAGC		

Table 27. Detection of bla_{IMP}, bla_{VIM} and bla_{OXA-48} genes (Reaction 4, annealing temperature: 59°C)

Target genes	Primer pairs	Expected Amplicon size (bp)	Reference
IMP2	F - GGAATAGAGTGGCTTAAYTCTC R - GGTTTAAAYAAAACAACCACC	232 bp	Poirel <i>et al.</i> , 2011
VIM	F - GATGGTGTGGTGGTCGCATA R - CGAATGCGCAGCACCAG	390 bp	Dallenne <i>et al.</i> , 2010
OXA-48	F - GCTTGATCGCCCTCGATT R - GATTTGCTCCGTGGCCGAAA	281 bp	Poirel <i>et al.</i> , 2011

Genotyping of *Staphylococcus* species:

Genotypic confirmation of antibiotic resistance

Table 28. PCR for the detection of antibiotic resistance genes.

Target genes	Primer pairs	Expected amplicon size (bp)	Reference
<i>mec A</i>	F- TGGCATTTCGTGTCACAATCG R- CTGGAACCTTGTGAGCAGAG	310	Zhang <i>et al.</i> , 2004
<i>mup A</i>	F - TATATTATGCGATGGAAGTTGG R- AATAAAATCAGCTGGAAAGTGTG	457	Seah <i>et al.</i> , 2012
<i>mup B</i>	F- CTAGAAGTCGATTTTGGAGTAG R- AGTGTCTAAAATGATAAGACGATC	674	Seah <i>et al.</i> , 2012
<i>ermA</i>	F- GCGGTAAACCCCTCTGAG R- GCCTGTGCGGAATTGG	434	Schlegelova <i>et al.</i> , 2008
<i>ermB</i>	F- CATTAAACGACGAAACTGGC R- GGAACATCTGTGGTATGGCG	425	
<i>ermC</i>	F- ATCTTTGAAATCGGCTCAGG R- CAAACCCGATTCCACGATT	295	
<i>vanA</i>	F-GGGAAAACGACAATTGC R-GTACAATGCGGCCGTTA	732	Dutka-Malen <i>et al.</i> , 1995

Storage of isolates:

1. Lyophilisation, where facilities are available.
2. Alternately, where lyophilization facilities are not available, cultures can be stored in 50% glycerol stock at -20°C/-80°C.
(50% glycerol stock- 1 ml of sterile glycerol + 1 ml of overnight Luria Bertani broth culture of the isolates).

Table 29. Genotypic confirmation of antibiotic resistance in Enterococci:

Gene	Primers(5'-3')	Expected amplicon size (bp)	Reference
vanA	F-GGGAAAACGACAATTGC R-GTACAATGCGGCCGTTA	732	Duttka- Malen <i>et al</i> , 1995
vanB	F-ATGGGAAGCCGATAGTC R-GATTCGTTCTCGACC	635	
vanC-1	F-GGTATCAAGGAAACCTC R-CTTCCGCCATCATAGCT	822	
vanC2/C3	F-CTCCTACGATTCTCTTG R-CGAGCAAGACCTTTAAG	439	
<i>aac(6)-Ie-aph(2)-Ia</i>	F-CAGAGCCTTGGGAAGATGAAG R-CCTCGTGTAATTCATGTTCTGGC	348	Vakulenko <i>et al</i> , 2003
<i>aph(2)-Ib</i>	F-CTTGGACGCTGAGATATGAGCAC R-GTTTGTAGCAATTCAGAAACACCCTT	867	
<i>aph(2)-Ic</i>	F-CCACAATGATAATGACTCAGTTCCC R-CCACAGCTTCCGATAGCAAGAG	444	
<i>aph(2)-Id</i>	F-GTGGTTTTTACAGGAATGCCATC R-CCCTCTTCATACCAATCCATATAACC	641	
<i>aph(3)-IIIa</i>	F-GGCTAAAATGAGAATATCACCGG R-CTTTAAAAAATCATACAGCTCGCG	523	
<i>ant(4)-Ia</i>	F-CAAACCTGCTAAATCGGTAGAAGCC R-GGAAAGTTGACCAGACATTACGAACT	294	

External Quality Assurance Scheme (EQAS)

External Quality Assurance Scheme (EQAS)

Purpose:

The objectives of this SOP are:

1. To ensure that the EQAS packs are sent at regular pre-defined intervals to all network sites to get a feedback on the performance of the laboratories.
2. To ensure that all the network sites participate in EQAS program and report the results on time.
3. To ensure that summary report for all sites is generated and sent on time.

Scope of this SOP:

This document applies to participating microbiology laboratories in the network:

Responsibilities:

1. The research officer at reference laboratory will be responsible for sending the EQAS packs as per schedule and preparing the report after the results are received. The research officer will also be responsible for giving a feedback to individual sites about their performance and follow up with sites in case the results are not received on time.
2. The site microbiologists and site coordinators will be responsible for performing the required tests and sending the results on time.
3. The Co-ordinator will be responsible for overall functioning of EQAS in a timely manner.

Procedures:

1. An EQAS pack containing 4 challenge strains will be sent to each participating microbiology laboratory from the laboratories at JIPMER, Puducherry (*Staphylococcus* and *Enterococcus* spp) and CMC, Vellore (Gram negative bacteria) every 3 months.
2. The packs will be sent by courier.
3. Each pack will be accompanied by instructions for recovery of lyophilized cultures and a hard copy of the EQAS Data Collection Sheet for entering the results. The sites will also be provided with soft copies of data collection forms.
4. The research officer at reference laboratory will record the dates when the pack is sent out to each site, in EQAS Tracking Log.
5. The site coordinator/ microbiologist on site will be requested to acknowledge the receipt of pack.
6. The research officer at Reference Laboratory will note down the dates of receipts of packs at each site in EQAS Tracking Log.
7. Each site will be given 15 days after the receipt of pack for performing the required tests and reporting the results.
8. The Research Officer at reference laboratory will calculate the dates when results are expected from each site based on the dates of receipts of packs at each site. The expected dates will be entered in the EQAS Tracking Log.
9. The microbiologist on site will follow the instructions given for recovery of lyophilized cultures and perform the required tests for identification of strains depending on the site

of isolation as provided with the specimen.

10. The identification of strains should be till the species level.
11. Identification of subtypes and groups should be done if possible.
12. After identification, the microbiologist will isolate the organism and perform the antimicrobial susceptibility test (AST).
13. The AST will be done by disk diffusion as well as minimum inhibitory concentration (MIC).
14. The resistance mechanisms (like ESBL) if detected should also be reported.
15. The test results will be entered in the data collection sheet by site microbiologist.
16. The site coordinator will check the data collection sheet for completeness before it is sent to reference laboratory.
17. The data collection sheets will be sent to the reference laboratory by e-mail. A copy of the email will be marked to Co-ordinator. The research officer will compile the results from all sites into a summary report format.
18. The research officer will send a feedback to individual sites by e-mail regarding their performance. A comparison of the results obtained by site and the results obtained by reference laboratory will be sent in a form format.

Procedures for follow up in case of deviations from required time lines:

1. If no acknowledgement for receipt of pack is received from sites within 4 days of sending the pack, the research officer will send an email to the concerned site to find out whether the samples have been received.
2. If no acknowledgement is received from sites within one week of sending packs, the research officer will send email to concerned sites to find out whether the samples have been received.
3. The research officer will send an e-mail reminder to site if the results are not received by expected date. A copy of the e-mail will be marked to Co-ordinator.
4. The Co-ordinator will contact the site microbiologist by phone in case no reply to the email is received within 5 days.
5. The follow up with sites will be continued until the results/responses are obtained. Copies of second and subsequent reminders will be marked to project coordinators.
6. The Co-ordinator will follow up with the research officer if the EQAS report is not sent out by the expected date.
7. All correspondence related to EQAS will be saved for future reference.

Contact Addresses for EQAS Scheme

1. Gram negative bacteria -Dr V Balaji

Mail ID- vbalaji@cmcvellore.ac.in

2. Staphylococci and enterococci – Dr SC Parija

Mail ID- subhashparija@yahoo.co.in

**The Microbiology External Quality Assessment Reference Unit
Proficiency Testing Programme
Bacteriology Survey**

Laboratory Region			
Survey shipment date:		Date received by participant:	
Survey closing date :		Date returned to Reference Lab:	
		Date evaluated :	
<u>Specimen no</u>	Participating Lab result	Expected result	Score
Microscopy			
Final identification			
Guideline			
AST choice			
AST results			
MIC			
MIC results			
Assessment			
Grading Total :		Percentage :	

Scoring System followed in the proficiency testing

Grading categories	Mark Allocation
Microscope (Sample and culture)	4,3,1,0
Culture and ID , Final organism identification	4,3,1,0
Choice of antibiotics	4,3,1,0
Susceptibility results	4,3,1,0
Total (Max score)	16 (4 x 4)
Percentage	100

Numerical grading scheme guideline

Score	Interpretation	Definition
4	Full value	Accepted by the committee as the correct answer either in terms of current nomenclature or in terms of appropriate clinical relevancy
3	Essentially correct or acceptable	A nomenclature or susceptibility error, generally at the species level not technically correct but would have little or no clinical impact. A deviation from what is considered the most clinically relevant result
2	Separator	To augment the difference between two grading groups
1	Incorrect or unacceptable	A nomenclature error that would be wrong at the species level but by reporting may have an impact on clinical interpretation and potentially a treatment error. A major susceptibility error. A clinically relevant result that could lead to diagnosis or treatment error.
0	Very incorrect or very unacceptable	A nomenclature error that would be wrong at either genus or the species level or major susceptibility error that could result in significant interpretation and or treatment error. A clinically relevant result that could lead to major diagnosis or treatment error

Recovery of Lyophilized Cultures

Opening of an ampoule

1. 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.
2. 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.
3. The plug should be removed with forceps. The discarded plug and the pointed end of the ampoule should be put into a lotion jar.

Rehydration and recovery

1. Flame the open end of the tube and add about 0.3-0.5 ml of nutrient broth / trypticase soya broth / chocolate broth using a sterile Pasteur pipette.
2. Mix the contents carefully so as to avoid frothing.
3. Subculture a loop-full of broth suspension onto appropriate media.
4. Transfer the rest to a tube containing 1 ml of appropriate broth.
5. Incubate both broth and plate cultures in aerobic / CO₂ tin/incubator at 35-37°C.

**Surveillance of Carriage of MDR
Enterobacteriaceae in the
General Population**

Surveillance of Carriage of MDR Enterobacteriaceae in the General Population

Subjects and sites of collection of samples

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

1. One hundred patients being admitted to Nehru Hospital, PGIMER, Chandigarh on admission (day zero of hospital stay)
2. One hundred patients attending the outpatient department of PGIMER for unrelated complaints (hypertension, gynaecology and obstetrical conditions and dermatology, etc)
3. 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:
 - a. Primary health care settings include private practitioners based in Panchkula
 - b. Secondary care setting includes patients presenting at ESI hospital, Ram Darbar, Chandigarh
 - c. Field setting (Anganwadi 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 Anganwadis).

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

1. Diabetes mellitus
2. Pregnancy
3. Any immunosuppressive disorder or medication history
4. History of recent (within 3 months) consumption of antibiotics
5. 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 tests¹⁴

**Surveillance of Antimicrobial Resistance
in Environmental Isolates (Nodal Center
and Optional for Regional Centers)**

Surveillance of Antimicrobial Resistance in Environmental Isolates (Nodal Center and Optional for Regional Centers)

Sites from which environmental samples to be collected

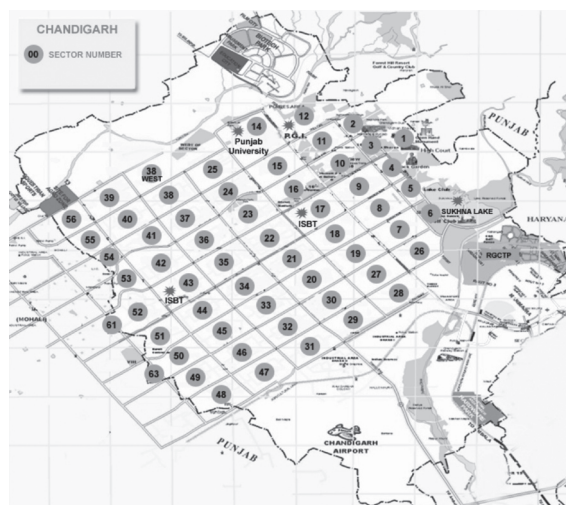
Chandigarh is divided into 62 sectors (numbered 1-63 excluding 13). Sewage samples will be collected from major drainage points of sector 12 (drains PGIMER, Chandigarh), sector 4, sector 17, sector 22 (drains a major bus terminal of Chandigarh), sector 32 (drains Government Medical College and Hospital, 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 cm×150 cm 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.¹⁵ 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 heavy paper 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 in place 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.¹⁴

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 -80° C in trypticase soya broth with glycerol for long term storage.

Annexure – 1

Annexure – 1

Important biochemical reactions

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 35°C 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. The control should remain yellow.

ONPG positive:

Escherichia coli

Salmonella choleraesuis subsp. *arizonae*

Neisseria lactamica

ONPG negative:

Proteus mirabilis

Neisseria meningitidis

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 ₂ PO 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 121°C for 15 minutes. Sterilize the glucose solution by filtration and add 0.25 ml to each tube (final concentration 0.5%).

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 4°C overnight. Warm to 45°C to dissolve the gelatin. Adjust to pH 8.4 and steam for 10 minutes. Cool quickly to 45°C 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 115°C. Remove from autoclave as quickly as possible and keep at low temperature (< 22°C).

(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

S.aureus

P.mirabilis

P.vulgaris

Negative

Listeria monocytogenes

Indole

Use:

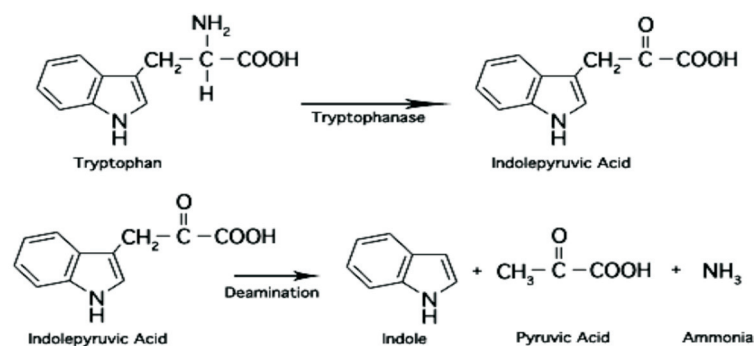
To determine the ability of an organism to split indole from tryptophan.

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 (NH₂) group of the tryptophan molecule is removed. Final products of the reaction are indole, pyruvic acid, ammonia (NH₃) and energy. Pyridoxal phosphate is required as a coenzyme.



Procedure:

Inoculate peptone broth with the test organism and incubate at 35°C 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.

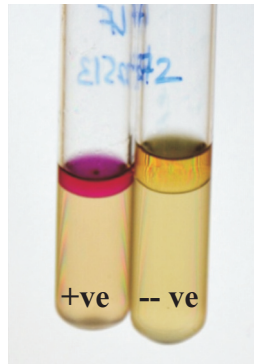
Result:

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*E.coli**P. vulgaris**P. rettgeri***Negative***Klebsiella**Citrobacter freundii***Methyl red****Use:**

The medium most commonly used is methyl red-Voges Proskauer broth, as formulated by Clark and Lubs. This medium also serves for the performance of the VP test.

Ingredients and preparation:

Polypeptone	7.0 g
Glucose	5.0 g
Dipotassium phosphate	5.0 g
Water	1.0 litre
pH	6.9
Methyl red pH indicator	
Methyl red, 0.1g in 300 ml of 95% ethyl alcohol	
Distilled water, 200 ml	

Principle and interpretation:

Methyl red is a pH indicator with a range between 6.0 (yellow) and 4.4 (red). The pH at which methyl red detects acid is considerably lower than the pH for other indicators used in bacteriologic culture media. Thus, to produce a color change, the test organism must produce large quantities of acid from the carbohydrate substrate being used.

The methyl red test is a quantitative test for acid production, requiring positive organisms to produce strong acids from glucose through the mixed acid fermentation pathway. Because many species of the Enterobacteriaceae may produce sufficient quantities of strong acids that can be detected by MR indicator during the initial phases of incubation, only organisms that can maintain this low pH after prolonged incubation, overcoming the pH –buffering system of the medium, can be called MR—positive.

The development of stable red color in the surface of the medium indicates sufficient acid production to lower the pH to 4.4 and constitutes positive test.

Procedure and interpretation:

Inoculate the glucose phosphate broth with a pure culture of the test organism. Incubate the broth at 35°C 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**

E.coli

Shigella spp.

Negative

Enterobacter aerogenes

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

Enterobacter aerogenes

Escherichia coli

Proteus mirabilis

Negative

Staphylococcus aureus

Shigella sonnei

Klebsiella pneumoniae

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 culture on agar medium and incubate at 35°C 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

Enterobacteriaceae

M.tuberculosis

Negative

Neisseria gonorrhoeae

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 121°C 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 appearance of yellow colour.

Oxidative

Alkaligenes spp.

Pseudomonas spp.

Fermentative

Aeromonas spp

Staphylococcus spp (except *S. saprophyticus*)

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.

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°C 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

Proteus vulgaris

Providencia spp

Negative

E.coli

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 ₄ H ₂ PO 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 121°C 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

Klebsiella

Salmonella

(except *S. Typhi* and *S. Paratyphi A*)

Proteus rettgeri

Negative:

E.coli

Shigella

Edwardsiella



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%
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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.

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 121°C 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

**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

Proteus

K. pneumoniae (weakly)

Negative

E.coli

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:

1) α -naphthol 5% color intensifier

α -naphthol	5.0 g
Absolute alcohol	100 ml

2) 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°C. 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

Enterobacter aerogenes

Negative

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 – 2

Annexure – 2

Preparation of Bacteriological Media and Reagents

a. 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 121°C for 15 minutes. Add 30 ml to each bottle under strict aseptic conditions. Incubate all bottles at 37°C for 48 hours to check sterility.

Use:

This medium is used for direct inoculation of blood for culture.

b. 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.

c. 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 4°C 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 121°C for 15 minutes. Filter through filter paper and distribute in screw capped prescription bottles in 50 to 100 ml amounts. Autoclave once more at 115°C 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 121°C for 20 minutes.

Medium	Difco		Hi-Media	
	Grams per Litre	Code	Grams per Litre	Code
Brain heart infusion agar	52	241830	52	M211
Brain heart infusion broth	37	237500	37	M210

Use:

This is used for direct inoculation of whole blood, bone marrow and body fluids for culture.

d. 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 50°C add the blood and mix well. After the addition of blood, heat in a water bath. Slowly bringing up the temperature to 75°C 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 50°C 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*.

e. 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.

f. 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:

NAD	50.0 mg.
Dist. Water	10.0 ml

Sterilize by filtration.

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 .

g. 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 121°C 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.

h. 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 121°C 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.

i. Nutrient agar (NA)

Agar powder	1.5 to 1.8 g
KH ₂ PO ₄	0.05 g
NaH ₂ PO ₄	0.05 g
Nutrient broth	100.0 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.

j. Nutrient Broth (NB)

Peptone	1.0 g
Beef extract (Lab Lemco)	0.4 g
Sodium chloride	0.5 g
Distilled water	100.0 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 121°C for 20 minutes.

Mediumcomplete	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.

k. 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 ± 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°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 121°C 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.

l. 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.

m. Trypticase soya broth (TSB)

TSB is made from commercially available dehydrated medium, Difco™ 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 121°C 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.

n. 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*.

o. 10% skim milk and 15% glycerol solution

10% skim milk and 15% glycerol solution is used for long term preservation of isolates by freezing at -70°C.

Media preparation

1. Place 10 g dehydrated skim milk and 85 ml distilled water into flask A. Swirl to mix.
2. Place 15 ml of glycerol into flask B.

3. Autoclave both flasks at 115°C for 10 minutes, and exhaust the pressure carefully.
4. While still hot, pour the contents of flask A into flask B in a safety cabinet.
5. 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% CO₂ (or in a candle-jar) to verify the sterility of the solution.

Passing result: no growth should be observed.

p. 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.

For quality control check for all the above media see chart for quality control in this section

q. 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 resistant 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 (for checking of 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)

Staphylococcus aureus ATCC 38591 (beta-lactamase positive)

Staphylococcus aureus ATCC 43300 (oxacillin resistant)

Staphylococcus aureus ATCC 29213 (beta-lactamase positive)

Stock cultures should be kept at -70°C 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.

r. McFarland standard

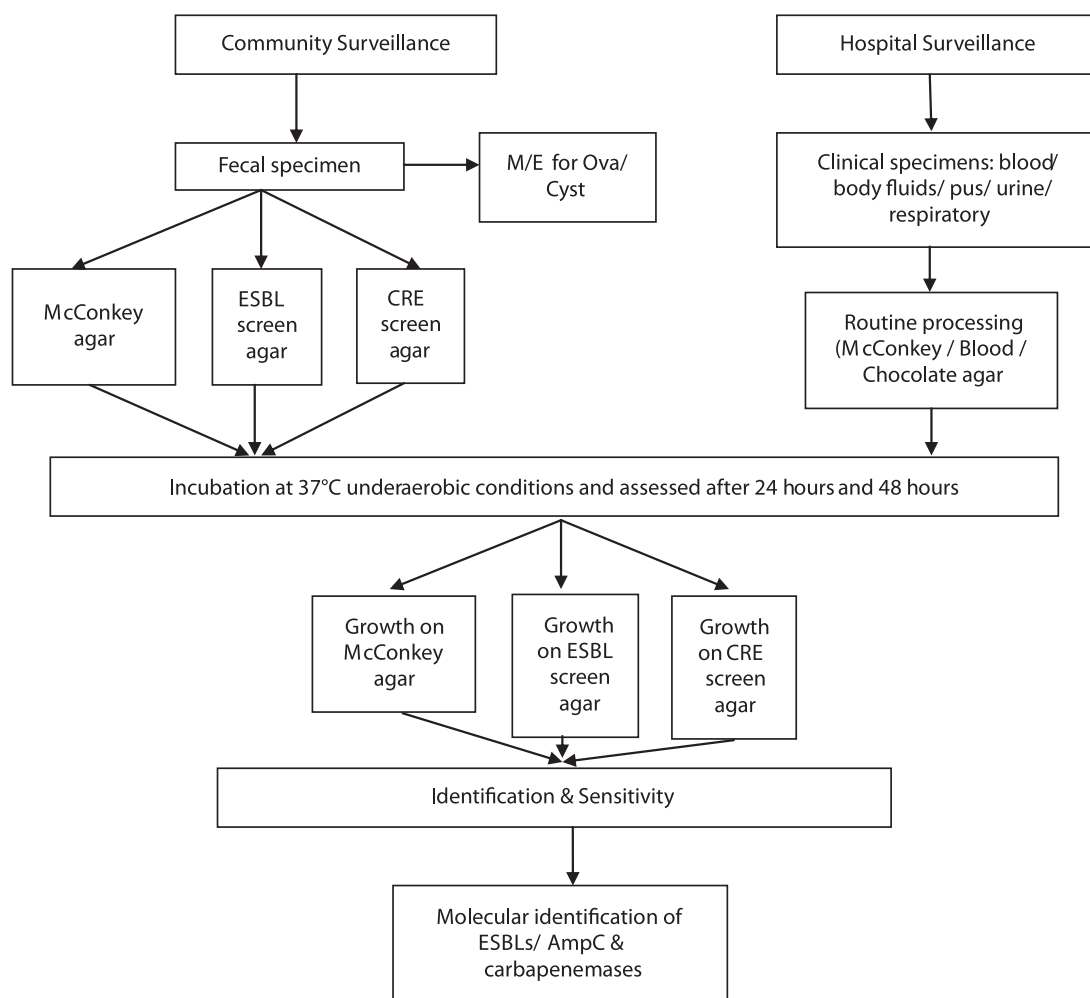
A barium chloride 0.5 McFarland density standard solution is prepared by adding 0.5 ml of a 1.175% (v/v) barium chloride dihydrate ($\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$) to 99.5 ml of 1% sulfuric acid. The resulting mixture is placed in a tube identical to that used for preparing the dilution for 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.

Note: These kits are also suitable to use. The protocol differs slightly between different manufacturer's kits hence a strict adherence to kit instruction must be followed.

s. Quality Control of Media

MEDIA	ORGANISMS	EXPECTED RESULTS
Blood agar	<i>S. pyogenes</i>	Small colonies with beta haemolysis
	Viridans streptococcus	Small colonies with alpha haemolysis
	<i>S. pneumoniae</i>	Alpha haemolytic smooth/mucoid colonies
Chocolate agar	<i>H. influenzae</i>	Translucent colonies
McConkey agar	<i>E. coli</i>	Non-mucoid lactose fermenting colonies
	<i>P. mirabilis</i>	Non-lactose fermenting colonies; no swarming
	<i>E. faecalis</i>	Magenta pink colonies
	<i>V. cholerae</i>	Non lactose fermenting colonies with dew drop appearance.
Mueller-Hinton Agar	<i>S. aureus</i>	Good growth/ATCC 29212 <i>E. faecalis</i> – sxt>20mm satisfactory
Mueller-Hinton blood agar/TSBA	<i>S. pneumoniae</i>	Large alpha haemolytic colonies with pitting
Nutrient agar	<i>S. aureus</i>	Pigmented colonies
Nutrient broth	<i>E. coli</i>	Growth
	<i>S. aureus</i>	Growth
	<i>P. aeruginosa</i>	Growth with pellicle
Thioglycollate broth	<i>B. fragilis</i>	Look for growth and smear morphology
	<i>C. perfringens</i>	
BPMM	<i>E. coli</i>	Non-mucoid lactose fermenting colonies
	<i>S. Typhi</i>	Non Lactose fermenting colonies
HTM	<i>H. influenzae</i>	Small translucent colonies

Flowchart of Workflow for Enterobacteriaceae



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