STANDARD OPERATING PROCEDURES

BACTERIOLOGY

2025

Government of Nepal
Ministry of Forests and Environment
Department of Environment
Bararmahal, Kathmandu

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Disclaimer

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Foreword

Government of Nepal
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Ref. No: Date:

Foreword

Antimicrobial resistance (AMR) is a critical and growing threat to public health, animal health food safety, and the environment globally. Nepal is not immune to its impacts. In line with the National Action Plan on AMR (NAP-AMR), the Government of Nepal has prioritized the establishment of a robust AMR surveillance system across human, animal, food, and environment sectors. As part of this multi-sectoral approach, the Department of Environment is planning to initiate AMR surveillance in the environmental sector.

Recognizing the need for standardized and reliable laboratory practices in this emerging area, the Department of Environment has developed and finalized the *Standard Operating Procedure (SOP) – Bacteriology in Environment Sector*in 2025. This SOP is a foundational step toward enabling systematic surveillance of AMR in environmental samples, particularly focusing on effluents from pharmaceutical industries and hospitals that serve as potential reservoirs and transmission routes for resistant pathogens.

The SOP outlines essential methods for the collection, processing, isolation, identification, and antimicrobial susceptibility testing of bacterial isolates from environmental sources. It is designed to support consistency, accuracy, and quality in laboratory operations, ensuring data generated through environmental AMR surveillance contributes meaningfully to the national evidence base.

Great thanks to Fleming Fund Country Grant for Nepal/FHI 360 for the technical assistance in preparation of this SOP.

I am confident that this SOP will serve as a key resource for laboratory professionals and institutions working in the environmental sector and will play an important role in advancing Nepal's AMR surveillance efforts under the One Health approach.

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Contents

Abbreviations and Acronyms

A/C	Air conditioners
AMC	Annual Maintenance Contract
AMR	Antimicrobial Resistance
AST	Antibiotic Susceptibility Test
BAA	Bile Aesculin Agar
BHI	Brain Heart Infusion
BPA	Baird Parker Agar
BPW	Buffered Peptone Water
BSC	Biosafety Cabinet
BTB	Bromothymol Blue
BSS	Biosafety and Biosecurity
CMA	Cetyl Milk Agar
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standards Institute
CO2	Carbon dioxide
CONS	Coagulase Negative Staphylococcus Species
DW	Distilled Water
EMB	Eosin Methylene Blue
GN	Gram Negative
H2S	Hydrogen Sulfide
ISO	International Organization for Standardization
HCWM	Healthcare Waste Management
HEPA	High Efficiency Particulate Air
LF	Lactose Fermenter
LIA	Lysine Iron Agar
LPF	Low Power Field

MA	MacConkey Agar
MHA	Mueller Hinton Agar
MIC	Minimum Inhibitory Concentration
MR	Methyl Red
MRD	Maximum Recovery Diluent
MRSA	Methicillin-Resistant Staphylococcus aureus
MSA	Mannitol Salt Agar
NB	Nutrient broth
NA	Nutrient Agar
NLF	Non-Lactose Fermenter
OIF	Oil Immersion Field
PPE	Personal Protective Equipment
PW	Peptone Water
QC	Quality Control
QCM	Quality Control Management
RT	Room Temperature
SIM	Sulfide Indole Motility
SOP	Standard Operating Procedure
TSI	Triple Sugar Iron
VP	Voges-Proskauer
WHO	World Health Organization
XLD	Xylose Lysine Deoxycholate
ZOI	Zone of Inhibition

Government of Nepal Ministry of Forests and Environment Department of Environment	Document Code: SOP 1	Page
Subject Title: Good Laboratory Practices in Environment Microbiology Laboratory		
Effective date: April 2025		Version no. 1
Prepared by: Anjani Kumar Adhikari	Reviewed and Approved by: Sailesh Kumar Jha	

1. Purpose

Good Microbiology Laboratory Practices guide the laboratory professional to follow the good microbiological practices to generate quality laboratory results. Standard microbiological laboratory practices are crucial for reliable identification of bacteria in environment samples. The standard laboratory practices need to be followed by all the staff working in the environmental microbiology laboratory.

2. Scope

Microbiological laboratory procedures are highly specialized and need strict adherence to good microbiological laboratory practices and stringent quality control. The laboratory personnel must be conscientious in minimizing the health hazards, the good laboratory practices to generate quality results. The SOP applies to all laboratory procedures conducted at environmental microbiology laboratory.

3. Requirements

3.1. Personal Protective Equipment (PPE): Laboratory coat, mask, gloves, safety goggles, boot, or disposable shoe cover, disposable head cover, face shield or visor.
3.2. First aid box
3.3. Spillage tool kit
3.4. Color-coded bins, biohazard sign label
3.5. Hand wash
3.6. Sanitization point
3.7. Eye wash station

4. Procedure

4.1. Standard microbiological safety practices

4.1.1. Display the standard biohazard warning sign on the doors of the rooms.

Note: Posted information must include: the laboratory's biosafety level, the supervisor's name (or other responsible personnel), telephone number, and required procedures for entering and exiting the laboratory.

4.1.2. Allow only authorized persons to enter the laboratory working areas.
4.1.3. Keep the laboratory doors closed.
4.1.4. Keep the laboratory locked when not in use.

4.2. Personal protection

4.2.1. Always wear the properly fitting, appropriate laboratory apron/gown during work in the laboratory.

Note: This protective clothing must be removed and kept in the laboratory before leaving for non-laboratory areas, such as offices or cafeteria.

4.2.2. Wear appropriate gloves for all procedures that may involve director accidental contact with any potentially infectious materials
4.2.3. Remove the gloves carefully after use and dispose in suitable color-coded bins and wash hands with soap and water.

Note: 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. Avoid touching the telephone, computer, or open doors with gloves that have been used in laboratory procedures.

4.2.4. Wash hands after handling infectious materials, and before leaving the laboratory working area.
4.2.5. Wear safety glasses, face shields (visors) or other protective devices when it is necessary to protect the eyes and face from splashes and impacting objects.
4.2.6. Wear closed-toed foot wear in laboratories.
4.2.7. Hair must be tied
4.2.8. Do not eat, drink, smoke or apply cosmetics in the laboratory working areas.
4.2.9. Do not store food or drink in the laboratory working areas

Note: Specimens should be assumed to be positive for pathogens and all procedures requiring handling of infectious materials, potentially infectious materials or specimens should be performed wearing appropriate PPE.

4.3. Technical procedures

4.3.1. Donot pipette by mouth.
4.3.2. Donot keep materials in the mouth or lick labels.
4.3.3. Minimize formation of aerosols and droplets during performance of all technical procedures.
4.3.4. Report all spills, accidents and overt or potential exposures to infectious materials to the laboratory supervisor. Keep a written record of such accidents in incident reports.
4.3.5. Use the spillage toolkit to contain any spillage of chemicals, culture media.
4.3.6. Decontaminate the contaminated liquids (chemically or physically) before disposing to the sanitary sewer.
4.3.7. Carry out all procedures in a way to minimize the risks of spills, splashes and the production of aerosols.

4.4. Laboratory working areas

4.4.1. Keep the laboratory neat, clean and free of materials that are not pertinent to work.
4.4.2. Decontaminate the work surfaces 5% Lysol or 10% bleach + 70% ethanol after any spills of potentially infectious material and after work is completed at the end of the working day.
4.4.3. Decontaminate all contaminated materials, specimens and cultures before disposal or cleaning for reuse.

4.4.4. Comply with triple layer packaging as per the national or international regulations for specimen packaging and transport.
4.4.5. Allocate separate area for keeping documents such as for recording and reporting.
4.4.6. Specify the regulations.
4.4.7. Triple layer packaging for infectious.

4.5. Biosafety management

4.5.1. Ensure that regular training/ orientation on laboratory safety is provided to all personnel working in the in laboratory.
4.5.2. Keep a copy of safety or operations manual in the laboratory in an area accessible to all.
4.5.3. Orient on special hazards, safety operations manual, standard practices and procedures.
4.5.4. Ensure that arthropod and rodent control programs are in place.
4.5.5. Provide appropriate medical evaluation (baseline titers), vaccination and treatment for all personnel and maintain adequate medical records.

Note: It is the responsibility of the laboratory head/Biosafety and Biosecurity (BSS) focal person to ensure the development and adoption of a biosafety management plan and a safety or operations manual.

4.6. Laboratory infrastructure

4.6.1. Provide ample space for the safe performance of laboratory work and for cleaning and maintenance.
4.6.2. The laboratory should have smooth walls, ceilings and floors which should be easy to clean, impermeable to liquids and resistant to the chemicals and disinfectants.
4.6.3. The laboratory should have slip-resistant floors.
4.6.4. The laboratory should have bench tops impervious to water and resistant to disinfectants, acids, alkalis, organic solvents and moderate heat.
4.6.5. There should be adequate illumination/light for all activities

Note: Undesirable reflections and glare should be avoided

4.6.6. Do not use fans in laboratory areas.

Note: Air-conditioners (A/C) are recommended for maintaining room temperature. A/C with HEPA filter is preferred for microbiology laboratory.

4.6.7. Use sturdy laboratory furniture.

Note: wooden furniture is not preferred in the microbiology lab.

Note: Open spaces between and under benches, cabinets and equipment should be accessible for cleaning. Storage space must be adequate to hold supplies for immediate use and thus prevent clutter on bench tops and in aisles.

4.6.8. Ensure that space and facilities are available for the safe handling and storage of solvents, radioactive materials, compressed and liquefied gases.
4.6.9. There should be provision of facilities for storing outer garments and personal items outside the laboratory working areas.
4.6.10. Do not eat, drink and rest inside the laboratory working area.
4.6.11. There should be provision of hand-washing basins, with running water facilities in each laboratory room, preferably near the exit door.
4.6.12. Use doors with vision panels, appropriate fire ratings and preferably self-closing.

4.6.13. Keep autoclave or other means of decontamination in appropriate proximity to the laboratory.
4.6.14. There should be availability of safety systems such as emergency shower and eyewash stations and facilities against fire, electrical emergencies.
4.6.15. Ensure the First-aid kit is readily accessible and refilled from time to time.
4.6.16. Ensure regular supply of good quality water.
4.6.17. Ensure the storage racks and cabinets are secured to the wall to avoid any accidents/injury during any natural disaster.
4.6.18. Microscope storage cabinet with sufficient light source.
4.6.19. Dark cabinet for media storage.

4.7 Laboratory equipment

4.7.1. Compile the equipment inventory with detailed information record (equipment installation, servicing and Annual Maintenance Contract (AMC).
4.7.2. Conduct the annual calibration and intermediate check of the equipment.
4.7.3. Maintain the equipment usage log form (Annex I) and enter the date, name, and initials of the operator with operating time.
4.7.4. Maintain temperature monitoring log/record for required equipment (Annex VI).

4.8. Laboratory waste management

4.8.1. Collect and segregate the laboratory waste in appropriate color-coded bins
  4.8.1.1. Red: Contaminated plastic items including used Petri dishes, used gloves, Tubes, pipette tips, and plastic containers.
  4.8.1.2. Blue: Non-contaminated glassware including Broken glassware, slides, and uncontaminated glass equipment.
  4.8.1.3. Green: Organic waste including biodegradable materials

4.8.2. Ensure that provision for decontaminating all laboratory wastes are available in the facility (e.g., autoclave, chemical disinfection or other validated decontamination method).
4.8.3. Decontaminate all cultures, stocks, and other potentially infectious materials before disposal using an effective method. Use following methods for decontamination depending on where it will be performed prior to transport:
  4.8.3.1. Place materials to be decontaminated outside of the immediate laboratory in a durable, leak proof container and secure for transport.
  4.8.3.2. Pack materials to be removed from the facility for decontamination in accordance with applicable local, state, and federal regulations.

4.9. Emergency measures: Mishaps with infective material

4.9.1. Spillage (minor spills)

4.9.1.1. Put on gloves.
4.9.1.2. Cover the spill with a cloth or tissue soaked in disinfectant (Sodium hypochlorite: (0.5-1%) for small spills and 4-5% for large spills) or 10% bleach+70% ethanol solution, leave for 10 minutes for small spills and up to 30 minutes for large spills and then mop up.
4.9.1.3. For risk group two organisms, disinfect and clean up. For risk group three organisms, fumigate (using formaldehyde) the room after proper disinfection and cleaning.
4.9.1.4. Deal with spillages in Bio-safety cabinets (BSC) by disinfecting (Spirit swab/70% alcohol) the affected surfaces in the cabinet and fumigate the cabinet.

4.9.2. Injuries/cuts/accidents

4.9.2.1. Allow 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 soap and water.

4.9.2.2. Report all injuries or unusual incidents immediately to the supervisor and document it properly.

4.9.2.3. When cuts or puncture wounds from potentially infected needles or glassware occur, wash the affected area promptly with disinfectant soap and water.

4.9.3 Training

4.9.3.1. Ensure appropriate training is provided to laboratory personnel regarding their duties, necessary precautions to prevent exposures and exposure management procedures and maintain all the training records or certification.

4.9.3.2. Provide annual updates or additional training/orientation when procedural or policy changes occur.

4.9.3.3. Following policy change, required laboratory training should be provided.

4.10. Special Practices

4.10.1. Advise all persons entering the laboratory about the potential hazards and meet specific entry/exit requirements.

4.10.2. Prepare and adopt laboratory-specific biosafety SOP/manual. The biosafety manual must be available and accessible.

4.10.3. Place the potentially infectious materials in a durable, leak proof container during collection, handling, processing, storage or transport within a facility.

4.10.4. Decontaminate the laboratory equipment routinely, and after spills, splashes, or other potential contamination.

4.10.5. Decontaminate equipment before repair, maintenance or removal from the laboratory.

4.10.6. Conduct all procedures involving the manipulation of infectious materials that may generate an aerosol within a BSC or other physical containment devices.

4.10.7. Document all laboratory incidents and actions taken.

Government of Nepal Ministry of Forests and Environment Department of Environment	Document Code: SOP-02	Page
Subject Title: Collection and Transportation of Hospital and Pharmaceutical Effluent Samples		
Effective date: April 2025		Version no.
Prepared by: Anjani Kumar Adhikari	Reviewed and Approved by: Sailesh Kumar Jha	

1. Purpose

This document aims to establish consistent procedures for sampling hospital and pharmaceutical effluent to isolate bacteria, including *Escherichia coli*, *Salmonella* species, *Shigella* species, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Enterococcus* species. It provides guidance for the proper collection, storage, and transportation of hospital and pharmaceutical effluents in a safe and professional manner, adhering to appropriate techniques for microbial culture.

2. Scope

This SOP focuses the collection and transportation of effluent samples from hospitals and pharmaceuticals using the grab sampling method. The purpose is to isolate various potential pathogenic bacteria of human concern. Grab samples consist of a single sample or individual samples collected over a period of fifteen minutes or less. The quantity of samples is determined by the specific analytical parameters. Grab sampling is particularly suitable for non-continuous waste streams, including batch discharges and intermittent flows. Grab sampling may also be appropriate when the characteristics of the waste stream are known to be constant through time. Certain parameters necessitate the collection of grab samples. Collecting a series of grab samples during continuous discharge provides valuable data on maximum and minimum concentrations, which cannot be achieved through the compositing process.

3. Requirements

3.1. Non-powdered disposable gloves
3.2. Camera
3.3. Sterilized glass or plastic bottles with cap (100ml or more)
3.4. Disinfectant (Alcohol/ethanol)
3.5. Labelling tape/Marker
3.6. Insulated cold box
3.7. Tissue paper/Paper towel
3.8. Bucket
3.9. Rope
3.10. Funnel
3.11. Ice packs
3.12. Zip locks
3.13. Sampling tool

4. Procedure for Grab sampling of effluent

4.1. Select sites for sample collection
4.2. Wear a clean pair of gloves.
4.3. Maintain optimum precautions during sampling.
4.4. Remove the cap of the bottle at the time of collection of samples.
4.5. Avoid contaminating the mouth and cap of the bottle.
4.6. Hold the bottle near the base and plunge with the neck of the bottle partially below the surface with the mouth of bottle directed against the current of the water flow

Note: *If there is no current, as in the case of a reservoir, create a current artificially by pushing bottle forward horizontally in a direction away from the hand.*

4.7. Leave air space in the bottle (2.5 cm) to facilitate mixing of sample prior to processing.
4.8. Recap the bottle using aseptic technique.
4.9. Wipe to clean the outer surface of the bottles
4.10. Label the bottles clearly indicating site, date and time of collection
4.11. Place the bottles into insulated cold box with ice packs.
4.12. Record sample details and take a photograph of the sample and sampling site.
4.13. Maintain a sampling logbook with this SOP

Note: Effluent samples should be collected from the most representative site prior to discharge into the receiving waters

Take three-point grab samples with at least five minutes interval between each sample

Larger volume of sampling bottles can be used

Sampling pole can be used to collect sample when it is not feasible to collect the sample from the site using hand

Sub-surface samples from shallower depth can be taken if the water being sampled is too shallow. Collect the sample using smaller container and transfer the sample using the sterilized funnel to the bottle and close the bottle.

Sterile sample bottles of appropriate volume, made of suitable material, containing sufficient sodium thiosulphate pentahydrate to give a final concentration in the sample of not less than 18 mg/l (for example, 0.1 ml of a 1.8 % m/v solution of $Na_2S_2O_3.5H_2O$ per 100 ml of sample, or equivalent) is used for sampling chlorinated wastewater effluent

5. Transportation to lab

Transport the collected samples as soon as possible to the lab maintaining cold chain.

6. Holding Time and temperature

Place the collected samples below 10°C during transport and until time of analysis and do not exceed 24 hours holding time.

7. Receipt of sample

7.1. Surface sterilization with 70% ethanol is done before opening.
7.2. Check temperature to ensure cold chain.
7.3. Record the time and date of sample receipt
7.4. Maintain the sample entry logbook.
7.5. Reject sample if leakage and breakage.

Government of Nepal Ministry of Forests and Environment Department of Environment	Document Code: SOP-03	Page
Subject Title: Methods for Isolation and enumeration of Bacteria from Effluent Samples		
Effective date: April 2025		Version no.
Prepared by: Anjani Kumar Adhikari	Reviewed and Approved by: Sailesh Kumar Jha	

1. Purpose

This document will provide standardized guidance on the different methods for isolation and enumeration bacteria from effluent samples.

2. Scope

This SOP describes common methods used for the isolation of bacteria from the effluent sample. The methods utilized differs according to intended bacterial isolation.

3. Isolation and enumeration methods

Isolation methods used for isolation and enumeration of bacteria depends on bacterial load and turbidity of effluent. Commonly used methods for isolation of bacteria from effluent includes membrane filtration, MPN method, streak plate method and pour plate method and spread plate method

4. Membrane filtration

In membrane filtration, a measured volume of sample is filtered, under vacuum, through a suitable membrane of uniform pore diameter, usually 0.45 µm and 0.22 µm. Bacteria are retained on the surface of the membrane which is placed on a suitable selective medium and incubated at an appropriate temperature.

When small quantities of sample (for example, of sewage effluent or of grossly polluted surface water) are to be tested, it is necessary to dilute a portion of the sample in sterile diluent to ensure that there is sufficient volume to filter across the entire surface of the membrane.

4.1. Requirements

4.1.1. Membrane filtration set
4.1.2. Sample
4.1.3. Sterile membrane filter (0.45/ 0.22 µm)
4.1.4. Culture media plates
4.1.5. Colony counter
4.1.6. Forceps

4.2. Procedure

4.2.1. Place the sterile or disinfected filtration apparatus in position and connect to a source of vacuum.
4.2.2. Remove the funnel.
4.2.3. Holding the edge of the sterile membrane filter with sterile smooth-tipped forceps place it, if gridded grid-side upwards, onto the porous disc of the filter base.
4.2.4. Replace the funnel securely on the filter base.
4.2.5. Pour or pipette the required volume of sample, or diluted sample, into the funnel.
4.2.6. When the volume of sample to be filtered is less than 10 ml, add 10 - 20 ml of sterile phosphate buffered dilution water or buffered peptone water as diluent to the funnel before addition of the sample.

This aids the dispersion of the bacteria over the entire surface of the membrane filter during the filtration process.

4.2.7. Apply a vacuum not exceeding 65 kPa and filter the sample slowly through the membrane filter.
4.2.8. Disconnect the vacuum as soon as the sample has been filtered.
4.2.9. Remove the funnel and transfer the membrane filter carefully onto the surface of culture media in Petri dish. (Keep grid side up)

Note: The surface of the medium should be dry and free of any surplus water. Ensure that no air bubbles are trapped between the membrane filter and the medium.

4.2.10. Cover the petri dich with its cover and incubate it at optimum temperature specified for bacteria under study
4.2.11. Observe and enumerate bacterial colonies on the surface of membrane filter after incubation
4.2.12. Calculate the number of colonies per milliliter as:

Colony forming units/ml = **No. of colonies X Dilution factor/Volume of sample filtered**

Note: The volumes, and dilutions, of samples should be chosen so that the number of coloniesto be counted on the membrane filter lies, if possible, between 20 and 80.

4.3. Suggested volumes to be filtered for water from different sources are as follows

Table 1: Volumes to be filtered for water from effluent are as follows

Sample type	Sample volume (ml)					
	100	10	1[1]	0.1[1,2]	0.01[1,2]	0.001[1,2]
Treated drinking water	x					
Partially treated drinking water	x	x				
Recreational water			x	x		
Protected source water		x	x			
Surface water			x	x		
Wastewater			x	x	x	
Discharge from sewage treatment plant			x	x	x	
Ponds, rivers, stormwater runoff				x	x	x

Source: UNEP/WHO (1996)

Note: [1] Small volume should be added to the filtration apparatus together with a minimum of 9 ml of sterile diluent to ensure adequate dispersal across the surface of the filter membrane
[2] 1.0, 0.1, 0.01 and 0.001-ml volumes are filtered after first preparing serial dilutions of the sample.

To filter:

4.3.1. 1.0 ml of sample, use 10 ml of 1/10 dilution
4.3.2. 0.1 ml of sample, use 10 ml of 1/100 dilution
4.3.3. 0.01 ml of sample, use 10 ml of 1/1,000 dilution
4.3.4. 0.001 ml of sample, use 10 ml of 1/10,000 dilution

4.4. Diluent

Phosphate buffered dilution water or Buffered peptone water is used as diluent used for diluting effluent sample. (See Annex V for composition of diluent)

5. MPN method

5.1. Requirements

- Effluent sample
- Sterile test tubes
- Selective/differential media (**MacConkey broth** for coliforms)
- Durham tubes (to detect gas production)
- Sterile pipettes
- Incubator (35–37°C)
- MPN Table (standard statistical table)

5.2 Procedure

5.2.1. Collect effluent sample in a sterile container, ideally on ice, and process within 6 hours.
5.2.2. Prepare 10-fold serial dilutions (e.g., 10^{-1}, 10^{-2}, 10^{-3}) of the sample using sterile water or saline.
5.2.3. Inoculate 3 or 5 tubes for each dilution level with 1 mL of the respective dilution.
5.2.4. Add Durham tubes if gas production is a growth indicator (e.g., for coliforms).
5.2.5. Incubate the tubes at 35–37°C for 24–48 hours, depending on the target bacteria.
5.2.6. Look for signs of growth:
    5.2.1.1. Turbidity
    5.2.1.2. Gas production
    5.2.1.3. Color change (based on indicator in the medium)
5.2.7. Record the number of positive tubes at each dilution.
5.2.8. Refer to a standard MPN table to estimate the number of bacteria per mL or 100 mL

6. Pour plate method

Pour plate method is usually the method of choice for isolation and counting the number of colony-forming bacteria present in diluted (using phosphate-buffered dilution water) or undiluted liquid specimen. In this method, a fixed amount of inoculum (generally 1 ml) from sample is placed in the center of a sterile Petri dish using a sterile pipette. Molten cooled agar (approximately-15mL) is then poured into the Petri dish containing the inoculum and mixed well. After the solidification of the agar, the plate is inverted and incubated at appropriate temperature for 24-48 hours. Bacteria will grow both on the surface and within the medium. Colonies that grow within the medium generally are small in size and maybe confluent; the few that grow on the agar surface are of the same size and appear like those on a streak plate. Each (both large and small) colony is carefully counted. Each colony represents a "colony-forming unit" (CFU). For accurate counts, the optimum count should be within the range of 30-300 colonies/plate. To ensure a countable plate a series of dilutions should be plated. The pour plate method of counting bacteria is more precise than the streak plate method, but, on average, it will give a lower count as heat-sensitive microorganisms may die when they come in contact with hot, molten agar medium

6.1 Requirement

6.1.1. Effluent sample
6.1.2. Molten agar medium
6.1.3. Hot water bath 44°C to 46°C
6.1.4. Sterile Petri dishes
6.1.5. Bunsen burner/electric loop sterilizer
6.1.6. Colony counter with magnifying glass
6.1.7. Sterile capped test tubes
6.1.8. Sterile Pipettes of various sizes (e.g. 01, 1.0 and 2.0 mL) or micropipettes

6.2. Procedure

6.2.1. Prepare the dilution of the test sample expected to contain between 30-300 CFU/mL
6.2.2. Aseptically inoculate labeled empty sterile petri dish with specified mL (0.1 or 1.0 mL) of diluted specimen
6.2.3. Collect the media bottle of sterile molten agar medium from the water bath (44°C to 46°C) and pour at least 10 to 12 ml liquefied medium into each dish by gently lifting cover just high enough to pour.
6.2.4. Flame the neck of the bottle and replace the cap.
6.2.5. Gently swirl the plate on the bench top to mix the culture and the medium thoroughly. Ensure that the medium covers the plate evenly and do not slip the agar over the edge of the Petri dish.
6.2.6. Allow the agar to solidify without disturbing it, it will take approximately 10 minutes.
6.2.7. Incubate the plate in an inverted position at 37°C for 24-48 hours.

Note: Check sterility of medium and dilution water blanks by pouring control plates for each series of samples

7. Spread plate method

7.1. Requirement

7.1.1. Effluent sample (e.g., from sewage, industrial discharge, etc.)
7.1.2. Sterile distilled water or saline (for serial dilution)
7.1.3. Sterile test tubes
7.1.4. Pipettes or micropipettes with sterile tips
7.1.5. Nutrient agar (NA) or other selective media
7.1.6. Petri plates
7.1.7. Glass or metal spreader (e.g., L-shaped rod)
7.1.8. Bunsen burner or spirit lamp
7.1.9. Ethanol (for sterilizing spreader)
7.1.10. Incubator (set at 35–37°C)
7.1.11. Marker and notebook

7.2. Procedure

7.2.1. Collect the effluent sample in a sterile container.
7.2.2. Store at 4°C if immediate processing is not possible.
7.2.3. Prepare a series of 10-fold dilutions (e.g., 10^{-1} to 10^{-6}) by transferring 1 mL of effluent sample into 9 mL of sterile saline or water.
7.2.4. Pour sterile nutrient agar into sterile Petri dishes.
7.2.5. Allow the agar to solidify.
7.2.6. Pipette 0.1 mL of diluted sample (usually from dilutions 10^{-3} to 10^{-6}) onto the surface of the agar plate.

7.2.7. Use a sterile L-shaped spreader to evenly spread the sample over the agar surface.
7.2.8. Re-sterilize the spreader with ethanol and flame between samples.
7.2.9. Incubate the plates upside down at 35–37°C for 24–48 hours.
7.2.10. After incubation, count the colonies on plates with 30–300 colonies (CFUs).
7.2.11. Record the dilution factor and calculate the colony-forming units per mL (CFU/mL) of the original effluent sample using the formula:

CFU/mL= Number of colonies × Dilution factor/ Volume plated

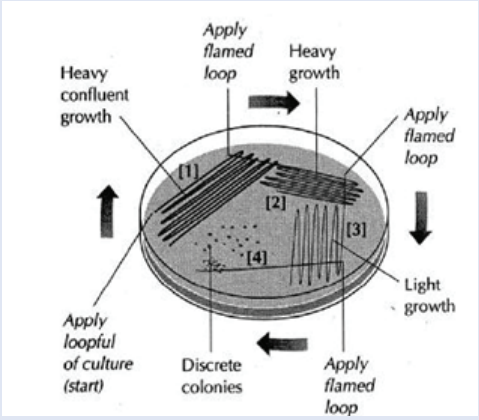
8. Streak plate method

Streak plate method is used for the isolation into a pure culture of the organisms, from a mixed population or after enrichment in specific enrichment media. The inoculum is streaked over the agar surface in such a way that it thins out the bacteria. While streaking in successive areas of the plate, the inoculum is diluted to the point where there is only one bacterial cell deposited every few millimeters on the surface of the agar plate. When these lone bacterial cells divide and give rise to thousands of new bacterial cells, an isolated colony is formed. Pure cultures can be obtained by picking well-isolated colonies and re-streaking these on fresh agar plates. The streaked plate is incubated at for 24-48 hours at appropriate temperature. The plate is examined carefully for the colonies to grow.

8.1. Requirements

8.1.1. A source of bacteria (stock culture, previously streaked agar plate or any other inoculum)
8.1.2. Inoculation loop
8.1.3. Bunsen burner
8.1.4. Lysol (10% v/v)
8.1.5. Agar plate

Figure 1: Streak plate method for isolation of bacteria



8.2. Procedure

8.2.1. Sterilize the inoculating loop in the Bunsen burner/electric loop sterilizer by putting the loop into the flame until it is red hot. Allow it to cool.
8.2.2. Pick an isolated colony from the agar plate or sample inoculated broth culture and spread it over the first quadrant (approximately 1/4 of the plate) using close parallel streaks or insert the loop into the tube/culture bottle and remove some inoculum.
8.2.3. Immediately streak the inoculating loop very gently over a quarter of the plate using a back-and-forth motion (see area 1).
8.2.4. Flame the loop again and allow it to cool. Going back to the edge of area 1 just streaked, extend the streaks into the second quarter of the plate (area 2).
8.2.5. Flame the loop again and allow it to cool. Going back to the area just streaked (area 2), extend the streaks into the third quarter of the plate (area 3).
8.2.6. Flame the loop again and allow it to cool. Going back to the area just streaked (area 3), extend the streaks into the center fourth of the plate (area 4).
8.2.7. Flame the loop once more.
8.2.8. Incubate the plate at appropriate temperature for appropriate period of time (depends with bacteria)
8.2.9. After incubation, observe the plate for isolated colony.

1. Purpose

This document will provide guidance to correctly prepare different culture media to support growth of microorganisms and biochemical media to observe the different characteristics specific to microorganisms which aids in their identification.

2. Scope

This SOP describes to prepare different culture and biochemical media required for culture isolation and identification of bacteria and conducting the antibiotic sensitivity testing (AST).

3. Requirements

3.1. The constituents and method of preparation of culture media varies depending on the type of media and manufacturer.

3.2. Media preparation by well-trained lab personnel (microbiologist).

4. Baird Parker Agar (BPA)

4.1. Purpose

Baird Parker Agar base is used for the selective isolation and enumeration of Coagulase Positive Staphylococci. It contains lithium chloride and potassium tellurite to inhibit the accompanying flora and glycine and pyruvate to facilitate the growth of staphylococci.

4.2. Procedure for preparation

4.2.1. Suspend 63 grams (or as directed by manufacturer) of the medium in one liter of distilled water.

4.2.2. Mix well. Heat with frequent agitation and boil for one minute until complete dissolution.

4.2.3. Sterilize in autoclave at 121°C at 15 lbs. /inch^2 for 15 minutes.

4.2.4. Cool to45°-50°C and add 10 ml. of a 1% potassium tellurite solution and 50 ml of an egg yolk emulsion.

4.2.5. Homogenize gently and pour into Petri dishes.

4.2.6. Perform sterility testing of the prepared media as described in section C

4.2.7. Label the side of each tube with date of preparation and batch number.

4.2.8. Refrigerate in sealed containers or in tubes or bottles with screw caps.

5. Bile Aesculin Agar (BAA)

5.1. Purpose

BAA is used for the isolation and presumptive identification of enterococci. Group D Streptococci grow well on this differential medium because the ox bile in the formula does not inhibit them while the other Gram-positive bacteria are inhibited. On the other hand, the hydrolysis of esculin to esculetin in this bile medium (differential test for enterococci) changes the medium to dark brown color. Tolerance to bile and the ability to hydrolyze esculin that reacts with the ferric citrate constitutes a reliable presumptive test for the identification of enterococci

5.2. Procedure for preparation

5.2.1. Suspend 64 grams (or as directed by manufacturer) of the medium in one liter of distilled water.
5.2.2. Mix well. Heat with frequent agitation and boil until completely dissolved.
5.2.3. Dispense into appropriate flask/container and sterilize at 121°C (15 lbs. sp.) for 15 minutes.
5.2.4. Overheating can cause darkening of the medium.
5.2.5. If tubes are used, allow to solidify in a slanted position
5.2.6. Cool to 45-50°C and dispense 20-25 ml of prepared media into sterile petri dishes (90mm).
5.2.7. Leave standing at room temperature to allow the media to solidify.
5.2.8. Perform sterility testing as described in Section C
5.2.9. Label the side of each tube with date of preparation and batch number.
5.2.10. Store the culture medium in upright position at 2-8°C sealed in plastic bags to reduce chances of contamination.

6. Brain Heart Infusion (BHI) Agar

6.1. Purpose

BHI Agar is used for the cultivation of fastidious microorganisms. Brain Heart Infusion Agar (BHI) is a solid medium rich in nutrients, suitable for the cultivation of several fastidious strains of bacteria, fungi, and yeasts. BHI agar is used for the cultivation of a wide variety of microorganisms

6.2. Procedure of preparation

6.2.1. Suspend 52 grams (or as directed by manufacturer)of the medium in one liter of distilled water.
6.2.2. Mix well.
6.2.3. Heat with frequent agitation and boil for one minute.
6.2.4. Dispense and sterilize at 121°C at 15 lbs. /inch^2 for 15 minutes.
6.2.5. Cool to 45-50°C.
6.2.6. Pour20-25 ml of the autoclaved medium in sterile petri dishes. Leave standing at room temperature to allow the media to solidify.
6.2.7. Perform sterility testing of the prepared medium as described in section C.
6.2.8. Label the bottom of each plate with date of preparation and batch number.
6.2.9. Store the culture media plates upside down at 2-8 °C sealed in plastic bags to reduce chances of contamination.

Note: Before pouring the medium swirl gently to distribute the possible precipitate

7. Brain Heart Infusion (BHI) Broth

7.1. Purpose

BHI broth is a general-purpose liquid medium used in the cultivation of fastidious and non-fastidious microorganisms, including aerobic and anaerobic bacteria, from a variety of clinical and non-clinical specimens. A supplemented pre-reduced formulation in tubes is especially recommended for the cultivation of anaerobes.

7.2. Procedure for preparation

7.2.1. Suspend 37.0 gm power (or as directed by manufacturer) in1liter of distilled water.
7.2.2. Heat if necessary, to dissolve the medium completely.
7.2.3. Dispense into bottles or tubes as desired. Sterilize by autoclaving at 121°C for 15 lbs. /inch2 for 15 minutes.
7.2.4. Cool to 45-50°C.
7.2.5. Perform sterility testing of prepared media as described in section C
7.2.6. Label the side of each tube with date of preparation and batch number.
7.2.7. Store the culture medium in upright position at 2-8 °C sealed in plastic bags to reduce chances of contamination.

8. Cetrimide Agar

8.1. Purpose

Cetrimide Agar Base promotes the production of pyocyanin a water soluble pigment as well as fluorescence, under ultraviolet light, of *Pseudomonas aeruginosa*, which constitutes a presumptive identification.

Cetrimide is the selective agent as it inhibits the growth of the accompanying microbial flora. Typical *Pseudomonas aeruginosa* colonies are greenish or yellowish green in color. Pyorubin-producing strains form reddish colonies.

8.2. Procedure of preparation

8.2.1. Suspend 45.3 grams (or as directed by manufacturer) of the medium in one liter of distilled water.
8.2.2. Add 10 ml of glycerol.
8.2.3. Heat agitating frequently, and boil for one minute. Dispense in appropriate container and sterilize and autoclave at to 121°Clbs. /inch^2 at for 15 minutes.
8.2.4. Cool to 45-50°C.
8.2.5. Pour20-25 ml of the autoclaved media in sterile petri dishes. Leave standing at room temperature to allow the media to solidify.
8.2.6. Perform sterility testing of the prepared media as described in section C.
8.2.7. Label the bottom of each plate with date of preparation and batch number.
8.2.8. Store the culture media plates upside down at 2-8 °C sealed in plastic bags to reduce chances of contamination.

9. Milk agar with acetyl trimethyl ammonium bromide

9.1. Purpose

Milk agar with acetyl trimethyl ammonium bromide is used for isolation of *Pseudomonas aeruginosa* from water sample.

9.2. Procedure of preparation

9.2.1. Add the acetyl trimethylammonium bromide and agar to 250 ml of sterile yeast extract broth, mix well and steam to dissolve.
9.2.2. Thoroughly mix the skimmed-milk powder with750 ml of water.
9.2.3. Autoclave the individual solutions separately at 121°C for 5 minutes.
9.2.4. Cool to approximately 50-55°C, add the skimmed-milk powder solution to the agar solution, mix thoroughly and pour into sterile Petri dishes.
9.2.5. Allow the medium to solidify, store at between 2-8°C, protected against dehydration, and use within one month

10. Eosin Methylene Blue (EMB) agar

10.1. Purpose

EMB agar is used for the study of enterobacteria. It is widely used in medical bacteriology, in techniques recommended by the American Public Health Association and for the detection and enumeration of coliforms, which can contaminate foods and drinking water

10.2. Procedure of preparation

10.2.1. Suspend 36 grams (or as directed by manufacturer) of the medium in one liter of distilled water.
10.2.2. Mix well.
10.2.3. Heat with frequent agitation and boil for one minute.
10.2.4. Sterilize in autoclave at 121°C at 15 lbs. /inch^2 for15 minutes.
10.2.5. Cool to 45-50°C.
10.2.6. Swirl gently, avoiding the formation of bubbles and pour 20-25 ml into sterile petri plates
10.2.7. Allow the media to solidify.
10.2.8. Perform sterility testing of the prepared medium as described in section C
10.2.9. Label the bottom of each plate with date of preparation and batch number.
10.2.10. Store the culture media plates upside down at 2-8 °C sealed in plastic bags to reduce chances of contamination.

11. Gram Negative (GN) broth

11.1. Purpose

GN stands from Gram Negative, as this medium is used for isolating and cultivating gram negative microorganisms. The GN enrichment broth encourages the growth of *Salmonella* and *Shigella* due to its content of mannitol, as it favors growth of mannitol-fermenting *Salmonella* and *Shigella* over mannitol non fermenting species such as Proteus. The gram-positive microorganisms are inhibited by the presence of citrate and deoxycholate

11.2. Procedure of preparation

11.2.1. Suspend 39 grams (or as directed by manufacturer) of the medium in one liter of distilled water.
11.2.2. Heat with frequent agitation to dissolve the medium completely.
11.2.3. Dispense into tubes and sterilize at 121°C at 15 lbs. /inch^2 for 15 minutes
11.2.4. Perform sterility testing of the prepared medium as described in section C
11.2.5. Label the bottom of each plate with date of preparation and batch number.
11.2.6. Store the culture media plates upside down at 2-8°C.

12. MacConkey Agar (MA)

12.1. Purpose

MacConkey agar is a differential medium to distinguish lactose fermenting bacteria from non-lactose fermenters

12.2. Procedure for preparation:

12.2.1. Suspend 51.1gm (or as directed by manufacturer) of MA powder in one liter of distilled or deionized water.
12.2.2. Heat until completely dissolved.
12.2.3. Sterilize in autoclave at 121°C for 15 minutes.
12.2.4. Cool to 45-50°C.
12.2.5. Pour 20-25 ml of the autoclaved media in sterile petri dishes. Leave standing at room temperature to allow the media to solidify.
12.2.6. Perform sterility testing of the prepared medium as described in section C
12.2.7. Label the bottom of each plate with date of preparation and batch number.
12.2.8. Store the culture media plates upside down at 2-8 °C sealed in plastic bags to reduce chances of contamination.

13. Mannitol Salt Agar (MSA)

13.1. Purpose

This is a selective medium prepared according to the recommendations of Chapman for the isolation of presumptive pathogenic staphylococci. Most of the other bacteria are inhibited by the high concentration of salt. The degradation of mannitol with the production of acid changes the color of the medium from rose to yellow. Due to its high content of sodium chloride, a heavy inoculum of the material in study can be used. The mannitol fermenting pathogenic staphylococci are larger and are surrounded by a yellow zone

13.2. Procedure of preparation

13.2.1. Suspend 111 grams (or as directed by manufacturer) of the medium in one liter of distilled water.
13.2.2. Mix well and heat with frequent agitation until complete dissolution.
13.2.3. Boil for one minute.
13.2.4. Sterilize in autoclave at 121°C at 15 lbs. /inch2for 15 minutes.
13.2.5. Pour 20-25 ml of the autoclaved media in sterile petri dishes. Leave standing at room temperature to allow the media to solidify.

13.2.6. Perform sterility testing of prepared medium as described in section C
13.2.7. Label the bottom of each plate with date of preparation and batch number.
13.2.8. Store the culture media plates upside down at 2-8°C sealed in plastic bags to reduce chances of contamination.

14. Membrane Enterococcus Agar (mEA)

14.1. Purpose

Membrane Enterococcus Agar is used for the isolation, cultivation, and enumeration of enterococci in water and sewage by the membrane filter method. It is also used for the direct plating of specimens for the detection and enumeration of enterococci.

14.2. Procedure of preparation

14.2.1. Add components to distilled/deionized water and bring volume to 1 liter (as directed by manufacturer)
14.2.2. Mix thoroughly.
14.2.3. Gently heat and bring to boiling.
14.2.4. Cool to 45°–50°C.
14.2.5. *Do not autoclave.*
14.2.6. Pour 20-25 ml into sterile Petri dishes and let the media solidify
14.2.7. Perform sterility testing of prepared medium as described in section C
14.2.8. Label the bottom of each plate with date of preparation and batch number.
14.2.9. Store the culture media plates upside down at 2-8°C sealed in plastic bags to reduce chances of contamination.

15. Mueller Hinton Agar (MHA)

15.1. Purpose

It is the medium recommended by CLSI for antibiotic sensitivity testing.

15.2. Procedure for preparation:

15.2.1. Suspend 38gm (or as directed by manufacturer) of the powder in one liter of distilled or deionized water
15.2.2. Mix thoroughly.
15.2.3. Heat with frequent agitation and boil for one minute to completely dissolve the powder. Autoclave at 121°C for 15 minutes.
15.2.4. Cool to 45-50 °C and dispense 20-25 ml of prepared media into sterile petri dishes (90mm).
15.2.5. Leave standing at room temperature to allow the media to solidify.
15.2.6. Perform sterility testing of the prepared medium as described in section C
15.2.7. Label the bottom of each plate with date of preparation and batch number.
15.2.8. Store the culture media plates upside down at 2-8 °C sealed in plastic bags to reduce chances of contamination.

16. Nutrient Agar (NA)

16.1 Purpose

Nutrient agar is used for the cultivation of a wide variety of non-fastidious bacteria. It is used as a basal medium for subculture and purification of isolates. Nutrient agar tube/slants are used primarily for the cultivation and maintenance of pure cultures.

16.2 Procedure for preparation:

16.2.1. Suspend 23gm (or as directed by manufacturer) of the powder in 1 liter of distilled or deionized water.
16.2.2. Heat with frequent agitation and boil for one minute to completely dissolve the powder. Autoclave 121 °C at 15 lbs. /inch^2 for 15 minutes.
16.2.3. Cool to 45 °C-50 °C and dispense 20-25 ml of prepared media into sterile petri dishes (90mm)
16.2.4. Leave standing at room temperature to allow the media to solidify.
16.2.5. If slants are to be prepared, pour the desired amount in glass tubes and leave to solidify by slanting at 30˚- 60˚ to produce the slope.
16.2.6. Perform sterility testing of the prepared medium as described in section C
16.2.7. Label the bottom of each plate with date of preparation and batch number.
16.2.8. Store the culture media plates upside down at 2-8 °C sealed in plastic bags to reduce chances of contamination.

17. Nutrient Broth (NB)

17.1. Purpose

Nutrient broth is used as a maintenance and enrichment medium for non-fastidious bacteria.

17.2. Procedure for preparation:

17.2.1. Dissolve 8 gm (or as directed by manufacturer) of the powder in one litre of purified water.
17.2.2. Heat with frequent agitation and boil for one minute to completely dissolve the powder.
17.2.3. Dispense into sterile screw capped tubes or bottles as necessary
17.2.4. Autoclave at 121°C at 15 lbs. /inch^2 for 15 minutes.
17.2.5. Perform sterility testing of the prepared medium as described in section C
17.2.6. Label the bottom of each plate with date of preparation and batch number.
17.2.7. Store the culture media plates upside down at 2-8 °C sealed in plastic bags to reduce chances of contamination.

18. Pseudomonas Agar

18.1. Purpose

This medium is designed for the presumptive identification of Pseudomonas aeruginosa and promotes pyocyanin production.

18.2. Procedure of preparation

18.2.1. Dissolve the solid ingredients, except the acetyl trimethylammonium bromide and nalidixic acid, in water.

18.2.2. To dissolve the ingredients, it will be necessary to heat boiling.
18.2.3. Add 10 ml of glycerol and mix well.
18.2.4. Dispense the resulting solution in appropriate volumes into suitable containers and sterilize by autoclaving at 121 °C at 15 lbs. /inch2for 15 minutes.
18.2.5. After autoclaving, the pH of the base medium should be checked to confirm a pH of 7.1 ± 0.2.
18.2.6. Cool the molten base medium to approximately 50 °C and add the acetyl trimethylammonium bromide and nalidixic acid as filter-sterilized aqueous sterile solutions to give final concentrations of 200 mg/l and 15 mg/l respectively.
18.2.7. Mix thoroughly and dispense into sterile Petri dishes.
18.2.8. Allow the complete medium to solidify, store at between 2 - 8 °C, protected from dehydration, and use within one month

19. Selenite-F-Broth

19.1. Purpose

Selenite-F broth is used as an enrichment medium for the isolation of Salmonella from feces, urine, water, food and environment. Selenite is inhibitory to coliforms and certain other microbial species and hence proliferates the growth of *Salmonella*.

19.2. Procedure for preparation

19.2.1. Suspend 23 gm of the dehydrated powder in 1 liter of distilled or deionized water.
19.2.2. Heat to boiling. **Do not autoclave.**
19.2.3. Dispense required volume (10-20 ml) in screw capped tubes, filling the tubes to about three-quarters.
19.2.4. Perform sterility testing of the prepared medium as described in section C
19.2.5. Label the bottom of each plate with date of preparation and batch number.
19.2.6. Store the culture media plates upside down at 2-8 °C sealed in plastic bags to reduce chances of contamination.

20. Tetrathionate broth

20.1. Purpose

Tetrathionate Broth Base is used as a selective enrichment for the cultivation of Salmonella species that may be present in small numbers and compete with intestinal flora. It is also used in processing fecal cultures for bacteria

20.2. Procedure of preparation

20.2.1. Suspend 46 grams (or as directed by manufacturer) of the medium in one liter of distilled water.
20.2.2. Mix well and heat to boiling.
20.2.3. Cool and dispense 10 ml in tubes continually swirling the flask to maintain homogeneity.
20.2.4. Add 20 ml per liter of iodine solution to the amount of medium to be used on the same day.
20.2.5. Prepare the solution by dissolving 6 gm of iodine and 5 gm of potassium iodide in 20 ml of distilled water.
20.2.6. Once the medium is prepared, store refrigerated.
20.2.7. Label the side of each tube with date of preparation and batch number.
20.2.8. Perform sterility testing of prepared medium as described in section 5.2
20.2.9. Store at 2-8°C

21. Xylose Lysine Deoxycholate (XLD) Agar

21.1. Purpose

XLD Agar is a moderately selective medium recommended for isolation and differentiation of enteric pathogens, especially *Shigella* species.

21.2. Procedure for preparation:

21.2.1. Suspend 55 gm (or as directed by manufacturer) of the powder in 1 liter of purified water. Mix thoroughly.
21.2.2. Heat with agitation just until the medium boils. DO NOT OVERHEAT. DO NOT AUTOCLAVE.
21.2.3. Cool to 45-50°C in a water bath and use immediately. Overheating causes precipitation.
21.2.4. Dispense 20-25 ml of prepared media into sterile petri dishes (90mm).
21.2.5. Leave standing at room temperature to allow the media to solidify.
21.2.6. Perform sterility testing of the prepared medium as described in section C
21.2.7. Label the bottom of each plate with date of preparation and batch number.
21.2.8. Store the culture media plates upside down at 2-8 °C sealed in plastic bags to reduce chances of contamination.

Biochemical Media

22. Lysine Iron Agar

22.1. Purpose

For the rapid differentiation of enterobacteria, especially *Salmonella* and Arizona. Lysine Iron Agar is very useful for the rapid differentiation of *Salmonella* and Arizona from Citrobacter. It is used to differentiate the enterobacteria on the basis of lysine decarboxylation and deamination and H2S production. The strains which rapidly ferment the lactose produce a large quantity of acid, changing the original purple colour of the medium to yellow.

22.2. Procedure of preparation:

22.2.1. Suspend 33 grams (or as directed by manufacturer) of the medium in one liter of distilled water.
22.2.2. Mix well and dissolve while heating and boil for one minute.
22.2.3. Dispense in tubes and sterilize in autoclave at 121°C at 15 lbs. /inch^2 for 15 minutes.
22.2.4. Cool in a slanted position
22.2.5. Perform sterility testing of prepared medium as described in section C

23. MR-VP broth

23.1. Purpose

MR-VP Broth (Methyl Red-Voges Proskauer Medium/Broth, also known as Buffered Peptone Glucose Broth) are used for the differentiation of bacteria by means of the methyl red and Voges-Proskauer test.

23.2. Procedure for Preparation:

23.2.1. Dissolve 17 gm (or as directed by manufacturer) of the powder in 1 liter of purified water and mix thoroughly.

23.2.2. If necessary, heat slightly to dissolve.
23.2.3. Dispense in tubes and autoclave at 121°C at 15 lbs. /inch^2 for 15 minutes.
23.2.4. Test samples of the finished product for performance using stable, typical control cultures.

24. Oxidative fermentative medium

24.1. Purpose

Oxidative fermentative medium is used to determine if Gram-negative bacteria metabolize carbohydrates oxidatively, by fermentation, or are non-saccharolytic (have no ability to use the carbohydrate in the media). During the anaerobic process of fermentation, pyruvate is converted to a variety of mixed acids depending on the type of fermentation. The high concentration of acid produced during fermentation will turn the bromothymol blue indicator in Oxidative fermentative media from green to yellow in the presence or absence of oxygen.

24.2. Procedure for preparation:

24.2.1. Suspend 9.4 gm (or as directed by manufacturer) of the powder in one litre of distilled water. Mix thoroughly.
24.2.2. Heat with frequent agitation and boil for one minute to completely dissolve the powder.
24.2.3. Autoclave at 121°C at 15 lbs. /inch^2 for 15 minutes. Add 1% carbohydrate before or after autoclaving depending on heat lability.
24.2.4. Test samples of the finished product for performance using stable, typical control cultures.

25. Simmon's Citrate agar

25.1. Purpose

Simmons Citrate Agar is used for the differentiation of Gram-negative bacteria on the basis of citrate utilization.

25.2. Procedure for preparation

25.2.1. Suspend 24.3 gm (or as directed by manufacturer) of the powder in one liter of purified water and mix thoroughly.
25.2.2. Heat with frequent agitation and boil for one minute to completely dissolve the powder.
25.2.3. Dispense in tubes and autoclave at 121°Cat 15 lbs. /inch^2 for 15 minutes.
25.2.4. Allow to cool in a slanted position for use as slants.
25.2.5. Test samples of the finished product for performance using stable, typical control cultures.

26. Sulphide indole motility medium

26.1. Purpose

Sulphide indole motility Medium enables determination of three characteristics by which enteric bacteria can be differentiated. Peptonized iron and sodium thiosulphate are the indicators of H_2S production. This H_2S reacts with peptonized iron to form black precipitate of ferrous sulphide. Motile organisms grow away from line of inoculation showing diffused growth while non-motile organisms grow along the stab line. Motility detection is possible due to the semisolid nature of the medium. Growth radiating out from the central stab line indicates that the test organism is motile. Indole production is detected by the addition of Kovac's reagents (isoamyl para-dimethylaminobenzaldehyde) following the incubation period.

26.2. Procedure for preparation:

26.2.1. Suspend 30 gm (or as directed by manufacturer) of the powder in one litre of distilled water. Mix thoroughly.
26.2.2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
26.2.3. Dispense and autoclave at 121°C at 15 lbs. /inch^2 for 15 minutes.
26.2.4. Test samples of the finished product for performance using stable, typical control cultures.

27. Triple Sugar Iron agar

27.1. Purpose

Triple Sugar Iron (TSI) agar is used for the differentiation of gram-negative enteric bacilli based on carbohydrate fermentation and the production of hydrogen sulfide.

27.2. Procedure for preparation:

27.2.1. Suspend 65 g (or as directed by manufacturer) of the powder in one liter of purified water and mix thoroughly.
27.2.2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
27.2.3. Dispense and autoclave at 121°C at 15 lbs. /inch2 for 15 minutes.
27.2.4. Cool in a slanted position so that deep butts are formed.
27.2.5. Test samples of the finished product for performance using stable, typical control cultures.

28. Urea Agar

28.1. Purpose

Urea Agar or Urease Test Broth are used for the differentiation of organisms, especially the Enterobacteriaceae, on the basis of urease production.

28.2. Procedure for preparation:

28.2.1. Suspend 2.4 gm in 95 ml of distilled water (or as directed by manufacturer) and mix thoroughly.
28.2.2. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C at 15 lbs. /inch2 for 15 minutes.
28.2.3. Cool to 50°C and aseptically introduce 5ml of sterile 40% urea solution (40 gm in 100 ml autoclaved water)
28.2.4. Mix well and distribute 10ml amounts into sterile containers and allow to set in the slope position.
28.2.5. Test samples of the finished product for performance using stable, typical control cultures.

Note: The procedure is based on preparation of Christensen's urea agar, which requires addition of urea solution after autoclaving. If the base already contains urea, prepare according to manufacturer's instructions.

28.3. Dispensing culture media

Media should be dispensed in a clean, draught-free room. Most fluid media are dispensed into screw-capped bottles or tubes, and then sterilized by autoclaving. Sterile media must be dispensed into sterile petri dishes, tubes, or bottles using an aseptic technique.

Note: There should not be frequent movement of people in and out of the media room to prevent contamination.

Procedure for dispensing sterile media into petri dishes

28.3.1. Layout the sterile petri dishes on a level, clean (disinfected) bench surface or inside a laminar flow clean bench.

28.3.2. Mix the medium gently rotating the flask or bottle. Avoid forming air bubbles.

28.3.3. Flame-sterilize the neck of the flask or bottle using Bunsen burner.

28.3.4. Pour 20-25 ml of medium into each dish (90-100 mm diameter). If air bubbles enter while pouring, rapidly flame the surface of the medium before gelling occurs.

28.3.5. Rotate the dish on the surface of the bench to ensure an even layer of agar. Agar plates should be of even depth (not less than 4 mm) and of a firm gel.

28.3.6. When the medium has solidified and cooled, stack the plates in inverted position and seal them in plastic zip lock bags to prevent loss of moisture and reduce the risk of contamination.

Note: Do not leave the plates exposed to bright light especially sunlight.

28.3.7. Label properly and store at 2-8°C.

28.4. Quality control of prepared media

Quality control tests should be carried out before using the media plate to ensure that the performance characteristics of the medium are within specification and that the methodology of medium preparation is satisfactory. Each lot/batch of prepared medium should be subjected to a minimal testing (such as pH, sterility testing, performance testing, stability) and maintain the media preparation, logbook and record keeping. which will ensure that it is acceptable and will demonstrate a typical bacterial performance.

28.4.1. pH testing

The pH of culture media is an essential factor affecting its quality. pH testing can be done during media preparation either before or after autoclaving using a well calibrated pH meter or if unavailable using narrow range pH papers. The prepared media should have a specific pH within its pH limit. The desired pH level may be achieved by adding either a dilute acid or base.

28.4.2. Procedure for checking the pH of a culture media using a pH meter

28.4.2.1. Switch on the pH meter and allow it to warmup according to manufacturer's instructions. If the electrode is made of glass, rinse it with distilled water.

28.4.2.2. Calibrate the pH meter by dipping it in buffers of pH 7, 4 and 9.2 consecutively and take the reading after leaving the electrode for 1-2 minutes in the solution.

28.4.2.3. Rinse the electrode with distilled water and blot dry using a tissue paper after each procedure.

28.4.2.4. Dip the pH meter in molten media and allow to stand for 1-2 minutes and record the pH.

28.4.2.5. Rinse the pH meter electrode after use and blot dry

28.4.3. Procedure for checking the pH of a culture media using a pH paper

28.4.3.1. If using pH paper, dip it into a sample of the medium when it is at room temperature and compare the color of the paper against the pH color chart provided.

28.4.3.2. Test an agar medium by pouring a sample of the molten medium into a small beaker or petri dish and when it has solidified, laying a narrow range pH paper on its surface. Compare the color of the paper against the pH color chart provided.

Note: The pH of a commercial dehydrated medium should not require adjustment providing it has been prepared correctly using pure water and clean equipment, and it has not been over-autoclaved. Manufacturer's instructions must be followed strictly.

28.4.3.3. The pH of the media should be adjusted as directed in the method of preparation. Minor adjustments should be carried out using 0.1mol/L (N/10) sodium hydroxide when the medium is too acidic, and 0.1 mol/L(N/10) hydrochloric acid when too alkaline.

28.4.3.4. When adjusting the pH of a large volume of medium it is best to measure the amount of acid that needs to be added to adjust 10 ml of the medium, and then calculate the amount required to adjust the remaining volume.

28.5. Sterility testing of culture media

All prepared media should be subjected to routine sterility testing. At least 5-10 % randomly selected plates/bottles (5% if a small batch is prepared like 50 plates and 10% if a large batch is prepared 100-200 plates or tubes) of a new batch prepared should be incubated at appropriate temperature e.g. 37°C for 18-24 hours to ascertain that there is no growth or contamination.

28.5.1. Sterility testing of liquid medium

28.5.1.1. For sterile liquid media in screw-cap tubes or bottles, incubate 5% of the batch at 35-37°C overnight.

28.5.1.2. Observe for visible turbidity or pellicle/film formation as it is an indication of contamination and discard the whole batch if contamination occurs.

28.5.2. Sterility testing of plated medium

28.5.2.1. Incubate 5% of each batch of uninoculated prepared plated medium at 35-37°C for 18-24 hrs.

28.5.2.2. Examine for any growth or contamination. Discard the whole batch if contamination occurs.

Note: There should be no evidence of microbial growth after incubation. Do not use the prepared lot if growth/contamination is observed.

28.6. Performance testing

Performance testing of prepared media is performed by inoculating, about 2% of each batch with control organisms (refer to Table 2) whose growth should be supported on the tested media.

28.7. Stability testing

All the prepared media, if stored aseptically have a certain shelf life. To check whether the media is still working, periodically perform sterility testing and growth performance to determine whether the storage conditions will give optimal results.

Table 2: Quality control parameters of some basic culture media are described below:

Culture Medium	pH at Room temperature (25°C)	Control strain for performance testing	Desired result of performance Testing	Stability/Shelf life (2-8°C)
Baird Parker agar	6.8 ± 0,2	*Staphylococcus aureus*	Black, shiny, convex and surrounded by a clear zone	
Bile Aesculin Agar	7.1 ± 0.2	*Streptococcus faecalis*	Brown coloration around the colony	One month (if protected against dehydration)
Cetrimide agar	7.2 ± 0,2	*Pseudomonas aeruginosa*	Greenish or yellowish-green or reddish colony	
EMB agar	7,2 ± 0,2	*Escherichia coli*	Green with metallic sheen	
GN broth	7,0 ± 0,2	*Shigella flexneri*		One month

Culture Medium	pH at Room temperature (25°C)	Control strain for performance testing	Desired result of performance Testing	Stability/Shelf life (2-8°C)
MacConkey agar	7.1 +/- 0.2	*Escherichia coli* *Salmonella spp*	Pink colonies Pale colonies	Not more than 7 days
Mannitol salt agar	7.4 ± 7.6	*Staphylococcus aureus*	Yellow colonies	Not more than 15 Days
Membrane enterococcus agar	7.1 ± 0.2	*Enterococcus faecalis*	Red, maroon or pink colonies	Not more than 30 days
Mueller Hinton Agar	7.3 +/- 0.1	*Escherichia coli*	Creamy colonies	Not more than 7 days
Nutrient agar	6.8 +/- 0.2	*Escherichia coli*	Creamy pale colonies	Not more than 7 days
Pseudomonas agar	7.1 ± 0.2	*Pseudomonas aeruginosa*	Blue-green, greenish brown or brown colonies	One month
Selenite F broth	7.1 ± 0.2	*Salmonella spp*	Turbidity	Not more than15 days
Tetrathionate broth	8,4 ± 0,2	*Salmonella Typhi*	Turbidity	Not more than15 Days
XLD agar	7.4±0.2	*Shigella spp*	Pink colonies	Not more than7 days

28.8. User Quality Assurance Practices

28.8.1. When a commercial supply of dehydrated media is obtained, maintain an inventory with the following details: date of receipt, opening date, product batch number, manufactured date, expiry date, condition upon delivery and size of delivery.

28.8.2. The laboratory personnel responsible for media preparation must fill in a log (Media Preparation Log) indicating date, name of media prepared, batch number, and name of the personnel.

28.8.3. Document QC failures after sterility testing, performance testing, and pH testing, using the log "QC of Culture Media".

28.8.4. DO NOT use the media beyond the expiration date. Use the prepared media within the allowed/ stated duration of weeks or months, or within the manufacturer's reagent expiration date, whichever comes first.

28.8.5. Discard the expired media following healthcare waste management SOP.

28.8.6. Before inoculating, examine the media physically for any growth or turbidity, dehydration, discoloration, sloped or uneven surface of petri plates, crystalline pattern on surface of the medium (indicative of freezing) etc. by the user.

Government of Nepal Ministry of Forests and Environment Department of Environment	Document Code: SOP 05	Page
Subject Title: Isolation and identification of *Escherichia coli from effluent*		
Effective date: April 2025		Version no.
Prepared by:	Reviewed and Approved by:	

1. Purpose

The document will provide guidance to isolate and identify *Escherichia coli* from effluent sample following standard microbiological procedure. *Escherichia coli* a member of the *Enterobacteriaceae* family and the fecal coliform group, indicates fecal contamination when present.

2. Scope

This SOP describes the microbiological procedure for isolation and identification of *E. coli* from hospital and pharmaceutical effluent

3. Requirements

3.1. Equipment

3.1.1. Membrane filtration unit with vacuum pump
3.1.2. Biosafety Cabinet class A2
3.1.3. Microscope
3.1.4. Incubator
3.1.5. Hot air oven
3.1.6. Autoclave
3.1.7. Electronic weighing balance (0.01)
3.1.8. Binoculars

3.2. Media

3.2.1. EMB agar
3.2.2. Nutrient agar
3.2.3. Biochemical test media (Sulphide indole motility, Simmon's Citrate, Christensen's Urease, Triple sugar iron agar)
3.2.4. Peptone water

3.3. Reagents

3.3.1. Normal saline
3.3.2. Gram staining reagents
3.3.3. Oxidase disc/reagent
3.3.4. Catalase reagent (3% hydrogen peroxide)
3.3.5. Kovac's reagent

3.4. Others

3.4.1. Glass slides
3.4.2. Sterile membrane filters, for example, white 47 mm diameter, cellulose-based
3.4.3. PPE (gloves, masks, aprons)
3.4.4. Inoculating loops, straight wire
3.4.5. Staining rack
3.4.6. Distilled water
3.4.7. Wash bottles

4. Procedure for Isolation of *E. coli*

4.1. Three different volumes of effluent sample i.e.1, 0.1, 0.01 ml or 0.1, 0.01 and 0.001ml (according to level of turbidity) are filtered through membrane filtration by preparing serial dilution to make total volume of 10 ml for each sample.
4.2. Each of the membrane filter is aseptically transferred to EMB agar
4.3. Media plates are then incubated at 35 ± 0.5°C for 18-24 hours under aerobic condition

5. Colony Morphology

E. coli produces colonies which are elevated or slightly convex, 2-3 mm in diameter, with blue-black center with a narrow, clear edge in transmitted light and blue-green metallic sheen in reflected light.

6. Enumeration

Enumerate the number of presumptive *E. coli* colonies on membrane filter with colony morphology described in section 4.1 and express as:

Presumptive cfu/ml = No. of colonies on membrane filter X DF / Volume of sample filtered

Where DF is dilution factor, if appropriate

The number of confirmed cfu/ml *E. coli* is calculated by multiplying the number of presumptive E. coli by proportion of isolates that are confirmed by the proportion of the isolates that are lactose-positive, produce indole at 44°C, and are oxidase-negative

7. Culture purification

7.1. Obtain a pure culture by carefully picking a well-isolated colony with typical colony characteristics on membrane filter
7.2. Streak the colony on nutrient agar plate.

Better distribution of colonies in the subculture is obtained if a portion of the picked colony is emulsified in peptone broth or physiological saline (0.85% w/v)

7.3. Incubate the NA plate at 35 ± 1 °C for 24 h and perform gram staining and biochemical tests for identification of bacteria. Follow SOP 4 for biochemical tests.

8. Identification of bacteria

8.1. Microscopy:

Grams stain

Perform Gram stain from the isolated colony. E. coli are gram-negative non-spore-forming rods.

8.2. Biochemical Identification

Catalase and Oxidase: Perform catalase and oxidase tests. If catalase is positive and oxidase is negative, proceed for further.

Inoculate the organism from a well-isolated colony into peptone water and then transfer it to the biochemical test media: SIM, MR-VP broth, Simmons' citrate, TSI, and Christensen's urease

9. Interpretation

E. coli gives results as shown in the table given below

Table 3: Characteristics of *E. coli*

Tests	Result
Gram's stain	Gram negative straight rods, non-spore forming
Catalase	Positive
Oxidase	Does not produce cytochrome c oxidase- Negative
Motility	Motile, very few strains non motile
Indole production at 44 °C	Positive
Hydrogen sulphide (H_2S) production	Does not produce H_2S
Simmons' Citrate Test	Citrate not utilized
TSI	A/A, produces gas, does not produce H_2S
Urease Test	(Few strain does not produce gas)
MR	Does not produce urease- Negative
VP	Positive
Urease Test	Negative

Government of Nepal Ministry of Forests and Environment Department of Environment	Document Code: SOP-06	Page
Subject Title: Isolation and identification of *Shigella* species from effluents		
Effective date: April 2025		Version no.
Prepared by:	Reviewed and Approved by:	

1. Purpose

The document will provide guidance to isolate and identify *Shigella* species from effluent sample following standard microbiological procedure. Four species or sero groups of the genus *Shigella* i.e. *dysenteries* (Group A), *Shigella flexneri* (Group B), *Shigella boydii* (Group C), and *Shigella sonnei* (Group D) are responsible for *Shigellosis*. When outbreaks occur, they are usually associated with fecal contamination of food and, less frequently, water. *Shigellae* are sensitive to chlorination at normal levels, and they do not compete favorably with other organisms in the environment. Their survival time is measured in hours and days, and is a function of the extent of pollution, as well as physical conditions such as temperature and pH.

2. Scope

This SOP describes the microbiological procedures for isolation and identification of Shigella species from hospital and pharmaceutical effluent.

3. Requirements

3.1 Equipment

3.1.1. Centrifuge
3.1.2. Biosafety cabinet class A2
3.1.3. Microscope
3.1.4. Incubator
3.1.5. Hot air oven
3.1.6. Autoclave
3.1.7. Micropipette

3.2. Media

3.2.1. GN broth
3.2.1. SS broth/ GN broth
3.2.1. MacConkey Agar
3.2.1. XLD agar, SS agar, DCA
3.2.1. Nutrient agar
3.2.1. Biochemical test medium (O/F, SIM, MR/VP, Simmon's Citrate, Christensen's Urease, TSI agar, ONPG)

3.3. Reagents

3.3.1. Normal saline
3.3.2. Gram staining reagents
3.3.3. Oxidase disc/reagent
3.3.3. Catalase reagent (3% H2O2)
3.3.4. Kovac's reagents
3.3.5. Methyl red
3.3.6. VP reagent (5% alpha napthol 40% KOH)
3.3.7. ONPG disc/ reagent

3.4. Others

3.4.1. Glass slides
3.4.2. PPE (Gloves, masks, aprons)
3.4.3. Inoculating loops, straight wire
3.4.4. Staining rack
3.4.5. Distilled water
3.4.6. Wash bottles

4. Procedure for Isolation of *Shigella* species

4.1. Centrifuge 200-250 mL water samples at 1520 × g for 15 min and pour off all but last 2 mL of supernatant (pipette).
4.2. Resuspend the pellet and add 8 mL SS broth or GN broth.
4.3. Incubate suspension for 18- 24 h at 35 ±1°C.
4.4. Mix suspension and inoculate one loopful (4 mm loop) to each of MacConkey and XLD/DCA plates by streak plate method
4.5. Incubate plates at 35 ±1 °C for 18-24 hours
4.6. Pick suspect colonies and inoculate to biochemical media.
4.7. Cultures that are presumptively identified as *Shigella* spp. are sero grouped by slide agglutination test using polyvalent and group specific antisera.

5. Colony Morphology

MacConkey agar: After 18-24 hours of aerobic incubation at 35 ± 1°C, *Shigella* species produce colonies which are colorless, non-lactose fermenting, 1–2 mm in diameter. On prolonged incubation, *Shigella sonnei* forms pink colonies (late lactose fermenter).

XLD agar – After 18-24 hours of aerobic incubation at 35 ± 1°C, *Shigella* species produces colonies which are 1- 2 mm in diameter, pink red in color without black center. Some strains may have a pink or yellow periphery.

DCA Agar: After 24-48 hours of aerobic incubation at 35 ± 1°C, *Shigella* species produces colonies which are 1- 2 mm in diameter colorless or translucent, circular, convex, smooth, and without a black center.

6. Culture purification

Subculture typical colonies on nutrient agar and incubate at 35±1 °C for 18-24 hours

Note: Better distribution of colonies in the subculture is obtained if a portion of the picked colony is emulsified in peptone broth or physiological saline (0.85% w/v)

7. Identification of bacteria

Identification is done on the basis of gram staining, biochemical test and serological test.

7.1. Microscopy

7.2. Grams staining

Gram negative rods, arranged singly, or in pairs.

7.3. Biochemical Identification

7.3.1. Perform Catalase and Oxidase test.

7.3.2. If catalase is positive and oxidase is negative, inoculate the colonies on biochemical test media- O/F media, TSI, SIM, Simmon's Citrate, Christensen's Urea and MR-VP

Note: Shigella dysenteriae type 1 is catalase-negative

7.3.3. Incubate the tubes at 35±1°C for 24 hours (48 hours for VP) and interpret the results as per the table given below.

8. Interpretation

Shigella species gives results as shown in able given below

Table 4: Characteristics of *Shigella* species

Tests	*Shigella* spp.
Gram stain	Gram negative rods (non spore forming)
Oxidase	Negative
Catalase	Positive (Except *Shigella dysenteriae* Type1)
Indole	Differential (*Shigella sonnei* is Negative)
MR	Positive
Lysine decarboxylase	Negative
VP	Negative
Citrate Utilization	Citrate is not utilized
O/F	Fermentative
Motility	Non-motile
TSI/Gas/H2S	(Alkali/Acid), Gas: Negative, H_2S negative
Urea Hydrolysis	Negative
ONPG	Positive for *Shigella sonnei*

9. Serological confirmation

Colonies presumptively identified as *Shigella* species by biochemical reactions and can further be confirmed by serological agglutination tests.

Note: *Shigella* species are non-motile, therefore only the somatic (O) antigens are utilized for the determination of serotype. However, some *Shigella* strains possess envelope surface antigens (K antigens) that prevent the

somatic antisera from coming in contact with somatic antigens and thus do not show agglutination even when the biochemical tests show positive indication of *Shigella*. In such cases, heat a saline suspension of the organism in a container of boiling water for 20 minutes to inactivate the surface antigens. Allow the suspension to cool, centrifuge and test a fresh saline suspension of sedimented organism.

9.1. Serotyping

Serotyping is a method based on the immuno-reactivity of various antigens. *Shigella* species are non-motile, as such, only the somatic (O) antigens are utilized for the determination of serotype. Flagellar (H) antigens are not expressed. Since there are no Polyvalent O antisera for *Shigella*, serotyping should be done with individual group specific Polyvalent as PolyA, PolyB, PolyC, PolyD and further processed for subtypes

9.2. Procedure for serotyping:

9.2.1. Before beginning serotyping, emulsify the strains from a nutrient agar medium in normal saline and observe for auto agglutination. Do not proceed if there is clumping in saline suspension.

9.2.2. Take a clean and grease free slide and mark two circles in it.

9.2.3. Add 10µl or two loopful of normal saline in those two circles and emulsify suspected colonies in it to form a milky suspension.

9.2.4. Add 10µl of desired antisera on one well, gently rock the slide back and forth and observe for visible agglutination within 30 seconds.

Table 5: Serotyping of *Shigella* species

Group	Polyvalent	Polyvalent Antisera	Factor Antisera
A	*Polyvalent A (Shigelladysenteriae)*	*Shigella dysenetriae* type 1&2	A1, A2
		Shigella dysenetriae type 1-4	A1, A2, A3, A4
		Shigella dysenetriae type 5-8	A5, A6, A7, A8
		Shigella dysenetriae type 9-12	A9, A10, A11, A12
B	*Polyvalent B (Shigella flexneri)*	*Shigella flexneri* B (1-6),Var X,varY, varZ	
		Shigella flexneri type 1	B1
		Shigella flexneri type 2	B2
		Shigella flexneri type 3	B3
		Shigella flexneri type 4	B4
		Shigella flexneri type 5	B5
		Shigella flexneri type 6	B6
		Shigella flexneri group 3,4	VarY
		Shigella flexneri group 6	VarX
		Shigella flexneri group 7	VarZ
C	Polyvalent C (*Shigella boydii*)	*Shigella boydii* type 1-18	C1-18
		Shigella boydii type 1-5	C1, C2, C3, C4, C5
		Shigella boydii type 6-10	C6, C7, C8, C9, C10
		Shigella boydii type 11-14	C11, C12, C13, C14
		Shigella boydii type 15-18	C15, C16, C17, C18
D	Polyvalent D (Shigella sonnei)		
		Shigella sonnei phase 1and 2	D1 and D2
		Shigella sonnei phase 1	D1
		Shigella sonnei phase 2	D2

Government of Nepal Ministry of Forests and Environment Department of Environment	Document Code: SOP 07	Page
Subject Title: Isolation and identification of *Salmonella* species from effluents		
Effective date: April 2025		Version no.
Prepared by:	Reviewed and Approved by:	

1. Purpose

The document will provide guidance to isolate and identify *Salmonella enterica* from effluent samples following standard microbiological procedure. Many different serotypes of *Salmonella* species are present, to varying extents, in humans, animals and birds. All members of the genus are potentially pathogenic. The low numbers of *Salmonella* species found in water mainly originate from sewage and sewage effluents. The numbers of Salmonella present in water are, generally, much lower than those of other micro-organisms.

2. Scope

This SOP describes the microbiological procedures for isolation and identification *Salmonella* species from hospital and pharmaceutical effluent

3. Requirements

3.1. Equipment

3.1.1. Biosafety cabinet (Class A2)
3.1.2. Microscope
3.1.3. Incubator
3.1.4. Hot air oven
3.1.5. Autoclave
3.1.6. Binoculars

3.2. Media

3.2.1. Tetrathionate broth, RBS, SS broth
3.2.2. XLD agar, SS agar
3.2.3. MacConkey agar
3.2.4. Nutrient agar
3.2.5. BPN
3.2.6. Biochemical test (Oxidative fermentative, SIM, MR/VP, Simmon's Citrate, Christensen's Urease, Triple sugar iron agar, lysine decarboxylase)

3.3. Reagents

3.3.1. Normal saline
3.3.2. Gram staining reagents

3.3.3. Oxidase disc/reagent
3.3.4. Catalase reagent (3% H2O2)
3.3.5. Kovac's reagents
3.3.6. Methyl red
3.3.7. VP reagent (5% alpha napthol 40% KOH)
3.3.8. Antisera: Salmonella polyvalent and monovalent antisera

3.4. Others

3.4.1. Glass slides
3.4.2. PPE (gloves, masks, aprons)
3.4.3. Inoculating loops, straight wire
3.4.4. Staining rack
3.4.5. Distilled water
3.4.6. Wash bottles

4. Procedure for isolation of *Salmonella* species

4.1. Pre-enrichment in BPW (225 ml) for 24 hours at 35°C + 25 ml effluent.
4.2. Add 1 ml of pre-enriched sample each in two different double strength tetrathionate broth and incubate one at 37°C and for RBS at 42°C for 24-48 hours to increase the recovery of *Salmonella* from effluent sample
4.3. After enrichment, streak a loopful of suspension on MacConkey agar and XLD, SS/DCA and incubate plates at 35°C for 24 hours

Note: Streaking duplicate plates, one heavily and one lightly streaked, often aids in recognition of enteric pathogens in the presence of large numbers of interfering organisms

5. Colony Morphology

5.1. *Salmonella* species appear as non-lactose fermenting, pale, small, 0.5-1 mm diameter, convex colonies with an entire margin on MacConkey agar
5.2. *Salmonella* species produce pink colonies with black center (*Salmonella* enterica Paratyphi A does not produce black center) on XLD agar, S. typhimurium produces whole black colonies, colonies on SS and DCA

6. Culture purification

6.1. Obtain a pure culture by carefully picking a well-isolated colony with typical colony characteristics on isolation agar (XLD/SS/DCA/MacConkey agar)
6.2. Streak the colony on nutrient agar plate.

Note: Better distribution of colonies in the subculture is obtained if a portion of the picked colony is emulsified in peptone broth or physiological saline (0.85% w/v)

6.3. Incubate the nutrient agar plate at 35 ± 1 °C for 18- 24 hours and perform Gram staining and biochemical tests for identification of bacteria

7. Identification of bacteria

7.1. Gram staining:

Salmonella species are Gram-negative, rod-shaped, arranged singly or in pairs.

7.2. Biochemical identification

7.2.1. Catalase and oxidase: Perform catalase and oxidase tests. If catalase is positive and oxidase negative, proceed for further identification.

7.2.2. Inoculate the organism from isolated colony in biochemical test media – Oxidative fermentative, SIM, MR/VP, Simmons' citrate TSI, lysine decarboxylase test, and Urea broth

8. Interpretation

Salmonella species gives the following biochemical reactions

Table 6: Characteristics of *Salmonella* species

Tests	*Salmonella* spp.
Gram stain	Gram negative rods
Oxidase	Negative
Catalase	Positive
Indole, sulfide, motility	Negative, variable, some motile
MR	Positive
VP	Negative
Citrate	+/-#
O/F	Fermentative
Motility	**Motile**
TSI/Gas/H_2S	Alkali/Acid, Gas Variable*, H_2S variable* (serotype specific)
Urea Hydrolysis	Negative

* *Salmonella* Typhi : TSI shows H_2S positive (in the line of inoculation)
**Salmonella* Paratyphi A: TSI shows No H2S and weak gas+
**Salmonella* Paratyphi B: TSI shows H2S + abundant, gas+
Salmonella enterica Typhi and Paratyphi A are citrate negative

Upon completion of the recommended biochemical tests, perform serotyping of salmonella from nutrient agar.

9. Serological confirmation

Serotyping is based on somatic antigens (O), flagellar antigens (H) and capsular antigens.

9.1. Take a clean and grease free slide and mark two circles on it.

9.2. Add 10 µl normal saline in both circles and emulsify suspected colonies using a sterile inoculating wire in both.

9.3. Look for auto agglutination. Do not proceed if there is clumping in saline suspension.

9.4. Add 10µl of desired antisera in first circle only, mix with a sterile applicator or loop, gently rock the slide back and forth and observe for visible agglutination within 30 secs. (Begin serotyping with polyvalent O and follow group specific antisera as shown in the table below).

Table 7: Kauffman-White Scheme for classification of *Salmonella*

"O"-group	Serovar	"O" antigens	"H" antigens	
			Phase 1	**Phase 2**
Group A	*S. ParatyphiA*	1,2,12	A	no phase 2 antigen
	S. Paratyphi A var. Durazzo	2,12	A	no phase 2 antigen
Group B	*S. Paratyphi B*	1,4,5,12	B	1,2
	S.Paratyphi B var. Odense	1,4,12	B	1,2
	S. Java	1,4,5,12	B	(1,2)
	S. Limete	1,4,12,27	B	1,5
	S. Typhimurium	1,4,5,12	I	1,2
	S. Typhimurium var. Copenhagen	1,4,12	I	1,2
	S. Agama	4,12	I	1,6
	S. Abortus-equi	4,12	no phase 1 antigen	e,n,x
	S. Abortus-ovis	4,12	C	1,6
	S. Agona	4,12	f,g,s	no phase 2 antigen
	S. Brandenburg	4,12	l,v	e,n,z
	S. Bredeney	1,4,12,27	l,v	1,7
	S. Derby	1,4,5,12	f,g	no phase 2 antigen
	S. Heidelberg	1,4,5,12	R	1,2
	S. Saintpaul	1,4,5,12	e,h	1,2
	S. Salinatis	4,12	d,e,h	d,e,n,z
	S. Stanley	4,5,12	D	1,2
C	*S. ParatyphiC*	6,7,	C	1,5
	S. Choleraesuis	6,7	C	1,5
	S. Choleraesuisvar. K unzendorf	6,7	(c)	1,5
	S. Decatur	6,7	C	1,5
	S. Typhisuis	6,7	C	1,5
	S. Bareilly	6,7	Y	1,5
	S. Infantis	6,7	R	1,5
	S. Menston	6,7	g,s,t	no phase 2 antigen
	S. Montevideo	6,7	g,m,s	no phase 2 antigen
	S. Oranienburg	6,7	m,t	no phase 2 antigen
	S. Thompson	6,7	K	1,5
C	*S. Bovismorbificans*	6,8	R	1,5
	S. Newport	6,8	e,h	1,2
D	*S. Typhi*	9,12,Vi	D	no phase 2 antigen
	S. Ndolo	9,12	D	1,5
	S. Dublin	1,9,12	g,p	no phase 2 antigen
	S. Enteritidis	1,9,12	g,m	no phase 2 antigen

	S. Gallinarum	1,9,12	no phase 1 antigen	no phase 2 antigen
	S. Pullorum	(1),9,12	no phase 1 antigen	no phase 2 antigen
	S. Panama	1,9,12	l,v	1,5
	S. Miami	1,9,12	A	1,5
	S. Sendai	1,9,12	A	1,5
E	*S. Anatum*	3,10	e,h	1,6
	S. Give	3,10	l,v	1,7
	S. London	3,10	l,v	1,6
	S. Meleagridis	3,10	e,h	l,w
E	*S. Cambridge*	3,15	e,h	l,w
	S. Newington	3,15	e,h	1,6
E	*S. Minneapolis*	(3), (15),34	e,h	1,6
E	*S. Senftenberg*	1,3,19	g,s,t	no phase 2 antigen
	S. Simsbury	1,3,19	no phase 1 antigen	Z
F	*S. Aberdeen*	11	I	1,2
G	*S. Cubana*	1,13,23	Z	no phase 2 antigen
	S. Poona	13,22	Z	1,6
H	*S. Heves*	6,14,24	D	1,5
	S. Onderstepoort	1,6,14,25	e,h	1,5
I	*S. Brazil*	16	A	1,5
	S. Hvittingfoss	16	B	e,n,x
Others	*S. Kirkee*	17	B	1,2
	S. Adelaide	35	f,g	no phase 2 antigen
	S. Locarno	57	Z	Z

Government of Nepal Ministry of Forests and Environment Department of Environment	Document Code: SOP 08	Page
Subject Title: Isolation and identification of *Pseudomonas aeruginosa* from effluents		
Effective date: April 2025		Version no.
Prepared by:	Reviewed and Approved by:	

1. Purpose

Pseudomonas. aeruginosa is a type of bacteria found in the environment, especially in soil and on plants. This bacterium can grow in water with very low nutrient levels and should not be present in drinking water. Although it is often found in small amounts in the normal gut bacteria of humans and animals, it should not be used to indicate fecal pollution.

2. Scope

The document will provide guidance to isolate and identify *Pseudomonas aeruginosa* (*P. aeruginosa*) from effluent sample following standard microbiological procedure

3. Requirements

3.1. Equipment

3.1.1. Membrane filtration unit with vacuum pump
3.1.2. Biosafety cabinet Class A2
3.1.3. Ultraviolet lamp
3.1.4. Microscope
3.1.5. Incubator
3.1.6. Hot air oven
3.1.7. Autoclave
3.1.8. Electronic balance
3.1.9. Binoculars

3.2. Media

3.2.1. Pseudomonas agar
3.2.2. Cetrimide agar
3.2.3. Nutrient agar
3.2.4. Biochemical test medium (O/F, SIM, MR/VP, Simmon's Citrate, Christensen's Urease, TSI agar)

3.3. Reagents

3.3.1. Normal saline
3.3.2. Gram staining reagents

3.3.3. Oxidase disc/reagent
3.3.4. Catalase reagent (3% H_2O_2)
3.3.5. Kovac's reagents
3.3.6. Methyl red (MR)
3.3.7. Voges Proskauer reagent (5% alpha naphthol 40% KOH)

3.4. Others

3.4.1. Glass slides
3.4.2. Sterile membrane filters, Mixed cellulose ester (MCE) 0.22µm nominal pore size
3.4.3. PPE (gloves, masks, aprons)
3.4.4. Inoculating loops, straight wire
3.4.5. Staining rack
3.4.6. Distilled water
3.4.7. Wash bottles

4. Procedure for Isolation of *Pseudomonas aeruginosa*

4.1. Dilute the sample

4.1.1. Filter three different volumes of effluent samples, such as 1 ml, 0.1 ml, and 0.01 ml, or 0.1 ml, 0.01 ml, and 0.001 ml (depending on the level of turbidity), through Whatman filter paper using the membrane filtration method, preparing serial dilutions to achieve a total volume of 10 ml for each sample.
4.1.2. Carefully transfer the membrane filter to a Petri dish containing Pseudomonas agar and/or cetrimide agar.
4.1.3. Ensure that no air bubbles are trapped between the membrane filter and the medium.
4.1.4. Cover the membrane filter with the lid of the Petri dish.
4.1.5. Incubate the plates at 35±1 °C for 48 hours.
4.1.6. Examine the filter under a UV lamp for fluorescent colonies.

Note: 'Rolling' the membrane filter onto the medium minimizes the likelihood of air bubbles becoming trapped.

4.2. Colony Morphology

P. aeruginosa produces pale colonies and green colored (due to pyocyanin production) pigmentation on incubation at 35±1 °C for 24-48 hours.

Note: Colonies may also be blue-green, greenish brown or brown in colour. Those colonies, which may or may not be pigmented, should also be considered as presumptive Pseudomonas aeruginosa

P. aeruginosa produces 4 distinct types of pigments, namely pyocyanin, pyoverdine, pyorubrin and pyomelanin, distinctly visible in Nutrient Agar and Mueller Hinton Agar plates and gives a fruity or pungent smell.

Six different types of colonies may be observed:

4.2.1. **Type I:** Large, low convex, rough and leafy type and sometimes surrounded by a serrated skirt of growth. These colonies are common in clinical isolates.
4.2.2. **Type II:** Smooth, circular, domed and described as coliform type.
4.2.3. **Type III:** Small and rough.
4.2.4. **Type IV:** Small and rugose.

4.2.5. **Type V:** The mucoid alginate-producing type V is very striking; the copious exopolysaccharide produced may result in merging colonial growth and may eventually drip onto the lid of the petri dish.

4.2.6. **Type VI:** Dwarf and smallest; dwarfs are usually variants of mucoid form and may appear slightly mucoid.

Cetrimide agar

Cetrimide agar is a selective medium used to isolate *P. aeruginosa*. This medium contains cetrimide, which inhibits the growth of most other bacteria while allowing *P. aeruginosa* to grow.

When cultured on cetrimide agar, *P. aeruginosa* typically produces:

Fluorescent colonies: These colonies can appear greenish due to the production of pyocyanin and pyoverdine pigments.

Clear zones: The bacterium may produce an area of clearing around the colonies due to the breakdown of nutrients in the medium.

These characteristics help in identifying *P. aeruginosa* in laboratory settings.

4.3. Enumeration

Enumerate the number of presumptive P. aeruginosa colonies on membrane filter with colony morphology described in section 3.1 and expressed as:

Presumptive cfu/ml = No of colonies on membrane filter× DF/ Volume of sample filtered

Where DF is dilution factor, if appropriate

V is Volume of sample filtered

The number of confirmed cfu/ml P. aeruginosa is calculated by multiplying the number of presumptive P.aeruginosa by proportion of isolates that are confirmed by the proportion of isolates that hydrolysed casein on cetyl trimethylammonium bromide, CMA.

4.4. Culture purification

Sub-culture target colonies from *Pseudomonas* agar to nutrient agar and incubate at 35±1 °C for 24 hours.

Note: Better distribution of colonies in the subculture is obtained if a portion of the picked colony is emulsified in peptone broth or physiological saline (0.85% w/v)

5. Identification of bacteria

5.1. Procedure

5.1.1. Sub-culture each pigmented and/or fluorescent colony to be tested from *Pseudomonas* agar to milk agar with acetyl trimethylammonium bromide (CMA) and spread so as to obtain single colonies.

5.1.2. Incubate the samples at 35±1 °C overnight. Colonies that are 2 to 4 mm in diameter, show typical pigment production, and have an "area of clearing" around them where casein has been broken down are recorded as confirmed *P. aeruginosa*.

5.1.3. Also perform microscopy and biochemical tests of the bacteria from nutrient agar

5.2. Microscopy:

Gram negative rods, straight or curved with an average size of 0.5 to 0.8 µm in width and 1.5 to 3.0 µm in length

5.3. Biochemical Identification

5.3.1. Perform Catalase and Oxidase tests; *P. aeruginosa* shows Catalase positive and Oxidase positive reactions.

5.3.2. Inoculate in biochemical tests media (Oxidative fermentative, SIM, MR/VP, Simmons' citrate, Urease hydrolysis, TSI agar) and incubate for 18-24 hours at 35±1°C.

6. Interpretation

P. aeruginosa gives following results.

Table 8: Characteristics of *P. aeruginosa*

Tests	Result
Gram stain	Gram negative straight rods, non-sporing
Catalase	Positive
Oxidase	Positive
Oxidative/Fermentative	Oxidative (Weak)
Simmons' Citrate utilization Test	Utilized
Urea hydrolysis	Not hydrolysed
Triple Sugar Iron	Alkaline (red) slant, no change in butt, H_2S not produced, gas not formed.
Sulphur Indole Motility	H_2S not produced, Indole not produced, motile

Quality control: Refer to SOP-12 Quality Assurance in Microbiology

Government of Nepal Ministry of Forests and Environment Department of Environment	Document Code: SOP 09	Page
Subject Title: Isolation and identification of *Staphylococcus aureus*		
Effective date: April 2025		Version no.
Prepared by:	Reviewed and Approved by:	

1. Purpose

The document will provide guidance to isolate and identify *Staphylococcus aureus* from effluent sample following standard microbiological procedure. *Staphylococcus aureus* are Gram positive cocci of uniform size, occurring characteristically in groups but also singly and in pairs. They are non-motile and non-capsulated. *Staphylococcus aureus* is widespread and often a part of human flora. However, tissue invasion of *Staphylococcus aureus*, could cause skin and soft tissue infection to bacteremia and septicemia. *Staphylococcus aureus* is a major cause of health care associated infections, typically transmitted by health care-providers or other patients. *Staphylococcus aureus* is also commonly involved in infections associated with recreational water

2. Scope

This SOP describes the microbiological procedure for isolation and identification of *Staphylococcus aureus* from waste water samples

3. Requirements

3.1. Equipment

3.1.1. Membrane filtration unit with vacuum
3.1.2. Biosafety cabinet
3.1.3. Microscope
3.1.4. Incubator
3.1.5. Hot air oven
3.1.6. Autoclave
3.1.7. Micropipette

3.2. Media

3.2.1. Mannitol salt agar
3.2.2. Baired Parker Agar (BPA)
3.2.3. Nutrient agar
3.2.4. DNAse media

3.3. Reagents

3.3.1. Normal saline

3.3.2. Gram staining reagents
3.3.3. Oxidase disc/reagent
3.3.4. Catalase reagent (3% H_2O_2)
3.3.5. Kovac's reagents
3.3.6. Citrated rabbit plasma or pooled fresh human plasma
3.3.7. Cefoxitin disc (30 µg)

3.4. Others

3.4.1. Glass slides
3.4.2. Sterile membrane filters, for example, white 47 mm diameter, cellulose-based whereas 0.22 µm diameter pore size in case of chlorinated effluent.
3.4.3. PPE (gloves, masks, aprons)
3.4.4. Inoculating loops, straight wire
3.4.5. Brute Forceps
3.4.6. Staining rack
3.4.7. Distilled water
3.4.8. Wash bottles

4. Procedure for Isolation

A. Three different volumes of effluent sample i.e.1, 0.1, 0.01 ml or 0.1, 0.01 and 0.001ml (according to level of turbidity) are filtered through membrane filter by preparing serial dilution to make total volume of 10 ml for each sample.
B. Place membrane filter on Baird-Parker agar and incubate at 35 ± 0.5°C for 48 ± 4 h in Mannitol Salt Agar and incubate at 35 ± 0.5°C for 24-48 h

4.1. Enumeration

Enumerate the number of presumptive *Staphylococcus aureus* colonies on membrane filter with colony morphology described in section 4.2 and expressed as:

Presumptive cfu/100ml = No of colonies on membrane filter × DF/ Volume of sample filtered

Where DF is dilution factor, if appropriate

The number of confirmed cfu/ml *Staphylococcus aureus* is calculated by multiplying the number of presumptive *Staphylococcus aureus* by proportion of isolates that are confirmed by catalase positive, oxidase negative and coagulase positive.

4.2. Colony Morphology

Staphylococci typically form slate gray to jet black, smooth, entire colonies in BPA.

If *S. aureus* is present egg yolk clearing may be observed in MSA, S. aureus produces yellow colonies

4.3. Culture purification

4.3.1. Obtain a pure culture by carefully picking a well-isolated colony with typical colony characteristics on membrane filter.
4.3.2. Streak the colony on nutrient agar plate.

Note: Better distribution of colonies in the subculture is obtained if a portion of the picked colony is emulsified in peptone broth or physiological saline (0.85% w/v)

5. Identification of bacteria

Incubate the subculture at 35 ± 1°C for 18-24 h and perform gram staining and biochemical tests for identification of bacteria

5.1. Microscopy

Gram staining: *Staphylococcus species* appear as Gram positive cocci arranged in grape-like clusters.

5.2. Biochemical Tests

Perform catalase test, oxidase test, O/F test and coagulase test (slide followed by tube test) and mannitol fermentation test (using mannitol salt agar)

Note: If slide coagulase test is positive (shows clumping) proceed to tube coagulase test. If positive, proceed for MRSA detection.

Table 9: Characteristics of *Staphylococcus aureus*

Tests	Result
Gram stain	Gram positive cocci arranged in clusters
Catalase	Produces catalase 'positive'
Oxidase	Produces cytochrome c oxidase, 'positive'
Coagulase	Produces coagulase, 'positive'
Oxidative/Fermentative	Fermentative
Mannitol fermentation	Ferments mannitol
DNase test	Positive

6. MRSA Detection

6.1. MRSA can be phenotypically detected by using Cefoxitin (30µg) disc (CLSI 2021).
6.2. Prepare a 0.5 McFarland inoculums of *S. aureus* in normal saline.
6.3. Prepare a lawn culture on Mueller Hinton agar by swabbing the plate with a 0.5 McFarland standard inoculums of S. aureus.
6.4. Leave at room temperature for 10-15 minutes.
6.5. Using sterile forceps, place a cefoxitin disc (30µg) on the swabbed media and incubate the plates at 35°C for 18-24 hrs.

7. Interpretation

If a zone of inhibition diameter of 21 mm or less is observed, the *S. aureus* strain is considered as MRSA.*

Note: Cefoxitin is tested as a surrogate for oxacillin. Isolates that test resistant by cefoxitin, cefoxitin disk, or oxacillin should be reported as oxacillin resistant. If testing only cefoxitin, report oxacillin susceptible or resistant based on the cefoxitin result.

Government of Nepal Ministry of Forests and Environment Department of Environment	Document Code: SOP-10	Page
Subject Title: Isolation and identification of *Enterococcus* species		
Effective date: April 2025		Version no.
Prepared by:	Reviewed and Approved by:	

1. Purpose

This document will provide guidance to isolate and identify *Enterococcus* species from effluent samples following standard microbiological procedure. *Enterococcus* species are Gram positive cocci, occurring in pairs or short chains. They are non-capsulated and the majority are non-motile. *Enterococcus faecalis* (formerly classified *Streptococcus faecalis*) is the main pathogen in the genus *Enterococcus*, causing about 95% of enterococcal infections including infections of the urinary tract, biliary tract, ulcers (e.g. bed sores), wounds (particularly abdominal) and occasionally endocarditis or meningitis.

2. Scope

This document describes the microbiological procedures for isolation and identification of *Enterococcus* species from hospital and pharmaceutical effluent

3. Requirements

3.1. Equipment

3.1.1. Membrane filtration unit with vacuum pump
3.1.2. Biosafety Cabinet class A2
3.1.3. Microscope
3.1.4. Incubator
3.1.5. Hot air oven
3.1.6. Autoclave
3.1.7. Electronic weighing balance (0.01)
3.1.8. Binoculars

3.2. Media

3.2.1. Membrane enterococcus agar (mEA)
3.2.2. Bile aesculin agar(BAA)
3.2.3. Nutrient agar
3.2.4. Brain Heart Infusion agar
3.2.5. 40 % bile agar or MacConkey agar
3.2.6. Glucose phenolphthalein broth
3.2.7. Sheep blood agar

3.3. Reagents

3.3.1. Normal saline
3.3.2. Gram staining reagents
3.3.3. Oxidase disc/reagent
3.3.4. Catalase reagent (3% H_2O_2)

3.4. Others

3.4.1. Glass Slides
3.4.2. Sterile membrane filters, for example, white 0.45µm diameter, cellulose-based
3.4.3. PPE (gloves, masks, aprons)
3.4.4. Inoculating loops, Straight wire
3.4.5. Staining rack
3.4.6. Distilled water
3.4.7. Wash bottles

4. Procedure for isolation of *Enterococcus* species

4.1. Three different volumes of effluent sample i.e.1, 0.1, 0.01 ml or 0.1, 0.01 and 0.001ml (according to level of turbidity) are filtered through membrane filtration by preparing serial dilution to make total volume of 10 ml for each sample.
4.2. Transfer the membrane filter carefully to a Petri dish containing mEA and incubate at 35.0 ± 1.0 °C for 4.0 ± 0.5 hours followed by 44.0 ± 0.5 °C for 40 ± 4 hours.

4.1. Enumeration

Enumerate the number of presumptive *Enterococcus* colonies on membrane filter with colony morphology described in section 4.2 and expressed as:

Presumptive cfu/100ml = No of colonies on membrane filter X DF/ Volume of sample filtered

Where DF is dilution factor, if appropriate

The number of confirmed cfu/ml Enterococcus species is calculated by multiplying the number of presumptive Enterococcus species by proportion of isolates that are confirmed by sub-culturing from mEA to Bile esculin agar (BAA) and incubating at 44 ± 0.5 °C for up to 18 hours. Enterococci should produce discrete colonies surrounded by a brown or black halo resulting from esculin hydrolysis

4.2. Colony Morphology

Enterococci produce red, maroon or pink colonies on mEA.

4.3. Culture purification

Colonies can be sub-cultured to nutrient agar, BHI agar and incubated at 35±1°C for 18-24 hours.

5. Identification of bacteria

Enterococcus species can be identified by gram staining, catalase test, also can be differentiated from other streptococci by their ability to grow in nutrient broth containing 6.5 % sodium chloride, and in glucose phenolphthalein broth modified to pH 9.6, and their resistance to heating at 60 °C.

5.1. Microscopy:

Grams stain: *Enterococcus* species are Gram positive cocci, occurring in pairs or short chains

5.2. Bile tolerance

From nutrient agar, sub-culture a portion of colony to a Petri dish or tube containing 40 % bile esculin agar and incubate at 35 °C for 24 - 48 hours. Growth on this medium indicates tolerance of bile salts. Alternatively, sub-culture to a Petri dish or tube containing MacConkey agar and incubate at 35 °C for 24 - 48 hours to show growth in the presence of bile salts.

Enterococci form small deep red colonies on MacConkey agar. Include control tests with organisms, of which one species is known to grow in the presence of 40 % bile (for example, *Enterococcus faecalis*) and one species is known not to grow in the presence of 40 % bile (for example, *Streptococcus pneumoniae*)

5.3. Heat resistance

From nutrient agar, transfer a portion of colony to 1 ml of the nutrient broth culture to a small test tube. Place the test tube in a water bath at 60 °C for 30 minutes. Cool the tube rapidly by immersing the tube in water and incubate at 35 °C for 18-24 hours. Sub-culture the broth to a Petri dish containing 5% sheep blood agar. Incubate overnight at 35 °C and examine for growth. Include control tests with organisms, of which one species is known to survive this heat treatment (for example, *Enterococcus faecalis*) and one species is known not to survive (for example, *Streptococcus bovis* or *Streptococcus equinus*).

5.4. Growth at pH 9.6

Transfer a colony from the nutrient agar and inoculate into a tube of glucose phenolphthalein broth modified to pH 9.6 and incubate at 35 °C for 18-24hours. Tolerance to this solution (at pH 9.6) is demonstrated by the heavy growth of organisms and by the decolorization of the medium from pink (red) to colourless. Include control tests with organisms, of which one species is known to grow at pH 9.6 (for example, *Enterococcus faecalis*) and one species is known not to grow at pH 9.6 (for example, *Streptococcus bovis* or *Streptococcus equinus*).

5.5. Salt tolerance

Transfer a colony from the nutrient agar and inoculate into a tube of nutrient broth containing 6.5% sodium chloride and incubate at 35 °C for 24 - 48 hours. Examine for growth. Include control tests with organisms, of which one species is known to grow in the presence of 6.5 % salt (for example, *Enterococcus faecalis*) and one species is known not to grow in the presence of 6.5 % salt (for example, *Streptococcus bovis* or *Streptococcus equinus*). Observe the turbidity of broth.

6. Interpretation

Table 10: Characteristics of *Enterococcus* species

Tests	Result
Gram stain	Gram positive cocci in pair or short chain
Catalase	Negative
Aesculin hydrolysis	Positive
40% bile tolerance (40%)	Tolerant
Heat resistance (600C)	Resistant
Growth at pH 9.6	Positive
Growth in 6.5 % NaCl	Positive

Government of Nepal Ministry of Forests and Environment Department of Environment	Document Code: SOP-11	Page
Subject Title: Antibiotic Susceptibility testing		
Effective date: April 2025		Version no.
Prepared by:	Reviewed and Issued by:	

1. Purpose

Determining the susceptibility of a bacterial strain to Revise appropriate antibiotics is done using modified Kirby Bauer disc diffusion method.

2. Scope

This SOP describes the procedure for antibiotic susceptibility testing for aerobic bacteria by modified Kirby Bauer's standard disc diffusion method

3. Materials and Reagents

3.1. Equipment

3.1.1. Biosafety Cabinet Class A2
3.1.2. Freezer -80°/-20°C/Refrigerator (2-8°C)
3.1.3. Incubator 35-37°C
3.1.4. Bunsen burner
3.1.5. Automatic disk dispenser/Forceps
3.1.6. Ruler/Vernier Calipers
3.1.7. Densitometer

3.2. Media

Mueller Hinton Agar

3.3. Reagents

3.3.1. Sterile swab sticks
3.3.2. Normal saline
3.3.3. 0.5 McFarland standard
3.3.4. Antibiotic discs as per CLSI recommended

3.4. Others

3.4.1. PPE (Gloves, Masks, Aprons)
3.4.2. Inoculating loops, Straight wire

4. Procedure

4.1. Preparation of MHA: 4mm depth (25 ml in 90 mm plates)

4.2. Preparing the inoculum

4.2.1. Make a smooth suspension of 0.5 McFarland turbidity (equivalent to a growth of 1.5-2x10^8 CFU/mL for E. coli ATCC 25922) by emulsifying 3-5 isolated colonies with identical morphology in normal saline.

4.2.2. Check the turbidity visually by comparing with the 0.5 McFarland standard or read in a densitometer/ turbidimeter.

NOTE: Using a sterile inoculating wire, pick only well-isolated colonies from the plate to avoid testing mixed cultures. If well-isolated colonies are not present, subculture the organism onto a fresh plate.

4.3. Inoculation of the agar plate:

4.3.1. Dip a sterile swab into the bacterial suspension and rotate the swab several times while pressing it firmly on the inside of the tube wall above the fluid level to remove the excess fluid from the swab. The swab should not be dripping wet.

4.3.2. Inoculate the AST agar plate with the swab on one quadrant of the plate. (Refer to table below for choice of media for AST). Repeat the streaking two times, rotating the plate 600 each time to ensure an even distribution 1of the inoculum. Rim the plate with the swab to pick up any excess liquid. Discard the swab into appropriate container with disinfectant

4.3.3. The inoculum is left to dry for 3-5 minutes at room temperature leaving the lid slightly agar (no more than 15 min.) for the surface of the plate to dry before proceeding to next step.

Table 11: Media and Incubation Condition for Antibiotic Susceptibility Test

Media	Organism	Incubation conditions
Mueller Hinton Agar	*Staphylococcus aureus Enterococci, Escherichia coli, Salmonella* species, *Shigella* species, *Pseudomonas aeruginosa*	35°C for 18-24 h

4.4. Preparing the disk diffusion test

This section explains how to place the antibiotic susceptibility disks on the inoculated agar plate following the disk-diffusion method.

Remove the required antibiotic disks from the refrigerator to attain room temperature before opening the container (to avoid condensation and subsequent deterioration).

Note: Antibiotic disc containers must contain an active desiccant (Silica gel will turn from blue to pink when fully saturated with moisture). Replace the disks and container in the refrigerator after use as soon as possible. Do not use disks past their expiry date.

All Petri-dishes and antibiotic disks/strips must be at room temperature before use.

4.4. Application of disks

4.4.1. Place disks of the appropriate antibiotics for the species in question on the plate using the automatic disk dispenser or manually using sterile forceps /needle. Use forceps to handle single disks. (Sterilize the forceps by cleaning them with a sterile alcohol pad & allowing to air dry or by flaming).

4.4.2. Apply the discs evenly on the agar surface; press gently on the disk after application.

Note: Do not relocate a disk once it has come into contact with the agar surface, as some antibiotics diffuse almost instantaneously. Instead, place a new disk in another location on the agar.

4.4.3. Keep a minimal distance of 24mm between the centers of adjacent disks (unless explicitly mentioned otherwise), this corresponds with maximal 6 disks on a 90 mm Petri dish and maximum 12 discs on a 150 mm Petri dishInvert the plates and incubate in the appropriate atmosphere for the appropriate time as indicated in Table 1.

4.4.4. Do not place agar plates in stacks of more than 10, because the middle plates will take longer to reach the incubator temperature. This delay could cause larger zones.

4.5. Reading the results

This section explains how to interpret and report the results. Before reading the results:

4.5.1. If individual colonies are seen, the suspension was not dense enough (< 0.5 McFarland) and the test must be repeated.

4.5.2. If the growth is excessive, the test suspension was too dense, and the test must be repeated.

4.5.3. In case of translucent culture medium, place the ruler against the bottom of the petri dish.

4.5.4. In case of non-translucent media (blood containing media), remove the lid from the plate and hand measure the diameter of the inhibition zone at the surface of the culture medium (Do not measure the haemolysis zone).

4.5.5. Following incubation measure the zone sizes to the nearest millimeter using a ruler or caliper. Include the diameter of the disk in the measurement. All measurements are made with the unaided eye. The end of the zone should be taken as the area showing no obvious visible growth that can be detected with unaided eyes. Ignore faint growth of tiny colonies that can only be detected with a magnifying lens at the edge of the zone of inhibited growth. Hold the plate a few inches above the bench, non-reflecting surface illuminated with reflected light.

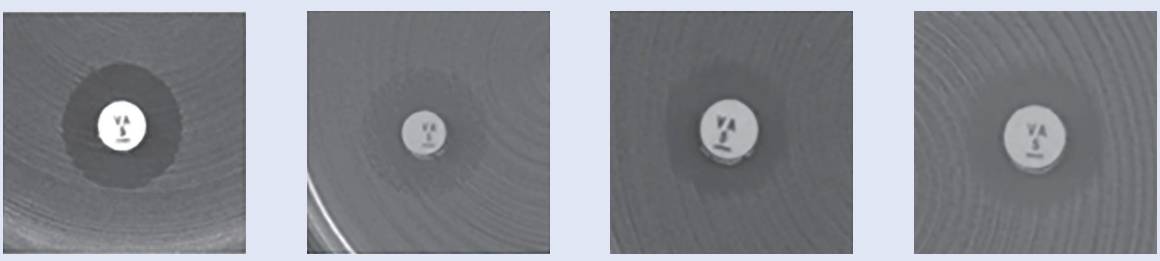
Note: for co-trimoxazole and ampicillin, the diameter is measured from the clear end of growth, and not from the start of the haze (haze must be ignored).

4.5.6. If a double inhibition zone is visible the inner inhibition zone is reported.

4.5.7. Distinct, discrete colonies within an obvious ZOI could be either mutants that are resistant to the drug being tested or the culture was not pure and they are different organism. If it is determined by repeat testing that the phenomenon repeats, the organism must be considered as resistant to that drug.

4.5.8. When testing Enterococcusspecies for vancomycin, the zone edge must be sharp and there can be no colonies inside the zone to categorize as susceptible. If the zone edge is hazy and/or there arecolonies inside the zone, the isolate is categorized as resistant (VRE).

Figure 2 : Example of inhibition zones for Enterococci with vancomycin (a; only zone with sharp edge indicates susceptibility, b; colonies inside zone indicates resistant, c & d; hazy edges indicate resistant)



Note: If the placement of the disk or the size of the zone does not allow to read the diameter of the zone, measure from the center of the disk to point on the circumference of the zone where a distinct edge is present (the radius) and multiply the measurement by 2 to determine the diameter.

4.5.9. Compare the measured zone size with that for the species and antibiotic combinations.

Note: Disk diffusion testing is not reliable for some organism/antibiotic combinations (e.g.E. coli and colistin, S. aureus and vancomycin.). In these circumstances an Etest (not recommended)/MIC may need to be undertaken.

5. Interpretation and reporting of results

Susceptibility to antibiotics is reported in code of interpretation:

S Susceptible

I Intermediate

R Resistant,

Using the latest published CLSI guidelines, determine the S, I or R of the organism to each drug tested. For each drug, indicate on the recording sheet whether the zone size falls under Susceptible (S), Intermediate (I) or resistant(R) criteria (Annex 3). Refer to Annex 7 for intrinsic resistance.

6. Quality control:

Refer to SOP-10Quality Assurance in Microbiology Laboratory

7. Waste disposal:

Refer to SOP-11 Waste management in microbiology laboratory

Government of Nepal Ministry of Forests and Environment Department of Environment	Document Code: SOP 12	Page
Subject Title: Quality assurance in Microbiology		
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1. Purpose

Microbiology procedures in the laboratory are highly specialized and needs stringent quality control. Quality control management (QCM) at each step of microbiology procedure will ensure the generation of standard reproducible reports. The laboratory must be consistent to pay attention to the following areas to generate quality reports.

2. Scope

This SOP describes quality assurance in microbiology laboratory. The manual describes the QCM under following headings:

2.1. Environmental control
2.2. Biosafety in microbiology lab
2.3. Sterilization/Disinfection control
2.4. Media control
2.5. Instrument control and calibration
2.6. Control of staining techniques
2.7. Controls for antibiotic susceptibility testing
2.8. Intra-laboratory quality assessment
2.9. Inter laboratory quality assessment
2.10. External quality assessment
2.11. Internal quality assurance audit
2.12. Maintenance of records

3. Requirements

3.1. Biosafety cabinet level 2
3.2. Microscope (compound), 10X, 40X and 100X
3.3. Incubator (25-30/ 37 °C)
3.4. Refrigerator and freezers (4°C/ -20°C/ -80°C)
3.5. Autoclave (preferably horizontal)
3.6. Hot air oven
3.7. pH meter (Bench Top)
3.8. PPE set
3.9. Autoclave indicators (Biological/Chemical)
3.10. QC strains
3.11. Centrifuge Vertex

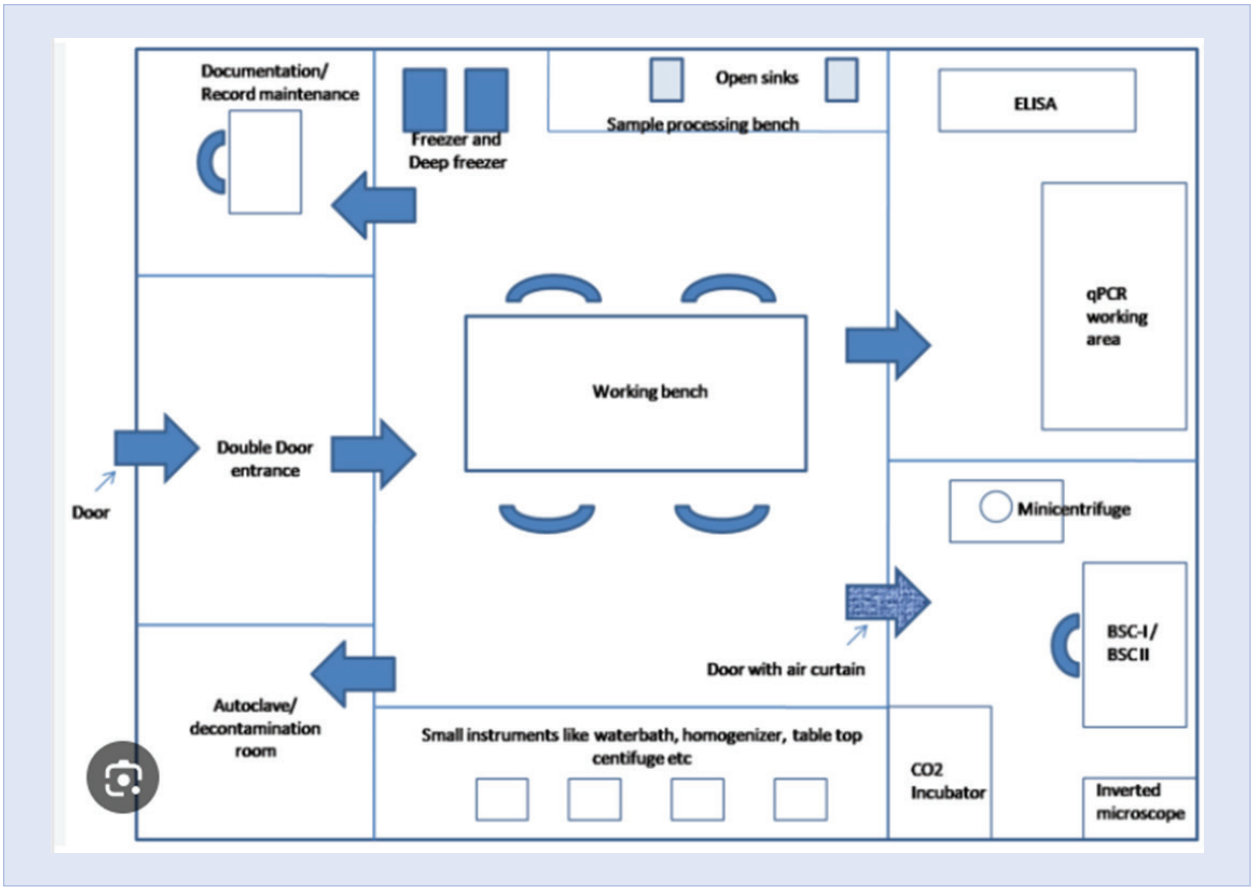
4. 4. Environmental control in microbiology laboratory

4.1. Laboratory structure

The microbiology lab structure includes:

4.1.1. Lab in-charge/ Lab managers room /documentation and record maintenance
4.1.2. Microbiology lab with sample receiving counter
4.1.3. Media /reagents preparation room
4.1.4. Specimen processing room
4.1.5. Storeroom
4.1.6. Washing room/Autoclave/decontamination room

Figure 3 : Design of physical spaces in microbiology laboratory



4.2. Purpose of different sectors of the microbiology laboratory

All samples and requisitions related to microbiology works are received in sample receiving counter. The samples for immediate work are shifted to sample processing area by laboratory staff. Samples that required to be stored are kept in refrigerator designated for sample storage. Any microbiological procedure, which can lead to aerosol formation, is not allowed in the routine laboratory. It requires stringent containment and should be processed in BSL-II and above laboratory.

Primary cultures of all specimens and handling are strictly, performed in a certified biosafety cabinet.

Preparation and storage of media is done in the sterile area aseptically (Laminar flow/ safety cabinet). Media preparation room is always kept clean and unnecessary entry into the room is restricted. Specially trained housekeeping workers disinfect and clean all culture plates/tubes in the washing room. All plates and infected items for routine microbiology workup are autoclaved prior to discarding or washing.

4.3. Room temperature:

Maintain laboratory temperature as follows:

4.3.1. Sample storage room: 20-25°C
4.3.2. Media room/Sterile room: 20-25°C

Sample processing area/room should not be connected with central AC. Individual AC is put-and temperature maintained at 20-25°C.

4.4. Room humidity

4.4.1. Sample storage room: below 60%
4.4.2. Media room/Sterile room: below 60%
4.4.3. Sample processing area/room: below 60 %

4.5. Air and surface monitoring

Air and surface can be monitored through

4.5.1. Observation for regular cleaning, scrubbing and dusting
4.5.2. Air colony count (TSA/PCA and PDA), MA

Perform cleaning and dusting thrice daily unless otherwise specified. Cleaning is done with plain water and mops normally except during some special situation like spilling.

4.6. Air colony count

Regular air colony count helps to check the quality of internal air. The air shouldn't contain large number of dust particles, which are common reservoir of many organisms. The settling of large particle in the air on sterile PCA and PDA/MA is measured at working level. Clean air reduces the chance of contamination during routine work and gives a healthy environment for the laboratory personnel.

Air sampler can be used to check the quality of internal air. In settling plate technique, one sterile PCA and PDA/MA plate is kept open on the working table for 1 hour. After exposure the plate should be closed and incubated. The PCA and MA plate is incubated at 37°C for 48 hours and PDA is incubated for up to 7 days at 25 °C.

Bacterial count: Acceptable limit up to 10 CFU in PCA/PDA/MA.

Fungal count: Acceptable limit: There should not be any fungal growth in the media.

5. Biosafety in microbiology lab

5.1. Sterilization and Disinfections control

5.1.1. Performance verification of autoclaves

The recommended operating conditions for the steam autoclave for heat insensitive instruments are 121°C at 151b/inch^2. Appropriate wrapping materials should therefore be used if instruments are to be stored after sterilization.

5.1.1.1. **Physical:** A simple method of monitoring is observation of the autoclave gauges and timers during the operational cycle, recording the temperature, time and pressure of operation in a logbook.

5.1.1.2. **Chemical:** For every load, use a strip of chemical indicator on each packed item. Validity is assured by color change after autoclave (as mentioned in manufacturer's instruction). The indicators only change color at specific temperature and/or time cycles and cannot therefore be an absolute guarantee of sterility.

5.1.1.3. **Biological indicator:** Use biological thermocouple containing 10^6 CFU of *Geobacillusstearothermophillus* spore to check performance of autoclave. Perform the test at 7 days interval or after running of 15 cycles, whichever is earlier. Place the indicator at one corner most area where chance of steam penetrability is least. Take out the indicator after the run, break the glass tube to release the media, and incubate it at 56°C for 48h.

Note: Follow manufacturer guidelines for each batch of biological indicator.

5.2. Performance verification of pre-sterilized laboratory supplies

Ensure that the containers for microbiological work-up are sterile. Use commercial wide mouth sterile containers for environmental sample collection. **Performance assessment of sterility check of prepared media and reagent.**

Check1-2% of plates or tubes randomly from every batch of media for sterility by keeping that plate/ tube in incubator for 24 hours (the temperature and time depending on the type of culture media). If media sterility is not satisfactory another sample from the same batch is incubated in a similar way. If the second media do not show sterility, the whole batch is discarded.

5.3. Media control

Media control is necessary to check sterility and growth promoting ability of the media either prepared in- house or procured commercially. The quality of the lot and each batch of media must be maintained with appropriate growth of control organisms. The quality control of media can be divided into two parts:

5.3.1. Physical characteristics

5.3.1.1. Visual test for color and clarity/ Texture

5.3.1.2. Gel strength-neither over hard nor over soft but firm and usable.

5.3.1.3. pH of the finished media

5.3.1.4. Check for damage such as cracks and defects.

5.3.1.5. General appearance/color/ non-hemolytic nature (blood containing media)

5.3.2. Sterility testing of culture media

Sterility test should be performed routinely for all types of media. For sterile media in screw-capped tubes or bottles, the simplest way to test for contamination is to incubate 5% of the batch at 35-37°C overnight. Contamination by microorganisms capable of overnight growth will be shown by turbidity in a fluid medium and growth on or in a solid medium. Media in Petri dishes are best examined for contamination immediately before use.

All media, even those that have been sterility tested at the time of preparation, should always be checked visually immediately before being inoculated for any change in appearance that could indicate contamination or deterioration.

5.4. Performance testing

Quality control testing of prepared media is performed by inoculating 2% of each batch with control organisms whose growth should be supported on the tested media. For the following media and organisms, testing is performed at 35-37°C incubation in 5-10 % CO_2/aerobically depending on the organism and media.

Table 12: Quality control of commonly used culture media

Culture medium	Recommended control species	Performance indicator	Incubation Environment
Baird Parker agar	*Staphylococcus aureus ATCC 25923* *Escherichia coli ATCC 25922*	Black colony, clear zone around colony No growth	Aerobically
Brain Heart Infusion agar	*Streptococcus pyogenes ATCC 19615*	Good growth	
EMB agar	*Escherichia coli ATCC 25922* *Enterobacter aerogenes ATCC 13048* *Pseudomonas aeruginosa ATCC 10145*	Green with metallic sheen Pink Colorless	Aerobically
Bile Aesculin Agar	*Enterococcus faecalis* (ATCC 29212) *Streptococcus pyogenes* ATCC 12344	Dark brown color around the colony No growth	
Simmons Citrate agar slant	*Pseudomonas aeruginosa* (ATCC® 27853) *Escherichia coli* (ATCC® 25922)	Deep blue color No change	Aerobically
Cetrimide agar	*Escherichia coli* ATCC 25922 *Pseudomonas aeruginosa* ATCC 27853	No growth Satisfactory growth	Aerobically
Lysine Iron Agar (LIA)	*Escherichia coli ATCC 25922* *Citrobacter freundii ATCC 8090*	Red-Purple/Red-purple, H2S- Red-Purple/Yellow, H2S+	Aerobically
MacConkey agar	*Escherichia coli* (ATCC 25922) *Proteus vulgaris ATCC 13315*	Pink colony (Lactose fermenter) Pale colony (Non- Lactose Fermenter)	Aerobically
Mueller HintonAgar	*Enterococcus faecalis* (ATCC 29212) *Pseudomonas aeruginosa* (ATCC 27853)	Acceptable QC Limits of zone diameters for Non fastidious Organisms*	Aerobically
Membrane enterococcus agar	*Enterococcus faecalis (ATCC 29212)*	Red, maroon or pink colonies	Aerobically
Mannitol saltsgar	*Staphyococcus aureus* (ATCC 25923) *Enterococcus faecalis* (ATCC 29212)	Yellow colony Pink colony/limited growth	Aerobically
Pseudomonas agar	*Pseudomonas aeruginosa* (ATCC 27853) *Pseudomonas aeruginosa* ATCC 25619 *Pseudomonas aeruginosa* ATCC 10145	Blue Blue green No color	Aerobically
SIM	*Escherichia coli* (ATCC 25922) *Klebsiella pneumoniaeProteus mirabilis*	Motile/Indole+ Non-Motile/Indole- H_2S +	Aerobically
TSI	*Pseudomonas aeruginosa* (ATCC 27853) *Escherichia coli* (ATCC 25922) *Salmonella* Typhi	Alkaline-/No change Acid/Acid Alkaline/Acid H_2S+	Aerobically

O/F media (with glucose)	*Escherichia coli ATCC 25922* *Pseudomonas aeruginosa ATCC 27853*	Yellow (in tube with oil and without oil) Yellow (in tube without oil) and green in tube with oil	Aerobically
Tetrathionate broth	*Escherichia coli* ATCC 25922 *Salmonella* Typhi 6539	Scarce or no growth Satisfactory	Aerobically
Urea Agar (Slant)	*Escherichia coli ATCC 25922* *Klebsiella pneumoniae ATCC 13883*	No change in color Pink Color	Aerobically
Xylose Lysine Deoxycholate (XLD) agar	*Salmonella* Typhimurium *ATCC 14028* *Staphylococcus aureus ATCC 25923*	Transparent red (black center) No growth	Aerobically

*Each batch of Mueller Hinton Agar is tested with at least twelve antibiotic disks that are representative of the major classes of antibiotics.

*The zone size control limits are listed in "Acceptable QC limits of zone diameters for non- fastidious organisms" as recommended by most recent CLSI criteria.

Table 13: Maintenance of quality control strains and preservation of stock cultures

Recommended control Species	Storage of Stock Culture	Maintenance of Stock culture for day to day use
Streptococcus pyogenes (ATCC 19615)	-70ºC in Cryovials with beads	Sub-culture in suitable plate every week and storage at 2-8ºC
Enterococcus faecalis (ATCC 29212)	-70ºC in Cryovials with beads	Sub-culture in suitable plate everyweek and storage at 2-8ºC
Escherichia coli (ATCC 25922)	-70ºC in Cryovials with beads	Sub-culture in suitable plate every week and storage at 2-8ºC
Klebsiella pneumoniae ATCC 13883	-70ºC in Cryovials with beads	Sub-culture in suitable plate every week and storage at 2-8ºC
Proteus vulgaris ATCC 13315	-70ºC in Cryovials with beads	Sub-culture in suitable plate every week and storage at 2-8ºC
Pseudomonas aeruginosa (ATCC 27853)	-70ºC in Cryovials with beads	Sub-culture in suitable plate every week and storage at 2-8ºC
Pseudomonas aeruginosa ATCC 25619	-70ºC in Cryovials with beads	Sub-culture in suitable plate every week and storage at 2-8ºC
Pseudomonas aeruginosa ATCC 10145	-70ºC in Cryovials with beads	Sub-culture in suitable plate every week and storage at 2-8ºC
Salmonella Typhi 6539	-70ºC in Cryovials with beads	Sub-culture in suitable plate everyweek and storage at 2-8ºC
Salmonella Typhimurium *ATCC 14028*	-70ºC in Cryovials with beads	Sub-culture in suitable plate every week and storage at 2-8ºC
Staphylococcus aureus ATCC 25923	-70ºC in Cryovials with beads	Sub-culture in suitable plate every week and storage at 2-8ºC
Streptococcus pneumoniae (ATCC 49619)	-70ºC in Cryovials with beads	Subculture in suitable plate every third day and storage in incubator

*Each vial contains approximately 20-30 individual CryoBeads, along with a cryo-preservative liquid (Triyptic Soy Broth with Glycerol). Microorganisms readily adhere to the surface because of the beads porous nature. For extended storage, after inoculation, vials are kept frozen (-70ºC) or below.

Every month, a single bead will be removed by forceps without allowing of thawing of the liquid and utilized to directly inoculate a suitable bacteriological medium and viability of the organism will be checked. The viable organism will be maintained as stock for day to day use as outlined in the above table.

Table 14: Disinfectants recommended in microbiology lab

Disinfectant	Concentration	Purpose
Lysol	5 %	Disinfection of surfaces/working area
Hypochlorite solution	1 %	Spill management
Glutaraldehyde	2 %	Surface disinfectant Sharp container
Ethyl alcohol + Chlorhexi-dine	70% + 2.5% respectively	Hand rub

6. Instrument control and calibration

For each instrument used in Microbiology Laboratory, following aspects need to be maintained.

6.1. Installation date/calibration date
6.2. Company name and contacts with phone number
6.3. Preventive maintenance
6.4. Breakdown register & follow-up
6.5. Calibration reports

7. Work instructions

Note and inform the respective laboratory head for any breakdown/performance outranging desired value.

7.1. All corrective minor adjustments have to be done immediately after discussion with Consultants, Senior Lab Staff or Senior Technician.
7.2. Inform all critical errors immediately to laboratory head and laboratory manager.
7.3. Perform calibration every time the machine needs shifting from the lab for repairing purpose.
7.4. Calibrate all the pipettes and centrifuges in every six months.

8. Control of staining techniques

Routine control of stain and staining technique is required for appropriate identification and reporting of organisms. Check each fresh batch of any component of a stain with staining of control organisms. If the staining quality is satisfactory the component can be used for further staining. Report any signs of deterioration of abnormal turbidity and prepare fresh staining solution.

Table 15 : Control organisms used in staining

Stain used in Microbiology Lab	Control organism	Expected result
Gram stain	*Staphylococcus aureus ATCC25923* *E.coli ATCC25922*	Gram positive (Purple) cocci Gram (Pink) negative bacilli

9. Control of antibiotic susceptibility testing

Quality control of antibiotic susceptibility tests has several components including quality and preparation of Mueller Hinton agar, media thickness and quality of antibiotic disks.

Table 16: Quality control measures for antibiotic susceptibility test

Quality	Interval	Method
New lot of disk	Each new lot	*E. coli* ATCC 25922 *S. aureus* ATCC 25923 Any of these organisms is tested and zone of inhibition is compared with CLSI guidelines.
Inoculum Preparation	Each organism	Matched with 0.5 MacFarland standards or densito meter
Media thickness	Each plate	Should be 4 mm
Disk	Weekly	Potency check of the day to day use disks must be checked by using: *E. coli* ATCC 25922 *S. aureus* ATCC 25923 Any of these organisms is tested and zone of inhibition is compared with CLSI guidelines.

10. Intra-laboratory/inter-departmental quality assessment

Intra laboratory/interdepartmental quality assessment is done to find the consistency of the results. This is usually checked at regular basis and whenever required as mentioned in the quality plan.

11. External quality assessment scheme

The laboratory participates in the external quality assessment scheme, which must verify the laboratory performance at least once in every 4 months. The performances are analyzed, recorded and corrective actions are taken.

12. Inter laboratory assessment

The laboratory must participate in the inter laboratory assessment for those parameters not covered by external quality assessment at least twice ina year.

13. Internal quality assurance audit

Conduct one internal quality audit in a year. The internal audit is done to identify any non-conformity, implementation of policies and protocols and for the continuous improvement of the lab followed by management review meeting (MRM).

14. Biosafety in microbiology laboratory

Biosafety is a major issue in microbiology as it deals with live organisms. Safety measures arespecifically important for the technicians who handle the cultures, process samples and conduct tests.Safety measures are equally important to avoid spread of organism from the laboratory to the outside environment. For biosafety the laboratory must follow some procedures such as:

14.1. There is a biosafety policy in the microbiology laboratory. Any person who works/observes in the laboratory must follow the biosafety guidelines. Biosafety guidelines are described in the quality system.
14.2. All microbiological work-up where there is a chance of aerosol transmission must be conducted in the biological safety cabinet.
14.3. The cabinet should be cleaned with ethanol after every task each day.
14.4. Air circulation in the infectious area must be separate from the other areas of the laboratory (No central A/C, only dedicated A/C to be used).
14.5. There must be adequate facility of handwashing in the infectious area.
14.6. There should be continuous supply of gloves, aprons and masks in the laboratory.
14.7. Unauthorized access should be prohibited/restricted.
14.8. For sample transportation outside the laboratory, the packaging norms according to the international standards should be followed and bio-hazard symbol must be utilized.

15. Maintenance of records

All quality control documents must be maintained for certain period of time according to laboratory's policy. Any non-conformity in the QC policy must be recorded. If similar kind of non-conformity occurs repeatedly, the corrective action must be taken or the quality policy must be reviewed.

16. Handling outliers of internal quality control

Outliers of internal quality controls should be recorded along with its report and its lot number verified. The batch or lot of the consumables outlier will be taken out of the laboratory circulation and disposed of.

17. Training of staff

Qualified and well-trained staff are crucial for ensuring reliable analytical results. The Laboratory Manager is responsible for making sure all personnel have the necessary knowledge, skills, and abilities for their roles. Laboratory analysts follow a structured training program as per the laboratory's training procedures. All training activities are recorded in the individual's training file. Training need identification should be done.

18. Handling outliers of internal quality control

Outliers of internal quality controls should be recorded along with its report and its lot number verified. The batch or lot of the consumables outlier will be taken out of the laboratory circulation and disposed of.

Annexes

Annex I: Equipment usage log sheet

Site:

Department:

SN	Equipment/Serial Number	Date	Time of use	Remarks	Used by	Signature

Annex II: List of antibiotic discs for AST

Pathogens	Basic Set	Extended Set
Escherichia coli	Ampicillin 10mcg Co-trimoxazole 1.25/23.75mcg Ciprofloxacin 530mcg/Ofloxacin 5mcg Ceftriaxone 30mcg, Cefotaxime 30mcg or Ceftazidime 30mcg Cephazolin 30mcg Gentamicin 10mcg Nitrofurantoin 300mcg	Tetracycline 30mcg/Doxycycline 30mcg, Chloramphenicol 30mcg Levofloxacin 5mcg Amoxycillin-clavulanic acid 20/10mcg/ Piperacillin-tazobactam 100/10mcg/ Ampicillin-sulbactam10/10mcg Amikacin Cefepime 30mcg Imipenem or meropenem 10mcg
Staphylococcus aureus	Penicillin 10 IU Cefoxitin 30mcg *, Cotrimoxazole 1.25/23.75mcg Clindamycin 2mcg Ciprofloxacin 5mcg or Ofloxacin 5mcg Erythromycin 15mcg Tetracycline 30mcg For urine isolates: Nitrofurantoin 300mcg *For MRSA screening purpose only. Erythromycin 15mcg to be kept next to Clindamycin 2mcg to test for D-test;	Azithromycin 15mcg Linezolid 30mcg Levofloxacin 5mcg Ceftaroline 30mcg Teicoplanin 30mcg Vancomycin (MIC) Chloramphenicol 30 mcg
Salmonella spp.	Ampicillin 10mcg Chloramphenicol 30mcg Co-trimoxazole 1.,25/23.75mcg Ciprofloxacin 5mcg Cefixime 30mcg/Ceftriaxone 30mcg/ Cefotaxime 30mcg	Pefloxacin 5mcg Azithromycin* 15mcg *For S. Typhi only
Shigella spp.	Ampicillin 10mcg Co-trimoxazole 1.25/23.75 Ciprofloxacin 5mcg	Levofloxacin 5mcg Azithromycin* 15mcg *Azithromycin for S. flexneriionly
Pseudomonas aeruginosa		
Enterococcus spp.		

Antimicrobial Agent	Organism	Resistant (< or = mm)	Intermediate (mm)	Susceptible (= or > mm)
Ampicillin 10mcg	Staphylococci Enterococci Enterobacteriaceae *Haemophilus influenza*	28 16 13 18	- - 14-16 19-21	29 17 17 22
Ampicillin-sulbactam 10/10mcg	Enterobacteriaceae, Acinetobacter *Haemophilus influenza*	11 19	12-14 -	15 20
Amoxicillin clavulanic acid(20/10 mcg)	Enterobacteriaceae *Haemophilus influenza*	13 19	14-17 -	>18 20
Amikacin 30 mcg	Enterobacteriaceae, Acinetobacter spp.	14	15-16	17
Azithromycin 15 mcg	Salmonella Typhi Staphylococci, *S. pneumoniae* *Haemophilus influenza*	12 13 -	- 14-17 -	13 18 12
Cefoxitin 30mcg	Enterobacteriaceae *Staphylococcus aureus* *Neisseria gonorrhoeae*	14 21 23	15-17 - 24-27	28 22 28
Cefoperazone 75mcg	Enterobacteriaceae	15	16-20	21
Ceftazidime 30 mcg	Enterobacteriaceae Acinetobacter spp. *Haemophilus influenza*	18-20 14 -	- 15-17 -	21 18 26

Cefotaxime 30mcg	Enterobacteriaceae Acinetobacter *Haemophilus influenza* *Neisseria gonorrhoeae*	22 14 - -	23-25 15-22 - -	26 23 26 31
Ceftriaxone 30mcg	Enterobacteriaceae Acinetobacter *Haemophilus influenza* *Neisseria gonorrhoeae*	19 13 - -	20-22 14-20 - -	23 21 26 35
Cefepime 30 mcg	Enterobacteriaceae Acinetobacter *Haemophilus influenza* *Neisseria gonorrhoeae*	18 14 - -	19-24 15-17 - -	25 18 26 31
Chloramphenicol 30mcg	Enterobacteriaceae, Enterococci, Staphylococci *Haemophilus influenza* *Streptococcus pneumoniae*	12 25 20	13-17 26-28 -	18 29 21
Ciprofloxacin 5mcg	Enterobacteriaceae except Salmonella, Acinetobacter, Staphylococci, Enterococci Salmonella *Haemophilus in luenzae* *Neisseria gonorrhoeae*	21 15 20 - 27	22-26 16-20 21-30 - 28-40	26 21 31 21 41
Clindamycin 2mcg	Staphylococci	14	15-20	21
Erythromycin 15mcg	Staphylococcus, Enterococcus *Streptococcus pneumoniae*	13 15	14-22 16-20	23 21
Ertapenem 10 mcg	Enterobacteriaceae *Haemophilus influenza*	18 -	19-21 -	22 19
Gentamycin 10mcg	Enterobacteriaceae, Acinetobacter, Staphylococcus	12	13-14	15
Imipenem 10mcg	Enterobacteriaceae Acinetobacter *Haemophilus influenza*	19 18 -	20-22 19-21 -	23 22 16
Levofloxacin 5mcg	Enterobacteriaceae except Salmonella Acinetobacter, Enterococci, *S. pneumoniae* Staphylococcus *Haemophilus influenza*	16 13 15 -	17-20 14-16 16-18 -	21 17 19 17
Meropenem 10mcg	Enterobacteriaceae Acinetobacter *Haemophilus influenza*	19 14 -	20-22 15-17 -	23 18 20
Nalidixic acid 30mcg	Enterobacteriaceae	13	14-18	19
Nitrofurantoin 300mcg (urinary)	Enterobacteriaceae, Staphylococci, Enterococci	14	15-16	17
Ofloxacin 5mcg	Enterobacteriaceae except Salmonella, *S. pneumo-niae* Staphylococci *Haemophilus in luenzae*	12 14 -	13-15 15-17 -	16 18 16
Penicillin 10U	Enterococcus Staphylococci *Neisseria gonorrhoeae*	16 28 26	- - 27-46	17 29 47
Piperacillin tazobactam	Enterobacteriaceae, Acinetobacter	17	18-20	21
100/10mcg	*Haemophilus influenza*	-	-	21

Sulfamethoxazole-tri- methoprim 1.25/23.75mcg	Enterobacteriaceae,Acinetobacter, Staphylococci, *Haemophilus influenzae* *Streptococcus pneumoniae*	10 15	11-15 16-18	16 19
Spectinomycin 100mcg	*Neisseria gonorrhoeae*	14	15-17	18
Tetracycline 30mcg	Enterobacteriaceae Acinetobacter spp. Staphylococci, Enterococci *Haemophilus influenza* *Neisseria gonorrhoeae* *Streptococcus pneumoniae*	11 14 25 30 24	12-14 15-18 26-28 31-37 25-27	15 19 29 38 28
Tobramycin 10mcg	Enterobacteriaceae, Acinetobacter	12	13-14	15
Vancomycin 30mcg	Enterococci	14	15-16	17

Annex III: Composition of Media

Baird Parker agar (BPA)

Composition

Formula in grams per liter

Glycine.....12.00

Casein Pancreatic Digest 10.00

Sodium Pyruvate.....10.00

Beef Extract..... 5.00

Lithium Chloride 5.00

Yeast Extract..... 1.00

Bacteriological Agar 20.00

Final pH 6.8 ± 0.2 at 25ºC

Bile Aesculin Agar (BAA)

Composition

Formula in grams per liter

Ox Bile..... 40.00

Peptone Bacteriological 5.00

Beef Extract 3.00

Esculin 1.00

Ferric Citrate..... 0.50

Bacteriological Agar 15.00

Final pH 6.6 ± 0.2 at 25ºC

Brain Heart Infusion (BHI) Agar

Composition

Formula in grams per liter

Peptone mixture 10.00

Beef Heart Infusion.....10.00

Calf Brain Infusion..... 7.50

Sodium Chloride.....5.00

Dipotassium Phosphate..... 2.50

Dextrose 2.00

Bacteriological Agar..... 15.0

Final pH 7.4 ± 0.2 at 25ºC

Brain Heart Infusion (BHI) Broth

Composition

Formula in grams per liter

Gelatin peptone.....10.00

Beef Heart Infusion..... 10.00

Calf Brain Infusion.....7.50

Sodium Chloride.....5.00

Disodium Phosphate.....2.50 Dextrose..... 2.00

Final pH 7.4 ± 0.2 at 25°C

Cetrimide Agar

Composition of Media

Formula in grams per liter

Gelatin Peptone..... 20.00

Potassium Sulfate 10.00

Magnesium Chloride 1.40

Cetrimide 0.30

Bacteriological Agar..... 13.60

Final pH 7.2 ± 0.2 at 25°C

Milk agar with cetyl trimethylammonium bromide

Skimmed-milk powder……100.0 gm

Yeast extract broth…….. 250 ml

Agar ……..15.0 gm

Cetyl trimethylammonium bromide…….300 mg

Water……750 ml

Yeast extract broth

Formula in grams per liter

Bacteriological peptone…… 10.0

Yeast extract……..3.0

Sodium chloride ……....5.0

Eosin Methylene Blue (EMB) agar

Composition

Formula in grams per liter

Bacteriological Peptone.....10.00

Lactose..... 5.00

Sucrose 5.00

Dipotassium Phosphate..... 2.00

Eosin Y 0.40

Methylene Blue 0.065

Bacteriological Agar 13.50

Final pH 7.2 ± 0.2 at 25°C

Gram Negative (GN) broth

Composition

Formula in grams per liter

Tryptose.....20.00
Sodium chloride 5.00
Sodium citrate 5.00
D-Mannitol..... 2.00
Dipotassium Hydrogen phosphate 4.00
Potassium Dihydrogen phosphate..... 1.50
Dextrose 1.00
Sodium deoxycholate 0.50
Final pH 7.0 ± 0.2 at 25ºC

MacConkey Agar (MA)

Composition

Formula in grams per liter
Pancreatic Digest of Gelatin.....17.00
Lactose monohydrate.....10.00
Sodium Chloride..... 5.00
Peptone Mixture 3.00
Bile Salts nº 3..... 1.50
Neutral Red 0.03
Crystal Violet..... 0.001
Bacteriological Agar 13.50
Final pH 7.1 ± 0.2 at 25ºC

Mannitol Salt Agar (MSA)

Composition

Formula in grams per liter
Sodium Chloride.....75.00
Peptone Mixture..... 10.00
D-Mannitol 10.00
Beef Extract..... 1.00
Phenol Red.....0,025
Bacteriological Agar..... 15.00
Final pH 7.4 ± 0.2 at 25ºC

Membrane Enterococcus Agar (mEA)

Composition

Formula in grams per liter
Tryptose…… ……..20.0
Yeast extract…….. ……5.0
Glucose…….. ……2.0
Dipotassium hydrogen phosphate…… 4 .0
Sodium azide……..……0.4
Agar……..……..10.0

2, 3, 5-triphenyltetrazolium chloride (TTC) ……..0.1

Final pH is 7.2 ± 0.2

Mueller Hinton Agar (MHA)

Composition

Formula in grams per liter

Beef Infusion 2.00

Casein Peptone H..... 17.50

Starch 1.50

Bacteriological Agar..... 17.00

Final pH 7.4 ± 0.2 at 25°C

Nutrient Agar (NA)

Composition

Formula in grams per liter

Gelatin Peptone..... 5.00

Beef Extract 3.00

Bacteriological Agar..... 15.00

Final pH 6,8 ± 0,2 at 25°C

Nutrient Broth (NB)

Composition

Same as Nutrient agar except agar

Pseudomonas Agar

Composition

Formula in grams per liter

Gelatin peptone ……16 g

Casein hydrolysate ……...10 g

Potassium sulphate ……...10 g

Magnesium chloride ……1.4 g

Glycerol ……10 ml

Cetyl trimethylammonium bromide ……..0.2 g

Nalidixic acid, sodium salt ……0.015 g

Agar ……11 g

Final pH of 7.1 ± 0.2

Selenite-F-Broth

Composition

Formula in gram per liter

Sodium acid selenite…… 4.0

Peptone ……..5.0

Lactose ……..4.0

Disodium hydrogen phosphate ……..9.5

Sodium dihydrogen phosphate ……..0.5

Final pH of the media is 7.1 ± 0.2

Tetrathionate broth

Composition

Formula in grams per liter

Peptone mixture..... 5.00

Bile Salts.....1.00

Calcium Carbonate..... 10.00

Sodium Thiosulfate.....30.00

Final pH 8.4 ± 0.2 at 25ºC

Xylose Lysine Deoxycholate (XLD) Agar

Formula in grams per liter

Xylose 3.50

L-Lysine 5.00

Lactose Monohydrate..... 7.50

Sucrose.....7.50

Sodium Chloride..... 5.00

Yeast Extract 3.00

Phenol Red..... 0.08

Bacteriological Agar 13.50

Sodium Deoxycholate..... 2.50

Sodium Thiosulfate.....6.80

Ferric Ammonium Citrate 0.80

Final pH 7.4 ± 0,2 at 25ºC

Biochemical Media

Lysine Iron Agar

Composition

Formula in grams per liter

L-Lysine.....10.00

Gelatin Peptone 5.00

Yeast Extract.....3.00

Dextrose..... 1.00

Ferric Ammonium Citrate.....0.50

Sodium Thiosulfate..... 0.04

Bromcresol Purple.....0.02

Bacteriological Agar..... 13.50

Final pH 6.7 ± 0.2 at 25ºC

MR-VP broth

Composition

Formula in grams per liter

Peptone mixture..... 7.00

Dextrose..... 5.00

Potassium Phosphate..... 5.00

Final pH 6.9 ± 0.2 at 25°C

Oxidative fermentative medium

Composition

Formula in grams per liter

Casein Peptone..... 2.00

Sodium Chloride.....5.00

Dipotassium Phosphate..... 0.30

Bacteriological Agar 2.50

Bromothymol Blue 0.03

Final pH 7.1 ± 0.2 at 25°C

Simmon's Citrate agar

Composition

Formula in grams per liter

Ammonium Dihydrogen Phosphate 1.00

Dipotassium Phosphate..... 1.00

Sodium Chloride.....5.00

Sodium Citrate..... 2.00

Magnesium Sulfate 0.20

Bacteriological Agar..... 15.00

Bromothymol Blue.....0.08

Final pH 6.9 ± 0.2 at 25°C

Sulphide indole motility medium

Composition

Formula in gram per liter

Peptone ……..…….30.0

Beef extract ……3.0

Ferrous ammonium sulphate ……..0.2

Sodium thiosulphate ……..0.025

Agar ……...4.0

Distilled water to 1000 ml

Final pH of the media is 7.2

Triple Sugar Iron agar

Composition

Formula in grams per liter

Peptone Mixture.....20.00

Lactose..... 10.00

Sucrose 10.00

Sodium Chloride..... 5.00

Beef Extract.....3.00

Yeast Extract.....3.00

Dextrose 1.00

Ferrous Ammonium Citrate 0.30

Sodium Thiosulphate 0.30

Phenol Red..... 0.025

Bacteriological Agar 12.00

Final pH 7.4 ± 0.2 at 25ºC

Urea Agar

Composition

Formula in grams per liter

Gelatin Peptone..... 1.00

Dextrose 1.00

Sodium Chloride..... 5.00

Monopotassium Phosphate 2.00

Urea 20.00

Phenol Red.....0.012

Final pH 6.8 ± 0.2 at 25ºC

Annex IV: Procedure for Gram staining and biochemical test

1.1.1 Gram's staining

This differential staining procedure separates most bacteria into two groups (Gram positive and Gram negative) on the basis of cell wall composition.

Principle:

Gram positive cell wall contains thick layer of peptidoglycan with numerous teichoic acid. While using primary stain crystal violet that penetrate through the wall and membrane of both Gram-positive and Gram negative cells. The crystal violet interacts with negatively charged components of bacterial cells, staining the cells purple. When added, iodine or iodide interacts with crystal violet to form large crystal violet iodine (CV-I) complexes within the cytoplasm and outer layers of the cell. The decolorizing agent (acetone/alcohol/acetone-alcohol solution) interacts with the lipids of the membranes of both Gram-positive and Gram negative bacteria. The outer membrane of the Gram-negative cell (lipopolysaccharide layer) is lost from the cell, leaving the peptidoglycan layer exposed. After decolorization, the Gram-positive cell remains purple in color, whereas the Gram-negative cell loses the purple color and is only revealed when the counterstain, the positively charged dye safranin, is used.

Requirements

Equipment

Microscope with oil immersion objective

Bunsen burner

Reagents

Commercial Gram Stain Kit containing

Crystal violet

Iodine acetone and/or acetone alcohol (95%)

A decolorizer made of acetone and alcohol (95%)

Safranin.

Specimen

Colonies growing on solid media or broth culture.

Procedure

Smear preparation (Culture isolate)

Colony from solid media: Emulsify a colony in a drop of sterile normal saline (preferred) or distilled water on the slide and make a thin preparation by spreading.

Broth culture: Transfer a loopful to a slide and make a thin preparation by spreading uniformly in a circle.

Smear fixation

Air dry the smear.

Heat fix air dried smear by passing 2-3 times through flame (Do not overheat as this causes deterioration of morphology).

Allow the slide to cool before staining.

Staining

Flood air-dried, heat-fixed smear with crystal violet staining reagent. Let is stand for 20 seconds.

Wash slide in a gentle and indirect stream of tap water in 5 seconds. Shake off excess water.

Flood slide with the mordant (Gram's iodine). Wait for 1min.

Wash slide in a gentle and indirect stream of tap water for in 5 seconds.

Shake off excess water.

Flood slide with decolorizing agent.

Wait 1-5 seconds or add drop by drop to slide until decolorizing agent running from the slide runs clear.

Do not over or under decolorized.

Excessive decolorization may give false Gram negative, insufficient decolorization may give false Gram positive.

Remove excessive decolorizer with gentle flow of tap water for 5 seconds. Shake off excess water.

Flood slide with counterstain (safranin). Wait for 20 seconds.

Wash slide in a gentle and indirect stream of tap water until no color appears in the effluent and then air dry or blot dry with absorbent paper filter paper.

Microscopic examination

Evaluate general nature of smear under low power (10X): e.g. smear thickness, staining,

decolorization.

Observe the smear under oil immersion (100x) using a bright field microscope and note color, shape and arrangement of bacteria

a) Gram-positive: bacteria will stain purple or deep violet.
b) Gram-negative: bacteria will stain pink/red and.

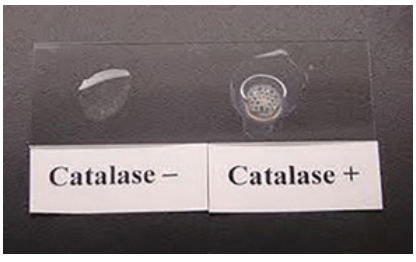
1.1.2 Catalase test

Principle

During aerobic respiration, in the presence of oxygen, microorganisms produce hydrogen peroxide, which is lethal to the cell itself. Catalase enzyme breaks down hydrogen peroxide into water and oxygen. The enzyme catalase is present in most cytochrome containing aerobic and facultative anerobic bacteria.

Procedure

Take a small amount of a culture from Nutrient Agar plate in a clean glass slide and add about 2-3 drops of 3% H2O2 on the surface of the slide.



Catalase test

Interpretation

The positive test is indicated by the formation of active bubbling of the oxygen gas. A false positive reaction may be obtained if the culture medium contains catalase (e.g., Blood Agar) or if an iron wire loop is used.

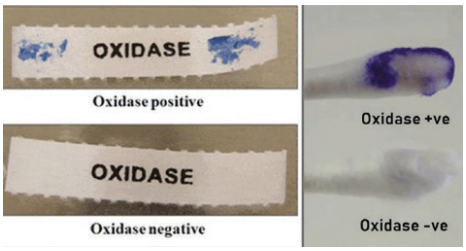
1.1.3 Oxidase test

Principle

This test is performed for the detection of cytochrome oxidase in bacteria which catalyzes the transport of electron donors. In the presence of redox dye such as Tetramethyl-p-phenylene diamine dihydrochloride (TMPD), the cytochrome oxidase oxidizes it into a deep purple colored end product indophenol which is detected in the test.

Procedure

I. Soak a piece of filter paper with few drops of oxidase reagent (TMPD) or commercially available oxidase discs can be used(take care not to expose to air for longer duration).
II. Smear the colony of the test organism on the filter paper/disc using a wooden stick.



Oxidase test

Interpretation

The positive test is indicated by the appearance of blue-purple color within 10 seconds.

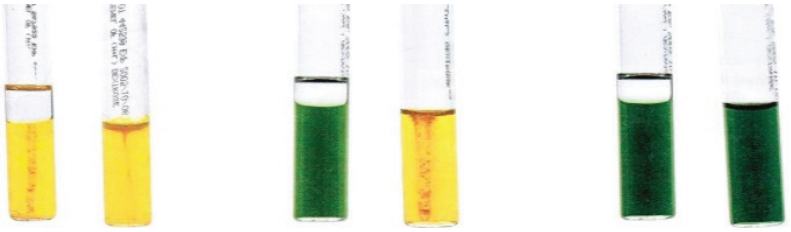
1.1.4 Oxidation-Fermentation test

Principle

This test is done to determine the oxidative or fermentative metabolism of carbohydrate resulting in production of various organic acids as end product. Some bacteria are capable of metabolizing carbohydrates (as exhibited by acid production) only under aerobic conditions, while others produce acid both aerobically and anaerobically.

Procedure

I. Stab the test organism into the bottom of two sets of tubes with Hugh and Leifson's (OF) media containing bromothymol blue indicator.
II. Cover the inoculated medium in one of the tubes with a 10mm deep layer of sterile paraffin oil.
III. Incubated the tubes at 37°C for 24 hours and examine for carbohydrate utilization as shown by acid production.


(a) Fermentative and oxidative **(b) Oxidative** **(c) Glucose not metabolized**

Interpretation

Fermentative organism utilize the carbohydrate in both the open and sealed tubes as shown by a change in colour of the medium from green to yellow. Oxidative organisms, however, are able to use the carbohydrate only in the open tube.

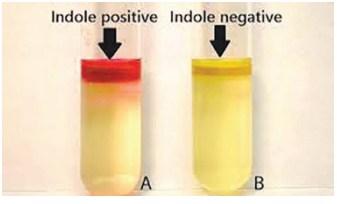
1.1.5 Indole Production test

Principle

This test detects the ability of the organism to produce an enzyme: 'tryptophanase' which oxidizes tryptophan to form indolic metabolites: indole, skatole (methyl indole) and indoleacetic acid. Indole if present combines with the aldehyde present in the reagent to give a red color in the alcohol layer. The color reaction is based on the presence of the pyrole structure present in indole.

Procedure

I. Stab a smooth bacterial colony on SIM (Sulphide Indole Motility) medium or sterile peptone water using a sterile straight wire.
II. Incubate the inoculated medium at 37°C for 24 hours.
III. After 24 hours incubation, add 0.5 ml of Kovac's reagent .



Indole Production test

Interpretation

Appearance of red color ring on the top of media indicates indole positive.

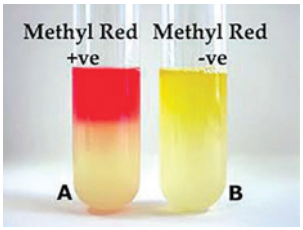
1.1.6 Methyl Red test

Principle

This test is performed to test the ability of an organism to produce sufficient acid from the fermentation of glucose to give a red color with the indicator methyl red (denotes changed in degree of acidity by color reactions over a pH range of 4.4-6.0).

Procedure:

I. Inoculate a pure colony of the test organism into 2 ml of MRVP medium.
II. Incubate the inoculated medium at 37°C for 48 hours.
III. After incubation, add about 5 drops of methyl red reagent and mix well.



Methyl Red test

Interpretation

The positive test is indicated by the development of bright red color, indicating acidity.

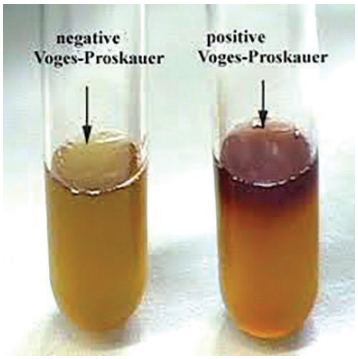
1.1.7 Voges Proskauer (VP) test

Principle

This test is employed to detect the production of acetyl methyl carbinol (a neutral end product) or its reduction product 2,3-butanidiol during fermentation of carbohydrates.

Procedure:

I. Inoculate a pure colony of the test organism into 2 ml of MRVP medium.
II. Incubate the inoculated medium at 37°C for 24 hours.
III. After incubation, add about 5 drops of Barritt's reagent and mix well for maximum aeration.



VP test

Interpretation

Positive test is indicated by the development of pink, red color.

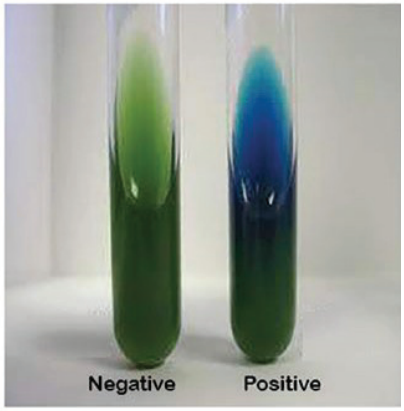
1.1.8 Citrate Utilization test

Principle

This test is employed to detect whether an organism utilizes citrate as a sole source of carbon for metabolism. Organism capable of utilizing citrate as its sole carbon source also utilizes the ammonium salts present in the medium as its sole nitrogen source, the ammonium salts are broken down to ammonia with resulting alkalinity.

Procedure

I. Inoculate (streak) a pure culture of test organism on the slant of Simmon's Citrate Agar medium and incubate at 37°C for 24 hours.



Citrate Utilization test

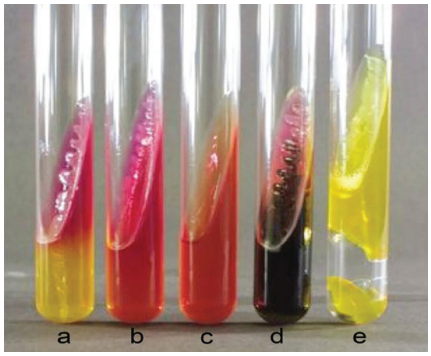
Interpretation

A positive test is indicated by the growth of organism and change in the color of media from green to blue, due to alkaline reaction. The pH indicator bromothymol blue has a pH range of 6.0-7.6, i.e. above pH 7.6; a blue color develops due to alkalinity of the medium.

1.1.9 Triple sugar iron (TSI) agar test

Principle

The TSI agar is used to determine the ability of an organism to utilize specific carbohydrates incorporated in the medium (glucose, sucrose and lactose in concentrations of 0.1%, 1.0% and 1.0% respectively), with or without the production of gas (indicated by bubbles or cracks in the media as well as an air gap at the bottom of the tube) along with determination of possible hydrogen sulfide production (detected by production of black color in the medium).



TSI test

Procedure

Streak the test organism on the surface and stab into 2/3rd of the medium of TSI agar slant and incubated at 37°C for 18-24 hours.

Note: Do not stab till the bottom of the tube as this results in false negative result for gas production.

Interpretation:

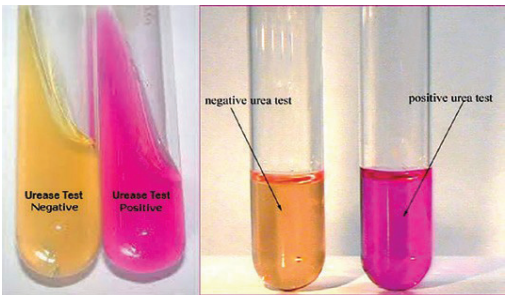
Acid production limited only to the butt region of the tube is indicative of glucose utilization, while acid production in slant and butt indicates sucrose or lactose fermentation. Phenol red is the pH indicator which gives yellow reaction at acidic pH, and red reaction to indicate an alkaline surrounding. Interpret the result as follows:

S.N.	Result	Interpretation
1	*Red (Alkaline)/Yellow (Acid), No Gas, No H_2S*	*Non lactose/Non-Sucrose fermenter, not aerogenic, No H_2S producer*
2	*Red (Alkaline)/No Change*	*Glucose, Lactose and Sucrose non-fermenter.*
3	*No Change/No Change*	*Non-fermenter*
4	*Red (Alkaline)/Yellow (Acid), No Gas, H_2S*	*Glucose, Lactose and Sucrose non-fermenter, H_2S producer*
5	*Yellow (Acid)/Yellow (Acid), Gas, No H_2S*	*Lactose/Sucrose fermenter, H_2S non producer*

1.1.10 Urea Hydrolysis test

Principle

This test demonstrates the urease activity present in certain bacteria which decomposes urea, releasing ammonia and carbon dioxide. Ammonia thus produced changes the color of indicator incorporated in the medium to pink.



Urea Hydrolysis test

Procedure

I. Inoculate the test organism in Christensen's urease medium/broth.
II. Incubate the inoculated medium at 37°C for 18-24 hours.

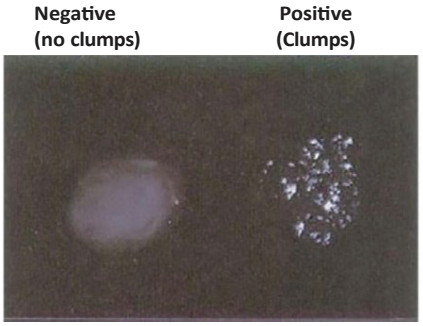
Interpretation

Positive organism shows pink or red color due to the breakdown of urea to ammonia. With the release of ammonia, the medium becomes alkaline as shown by a change in color of the indicator to pink.

1.1.11Coagulase test

Principle

This test is used specifically to differentiate species within the genus Staphylococci: S. aureus (usually positive) from *S. saprophyticus* & *S. epidermidis* (negative). A positive coagulase test is usually the final diagnostic criterion for the identification of S. aureus. Free coagulase and bound coagulase are the two types of coagulase possessed by this organism; most strains possess both free and bound coagulase.



Slide Coagulase Test

Slide Test

Bound coagulase (Clumping Factor) is detected by slide test. The bound coagulase is bound to the bacterial cell wall and reacts directly with fibrinogen. This results in alteration of fibrinogen so that it precipitates on the staphylococcal cell, causing the cells to clump when a bacterial suspension is mixed with plasma.

Procedure

I. For slide coagulase test, place a drop of physiological saline at three places on a clean slide.
II. Emulsify a colony of the test organism in the first two drops and a known strain of *Staphylococcus aureus* on the third drop to make thick suspensions.
III. Add a drop of plasma to the suspensions on the first (test) and third (positive control) suspension and mix gently.

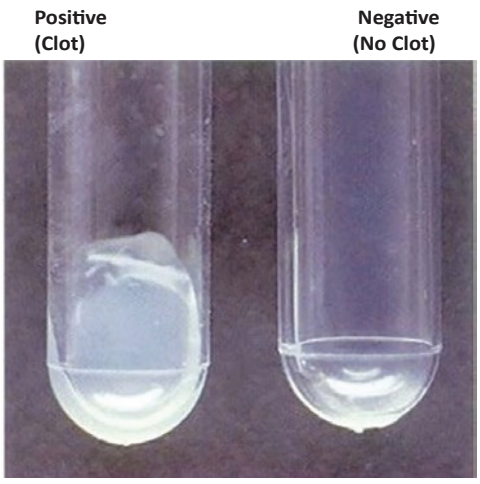
Note: Bring the plasma to room temperature as cold plasma leads to delayed coagulaton and false negative results.

Interpretation

A clumping observed within 10 seconds in the test suspension indicates the positive coagulase test. No plasma was added in second suspension. This is used for the differentiation of any granular appearance of the organism from true coagulase clumping. The third drop of saline is used for a known strain of coagulase positive staphylococci (positive control). As slide test only detects bound coagulase, negative result should be confirmed with a tube coagulase test.

Tube Test

Tube coagulase test is carried out to detect the production of free coagulase. Plasma contains coagulase reacting factor (CRF) which activates free coagulase. The activated coagulase acts upon prothrombin thus converting it to thrombin. Thrombin converts fibrinogen into fibrin which is detected as a firm gel (clot) in the tube test. Tube test is performed when negative or doubtful results are obtained in the slide coagulase test. In the tube coagulase test, plasma is diluted 1:10 in physiological saline.



Tube Coagulase Test

Procedure

I. Take four small tubes, one for test organism, one for positive control, one for negative control, and one to observe self-clotting of plasma
II. Add 0.5 ml of the diluted plasma into each tube.
III. Add 0.1 ml of test organism (18-24 hours broth culture), 0.5 ml positive control (*S. aureus* culture) and 0.5 ml negative control (*S. epidermidis* culture) to test, positive control, and negative control tubes respectively and mix gently.
IV. Add 0.1 ml sterile broth to the fourth tube.
V. Incubate all tubes at 37°C in a water bath for 4-6 hours

If no coagulation is observed for hours, then remove the tube from the water bath and leave it room temperature.

Interpretation

The clotting observed by gently tilting the tube indicates positive coagulase test. Definite clot formation indicates a positive test, granular or ropy growth is regarded as doubtful and requires retesting.

Annex V: Composition and preparation of Diluent

Buffered water: To prepare stock phosphate buffer solution, dissolve 34.0 g potassium dihydrogen phosphate (KH2PO4), in 500 mL reagent-grade water, adjust to pH 7.2 ± 0.5 with 1N sodium hydroxide (NaOH), and dilute to 1 Liter with reagent-grade water.

Add 1.25 mL stock phosphate buffer solution and 5.0 mL magnesium chloride solution (81.1 gm MgCl2·6H2O/L reagent-grade water) to 1 Liter reagent-grade water. Dispense in amounts that will provide 99 ± 2.0 mL or 9 ± 0.2 mL after autoclaving for 15 minutes

Buffered peptone water

Table 2: Composition of buffered peptone water

Peptone	10.0 gm
Sodium chloride	5.0 gm
Disodium hydrogen phosphate	3.5 gm
Potassium dihydrogen phosphate (KH_2PO_4)	1.5 gm
Water	1000 ml

Note: Do not suspend bacteria in any dilution water for more than 30 minutes at room temperature because death or multiplication may occur.

Dilution water is sterilized by autoclaving at 121°C for 15 minutes at 15lb/inch^2

Make serial dilutions with sterile reagent-grade water (RGW)

Note: Use buffered peptone dilution water rather than buffered water when preparing dilutions of samples containing heavy-metal ions

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