Table of Contents

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 01
Title: SOP for sample collection, packaging and transportation for Bacteriology Tests
Version No: 2026.01, Total Pages 8
Issue Month/Effective Date: May 2026
Revision Summary: 2024.01 Revised and updated
Supersedes Version No: 2024.01
Prepared by: LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, RVH&ECs, SVL & DVLs

1. Scope

This SOP describes the procedures for collecting various samples, packaging and transportation for different bacteriology tests.

2. Objective

To guide the laboratory technicians in collecting and preserving appropriate samples for laboratory investigations of bacterial infections in the animals using aseptic measures.

3. Equipment and Consumables

3.1 Sterile plastic bottles and vials are used for collection of fluids, discharges and small lesions.
3.2 Plastic bags- These are used of larger specimen organs with a minimum thickness of 0.1mm. All bags should be securely sealed, by double folding and as there is often the risk of tearing or leaking, the bag should be packed inside a second bag.
3.3 Swabs-Plain sterile cotton wool swabs are very much suitable for transporting specimens for culture. Swabs with culture media as transport swabs are available for long distance transportation.
3.4 Microscopic slides- Standard slides with grease and moist free are used to make smears from the pathological specimens.
3.5 Milk sample bottles-Sterile (30ml) bottles with screw-topped plastic bottles are used for milk samples for culture.
3.6 Pipettes for collecting vaginal mucus from cows and preputial scrapings from bulls- These pipettes should be sterile and used for collecting discharges or exudates from the area of pathological lesions or for screening of specific diseases in livestock.

4. Procedure

4.1 Abortion cases

4.1.1 A whole fetus should be submitted if possible. If not, fetal abomasal contents (ruminants), lung, liver and a sample of any gross lesions on the fetus should be sent.
4.1.2 A piece of obviously affected placenta and two or more cotyledons from cattle and sheep.
4.1.3 Uterine discharge (especially if no placenta is available).
4.1.4 If leptospiral abortion is a possibility, 20ml of mid-stream urine from the dam preserved with 1.5ml of 10% formalin should be submitted.
4.1.5 Serum from the dam for serological tests. Acute and convalescent serum samples should be considered for endemic diseases.

4.2 Faecal samples and rectal swabs

4.2.1 Collect approximately 30g of faeces directly from the rectum into the sterile jar.
4.2.2 Rectal swabs, heavily impregnated with faeces should be submitted in charcoal transport medium or other relevant medium with similar character.

4.3 Smears of intestinal mucosa and pathological lesions

4.3.1 A number of smears from the intestinal mucosa and other pathological lesions are to be prepared.
4.3.2 These smears are air dried and wrapped separately.
4.3.3 Always leave one end of the slide free for handling and labelling.
4.3.4 Smears should be clearly labelled, preferably with a glass marking pencil.
4.3.5 The smear should be transported only after proper wrapping.

4.4 Milk sample collection

It is vital that a milk sample for microbiology is taken so as to ensure that the potential pathogen(s) in the sample came from inside of the mammary gland and not from dust or faecal particles on the udder surface. The following are the main points for the good collection techniques:

4.4.1 Wipe the teat thoroughly with 70% ethyl alcohol, paying particular attention to the teat orifice.

4.4.2 Hold the sterile collection bottle nearly horizontal and keep the lid close by the little finger so that the lid does not become contaminated.

4.4.3 Carry out the collection as swiftly as possible.

A composite milk sample is satisfactory unless it is necessary to investigate the quarters separately. The first stream of the milk, from the teat canal, usually has a higher cell count and bacterial population than that in the mammary gland. As the results from the examination of the 'fore-milk 'are more a reflection of the condition existing within the teat than the mammary gland itself, it is usually recommended the first few squirts of milk from each quarter be discarded. The milk sample should, ideally, be kept refrigerated from the time of collection to the time of bacteriological examination. If Mycoplasma mastitis is suspected a simple transport medium is the milk sample itself with Ampicillin added at 5mg/ml. A second milk sample should be submitted, without Ampicillin, as a check for other mastitis-producing pathogens.

4.5 Abscesses

If possible, about 3ml of pus should be collected, together with scrapings from the wall of the abscess. Pus at the center of an abscess is often sterile. Pus from recently formed abscesses will yield the best cultural results.

4.6 Specimens for anaerobic culture

A good collection method is essential, because many anaerobes do not survive frank exposure to the oxygen in the air for more than 20 minutes. It is important not to contaminate the samples by contact with adjacent mucosal surfaces as these have a resident anaerobic flora. Specimens from animals that have been dead for more than 4 hours are usually unsuitable because of the rapid post mortem invasion of the animal body by anaerobes from the intestinal tracts. Bone marrow is a good specimen to collect for the diagnosis of blackleg or malignant oedema, as bone marrow appears to be one of the last tissues to be invaded by contaminating bacteria. A piece of rib stripped of the periosteum could be submitted to the laboratory for the extraction of the bone marrow. Any specimens for the attempted isolation of anaerobes must arrive at the laboratory as soon as possible. Collection of samples for anaerobic culture on ordinary swabs is usually of no value. Acceptable samples include blocks of tissue (4 cm3) placed in a sterile closed container, tissues contained in commercial 'anaerobe specimen collectors', and, for liquid exudates, the sample can be collected in a disposable syringe. Air is expelled from the syringe and the needle bent back on itself and

plugged.

In suspected enterotoxaemia cases, where the demonstration of a specific toxin is required, at least 20ml of ilial contents should be submitted. A loop of ileum with contents, tied off at each end, is acceptable or the ilial contents may be drained into a screw-capped bottle.

In case of suspected foot rot select the animal most likely to be infected, preferably early active cases. Five cases will be adequate for most investigations. Collect a scraping with a sterile scalpel blade or sterile wooden applicators from the moist inter digital area. The creamy/white dead tissue seen in the inter digital or skin/horn junction area provide the best samples, especially in early cases. For more advanced cases, take scrapings from the leading edge of the lesion.

Embed the scraping in the TOP 1-2 mm of the Modified Stuart's Transport Medium (STM gel). Do not put swabs, sticks, scalpel blades and large tissue samples into the transport STM as they push the lesion material to the bottom of the STM making it difficult to find. Furthermore, swabs introduce oxygen into the medium and thus reduce the survival of anaerobes such as *Dichelobacter nodosus.*

4.7 Urine samples

Urine samples may be submitted for urinalysis, bacterial microscopy and culture or for a viable bacterial count to establish whether a clinical bacterium is present. For bacteriological procedures the preferred methods of collection are by cystocentesis, by catheter or mid – stream urine samples.

4.8 Samples from Skin lesions

If intact pustules or vesicles are present, the surface should be disinfected with 70% ethyl alcohol, allowed to dry, and materials aspirated from the lesion with a sterile syringe and fine needle. Plucked hairs, skin scrapings (including the scalpel blade itself) and any scab material that is present should be submitted. These specimens will also allow detection of mange or a bacterial infection, if present.

4.9 Blood cultures

These are used if a bacteremia is suspected. Strict aseptic precautions should be taken when collecting the blood. The area over the site of vein puncture must be shaved, cleaned thoroughly with a detergent, dried and 70% ethyl alcohol applied to the skin and allowed to act for at least 30 seconds. As bacteremia can be intermittent, it is advisable to take more than one sample within a 24-hour period. The blood should be added aseptically and without delay to one of the special commercial blood-culture bottles and then sent to the laboratory.

4.10 Samples containing bacteria that require transport media

Streptococcus species are particularly susceptible to desiccation especially if collected on a dry swab. Commercial swabs supplied with transport medium are satisfactory. For the isolation of *Moraxella bovis*, ideally lachrymal secretions should be plated on blood agar immediately after

collection. If this is not possible, the swab should be placed in a commercial transport medium and delivered to the laboratory within 2 hours of collection. Cervical or vaginal mucus from infertile cows for the isolation of *Campylobacter* species and samples that might contain *Mycoplasma* species should be placed in a special transport medium. The diagnostic laboratory should be consulted before collecting samples for the isolation of these pathogens.

4.11 Nasal discharge

Samples may be taken with cotton or gauze swabs. It may be helpful if the swab is first moistened with a transport medium. The swab should be allowed to remain in contact with the secretions for up to 1 minute, then place in the transport medium and sent to the laboratory without delay at 4 degrees Celsius. Long protected nasopharyngeal swab should be used to collect samples for some suspected viral infections.

4.12 Preparing bacterial smears

Microscopic slides are not always clean enough to use directly from the supplier. Rubbing with a clean, soft cloth and a flick through the Bunsen flame may be sufficient to remove greasy film. If not, a mildly abrasive liquid cleaner can be used followed by rinsing the slide thoroughly and wiping it dry with a clean cloth. A scalpel and forceps should be kept in a container of 70% ethyl alcohol. The instruments are flamed and cooled before use. Afterwards they should be placed into a container of disinfectant. When making a smear from tissue lesions, the specimen is held firmly with the forceps and the scalpel is used to scrape deep into the material. A small amount of the scrapings is placed on the clean microscopic slide. Another clean slide is used with a scissor action to prepare a thin smear. With liquid or semi-liquid specimen, the sample is placed on the slide with a sterile swab. The contents of the swab are smeared over the surface of the slide, with the aim of having thick and thin areas of specimen present. The smears are allowed to dry thoroughly before proceeding further.

4.13 Fixing the smears

The reasons for fixing the smears include killing the vegetative bacteria, rendering them permeable to the stain and ensuring that the material is firmly fixed to the slide. Fixed and stained smears should be handled carefully as not all bacteria, especially endospores, may have been killed. After use, the stained smears should be autoclaved or soaked in reliable disinfectants (24-48 hours) before discarding. For routine staining the smears are fixed by passing the slide, smear side up, quickly through the Bunsen flame two to three times, taking care not to overheat the smears. This can be tested on the back of the hand; the slide should feel warm but not hot enough to burn.
Dried smears to be stained by the Giemsa stain are first fixed in absolute methyl alcohol for 3 minutes and then dried.

4.14 Preputial and cervical vaginal washing

For preputial washing, 20-30 ml of the sterile PBS is introduced into the preputial sac. After vigorous massage for 15-20 seconds, the infused liquid is collected in a sterile flask, while sealed immediately and then sent to the laboratory.

In females after cleaning the vulval region the vaginal cavity is washed by infusing 20-30 ml of sterile PBS into the cavity through a syringe attached to a sterile catheter. The fluid is sucked out and re-infused four or five times before being collected in a sterile flask, which is then sealed immediately and sent to the laboratory.

4.15 Blood smear in case of suspected bacteremia

In case of suspected bacteremia cases like in Hemorrhagic septicemia, anthrax and other diseases, blood smear preparation and observation under microscope can give a fair line of diagnosis. The blood smear preparation is as shown in the parasitology section followed by proper fixation and staining methods.

4.16 Body fluids

Fluids for culture (e.g. body cavity fluids, pericardial fluid, joint aspirates) should be submitted in a sealed sterile tube, in as large a volume as is available (up to ~10 ml), since the concentration of organisms may be very low in these samples. Fluids may be submitted in blood culture bottles or Isolator tubes (keeping in mind that Isolator tubes must be processed in the lab within 24 hrs) for highest sensitivity. Frontal fluids (thoracic or peritoneal fluids or heart blood) to be examined for Leptospira sp. by FA test are best submitted in a sealed sterile tube to which 10% buffered formalin is added at a rate of 1.5 ml per 20 ml fluid. Never submit fluid in syringes, which tend to leak in transit and contaminate packaging. Never submit fluids or other specimens for bacteriologic culture in EDTA (purple top) Vacutainer tubes, as EDTA is highly toxic to many bacterial species.

4.16 Environmental samples

4.16.1 Water sampling: Rinse bottle (except if pre-sterlized) three times with sample water and fill to within 1-2 inches of the top.

4.16.2 Soil/sediment sampling: Collect samples at a depth of 30 cm using a sterile shovel and transfer 20-40gm of soil to sterile containers.

4.16.3 Surface/swab sampling: Use sterile sponges or swabs for hard surfaces, drains, or equipment. Collect samples from hard-to-clean areas to detect pathogens like *Salmonella spp.*

5. Safety

The samples should be considered infectious since some of the bacterial pathogens could be zoonotic in nature.

6. Waste Disposal

All the waste generated during sample collection should be disposed appropriately based on the zoonotic and non-zoonotic nature of the cases.

7. Sample Packaging

To establish safe, standardized procedures for packaging animal disease bacteriological samples (diagnostic specimens) for transportation to laboratories, ensuring the safety of personnel and the integrity and viability of samples.

7.1 Materials required

- Primary containers: Leak-proof, sterile containers (screw-capped tubes, & sealed plastic bags.
- Absorbent material: Cotton wool or paper towels, sufficient to absorb all liquid.
- Secondary Packaging: Watertight container (zip-lock bag, plastic canister).
- Outer Packaging: Rigid shipping box (cardboard or plastic).
- Labeling/Documentation: Parafilm, shipping labels, biohazard stickers, sample referral forms.
- Coolant: Ice packs or dry ice if required for maintenance of cold chain.

7.2 Procedure-Triple Packaging System

7.2.1 **Primary Receptacle (Inner)**

- Collect samples (tissue, blood, swab) directly into a sterile, leak-proof container.
- Seal the cap with parafilm to prevent leakage.
- Label the container with a sample ID, animal ID, and date using a waterproof marker.

7.2.2 **Secondary Packaging (Immediate)**

- Wrap the primary container in absorbent material enough to soak up all liquid in case of breakage.
- Place the wrapped container inside a secondary watertight plastic bag or container and seal it.
- Place sample submission form in a separate bag outside the secondary container to keep it dry.

7.2.3 **Outer Packaging (Shipping)**

- Place a secondary container inside a rigid, protective outer container (styrofoam box within a cardboard box).
- Place cold packs around the secondary container if the sample requires maintenance of the cold chain.
- Seal the outer container tightly with strong adhesive tape.

7.3 Labelling and Marking

● Clearly label the outer package with the shipping address and contact numbers of the both sender and receiver.
● Ensure the package is marked with proper shipping names (e.g., Biological substance)
● Apply a biohazard symbol if required.

7.4 Transport and Safety Precautions

● Samples should be sent as soon as possible after collection to maintain integrity and viability of samples.
● Ensure all samples are transported between 4-8 degree Celcius using a cold chain.
● Samples must be always transported in designated pool vehicles.

8. References

● Clinical Veterinary Advisor, 2nd Ed. 2011
●https://www.msdvetmanual.com/clinical-pathology-and-procedures/collection-and-submission- of-laboratory-samples/overview-of-collection-and-submission-of-laboratory-samples accessed on 04/10/2018
●World Organization for Animal Health (OIE) (2018) Manual 7-Sample Collection and transport. Available at: https://rr-asia.woah.org/app/uploads/2020/02/seacfmd-manual-7.pdf

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 02
Title: SOP for Culture techniques
Version No: 2026.01, Total Pages:4
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&ECs

1. Scope

Culture techniques help to isolate bacteria from clinical, animal, and environmental samples to obtain pure cultures.

2. Objective

Culture techniques are fundamental laboratory methods that enable accurate isolation, identification, and characterization of bacteria, supporting effective diagnosis, treatment decisions,

and surveillance of infectious diseases.

3. Principles

The processing of clinical specimens for bacteriological examination involves a systematic approach with several key decision-making steps. First, the type of specimen and its anatomical origin are evaluated to determine whether pre-treatment procedures such as centrifugation or homogenization are required before culture. Second, appropriate primary isolation media are selected to support the growth of the suspected bacteria. Third, suitable incubation conditions including temperature, atmospheric environment, and duration are chosen to promote optimal bacterial growth.

4. Equipment and Consumables

4.1 Biological safety cabinet / Laminar airflow cabinet
4.2 Incubator
4.3 Autoclave
4.4 Hot air oven
4.5 Refrigerator / Freezer
4.6 Bunsen burner
4.7 Anaerobic jar
4.8 Vortex mixer
4.9 Culture media
4.10 Petri dishes (sterile)
4.11 Inoculating loops and needles
4.12 Sterile swabs
4.13 Pipettes and micropipette tips
4.14 Test tubes and culture tubes
4.15 Sterile normal saline or buffer solutions
4.16 Disposable gloves, masks, and laboratory coats
4.17 Marker pens and labeling materials
4.18 Biohazard bags and disinfectants

5. PROCEDURE

5.1 Specimen pre-treatment

5.1.1 Surface sear method - The surface of the tissue is seared with a hot spatula. The seared surface is cut with a sterile scalpel blade and then the cut surface is swabbed for culture.
5.1.2 Sterile scalpel method - Using a sterile scalpel and a sterile petri dish, mince tissue until it is homogenous in consistency. Remove the specimen with a sterile pipette or swab.

5.2 Inoculation

5.2.1 Where more than one culture medium plate is to be inoculated, it is best to start with the non-

inhibitory medium, such as Sheep blood agar, and then streak on to any inhibitory or selective media, such as MacConkey agar.

5.2.2 Label the plates on the agar side with the laboratory submission number and date of inoculation/culture using marker pen.

5.2.3 Samples submitted as swabs must be moist or shipped using transport media. The swab is rotated over one quarter to one third of the required agar plate or plates. The plate or plates are then streaked out by loop. Then the swab is placed into broth medium if required and the top of the swab removed by breaking it against the side of the bottle or tube.

5.2.4 The essence of streaking agar plates is to obtain isolated colonies and it is best to use the entire plate on initial culture. Half plates or quarter plates are also acceptable.

5.2.5 The process of streaking requires that at least three sets of streaks are made out from the lawn with a disposable sterile plastic loop that is discarded into disinfectant immediately after use or platinum loop that can be sterilized with flame.

5.2.6 Samples from pre-treatment processing and samples submitted as liquids (such as urine, milk, etc.) may have a drop of the liquid/loop full placed onto one quarter of the required agar plate/s before streaking. If there is observation of mixed growth on the plate, then it's advisable for subculture for identification of pathogen.

6. Incubation

6.1.1 Once the media has been selected for culture of the sample, temperature and incubation time must be considered along with the gaseous atmosphere under which culture plates are incubated.

6.1.2 General guidelines are available and these are: Normal atmosphere (aerobic) for most pathogenic bacteria and all fungi 18-24 hours - Most of the rapidly growing bacteria. 24-48 hours The rapidly growing bacteria when plated on selective agar. 4-7 days - *Brucella spp., Campylobacter spp., Nocardia asteroids, Mycoplasma spp.*

7. Result Interpretation

7.1 The basic information required for the identification of bacterial isolates is knowledge of some or all of the following reactions: Colony description including the form of hemolysis on sheep blood agar plates, growth on MA (Lactose fermenting, nonlactose fermenting and no growth), Gram reaction, catalase, oxidase, motility and O/F reaction.

7.2 From these qualities, one can decide which secondary tests are required for a more definitive identification of the isolate.

8. Quality Control

Quality control in culture techniques helps maintain accuracy, reliability, and reproducibility of bacteriological results, ensuring correct isolation and identification of bacteria.

9. Waste Disposal

9.1 Proper waste disposal in culture techniques is essential to prevent contamination, protect laboratory personnel, and ensure biosafety when handling bacterial cultures and clinical specimens.

9.2 All bacterial cultures, contaminated media, plates, and tubes should be sterilized by autoclaving (usually at 121 °C for 15–20 minutes) before disposal.

10. References

● SOP Version 2013, NCAH, Serbithang, Bhutan
● SOP Version 2028.1, NCAH, Serbithang, Bhutan
● Barrow GI, Feltham R K A, editors. Cowan and Steel's Manual for the Identification of Medical Bacteria. 3rd ed. Cambridge: Cambridge University Press; 1993. p. 226
● Department of Veterinary Pathology and Public Health. Laboratory Manual for Veterinary Microbiology. Queensland.
● Microbiology Laboratory. London: Public Health Laboratory Service; 1999. p. 147-8

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 03
Title: SOP for Gram staining
Version No: 2026.01, Total Pages:4
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&ECs

1. Scope

Gram staining in bacteriology is used to differentiate Gram-positive and Gram-negative bacteria, observe morphology and guide further culture and antimicrobial testing.

2. Objective

- To differentiate bacteria into Gram-positive and Gram-negative groups.
- To observe bacterial morphology (shape) and arrangement.
- To provide preliminary identification of bacterial genus.

3. Principles

The Gram staining reaction is based on the differences in the chemical composition of bacterial cell walls. Gram-positive bacteria have thick peptidoglycan layers that retain the crystal violet-iodine complex and appear purple. Gram-negative bacteria have thin peptidoglycan layers and lose the primary stain during decolorization, taking up the counterstain and appearing pink/red.

4. Equipment and Consumable

4.1 Clean grease-free glass slides
4.2 Bio-safety cabinet
4.3 Inoculating loop or sterile swab
4.4 Bunsen burner
4.5 Staining rack
4.6 Wash bottle with Sterile distilled water
4.7 Microscope (oil immersion objective)
4.8 Blotting paper
4.9 Immersion oil
4.10 Platinum loop
4.11 PPE (Gloves, Mask, Lab. Coat)
4.12 70% Alcohol
4.13 Fresh culture.
4.14 Sterile distilled water

5. Reagents

5.1 Crystal Violet – Primary stain
5.2 Gram's Iodine – Mordant
5.3 Decolorizer (95% ethanol or acetone-alcohol)
5.4 Safranin – Counterstain

6. Procedure

6.1 Smear preparation:

6.1.1 Take a grease free dry slide and add a drop of distilled water
6.1.2 Sterilize the inoculating loop on a flame of a Bunsen burner.
6.1.3 Transfer a loopful of culture (or the specimen) by sterile loop and make a smear at the centre. Smear should not be very thin or very thick.
6.1.4 Allow the smear to dry in the air.
6.1.5 Fix the dry smear by passing the slide 3-4 times through the flame quickly with the smear side facing up.

6.2 Gram staining

6.2.1 Place the slides on the staining rods.
6.2.2 Cover the smear with crystal violet stain and leave for 1 minute.
6.2.3 Wash carefully under running tap water.
6.2.4 Flood the smear with Gram's iodine solution and leave for 1 minute.
6.2.5 Drain off the iodine and wash the slide in a gentle stream of tap water.
6.2.6 Flood the slide with the decolorizing agent then wait for 20-30 seconds.
6.2.7 Gently wash the slide under running tap water and drain completely.
6.2.8 Counter stain with safranin and wait for about 30 seconds to 1 minute.
6.2.9 Wash the slide in a gentle/indirect stream of tap water until no colour appears in the effluent and then blot dry with absorbent paper.
6.2.10 Observe under oil microscope (100 x objectives).

7. Result Interpretation

Purple / Violet cells: Gram-positive bacteria
Pink / Red cells: Gram-negative bacteria

8. Quality Control

Gram-positive control: *Staphylococcus spp. (*ATCC-25923)
Gram-negative control: *Escherichia coli* (ATCC-25922)

9. Waste Disposal

- Waste generated during the Gram staining procedure should be disposed of following laboratory biosafety and waste management guidelines.
- Used slides should be disinfected in an appropriate disinfectant such as 0.5–1% sodium hypochlorite before cleaning or disposal.
- Contaminated materials including swabs, gloves, and blotting paper should be discarded in biohazard waste containers for proper treatment such as autoclaving,
- While liquid staining reagents should be collected in designated chemical waste containers for safe disposal Safety Precautions

10. References

- SOP version 2018.01, NCAH, Serbithang
- Department of Veterinary Pathology and Public Health. Laboratory Manual for Veterinary Microbiology. Queensland.
- Rajiv Gandhi College of Veterinary & Animal Sciences. Preventive Veterinary Medicine Manual. India. Puducherry.
- WHO. Guidelines on Standard Operating Procedures for Microbiology. Regional Office for South-East Asia. New Delhi. 2000. p. 1-90

- https://laboratoryinfo.com/gram-staining-principle-procedure-interpretation-and-animation/accessed on 17th September 2018

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 04
Title: SOP for Catalase test
Version No: 2026.01, Total Pages:4
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&ECs

1. Scope

The catalase test is a simple biochemical test used in microbiology to detect the presence of the catalase enzyme produced by certain bacteria.

2. Objectives

To detect the ability of organisms to produce the catalase enzyme and to differentiate catalase-positive organisms like Micrococci and Staphylococci from catalase-negative organisms like Streptococci.

3. Principle

The metabolic activity of aerobic and facultative anaerobic microorganisms produces toxic byproducts like hydrogen peroxide and superoxide radical (O2-). These products are toxic to the organisms and might even result in cell lysis if not broken down. In the case of pathogenic organisms, different mechanisms are found that break down these products to non-toxic substances. Bacteria capable of synthesizing the enzyme catalase hydrolyze hydrogen peroxide into water and gaseous oxygen, which results in the liberation of gas bubbles.

The production of catalase thus protects the organism against the lethal effect of hydrogen peroxide accumulated at the end of the aerobic metabolism. The presence of the catalase enzyme can be demonstrated by adding hydrogen peroxide to the bacterial inoculum, which results in the rapid liberation of oxygen bubbles. The lack of enzyme is demonstrated by the absence of such bubbles.

$$H_2O_2 \xrightarrow{\text{Catalase}} H_2O + O_2$$

4. Equipment and Consumables

4.1 Sterile petri dishes
4.2 Sterile pipette
4.3 Biohazard bags
4.4 Inoculating loops
4.5 Biological safety cabinet
4.6 Bunsen burner
4.7 Microscope slides
4.8 Tissue paper
4.9 Marker pens
4.10 Culture plates
4.11 30% H_2O_2 for Neisseria
4.12 15% H_2O_2 for anaerobes
4.13 3% H_2O_2 for other bacteria (purchase or dilute 30% 1:10 in deionized water prior
4.14 70% Alcohol

5. Procedure

5.1. Slide Method

- A microscope slide is placed inside a petri dish. The use of a petri dish is optional and is used to limit catalase aerosols, which might carry viable bacterial cells.
- A small amount of organism is collected from a well-isolated 18 to 24 hours colony with a sterile inoculating loop or wooden applicator stick and placed onto the microscope slide.
- However, no agar must be picked up with the colony, especially when the culture is picked up from blood agar.

- A drop of 3% H2O2 onto the organism on the microscope slide by using a dropper or Pasteur pipette. The formation of bubbles is observed against a dark background to enhance readability.

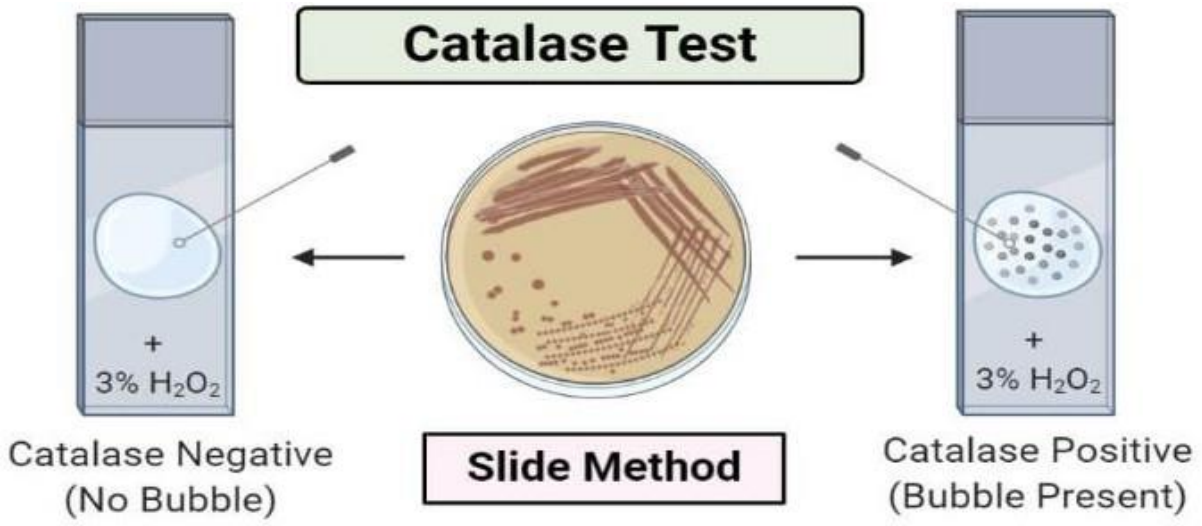


Image: Catalase test-slide method

5.2. Tube Method

- About 4 to 5 drops of 3% H2O2 are added to a test tube.
- Using a wooden applicator stick, a small amount of organisms from a well-isolated 18 to 24 hours colony is collected and placed into the test tube.
- The tube is placed against a dark background and observed for immediate bubbles.

5. Result and Interpretation

Positive: Immediate appearance of bubbles.
Weak positive: Appearance of 1-2 bubbles
Negative: No bubbles or few bubbles after 20 seconds.

6. Quality Control

The following organisms can be used for positive and negative quality control results.
Catalase positive: *Staphylococcus aureus*
Catalase-negative: *Streptococcus pyogenes*

7. Waste Disposal

- Used glass slides and plastic loops/applicator sticks should be placed directly into a biohazard sharps container or a specialized biohazard disposal bin for autoclaving.
- Small amounts of 3% hydrogen peroxide used in the test can generally be disposed of down the sink with ample running water. However, large volumes should be treated as chemical waste.

- Contaminated Materials: Any waste, such as paper towels used to clean spills or contaminated pipette tips, should be placed in biohazardous waste bags.
- Waste Treatment: Autoclaving (sterilization) is required for all materials containing microorganisms, particularly when testing pathogenic species.

8. References

- Biochemical Tests for the Identification of Aerobic Bacteria. (2016). Clinical Microbiology Procedures Handbook 3.17.1.13.17.48.3.DOI: 10.1128/9781555818814.ch3.17.1
- Karen Reiner. Catalase Test Protocol. Created: Thursday, 11 November 2010. American Society for Microbiology. 2016.
- SOP Version 2013, NCAH, Serbithang, Bhutan
- SOP Version 2028.1, NCAH, Serbithang, Bhutan
- Barrow GI, Feltham R K A, editors. Cowan and Steel's Manual for the Identification of Medical Bacteria. 3rd ed. Cambridge: Cambridge University Press; 1993. p. 226
- Department of Veterinary Pathology and Public Health. Laboratory Manual for Veterinary Microbiology. Queensland.
- Microbiology Laboratory. London: Public Health Laboratory Service; 1999. p. 147-8

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 05
Title: SOP for Oxidase test
Version No: 2026.01, Total Pages:3
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&ECs

1. Scope

The oxidase test is used in microbiology to identify bacteria by detecting the presence of the enzyme cytochrome c oxidase.

2. Objective

To detect the presence of the enzyme cytochrome oxidase in bacteria and differentiate oxidase-positive and oxidase-negative bacteria for identification purposes.

3. Principles

Organisms that contain cytochrome enzymes produce an oxidase enzyme that helps in electron transfer during respiration. These organisms can oxidize the test reagent, turning it blue or purple, and are called oxidase-positive. Those lacking this system cannot change the reagent color and are oxidase-negative. The reagent used acts as an artificial electron acceptor and forms a blue-colored compound when oxidized. This cytochrome system is mainly found in aerobic organisms that use oxygen as the final electron acceptor, producing water or hydrogen peroxide as end products.

4. Equipment and Consumables

4.1 Clean grease-free glass slides
4.2 Sterile pipettes/tips
4.3 Bio-bags
4.4 Inoculating loops
4.5 Biological safety cabinet
4.6 Bunsen burner
4.7 Microscope
4.8 Marker pens

5. Media and reagents

5.1 Oxidase disc
5.2 Fresh culture

6. Procedure

Scrape fresh culture using sterile inoculating loop /pipette tip & smear on impregnated oxidase disc.

7. Result interpretation

Positive: Development of a deep purple-blue colour within 5–10 seconds (presence of oxidase enzyme)

Negative: No colour change (absence of oxidase enzyme)

8. Quality Control

Gram-positive control: *Staphylococcus spp.(*ATCC-25923)
Gram-negative control: *Escherichia coli* (ATCC-25922)

9. Waste Disposal

- Dispose used swabs, filter papers, and culture materials in a biohazard waste bag.
- Autoclave all biohazard waste before final disposal.
- Discard oxidase reagent in a designated chemical waste container.

- Do not pour reagent directly into the sink.
- Disinfect contaminated glassware with suitable disinfectant (e.g., 1% sodium hypochlorite).
- Wash and sterilize glassware before reuse.
- Perform proper hand hygiene after handling materials.

10. References

- SOP version 2018.01, NCAH, Serbithang
- Advisory Committee on Dangerous Pathogens. 2004 Approved List of Biological Agents.
- http://www.hse.gov.uk/pubns/misc208.pdf. p. 1-17
- Barrow GI, Feltham R K A, editors. Cowan and Steel's Manual for the Identification of Medical Bacteria. 3rd ed. Cambridge: Cambridge University Press; 1993. p. 226
- Control of Substances Hazardous to Health Regulations 2002. General COSHH. Approved Code of Practice and Guidance, L5. Suffolk: HSE Books; 2002.
- Department of Veterinary Pathology and Public Health. Laboratory Manual for Veterinary Microbiology. Queensland.
- Health and Safety Executive. 5 steps to risk assessment: a step-by-step guide to a safer and healthier workplace, IND (G) 163 (REVL). Suffolk: HSE Books; 2002.
- Health and Safety Executive. A guide to risk assessment requirements: common provisions in health and safety law, IND (G) 218 (L). Suffolk: HSE Books; 2002.
- Health Services Advisory Committee. Safety in Health Service laboratories. Safe working and the prevention of infection in clinical laboratories and similar facilities. 2nd ed. Suffolk: HSE Books; 2003.
- Holliday MG, Ford M, Perry JD, Gould FK. Rapid identification of Staphylococcus aureus by using fluorescent staphylocoagulase assays. J Clin Microbiol 1999; 37:1190-2.
- Toowoomba Veterinary Laboratory: Biochemical tests. Laboratory Methods Manual. Issue 4. Queensland; Australia: 2004. P. 1-8
- MacFaddin JF, editor. Biochemical Tests for Identification of Medical Bacteria. 3rd ed. Philadelphia: Lippincott Williams & Wilkins; 2000. p. 105-19
- Public Health Laboratory Service Standing Advisory Committee on Laboratory Safety. Safety Precautions: Notes for Guidance. 4th ed. London: Public Health Laboratory Service (PHLS); 1993.
- Snell JJS, Brown D F J, Roberts C, editors. Quality Assurance Principles and Practice in the Microbiology Laboratory. London: Public Health Laboratory Service; 1999. p. 147-8
- Subcommittee on taxonomy of staphylococci and micrococci - minutes of first meeting. International bulletin of bacteriological nomenclature and taxonomy 1965; 15:107-8
- Williams REO HG. Determination of Coagulase and Alpha-Haemolysin Production by Staphylococci. Br J Exp Pathol 1946; 27:72-81.

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 06
Title: SOP for Motility Test
Version No: 2026.01, Total Pages:3
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&ECs

1. Scope

The motility test is used to determine whether a microorganism is capable of active movement, typically by means of flagella.

2. Objective

To determine the motility of bacterium and differentiate between motile and non-motile bacteria.

3. Principle

Sulfide Indole Motility (SIM) medium has a soft consistency that allows motile bacteria to migrate through the agar, resulting in a cloudy or diffuse appearance. The inoculum is introduced by stabbing the center of the semi-solid agar deep. Motility is indicated by a diffuse zone of growth extending outward from the line of inoculation. Some organisms may spread throughout the entire medium, while others form lateral growths or small nodular extensions from the stab line.

4. Equipment and Consumables

4.1 Glass slide
4.2 Cover slip
4.3 Sterile petri dishes
4.4 Sterile pipette
4,5 Biohazard bags
4.6 Inoculating loops
4.7 Biological safety cabinet
4.8 Bunsen burner
4.10 Tissue paper
4.11 Marker pens
4.12 Culture plates
4.13 70% alcohol
4.14 Distilled water

5. PROCEDURE

5.1 Semi-solid agar method

5.1.1 With a sterile straight needle, touch a colony of a young (18 to 24 hours) culture growing on agar medium.
5.1.2 Single stab down the center of the tube to about half the depth of the medium.
5.1.3 Incubate at 35°-37°C for 18-24 hours.

5.2 Direct Smear method

5.2.1 Place a small drop of liquid bacterial culture in the centre of a cover slip.
5.2.2 Place a small drop of water at each corner of the cover slip.
5.2.3 Invert a slide with a central depression over the cover slip.
5.2.4 Cover slip will stick to slide and when the slide is inverted the drop of bacterial culture will suspend in the well
5.2.5 Examine microscopically (X400) for motile organisms.

6. Result Interpretation

6.1 Semi-solid agar method

Positive: Organism can be recovered from the outer section of the medium.

Negative: Organism remains in the inner tube.

6.2 Hanging drop method

Positive: A darting, zig-zag, tumbling or other organized movement.

Negative: No movement or Brownian motion only.

7. Quality Control

Positive: *Escherichia coli* (ATCC25922)

Negative: *Staphylococcus aureus* (ATCC25923)

8. WASTE DISPOSAL

- Decontamination of cultures: All used motility test media containing bacterial growth should be considered biohazardous and must be sterilized, typically by autoclaving at 121 °C for 15–20 minutes before disposal.
- Disposal of culture media: After autoclaving, the decontaminated semi-solid agar media can be discarded according to laboratory waste management protocols (e.g., in designated biohazard waste bins).
- Liquid waste: Any liquid cultures or reagents should be disinfected using appropriate chemical disinfectants (e.g., 1% sodium hypochlorite) before disposal.

9. References

- Tille P.M. 2014. Bailey and Scott's diagnostic microbiology. Thirteen edition. Mosby, Inc., an affiliate of Elsevier Inc. 3251 Riverport Lane. St. Louis. Missouri 63043
- Pelczar MJ, Chan E.C.S, Krieg NR. 1958. Microbiology. Tata McGraw Hill Education Private Limited. 7 West Patel Nagar. New Delhi 110 008.
- Barrow GI, Feltham R K A, editors. Cowan and Steel's Manual for the Identification of Medical Bacteria. 3rd ed. Cambridge: Cambridge University Press; 1993. p. 226
- SOP Version 2013, NCAH, Serbithang, Bhutan
- SOP Version 2028.1, NCAH, Serbithang, Bhutan

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 07
Title: SOP for Oxidation/Fermentation of glucose test
Version No: 2026.01, Total Pages:3
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&ECs

1. Scope

Used to differentiate bacteria based on whether they metabolize glucose by oxidation, fermentation, or not at all, aiding in bacterial identification.

2. Objective

To determine whether a microorganism metabolizes glucose by oxidation, fermentation, or not at all, help differentiate and identify bacterial species based on their carbohydrate utilization patterns, and assess the metabolic characteristics of aerobic and facultative anaerobic bacteria.

3. Principle

- **Oxidative:** The organism produces acid only in the presence of oxygen (open tube).
- **Fermentative:** The organism produces acid in both aerobic (open) and anaerobic (sealed) tubes.
- **Non-saccharolytic:** The organism cannot utilize the carbohydrate; no acid is produced.

4. Equipment and Consumables

4.1 Sterile Screw cap Glass tubes
4.2 Inoculating loops
4.3 Biological safety cabinet
4.4 Incubators(37°C)
4.5 Bunsen burner
4.6 Bio-bag
4.7 Marker pen

5. Media and Reagents

5.1 Sucrose, Dextrose & lactose
5.2 O/F basal media
5.3 Fresh culture
5.4 70% alcohol
5.5 Liquid paraffin

6. Procedure

- Inoculate two tubes of O/F glucose medium with the test organism.
- Add sterile paraffin oil to one tube (anaerobic), leave the other open (aerobic).
- Incubate at 35–37°C for 24–48 hours.

7. Result interpretation

Yellow in both tubes (aerobic & anaerobic): Fermentative (F), organism ferments glucose.
Yellow in aerobic tube only: Oxidative (O), organism oxidizes glucose.
No color change (green/blue) in both tubes: Non-reactive, organism does not utilize glucose.

8. Quality Control

Gram-positive control: *Staphylococcus spp.* (ATCC-25923)
Gram-negative control: *Escherichia coli* (ATCC-25922)

9. Waste Disposal

- All inoculated O/F tubes and media should be autoclaved before disposal.
- Disposable tools (loops, pipettes) go into biohazard containers; reusable ones must be

sterilized.

- Follow lab safety: wear gloves, lab coat, and never pour live cultures down the sink.

10. References

- SOP version 2018.01, NCAH, Serbithang, Bhutan
- Clinical Veterinary Microbiology by P.J. Quinn, M.E. Carter, B. Markey and G.R. Carter.

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 08
Title: SOP for Lactophenol Cotton Blue staining
Version No: 2026.01, Total Pages:3
Issue Month/Effective Date: May 2026
Revision Summary: 2022.01 Revised and updated
Supersedes Version No: 2022.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&ECs

1. Scope

Lactophenol Cotton Blue staining is used for the microscopic examination, visualization, and identification of fungi**.** It helps in observing fungal structures such as hyphae, spores, and conidia from clinical, environmental, and culture samples, supporting routine diagnosis and laboratory studies.

2. Objective

To stain and clearly visualize fungal structures such as hyphae, spores, and conidia under the microscope, and to aid in the identification of fungi based on their morphological characteristics.

3. Principle

Lactophenol cotton blue is composed of lactophenol, which serves as the mounting fluid, and the dye, cotton blue. Organisms suspended in this mounting fluid are rapidly killed by the presence of the phenol which acts as a gross cytoplasmic poison, precipitating cellular proteins and inactivating essential enzyme system. At high concentrations of phenol, cells are not lysed due to the inactivation of lytic cellular enzymes. Cotton blue is an acid which stains chitin and cellulose. Staining of fungi by cotton blue is due to the presence of chitin in their cell walls. This stain is supplied in a hermetically sealed Ampule for improved stability of the reagent prior to use.

4. Equipment and Consumables

4.1 Clean and grease free glass slides
4.2 Cover slips
4.3 Inoculating loop
4.4 Pipettes tips/ Needle
4.5 Bunsen burner
4.6 PPE (Gloves, Apron, Mask)
4.7 Tissue Paper
4.8 Marker pens

5. Media and Reagents

5.1 Lacto phenol cotton blue stain
5.2 Culture plate
5.3 70% alcohol

6. Procedure

6.1 Place a drop of lactophenol cotton blue stain on a clean slide.
6.2 With help of a needle/surgical blade remove a portion of the fungal colony and keep it in the dye solution or spread gently with sterilized platinum loop.
6.3 Apply a clean cover slip over the stain without formation of any air bubbles. Remove excess stain from the edge of the cover slip using blotting paper.
6.4 Allow the stain to act for 5-10 minutes.
6.5 Examine the slide for studying the morphology of the fungus

7. Result Interpretation

Positive result**:** Fungal elements are clearly visible as blue-stained structures (hyphae, spores, conidia).
Negative result: No fungal structures observed.

8. Quality Control

- Use known fungal culture as positive control.
- Check stain quality (should be clear blue, not precipitated).

- Use clean slides and coverslips to avoid contamination.
- Ensure proper sample preparation (thin, well-spread).

9. Waste Disposal

- Dispose of used slides and coverslips in a sharp container.
- Place fungal cultures and contaminated materials in biohazard bags and autoclave before disposal.
- Discard used needles in puncture-proof containers.
- Liquid waste containing stain should be disposed of as chemical waste according to lab guidelines.
- Follow standard biosafety procedures.

10. References

- SOP version 2022.01, NCAH, Serbithang, Bhutan.
- Department of Veterinary Pathology and Public Health. Laboratory Manual for Veterinary Microbiology. Queensland.
- Rajiv Gandhi College of Veterinary & Animal Sciences. Preventive Veterinary Medicine Manual. India: Puducherry.
- WHO. Guidelines on Standard Operating Procedures for Microbiology. Regional Office for South-East Asia. New Delhi. 2000. p. 1-90.

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 09
Title: SOP for Acid Fast Staining
Version No: 2026.01, Total Pages:3
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&ECs, SVL, DVL

1. Scope

Acid-fast staining is an essential technique for the detection, identification, and study of clinically important microorganisms.

2. Objectives

The main aim objective of this stain is to differentiate bacteria into acid fast group and non-acid fast groups.

3. Principle

Acid-fast mycobacteria contain mycolic acid in their outer membrane, making the cells waxy and resistant to staining with aqueous based stains. The primary stain, carbolfuchsin is applied to the cells, and heat and phenol are used to allow the stain to penetrate into the waxy surface of acid-fast microorganisms. The excess stain is removed with treatment by acid alcohol (ethanol and hydrochloric acid). A secondary stain, methylene blue, is then applied to the cells.

4. Equipment and Consumables

4.1 Glass slide
4.2 Blotting paper
4.3 Microscope (Leica)
4.4 Lint free tissue paper
4.5 Pipette/pipette tip
4.6 Bunsen burner
4.7 Platinum loop
4.8 Staining rack
4.9 ZN Acid Fast Stains (Primary stain:add 30ml HCL to 1 liter of 95% denatured 0.3% Carbol-fuchsin, Counter stain:0.3%methylene blue, dissolve 3 gm methylene blue in 1 liter distilled water) 0.3% Carbol-fuchsin)
4.10 Sterile distilled water
4.12Immersion oil
4.12 70% alcohol

5. Procedure

5.1 Prepare and fix the specimen smear prior to staining.
5.2 Place a small strip of blotting or filter paper over the top of the specimen, and place the slide over a boiling hot water bath on a mesh surface.
5.3 Cover the filter paper with the primary stain, carbolfuchsin. Leave the slide on the water bath for 3 to 5 minutes. Continue to apply stain if the filter paper begins to dry.
5.4 Remove the filter paper and rinse the slide with water until the solution runs clear.
5.5 Run acid-alcohol decolorizer over the slide for approximately 10 to 15 seconds.
5.6 Rinse the slide with water.
5.7 Cover the smear with the secondary or counterstain, methylene blue, for 1 minute.
5.8 Gently rinse the slide with water.
5.9 Blot the slide dry with bibulous paper.

6. Result Interpretation

Acid Fast: Bright red to intensive purple, Red, straight or slightly curved rods, occurring singly or in small groups, may appear beaded.
Non-Acid Fast: Blue color and in addition, background material should stain blue.

7. Quality Control

Positive control: acid-fast organism (e.g., *Mycobacterium* spp.)

Negative control: non–acid-fast organism (e.g., *Escherichia coli*)

8. Waste disposal

- Contaminated slides and smear: All stained slides containing clinical specimens (e.g., suspected Tuberculosis samples) should be considered infectious. These should be immersed in disinfectant (e.g., 1% sodium hypochlorite) and then autoclaved before disposal or reuse.
- Biological waste (Specimens)**:** Residual sputum or other clinical samples must be disinfected (e.g., with bleach) or autoclaved at 121 °C for 15–20 minutes before disposal.
- Used reagents and stains**:** Carbol-fuchsin and methylene blue solutions should be collected in designated chemical waste containers. Acid-alcohol (flammable) must be disposed of according to chemical safety guidelines and not poured directly into drains.

9. References

- Department of Veterinary Pathology and Public Health. Laboratory Manual for Veterinary Microbiology. Queensland.
- Rajiv Gandhi College of Veterinary & Animal Sciences. Preventive Veterinary Medicine Manual. India: Puducherry.
- WHO. Guidelines on Standard Operating Procedures for Microbiology. Regional Office for South-East Asia. New Delhi. 2000. p. 1-90.

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 10
Title: SOP for Spore staining
Version No: 2026.01, Total Pages:3
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: LSU(NCAH), RLDC, RVH&ECs, SVL & DVL

1. Scope

Spore staining is used to detect endospores and differentiate spore-forming bacteria such as Bacillus and Clostridium from non-spore-forming bacteria.

2. Objective

To detect and differentiate endospore-forming bacteria such as *Bacillus* and *Clostridium* from non-spore-forming bacteria.

3. Principle

In Schaeffer–Fulton's method, the primary stain malachite green is driven into the spores by applying heat (steam). Since malachite green is water-soluble and has low affinity for cellular components, it is easily washed out from vegetative cells with water. The vegetative cells are then counter stained with safranin, while the spores retain the green color.

4. Equipment and Consumables

4.1 Clean grease-free glass slides
4.2 Inoculating loop
4.3 Beaker
4.4 Microscope
4.5 Distilled water
4.6 Bunsen burner
4.7 Blot paper

5. Media and Reagents

5.1 Schaeffer and Fulton's Spore Stains Kit
5.2 70% alcohol
5.3 Immersion oil

6. Procedure

6.1 A small amount of culture is placed on a clean slide, mixed with a drop of water, and spread to form a thin smear.
6.2 The smear is air-dried and heat-fixed by gently passing over a flame.
6.3 The slide is positioned over a beaker of boiling water with the smear facing upward; once condensation appears underneath, the smear is flooded with Schaeffer–Fulton Spore Stain.
6.4 The slide is steamed for 3–5 minutes and then rinsed with running water.
6.5 It is counterstained with Schaeffer–Fulton Spore Stain B for 1 minute.
6.6 Finally, the slide is washed, dried, and examined under the oil immersion lens.

7. Result interpretation

Mature spores stain green, regardless of being free or within the vegetative sporangium, whereas the vegetative cells and sporangia stain red.

8. Quality Control

Positive Control: Use a known spore-forming bacterium, such as *Bacillus* or *Clostridium*, to ensure spores stain green.
Negative Control: Use a non-spore-forming bacterium to confirm that no green spores appear.

9. Waste Disposal

- Place used slides, swabs, and culture materials in a biohazard bag and autoclave before disposal.
- Collect leftover malachite green and safranin in a chemical waste container; do not pour into the sink.
- Disinfect contaminated glassware with suitable disinfectant before washing.
- Treat all materials that contacted bacterial cultures as bio-hazardous.
- Wash hands thoroughly after handling staining materials.

10. References

- SOP version 2018.0, NCAH, Serbithang, Bhutan
- Department of Veterinary Pathology and Public Health. Laboratory Manual for Veterinary Microbiology. Queensland.
- Rajiv Gandhi College of Veterinary & Animal Sciences. Preventive Veterinary Medicine Manual. India: Puducherry.
- WHO. Guidelines on Standard Operating Procedures for Microbiology. Regional Office for South-East Asia. New Delhi. 2000. p. 1-90.

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 11
Title: SOP for Methylene blue staining
Version No: 2026.01, Total Pages:3
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: LSU(NCAH), RLDC, RVH&ECs, SVL & DVL

1. Scope

Applicable for identifying bacterial shape (cocci, bacilli) and arrangements in microbiology laboratories.

2. Objective

To visualize bacterial cells, particularly their morphology and arrangement, using simple staining with methylene blue.

3. Principles

Methylene blue is a cationic (basic) dye that attaches to the negatively charged bacterial cell structures. This staining makes the cells appear blue under the microscope, allowing observation of their morphology and arrangement, while the background remains lightly colored.

4. Equipment and Consumables

4.1 Clean grease-free glass slides
4.2 Inoculating loop
4.3 Microscope
4.4 Distilled water
4.5 Bunsen burner
4.6 Blot paper

5. Media and Reagents

5.1 Methylene blue solution
5.2 70% alcohol

6. Procedure

6.1 Place a small amount of the culture on a clean slide, add a drop of water, and spread it into a thin smear.
6.2 Allow the smear to air dry, then briefly pass it over a flame to fix the cells.
6.3 Apply methylene blue stain for 1–2 minutes, dilute with an equal volume of distilled water, and let it stand for 10 minutes.
6.4 Rinse gently with water, then blot or let the slide air dry.
6.5 Observe under oil immersion (100×) to examine the bacterial shape and arrangement.

7. Result interpretation

Bacteria are stained blue and in the case of *Bacillus anthracis* capsular material takes a pink stain.

8. Quality Control

Gram-positive control: *Staphylococcus spp.* (ATCC-25923)
Gram-negative control: *Escherichia coli* (ATCC-25922)

9. Waste Disposal

- Used slides & contaminated materials: Dispose in biohazard containers.
- Excess stain & rinse water: Collect in labeled chemical waste container.
- Surfaces: Wipe with 10% bleach solution.

10. References

- SOP version 2018.01, NCAH, Serbithang, Bhutan
- Department of Veterinary Pathology and Public Health. Laboratory Manual for Veterinary Microbiology. Queensland.
- Rajiv Gandhi College of Veterinary & Animal Sciences. Preventive Veterinary Medicine Manual. India: Puducherry.
- WHO. Guidelines on Standard Operating Procedures for Microbiology. Regional Office for South-East Asia. New Delhi. 2000. p. 1-90.

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 12
Title: SOP for Capsule staining test
Version No: 2026.01, Total Pages:3
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, RVH&ECs, SVL & DVLs

1. Scope

Capsule staining is a specialized technique used to visualize the presence of a capsule, a gelatinous outer layer surrounding certain microorganisms.

2. Objective

- To prepare a smear of an encapsulated bacterium and stain its capsule using the Anthony capsule stain.
- To visualize the capsule and differentiate it from the cell body.

3. Principle

Capsule staining is a differential staining technique used to visualize capsules surrounding bacterial cells. Capsules are composed of polysaccharides or proteins and are easily damaged by heat or water; therefore, heat fixation is avoided and water is not used for rinsing.

The primary stain, crystal violet, stains both the bacterial cell and the capsule. This is followed by 20% copper sulfate, which acts as both a decolorizer and counterstain, removing the stain from the capsule while staining it lightly.

4. Equipment and Consumables

4.1 Glass slide
4.2 Blotting paper
4.3 Microscope
4.5 Lint free tissue paper
4.6 Pipette/pipette tip
4.7 Bunsen burner
4.8 Platinum loop
4.9 Capsule Staining - Capsule Stains Kit
4.10 Immersion oil
4.11 70% alcohol
4.12 Distilled water

5. Procedure

5.1 Prepare thin smears of bacterial culture on a microscope slide.
5.2 Allow the smear to only air-dry. Do not heat-fix as this will cause the capsule to shrink or be destroyed.
5.3 Apply 1% crystal violet and allow it to remain on the slide for 2 minutes.
5.4 With the slide over the proper waste container provided, gently wash off the crystal violet with 20% copper sulfate. Caution: Do not wash the copper sulfate and stain directly into the sink.
5.5 Blot the slide dry with bibulous paper.
5.6 Observe with the oil immersion lens.

6. Result Interpretation

Capsule: Clear halos zone against dark background
No Capsule: No Clear halos zone

7. Quality control

Positive control: Encapsulated bacteria (e.g., *Klebsiella pneumoniae*) to confirm capsule visualization.
Negative control: Non-encapsulated bacteria (e.g., *Escherichia coli*) to verify specificity of

staining.

8. Waste disposal

- Contaminated slides and smears: Treat all slides containing bacterial samples as infectious. Immerse in disinfectant (e.g., 1% sodium hypochlorite) and autoclave before disposal or reuse.
- Biological waste (specimens): Any residual bacterial cultures should be disinfected or autoclaved at 121 ℃ for 15-20 minutes before disposal.
- Used reagents: Crystal violet and copper sulfate solutions should be collected in designated chemical waste containers.
- Do not pour directly into drains.

9. REFERENCES

- Department of Veterinary Pathology and Public Health. Laboratory Manual for Veterinary Microbiology. Queensland.
- Rajiv Gandhi College of Veterinary & Animal Sciences. Preventive Veterinary Medicine Manual. India: Puducherry.
- WHO. Guidelines on Standard Operating Procedures for Microbiology. Regional Office for South-East Asia. New Delhi. 2000. p. 1-90.

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 13
Title: SOP for Nitrate test
Version No: 2026.01, Total Pages:3
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&ECs

1. Scope

The Nitrate Reduction Test is used to detect bacteria that can reduce nitrate to nitrite or other nitrogen compounds, aiding in identification and differentiation of bacterial species.

2. Objective

- To determine the ability of bacteria to reduce nitrate (NO_3^-) to nitrite (NO_2^-) or other nitrogenous compounds.
- To aid in the identification and differentiation of bacterial species based on nitrate reduction capability.

3. Principle

Certain bacteria can produce the enzyme nitrate reductase, which converts nitrate (NO_3^-) to nitrite (NO_2^-) during anaerobic respiration. When nitrate reagents are added, the presence of nitrite forms a red-colored compound, indicating a positive result. If no color develops, it may mean nitrate was not reduced (negative) or was further reduced to nitrogen gas or other compounds, which can be confirmed by adding zinc dust

4. Equipment and Consumables

4.1 Clean and grease free sterile screw cap test tubes
4.2 Inoculating loop
4.3 Bunsen burner
4.4 PPE (Gloves, Apron, Mask)
4.5 Tissue Paper
4.6 Marker pens

5. Media and Reagents

5.1 Fresh Culture plates
5.2 Nutrient broth containing 0.1% KNO3
5.3 Solution A - 0.8% sulphanilic acid in 5N acetic acid
5.4 Solution B - 0.5% alpha-naphthylamine in 5N acetic acid
5.5 70% alcohol

6. Procedure

6.1 Inoculate a loopful of bacterial culture into nitrate broth.
6.2 Incubate at 35–37°C for 24–48 hours.
6.3 After incubation, add Nitrate Reagent A (sulfanilic acid) and Reagent B (α-naphthylamine).
6.4 Mix gently and observe the colour change.

7. Result Interpretation

Positive: Red color after addition of reagent (nitrate reduced to nitrite).
Negative: No color changes
Confirmation (if no color develops):
Add a small amount of zinc dust:
Red color after zinc → Negative (nitrate still present)
No color after zinc → Positive (nitrate reduced beyond nitrite to nitrogen gas or other compounds)

8. Quality Control

- Positive control: a known nitrate-reducing bacterium (e.g., *Escherichia coli*)
- Negative control: a bacterium that does not reduce nitrate (e.g., *Staphylococcus aureus*)
- Incubate for the recommended time and temperature
- Confirm proper reagent reaction by adding zinc when necessary
- Maintain aseptic technique to avoid contamination

9. Waste Disposal

- Used culture tubes and broths: Autoclave before disposal in biohazard bags.
- Contaminated loops and inoculation tools: Dispose in sharps containers after disinfection.
- Reagents (chemicals): Dispose according to chemical waste guidelines
- Follow laboratory protocols to prevent contamination or infection.
- Gloves and other disposables that contacted cultures should go into biohazard waste.

10. References

- SOP version 2022.01, NCAH, Serbithang, Bhutan
- Advisory Committee on Dangerous Pathogens. 2004 Approved List of Biological Agents. http://www.hse.gov.uk/pubns/misc208.pdf. p. 1-17
- Barrow GI, Feltham R K A, editors. Cowan and Steel's Manual for the Identification of Medical Bacteria. 3rd ed. Cambridge: Cambridge University Press; 1993. p. 226
- Control of Substances Hazardous to Health Regulations 2002. General COSHH. Approved Code of Practice and Guidance, L5. Suffolk: HSE Books; 2002.
- Department of Health NHS Executive: The Caldicott Committee. Report on the review of patient-identifiable information. London. December 1997.
- Department of Veterinary Pathology and Public Health. Laboratory Manual for Veterinary Microbiology. Queensland.
- Health and Safety Executive. 5 steps to risk assessment: a step by step guide to a safer and healthier workplace, IND (G) 163 (REVL). Suffolk: HSE Books; 2002.

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 14
Title: SOP for Indole production test
Version No: 2026.01, Total Pages:3
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC & RVH&ECs

1. Scope

The Indole test is a crucial biochemical test used in microbiology to determine the ability of a microorganism, particularly members of the Enterobacteriaceae family, to produce the enzyme tryptophanase.

2. Objective

To detect the presence of the enzyme tryptophanase in a bacterial isolate and identify the ability of bacteria to convert the amino acid tryptophan into indole.

3. Principle

Indole test is a biochemical test which differentiates the coliform from other members of Enterobacteriacee by detecting their ability to produce the enzyme tryptophanase. This enzyme hydrolyses the amino acid tryptophan into indole, pyruvic acid and ammonia. It is the intracellular enzyme (endoenzyme).

Tryptophan + H2O —(*Tryptophanase enzyme*)→ *Indole + Pyruvic acid + Ammonia*

4. Equipment and consumables

4.1 Sterile petri dishes
4.2 Biohazard bags
4.3 Inoculating loops
4.4 Biological safety cabinet
4.5 Incubators (37°C, CO2 and air)
4.6 Bunsen burner
4.7 Tube with screw cap(15ml)
4.8 Marker pens
4.9 Culture plates
4.10 70% alcohol.
4.11 Indole media
4.12 Kovac's reagent

5. Procedure

5.1 The broth medium or the agar medium in the tube is stabbed with the colony taken from an 18-24 hours culture.
5.2 For the liquid medium, a small portion of the inoculated broth is taken in a separate tube.
5.3 The tubes are then incubated at 37°C for 24 hours.
5.4 For Kovac's reagent, three drops of Kovac's reagent are added down the side of the tube, and the color change is observed at the meniscus.
5.5 For Ehrlich's reagent, 0.5 ml of xylene is added to the tube and inverted to mix well. Further, six drops of Ehrlich's indole reagent are added down the side of the tube, and the color is observed below the xylene layer.

6. Result Interpretation

Positive: Formation of pink-red coloration (cherry red ring) in the reagent at the point of contact between the reagent and the medium.
Negative: The absence of color or the appearance of slightly yellow color.

7. Quality Control

Positive control: *Escherichia coli* (should produce a red ring).
Negative control: *Klebsiella pneumoniae* (should show no red color).

8. Waste disposal

- Biological Waste: Inoculated media and bacterial cultures should be autoclaved.
- Chemical Waste (Kovac's/Ehrlich's Reagent): Collect in labeled chemical waste system and do not discard into sink. Dispose through approved chemical waste system.

9. Reference

- Department of Veterinary Pathology and Public Health. Laboratory Manual for Veterinary Microbiology. Queensland.
- WHO. Guidelines on Standard Operating Procedures for Microbiology. Regional Office for South-East Asia. New Delhi. 2000. p. 1-90.
- SOP Version 2028.1
- Quinn P.J., Carter M.E., Markey B. and Cater G.R. Clinical Veterinary Microbiology. 2000.

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 15
Title: SOP for Methyl Red (MR) test
Version No: 2026.01, Total Pages:4
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC & RVH&ECs

1. Scope

The methyl red (MR) test is a vital biochemical assay in veterinary microbiology used to differentiate Enterobacteriaceae, such as identifying *Salmonella* and *E. coli* by detecting stable acid end-products from glucose fermentation. It is part of the IMViC series, essential for diagnosing enteric infections and assessing food safety.

2. Objective

To differentiate two major types of facultative anaerobic enteric bacteria based on the production of acid and identify bacterial ability to produce stable acid end products by means of a mixed-acid

fermentation of glucose.

3. Principle

Methyl red test, commonly known as MR test is used to determine the ability of an organism to produce and maintain stable acid end products from glucose fermentation. MR test along with the VP test is performed simultaneously because they are physiologically related and are performed on MRVP broth. All members of the Enterobacteriaceae can convert glucose to pyruvic acid by the Embden-Meyerhof pathway, but bacteria can further metabolize pyruvic acid by two different pathways. Organisms metabolizing pyruvic acid by the mixed acid pathway will produce more acid end products, such as lactic acid and acetic acid, and maintain an acidic environment. The methyl red detects mixed acid fermentation that lowers the pH of the broth. The MR indicator is added after incubation which is red at pH 4.4 and yellow at pH 6.2. If the organism produces a large number of organic acids that include formic acid, acetic acid, lactic acid, and succinic acid from glucose fermentation, the broth medium will remain red after the addition of methyl red, a pH indicator. MR-negative organisms further metabolize the initial fermentation products by decarboxylation to produce neutral acetyl methylcarbinol (acetoin), which results in decreased acidity in the medium and raises the pH towards neutrality (pH 6.0 or above). For those organisms which do not produce the acid end products, the broth medium will change to yellow coloration indicating a negative test.

4. Equipment & Reagents

4.1 Sterile petri dishes
4.2 Biohazard bags
4.3 Inoculating loops
4.4 Biological safety cabinet
4.5 Incubators (37oC and air)
4.6 Bunsen burner
4.7 Tube with screw cap (10ml)
4.8 Marker pens
4.9 Fresh culture plates
4.10 70% alcohol
4.11 MR-VP broth
4.12 Methyl red reagent

5. Procedure

5.1 Inoculate MRVP broth with a pure culture of the organism.
5.2 Incubate at 35°-37°C for a minimum of 18-24 hours in ambient air.
5.3 Add 5 or 6 drops of methyl red reagent per 5 mL of broth.
5.4 Observe for the color change in the broth medium.

6. Result Interpretation

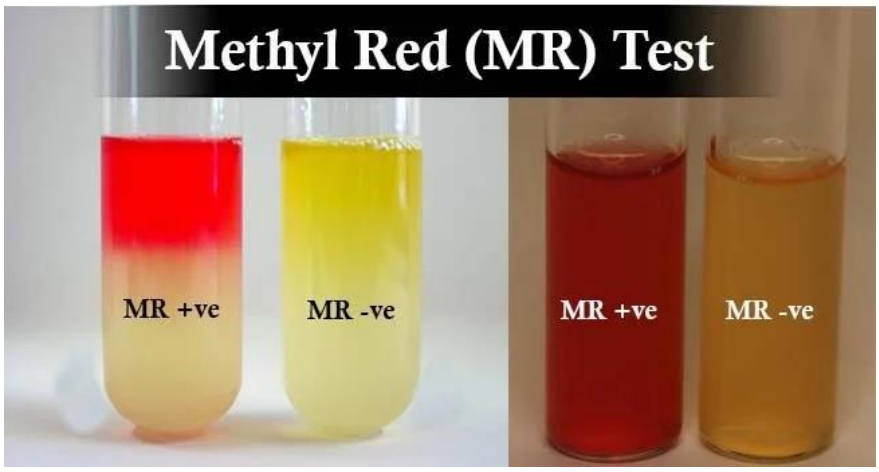


Image: Methyl Red (MR) test

Positive: Bright red colour
Positive (weak): Red-orange colour
Negative: Yellow colour

7. Quality Control

- **Positive control (MR positive):**
  Escherichia coli → produces stable acid → **Red color**

- · **Negative control (MR negative):**
  Enterobacter aerogenes (now *Klebsiella aerogenes*) → produces neutral end products → **Yellow/orange color**

8. Waste Disposal

-Includes MR-VP broth inoculated with organisms such as Escherichia coli or Enterobacter aerogenes:

- Collect all used cultures in **biohazard containers**
- **Autoclave at 121°C for 15–30 minutes**
- After sterilization, discard as **non-infectious liquid waste** according to lab policy

9. References

- Advisory Committee on Dangerous Pathogens. 2004 Approved List of Biological Agents. http://www.hse.gov.uk/pubns/misc208.pdf. p. 1-17.
- Barrow GI, Feltham R K A, editors. Cowan and Steel's Manual for the Identification of Medical Bacteria. 3rd ed. Cambridge: Cambridge University Press; 1993. p. 226
- Control of Substances Hazardous to Health Regulations 2002. General COSHH. Approved Code of Practice and Guidance, L5. Suffolk: HSE Books; 2002.
- Department of Health NHS Executive: The Caldicott Committee. Report on the review of patient-identifiable information. London. December 1997.
- Department of Veterinary Pathology and Public Health. Laboratory Manual for Veterinary Microbiology. Queensland.
- Health and Safety Executive. 5 steps to risk assessment: a step by step guide to a safer and healthier workplace, IND (G) 163 (REVL). Suffolk: HSE Books; 2002.
- Health and Safety Executive. A guide to risk assessment requirements: common provisions in health and safety law, IND (G) 218 (L). Suffolk: HSE Books; 2002.
- Health Services Advisory Committee. Safety in Health Service laboratories. Safe working and the prevention of infection in clinical laboratories and similar facilities. 2nd ed. Suffolk:HSE Books; 2003.
- Holliday MG, Ford M, Perry JD, Gould FK. Rapid identification of Staphylococcus aureus by using fluorescent staphylocoagulase assays. J Clin Microbiol 1999; 37:1190-2.
- MacFaddin JF, editor. Biochemical Tests for Identification of Medical Bacteria. 3rd ed. Philadelphia: Lippincott Williams & Wilkins; 2000. p. 105-19
- Public Health Laboratory Service Standing Advisory Committee on Laboratory Safety. Safety Precautions: Notes for Guidance. 4th ed. London: Public Health Laboratory Service (PHLS); 1993.
- Quinn P.J., Carter M.E., Markey B. and Cater G.R. Clinical Veterinary Microbiology. 2000. Snell JJS, Brown D F J, Roberts C, editors. Quality Assurance Principles and Practice in the
- Microbiology Laboratory. London: Public Health Laboratory Service; 1999. p. 147-8 Subcommittee on taxonomy of staphylococci and micrococci - minutes of first meeting.
- International bulletin of bacteriological nomenclature and taxonomy 1965; 15:107 Toowoomba Veterinary Laboratory: Biochemcial tests. Laboratory Methods Manual. Issue Queensland; Australia: 2004. P. 1-8
- Williams REO HG. Determination of Coagulase and Alpha-Haemolysin Production by Staphylococci. Br J Exp Pathol 1946; 27:72-81.
- https://microbeonline.com/methyl-red-mr-test-principle-procedure-results/ accessed on 29th August 2018

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 16
Title: SOP for Urease test
Version No: 2026.01, Total Pages:4
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC & RVH&Ecs

1. Scope

Urease test is used to identify organisms that are capable of hydrolyzing urea to produce ammonia and carbon dioxide. The test is particularly used for the presumptive identification of Proteus species for other members of the Enterobacteriaceae family. It also differentiates Proteus from the non-lactose-fermenting bacteria.

2. Objective

Used to determine the ability of an organism to split urea by production of the enzyme urease.

3. Principle

Urea medium, whether a broth or agar, contains urea and the phenol red as a pH indicator. Many organisms, especially those that cause urinary tract infections, produce the urease enzyme, which catalyzes the splitting of urea in the presence of water to release two molecules of ammonia and carbon dioxide. The ammonia combines with the carbon dioxide and water to form ammonium carbonate, which turns the medium alkaline, turning the indicator from its original orange-yellow color to bright pink. This test is performed as part of the identification of several genera and species of the Enterobacteriaceae family, including *Klebsiella*, *Proteus*, and some *Citrobacter* and *Yersinia* species, as well as some *Corynebacterium* species. The test is also useful to identify *Cryptococcus, Brucella, Helicobacter pylori*, and many other bacteria that produce the urease enzyme. Disks are available that combine urea and phenylalanine deaminase (PDA), allowing a one-disk test to identify *Proteus, Providencia*, and *Morganella* and to separate them from *Klebsiella* and *Yersinia enterocolitica*. The disk reactions are rapid and sensitive and allow for the rapid detection of agents of serious infections, e.g., *Brucella, and Cryptococcus.*

4. Equipment and Consumables

4.1 Sterile petri dishes
4.2 Biohazard bags
4.3 Inoculating loops
4.4 Biological safety cabinet
4.5 Incubators (37oC and air)
4.6 Bunsen burner
4.7 Tube with screw cap (15ml)
4.8 Marker pens
4.9 Fresh culture plates
4.10 70% alcohol
4.11 Christensen's/urea medium
4.12 Urea supplement
4.13 Distilled water

5. Procedure

- Inoculate slope heavily over the entire surface.
- Incubate inoculated slope at 35-37°C in an incubator.
- Examine slopes after 18-24 hours of incubation.

6. Result Interpretation

Positive: Pink color (Alkaline) indicating NH3 liberation from urea by the action of Urease.
Negative: Color of medium remains unchanged.

NOTE: Organisms that hydrolyze urea rapidly (e.g. *Proteus spp*) may produce positive reactions within 1 or 2 hours; less active species (e.g. *Klebsiella spp*) may require 3 or more days.

7. Quality Control

Use standard reference strains:

- **Positive control (Urease positive):**
  Proteus vulgaris → Rapid urease activity → **Pink color**
- **Negative control (Urease negative):**
  Escherichia coli → No urease activity → **No color change (yellow/orange)**

8. WASTE DISPOSAL

Includes urea agar/broth inoculated with organisms such as Proteus vulgaris or Escherichia coli:

- Collect all used cultures in **biohazard containers**
- **Autoclave at 121°C for 15–30 minutes**
- After sterilization, discard as **non-infectious laboratory waste** per institutional guidelines

9. References

- Advisory Committee on Dangerous Pathogens. 2004 Approved List of Biological Agents. http://www.hse.gov.uk/pubns/misc208.pdf. p. 1-17.
- Barrow GI, Feltham R K A, editors. Cowan and Steel's Manual for the Identification of Medical Bacteria. 3rd ed. Cambridge: Cambridge University Press; 1993. p. 226
- Control of Substances Hazardous to Health Regulations 2002. General COSHH. Approved Code of Practice and Guidance, L5. Suffolk: HSE Books; 2002.
- Department of Veterinary Pathology and Public Health. Laboratory Manual for Veterinary Microbiology. Queensland.
- Health and Safety Executive. 5 steps to risk assessment: a step by step guide to a safer and healthier workplace, IND (G) 163 (REVL). Suffolk: HSE Books; 2002.
- Health and Safety Executive. A guide to risk assessment requirements: common provisions in health and safety law, IND (G) 218 (L). Suffolk: HSE Books; 2002.
- Health Services Advisory Committee. Safety in Health Service laboratories. Safe working and the prevention of infection in clinical laboratories and similar facilities. 2nd ed. Suffolk: HSE Books; 2003.
- Holliday MG, Ford M, Perry JD, Gould FK. Rapid identification of Staphylococcus aureus by using fluorescent staphylocoagulase assays. J Clin Microbiol 1999; 37:1190-2.
- MacFaddin JF, editor. Biochemical Tests for Identification of Medical Bacteria. 3rd ed. Philadelphia: Lippincott Williams & Wilkins; 2000. p. 105-19
- Public Health Laboratory Service Standing Advisory Committee on Laboratory Safety. Safety Precautions: Notes for Guidance. 4th ed. London: Public Health Laboratory Service (PHLS); 1993.
- Quinn P.J., Carter M.E., Markey B. and Cater G.R. Clinical Veterinary Microbiology. 2000.

- Snell JJS, Brown D F J, Roberts C, editors. Quality Assurance Principles and Practice in the Microbiology Laboratory. London: Public Health Laboratory Service; 1999. p. 147-8
- Subcommittee on taxonomy of staphylococci and micrococci - minutes of first meeting. International bulletin of bacteriological nomenclature and taxonomy 1965; 15:107-8.
- Toowoomba Veterinary Laboratory: Biochemcial tests. Laboratory Methods Manual. Issue 4. Queensland; Australia: 2004. P. 1-8
- Williams REO HG. Determination of Coagulase and Alpha-Haemolysin Production by Staphylococci. Br J Exp Pathol 1946; 27:72-81.
- https://microbeonline.com/urease-test-principle-procedure-interpretation-and-urease-positive-organisms/ accessed on 29th August 2018

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 17
Title: SOP for Aesculin hydrolysis test
Version No: 2026.01, Total Pages:3
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC & RVH&ECs

1. Scope

It is used to detect aesculin-hydrolyzing bacteria, helping to identify *Enterococcus* and *Streptococcus* and differentiate them from other Gram-positive cocci.

2. Objective

To detect the ability of bacteria to hydrolyze aesculin to aesculetin and glucose, which helps identify organisms such as *Enterococcus* and *Streptococcus.*

3. Principle

Aesculin in the medium is hydrolyzed by bacteria producing the enzyme **β-glucosidase**, releasing aesculetin. Aesculetin reacts with ferric citrate in the medium to form a dark brown or black complex, indicating a positive result.

4. Equipment and Consumables

- Inoculating loop
- Biological safety cabinet
- Bunsen burner
- Incubators(37^{o}C)
- Tube with screw cap (15ml) & test tube (15ml)
- Marker pens
- Fresh culture plates
- Aesculin hydrolysis medium
- 70% alcohol

5. Procedure

6.1 Streak the test organism onto the surface of bile aesculin agar**.**
6.2 Incubate at 35–37°C for 18-24 hours.
6.3 Observe for blackening of the medium.

6. Result interpretation

Table: Result interpretation for Aesculin hydrolysis test

Observation	Result	Interpretation
Blackening of >50% of the medium	Positive	Bacteria hydrolyze aesculin
No blackening or <50% of medium	Negative	Bacteria do not hydrolyze aesculin

7. Quality Control

Positive control: *Enterococcus faecalis*-shows blackening of the medium.
Negative control: *Escherichia coli*-no blackening of the medium.

8. Waste Disposal

- Autoclave all used media and cultures at 121°C for 15minutes before disposal.
- Discard contaminated materials (loops, swabs) in biohazard bags and autoclave.
- Treat liquid waste with 1% bleach for 30 minutes before disposal.
- Sterilize reusable glassware before washing and reuse.

9. References

- SOP version 2018.01, NCAH, Serbithang, Bhutan
- Barrow GI, Feltham R K A, editors. Cowan and Steel's Manual for the Identification of Medical Bacteria. 3rd ed. Cambridge: Cambridge University Press; 1993. p. 226
- Department of Veterinary Pathology and Public Health. Laboratory Manual for Veterinary Microbiology. Queensland.
- Public Health Laboratory Service Standing Advisory Committee on Laboratory Safety. Safety Precautions: Notes for Guidance. 4th ed. London: Public Health Laboratory Service (PHLS); 1993.
- Quinn P.J., Carter M.E., Markey B. and Cater G.R. Clinical Veterinary Microbiology. 2000.
- Snell JJS, Brown D F J, Roberts C, editors. Quality Assurance Principles and Practice in the Microbiology Laboratory. London: Public Health Laboratory Service; 1999. p. 147-8

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 18
Title: SOP for Citrate utilization test
Version No: 2026.01, Total Pages:3
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC & RVH&ECs

1. Scope

To differentiate gram-negative bacteria based on their ability to utilize citrate as the sole carbon source and ammonium salts as the sole nitrogen source.

2. Objective

The Citrate Utilization Test (commonly performed using Simmons citrate agar) is used to determine the ability of bacteria to utilize citrate as the sole carbon source and ammonium salts as the nitrogen source.

3. Principle

- Utilization: Organisms capable of producing citrate transport proteins (permeases) bring citrate into the cell.
- Metabolism: Citrate is hydrolyzed by citrate lyase to oxaloacetate and acetate.
- Alkalinization: Metabolism produces sodium carbonate (when reacts with sodium ions) and ammonium hydroxide (from ammonia metabolism), raising the pH above 7.6.
- Indicator: Bromothymol blue acts as the pH indicator, changing from green (neutral, pH 6.9) to Prussian blue (alkaline, pH > 7.6).

4. Equipment and consumables

4.1 Sterile petri dishes
4.2 Biohazard bags
4.3 Inoculating loops
4.4 Biological safety cabinet
4.5 Incubators (37°C, CO2 and air)
4.6 Bunsen burner
4.7 Tube with screw cap(15ml)
4.8 Marker pens
4.9 Culture plates
4.10 70% alcohol
4.11 Distilled water
4.12 immons citrate agar

5. Procedure

5.1 Inoculation: Use a sterile inoculating needle to pick a pure, 18-24 hour colony and streak the surface of a Simmons Citrate Agar slant, then stab the butt.
5.2 Aeration: Ensure the cap is loose to allow for sufficient oxygen for aerobic metabolism.
5.3 Incubation: Incubate at 35-37°C for 24-48 hours, although some organisms may require up to 4-7 days.
5.4 Observation: Look for a change in color from green to Prussian blue and the presence of growth

6. Results

Positive: Growth on the slant and/or the medium turns blue (e.g., *Klebsiella pneumoniae, Enterobacter aerogenes*).
Negative: No growth and the medium remain green (e.g., *Escherichia coli*)

7. Quality Control

Positive control: *Klebsiella pneumoniae & Enterobacter aerogenes* (now known as *Enterobacter/Klebsiella aerogenes*)

Expected result: Growth with blue color change.

Negative control: *Escherichia coli*

Expected result: No growth; medium remains green.

8. Waste Disposal

All waste from the Citrate Utilization Test should be Segregated → Disinfected/Autoclaved → Safely discarded.

9. Reference

- Department of Veterinary Pathology and Public Health. Laboratory Manual for Veterinary Microbiology. Queensland.
- WHO. Guidelines on Standard Operating Procedures for Microbiology. Regional Office for South-East Asia. New Delhi. 2000. p. 1-90.
- SOP Version 2028.1, NCAH, Serbithang, Bhutan
- Quinn P.J., Carter M.E., Markey B. and Cater G.R. Clinical Veterinary Microbiology. 2000.

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 19
Title: SOP for Triple Sugar Agar iron (TSI) test
Version No: 2026.01, Total Pages:4
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC & RVH&ECs

1. Scope

The TSI test is used to differentiate enteric bacteria based on sugar fermentation, gas production, and hydrogen sulfide formation, aiding in the identification of the Enterobacteriaceae family.

2. Objective

To determine the fermentation of glucose, lactose, and sucrose, along with gas and hydrogen sulfide production, for identifying enteric bacteria.

3. Principles

The TSI test differentiates bacteria based on their ability to ferment glucose, lactose, and sucrose, produce gas, and generate hydrogen sulfide (H_2S); acid production from sugar fermentation turns the medium yellow, while H_2S reacts with iron salts to form a black precipitate, and unfermented sugars result in a red (alkaline) slant.

4. Equipment and Consumables

4.1 Inoculating loop
4.2 Distilled water
4.3 Bunsen burner
4.4 Incubators(37°C)
4.5 Tube with screw cap (15ml) & test tube (15ml)
4.6 Marker pens
4.7 Fresh culture plates
4.8 TSI medium
4.9 70% alcohol

5. Procedure

5.1 Using a sterile loop, pick a small amount of the bacterial culture.
5.2 Stab the butt of the TSI agar tube deeply with the loop to inoculate the organism at the bottom.
5.3 Streaked from the bottom up to the top and streak the surface of the slant while withdrawing the loop.
5.4 Incubate the tube at 37°C for 18–24 hours with a loosely capped lid, which is necessary for proper reactions in the medium.

6. Result Interpretation

Table: The TSI slant reading

Slant / Butt	Observation	Interpretation
Yellow / Yellow (A/A)	Acid slant and acid butt	Fermentation of glucose **and** lactose and/or sucrose
Red / Yellow (K/A)	Alkaline slant and acid butt	Fermentation of **glucose only**
Red / Red (K/K)	Alkaline slant and alkaline butt	No sugar fermentation
Black precipitate in butt	Blackening of medium	Hydrogen sulfide (H_2S) production
Bubbles, cracks, or lifted agar	Gas formation	Gas produced during fermentation

7. Quality Control

Positive Control: Use a known fermenter such as *Salmonella* to confirm sugar fermentation and gas production.

Negative Control: Use a non-fermenting bacterium like *Escherichia* to ensure no color change occurs.

8. Waste Disposal

- Place used TSI tubes, inoculating loops, and culture materials in a biohazard bag and autoclave before disposal.
- Collect any leftover medium or culture in a biohazard waste container; do not pour into the sink.
- Disinfect contaminated glassware with suitable disinfectant before washing.
- Treat all items that contacted bacterial cultures as biohazardous.

9. References

- SOP version 2018.01, NCAH, Serbithang, Bhutan
- AdvisoryCommitteeonDangerousPathogens.2004ApprovedListofBiologicalAgents. http://www.hse.gov.uk/pubns/misc208.pdf. p. 1-17.
- Barrow GI, Feltham R K A, editors. Cowan and Steel's Manual for the Identification of Medical Bacteria. 3rd ed. Cambridge: Cambridge University Press; 1993. p. 226
- Control of Substances Hazardous to Health Regulations 2002. General COSHH. Approved Code of Practice and Guidance, L5. Suffolk: HSE Books; 2002.
- Department of Health NHS Executive: The Caldicott Committee. Report on the review of patient-identifiable information. London. December 1997.
- Department of Veterinary Pathology and Public Health. Laboratory Manual for Veterinary Microbiology. Queensland.
- Health and Safety Executive. 5 steps to risk assessment: a step-by-step guide to a safer and healthier workplace, IND (G) 163 (REVL). Suffolk: HSE Books; 2002.
- Health and Safety Executive. A guide to risk assessment requirements: common provisions in health and safety law, IND (G) 218 (L). Suffolk: HSE Books; 2002.
- Health Services Advisory Committee. Safety in Health Service laboratories. Safe working and the prevention of infection in clinical laboratories and similar facilities. 2nd ed. Suffolk: HSE Books; 2003
- Holliday MG, Ford M, Perry JD, Gould FK. Rapid identification of Staphylococcus aureus by using fluorescent staphylo coagulase assays. J Clin Microbiol 1999; 37:1190-2.
- MacFaddin JF, editor. Biochemical Tests for Identification of Medical Bacteria. 3rd ed. Philadelphia: Lippincott Williams & Wilkins; 2000. p. 105-19

- PublicHealthLaboratoryServiceStandingAdvisoryCommitteeonLaboratorySafety. Safety Precautions: Notes for Guidance. 4th ed. London: Public Health Laboratory Service (PHLS); 1993.
- QuinnP.J.,CarterM.E.,MarkeyB.andCaterG.R.ClinicalVeterinaryMicrobiology. 2000.
- Snell JJS, Brown D F J, Roberts C, editors. Quality Assurance Principles and Practice in the Microbiology Laboratory. London: Public Health Laboratory Service; 1999. p. 147-8
- Subcommitteeontaxonomyofstaphylococciandmicrococci-minutesoffirstmeeting. International bulletin of bacteriological nomenclature and taxonomy 1965; 15:107-8.
- Toowoomba Veterinary Laboratory: Biochemcial tests. Laboratory Methods Manual. Issue 4. Queensland; Australia: 2004. P. 1-8
- WilliamsREOHG.DeterminationofCoagulaseandAlpha-HaemolysinProductionby Staphylococci. Br J Exp Pathol 1946; 27:72-81.
- https://microbeonline.com/triple-sugar-iron-agar-tsi-principle-procedure-and-interpretation/accessed on 29th August 2018

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 20
Title: SOP for Voges-Proskauer (*VP) test*
Version No: 2026.01, Total Pages:3
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&ECs

1. Scope

The Voges-Proskauer (VP) test is a biochemical test used to determine the ability of microorganisms to produce acetoin (acetylmethylcarbinol) from glucose fermentation via the butanediol fermentation pathway.

2. Objective

To differentiate bacteria based on their property to produce acetoin as the end product of glucose fermentation.

3. Principle

After glycolysis, the produced pyruvate can be metabolized through the butylene glycol pathway producing two neutral end products, acetylmethylcarbinol (also known as acetoin) and butanediol.

4. Equipment and Consumables

4.1 Sterile petri dishes
4.2 Biohazard bags
4.3 Inoculating loops
4.4 Biological safety cabinet
4.5 Incubators (370C, CO2 and air)
4.6 Autoclave
4.7 Bunsen burner
4.8 Refrigerator
4.9 Water bath
4.10 Tube with screw cap (10ml)
4.11 Marker pens
4.12 Culture plates
4.13 70% alcohol
4.14 Distilled water
4.15 Glucose phosphate peptone water (MR-VP broth)
4.16 5% alphanapthol in absolute ethyl alcohol,
4.17 40% KoH
4.18 Creatine

5. Procedure

5.1 Suspend a loop-full of the suspected colony in a sterile tube containing 5 ml of the VP medium and incubate at 37°C for 18-24 hrs.
5.2 After incubation add 2 drops of 0.3% creatine solution, 3 ml of 5% alpha-naphthol in absolute ethyl alcohol and then 1 ml of 40% KoH solution.
5.3 Shake after addition of each reagent and leave for 1-2 minutes. Positive reactions usually occur within 1 minute.

6. Result Interpretation

Positive: Formation of a pink-red color over the surface of the medium.
Negative: Lack of pink-red color over the surface of the medium or the formation of the copper color.

7. Quality control

Positive control (VP+): *Enterobacter aerogenes* (now *Klebsiella aerogenes*), *Klebsiella pneumoniae.*

Negative control (VP-): *Escherichia coli*

8. Disposal Waste

- All culture tubes and inoculated media should be autoclaved at 121°C for 15–20 minutes
- α-naphthol: Flammable and toxic and should be collected in labeled chemical waste container. Do not pour directly into sink.
- KOH (alkali): Corrosive → neutralize with dilute acid.

9. Reference

- Department of Veterinary Pathology and Public Health. Laboratory Manual for Veterinary Microbiology. Queensland.
- WHO. Guidelines on Standard Operating Procedures for Microbiology. Regional Office for South-East Asia. New Delhi. 2000. p. 1-90.
- SOP Version 2028.1, NCAH, Serbithang, Bhutan
- Quinn P.J., Carter M.E., Markey B. and Cater G.R. Clinical Veterinary Microbiology. 2000.

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 21
Title: SOP for Culture and identification of E. coli
Version No: 2026.01, Total Pages:4
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&ECs

1. Scope

The identification of *Escherichia coli* is important for diagnosing infections, detecting fecal contamination in food and water, and supporting public health, veterinary, and quality control activities.

2. Objective

To accurately isolate and identify *Escherichia coli* from clinical, food, or environmental samples for diagnosis, contamination detection, and quality control purposes.

3. Principles

The identification of *Escherichia coli* is based on its ability to grow on selective and differential media, where it produces characteristic colonies (such as lactose fermentation on MacConkey agar), along with confirmation through specific biochemical reactions and, when required, serological tests.

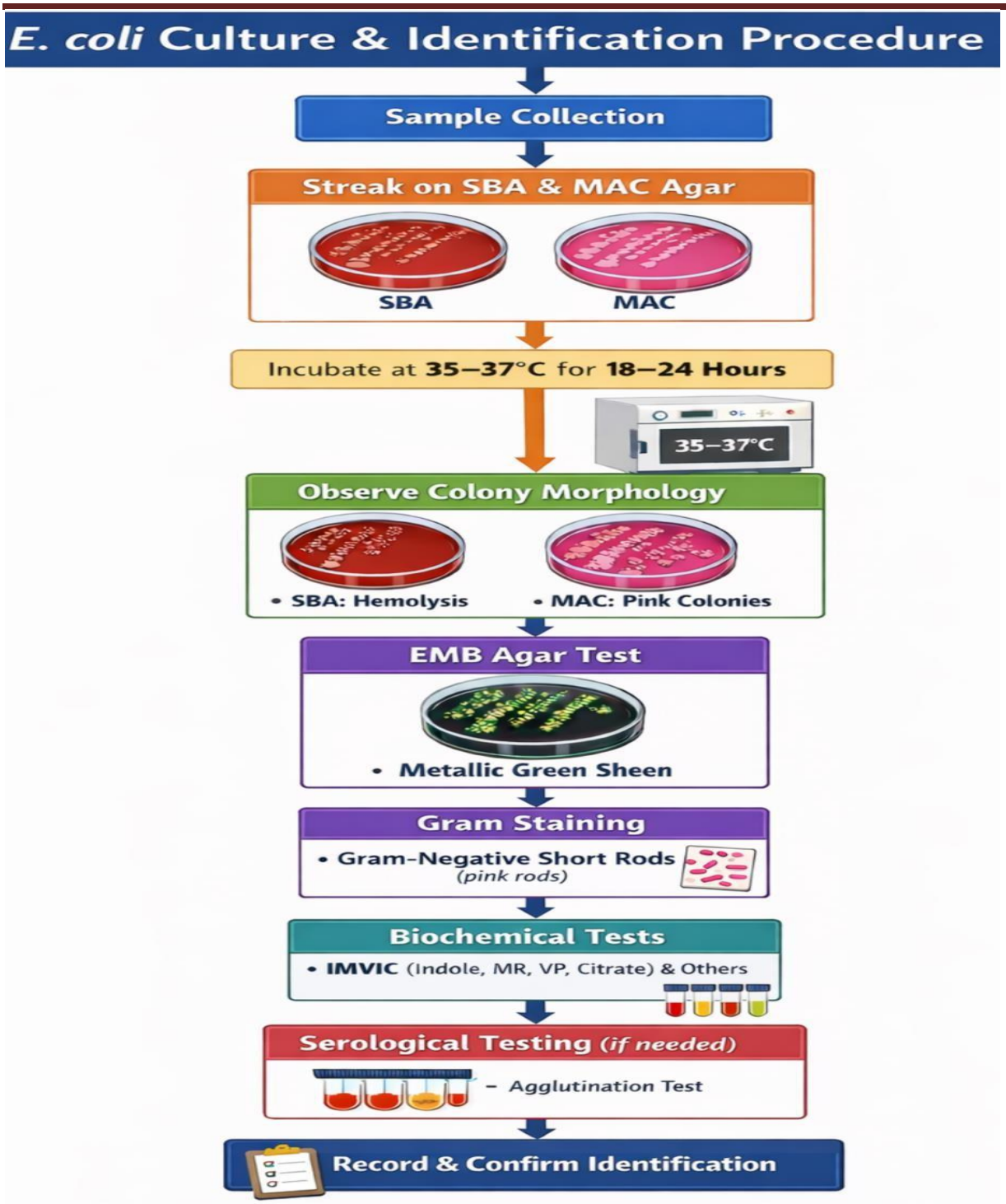
4. Equipment and Consumables

4.1 Inoculating loop
4.2 Biological safety cabinet
4.3 Bunsen burner
4.4 Incubators(37^{o}C)
4.5 Autoclave
4.6 Clean grease-free glass slides
4.7 Distilled water
4.8 PPE (Gloves, Apron, Mask)
4.9 Marker pens
4.10 Sheep blood Agar
4.10 MacConkey Agar
4.11 EMB Agar
4.12 Gram Stain kit
4.13 MR-VP Broth
4.14 Indole
4.15 Citrate
4.16 70% alcohol

5. Procedure

5.1 Streak the specimen on Sheep Blood Agar (SBA)**,** MacConkey Agar (MAC)**,** and Eosin Methylene Blue (EMB) Agar to obtain isolated colonies.
5.2 Incubate plates at 35–37°C for 18–24 hours under aerobic conditions.

5.3 Colony Observation
5.4 SBA: Observe size, shape, hemolysis.
5.6 MAC: Look for pink colonies indicating lactose fermentation.
5.7 EMB: *E. coli* typically produces metallic green sheen colonies due to strong lactose fermentation.
5.8 Gram Staining: Gram-negative short rods
5.9 Perform **IMViC** tests (Indole, Methyl Red, Voges-Proskauer, Citrate) and to confirm identification.
5.10 Serological Testing (if required): Test with specific antisera for pathogenic serotype identification as detailed in the flow chart below:


E. coli Culture & Identification Procedure
Sample Collection
Streak on SBA & MAC Agar
SBA
MAC
Incubate at 35–37°C for 18–24 Hours
35–37°C
Observe Colony Morphology
• SBA: Hemolysis
• MAC: Pink Colonies
EMB Agar Test
• Metallic Green Sheen
Gram Staining
• Gram-Negative Short Rods
(pink rods)
Biochemical Tests
• IMVIC (Indole, MR, VP, Citrate) & Others
Serological Testing (if needed)
– Agglutination Test
Record & Confirm Identification

6. Result interpretation

Table: Tests for identification of *E. coli*

Test/Media	Observation	Interpretation
Sheep Blood Agar (SBA)	Colonies: medium-sized, grayish, usually non-hemolytic	Typical colony morphology
MacConkey Agar (MAC)	Pink colonies	Lactose fermenter
Eosin Methylene Blue Agar (EMB)	Colonies with metallic green sheen	Strong lactose fermentation – characteristic of *E. coli*
Indole Test	Positive (red layer after adding Kovac's reagent)	*E. coli* produces indole from tryptophan
Methyl Red Test (MR)	Positive (red color)	Mixed acid fermentation – confirms *E. coli*
Voges-Proskauer Test (VP)	Negative (no color change)	Distinguishes *E. coli* from VP-positive organisms
Citrate Test	Negative (no growth/color change)	Cannot utilize citrate as sole carbon source
Serological Agglutination	Agglutination with specific antisera	Confirms pathogenic serotype

7. Quality Control

Positive control: *E. coli* ATCC 25922

8. Waste Disposal

- Autoclave all used media and cultures at 121°C for 15 minutes before disposal.
- Discard contaminated materials (loops, swabs) in biohazard bags and autoclave.
- Sterilize reusable glassware before washing and reuse.
- Work surfaces must be disinfected with 70% ethanol or appropriate disinfectant after use.
- Wash hands thoroughly after handling.

9. References

- SOP version 2018.01
- Advisory Committee on Dangerous Pathogens. 2004 Approved List of Biological Agents. http://www.hse.gov.uk/pubns/misc208.pdf. p. 1-17
- Clinical Veterinary Microbiology by P.J.Quinn, M.E.Carter, B.Markey and G.R.Carter.

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 22
Title: SOP for Culture and identification of Salmonella species
Version No: 2026.01, Total Pages:5
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&ECs

1. Scope

To definitively identify *Salmonella spp.* by biochemical testing of the isolated colonies in the laboratory.

2. Objective

- To detect, isolate, and identify *Salmonella* spp. from clinical or environmental specimens.
- To perform serotyping to identify specific serovars, crucial for epidemiology and outbreak investigations.
- To determine the antimicrobial susceptibility profile of isolated strains.

3. Principle

- The identification relies on the ability of Salmonella to grow on selective media and exhibit specific biochemical and antigenic characteristics:
- Selective Inhibition: Media containing bile salts, brilliant green, or Heokton inhibit gram-positive bacteria and many other gram-negative rods.
- Differential Characteristics: *Salmonella* are typically lactose-nonfermenters, produce hydrogen sulfide (H2S) and utilize lysine.
- Selective Enrichment: *Salmonella* are allowed to recover and multiply in non-selective broth (e.g., Buffered Peptone Water) followed by selective enrichment broths (e.g. Rappaport-Vassiliadis).
- Colonial Morphology: On Selective plate (Heokton/BGA), typical *Salmonella* produce red/pink colonies, often with black center

4. Equipment and Consumables

4.1 Autoclave
4.2 Sterile pipettes
4.3 Biohazard bags
4.4 Inoculating loops
4.5 Biological safety cabinet (ESCO)
4.6 Incubators (37^{o}C, 41^{o}C and air)
4.7 Bunsen burner
4.8 Marker pens
4.9 Wrapping material (Aluminium foil)
4.10 Graduated pipettes (10mL)
4.11 Test tubes (15mL)
4.12 Test tubes rack
4.13 Auto-pipette
4.15 Rubber bulb
4.16 Sterile Petri plates (15mmx90mm)
4.17 Buffered Peptone water
4.18 Rappaport-Vassiliadis (RV) broth, 10 mL
4.19 Brilliant green/ Heokton agar plates
4.20 Nutrient Aga/Sheep blood Agarr
4.21 MR/VP Medium
4.22 TSI
4.23 Urease
4.24 Indole
4.21 Salmonella agglutinating antisera, polyvalent O and H

5. Procedure

5.1 Sample (e.g., 25g food/swab) is added to a non-selective broth (225 mL or 10mL) Buffered Peptone Water (BPW) and incubated at 37°C for 18–24 hours.

5.2 Selective Enrichment: Transfer 0.1 mL of pre-enrichment culture to 10 ml of Rappaport-Vassiliadis (RV) broth.

5.3 Incubation: RV broth is incubated at 41 ^{0}C for 24 to 48 hours,

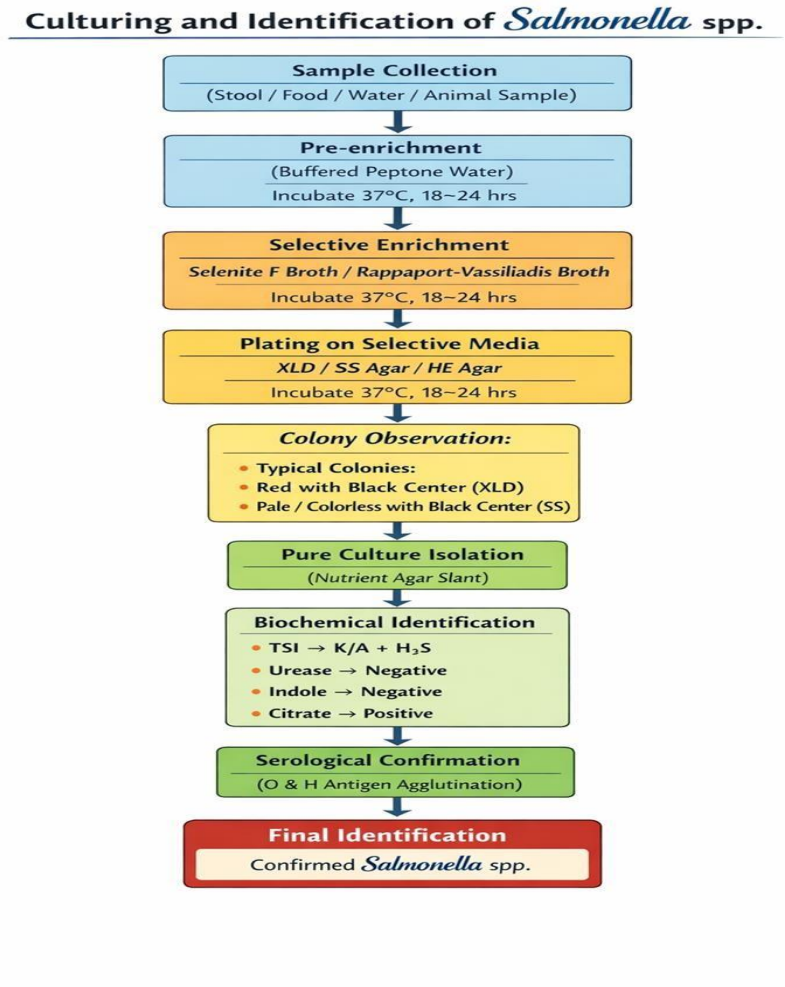
5.4 Streak the selective enrichment broths onto solid selective media plates: Heokton Agar and a second agar (e.g., XLD or Brilliant Green Agar) and incubate at 37^{0}C for 18-24hrs.

5.5 Colonial Inspection: Salmonella on XLD appears as red/pink colonies with black centers and Heokton appears as black colony.

5.6. Pick a well-isolated black colony and aseptically streak it onto Sheep Blood Agar or Nutrient Agar for further testing.

5.7 Biochemical Tests (Presumptive): Suspected colonies are tested using Triple Sugar Iron (TSI) agar (alkaline slant, acid butt, Gas+), Urease (negative), and Lysine Iron Agar (LIA) (positive), MR- Positive and VP-Negative.

5.7 Slide agglutination using polyvalent O and H antisera (Kauffmann-White scheme) to confirm genus and determine serogroup.



6. Result Interpretation

Table: Biochemical test for different *Salmonella spp.*

TESTS	*Salmonella typhi*	*Salmonella Typhimurium*	*Salmonella paratyphi*
Indole	Negative	Negative	Negative
MR	Positive	Positive	Positive
VP	Negative	Negative	Negative
H2S	Positive	Positive	Negative
Urea	Negative	Negative	Negative
TSI	Alkali/Acid	Alkali/Acid	Gas

7. Quality control

Positive control: *Salmonella typhimurium*
Expected: Growth on selective media and typical biochemical reactions.

Negative control*: Escherichia coli*
Expected: Different colony morphology and biochemical reactions.

8. Waste Disposal

Culture Plates and Media: Collect in biohazard bag, autoclave for 121°C for 15-30 minutes. Alternatively, treat with 1% sodium hypochlorite (30 minutes contact time) before disposal.

9. References

- Garcia, L.S., Clinical Microbiology Procedures Handbook, 3rd Edition, ASM, Washington, D.C., Volume 1, 2010.
- Murray (Chief Editor), Manual of Clinical Microbiology, 7th Edition, ASM Press, Washington D.C., 1999.
- Murray (Chief Editor), Manual of Clinical Microbiology, 8th Edition, ASM Press, Washington D.C., 2003.
- Blaser MJ, Lofgren JP. Fatal salmonellosis originating in a clinical microbiology laboratory. J Clin Microbiol 1981;13:855-8.

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 23
Title: SOP for Culture and identification of Pasteurella species
Version No: 2026.01, Total Pages:3
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&ECs

1. Scope

To describe the standardized method for isolation, culture, and identification of Pasteurella species from clinical and environmental samples.

2. Objective

- To isolate *Pasteurella spp.* from clinical samples.
- To identify the bacteria based on phenotypic, morphological, and biochemical characteristics.
- To distinguish *Pasteurella spp.* from similar organisms like *Haemophilus* or *Actinobacillus.*

3. Principle

- Growth Requirements: Pasteurella species are not fastidious and grow well on enriched media, such as blood agar (BA) or chocolate agar, at 37°C.
- Culture Characteristics: They typically appear as non-hemolytic, grey, mucoid/viscous colonies with a characteristic "musty" or "mousy" odor.
- Selective Inhibition: Pasteurella generally do not grow well on MacConkey agar, which helps differentiate them from members of the Enterobacteriaceae family.
- Biochemical Profiling: Key identification tests include positive oxidase, catalase, and indole tests.

4. Equipment and consumables

4.1 Incubator at 35–37°CBiological safety cabinet
4.2 Autoclave
4.3 Microscope
4.4 Blood agar (5% sheep blood)
4.5 Chocolate agar
4.6 MacConkey agar
4.7 Nutrient agar
4.8 Gram stain reagents
4.9 Oxidase and catalase reagents
4.10 Indole test reagents

5. Procedure

5.1 Inoculation: Streak the specimen onto 5% sheep blood agar (preferred) or chocolate agar.
5.2 Incubation: Incubate plates at 35–37°C in an aerobic atmosphere or with 5-10% CO2 for 18-24 hours.
5.3 Selective Medium (Optional): If samples are heavily contaminated, a selective medium containing antibiotic (e.g., neomycin, vancomycin) can be used.
5.4 Colony Morphology: Observe for small, gray, convex, non-hemolytic, shiny, and mucoid colonies.
5.5 Gram Stain: *Pasteurella* species are Gram-negative, appearing as small coccobacilli, often showing characteristic bipolar staining (darker staining at the poles).

6. Result interpretation

Table: Biochemical tests for *Pasturella spp.*

Test	Reaction	Result
Catalase	Bubbles formation	Positive
Oxidase	Purple or blue in color	Positive
MacConkey agar	No growth	Negative
MOTILITY	No zig-zag movement	Non-motile
INDOLE	Red in brim	Positive
UREA	No change	Negative

Species Identification: Further identification (e.g., for *P. multocida* subspecies) can be achieved using automated systems like Vitek 2.

7. Quality Control

Positive: *Pasteurella multocida* ATCC strain
Negative: *Escherichia coli* (for MacConkey growth comparison)

8. Waste Disposal

- Autoclave all used plates, swabs, and cultures.
- Dispose of biological waste as per institutional guidelines.
- Decontaminate work surfaces with disinfectant.

9. References

- Murray (Chief Editor), Manual of Clinical Microbiology, 7th Edition, ASM Press, Washington D.C., 1999.
- Murray (Chief Editor), Manual of Clinical Microbiology, 8th Edition, ASM Press, Washington D.C., 2003.
- SOP Version 2028.1, NCAH, Serbithang, Bhutan.
- Quinn P.J., Carter M.E., Markey B. and Cater G.R. Clinical Veterinary Microbiology. 2000.

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 24
Title: SOP for Culture and identification of Bacillus anthracis
Version No: 2026.01, Total Pages:6
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&ECs

1. Scope

This SOP applies to clinical specimens (blood, vesicular fluid, eschar swabs, stool, cerebrospinal fluid) and environmental samples (soil, water, animal products) suspected of containing *Bacillus anthracis.*

2. Objective

To provide a structured approach for the safe, reliable, and standardized culturing, presumptive identification, and characterization of *Bacillus anthracis* isolates to support diagnosis and surveillance.

3. Principle

Bacillus anthracis is a Gram-positive, non-motile, facultative anaerobic spore-forming rod. It grows readily on common laboratory media, but selective media are required for environmental or contaminated samples. Identification relies on characteristic colony morphology (Medusa head), lack of hemolysis, susceptibility to gamma phage, and specific staining (M'Fadyean capsule stain).

4. Equipment and consumables

4.1 Incubator set at 37°C
4.2 Light microscope
4.3 Dark ground microscope
4.4 Biohazard hood, Class 2
4.5 Disposable nitrile gloves
4.6 Disposable surgical face masks
4.7 Disposable petri dishes
4.8 Wooden swab sticks
4.9 Facial tissues
4.10 Wash bottle of distilled water
4.11 Disposable toxicology jar with screw cap for use as discard container
4.12 Glass microscope slides and cover slips
4.13 Coplin jar
4.14 Sterile swabs
4.15 Sterile inoculating loops
4.16 Sterile disposable transfer pipettes
4.17 Sheep blood agar (SBA) plates
4.18 McConkey agar (MA) plates
4.19 Spray bottle containing freshly prepared 10% sodium hypochlorite
4.20 Metal discard tray
4.21 Aluminium foil
4.22 Nutrient agar plates
4.23 Sheep blood agar (BSA) plates
4.24 Polymixin Lysozyme EDTA Thallous acetate (PLET) agar plates
4.25 Polychrome methylene blue (M'Fadyeans) stain
4.26 Spore stains kit
4.27 Capsule stains kit
4.28 Absolute alcohol
4.29 Sterile normal saline
4.30 Distilled H_2O

5. Procedure

5.1 Direct smear examination

- Make small incision into an ear vein and prepare two thin blood smears.
- Stain one with Methylene blue and another with Gram's stain (Refer SOP for Methylene blue and Gram staining techniques).

- Examine for Gram positive square ended rods with distinct capsule.

Note: In pigs and horses, smears can be made from any edematous lesions.

5.2 Culture and identification of *B. anthracis* from fresh specimens

- Blood is the primary clinical material to be examined. Swabs of blood, other body fluids or swabs taken from incisions in tissues or organs can be used.
- Primary isolation can be done on Sheep blood agar and Mac Conkey agar are the diagnostic medium of choice to culture and isolate *B. anthracis*. Blood agar is spread with the samples and incubated in air/CO_2 at 35-37ºC for 24-48 hours.
- Presumptive identification can be made from the growth characteristics and colony appearance.
- Gram staining can be done (Refer SOP for Grams staining techniques) to make presumptive identification by microscopic appearances.

5.3 Culture and identification of *B. anthracis* from old, decomposed specimens, processed materials and environmental samples including soil.

Identification of *B. anthracis* from old, decomposed specimens, processed materials, and environmental samples, including soil, is possible but these samples often have saprophytic contaminants that outgrow and obscure *B. anthracis* on non-selective agars.

The following procedure is suggested:

- The sample is blended in two volumes of sterile distilled or deionised water and placed in a water bath at 62.5 ± 0.5°C for 30-60 minutes.
- Tenfold dilutions to 10-2 or 10-3 are then prepared. From each dilution, 10-100 µl are plated on to blood agar and optionally 250-300 µl on to PLET agar. All plates are incubated at 37°C.
- Blood agar plates are examined for typical colonies after overnight incubation, and the PLET plates are examined after 40–48 hours. Confirmation of the identity of suspect colonies as *B. anthracis* is done as described for fresh samples above.
- PLET medium is prepared by using heart-infusion agar base (DIFCO) made up to the manufacturer's instructions with the addition of 0.25–0.3 g/litre EDTA and 0.04 g/litre thallous acetate. (*NOTE: thallous acetate is poisonous and should be handled with care*.) The mixture is autoclaved and uniformly cooled to 50°C before adding the polymyxin at 30,000 units/litre and lysozyme at 300,000 units/litre. After mixing thoroughly, the agar is dispensed into Petri dishes.
- Animal inoculation may be considered for recovery of *B. anthracis* if all other methods fail. However, due to the increasing concern to eliminate the use of animals for biological testing, this approach should be used as a last resort and only if justified.

5.4 Capsule visualization

- Virulent encapsulated *B. anthracis* is present in tissues and blood and other body fluids from animals that have died from anthrax. The bacteria should be looked for in smears of these specimens that have been dried, fixed and stained with polychrome methylene blue (M'Fadyean's reaction).
- Refer SOP for polychrome methylene blue staining.

- Gram and Giemsa stains do not reveal the capsule. The capsule is not present on *B. anthracis* grown aerobically on nutrient agar or in nutrient broths, but can be seen when the virulent bacterium is cultured for a few hours in a few millilitres of blood (defribrinated horse or sheep blood seems to work best).
- Alternatively, the capsule is produced when the virulent *B. anthracis* is cultured on nutrient agar containing 0.7% sodium bicarbonate and incubated in the presence of CO_2 (20% is optimal, but a candle jar works well). The encapsulated *B. anthracis* will form mucoid colonies and the capsule can be visualized by making thin smears on microscope slides, fixing, and staining with polychrome methylene blue.

5.5 Spore identification

When viewed unstained, endospores of living bacilli appear edged in black and are very bright and refractile. Endospores strongly resist application of simple stains or dyes and hence appear as nonstaining entities in Gram-stain preparations. However, once stained, endospores are quite resistant to decolorization. This is the basis of several spore stains such as the Schaeffer-Fulton staining method which also differentiates the spores from sporangia and vegetative cells.
Refer SOP for spore staining technique.

5.6 Additional tests

- **Lecithinase production**

Inoculate an egg yolk agar plate and incubate at 35-37ºC for 18-24h and then examine for a zone of egg yolk precipitation.

- **Motility**-Refer SOP for motility test.
- **Immunological detection and diagnosis**

Ascoli test and Immunofluorescence are two immunological methods to detect and diagnose *B. anthracis* but due to considerable constraints in using these methods as routine detection methodology, this is not applicable for veterinary laboratories in Bhutan.

- **Confirmation of virulence with PCR**

Full confirmation of virulence can be carried out using PCR if required and the samples will be forwarded to the Reference Laboratory.

6. Result Interpretation

Presumptive identification: May be made if appropriate growth characteristics, colonial appearance and Gram stain of the culture, are demonstrated.

- B. anthracis are observed as Gram positive square ended rods with distinct capsule.
- *B. anthracis* colonies are grey-white to grey, 0.3–0.5 mm in diameter, non-haemolytic, with a ground-glass moist surface, and very tacky when teased with an inoculating loop.
- Tailing and prominent wisps of growth trailing back toward the parent colony, all in the same direction, are sometimes seen. This characteristic has been described as a ‘medusa head’ appearance.
- Confirmation of *B. anthracis* should be accomplished by the demonstration of capsulated, spore-forming bacilli. The capsule stains pink, whereas the bacillus cells stain dark blue. The cells are found in pairs or short chains and are often square ended (the chains are sometimes likened to a set of railway carriages – so-called ‘box-car’ appearance).

- In the spore stain of a *Bacillus* species by Schaeffer-Fulton method, mature spores stain green, whether free or still in the vegetative sporangium and vegetative cells and sporangia stain red.

Confirmation of identification; May be made following Lecithinase activity, motility, penicillin susceptibility and commercial identification kit results and/or the Reference Laboratory report.

Lecithinase production - *B. anthracis, B. cereus, B. thuringiensis* and *B. mycoides* are positive.
Motility - All *Bacillus* species are motile with the exception of *B. anthracis* and *B. mycoides.*
Note-Further confirmation by VITEK 2

7. Quality control

- Eessential for maintaining safety, ensuring the viability of the agent, and accurately distinguishing it from closely related *Bacillus* species, such as *B. cereus* and *B. thuringiensis*. Given that *B. anthracis* is a potential bioterrorism agent (Category A).
- All laboratory work must be performed in accordance with high-level biosafety regulations (typically BSL-3), with strict controls on the storage, transport, and handling of strains.

8. Waste disposal

- **Primary Containment:** All contaminated materials (tips, loops, plates, tubes, swabs) must be placed directly into strong, leak-proof autoclavable bags or containers within the BSL-3 cabinet.
- **Decontamination (autoclaving):** All contaminated materials must be autoclaved at 121°C for at least 1 hour (longer than standard cycles) to ensure the destruction of highly resistant spores. It is strongly recommended that *B. anthracis* waste be autoclaved twice.
- **Liquid Waste:** Liquid cultures and contaminated fluids should be autoclaved or treated with a fresh 10% hypochlorite solution (10,000 ppm) for at least one hour.
- **Disinfection:** Benches and work areas must be wiped down with 10% hypochlorite solution (10,000 ppm) after work is finished.
- **PPE disposal:** Contaminated lab coats, gowns, and disposable gloves must be autoclaved before being laundered or disposed of.
- **Staining:** Staining tray runoff and washes should be collected into a beaker containing a 10% hypochlorite solution.
- **Microscope slides:** Slides stained for *B. anthracis* must be placed in a sharps container and autoclaved before incineration.

9. References

- OIE Terrestrial Manual: Anthrax. Sec: 2.1., Chapter 2.1.1. Paris, 2008. p. 135-143
- Turnbull PCB, Bohm R, Chizyuka HGB, Fujikura T, Hugh-Jones ME, Melling J. Guidelines for the Surveillance and Control of Anthrax in Humans and Animals. WHO/Zoon. /93.170; Geneva: World Health Organisation; 1993
- SOP Version 2013, NCAH, Serbithang, Bhutan.
- SOP Version 2028.1, NCAH, Serbithang, Bhutan.

- Barrow GI, Feltham R K A, editors. Cowan and Steel's Manual for the Identification of Medical Bacteria. 3rd ed. Cambridge: Cambridge University Press; 1993. p. 226
- Department of Veterinary Pathology and Public Health. Laboratory Manual for Veterinary Microbiology. Queensland.
- Microbiology Laboratory. London: Public Health Laboratory Service; 1999. p. 147-8.

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 25
Title: SOP for Culture and identification of Staphylococcus species
Version No: 2026.01, Total Pages:4
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&ECs

1. Scope

To isolate, culture, and identify *Staphylococcus* species from clinical or environmental samples using standard microbiological techniques.

2. Objective

- To isolate *Staphylococcus* spp. in pure culture from clinical or environmental samples.
- To differentiate *Staphylococcus* from other Gram-positive cocci (e.g., *Streptococcus*).
- To distinguish *S. aureus* (coagulase-positive) from CoNS (coagulase-negative), such as *S. epidermidis* and *S. saprophyticus*.
- To determine antimicrobial susceptibility (AMR) profiles, specifically for MRSA.

3. Principle

Staphylococcus spp. are Gram-positive cocci arranged in clusters. They grow on general and selective media and are identified by colony morphology, Gram staining, and biochemical tests such as catalase and coagulase.

4. **Equipment and Consumables**

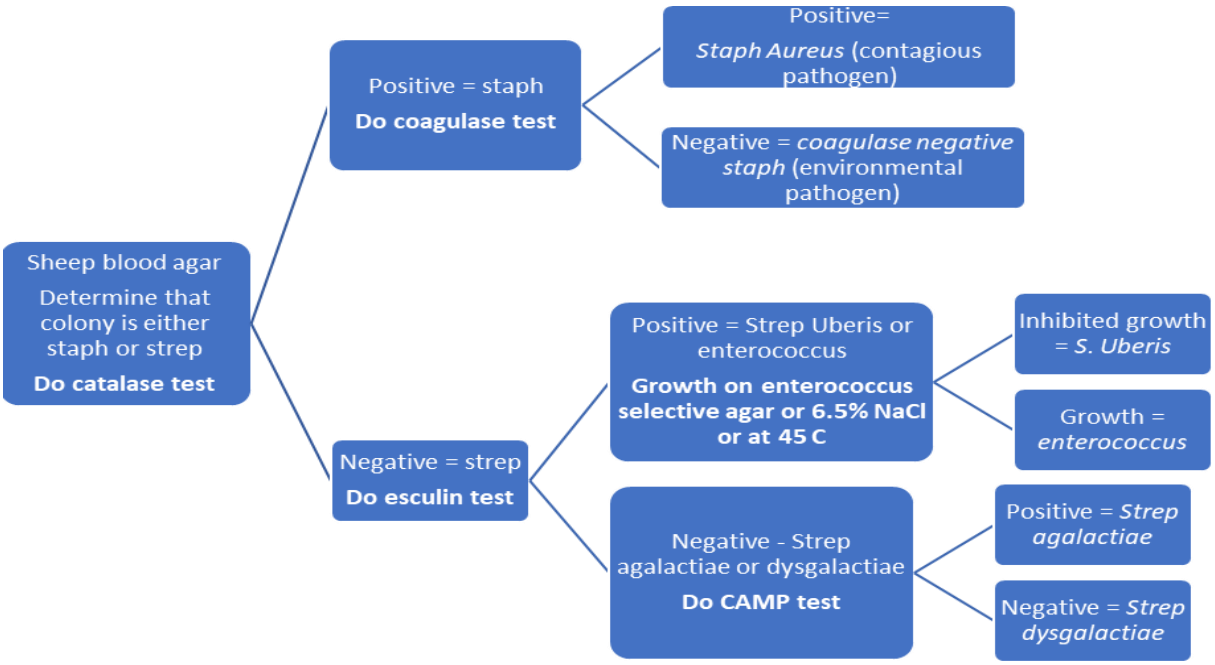
4.1 Incubator (35–37°C)
4.2 Biosafety cabinet
4.3 Inoculating loop
4.4 Microscope
4.5 Autoclave
4.6 Nutrient agar
4.7 Blood agar
4.8 Mannitol salt agar (MSA)
4.9 MacConkey agar
4.10 Gram stain reagents
4.11 Hydrogen peroxide (3%)
4.12 Coagulase plasma
4.13 Oxidase reagent

5. Procedure

5.1 Streak samples onto Sheep Blood Agar (non-selective, allows hemolysis check), MacConkey agar and Mannitol Salt Agar (selective/differential for *S. aureus*).
5.2 INCUBATION: Incubate aerobically at 35 to 37 ^{0}C for 18-24 hours.
5.3 Observation in blood agar: 1–3 mm creamy/white/yellowish colonies with or without Beta - hemolysis.
5.4 MSA**:** Yellow colonies (presumptive *S. aureus*) or red/pink colonies (other species).
5.5. MacConkey Agar- No growth observed.

Sub-culturing

Isolate pure subcultures of morphologically distinct colonies on Sheep Blood Agar before proceeding to bacterial identification.



1. Result Interpretation

Test	Reaction	Result
Gram's stain	Gram positive cocci in clusters	Positive
Catalase test	Rapid bubbles formation	Positive
Oxidase	Blue color develop	Positive
Coagulase test	Clumping	Positive

7. Quality Control

S. aureus- coagulase positive control.
S. epidermidis- coagulase negative control.

8. Waste Disposal

- Autoclave all biological waste at 121°C for 15–30 min.
- Dispose according to biomedical waste guidelines.
- Decontaminate work surfaces with disinfectant.

9. References

- Claxton, P. D., & Ryan, D. (1980). *Bovine Mastitis: Bacteriology*. Australian Standard Diagnostic Techniques for Animal Diseases. Arthur Webster Pty Ltd and NSW Agriculture
- Murray (Chief Editor), Manual of Clinical Microbiology, 8th Edition, ASM Press, Washington D.C., 2003.
- SOP Version 2028.1, NCAH, Serbithang, Bhutan.
- Quinn P.J., Carter M.E., Markey B. and Cater G.R. Clinical Veterinary Microbiology. 2000.

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 26
Title: SOP for Culture and identification of Streptococcus species
Version No: 2026.01, Total Pages:3
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&ECs

1. Scope

To detect, isolate, and identify Streptococcus *spp.* (including alpha-hemolytic, beta-hemolytic, and *S. pneumoniae*) using culture, microscopy, and biochemical techniques to guide clinical management.

2. Objective

- To isolate Streptococcus *spp.* from different samples.

- To identify Streptococcus *spp.* based on cultural, morphological, and biochemical characteristics**.**
- To differentiate major groups such as: Alpha-hemolytic Streptococci, Beta-hemolytic Streptococci, and Gamma (non-hemolytic) Streptococci.

3. Principle

Streptococcus *spp.* are fastidious, Gram-positive cocci that require enriched media such as blood agar for optimal growth. Their ability to produce hemolysins (streptolysins O and S) enables differentiation based on hemolytic patterns (alpha, beta, or gamma hemolysis).

4. Equipment and Consumables

4.1 Glass slide
4.2 Blotting paper
4.3 Microscope (Leica)
4.4 Lint free tissue paper
4.5 Pipette/pipette tip
4.6 Bunsen burner
4.7 Platinum loop
4.8 Staining rack
4.9 Petri-plates
4.10 Autoclave
4.11 SBA/MacConkey Agar
4.12 Reagents for First and Second stage tests
4.13 Fresh culture
4.14 Sterile distilled water
4.15 Immersion oil
4.160%alcohol

5. Procedure

5.1 Inoculate the specimen onto 5% Sheep Blood Agar and MacConkey agar using aseptic technique and quadrant streaking to obtain isolated colonies.
5.2 Incubate the plate at 35-37°C for 18-24 hours in a 5% CO_2 atmosphere (candle jar or CO_2 incubator).
5.3 Examine the plate for growth and hemolysis patterns (α, β, or γ).

6. Result Interpretation

Test	Result	Interpretation
Gram's stain	Gram + cocci in chains	Streptococcus spp.
Catalase	Negative	Confirms *Streptococcus*
Beta hemolysis + Bacitracin sensitive	Positive	*S. pyogenes*
Beta hemolysis + CAMP positive	Positive	*S. agalactiae*
Alpha hemolysis + Optochin sensitive	Positive	*S. pneumoniae*
Bile esculin positive	Positive	*Enterococcus spp.*

7. Quality Control

- *S. pyogenes*: Beta hemolysis, Bacitracin sensitive
- *S. agalactiae*: CAMP positive
- *S. pneumoniae*: Optochin sensitive
- Check media sterility and performance.
- Verify reagent expiry.

8. Waste disposal

- Dispose cultures in biohazard bags.
- Autoclave at 121°C for 15–30 minutes.
- Decontaminate work surfaces with disinfectant (e.g., 70% ethanol or bleach).

9. REFRENCES

- Quinn P.J., Carter M.E., Markey B. and Cater G.R. Clinical Veterinary Microbiology. 2000.
- Subcommittee on taxonomy of staphylococci and micrococci - minutes of first meeting. International bulletin of bacteriological nomenclature and taxonomy 1965; 15:107-8.
- Toowoomba Veterinary Laboratory: Biochemcial tests. Laboratory Methods Manual. Issue 4. Queensland; Australia: 2004. P. 1-8
- SOP Version 2013, NCAH, Serbithang, Bhutan
- SOP Version 2028.1, Serbithang, Bhutan
- Microbiology Laboratory. London: Public Health Laboratory Service; 1999. p. 147-8.

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 27
Title: SOP for Culture and identification of Enterococcus species
Version No: Nil, Total Pages:5
Issue Month/Effective Date: May 2026
Revision Summary: NA
Supersedes Version No: NA
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&ECs

1. Scope

Isolation and identification of *Enterococcus* species from clinical or environmental samples using culture, Gram staining, and basic biochemical tests.

2. Objective

To isolate and identify *Enterococcus* species from clinical or environmental samples using standard microbiological techniques.

3. Principles

Enterococcus are Gram-positive cocci arranged in pairs or chains, catalase-negative, able to grow in 6.5% NaCl, and hydrolyze esculin in the presence of bile. These characteristics distinguish them from other Gram-positive cocci.

4. Equipment and Consumables

4.1 Inoculating loop
4.2 Biological safety cabinet
4.3 Bunsen burner
4.4 Incubators(37^{o}C)
4.5 Autoclave
4.6 Clean grease-free glass slides
4.7 Distilled water
4.8 PPE (Gloves, Apron, Mask)
4.9 Marker pens
4.10 Sheep blood Agar
4.11 Azide Dextrose Broth
4.12 Buffer peptone water
4.13 Slanetz Bartley Agar
4.14 Bile Aesculin Agar
4.15 Gram Stain kit
4.16 6.5% NaCl
4.17 3% Hydrogen peroxide
4.18 70% alcohol

5. Procedure

Pre-enrichment:

5.1 Inoculate the specimen into Buffered Peptone Water (BPW).
5.2 Incubate at 35–37°C for 18–24 hours.
5.3 Transfer 1 mL of the pre-enriched culture into Azide Dextrose Broth (ADB).
5.4 Mix well and incubate at 35–37°C for 18–24 hours.
5.5 After enrichment, take a loopful of the Azide Dextrose Broth culture.
5.6 Streak onto: Sheep Blood Agar (SBA) and Slanetz & Bartley (or Slanetz) Agar.
5.7 Incubate the plates at 35–37°C for 24–48 hours.

5.1.1 Colony observation:

SBA: small, grayish colonies; alpha or gamma hemolysis may be seen.
Slanetz: red/pink to maroon colonies indicate Enterococcus growth.

5.1.2 Gram staining:

- Prepare a smear from isolated colonies and follow the gram stain procedure (SOP).
- Observe under oil immersion: Gram-positive cocci in pairs or chains.

Biochemical tests:

5.1.3 Catalase Test

- Place a drop of 3% H_2O_2 on a clean slide.
- Add a small amount of colony and mix.

5.1.4 Bile Esculin Test

11. Inoculate organism onto Bile Esculin Agar.
12. Incubate at 35–37°C for 24 hours.

5.1.5 6.5% NaCl Growth Test

- Inoculate organism into 6.5% NaCl broth.
- Incubate at 35–37°C for 24 hours.

Flow Chart

Sample
▼
Pre-enrichment in Buffered Peptone Water (BPW)
Incubate 35–37°C, 18–24 hours
▼
Selective Enrichment in Azide Dextrose Broth (ADB)
Transfer 1 mL from BPW, mix, incubate 35–37°C, 18–24 hours
▼
Isolation on Selective Media
- Sheep Blood Agar (SBA)
- Slanetz Agar
Incubate 35–37°C, 24 hrs
▼
Observation of Colonies
SBA: small gray, alpha/gamma hemolysis
Slanetz: red/pink colonies
▼
Gram Staining
Gram-positive cocci in chains/pairs
▼
Biochemical Tests
Catalase
6.5% NaCl
Bile Esculin
▼

6. Result Interpretation

Test	Result
Gram stain	Gram-positive cocci
Catalase	Negative
6.5% NaCl	Positive
Bile Esculin	Positive
Slanetz Bartley agar	Red/pink colonies

Table: Differentiation on the identification of the two different *Enteriococcus spp.*

Characteristic	*Enterococci faecalis*	*Enterococci faecium*
Slanetz-Barley	Deep red colonies with golden reflection	Pink/white colonies
Blood agar	Gamma / no haemolysis	Alpha haemolysis
Morphology	Gram positive cocci	Gram positive cocci
Catalase activity	Negative	Negative
Bile esculin test	Black medium	Black medium

7. Quality Control

Positive: *Enterococcus faecalis* ATCC 29212
Negative: *Staphylococcus aureus* ATCC 25923

8. Waste Disposal

- Autoclave all used media and cultures at 121°C for 15 minutes before disposal.
- Discard contaminated materials (loops, swabs) in biohazard bags and autoclave.
- Sterilize reusable glassware before washing and reuse.
- Wash hands thoroughly after handling**.**
- Liquid waste should be treated with disinfectant (e.g. 10% bleach for 30 minutes) before discarding.
- Work surfaces must be disinfected with 70% ethanol or appropriate disinfectant after use.
- Follow laboratory biosafety guidelines for infectious waste disposal.

9. References

- SOP version 2018.01. NCAH, Serbithang, Bhutan.
- Advisory Committee on Dangerous Pathogens. 2004 Approved List of Biological Agents. http://www.hse.gov.uk/pubns/misc208.pdf. p. 1-17
- Clinical Veterinary Microbiology by P.J.Quinn, M.E.Carter, B.Markey and G.R.Carter
- Barrow GI, Feltham R K A, editors. Cowan and Steel's Manual for the Identification of Medical Bacteria. 3rd ed. Cambridge: Cambridge University Press; 1993. p. 226
- Control of Substances Hazardous to Health Regulations 2002. General COSHH. Approved Code of Practice and Guidance, L5. Suffolk: HSE Books; 2002.
- Department of Health NHS Executive: The Caldicott Committee. Report on the review of patient-identifiable information. London. December 1997.
- Department of Veterinary Pathology and Public Health. Laboratory Manual for Veterinary Microbiology. Queensland.
- Health and Safety Executive. 5 steps to risk assessment: a step-by-step guide to a safer and healthier workplace, IND (G) 163 (REVL). Suffolk: HSE Books; 2002.

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 28
Title: SOP for Culture and identification of Clostridium species
Version No: 2026.01, Total Pages:4
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&ECs

1. Scope

Applicable in microbiology laboratories for detection of anaerobic, spore-forming Gram-positive bacilli.

2. Objective

To isolate and identify *Clostridium species* from clinical or environmental samples.

3. Principles

Clostridium spp. is obligate anaerobes that grow in oxygen-free conditions and produce characteristic colonies and biochemical reactions. Identification is based on morphology, anaerobic growth, and biochemical tests.

4. Equipment and Consumables

4.1 Inoculating loop
4.2 Biological safety cabinet
4.3 Bunsen burner
4.4 Incubators(37^{o}C)
4.5 Autoclave
4.6 Anaerobic jar
4.7 Gas Pack
4.8 Clean grease-free glass slides
4.9 Distilled water
4.10 PPE (Gloves, Apron, Mask)
4.11 Marker pens
4.12 Sheep blood Agar
4.13 MacConkey Agar
4.14 3% Hydrogen peroxide
4.15 Oxidase disc
4.16 MIL medium
4.17 O-F medium
4.18 Gram Stain kit
4.19 70% alcohol

5. Procedure

5.1 Take the specimen (clinical sample, environmental swab). Mix well if in transport medium.
5.2 Using a sterile loop, streak the sample on the surface of the blood agar plate and on MacConkey agar to check for Gram-negative enteric bacteria.
5.3 Incubate the cultured plate 40-48 hours in anaerobic condition at 35-37°C
5.4 Colony observation:
Blood Agar: Check for colony size, shape, hemolysis (alpha, beta, gamma).
MacConkey Agar: Observe lactose fermentation colonies.

5.6 Identification based on the test below:

- Gram stain - Prepare a smear from isolated colonies and follow the gram stain procedure (SOP)
- Oxidase test - Follow SOP on oxidase test
- Catalase test – Follow SOP on catalase test
- Motility test- Follow SOP on Motility test
- O-F test- Follow SOP on O-F test

6. Result Interpretation

Table: Differentiation on the identification of the two different *Clostridium species*

Species	Colony on Blood Agar	Hemolysis	Spore Position	Motility	Catalase	Oxidase	O/F Test	Key Notes
Clostridium perfringens	Large, irregular, opaque	Double-zone	Subterminal	Non-motile	Negative	Negative	Fermentative (F)	"Stormy fermentation" in milk; common in Gas gangrene
Clostridium tetani	Swarming growth, small round	Usually none	Terminal ("drumstick")	Motile	Negative	Negative	Fermentative (F)	Produces toxin causing Tetanus
Clostridium botulinum	Small, grayish colonies	Usually none	Sub terminal / central	Motile	Negative	Negative	Fermentative (F)	Produces toxin; causes Botulism
Clostridium difficile	Yellowish, irregular	Variable	Central / subterminal	Motile	Negative	Negative	Fermentative (F)	Causes Antibiotic-associated diarrhea
Clostridium sporogenes	Large, spreading	Usually none	Central	Motile	Negative	Negative	Fermentative (F)	Non-pathogenic; used as control organism

Note: for further confirmation by Vitek2

7. Quality Control

Positive control: *Clostridium perfringens*

8. Waste Disposal

- Autoclave all used media and cultures at 121°C for 15 minutes before disposal.
- Discard contaminated materials (loops, swabs) in biohazard bags and autoclave.
- Sterilize reusable glassware before washing and reuse.
- Wash hands thoroughly after handling.
- Liquid waste should be treated with disinfectant (e.g. 10% bleach for 30 minutes) before discarding.
- Work surfaces must be disinfected with 70% ethanol or appropriate disinfectant after use.

Note: Proper sterilization is essential as Clostridium forms spores.

9. References

- SOP version 2018.01, NCAH, Serbithang, Bhutan.
- Advisory Committee on Dangerous Pathogens. 2004 Approved List of Biological Agents. http://www.hse.gov.uk/pubns/misc208.pdf. p. 1-17
- Clinical Veterinary Microbiology by P.J.Quinn, M.E.Carter, B.Markey and G.R.Carter Barrow GI, Feltham R K A, editors. Cowan and Steel's Manual for the Identification of Medical Bacteria. 3rd ed. Cambridge: Cambridge University Press; 1993. p. 226
- Control of Substances Hazardous to Health Regulations 2002. General COSHH. Approved Code of Practice and Guidance, L5. Suffolk: HSE Books; 2002.
- Department of Health NHS Executive: The Caldicott Committee. Report on the review of patient-identifiable information. London. December 1997.
- Department of Veterinary Pathology and Public Health. Laboratory Manual for Veterinary Microbiology. Queensland.
- Health and Safety Executive. 5 steps to risk assessment: a step by step guide to a safer and healthier workplace, IND (G) 163 (REVL). Suffolk: HSE Books; 2002.
- Identification of Clostridium species, Issue no: 3 Issue date: 14.07.08 Issued by: Standards Unit, Evaluations and Standards Laboratory Page no: 1 of 14. BSOP ID 8i3

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 29
Title: SOP for Culture and identification of Corynebacterium species
Version No: 2026.01, Total Pages:4
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&ECs

1. Scope

The scope of *Corynebacterium* culture and identification is to isolate, identify, and differentiate pathogenic from non-pathogenic species using colony morphology, Gram staining, and biochemical tests to support clinical diagnosis.

2. Objective

To detect, isolate, and accurately identify *Corynebacterium* species, distinguishing pathogenic from non-pathogenic strains using microbiological and biochemical methods for clinical diagnosis.

3. Principles

Corynebacterium species are Gram-positive, non-spore-forming rods that can be isolated on selective or enriched media; identification is based on colony morphology, Gram staining, and biochemical reactions (catalase, sugar fermentation, nitrate reduction), allowing differentiation of pathogenic from non-pathogenic strains.

4. Equipment and Consumables

4.1 Inoculating loop
4.2 Biological safety cabinet
4.3 Scapel blades
4.4 Sterile forceps
4.5 Bunsen burner
4.6 Incubators(37^{o}C)
4.7 Autoclave
4.8 Clean grease-free glass slides
4.9 Distilled water
4.10 PPE (Gloves, Apron, Mask)
4.11 Marker pens
4.12 Water bath
4.13 Sheep blood Agar
4.14 MacConkey Agar
4.15 3% Hydrogen peroxide
4.16 Oxidase disc
4.17 MIL medium
4.18 Nitrate Broth
4.19 Reagent A & B for nitrate detection
4.20 O-F medium
4.21 Gram Stain kit
4.22 10% and 1% sodium hypochlorite
4.23 70% alcohol

5. Procedure

- Collect clinical specimens (throat swab, wound swab, nasal swab) aseptically in suitable medium.
- Culture on Sheep blood agar and MacConkey agar incubate for 16-48hours and incubate at 35-37°C.
- **Colony observation:**

Blood Agar**:** Check for colony size, shape, hemolysis (alpha, beta, gamma).
MacConkey Agar**:** Observe lactose fermentation colonies.

- **Identification based on the test below:**
- Gram stain - Prepare a smear from isolated colonies and follow the gram stain procedure (SOP).
- Observe for Gram-positive, club-shaped rods**,** often in "V" or "L" formations.
- Oxidase test - Follow SOP on oxidase test
- Catalase test – Follow SOP on catalase test
- Motility test- Follow SOP on Motility test
- Nitrate reduction test - Follow SOP on Nitrate reduction test
- CAMP Test- Follow SOP on CAMP test
- O-F test- Follow SOP on O-F test

6. Result interpretation

- Blood Agar**:** Small to medium, gray-white, smooth, slightly convex colonies, usually non-hemolytic; some pathogenic species may show slight hemolysis.
- MacConkey Agar**:** No growth.
- Gram Stain: Gram positive rods, pleomorphic, slightly curved with tapered or clubbed ends. Cells may occur singly or in pairs, often in a "V" formation (forming "Chinese letters"). Cells usually stain weakly and unevenly giving a beaded appearance.

Tabulated below the differentiation on the identification of the two different ***Corynebacterium species***

Feature / Test	***C. diphtheriae***	***C. ulcerans***	***C. pseudotuberculosis***
Gram Stain	Gram-positive, club-shaped rods, often in "V" or "L" formations	Gram-positive rods, club-shaped	Gram-positive rods, club-shaped
Catalase	+	+	+
Oxidase	–	–	–
Motility	–	–	–
Nitrate Reduction	–	+	+
O/F (Oxidation-Fermentation)	F	F	F
Reverse CAMP Test	–	+	+
Sugar Fermentation	Glucose +, Maltose +, Sucrose –	Variable	Variable

Note: For further confirmation by Vitek2

7. Quality Control

- Positive control: *Corynebacterium diphtheriae* (ATCC 13812)
- CAMP test control: *Staphylococcus aureus* (ATCC 25923)

8. Waste Disposal

- Autoclave all used media and cultures at 121°C for 15 minutes before disposal.
- Discard contaminated materials (loops, swabs) in biohazard bags and autoclave.
- Sterilize reusable glassware before washing and reuse.
- Dispose sharps in puncture-proof containers; autoclave if required.
- Liquid waste should be treated with disinfectant 10% bleach for 30 minutes before discarding.
- Work surfaces must be disinfected with 1% sodium hypochlorite or 70% ethanol after work.
- Wash hands thoroughly after handling

9. References

- SOP version 2018.01, NCAH, Serbithang, Bhutan.
- Advisory Committee on Dangerous Pathogens. Categorization of biological agents according to hazard and categories of containment, 4th ed. Suffolk: HSE books; 1995 (with supplements 1, 1998 and 2, 2000).
- Advisory Committee on Dangerous Pathogens. The management, design and operation of microbiological containment laboratories. Suffolk: HSE Books; 2001.
- BS EN 12469: 2000. Biotechnology - performance criteria for microbiological safety cabinets. London: British Standards Institution (BSI); 2000.
- BS 5726: 1992. Microbiological safety cabinets. Part 2. Recommendations for information to be exchanged between purchaser, vendor and installer and recommendations for installation. London: British Standards Institution (BSI); 1992.
- BS 5726: 1992. Microbiological safety cabinets. Part 4. Recommendations for selection, use and maintenance. London: British Standards Institution (BSI); 1992.
- Control of Substances Hazardous to Health Regulations 2002. General COSHH. Approved Code of Practice and Guidance, L5. Suffolk: HSE Books; 2002.
- Health and Safety Executive. 5 steps to risk assessment: a step-by-step guide to a safer and healthier workplace, IND (G) 163 (REVL). Suffolk: HSE Books; April 2002.
- Health and Safety Executive. A guide to risk assessment requirements: common provisions in health and safety law, IND (G) 218 (L). Suffolk: HSE Books; March 2002.

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 30
Title: SOP for Culture and identification of Campylobacter species
Version No: 2026.01, Total Pages:3
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No:2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&ECs

1. Scope

To provide standardized procedures for the isolation, culturing, and identification of Campylobacter species from clinical, food, or environmental samples.

2. Objective

The primary objective is the isolation and identification of thermophilic *Campylobacter spp.* from stool, food, or environmental samples to diagnose campylobacteriosis and determine the species responsible.

3. Principle

Microaerophilic Environment: Campylobacter requires a low oxygen (5-10% O2) and high carbon dioxide (5-10% CO2) atmosphere to grow, typically achieved using specialized gas-generation systems.

Thermotolerance: *C. jejuni* and *C. coli* are thermophilic, growing optimally at 42°C to 43°C .This high incubation temperature suppresses the growth of most competing flora.

Selective Media: Culture media (e.g., Modified Charcoal Cefoperazone Deoxycholate Agar - mCCDA) contain antibiotics (cefoperazone, vancomycin) that suppress fecal flora, allowing *Campylobacter* to form typical colonies.

Motility: *Campylobacter* has a characteristic corkscrew-like motion used for identification.

4. Equipment and Consumables

4.1 Incubator with microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) at 42°C
4.2. Biological safety cabinet
4.3 Autoclave
4,4 Water bath
4.4 Microscope (phase contrast preferred)
4.5. Cary-Blair transport medium (for stool)
4.6 Selective enrichment broth (e.g., Bolton or Preston broth)
4.8 Selective agar plates (e.g., Modified Charcoal Cefoperazone Deoxycholate Agar - mCCDA)
4.8 Blood agar (optional)
4.9 Sterile loops, pipettes, and tubes
4.10 Gram stain reagents
4.11 Oxidase test reagent

5. Procedure

5.1 Enrichment-Inoculate 1 g/mL of sample into Bolton broth /Preston (or other selective enrichment media). Incubate at 42°C for 24 hours under microaerophilic conditions.

5.2 Plating- Streak a loopful of enrichment broth onto mCCDA plates. Incubate plates at 42°C for 48 hours under anaerobic conditions. Then culture in Sheep Blood Agar at 42°C at 48hours under anaerobic condition for appears like *Camphylobacter.*

5.3 Colony Morphology-Typical colonies: gray, moist, flat or slightly raised, spreading. Small colonies may have butterfly or “seagull wing” appearance.

5.4 Microscopic Examination-Perform Gram staining- *Campylobacter spp.* appears as Gram-negative curved or spiral rods.

6. Result Interpretation

Test	Reaction	Result
Catalase	Positive	
Oxidase	Positive	
Hippurate hydrolysis	Positive	*Camphylobacter jejuni*
Hippurate hydrolysis	Negative	*Camphylobacter coli*

7. Quality Control

- Use reference strains (e.g., *C. jejuni* ATCC 33560) for positive controls.
- Incubators, media, and reagents should be checked regularly.
- Document all observations and plate records.

8. Waste Disposal

Autoclave all plates, enrichment broth, and consumables before disposal.
Disinfect spills immediately with 10% bleach solution.

9. Reference

- Murray (Chief Editor), Manual of Clinical Microbiology, 7th Edition, ASM Press, Washington D.C., 1999.
- Murray (Chief Editor), Manual of Clinical Microbiology, 8th Edition, ASM Press, Washington D.C., 2003.
- SOP Version 2028.1, NCAH, Serbithang, Bhutan
- Quinn P.J., Carter M.E., Markey B. and Cater G.R. Clinical Veterinary Microbiology. 2000.

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 31
Title: SOP for Fungal culture and identification
Version No: 2026.01, Total Pages:3
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No:2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&ECs

1. Scope

● Covers isolation, cultivation, and identification of fungi from clinical, veterinary, food, and environmental samples
● Includes yeasts (e.g., Candida spp.) and molds (e.g., Aspergillus spp.)
● Applied in diagnosis of fungal infections, contamination detection, and laboratory research.
● Performed under appropriate biosafety and laboratory standards.

2. Objective

- To isolate and obtain pure cultures of fungi from specimens.
- To identify fungi based on:
  - Colony morphology
  - Microscopic characteristics
  - Biochemical/physiological properties (when required)
- To differentiate pathogenic fungi from contaminants.
- To aid in diagnosis, treatment, and epidemiological studies.

3. Principle

Fungi are cultivated on selective media (e.g., Sabouraud Dextrose Agar) that supports fungal growth and inhibits bacteria. Under suitable temperature and incubation conditions, fungi utilize nutrients and form characteristic colonies.

Identification relies on:

- Macroscopic features (color, texture, growth rate)
- Microscopic morphology (hyphae, spores, yeast cells) using stains like Lactophenol Cotton Blue
- Additional identification may involve biochemical and physiological tests.
- Strict aseptic techniques ensure accurate isolation and prevent contamination.

4. Equipment and Consumables

4.1 Sterile petri dishes
4.2 Sterile pipettes
4.3 Sterile scalpel
4.4 Sterile artery forceps
4.5 Biohazard bag
4.6 Inoculating loop
4.7 Biological safety cabinet
4.8 Incubators (25°C and air)
4.9 Bunsen burner
4.10 Microscope
4.11 Microscope slides and cover slips
4.12 Marker pens
4.13 Wrapping material (Aluminum foil)
4.15 Surgical blades
4.16 Sabouraud dextrose (SBA) agar
4.17. Lactophenol cotton blue stain

5. Procedure

5.1 Sabouraud dextrose (SBA) agar is the standard medium for isolation of fungi and can be used for the successful isolation of dermatophytes.
5.2 The culture is allowed to incubate at room temperature (22° to 25°C).
5.3 Placement on an open shelf or counter allows daily observation for up to 8-10 days for growth.

5.4 Dermatophytes are identified on the basis of both their gross colony characteristics and microscopic morphologic characteristics. Rate of growth, texture, pattern of growth, color of the colony, and pigmentation of the reverse of the colony should be noted. Most dermatophyte colonies are white or light shades of apricot, yellow, or cream to tan.

5.5 Darkly colored brown or black fungi are likely to be contaminants.

6. Result Interpretation

Definitive identification of a dermatophyte and species identification require microscopic examination of wet tape mounts prepared in lactophenol cotton blue stain (refer SOP for Lactophenol cotton blue staining procedure). The slide is examined for microconidia, macroconidia, hyphae structures, and other identifying characteristics.

7. Quality Control

- Check sterility by incubating uninoculated plates.
- Verify pH (≈5.6) and appearance (no cracks, contamination, dehydration).
- Perform growth promotion test using control strains (e.g., Candida albicans, Aspergillus niger)

8. Waste disposal

- Autoclaving (Preferred method) 121°C for 15–30 minutes
- Chemical disinfection (if autoclave not available):
- 1% Sodium hypochlorite (30 minutes contact times)

8. References

- Quinn P.J., Carter M.E., Markey B. and Cater G.R. Clinical Veterinary Microbiology. 2000.
- Subcommittee on taxonomy of staphylococci and micrococci - minutes of first meeting. International bulletin of bacteriological nomenclature and taxonomy 1965; 15:107-8.
- Toowoomba Veterinary Laboratory: Biochemcial tests. Laboratory Methods Manual. Issue 4. Queensland; Australia: 2004. P. 1-8
- SOP Version 2013, NCAH, Serbithang, Bhutan
- SOP Version 2028.1, NCAH, Serbithang, Bhutan
- Microbiology Laboratory. London: Public Health Laboratory Service; 1999. p. 147-8

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 32
Title: SOP for California Mastitis Test
Version No: 2026.01, Total Pages:4
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No:2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, RVH&ECs, SVL & DVLs

1. Scope

To detect subclinical mastitis, monitor udder health, and screen milk for high somatic cell counts in dairy animals.

2. Objective

To identify subclinical mastitis in dairy animals by detecting elevated somatic cell levels in milk for early diagnosis and management.

3. Principle

The test detects subclinical mastitis by reacting a detergent-based reagent with somatic cell DNA in milk, causing gel formation, where the thickness of the gel indicates the level of somatic cells and severity of infection.

4. Equipment and Consumables

4.1 CMT Paddle
4.2 PPE (Gloves, Apron, Mask)
4.3 Tissue Paper
4.4 Marker pens
4.5 CMT reagents
4.6 70% alcohol

5. Procedure

5.1 Procedure for bulk milk sampling and testing

- Wear gloves
- Mark each well of the paddle with the farmer's name for identification purposes.
- Collect approximately 3mls of milk from the farmer's bulk milk- the ideal amount of milk is what remains in the paddle when it is tilted to nearly vertical, or to the line indicated in the CMT paddle if present.
- Add an equal amount of CMT reagent to each well in the paddle or to the line indicated on the paddle.
- Rotate the paddle in a circular motion to thoroughly mix the contents for no more than 10 seconds. The reaction will start to disintegrate after 20 seconds, and so a quick reading of results is required.
- The reaction is visually scored depending on the amount of gel that forms.
- Rinse paddle before next use.
- The details of the sample collection, testing and recording of the results is recorded in Bulk Milk Sample Submission Form.

5.2 Procedure for individual milk sampling and testing

- Wear gloves
- Hold the paddle in the same way each time to identify which wells correspond to each quarter.
- Foremilk each quarter and discard the first few streams of milk.
- Draw 2-3 squirts of milk from each quarter into the corresponding well-to the line on the CMT paddle or what remains in the paddle when it is tilted almost vertically.
- Add an equal amount of CMT reagent or until the line indicated on the CMT paddle.
- Rotate the paddle in a circular motion and read the results of the test quickly- within 10-20 seconds.
- The reaction is visually scored depending on the amount of gel that forms.
- Record the result on the recording form.
- Quarters that test positive to the CMT should have a sterile sample taken for culture.

- Rinse paddle before next use.

Image: Flow chart for CMT

6. Result Interpretation

Table: California Mastitis Test Result Interpretation

Score / Reaction	Gel Formation	Interpretation
Negative (–)	No gel; liquid remains watery	Healthy udder / no mastitis
Trace (T)	Slight thickening; almost watery	Possible early/subclinical mastitis
Weak Positive (+)	Mild gel formation; edges start to thicken	Subclinical mastitis
Distinct Positive (++)	Clear gel formation; viscous	Subclinical mastitis
Strong Positive (+++)	Thick gel; may adhere to paddle	Severe subclinical / clinical mastitis

7. Quality Control

Positive control: Use milk with known high somatic cell count to ensure the reagent produces gel formation.
Negative Control**:** Use milk with low somatic cell count (healthy udder) to confirm no gel forms.

8. Waste Disposal

- Dispose used milk and CMT-contaminated materials in biohazard waste or treat with 1% bleach**.**
- Rinse and disinfect paddles with bleach or 70% ethanol before reuse.
- Dispose gloves, stirrers, and paper towels in contaminated waste container**.**

9. References

- SOP version 2018.01, NCAH, Serbithang, Bhutan
- Mastitis surveillance plan documents 2024, NCAH, Serbithang, Bhutan
- Quinn P.J., Carter M.E., Markey B. and Cater G.R. Clinical Veterinary Microbiology. 2000.
- Rajiv Gandhi College of Veterinary & Animal Sciences. Preventive Veterinary Medicine Manual. India: Puducherry BS EN 12469: 2000.
- Biotechnology - performance criteria for microbiological safety cabinets. London: British Standards Institution (BSI); 2000

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 33
Title: SOP for Breed's direct smear method
Version No: 2026.01, Total Pages:4
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No:2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&ECs

1. Scope

The Breed direct smear method is used for the rapid estimation of bacterial cells in raw milk without the need for culturing. It is primarily applied in dairy microbiology and milk quality control.

2. Objective

- To quickly estimate the total bacterial population in a sample of milk.
- To identify the types of microorganism present (e.g., cocci, rods) to infer sources of contamination.
- To screen raw milk for bacterial quality and categorize it (good, fair, poor, very poor).

3. Principle

The method involves spreading a precise, small volume (0.01ml) of milk over a defined area (1 cm^2) on a microscope slide. The smear is dried, defatted, fixed, and stained (usually with methylene blue). Under a calibrated light microscope (oil immersion), the bacteria are counted in several fields, and the average count is multiplied by a "microscopic factor" to determine the total number of microorganisms per milliliter (1 mL) of the sample.

4. Equipment and Consumables

4.1 Clean microscope slides
4.2 Breed's Pipette- to deliver 0.01 ml
4.3 Bent point needle- to spread milk on the slide
4.4 Drying surface- to dry the smear
4.5 Slide holders
4.6 Staining jars
4.7 Compound microscope with oil immersion objective
4.8 Stage Micrometer- slide ruled in 1 and 0.01 mm
4.9 Ethanol
4.10 Toluene
4.11 Hasting stain
4.12 Xylene

5. Procedure

5.1 Preparation**:** Clean a glass slide thoroughly to make it grease-free. Use a template to mark a 1cm x1cm ($1cm^2$) square.
5.2 Sampling: Using a sterile pipette or calibrated loop, take 0.01 mL of a well-mixed milk sample.
5.3 Smearing: Spread the 0.01 mL of milk uniformly over the marked 1 cm^2 area.
5.4 Drying: Allow the smear to air dry on a level surface at a temperature of 45 to 45 ^0C
5.5 Defatting and Fixing: Immerse the slide in xylene for 1–2 minutes to remove fat, then fix the smear using methanol, which fixes and defats simultaneously.
5.6 Staining: Stain the slide with hasting stain for 3 minutes, rinse gently with water, and let it dry.
5.7 Microscopic Examination: Examine the slide under an oil immersion lens (total magnification 1000x)

Calculation:

$$\text{Count per ml} = \frac{N \times 4 \times 10^4}{\pi r^2}$$

Where N = average number of cells per field r = radius of field in mm.

The diameter of the field can be measured using a micron scale or by using the vernier micrometer stage adjustment of the microscope.

The radius of the oil immersion field is usually about 0.08 mm so that the area of the field will be 0.25 mm^2 and bacteria/ml = N X 4 X 10^4.

Examination of stained films

Count only those somatic cells with an identifiable stained nucleus. For polymorphonucleated cells, count as well any that has two or more discernible nuclear lobes; for other somatic cells, count any that has a nucleus that appears to be essentially intact. If in doubt about a cell, which may in fact be only a fragment, do not count.

No. of Cells	*Fields to be counted*
Less than 0.5	50
0.5 to 1.0	25
1 to 10	10
10 to 30	05

6. Result Interpretation

When used for milk from individual cows, if counts are greater than 500,000 cells/ml are considered significant for mastitis.

It is useful to note the type of cell which is predominant in the sample.

Cells	*Normal Lactation*	*Mastitis*
	Early Later	
Epithelial	+ +	+
Lymphocytes	- -	+
polymorphs	- -	+++
Total cell count/ml	Usually, 300.000 Usually 300,000	>500,000

A high polymorph count is considered especially characteristic of acute Streptococcal and Staphylococcal infections, or acute phases of lasting infections.

7. Quality Control

Quality control ensures accuracy, consistency, and reliability in estimating bacterial load in milk samples using the Breed's method.

8. Waste disposal

- Treat all milk samples as potentially infectious
- Follow biosafety level 2 (BSL-2) practices.
- Ensure segregation of biological, chemical, and sharps waste.

9. References

- Quinn P.J., Carter M.E., Markey B. and Cater G.R. Clinical Veterinary Microbiology. 2000.
- Subcommittee on taxonomy of staphylococci and micrococci - minutes of first meeting. International bulletin of bacteriological nomenclature and taxonomy 1965; 15:107-8.
- Toowoomba Veterinary Laboratory: Biochemcial tests. Laboratory Methods Manual. Issue 4. Queensland; Australia: 2004. P. 1-8
- SOP Version 2013, NCAH, Serbithang, Bhutan
- SOP Version 2028.1, NCAH, Serbithang, Bhutan
- Microbiology Laboratory. London: Public Health Laboratory Service; 1999. p. 147-8

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 34
Title: SOP for Tuberculin test using Purified Protein Derivative (PPD) test
Version No: 2026.01, Total Pages: 4
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, RVH&ECs, SVL & DVLs

1. Scope

The tuberculin test using Purified Protein Derivative (PPD) is the primary, internationally recognized in vivo diagnostic method for identifying *Mycobacterium bovis* infection (bovine tuberculosis) in live animals. It identifies infected animals by measuring delayed-type hypersensitivity at the injection site (caudal fold or cervical), allowing for the identification and removal of infected animals to control and eradicate tuberculosis in cattle, buffalo, bison, and other susceptible species.

2. Objective

To describe the standardized procedure for conducting the Tuberculin Skin Test (TST) in cattle using Purified Protein Derivative (PPD) for the detection of bovine tuberculosis.

3. Principle

The Tuberculin Skin Test is a delayed-type hypersensitivity reaction. Cattle previously sensitized to *Mycobacterium bovis* will develop a localized skin swelling at the site of intradermal injection of bovine PPD, which is measured after 72 hours.

4. Materials and Equipment

4.1. Bovine PPD (approved source, within expiry date)
4.2 Tuberculin syringe (1 mL) with fine needle (26–27 gauge)
4.3 Vernier caliper or skin thickness gauge
4.4 Clippers or scissors
4.5 70% alcohol or suitable antiseptic
4.6 Permanent marker
4.7 Animal identification records
4.8 PPE (gloves, protective clothing)

4.1.1 Storage and Handling of PPD

- Store PPD at 2–8°C (do not freeze)
- Protect from direct sunlight
- Use aseptic technique
- Discard unused PPD as per biohazard guidelines

4.1.2 Animal Selection and Preparation

- Test clinically healthy cattle
- Avoid testing animals that are severely stressed, sick, or within 4 weeks post-calving
- Properly restrain the animal
- Identify and record animal ID, age, sex, breed, and location

4.1.3 Test Site

- **Single Intradermal Test (SIT):** Middle third of the neck (lateral side)
- Clip hair over a circular area (~2–3 cm diameter)
- Clean the site with 70% alcohol and allow to dry

5. Procedure

5.1 Pre-injection Measurement

- Measure skin thickness at the test site using a vernier caliper
- Record the baseline skin thickness (in mm)

5.2 Injection of PPD

- Draw 0.1 mL of bovine PPD into the tuberculin syringe
- Inject intradermally at the prepared site
- A small pea-sized swelling should be visible immediately (confirm correct injection)
- Mark the injection site for easy identification

5.3 Post-injection Observation

- Do not massage the site
- Release the animal and ensure normal management

5.4. Reading of Test Results

- Read the test at 72 ± 4 hours post-injection
- Measure skin thickness again at the same site
- Calculate the increase in skin thickness (post-pre measurement)

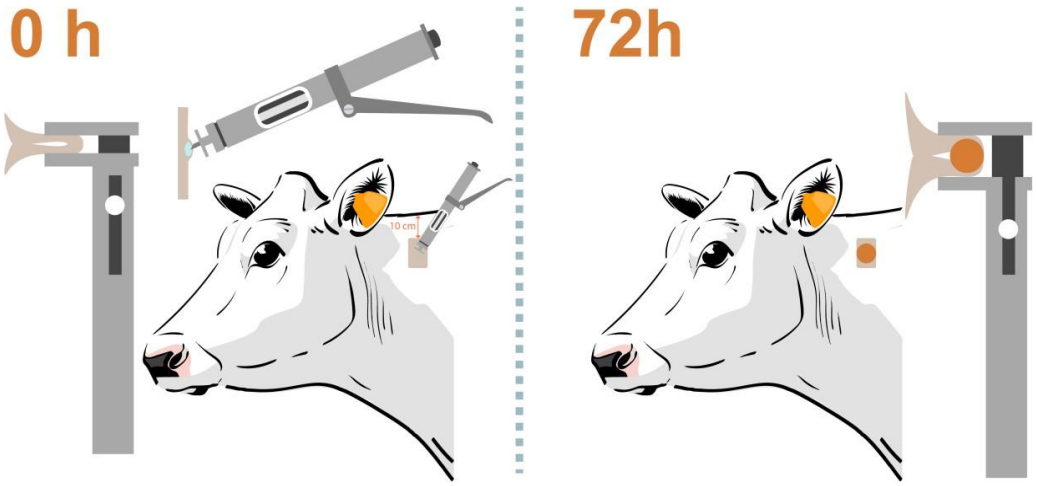


Image: Tuberculin test in Bovine

6. Interpretation of Results (Single Intradermal Test)

Increase in Skin Thickness	Interpretation
<2 mm	Ngative
2-4 mm	Inconclusive/ Suspect
≥ 4 mm	Positive

Note: Interpretation may vary as per national TB control guidelines

6.1. Recording and Reporting

- Record all measurements clearly
- Report positive and inconclusive cases to the competent authority
- Maintain records for surveillance and traceability

7. Quality Control

- Use only validated and non-expired PPD
- Ensure proper intradermal injection technique
- Calibrate measuring instruments regularly
- Conduct periodic refresher training

8. Biosafety and Waste Disposal

- Wear PPE during testing
- Dispose of needles and syringes in sharps containers
- Decontaminate waste as per biosecurity guideline

9. References

- Quinn P.J., Carter M.E., Markey B. and Cater G. R. Clinical Veterinary Microbiology. 2000.
- Rajiv Gandhi College of Veterinary & Animal Sciences. Preventive Veterinary Medicine Manual. India: Puducherry.
- SOP Version 2018.1, NCAH, Serbithang, Bhutan
- Microbiology Laboratory. London: Public Health Laboratory Service; 1999. p. 147-8
- Toowoomba Veterinary Laboratory: Biochemcial tests. Laboratory Methods Manual. Issue 4. Queensland; Australia: 2004. P. 1-8

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 35
Title: SOP for Slide Agglutination test for Salmonella species
Version No: 2026.01, Total Pages:3
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&Ecs

1. Scope

Rapid presumptive identification and serotyping of *Salmonella* cultures isolated from clinical samples and environmental sources.

2. Objective

- To confirm the presence of *Salmonella* species at the genus level using Polyvalent O or H antisera.
- To determine the specific serogroup or serotype of a Salmonella isolate using monovalent antisera.
- To provide rapid preliminary results within 1–2 minutes, allowing for fast screening.

3. Principle

When a suspected *Salmonella* colony (antigen) is mixed with specific *Salmonella* antiserum (antibody) on a slide, the antibodies cross-link the antigens (bacteria) if they are homologous, creating a visible clumping (agglutination). The test targets somatic (O) antigens on the cell wall or flagellar (H) antigens, which are identified using corresponding O or H antisera based on the Kauffmann-White scheme.

A negative control using normal saline is crucial to ensure the reaction is not due to auto-agglutination.

4. Equipments and Consumables

4.1 Light microscope (optional, for confirmation if needed)
4.2 Clean glass slides or agglutination tiles
4.3 Inoculating loop (wire or disposable)
4.4 Micropipette (optional)
4.5 Mixing sticks / applicator sticks
4.6 Timer or stopwatch
4.7 Biosafety cabinet (recommended for pathogen handling)
4.8 Incubator (for culture preparation)
4.9 Specific antisera (e.g., O and H antisera for *Salmonella*)
4.10 Sterile normal saline (0.85% NaCl)
4.11 Pure bacterial culture (18-24 hours old)
4.12 Disposable loops or sterile swabs
4.13 Lens tissue or blotting paper
4.14 Gloves and other PPE (lab coat, mask)
4.15 Disinfectant (e.g., 70% ethanol, 1% sodium hypochlorite)
4.16 Marker pen for labeling slides
4.17 Absorbent paper for cleaning spills

5. Procedure

5.1 Preparation of suspension**:** Place two separate drops of 0.85% sterile normal saline on a clean glass slide.

5.2 Emulsification**:** Using a loop, take a small portion of the suspect colony (grown on non-selective agar) and emulsify it in both saline drops to create a smooth, milky, and uniform suspension.

5.3 Antiserum application**:** Add one drop of specific *Salmonella* antisera (e.g., Polyvalent O or H) to one of the suspensions (Test).

5.4 Control application: Add one drop of normal saline (or nothing) to the other suspension (Control).

5.5 Mixing and observation: Mix the drops using separate tips and gently rock the slide back and forth for 1 minute.

5.6 Reading results: Observe for visible agglutination (clumping) against a dark background with indirect lighting.

6. Result Interpretation

Positive: Visible, distinct agglutination (clumping) within 1–2 minutes, with a clear suspension in the test area and no agglutination in the control.

Negative: No clumping or turbidity remains uniform in both test and control areas.

Invalid/auto-agglutination: Agglutination occurs in the saline control, indicating the strain is rough or self-agglutination.

7. Quality Control

- **Positive and Negative control:** Known smooth strains of Salmonella should be tested with the antiserum (positive control) and with saline (negative control) to verify the activity of the reagents.
- **Auto-agglutination check:** Before adding antiserum, a suspension of the test organism in 0.85% saline is observed. If the organism clumps in saline alone (auto-agglutination), it is considered "rough" and cannot be typed with this method; such tests must be discarded.
- **Reagent testing:** Agglutinating sera should be checked for potency and specificity using stock cultures. If using latex kits, a Reagent Control and Positive Control should be performed with every batch.
- **Media and temperature:** Ensure all media is properly prepared and autoclaved (121°C for 15 min), with reagents brought to room temperature before testing.
- Observation: Agglutination must be observed within 30-60 seconds to 2 minutes. Clear agglutination indicates a positive result.

8. Waste disposal

- **Decontamination:** Non-disposable apparatus and all contaminated materials should be autoclaved at 121°C for at least 15 minutes.
- **Slides and materials:** Used slides, pipettes, and inoculation sticks should be discarded into a biohazard bag or rigid sharps container.
- **Spillage management:** Any spills should be immediately treated with absorbent material and disinfected with 70% alcohol or a standard disinfectant.
- **Reagent disposal:** *Salmonella* antisera may contain preservatives like 0.5% phenol or 0.1% sodium azide, which are toxic. Contact with skin or eyes must be treated with extensive washing.

9. References

- Blaser MJ, Lofgren JP. Fatal salmonellosis originating in a clinical microbiology laboratory. J Clin Microbiol 1981;13:855-8.
- Quinn P.J., Carter M.E., Markey B. and Cater G.R. Clinical Veterinary Microbiology. 2000.
- Forbes BA, Sahm D F, Weissfeld A S, editors. Bailey and Scott's Diagnostic Microbiology. 11th ed. St. Louis: Mosby Inc; 2002. p. 206-7
- SOP Version 2013, NCAH, Serbithang, Bhutan
- SOP Version 2018., NCAH, Serbithang, Bhutan
- Microbiology Laboratory. London: Public Health Laboratory Service; 1999. p. 147-8

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 36
Title: SOP for Slide Agglutination test for Staphylococcus aureus
Version No: 2026.01, Total Pages:3
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&Ecs

1. Scope

To rapidly identify *Staphylococcus aureus* by detecting bound coagulase and differentiate it from coagulase-negative Staphylococci in clinical samples.

2. Objective

To rapidly detect bound coagulase (clumping factor) for identification of *Staphylococcus aureus*.

3. Principle

The slide coagulase test is based on the ability of *Staphylococcus aureus* to produce bound coagulase (clumping factor**)** on its cell surface. This factor reacts with fibrinogen present in plasma,

converting it into fibrin and causing visible clumping (agglutination) of bacterial cells on the slide. The presence of clumping indicates a positive result, confirming coagulase-positive Staphylococci.

4. Equipment and Consumables

- Biological safety cabinet
- Clean grease-free glass slides
- Normal saline
- Inoculating loop
- Bunsen burner
- PPE (Gloves, Apron, Mask)
- Tissue Paper
- Toothpick
- Marker pens
- Bacterial culture (*Staphylococcus* isolate)
- Distilled water
- Rabbit plasma (anti-coagulated)
- 70% alcohol

5. Procedure

5.1 Use a grease pencil to divide the slide into two areas, labeling them **"Test"** and **"Control."**
5.2 Place a small drop of distilled water on each area.
5.3 Pick 1–2 colonies of *Staphylococcus* from a blood agar plate and mix into each drop to make a smooth suspension.
5.4 To the Test drop**,** add a drop of rabbit plasma and mix gently with a toothpick.
5.6 Leave the Control drop without plasma to detect auto-agglutination.
5.7 Clumping within 5-10 seconds in the Test area indicates a positive reaction.

6. Result Interpretation

Result	Observation	Interpretation
Positive	Immediate clumping	*Staphylococcus aureus*
Negative	No clumping	Coagulase-negative Staphylococci

7. Quality Control

- **Positive control:** *Staphylococcus aureus* → clumping
- **Negative control:** *Staphylococcus epidermidis* → no clumping

8. Waste Disposal

- Used slides and applicators**:** Place in autoclave bags or disinfect with 1% sodium hypochlorite before disposal.
- Used plasma**:** Treat as bio-hazardous material; autoclave or disinfect before discarding.
- Gloves and Tissue paper**:** Dispose in contaminated/biohazard waste container.
- Work surfaces**:** Clean with 1% bleach or 70% ethanol after testing.

9. References

- SOP version 2018.01, NCAH, Serbithang, Bhutan
- Mastitis surveillance plan documents 2024
- Quinn P.J., Carter M.E., Markey B. and Cater G.R. Clinical Veterinary Microbiology. 2000.
- Rajiv Gandhi College of Veterinary & Animal Sciences. Preventive Veterinary Medicine Manual. India: Puducherry BS EN 12469: 2000.
- Biotechnology - performance criteria for microbiological safety cabinets. London: British Standards Institution (BSI); 2000
- MacFaddin JF, editor. Biochemical Tests for Identification of Medical Bacteria. 3rd ed. Philadelphia: Lippincott Williams & Wilkins; 2000. p. 105-19
- Public Health Laboratory Service Standing Advisory Committee on Laboratory Safety. Safety Precautions: Notes for Guidance. 4th ed. London: Public Health Laboratory Service (PHLS); 1993.
- Quinn P.J., Carter M.E., Markey B. and Cater G.R. Clinical Veterinary Microbiology. 2000.
- Snell JJS, Brown D F J, Roberts C, editors. Quality Assurance Principles and Practice in the Microbiology Laboratory. London: Public Health Laboratory Service; 1999. p. 147-8

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 37
Title: SOP for Tube Coagulase Test for Staphylococcus aureus
Version No: 2026.01, Total Pages:3
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&Ecs

1. Scope

To confirm the presence of *Staphylococcus aureus* by detecting free coagulase, differentiate coagulase-positive from coagulase-negative Staphylococci, and support the diagnosis of Staphylococcal infections in clinical samples.

2. Objective

The objective of the tube coagulase test is to detect the presence of free coagulase enzyme secreted by *Staphylococcus aureus,* which reacts with plasma to form a clot. This test serves to confirm the identity of coagulase-positive Staphylococci, differentiate them from coagulase-negative Staphylococcus species, and support the accurate diagnosis of staphylococcal infections in clinical specimens such as blood, pus, or other body fluids

3. Principle

The tube coagulase test is based on the ability of *Staphylococcus aureus* to produce free coagulase, an extracellular enzyme. Free coagulase reacts with coagulase-reacting factor (CRF) in plasma, converting fibrinogen to fibrin, which leads to clot formation in the test tube. The formation of a clot indicates a positive result, confirming the presence of coagulase-positive *S. aureus*.

4. Equipment and Consumables

- Biological safety cabinet
- Clean grease-free test tube
- Normal saline
- Inoculating loop
- Bunsen burner
- PPE (Gloves, Apron, Mask)
- Tissue Paper
- Marker pens
- Bacterial culture (*Staphylococcus* isolate)
- Distilled water
- Tryptic Soya Broth
- Rabbit plasma (anticoagulated)
- 70% alcohol

5. Procedure

5.1 Label three test tubes as “test”, “negative control” and “positive control”.
5.2 Fill each test tube with 1 ml of 1:6 dilution of rabbit plasma in normal saline.
5.3 Add 0.1 ml of overnight broth culture to the tube labelled as test.
5.4 Add 0.1 ml of overnight broth culture of known *S. aureus* to the tube labelled positive control and 0.1 ml of sterile broth to the tube labelled negative control.
5.5 Incubate all the tubes at 35-37oC for 4 hours.
5.6 Examine at 1, 2 and 4 hour for clot formation by tilting the tube through 90 degree.
5.7 Leave negative tubes at room temperature overnight and re-examine. (This step is essential, for some strains of *S. aureus*, including many MRSA, produce a delayed clot which is rapidly lysed at 37°C by the organism’s staphylokinase).

6. Result Interpretation

Result	Observation	Interpretation
Positive	Formation of a clot or gel in the plasma tube within 1-4 hours (may be up to 24 hours for some strains)	*Staphylococcus aureus*
Negative	Plasma remains liquid with no clot formation after 24 hours	coagulase-negative Staphylococci or other bacteria

7. Quality Control

- **Positive control:** *Staphylococcus aureus* → clumping
- **Negative control:** *Staphylococcus epidermidis* → no clumping

8. Waste Disposal

- Used tubes and plasma treat as biohazardous material, autoclave or disinfect with 1% sodium hypochlorite before disposal.
- Gloves and Tissue paper**:** Dispose in contaminated/biohazard waste container.
- Work surfaces**:** Clean with 1% bleach or 70% ethanol after testing.
- Wash hands thoroughly after handling.

9. References

- SOP version 2018.01, NCAH, Serbithang, Bhutan
- Mastitis surveillance plan documents 2024, NCAH, Serbithang, Bhutan
- Quinn P.J., Carter M.E., Markey B. and Cater G.R. Clinical Veterinary Microbiology. 2000.
- Rajiv Gandhi College of Veterinary & Animal Sciences. Preventive Veterinary Medicine Manual. India: Puducherry BS EN 12469: 2000.
- Biotechnology - performance criteria for microbiological safety cabinets. London: British Standards Institution (BSI); 2000
- MacFaddin JF, editor. Biochemical Tests for Identification of Medical Bacteria. 3rd ed. Philadelphia: Lippincott Williams & Wilkins; 2000. p. 105-19
- Public Health Laboratory Service Standing Advisory Committee on Laboratory Safety. Safety Precautions: Notes for Guidance. 4th ed. London: Public Health Laboratory Service (PHLS); 1993.
- Quinn P.J., Carter M.E., Markey B. and Cater G.R. Clinical Veterinary Microbiology. 2000.
- Snell JJS, Brown D F J, Roberts C, editors. Quality Assurance Principles and Practice in the Microbiology Laboratory. London: Public Health Laboratory Service; 1999. p. 147-8
- https://microbeonline.com/diagnostic-tests-biochemical-tests-coagulase-test/ accessed on 9th September 2018.

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 38
Title: SOP for Antimicrobial Sensitivity Test by CLSI
Version No: 2026.01, Total Pages:6
Issue Month/Effective Date: May 2026
Revision Summary: 2022.01 Revised and updated
Supersedes Version No: 2022.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&ECs

1. Scope

This SOP outlines the procedures for performing the CLSI agar disk diffusion test to determine the invitro antimicrobial susceptibility of aerobic bacteria, and includes:

- Testing conditions, such as inoculums preparation and standardization, incubation time, and temperature.
- Interpretation of results based on inhibition zones.
- Quality control (QC) procedures to ensure accuracy.
- Selection of antibiotic panels for small animals, ruminants, poultry, pigs, and horses.

2. Objective

- To determine the sensitivity or resistance of pathogenic aerobic and facultative anaerobic bacteria to various antibiotics.
- To assist veterinarians in selecting the most effective treatment for animal infections.
- To test bacteria on Mueller-Hinton agar using antibiotic-impregnated disks and assess zones of inhibition**.**
- To standardize antibiotic panels across veterinary laboratories for routine testing of bacterial isolates from different animal species.

3. Principle

A standardized inoculum of the organism is swabbed onto the surface of a Mueller-Hinton agar plate. Antimicrobial impregnated filter paper disks of standardized concentration are placed on the agar. The presence or absence of growth around the disks is observed and measured using a scale in millimeters after 24 hours of incubation. The diameter of the zone of inhibition is interpreted using latest CLSI guideline to obtain report as susceptible, intermediate or resistant.

4. Equipment and Consumables

4.1 Biological safety cabinet
4.2 Antibiotic disc dispenser or Forceps
4.3 Ruler to measure inhibition zones
4.4 Vortex mixer
4.5 Incubator
4.6 Turbidity meter for McFarland's turbidity determination.
4.7 Sterile petri plates
4.8 Sterile cotton swab
4.9 Inoculating loop
4.10 Bunsen burner
4.11 PPE (Gloves, Apron, Mask)
4.12 Tissue Paper
4.13 Marker pens
4.14 Fresh Bacterial culture
4.15 Distilled water
4.16 Tryptic Soya Broth
4.17 Mueller-Hinton Agar
4.18 Normal saline
4.19 Antibiotic discs
4.20 70% alcohol

5. Procedure

5.1. Preparation of Inoculum

- Using sterile loop or swab, transfer 1 or 2 isolated colonies of similar colony morphology grown overnight from non-selective medium to 3 ml sterile normal saline aliquot.

- Vortex for 15-20 seconds to mix well and adjust the turbidity to 0.5 McFarland turbidity standards, which is equal to 1-2 x 108 CFU/ml for *E.coli* ATCC 25922. Optimally use the inoculum within 15 minutes.
- Dip a sterile cotton swab into the inoculum and rotate it against the wall of the tube above the liquid to remove excess inoculum.
- Swab entire surface of agar plate three times, rotating plate approximately 60 degrees between streaking to ensure even distribution. Avoid hitting sides of petri plate and creating aerosols.
- Allow inoculated plate to stand for at least 3 minutes but no longer than 15 minutes before applying disks.

5.2. Application of Antimicrobial disks to agar plate

- After removing unopened containers of disks from freezer or refrigerator, allow them to equilibrate to room temperature (requires at least 1 hr.) prior to opening to minimize condensation. Do not use disks beyond expiry date.
- Apply disks to agar surface by using antibiotic disk dispenser or sterile forceps.
- Apply gentle pressure with sterile forceps to ensure complete contact of disk with agar.
- Do not place disks closer to each other than 24mm from center to center.
- Place no more than 9 disks on a 150-mm plate or place no more than 5 disks on a 90-mm plate.
- Do not relocate a disk once it has made contact with agar surface, because antimicrobial diffusion begins instantly.

5.3. Incubation

- Incubate plates within 15 minutes of disk application.
- Invert plates and stack them no more than 5 high.
- Incubate for 16 to 18 hours at 35±2˚C in an ambient air incubator.
- In case of *Campylobacter spp*. incubate in an anaerobic condition.

6. Result Interpretation

6.1 Reading plates

- Each plate should be examined after incubating for 16 to 18 hours, for confluent lawn of growth and circular zones of inhibition.
- The measuring device should be held on the back of the inverted petri dish, which is illuminated with reflected light located a few inches above a black, nonreflecting background.
- The diameters of the zones of complete inhibition, including the diameter of the disk, should be measured to the nearest whole millimeter with Vernier calipers or a ruler.
- If blood was added to the agar base (as with streptococci), the zones must be measured from the upper surface of the agar illuminated with reflected light and with the cover removed.
- For coagulase-negative Staphylococcus spp. with cefoxitin, 24 hours of incubation are needed before reporting as susceptible. Other agents should be read and reported at 16 to 18 hours. With cefoxitin, the zone of diameter needs to be read with plate held up to the light.
- When blood supplemented medium for testing streptococci is used, the zones of growth inhibition, not the zone of inhibition of hemolysis, should be measured.

6.2 Reference Range

- Use CLSI standard (Latest version) guidelines for interpretation, by referring to M100 tables 2A through 2I.

6.3 Reporting Format

- Report results as susceptible, intermediate, or resistant along with zone diameter according to laboratory practice and format.
- Report any multidrug resistant (MDR) isolate to National veterinary reference laboratory (NVL) for further confirmation. (MDR- if the isolate is resistant to three or more antibiotic class)
- Perform ESBL testing if the isolates are resistant to 3rd generation cephalosporin (Ceftriaxone)

7. Quality Control

7.1 QC Strains

- Reference strains used are recommended in CLSI Standard (Latest version).
- ❖ *Escherichia coli* ATCC 25922
- ❖ *Staphylococcus aureus ATCC 25923*
- Maintenance of QC strains.
- Maintain permanent stock cultures at –20˚C or –70˚C in tryptic soy broth with 15-20% glycerol.
- Maintain working stock cultures for up to 1 week. Subculture each week for no more than 3 successive weeks.

7.2 Frequency of QC Testing

- Perform QC daily until acceptable results from 20 (or 30) consecutive days of testing have been obtained.
- Proficiency in performing QC tests is confirmed if for each drug, no more than 1 out of 20 or 3 of 30 results are outside the accuracy limits.
- After validation of proficiency, frequency of QC testing can be reduced from daily to weekly.
- Results are reviewed for acceptability and recorded on the Quality Control log sheets before reporting results

7.3 Antibiotic Panels

Antibiotic panels are specific for animal species and selected based on antibiotic available for prescription.

Table: **Antibiotic panel for small animal**

SL.No	Gram positive panel	Gram negative panel
1	Cotrimoxazole (trimethoprim/sulfamethoxazole)	Ampicillin
2	Enrofloxacin (Ciprofloxacin)	Gentamicin
3	Tetracycline	Ciprofloxacin
4	Ceftriaxone	Cotrimoxazole
5	Penicillin-G	Tetracycline

6	Cloxacillin (Oxacillin)	Amoxicillin/Clavulanate
7	Amoxicillin/Clavulanate	Ceftriaxone

Table: **Antibiotic panel for ruminants**

Sl.no	Gram positive panel	Gram negative panel	Pasteurellaceae
1	Tetracycline	Ampicillin	Ampicillin
2	Ceftriaxone	Tetracycline	Tetracycline
3	Penicillin-g	Ceftriaxone	Ceftriaxone
4	Cloxacillin(oxacillin)	Sulfadimidine	Sulfadimidine
5	Sulfadimidine	Trimethoprim	Trimethoprim
6	Trimethoprim		Penicillin G

Table: **Antibiotic panel for poultry**

Sl.no	Gram positive panel	Gram negative panel
1	Tetracycline	Ampicillin
2	Ceftriaxone	Tetracycline
3	Ampicillin (Amoxycillin)	Ceftriaxone
4	Cotrimoxazole	Cotrimoxazole
5	Tylosin	Neomycin

Table: **Antibiotic panel for pigs**

Sl.no	Gram positive panel	Gram negative panel	Pasteurellaceae
1	Tetracycline	Ampicillin	Ampicillin
2	Ceftriaxone	Tetracycline	Tetracycline
3	Penicillin-G	Ceftriaxone	Ceftriaxone
4	Cloxacillin (Oxacillin)	Cotrimoxazole	Cotrimoxazole
5	Cotrimoxazole	Neomycin	Penicillin_G
6		Tylosin	

Table: **Antibiotic panel for horses**

Sl.no	Gram positive panel	Gram negative panel
1	Tetracycline	Tetracycline
2	Ceftriaxone	Ceftriaxone
3	Penicillin-G	Cotrimoxazole
4	Enrofloxacin (ciprofloxacin)	Gentamin
5	Cotrimoxazole	Enrofloxacin(ciprofloxacin)
6	Cephalexin	

8. Waste Disposal

- Used agar plates**:** Autoclave at 121°C for 15–30 minutes before disposal in biohazard waste

- Contaminated disks and consumables**:** Collect in biohazard bags and autoclave or disinfect with 1% sodium hypochlorite before disposal.
- Gloves, pipette tips, and paper towels**:** Dispose in contaminated/biohazard waste containers**.**
- Work surfaces and equipment: Clean with 1% bleach or 70% ethanol after testing.
- Sharps (if used**):** Dispose in puncture-proof sharps containers**.**

9. References

- SOP version 2022.01, NCAH, Serbithang, Bhutan.
- Harmonized Test Protocol for Isolation, Identification and ABST profiling of Salmonella in Human, Animal and Food products in Bhutan through One Health approach-AGISAR, WHO, Bhutan.
- CLSI Guidelines-M100S-Performance Standards for Antimicrobial Susceptibility Testing, 26th Edition.
- CLSI, M02 Performace standards for Antimicrobial Disk Susceptibility tests 13th edition, January 2018
- Standard Operating Procedures, Bacteriology, Antimicrobial Resistance Surveillance and Research Network, Indian Council of Medical Research, 2nd Edition

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 39
Title: SOP for Plate Count Test by pour plate technique
Version No: 2026.01, Total Pages:3
Issue Month/Effective Date: May 2026
Revision Summary: 2018.01 Revised and updated
Supersedes Version No: 2018.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&ECs

1. Scope

Used for enumeration of viable aerobic bacteria in samples, commonly applied in food, water, milk, and clinical samples and helps assess microbial load and hygienic quality.

2. Objective

To determine the total viable bacterial count (CFU/ml or CFU/g) in a sample and to estimate the level of contamination and monitor quality control.

3. Principle

A defined volume of test portion or series of decimal dilutions of the sample is mixed with the culture medium in petri dishes and incubated at 32℃ for 72 hours. The colonies are counted and the number of viable micro-organisms per milliliter or gram of sample is calculated.

4. Equipment and Consumables

4.1 Incubator at 30 ± 2℃
4.2 Water-bath at 56℃
4.3 Colony counting equipment (optional)
4.4 Petri dishes(sterile)-15mm x 90 mm diameter
4.5 Automatic pipette and associated pipette tips or total delivery pipettes, of 1 mL or 0.1 mL nominal capacity
4.6 Biosafety cabinet
4.7 Rubber bulb
4.8 Wide mouth bottle (250mL)
4.9 Test tube with screw cap (15mL)
4.10 Autoclave
4.11 Waste container
4.12 Plate count agar
4.13 Peptone saline diluent (Maximum recovery diluent)
4.14 Distilled water

5. Procedure

5.1 Sample Dilution: Perform serial dilutions (e.g., 10-1 to10-6) of the sample in sterile water or buffered saline to achieve a target of 30-300 colonies per plate.
5.2 Inoculation: Using a sterile pipette, transfer 1.0 mL (or 0.1 mL) of the desired dilution into the center of a sterile empty, labeled petri dish.
5.3 Pouring Agar: Pour ~15-20 mL of molten PCA (at 45-50°C) into the Petri dish containing the inoculum.
5.4 Mixing: Immediately and gently swirl the dish in a figure-eight or "S" motion to ensure thorough and uniform mixing of the sample and agar.
5.5 Solidification: Allow the plates to solidify on a level surface (approx. 10 minutes
5.6 Incubation: Invert the plates to prevent condensation dripping and incubate, commonly at 32°C for 72 hours.
5.7 Counting: After incubation, count all colonies (surface and embedded) in plates with 30-300 colonies using a colony counter.

6. Result Interpretation

Calculation of Results
Use counts from all plates containing up to 300 colonies. The number (N) of viable microorganisms per mL or g of sample is given by the formula:

$$N = \frac{\Sigma C}{(n1 + 0.1n2)\, d}$$

ΣC= Sum of the colonies counted

n1 = Number of plates counted at the first dilution
n2 = Number of plates counted at the second dilution
d = Dilution from which the first counts were obtained
Count the colonies in both plates. The number (N) of viable micro-organisms per milliliter or gram is given by the formula

$$N = \frac{\Sigma C \times 10}{2}$$

7. Quality Control

- Check appearance, color, and sterility of Plate Count Agar before use.
- Perform sterility test by incubating an uninoculated plate (no growth should occur).
- Verify performance using control organisms such as: *Escherichia coli & Staphylococcus aureus.*
- Ensure proper pH (≈7.0 ± 0.2).

8. Waste disposal

- All used plates, pipettes, and contaminated materials are treated as biohazard waste.
- Place in autoclavable biohazard bags.
- Autoclave at 121°C for 15-30 minutes before disposal.
- Alternatively, soak in disinfectant (e.g., 1% sodium hypochlorite) before discarding.

9. References

- Quinn P.J., Carter M.E., Markey B. and Cater G.R. Clinical Veterinary Microbiology. 2000.
- Subcommittee on taxonomy of staphylococci and micrococci - minutes of first meeting. International bulletin of bacteriological nomenclature and taxonomy 1965; 15:107-8.
- Toowoomba Veterinary Laboratory: Biochemcial tests. Laboratory Methods Manual. Issue 4. Queensland; Australia: 2004. P. 1-8
- SOP Version 2013, NCAH, Serbithang, Bhutan.
- SOP Version 2018.1, NCAH, Serbithang, Bhutan.
- Microbiology Laboratory. London: Public Health Laboratory Service; 1999. p. 147-8.

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 40
Title: SOP for Bacterial glycerol stock preparation and storage
Version No: 2026.01, Total Pages:4
Issue Month/Effective Date: May 2026
Revision Summary: 2022.01 Revised and updated
Supersedes Version No: 2022.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&ECs

1. Scope

This procedure applies to the long-term preservation of bacterial cultures in laboratories. It ensures that bacterial strains can be stored safely for extended periods without significant loss of viability or genetic characteristics.

2. Objective

- To prepare a stable bacterial stock using glycerol as a cryoprotectant.
- To store bacterial strains at low temperatures (typically −80°C) for long-term use.
- To maintain the integrity, viability, and genetic stability of the bacterial cultures.

3. Principle

Glycerol acts as a cryo-protectant, preventing the formation of ice crystals that can damage bacterial cells during freezing. Mixing bacterial cultures with sterile glycerol and storing them at ultra-low temperatures slows down metabolic activity and preserves the cells in a dormant state, allowing recovery of viable bacteria even after months or years.

4. Equipment and Consumables

4.1 Biological safety cabinet
4.2 Vortex mixer
4.3 Sterile cotton swab
4.4 Inoculating loop
4.5 Pipettes
4.6 Pipettes tips
4.7 Conical flask
4.8 Measuring cylinder
4.9 Bunsen burner
4.10 PPE (Gloves, Apron, Mask)
4.11 Cryo-labelling sticker
4.12 Tissue Paper
4.13 Marker pens
4.14 Fresh Bacterial culture
4.15 Distilled water
4.16 Normal saline
4.17 40% glycerol stock
4.18 LB Broth
4.19 Nutrient Agar
4.20 70% alcohol

5. Procedure

5.1 Preparation of 40% bacterial glycerol stock using LB broth:

- Obtain pure bacterial isolate from Nutrient agar plate or Sheep blood agar.
- Inoculate the pure colony in 5ml LB broth and to obtain bacteria at exponential phase for storage.

5.2 Glycerol stock preparation:

- Use sterile pipette tips and sterile micro centrifuge tubes/cryogenic tubes (1.5-2ml).
- From the 5 ml cultures prepared above add 0.50 ml/500ul of culture to a sterile 1.5 ml micro centrifuge tube. To this add 0.50 ml/500ul of sterile glycerol solution (40% of glycerol stock). Prepare in multiple/ duplicates.
- Mix the solution by inversion and quickly place into the -80°C freezer.
- Freeze in the -80°C only. The cells will die if left at -20°C.
- Label the tube with appropriate identity/bio repository number and information.
- Revive by scraping off splinters of solid ice with a sterile loop or pipette tip (be careful not to allow contents to thaw).

Note:

1. *Snap top tubes are not recommended as they can open unexpectedly at - 80°C.*
2. *Frequent freeze thaw reduces shelf life of bacteria; hence it is recommended to prepare the stock in multiples.*

5.3 Preparation of 20% bacterial glycerol stock using Nutrient Agar:

Produce a single pure colony after identification of bacteria in Nutrient agar plate after 24 hours of incubation at 37°C to obtain bacteria at exponential phase for storage.

Glycerol stock preparation:

- Use sterile pipette tips and sterile micro centrifuge tubes/cryogenic tubes (1.5-2ml).
- Pick few single colonies from the NA plate prepared above and mix it to 500ul of autoclaved distil water/appropriate broth (BHI broth/ LB broth) to obtain clear suspension of the culture in a 1.5ml/2ml cryogenic tube.
- Add 500ul of sterile 40% glycerol stock solution to the above suspension, to make a final concentration of 20 % bacterial glycerol stock.
- Prepare the bacterial glycerol stocks in multiples.
- Mix the solution by inversion and quickly place into the -80°C freezer.
- Freeze in the -80°C only. The cells will die if left at -20°C.
- Label the tube with appropriate identity and information
- Revive by scraping off splinters of solid ice with a sterile loop or pipette tip (be careful not to allow contents to thaw).

Note:

1. *Snap top tubes are not recommended as they can open unexpectedly at - 80°C.*
2. *Frequent freeze thaw reduces shelf life of bacteria; hence it is recommended to prepare the stock in multiples.*

6. Quality Control

6.1 **Viability Check**

- After freezing, periodically thaw a test vial and streak onto appropriate agar.
- Confirm growth to ensure bacteria remain viable.

6.2 **Purity Check**

- Examine recovered cultures for contamination by observing colony morphology.
- Optional: Perform Gram staining or relevant biochemical tests to confirm identity.

6.3 **Storage Conditions**

- Verify −80°C freezer temperature regularly.
- Avoid repeated freeze-thaw cycles to maintain bacterial integrity.

6.4 **Maintenance of QC strains**

- Maintain permanent stock cultures at –20˚C or –80˚C in tryptic soy broth with 20% glycerol.
- Maintain working stock cultures for up to 1 week. Subculture each week for no more than 3 successive weeks.

6.5 **Frequency of QC Testing**

- Perform QC daily until acceptable results from 20 (or 30) consecutive days of testing have been obtained.
- Proficiency in performing QC tests is confirmed if for each drug, no more than 1 out of 20 or 3 of 30 results are outside the accuracy limits.
- After validation of proficiency, frequency of QC testing can be reduced from daily to weekly.
- Results are reviewed for acceptability and recorded on the Quality Control log sheets before reporting results.

7. Waste Disposal

- Dispose of all used pipette tips, micro-centrifuge tubes, and cryovials in a biohazard sharps/container.
- Any leftover bacterial culture or glycerol mixture should be autoclaved at 121°C for 15-20 minutes before disposal.
- Contaminated gloves, paper towels, and other disposables should be placed in biohazard bags and autoclaved or incinerated according to institutional guidelines.
- Decontaminate any spills with 10% bleach or appropriate disinfectant and follow standard laboratory spill procedures.

8. References

- SOP version 2022.01, NCAH, Serbithang, Bhutan
- Harmonized Test Protocol for Isolation, Identification and ABST profiling of Salmonella in Human, Animal and Food products in Bhutan through One Health approach-AGISAR, WHO, Bhutan.
- CLSI Guidelines-M100S-Performance Standards for Antimicrobial Susceptibility Testing, 26th Edition.
- CLSI, M02 Performance standards for Antimicrobial Disk Susceptibility tests 13th edition, January 2018
- Standard Operating Procedures, Bacteriology, Antimicrobial Resistance Surveillance and Research Network, Indian Council of Medical Research, 2nd Edition
- Quinn P.J., Carter M.E., Markey B. and Cater G.R. Clinical Veterinary Microbiology. 2000. Bacteria Glycerol Stock Protocol, Provost & Wallert Research, University of San Diego.

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 41
Title: SOP for Extended-Spectrum-Beta-Lactamase (ESBL) test
Version No: 2026.01, Total Pages:4
Issue Month/Effective Date: May 2026
Revision Summary: 2022.01 Revised and updated
Supersedes Version No: 2022.01
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&ECs

1. Scope

ESBL test involves the identification and confirmation of ESBL-producing Enterobacterales (most commonly *E. coli, Salmonella* and *Klebsiella pneumoniae*) in clinical specimens (urine, blood, wound and rectal swabs) and environmental samples.

2. Objective

An ESBL test is to rapidly and accurately detect extended-spectrum beta-lactamase (ESBL) producing bacteria, which are a major concern due to their resistance to common antibiotics and the threat they pose to successful treatment of infections.

3. Principle

The detection of ESBL is based on the principle that these enzymes are inhibited by -lactamase inhibitors, such as clavulanic acid.

Synergy Method: The test demonstrates enhanced activity of an indicator cephalosporin (e.g., cefotaxime or ceftazidime) when combined with a -lactamase inhibitor (clavulanic acid) compared to the cephalosporin alone.

Phenotypic confirmation: The enzyme inactivates cephalosporins; however, if the inhibitor (clavulanic acid) blocks the enzyme, the *E. coli* becomes susceptible to the cephalosporin, resulting in a larger zone of inhibition in disc diffusion tests.

4. Equipment and Consumables

4.1 Mac conkey agar with CTX
4.2 Overnight culture of bacterial isolate on Sheep blood agar or Nutrient agar
4.3 Mueller Hinton Agar
4.4 Normal saline of 3ml
4.5 Nephalometer/densitometer
4.6 0.5Mc Farland standard
4.7 Sterile cotton swab
4.8 Sterile forceps
4.9 35°C to 37°C ambient- air incubator
4.10 Biohazard bag
4.11 Sterile pipettes
4.12 Inoculating loop
4.13 Biological safety cabinet
4.14 Bunsen burner
4.15 Marker pens
4.16 Antimicrobial agents
4.17 Disk diffusion

- Cefotaxime 30 µg
- Cefotaxime-clavulanate 30/10 µg
- Ceftazidime- 30 µg
- Ceftazidime-clavulanate-30/10 µg

4.18 Quality/Control strains- ESBL test E. coli 25922 should be used for routine QC (e.g., weekly or daily) and disk diffusion <2 mm increase in zone diameter for antimicrobial agent tested in combination with clavulanate vs the zone of diameter when tested alone.

5. Procedure

5.1 Inoculate the specimen onto MacConkey agar supplemented with cefotaxime (CTX) and incubate at 37)C for 18- 24 hrs.
5.2 Examine MacConkey agar with cefotaxime (CTX) for lactose-fermenting colonies, pick well-isolated colonies, and subculture onto Sheep Blood Agar; incubate at 37°C for 18–24 hours to obtain pure isolates for further testing.

5.3 Make a direct broth suspension of isolated colonies selected from an 18 to 24hrs sheep blood agar plate.
5.4 Adjust the turbidity of the culture with normal saline to achieve a turbidity equivalent to a 0.5 McFarland standard. Use the inoculum within 15 minutes.
5.5 Dip a sterile cotton swab into .5Mcfarlend adjusted suspensions and rotate the swab several times and press firmly on the inside wall of the tube above the fluid level.
5.6 Inoculate the dried surface of an MHA plate by streaking the swab over the entire sterile agar surface.
5.7 Repeat this procedure by streaking 2 more times, rotating the plate approximately 600 each time to ensure an even distribution of inoculum.
5.8 Place antibiotic disk (Cefotaxime 30 µg, Cefotaxime-clavulanate 30/10 µg, Ceftazidime- 30 µg and Ceftazidime-clavulanate-30/10 µg) on the surface of the inoculated plate.
5.9 Press each disk down to ensure complete contact with the agar surface.
5.10 Invert the plate and place them in an incubator set to 350C to 370C within 15 mins after the disks are applied for 16-18hrs.
5.11 Measure the zone diameter (mm) of the antibiotic disk.

6. Result Interpretation

Disk diffusion: >5 mm increase in a zone diameter for either antimicrobial agent tested in combination with clavulanate vs the zone diameter of the agent when tested alone = ESBL (e.g., Ceftazidime zone=16mm; Ceftrazidime-clavulanate zone=22mm).

7. Quality Control

- Reference Strains: Use standardized control strains such as E. coli ATCC 25922 (negative control/susceptible) and confirmed ESBL-producing strains (positive control) to validate testing materials.
- Detection Methods: Confirmatory tests, such as the phenotypic combination disc diffusion test, are used according to Clinical and Laboratory Standards Institute (CLSI) guidelines.
- Indicators: A reduction of 5 mm in the zone of inhibition for antibiotic-clavulanic acid disks compared to the antibiotic disk alone confirms ESBL production.

8. Waste disposal

- Sterilization: All biohazardous waste, including culture plates, contaminated consumables, and samples, must be autoclaved (1210Cfor 15-30 minutes) before disposal.
- Liquid Waste: Liquid waste should be treated with chemical disinfectants, such as 10% sodium hypochlorite (bleach), for a minimum of 30 minutes before disposal.

9. References

- Quinn P.J., Carter M.E., Markey B. and Cater G.R. Clinical Veterinary Microbiology. 2000.
- Subcommittee on taxonomy of staphylococci and micrococci - minutes of first meeting. International bulletin of bacteriological nomenclature and taxonomy 1965; 15:107-8.
- Toowoomba Veterinary Laboratory: Biochemcial tests. Laboratory Methods Manual. Issue 4. Queensland; Australia: 2004. P. 1-8
- SOP Version 2013, NCAH, Serbithang, Bhutan.

- SOP Version 2018.01, Serbithang, Bhutan.
- Microbiology Laboratory. London: Public Health Laboratory Service; 1999. p. 147-8
- CLSI. Performance Standards for antimicrobial Susceptibility Testing, 32 nd edition CLSI supplement M100 clinical and Laboratory Standard Institute:2022.
- CLSI performance Standards for Antimicrobial Susceptibility Testing. 13th.edition CLSI supplement M02 Clinical and Laboratory Standards Institute; 2018.

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 42
Title: SOP for CAMP Test
Version No: 2026.01, Total Pages:5
Issue Month/Effective Date: May 2026
Revision Summary: NA
Supersedes Version No: NA
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&Ecs

1. Scope

The CAMP test (named after Christie–Atkins–Munch-Petersen) is a specialized biochemical test used in microbiology laboratories to identify specific pathogenic bacteria based on their production of a diffusible extracellular protein known as the CAMP factor.

2. Objective

- To detect the ability of an unknown organism (usually beta-hemolytic streptococcus) to produce CAMP factor.
- To differentiate *Streptococcus agalactiae* (CAMP-positive) from other beta-hemolytic streptococci (CAMP-negative), such as Group A Streptococci (e.g., *S. pyogenes*)

3. Principle

The test is based on a **synergistic phenomenon**.

- *Staphylococcus aureus* produces a diffusible hemolysin known as **beta-lysin** (sphingomyelinase C).
- *Streptococcus agalactiae* produces a heat-stable extracellular protein called the **CAMP factor**.
- When both organisms are streaked perpendicular to each other on sheep blood agar without touching, the CAMP factor interacts with the beta-lysin from *S. aureus*, resulting in **enhanced lysis of erythrocytes** (red blood cells).
- This enhanced hemolysis appears as a distinct **arrowhead-shaped or "flame-shaped" zone** of hemolysis at the intersection.

4. Equipment and consumables

4.1 Blood Agar
4.2 Beta-lysin reagent
4.3 Culture of *S.aureus*
4.4 Commercial reagents : Disks containing beta-lysin of *aureus*
4.5 Sterile wooden applicator sticks or bacteriologic loops
4.6 Distilled water
4.7 Petri dish and slide

5. Procedure

5.1 S. aureus is streaked on the blood agar in a straight line across the center of the plate.
5.2 The unknown microorganism is then streaked in the same manner perpendicular to the Staphylococcus while avoiding the touching of the organism to previously streaked area.
5.3 The positive control organism is streaked parallel to and approximately 1 inch from the unknown organism.

5.4 Each streak on the plates is labelled on the back of the plate.

5.5 The plate is incubated overnight at 35°C in an incubator.

6. Result Interpretation

- A positive result in the standard assay is the formation of a distinct arrowhead of hemolysis at the intersection of the *Staphylococcus* and test organism streaks.
- A positive reverse CAMP or phospholipase D is indicated by a typical arrowhead of no hemolysis at the junction of the two hemolytic organisms.
- In the case of the disk test, a positive result is demonstrated by a distinct arc-shaped zone of complete hemolysis at the point of interaction of the disk with beta-lysin and the test microorganism.
- A lack of enhanced hemolysis near the colony being tested is a negative test

7. Quality Control

As a form of quality control for the CAMP test, two different organisms can be taken as a positive and negative control.

Control	Incubation	Results
Streptococcus agalactiae	Incubation for 24-48 hours at 37°C in the air with 5% CO_2	CAMP positive; formation of arrowhead hemolysis at the intersection of the streaks.
Streptococcus pyogenes	Incubation for 24-48 hours at 37°C in the air with 5% CO_2	CAMP negative; β-hemolysis with no enhanced arrowhead.

8. Waste Disposal

8.1 Biological Waste (Infectious Materials)

- Includes: inoculated **Sheep Blood Agar plates**, bacterial cultures (e.g., *Streptococcus agalactiae*, *Staphylococcus aureus*).
- Dispose in **biohazard bags**.
- Sterilize by **autoclaving at 121°C for 15–20 minutes** before final disposa

8.2 Work Surface Decontamination

- Clean benches with **70% ethanol or suitable disinfectant** after the procedure

8.3 Contaminated Glassware

- Petri dishes, slides:

  Soak in **disinfectant (e.g., 0.5–1% sodium hypochlorite)** for at least **30 minutes**.

  Wash and sterilize by **autoclaving** before reuse or disposal.

8.4 Liquid Waste

- Broths or suspensions:
  - Treat with **appropriate disinfectant (e.g., bleach)** for **30 minutes**.
  - Then discard according to laboratory biosafety guidelines.

8.5 Used Inoculating Tools

- **Loops, needles, swabs**:
  - If reusable: sterilize by **flaming or autoclaving**.
  - If disposable: discard in **biohazard sharps or infectious waste container**.

8.6 PPE (Gloves, Lab Coats, etc.)

- Disposable gloves:
- Discard in biohazard waste bags.
- Reusable lab coats:
- Send for proper laundering and disinfection

9. REFERENCE

- Biochemical Tests for the Identification of Aerobic Bacteria. (2016). *Clinical Microbiology Procedures Handbook, 3.17.1.1–3.17.48.3.* DOI: 10.1128/9781555818814.ch3.17.1.
- SOP Version 2013, NCAH, Serbithang, Bhutan
- SOP Version 2028.1, NCAH, Serbithang, Bhutan
- Barrow GI, Feltham R K A, editors. Cowan and Steel's Manual for the Identification of Medical Bacteria. 3rd ed. Cambridge: Cambridge University Press; 1993. p. 226
- Department of Veterinary Pathology and Public Health. Laboratory Manual for Veterinary Microbiology. Queensland.
- Microbiology Laboratory. London: Public Health Laboratory Service; 1999. p. 147-8

Proprietary Statement: *This document contains proprietary information on Laboratory service unit, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No: NCAH/LSU/BACTO 43
Title: SOP for VITEK 2 test
Version No: 2026.01, Total Pages:4
Issue Month/Effective Date: May 2026
Revision Summary: NA
Supersedes Version No: NA
Prepared by: Bacteriology Section, LSU, NCAH
Reviewed by: Dr.Rinzin Loday, Mr.Tenzinla & Mrs.Bindu Parajuli
Approved by:
Application/Distribution: NCAH (LSU), NVH, RLDC, & RVH&Ecs

1. Scope

VITEK 2 test is a fully automated system that performs bacterial identification (ID) and antimicrobial susceptibility test (AST) card for the automated identification and susceptibility testing of most clinically significant microorganisms.

2. Objective

- To rapidly and accurately identify bacteria and yeast.
- To determine antimicrobial susceptibility patterns.
- To standardize and automate microbiological testing.

3. Principle

Vitek is a system or test that helps identify bacteria. The identification is brought by the levels of nutrient usage and the biochemical reactions. Test results, which involve bacteria growth, are obtained within 18-72 hours.

4. Equipment and Consumables

4.1 VITEK® 2 COMPACT Instrument
4.2 Compact Workstation
4.3 PC Computer
4.4 Monitor, Keyboard, Mouse
4.5 Printer
4.6 Uninterruptible Power Supply (UPS)
4.7 DENSICHEK® Plus
4.8 VITEK® DENSICHEK® pod and base
4.9 Power conditioner
4.10 Dispensing saline
4.11 Cards (GP ID & GP AST)
4.12 Cards (GN ID & GN AST)
4.21 Pipettes
4.22 Pipettes tips
4.23 Tubes

5. Procedure

5.1 For testing one isolate take two un-sensitized tubes and label it with ID (Identification) and AST (Antimicrobial susceptibility) also mention Lab ID and place it cassette.
5.2 Take 3 mL suspension solution in each tube from dispensette.
5.3 Prepare the inoculums suspension in ID tube using a sterile loop or swab o transfer sufficient number of morphologically similar single isolated colonies from pure culture and calibrate the turbidity of ID tube by Densi CHECK PLUS.

6 Bacteria (GN & GP)	7 0.50-0.63 McFarland
8 Yeast and BCL	9 1.80- 2.20 McFarland
10 NH and ANC	11 2.70- 3.30 McFarland

5.4 Transfer 145 microliters (for GNB) and 280 microliters (for GPB) from ID tube to AST tube. Place ID card in ID tube and AST card in AST tube in cassette.
5.5 Fill in blank cassette worksheet with required information for data entry.
5.6 Age of suspension must not exceed 30minutes before inoculating card and loading the cassette.
5.7 Open the Fill door and place the cassette into the chamber.
5.8 Close the fill door.
5.9 Press the Start Fill button to begin the filling process.
5.10 After completion of the Fill cycle (Fill indicator blue arrow blinking), remove the cassette from the Filler Station and close the Fill door. The Load door unlocks.
5.11 Open the Load door and place the cassette into the Cassette Load/Unload Station.
5.12 Close the Load door. The cassette icon appears on the Status screen.
5.13 After processing of test cards unload the Cassette as a blinking, blue indicator LED at the load/Unload Station indicates that an empty cassette is at the station.
5.14 Open the Load door and remove the cassette from the instrument.
5.15 Close the Load door.

6. Result Interpretation

- Identification results: organism name with confidence level.
- AST results: Sensitive (S), Intermediate (I), Resistant (R)
- Review unusual or low discrimination results manually.

Serial No.	Range %	Interpretation
1	70%	Acceptable
2	70 to 80%	Good
3	80 to 90%	Very good
4	90 to 99%	Excellent

7. Quality Control

- Use reference strains (e.g., ATCC strains) regularly.
- Check McFarland standard accuracy, Card storage conditions, and Instrument calibration.
- Perform QC as per manufacturer guidelines.
- Document all QC results.

8. Waste Disposal

- Dispose of used cards and cultures as biohazard waste**.**
- Autoclave infectious materials before disposal.
- Discard liquid waste with disinfectant (e.g., 1% sodium hypochlorite).

9. References

- Quinn P.J., Carter M.E., Markey B. and Cater G.R. Clinical Veterinary Microbiology. 2000.
- Subcommittee on taxonomy of staphylococci and micrococci - minutes of first meeting. International bulletin of bacteriological nomenclature and taxonomy 1965; 15:107-8.

- Toowoomba Veterinary Laboratory: Biochemical tests. Laboratory Methods Manual. Issue 4. Queensland; Australia: 2004. P. 1-8
- Microbiology Laboratory. London: Public Health Laboratory Service; 1999. p. 147-8