



Abdisa Tagesu*

Jimma University, School of Veterinary Medicine, Jimma, Oromia, Ethiopia

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*Corresponding author: Abdisa Tagesu, Jimma University, School of Veterinary Medicine, Jimma, Oromia, Ethiopia, Tel: +251933681407, E-mail: abdisatagesu@gmail.com

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Research Article

Microbiology examination

Veterinary microbiology laboratory examination is required to establish the clinical samples, etiology and line treatment through antibacterial sensitivity testing. Veterinarian should have to go for antimicrobial sensitive test before treatment of patient with antibacterial drugs, without testing of antimicrobial sensitive, the bacteria or microorganism may develop resistance to that drugs. In microbiology laboratory examinations, the clinical samples can be examined in different ways, the ways are by direct examination and culturing on media and isolation of organism.

Direct examination in microbiology examination

The collected samples can be directly examined are tissues, organ swabs (nasal, fecal, vaginal, and prepuccial and conjunctival), urine, blood and exudate [1]. The sample can be collected for bacterial culture or direct examination from different organ and postmortem as it is detailed in table 1.

Staining and its principle in Microbiology diagnosis

Direct examination of the collected sample can be conducted by smearing of tissue or organ on clean and dry glass slide. Thin smear are prepared from blood, exudate and swabs, air dried and fixed on flame of Bunsen burner for a few seconds. A stain is a substance that adheres to a cell, giving the cell color. The presence of color gives the cells significant contrast so they are much more visible. Staining is an auxiliary technique used in microscopy to enhance contrast in the microscopic image [3]. The stain can be classified into simple and differential staining [4]. Simple staining are used to stain negatively charged particles, usually the bacterial cell wall components and nucleic acid carrying negative charge which strongly attract the basic stain with the positively charges chromogen.

The morphology and arrangement of the bacteria are visualized by simple staining. The basic stains commonly used are methylene blue, crystal violet and carbol fuchsin.

Whereas, differential staining uses three reagents like primary stain, decolourizer and counter stain. The primary stain imparts colour to all the cells, the decolourizer is used to establish a colour contrast and counter stains the cells that are decolourised [5]. Staining are adding the color to bacterial cell and used to determine bacterial morphology and to distinguish bacteria from different species by differential staining characteristics. Before staining all slides are fixed by heat, methyle alcohol and formalin (Figure 3). Fixation is important to make permeable staining to bacterial cell by killing vegetative bacteria and protoplasmic shrinkage [1].

Gram staining technique

Gram staining is used in bacteriological to differentiate Gram negative (stain red to pink) from Gram positive (stain purple to blue) bacteria. Cell wall, aging or death may cause Gram positive bacteria to appear Gram negative (Figure 1) [6]. The cell walls of the two groups are morphologically and chemically quite different. One explanation for the differential staining reaction emphasizes the higher lipid content of the cell walls in Gram-negative bacteria. During the decolorization

Table 1: Sample collection for bacterial isolation from sick animals and carcass [2].

Predominant clinical sign or post mortem lesions	Bacteria	Samples
Septicemia, toxemia, sudden death, hemorrhages, subcutaneous oedema, gas gangrene.	Bacillus anthracis, Clostridium Pasteurella, E. coli, Salmonella, Erysipelothrix, Staphylococcus	Blood, body fluids, parenchymatous organs, bone marrow, lymphnode
Enteritis, dysentery	E. coli, Salmonella, Mycobacterium paratuberculosis, Clostridium, Campylobacter, Brachyspira hyodysenterica	Tied off portions of affected intestine, mesenteric lymphnodes
Pneumonia, coughing	Pasteurella, Mannheimia, Bordetella, Histophilus, Mycoplasma	Affected lung, transtracheal aspirates, nasal swabs, mediastinal lymph nodes.
Nervous signs	Cl.botulinum, Cl.Tetani, L.monocytogenes, Acetobacterium pyrogenes, E. coli	Serum, contents of the rumen and large intestine, wound swab or tissue, brain, liver, spleen, CSF
Abscess, granuloma, arthritis	Staphylococcus, Streptococcus, Corynebacterium, Actinomyces, Actinobacillus, Brucella, Pseudomonas, Erysipelothrix, Fusobacterium, Mycobacterium, Nocardia, Mycoplasma	Pus from edge of lesion, swab, intact abscess, joint aspirate.
Mastitis	As for abscess, also Leptospira, Enterobacteria, yeasts, Mycobacteria	Milk, supramammary lymph nodes
Skin lesions	As for abscesses, also Dermatophilus congolensis and dermatophytes	Fresh scabs, skin biopsy, skin scraping
Abortion, infertility	Brucella, Leptospira, Campylobacter, Listeria, Chlamydia, Salmonella, Coxiella	Foetus, foetal membranes, foetal stomach, vaginal swabs, blood from dam

step, alcohol may extract the lipids, increasing the porosity or permeability of the cell walls. Thus, the crystal violet-iodine complex is easily lost.

The Gram-positive bacteria, however, do not have lipid-rich cell walls. Their cell walls become dehydrated during the alcohol treatment, decreasing the porosity so that the crystal violet-iodine complex is retained [7]. Gram-positive organisms are able to retain the crystal violet stain because of the high amount of peptidoglycan in the cell wall. Gram positive cell walls typically lack the outer membrane found in Gram-negative bacteria [8].

The cell walls of gram positive bacteria are thicker with less lipids substance than gram negative bacteria (Figure 2). The solvent dissolves the lipids, which combined with thinner cell walls, washes out or decolorizes the stain. Counter stain (safranin) is added to exaggerate the contrast with gram negative cells. However, in gram positive bacteria, when washed with solvent, the cell pores close becoming less permeable and are able to retain the stain and cell become purple. The crystal violet is primary stain, which stains everthings in the smear purple blue. Gram's iodine acts as a mordant that causes the crysatal violet to penetrate and adhere to the gram positive cell [8].

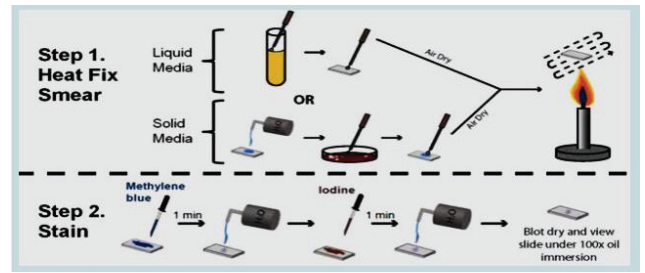


Figure 3: General staining techniques in veterinary microbiology (<http://microbeonline.com>).

The acetone alcohol mixture acts as the decolorizer that washes the stain away from everything in the smear except the gram positive organisms. The safranin is the counter stain that stains everything in the smear that has been decolorized in gram negative organisms [9]. The method of Gram stain is named after its inventor, the Danish scientist Hans Christian Gram 1853–1938, who developed the technique while working with Carl Friedländer in the morgue of the city hospital in Berlin in 1884. Gram devised his technique not for the purpose of distinguishing one type of bacterium from another but to make bacteria more visible in stained sections of lung tissue [10].

The following are procedure of gram staining (Figure 1,3) [2,11,12]:

First of all, presenting all material required for staining, like staining reagent, bacterial sample, Bunsen burner, slide, cover slide, water, pipette, loop, forceps.

- ✓ First sterile the loop and take specimen of bacteria or swabs in to slide
- ✓ Fixation of slide with the specimen by passing over heat (flame) several time us in forceps.
- ✓ Flood the fixed smear with crstal violet solution and allow remaining for 60 seconds.
- ✓ Rinse off the crstal violet with distilled or tap water
- ✓ Flood the slide with iodine solution and allow remain for 60 seconds.
- ✓ Rinse off the iodine solution with distilled or tap water
- ✓ Flood the slide with decolorize for 5 seconds
- ✓ Rinse off the decolorizer with distilled or tap water
- ✓ Flood the slide with safranin and allow to remains for 30 seconds.
- ✓ Rinse off the Safranin with distilled or tap water.
- ✓ Dry the slide on absorbent paper and place in an upright position.
- ✓ Put stained slide under microscope at 100 x objectives and observe the color change of bacterial specimen, and the gram positive (Stain deep violet to blue) and negative bacteria (stain pink to red) will be observed.

Gram Staining Procedure		Gram Positive Cell Wall		Gram Negative Cell Wall	
Process of test	Appearance of Cells	Effect of Step	Effect on Cell Wall	Effect of Step	Effect on Cell Wall
Step 1: Begin with heat fixed cells		Step 1: Cell wall remains clear.		Step 1: Cell wall remains clear.	
Step 2: Flood slide with crystal violet dye for 1 min.		Step 2: Peptidoglycan cell wall is flooded with crystal violet and appears purple.		Step 2: Cell wall is stained purple from the crystal violet dye.	
Step 3: Add iodine solution for 1 min.		Step 3: A crystal violet-iodine complex is formed within the peptidoglycan cell wall trapping the purple stain.		Step 3: A crystal violet-iodine complex is formed but does not adhere to the cell wall due to the thin layer of peptidoglycan.	
Step 4: Wash slide with alcohol for 20sec.		Step 4: The crystal violet-iodine complex is trapped with the peptidoglycan cell wall and doesn't wash out.		Step 4: The crystal violet-iodine structure is washed out of the thin peptidoglycan layer.	
Step 5: Counter stain with safranin.		Step 5: As the peptidoglycan cell wall remains stained purple the red safranin has no effect.		Step 5: The red safranin stains the washed gram negative cells.	

Figure 1: The procedure of gram staining (http://www.wikiwand.com/en/Gram_stain).

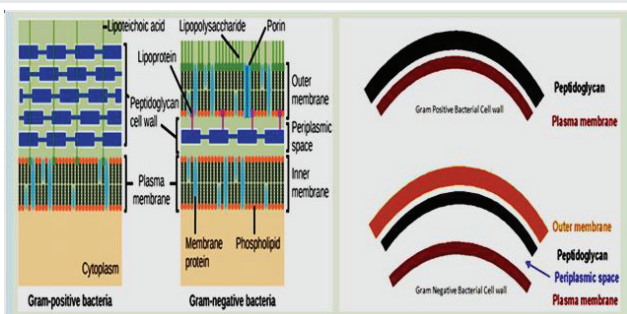


Figure 2: Structure of gram positive and negative bacteria (<https://bio.libretexts.org>).

Acid fast staining (The Ziehl–Neelsen stain)

The outer layer of the mycobacterial cell wall consists mainly of long-chain fatty acids called mycolic acids [13]. These mycolic acids comprise approximately 40% of the dry weight of the bacterium and are partially responsible for its acid fastness and the relative impermeability of the cell wall, including impermeability to antibacterial agents [14,15].

The Ziehl – Neelsen stain was first described by two German doctors; Franz Ziehl (1859 to 1926), a bacteriologist and Friedrich Neelsen (1854 to 1894) a pathologist. In this type some bacteria resist decolorization by both acid and alcohol and hence they are referred as acid fast organisms. This staining technique divides bacteria into two groups namely acid-fast and non acid-fast [16]. Zhiel neeslen is used to detect mycobacteria, chlymdia, nocardia and brucella spp [17]. Once stain penetrated and combine with the mycolic acid, and the cells resist decolorization even when a dilute acid alcohol solution is applied. Therefore organism said to be acid resistance or acid fast [18].

Preparation of reagent before staining [11]:

- ✓ **Ziehl neelsen carbolfuchsin:** Dissolve 3 g basic fuchsin in 100 ml 95% ethyl alcohol. Prepare a 5% phenol solution by dissolving 5g phenol in 100 ml distilled water. Prepared the Ziel Neelsen carbolfuchsin by mixing 10 ml alcoholic basic and 90 ml 5% phenol and allowing the mixture to stand for 24 hr. Filter the solution prior to use.
- ✓ **Acid alcohol:** Mix 2 ml concentrated hydrochloric acid & 98 ml 95% ethyl alcohol.
- ✓ **Methylene blue:** Prepare solution of methylene blue by adding 1.5 g powdered methylene blue to 100 ml 95% ethyl alcohol. Slowly add the alcohol to dissolve the powder. Add 30 ml saturated alcoholic solution for methylene blue to 100ml distilled water and 0.1 ml 10% potassium hydroxide. Filter and dilute it 1: 20 with distilled water to prepare the final methylene blue counterstain.

Staining Procedure

The acid fast staining can be conducted as the following procedure for bacteria which have mycolic acid. The procedure is started by applying the primary dye (carbolfuchsin or malachite green) and putting the smear to heat. The importance of heat is to enhance penetration and retention of the dye into the cells of bacteria. The general use of the reagents are like; Acid alcohol is decolorize removes the red stain from bacteria that are non-acid fast. Acid fast organism retains the red colour since carbolfuchsin is more soluble in the cell wall waxes than acidic alcohol. But, in non-acid fast bacteria cell wall lack of wax components and carbolfuchsin remove rapidly and cell become colorless [11,17,19]:

- ✓ Allow the film to air dry and then gently heat and fix it.

- ✓ Add additional stain (carbol fuchsin) and heated stain remain on slide for 2–5 minute
- ✓ Wash off the stain with clean water
- ✓ Cover the smear with 3% acid alcohol or 20% sulfuric acid for 2–5 minutes, until the smear is sufficiently decolorized (pale pink).
- ✓ Add a bit more decolorizer for very thick slides or to those continue to red dye
- ✓ Wash well with water
- ✓ Cover the stain with malachite green stain or methylene for 1–2 minutes
- ✓ Wash off stain with clean water
- ✓ Wipe the back of the slide clean, and place it in a draining rack for smear to air dry
- ✓ Examine the smear microscopically, using 100x oil immersion and identify bacterial whether acid fast or non-acid fast.
- ✓ Interpretation: Acid Fast Bacilli: Red, straight or slightly curved rods, occurring singly or in small groups, may appear beaded. Cells: Green (malachite green) or Blue (methylene blue). Background material: Green (malachite green) or Blue (methylene blue). The color is depend on the reagent which used in procedure as table 2.

Spore staining

Bacterial spores are bacterial mechanism that is intentionally set in an attempt to secure themselves to the adverse effects of the external environment (Figure 4). Spore is extremely resistance to harsh environment and disinfectants [20,21]. Endospore staining is the type of staining to recognize

Table 2: The interpretation of acid fast staining of bacteria (<http://microbeonline.com>).

Reagent	Acid Fast	Non-Acid Fast
Carbol Fuchsin with heat	Red (Hot Pink)	Red (Hot Pink)
Acid Alcohol	Red	Colorless
Methylene Blue/Malachite Green	Red	Blue/Green

the presence spore in bacterial vegetative cells and can penetrated wall thickness of spore bacteria. Spores may be located in the middle of the cell, at the end of the cell, or between the end and middle of the cell (Figure 5). Spore shape may also be of diagnostic use. Spores may be spherical or elliptical [22]. There are two major pathogenic spore forming genera, Bacillus and Clostridium [23].

Procedure of spore staining (Figure 6) [24,25]:

- ✓ Prepare a smear of the acid fast species of bacteria and make it air dry and heat fix.
- ✓ Put a beaker of water on the hot plate and boil until

steam is coming up from the water. Then turn the hot plate down so that the water is barely boiling.

- ✓ Place the wire stain rack over the beaker which now has steam coming up from the boiled water.
- ✓ Cut a small piece of paper towel and place it on top of the smear on the slide. The towel will keep the dye from evaporating too quickly, thereby giving more contact time between the dye and the bacterial walls.
- ✓ Flood the smear with the primary dye, malachite green or carbonfuchsin, and leave for 5 minutes. Keep the paper towel moist with the malachite green. Do not let the dye dry on the towel.
- ✓ Remove and discard the small paper towel piece.
- ✓ Wash really well with water and move the slide and wire rack from the boiling
- ✓ Water to the regular stain tray to finish up the last step in the procedure.
- ✓ Place the smear in the stain jar or flood the smear with the counterstain dye, safranin, and leave for 30 seconds-60 seconds.

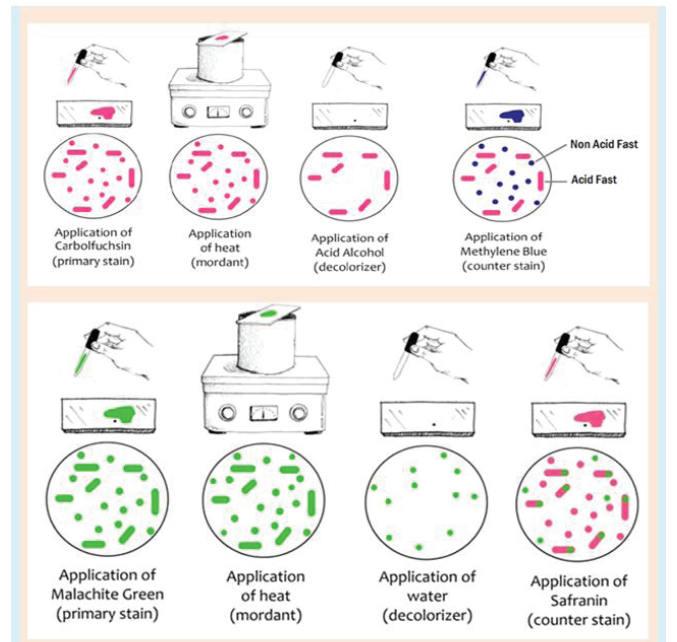


Figure 6: Procedure of spore staining. <http://laboratoryinfo.com> and <http://microbiologyinfo.com>.

- ✓ Wash well with water. Blot dry with bibulous paper.
- ✓ Examine the slide under microscope for the presence of endospores. Endospores are bright green and vegetative cells are brownish red to pink which picked up form safranin dye. The spore can be one terminal (*Cl. Tetani*), central (*Bacillus anthracis*) and subterminal (*Cl.perfringe*) location.

Capsule staining

The capsule stain is a type of differential stain which selectively stains bacterial capsules. A capsule is a substance that is synthesized in the cytoplasm and secreted to the outside of the cell where it surrounds the bacterium. Capsules can be polysaccharide, polypeptide, or glycoprotein. Capsules are associated with virulence in several microorganisms, including *Streptococcus pneumonia* and *Neisseria meningitidis*, because capsules provide a mechanism for these pathogens to evade the host immune system. Because of their structure and composition, heat and water will dislodge capsules from bacteria during laboratory procedures. In the capsule staining procedure, the primary stain is crystal violet, and all parts of the cell take up the purple crystal violet stain. There is no mordant in the capsule staining procedure. A 20% copper sulfate solution serves a dual role as both the decolorizing agent and counter stain. It decolorizes the capsule by washing out the crystal violet, but will not decolorize the cell. As the copper sulfate decolorizes the capsule, it also counter stains the capsule. Thus, the capsule appears as a faint blue halo around a purple cell [26].

Procedure of capsule staining [27]:

- ✓ Obtain a clean glass slide. Choose one of the above broth cultures, and agitate your broth culture to disperse the bacteria.

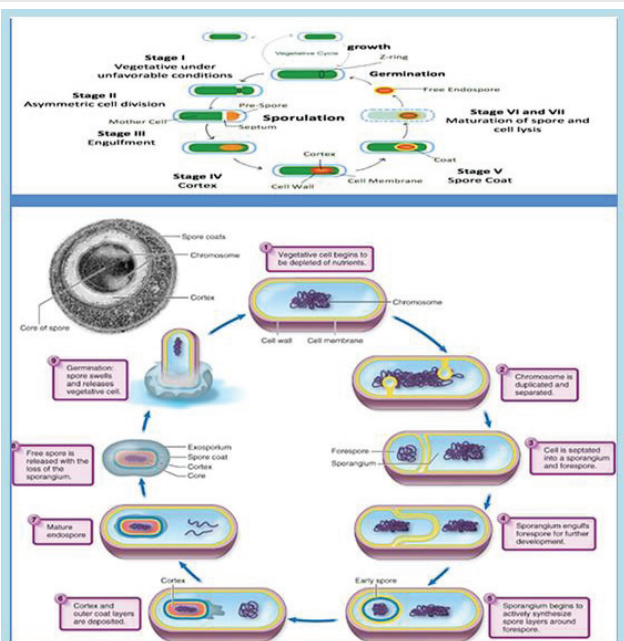


Figure 4: Sporulation of bacteria while change from unfavorable condition. [http://commons.wikimdia.org/wiki-File :Sporulation_in_Bacillus_subtilis.jpg](http://commons.wikimdia.org/wiki-File:_Sporulation_in_Bacillus_subtilis.jpg), http://online.science.psu.edu-micrb106_wd/node/6057.

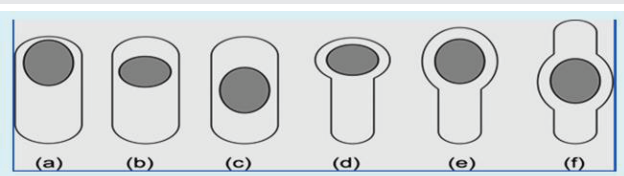


Figure 5: Morphology of endospores : Terminal(a,d,e); Subterminal (b) ; Central(c,f) ; circular(b,d), ellipsoid(a,c,e,f).<http://ttkamop.elte.hu>.

- ✓ Prepare a smear using 2–3 loopfuls of the broth culture. Allow the smear to air-dry, but do not heat fix this slide! Heat will cause the capsule to dislodge.
- ✓ Cover the smear with crystal violet, and stain the smear for 2 minutes.
- ✓ Tilt the slide and rinse with 20% copper sulfate solution. Do not rinse with water! Water will remove the capsule from the cell.
- ✓ Let the slide air dry for a few minutes. Do not blot the slide! Blotting will remove the bacteria from the slide and/or distort the capsule.
- ✓ Observe the slide under oil immersion, and Look for purple cells surrounded by a clear or faint blue halo on a purple background. (The halo is the capsule.) You may need to decrease the amount of light in order to make the capsule easier to see.
- ✓ Clean your microscope with lens cleaner, removing all oil from lenses and the capsule will be observed as figure 7.

Cultural methods

The purposes of culturing media in veterinary clinical samples are to cultivate the organism and to obtain the discrete colonies for isolation of organism in the pure culture. The cultural media which used in veterinary laboratory are classified in to four groups, like simple, enriched, selective and differential or biochemical media [26,28].

Simple media: It is also called general media or universal media. It is a media which used for bacteriological examinations of the clinical specimens and support the microorganisms that do not require special nutrients. Example: nutrient broth, nutrient agar.

Enriched media: The cultured media that enriched with whole blood, serum, vitamins, special extraction which support for growth of fastidious organisms. Example: Blood agar, serumagar, chocolate agar.

Selective media: It allows the growth of certain types of organisms and prevents or slows down growth of bacteria other than pathogens for which media are intended. Example: MacConkey agar, Salmonella shigella agar.

Biochemical or differential media: It is the media that used to identification of one bacterial species from other. The media which indicator substances are added to differentiate bacteria. Example: TCBS agar differentiates sucrose fermenter to non-sucrose fermenter. The routine bacteriological examinations are carried out by using blood agar or tryptose soya agar plates/ MacConkey agar plates. Example: mannitol salt agar, DNase agar, blood agar, MacConkey agar and eosin-methylene blue agar.

MacConkey Agar is both selective and differential [29–31]. MacConkey agar is a selective culture medium, which

is used to grow gram negative bacteria and to identify those which ferment lactose and also differential, meaning that this medium differentiates or distinguishes between groups of bacteria on the basis of a color change reaction. MacConkey's contains two additives that make it differential; neutral red (a PH indicator) and lactose (a disaccharide) [29–31].

MacConkey Agar media contains crystal violet and bile salts, which inhibit most gram-positive organisms and select for gram-negative organisms (Figure 8). It also contains the substrate lactose and the pH indicator neutral red, which allow differentiation among gram-negative bacteria based on their ability to ferment lactose. If an organism is unable to ferment lactose, the colonies will be colorless, taking on the color of the medium.

Lactose fermenter bacteria are like *Citrobacter* spp, *Klebsiellasp*, *Escherichia coli*, and *Serratia* spp. and lactose fermenter are like *Proteus* spp, *Shigella* spp, *Yersinia* spp, *Salmonella* spp, *Edwardsiella* spp, *Hafnia* spp, *Morganella* spp, *Providencia* spp and Gram positive bacteria are nor growth on mackonkey [32,33]. Growth on MacConkey agar indicates the organism is resistant to crystal violet and bile salts, and is likely to be gram-negative. Enteric bacteria that have the ability to ferment lactose can be detected using the carbohydrate lactose, and the pH indicator neutral red. Growth which is a pinkish-red color indicates the organism has the ability to ferment lactose [33].

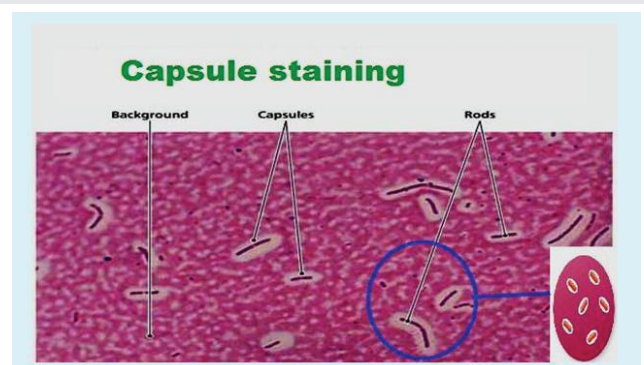


Figure 7: Capsule staining. Capsule staining is negative staining techniques due to its stain only bacterial cell and background, but capsule stay in unstained around bacterial cell [27].



Figure 8: Macconkey Agar : (<http://chrysacsiovi.tk>).

Eosin Methylene Blue (EMB) agar is both selective and differential. Eosin methylene blue is selective for gram negative bacteria, the dyes eosin and methylene blue found in medium which inhibit growth of gram positive bacteria [33,34]. It also contains lactose, allowing differentiation between organisms which ferment lactose and produce acid end-products, and organisms that do not ferment lactose. Small amounts of acid production result in a pink colored growth (e.g. *Enterobacter aerogenes*) while large amounts of acid cause the acid to precipitate on the colony, resulting in a characteristic greenish, metallic sheen (e.g. *E. coli*). Organisms which do not ferment lactose will be colorless, taking on the color of the medium [34].

Blue agar indicates the organism can grow in the presence of the dyes eosin and methylene blue and is likely a gram-negative. Growth which is a pink color indicates the organism can ferment lactose to form weak acid end-products, and growth which exhibits a green metallic sheen indicates the organism can ferment lactose to form strong acid end-products [35] (Figure 9).

Mannitol salt agar (7.5% NaCl) is a medium selective for staphylococci and differential with respect to mannitol fermentation. It contains a high concentration (about 7.5%-10%) of salt (NaCl), making it selective for Gram-positive bacteria (*Staphylococcus* and *Micrococcaceae*) since this level of salt is inhibitory to most other bacteria. *Staphylococcus aureus* produces yellow colonies with yellow zones, whereas other coagulase-negative staphylococci produce small pink or red colonies with no colour change to the medium. If an organism can ferment mannitol, an acidic byproduct is formed that causes the phenol red in the agar to turn yellow [36].

Fermentation of mannitol is only seen in the pathogenic species of *Staphylococcus* and is signaled by the production of acidic products leading phenol red in the media to change from a neutral red-orange to bright yellow. Non-pathogenic staphylococci produce small colonies surrounded by red or pink- purple zones due to the production of basic by products due to metabolism^{36,37}. A change in the color of the agar from pink to yellow indicates the organism has the ability to ferment mannitol. Yellow coloration can usually be seen around the sides of the bacterial growth [38].

Blood Plates: All organisms could growth on the blood plates, but they are not selective. Blood agar is a rich medium that has been supplemented with fresh 5-10% blood. The hemolytic response can be dependent upon the type of blood. Sheep blood is commonly used, but some organisms require rabbit or bovine blood [39,40]. Blood agar is a differential medium. It is also commonly used as an enriched medium for growing fastidious bacteria. Some bacteria produce exotoxins called hemolysins that cause lysis of red blood cells.

The degree of the hemolysis is an especially useful tool for differentiation among Gram-positive cocci. The three types of hemolysis are (Figure 10) [39,39]:

- **Beta hemolysis:** When the bacteria cause complete lysis of red blood cells and hemoglobin, this results in complete clearing of the blood around colonies.

- **Alpha hemolysis:** when bacteria causes partial lysis of red blood cells and hemoglobin, which results in a greenish-grey or greenish-yellow discoloration of the blood around the colonies.
- **Gamma hemolysis:** when the bacteria couldn't causes the hemolysis of the red blood cells and hemoglobin. Then no any change in the medium.

Culture transfer techniques

Various media types used in microbiology labs include agar slants, agar deeps, agar plates, and broths. An agar slant is a solid media in a test tube with a slanted surface on which to culture the microorganism. These are typically inoculated by streaking the surface of the slant with a sterile loop. An agar deep is a solid media in a test tube which does not have a slanted surface. These are typically inoculated by stabbing the media with a sterile needle. An agar plate is a solid media which is contained in a Petri plate, providing an optimal surface on which to culture microorganisms. Like the agar slants, these are inoculated by streaking the surface with a sterile loop. Broth tubes are a liquid medium which can be inoculated by a sterile loop, needle, or pipette [26].



Figure 9: The eosin methylene blue. Quadrant 1 : Growth on the plate indicates the organism, *Escherichia coli*, is not inhibited by eosin and methylene blue and is gram-negative bacterium. The green metallic sheen indicates *E. coli* is able to ferment lactose to produce strong acid end-products. Quadrant 2 : Growth on the plate indicates the organism, *Pseudomonas aeruginosa*, is not inhibited by eosin and methylene blue and is a gram-negative bacterium. The absence of color in the bacterial growth indicates *P. aeruginosa* is unable to ferment lactose. Quadrant 3 : Growth on the plate indicates the organism, *Enterobacter aerogenes*, is not inhibited by eosin and methylene blue and is a gram-negative bacterium. The pink color of the bacterial growth indicates *E. aerogenes* is able to ferment lactose to produce weak acid end-products. Quadrant 4 : Absence of growth indicates the organism, *Staphylococcus aureus*, is inhibited by eosin and methylene blue and is a gram positive bacterium.

(<http://iws2.collin.edu/dcairn/CCCCC%20Micro/embagar.htm>).

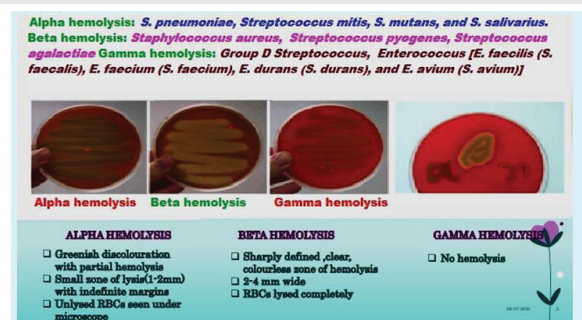


Figure 10: Types of hemolysis on blood agar.

Procedure

- ✓ Label each tube appropriately.
- ✓ Using a sterile needle, obtain a small amount of culture from the broth tube containing
- ✓ Use the needle to inoculate a nutrient agar deep tube by stabbing the needle into the agar deep.
- ✓ Using a sterile loop, obtain a loop full of culture from the broth tube containing cultured bacteria. Use the loop to inoculate a nutrient agar slant tube by streaking the agar slant with a zig-zag motion.
- ✓ Using a sterile loop, obtain a loop full of culture from the slant of cultured bacteria
- ✓ Use the loop to inoculate a nutrient broth tube by gently swishing the loop around in the liquid broth.
- ✓ Make sure all caps are loose, but secure.
- ✓ Incubate at 30 °C for 48 hours.

Isolation of pure colony

A colony forms on a plate when a single microbe is inoculated onto the surface of the plate and reproduces until there are enough cells to form a visible colony. Since a colony theoretically forms from a single cell, a colony should then represent a pure culture. One way to obtain single, isolated colonies is using the quadrant streak method [41]. The quadrant streak plate method allows sequential dilution of the original microbial material over the entire surface of a fresh plate (Figure 11). After the original sample is diluted by streaking it over successive quadrants, the number of organisms will be decreased. Usually by the third or fourth quadrant only a few organisms are transferred, and these produce single, discrete colonies.

Procedure [41-143]:

- Label different nutrient agar plates.
- Divide the agar plate into 4 quadrants.
- Place a loopful of culture onto the plate in Quadrant 1 with a sterile loop and streak the loop very gently using a back and forth motion.
- Sterilize loop. Go back to the edge of Quadrant 1 and extend the streaks into Quadrant 2, going back into Quadrant 1 twice.
- Sterilize loop. Go back to the edge of Quadrant 2 and extend the streaks into Quadrant 3, going back into Quadrant 2 twice.
- Sterilize loop. Go back to the edge of Quadrant 3 and extend the streaks into Quadrant 4, going back into Quadrant 3 twice. Be careful NOT to go back into Quadrant 1!

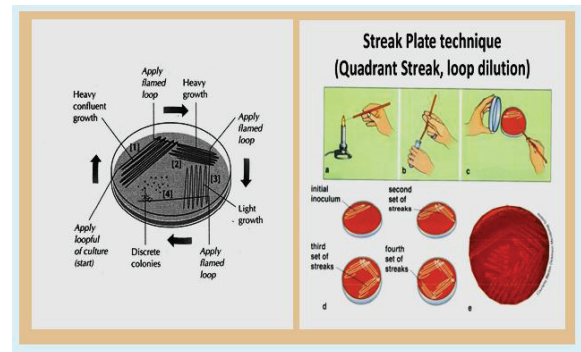


Figure 11: Four streaking techniques in isolation and inoculating method of bacteria.

- Tape plate closed on both sides. Make sure the plate is labeled with your name, date, and the organism(s), and incubate upside down (to prevent condensation from getting on to agar) at 30 °C.

Serial dilution

The purpose of serial dilution in veterinary microbiology laboratory is to quantifying the number of bacteria in a broth culture and also necessary to quantify the number of living bacteria in a particular sample. A Pure culture may be obtained by serially diluting the sample with sterile water to the point of extinction in number of cells. This method is used to isolate the organisms, if it is present in large number in the mixture [42]. The liquid culture needs to be diluted, often 1-million-fold, before it can be plated. When such a large dilution is required, an accurate dilution cannot be made in a single dilution step and it is necessary to make serial dilutions. Serial dilutions are a step-wise set of dilutions which sequentially dilute the bacterial culture. One or more of the dilutions are then plated on the agar plates to determine the number of colonies present in the original culture.

Only plates containing between 30 and 300 colonies are counted to ensure statistically significant data. To estimate the number of bacterial in the original culture, the number of colonies on the plate is multiplied by the total dilution plated. For example, suppose 0.1 ml of a 10^{-6} dilution was plated, and 123 colonies were counted following incubation. The total dilution plated would be 10^{-7} (since only 0.1 ml was plated), and the number of bacteria/ml of the original culture would be: $(123) \times 1/10^{-7} = 1.23 \times 10^9$ CFU/ml. Note that the results are expressed as “colony forming units (CFU)” per ml [42,43] (Figure 12).

Calculate the number of bacteria (colony forming units) per milliliter or grams of sample by dividing number of colonies by dilution factor multiplied by amount of specimen added to agar plate, the equation can be:

$$C = \frac{N}{S * D}$$

$$C = \text{concentration} \left(\frac{\text{CFU}}{\text{mL}} \right)$$

$N = \text{Numbers of colonies}$

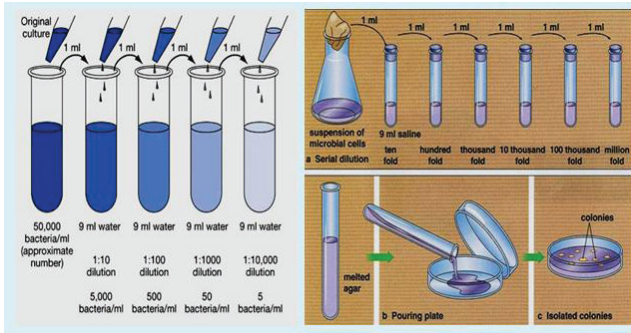


Figure 12: Serial dilution methods in bacterial isolation.

$D =$ Dillutions blank factor

$S =$ Volume transferred to plate

Procedure how to dilute [43]:

- ✓ Prepare serial dilutions of the broth culture as shown below. Be sure to mix the nutrient broth tubes before each serial transfer. Transfer 0.1 ml of the final three dilutions ($10^{-5}, 10^{-6}, 10^{-7}$) to each of three nutrient agar plates, and label the plates.
- ✓ Position the beaker of alcohol containing the glass spreader away from the flame. Remove the spreader and very carefully pass it over the flame just once (lab instructor will demonstrate). This will ignite the excess alcohol on the spreader and effectively sterilize it.
- ✓ Spread the 0.1 ml inoculum evenly over the entire surface of one of the nutrient agar plates until the medium no longer appears moist. Return the spreader to the alcohol.
- ✓ Repeat the flaming and spreading for each of the remaining two plates.
- ✓ Invert the three plates and incubate at room temperature until the next lab period.

The effective on growth of bacteria

Bacterial growth is depending on the temperature may affect bacterial enzymes, membrane fluidity. The enzymes are less active at low temperatures due to low kinetic energy and increasingly as temperature increase. However, when temperature becomes too high, enzymes become denatured and will cease its functions. At low temperatures, the lipids in a cell membrane can pack too tightly and solidify this prohibiting membrane from proper functions. When temperatures become excessively high, the lipid bilayer can become too fluid and lose its integrity. Bacteria can be divided into four depending on ability to survive in temperature environment; all prokaryotes can be classified into four general groups depending on their temperature requirements [44].

- ✓ Psychrophile: Those bacteria grows at ranging from -5 to 20 °C

- ✓ Mesophile: Those bacteria grow at ranging from 20 to 45 °C. Example, *Staphylococcus aureus* (45°C) and *Staphylococcus epidermidis* (40°C).
- ✓ Thermophile: Those bacteria grows at ranging from 45 to 80 °C
- ✓ Hyperthermophile: Those bacteria grows at ranging from 80 to 105 °C
- ✓ Bacterial growth is also dependent on the presence of oxygen in the environment, as different bacteria have different oxygen requirements depending on the types of enzymes they possess. The major bacterial oxygen classes are aerobes, microaerophiles, obligate anaerobes, aerotolerant anaerobes, and facultative anaerobes [44,45] (Figure 13).

- Aerobes require atmospheric O₂ (20%), and use O₂ as the final electron acceptor in the electron transport system.
- Microaerophiles require O₂ at below atmospheric concentrations, typically 2-10%. Microaerophiles have a limited ability to neutralize toxic oxygen, so excess O₂ will kill the bacteria. However, microaerophiles do use O₂ as final electron acceptor in the electron transport system. Example: *Campylobacter jejuni*, *Helicobacter pylori*.
- Obligate Anaerobes cannot survive in the presence of any oxygen. Obligate anaerobes lack the enzymes necessary to break down the toxic by-products of oxygen. Examples are like: *Clostridium*, *Bacteroides*.
- Aero tolerant: Anaerobes grow equally well in the presence or absence of oxygen. They do possess enzymes necessary to neutralize toxic oxygen by-products, but

	a. Obligate Aerobes	b. Facultative Anaerobes	c. Obligate Anaerobes	d. Aerotolerant Anaerobes	e. Micro-aerophiles
Effect of Oxygen on Growth	Only aerobic growth; oxygen required.	Both aerobic and anaerobic growth; greater growth in presence of oxygen.	Only anaerobic growth; ceases in presence of oxygen.	Only anaerobic growth; but continues in presence of oxygen.	Only aerobic growth; oxygen required in low concentration.
Bacterial Growth in Tube of Solid Growth Medium					
Explanation of Growth Patterns	Growth occurs only where high concentrations of oxygen have diffused into the medium.	Growth is best where most oxygen is present, but occurs throughout tube.	Growth occurs only where there is no oxygen.	Growth occurs evenly; oxygen has no effect.	Growth occurs only where a low concentration of oxygen has diffused into medium.
Explanation of Oxygen's Effects	Presence of enzymes catalase and superoxide dismutase [SOD] allows toxic forms of oxygen to be neutralized; can use oxygen.	Presence of enzymes catalase and SOD allows toxic forms of oxygen to be neutralized; can use oxygen.	Lacks enzymes to neutralize harmful forms of oxygen; cannot tolerate oxygen.	Presence of one enzyme, SOD, allows harmful forms of oxygen to be partially neutralized; tolerates oxygen.	Produce lethal amounts of toxic forms of oxygen if exposed to normal atmospheric oxygen.

Figure 13: Classification of bacteria depending on their environment (requirement of oxygen).

they never use O₂ as a final electron acceptor. Example: *Enterococcus faecalis*, some *Lactobacillus*

- Facultative Anaerobes are also able to live either in the presence or absence of oxygen, but they prefer oxygen so they can carry out aerobic respiration with O₂ as final electron acceptor to maximize ATP yields. These organisms can use other electron acceptors if O₂ is not available, such as fumarate and nitrate. They can also utilize fermentative metabolism in the absence of oxygen. Example: All *Enterobacteriaceae* (*E.coli*), some *Bacillus*, *Staphylococcus aureus*.
- Obligate aerobic bacteria: They have respiratory enzymes and lack the capacity for fermentations. Example: *Pseudomonas*, some *Bacillus*, *Mycobacterium tuberculosis*
- Capnophilic bacteria require increased concentration of carbon dioxide (5% to 10%) and approximately 15% oxygen. This condition can be achieved by a candle jar (3% carbon dioxide) or carbon dioxide incubator, jar or bags. (*Haemophilus influenzae*, *Neisseria gonorrhoeae*).

Cultivation of bacteria on anaerobic condition

Specialized methods are necessary to culture organisms anaerobically. One such method is the use of fluid thioglycollate broth, which is a reducing medium. It contains sodium thioglycollate, which reacts with molecular oxygen keeping free oxygen levels low. The sodium thioglycollate in the broth creates a redox potential in the tube, with higher levels of oxygen at the top of the tube, and a complete absence of oxygen at the bottom of the tube. Fluid thioglycollate broth also typically contains a redox potential indicator such as resazurin, which produces a pink coloration in an oxidized environment.

A second method used to culture organisms anaerobically is the use of a GasPak Jar and candle method [45] (Figure 14). This is a specialized culture vessel in which an anaerobic environment is generated after inoculated media are sealed into the chamber. Anaerobic conditions are created by adding water to a gas generator envelope that is placed in the jar just before sealing. There are two chemical tablets in the envelope, sodium borohydride and sodium bicarbonate. Water reacts with these chemicals, producing hydrogen gas from the sodium borohydride and carbon dioxide from the sodium bicarbonate. The hydrogen gas combines with free oxygen in the chamber to produce water, thus removing all free oxygen from the chamber. This reaction is catalyzed by the element palladium, which is attached to the underside of the lid of the jar. The carbon dioxide replaces the removed oxygen, creating a completely anaerobic environment [45].

Biochemical test

There are two levels of biochemical tests. The Primary Identification test in which, once a pure culture is obtained, the results from a few comparatively simple tests can often identify the bacterium to a generic level. The Secondary Identification of Bacteria occurs once the bacterium has been identified to a

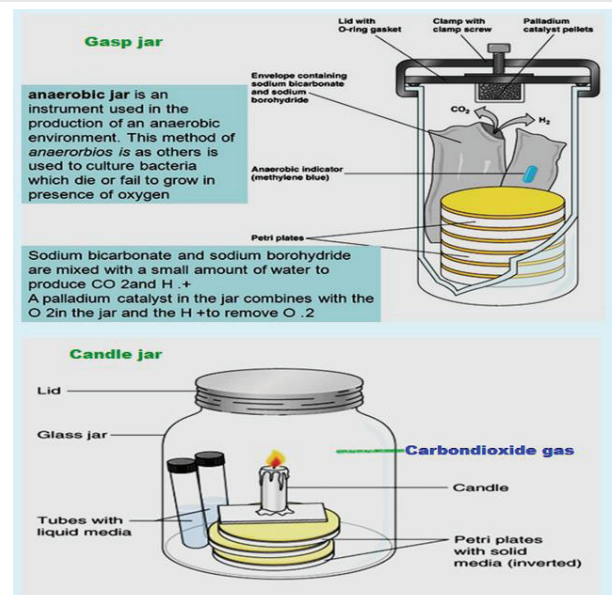


Figure 14: Creating anaerobic environment in culturing of bacteria by using jar and candle jar.

generic level, some of the tests can be carried out to identify the species. Secondary biochemical tests are: Fermentation of carbohydrates, Citrate utilization, Decarboxylation of amino acids, gelatin liquefaction, hydrogen sulfide test, indole test, Methyl red test, nitrate reduction test, ONPG test, Urease test and Voges Proskauer test [46]. The bacteria generally classified based on their biomedical test and staining and morphological structure is detailed in figure 15.

Catalase test

Catalase is the enzyme which present in most cytochrome containing aerobic and facultative anaerobic bacteria used to break hydrogen peroxide into oxygen (O₂) and water (H₂O). When the hydrogen peroxide is added to bacterial sample, it produces some bubble. When bubble is formed it shows, that bacteria is catalase positive [47] (Figure 16).

- ✓ Catalase positive:
- ✓ *Staphylococcus aureus*,
- ✓ *Micrococcus*,
- ✓ *Bacillus*,
- ✓ *Listeria monocytogenes*,
- ✓ *Enterobacteriaceae*,
- ✓ *Gonococcus* & *Meningococcus*,
- ✓ *Vibrio cholerae*,
- ✓ *Pseudo/Aero/Plesiomonas*,
- ✓ Catalase negative:
- ✓ *Streptococcus* spp
- ✓ *Clostridium*

Triple Sugar Iron Agars test

Triple Sugar Iron Agars (TSI) is used to determine if bacteria can ferment glucose and/or lactose and if they can produce hydrogen sulfide or other gases (Figure 17) [48]. This method used to differentiate various *Enterobacteriaceae*, including *Salmonella* and *Shigella*, which are intestinal pathogens. TSI contains three sugars: glucose, lactose and sucrose. Lactose and sucrose occur in 10 times the concentration of glucose (1.0% versus 0.1%). Ferrous sulfate, phenol red (a pH indicator that is yellow below pH 6.8 and red above it), and nutrient agar are also present. The tube is inoculated by stabbing into the agar butt (bottom of the tube) with an inoculating wire and then streaking the slant in a wavy pattern. Results are read at 18 to 24 hours of incubation [38,49,50].

Procedure:

Preparation of media: Add 3.0 gram of Beef extract, 3 gram of yeast extract, 15 gram of peptone, 5 grams of protease peptone, 10.0 grams of lactose, 10.0 gram of saccharose, 1.0 gram of glucose, 0.2 gram of ferrous sulphate, 5.0 gram of

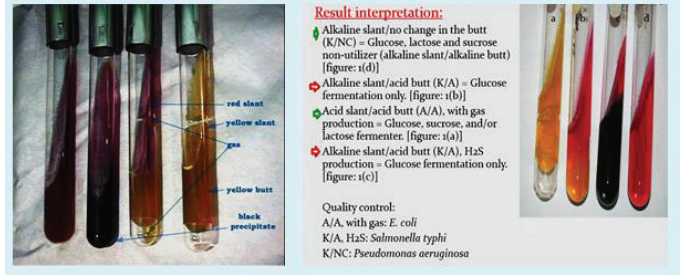


Figure 17: Triple sugar test and its interpretation.

sodium chloride, 0.3 gram of sodium thiosulphate, 0.024 gram of phenol red and 12 gram of agar and make the mixture up to 1000ml with distilled water. The Peptone mixture and the Beef and Yeast extracts provide the nutrients essential for growth. Sodium chloride maintains the osmotic balance of the medium. The Bacteriological agar is the solidifying agent.

The procedures are performed as below [51]:

- Sterilize the inoculating needle in the blue flame of the bunsen burner till red hot and then allowed to cool.
- From the rack, take the Trypticase soy broth tube containing the 24–48 hour culture, remove the cap and flame the neck of the tube.
- Using aseptic technique, take the culture of the organism from the TSB (tryptic soy broth) tube with the needle.
- Again flame the neck of the tube and replace the tube in the test tube rack.
- Take a sterile TSI slant tube from the rack, remove the cap and flame the neck of the tube.
- Stab the needle containing the pure culture into the medium, upto the butt of the TSI tube, and then streak the needle back and forth along the surface of the slant.
- Again flame the neck of the TSI tube, cap it and place it in the test tube rack.
- Incubate at 37°C for 18 to 24 hours.

Interpretation:

- A yellow slant on TSI indicates the organism ferments sucrose and/or lactose.
- A yellow butt shows that the organism fermented glucose.
- Black precipitate in the butt indicates hydrogen sulfide production (*Salmonella typhi*).
- Production of gases other than hydrogen sulfide is indicated either by cracks or bubbles in the media or the media being pushed away from the bottom of the tube.

Urease test

Urease test is used to differentiate urease positive proteus

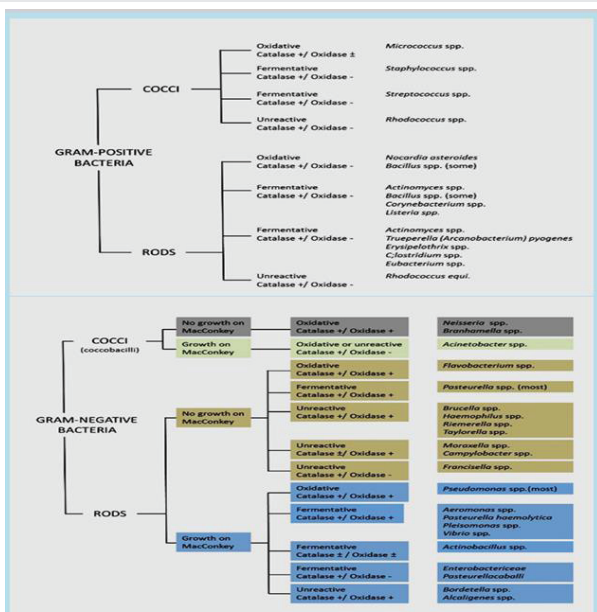


Figure 15: Classification of bacteria depending on their morphological biochemistry (www.afrivip.org).

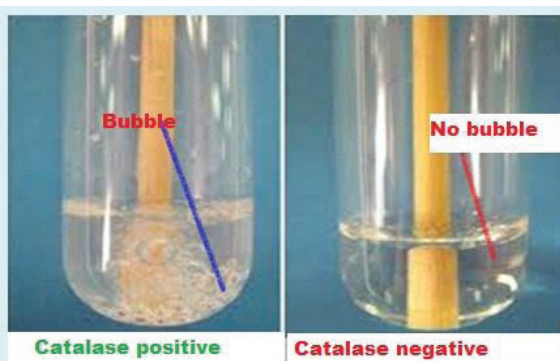


Figure 16: Catalase test and its interpretation.

species from other members of enterobacteriaceae. Some species of bacteria possess the enzyme urease and able to hydrolyze urea with the release of ammonia and carbon dioxide. Urea broth is a yellow orange color. The enzyme urease will be used to hydrolyze urea to make ammonia. If ammonia is made, the broth turns a bright pink color and it is positive and in negative result no color change. The result of interpretation positive test is magenta to pink colour (Figure 18) [38,52].

Motility test

Major bacteria are motile by flagella. Motility can be temperature dependent and some bacteria tend to be motile at ambient temperature but not at 37 °C. Semisolid motility media are important with Terazolium salts can be added to these media aid in detection of motility. Before autoclaving the motility medium, 0.05 g of 2, 3, 5-triphenylterazolium chloride is added per litre of medium. Terazolium is colorless, but a bacterium is grown the dye is incorporated into bacterial cells where it is reduced to an insoluble red pigment, formazan. The red colour forms only in the area of medium where the bacterium is growing. Motility media are prepared in test tube, two test tubes of the medium are stab-inoculated using a straight wire. One tube is incubated at room temperature and the other at 37°C. The tubes are examined for motility after 24 and 48 hrs. Motile bacteria migrate through semisolid medium which become turbidity. If TTC has been incorporated into the medium, the motility is demonstrated by a red colour throughout the agar. The growth of non-motile bacteria is confined to the stab line [53] (Figure 19).

Coagulase Test

The coagulase test usually relates with pathogenicity, some staphylococci can be negative to the slide coagulase test, but positive to the tube test.

Slide coagulase test: Heavy loopful of the staphylococci culture is emulsified in a drop of water on a microscope slide. A loop of rabbit plasma is added and mixed well with the bacterial suspension. The slide is gently rocked and a positive reaction is indicated by clumping within 10 seconds (Figure 20).

Tube coagulase test: 0.5 ml of rabbit plasma is placed in a small test tube. A suspension (0.01) of an overnight broth culture is added to the rabbit plasma test tube. The tube is rotated gently to mix the contents and then incubated at 35–37°C, preferably in a water bath. Alternatively one to three large well isolated colonies can be transferred into 0.05 ml of rabbit plasma in a tube and incubated at 35–37°C. A positive with degree of clotting of the plasma can occur in 2–4 hours. Many weak coagulase positive strains will coagulate at the plasma only after overnight incubation [17,38] (Figure 20).

Citrate utilization test

Citrate utilization test is commonly employed as part of a group of tests, the IMViC (Indole, Methyl Red, VP and Citrate) tests, that distinguish between members of the Enterobacteriaceae family based on their metabolic by-products [54].

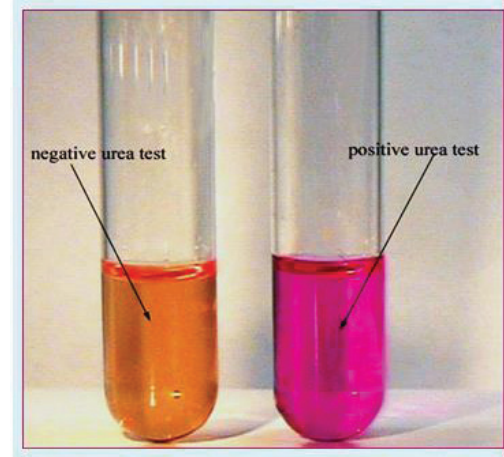


Figure 18: Urease test and its interpretation.

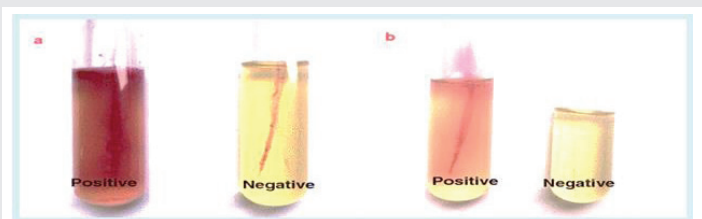


Figure 19: Motility test medium tubes containing TTC inoculated with *Escherichia coli* (a) and *Bacillus cereus* (b). Positive before treatment, negative after treatment with prodigiosin [53].

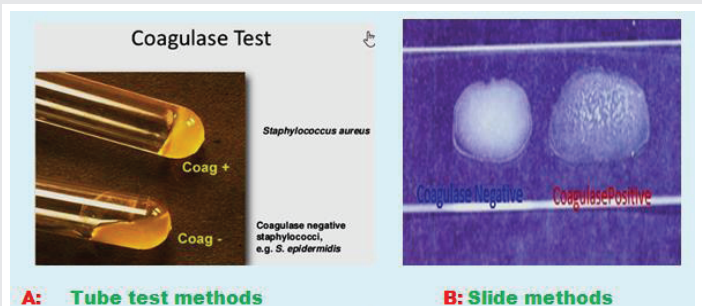


Figure 20: Coagulase test by two methods.

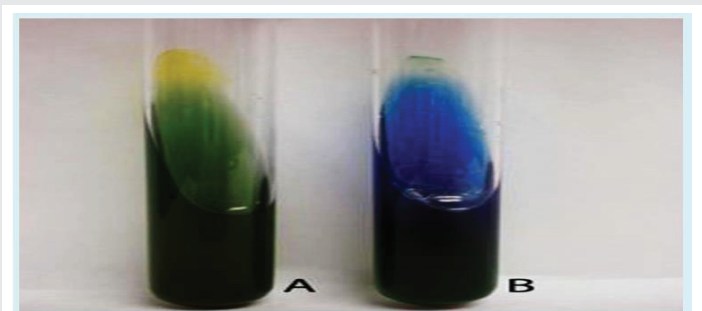


Figure 21: Citrate test to differentiate enterobacteriaceae spp. A : Citrate negative : No growth and no color change will be visible, the medium remains the deep forest green color of uninoculated agar. Only bacteria that can utilize citrate as the sole carbon and energy source will be able to grow on the Simmons citrate medium, thus a citrate-negative test culture will be virtually indistinguishable from an uninoculated slant. B : Citrate positive : Growth of bacteria will be visible on the slant and medium will be an intense prussian blue. The alkaline carbonates and bicarbonates produced as by-products of citrate catabolism raise the pH of the medium to above 7.6, causing the bromothymol blue to change from the original green color to blue.

Procedure of citrate utilization test:

- Inoculate Simmons citrate agar lightly on the slant by touching the tip of a needle to a colony that is 18 to 24 hours old.
- Incubate at 35°C to 37°C for 18 to 24 hours. Some organisms may require up to 7 days of incubation due to their limited rate of growth on citrate medium.
- Observe the development of blue color; denoting alkalinization (Figure 21).

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