MCB 301 E-NOTE TEMPLATE

COURSE CODE: MCB 301

COURSE TITLE: Bacteriology

NUMBER OF UNITS: 3 Units

COURSE DURATION: Three hours per week

COURSE DETAILS:

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Other Lecturers: Prof. Bankole, M.O. and Dr. Shittu, O.B.

COURSE CONTENT:

A detailed coverage of classification and characteristics of bacteria – the morphology, life cycle and biochemical characteristics of bacteria and other eukaryotes to include their isolation and identification. The significant role of bacteria in agriculture, industry, medicine, pharmaceuticals and foods. Bacterial infections and methods in diagnostic bacteriology; aspects of molecular bacteriology.

COURSE REQUIREMENTS:

Departmental course for B.Sc Microbiology

READING LIST:

1. Banor, J.D., Akermann, P.G., Toro, G.: Clinical Laboratory Methods. 8th Edition. The C.V. Mosby Company. Saint Louis, U.S.A. 1974
2. Brooks, G., Balel, J., Morge, S.: In: Jaetz, Melnick and Adelberg (Editor). Medical Microbiology. 21st Ed. Aphenton and Lange. Stanford, California. 1998
3. Davidson, I. and Henry, J.B.: Clinical Diagnosis. 15th Ed. W.B. Saunder Company. 1974

LECTURE NOTES:

CLASSIFICATION OF PRINCIPAL GROUPS OF BACTERIA

Three major groups of bacteria can be recognized on the basis of the mechanism of movement and the character of the cell wall: Myxobacteria, spirochetes and eubacteria.

MYXOBACTERIA: The myxobacteria are thin-walled, flexible, rod-shaped organisms. They propel themselves along solid surfaces by an unknown gliding mechanism: no motility organelles are detectable. There are no human pathogens in this group.

SPIROCHETES: The spirochetes are thin- walled, flexible, helical rods. They propel themselves by undulation of an axial filament which is wound about the cell body. Three genera contain important pathogens for human:

EUBACTERIA: The eubacteria include (1) stalked (2) budding (3) mycelia organisms and (4) simple unicellular forms.

Usually (1) stalked and (2) budding organism are not pathogenic.

(3) Mycelia forms include pathogens such as Actinomycetes -Norcadia-acid fast and aerobic.

Mycobacteria-genus Mycobacterium which include agents of tuberculosis and are acid-fast organisms.

(4) Unicellular forms-These include spheres (cocci) and straight rods (bacilli). The majority of the bacteria pathogenic for human fall into this group

Stains combine chemically with bacterial determines one of the most important bacterial characteristics, their response to gram stain. During the staining procedure, gram-positive cells retain the crystal violet-iodine complex, remaining blue, whereas gram-negative cells are

completely decolorized by alcohol and take on the red color of counter-stain (safranin). Gram-positive bacteria contain less lipids than gram-negative bacteria.

Bacteria produce various enzymes that are able to split food elements and are responsible for fermentation reaction of bacteria. Some enzymes are characteristic of certain organism e.g. coagulase production of pathogenic Staphylococci and Streptokinase synthesis of Streptococci.

EUCARYOTES AND PROCARYOTES

Ecaryotes - an organism whose cells have a true nucleus bounded by a clear membrane and exhibit mitosis.

Procaryotes - an organism e.g. bacterium that does not have a true nucleus, the nuclear material being scattered in the cytoplasm of the cell and that reproduce by cell division.

ENDOTOXINS OF GRAM-NEGATIVE BACTERIA

The endotoxins of gram-negative bacteria are complex lipopolysaccharides derived from bacterial cells and often liberated when bacteria lyse. The substances are heat-stable, with molecular weight variously extended to between 100,000 and 900,000.

The pathophysiologic effects of all endotoxins are similar regardless of their origin. The administration of endotoxin to animals or human result in series of events as the endotoxin is taken up by reticuloendothelial or endothelial cells, degraded or neutralized.

The following are prominently observed clinically or experimentally: fever, leukemia and hypoglycemia, hypotension and shock, impaired perfusion and acidosis of essential organs, activation of C3 and complement cascade, intravascular coagulation and death.

It has also been documented tha pregnant women with active urinary tract infection caused by gram-negative bacteria may have premature labor and consequently a high perinatal mortality rate offspring. This may be caused by endotoxins originating in the urinary tract.

ENDOTOXINS PRODUCED BY AEROBIC GRAM-NEGATIVE ENTERIC BACTERIA

Many entric bacteria in addition to their cotent of endotoxin, produce exotoxin of considerable medical importance.

***Escherichia coli* endotoxin-** Some strains of *E. coli* produce a heat-labile exotoxin tha is under the genetic control of a transmissible plamid and some strainsproduce a heat-stable enterotoxintha is under the genetic control of a heterogeneous group of plasmids. The heat-stable and heat-labile endotoxin can cause diarrhea, especially in children.

***Klebsiella pneumoniae* endotoxin-** Some strains of *K.pneumonia* produce a heat-stable enterotoxin that induce hypersecretion of fluids and electrolytes into the lumen of the small intestine and thus gives rise to diarrhea.

***Pseudomonas aeruginosa* exotoxin-** Many strains of *Pseudomonas aeruginosa* can produce an exotoxin in vitro and also in vivo that markedly inhibits proteiinsynthesis and causes tissue necrosis.

***Shigella dysenteriae* exotoxin-** *Shigella dysenteriae* type I produces a heat-labile exotoxin that affects both the gut and the CNS.Itproduces diarrhea. In human, it also inhibits sugar and amino acid absorption in the small intestine. Acting as a neurotoxin, thus materialmay contribute to the extreme severity ant fatal nature of *Shigella dysenteriae* infection and to the CNS reactions (meningitis, coma) observed in them.

***Vibrio cholerae* enterotoxin –** *Vibrio cholerae* and related vibrios produce the prototype pf a heat-labile enterotoxin that act by stimulating adenyl cyclase activity and thus raising concentration of cAMP in the mucosa of the small intestine (AMP =Adenine monophosphate).

The resulting hypersecretion of water and electrolytes cause massive diarrhea, which can lead to severe dehydration, acidosis and death.

GROWTH OF BACTERIA

When bacteria are inoculated in a suitable medium and placed in a correct environment, they will grow at a rapid rate. There are four main phases of growth in a typical growth curve:

1. **LAG PHASE:** This is a period when the bacteria adapt themselves to their new environment. The time involved in this reorganization of cell functions varies depending on the number of organisms inoculated and the conditions at that time.
2. **LOG OR EXPONENTIAL PHASE:** At this stage the cells divide at a constant rate and since reproduction is by binary fission, the relationship of time and number of cells (expressed as logarithm or number of cells) is linear. At this stage the cells are most active metabolically, making them more susceptible to antimicrobial agents.
3. **STATIONARY PHASE:** The production of toxic waste products as well as depletion of certain nutrient substances cause a decrease in the rate of growth to such an extent that the number of cells remains relatively constant.
4. **DEATH PHASE OR PHASE OF DECLINE:** At this stage cells begin to die, the cause being a continuation of events leading to the stationary phase. The number of viable bacteria decreases.

FACTORS AFFECTING BACTERIA GROWTH

The chemical composition of bacteria varies according to the species and the media on which they are grown. The growth of bacteria in culture media is determined by environmental factors such as moisture, composition of air, PH, temperature, salt, availability of carbon and

nitrogen and growth factors such as the presence of thiamine. The gas requirements vary: Obligate aerobes grow only in an free oxygen and obligate anaerobes grow only in oxygen-free atmosphere. Aerobes and anaerobes that do not clearly belong to either group are called facultative aerobes or anaerobes. Most bacteria grow better with the addition of carbon dioxide. The optimal PH is close to neutral or is slightly alkaline. The optimal temperature for most pathogens is 37oC and the optimal salt concentrations below 1%.

SYNTHESIS OF PROTOPLASM

It is readily apparent that in order to reproduce all bacteria must be able to synthesize their proteins, carbohydrate, fats and nucleic acids, the constituents of protoplasm. But the complexity of the raw materials that bacteria utilize to accomplish this synthesis varies considerably with the particular species. Some organisms have extraordinary process of synthesis and are able to reproduce using only inorganic materials in their cellular nutrition. On the other hand, some parasitic bacteria can not be made to grow in the laboratory unless they are supplied with highly complex organic materials such as whole blood. Such bacteria have a limited enzymatic endowment and hence, an equally limited ability to synthesize. Bacterium that can not synthesize any one of these essential building block must have them supplied or it can not grow. Such requirements are collectively termed ESSENTIAL METABOLITE. Another category of essential metabolites is vitamins: nicotinic acid, thiamine, riboflavin and pantothenic acid. Many of these vitamins are necessary to form the coenzymes necessary for enzymatic action. That is when vitamins required are supplied in suboptimal concentrations, the amount of bacterial growth is directly proportional to the amount of the vitamin added

THE BACTERIA CELL WALL STRUCTURE

The cell wall is the layer, usually fairly rigid, that lies just outside the plasma membrane. It is one of the most important prokaryotic structures for several reasons: (1) it helps determine the shape of the cell,(2) it helps protect the cell from osmotic lysis (3) it can protect the cell from toxic substances and (4) in pathogens it can contribute to pathogenicity. The bacteria cell wall also is the site of several antibiotics.

After Christian Gram developed the Gram stain in 1884, it soon became evidence that most bacteria could be divided into two major groups based on their response to Gram stain procedure. Gram positive bacteria stain purple, whereas gram negative bacteria were colored pink or red by the technique. The true structural difference between these two groups did become clear until the advent of the transmission of electron microscope. The gram positive cell wall consist of a single 20 to 80 nm thick homogeneous layer of peptidoglycan(murein) lying outside the plasma membrane. In contrast, the gram negative cell wall is quite complex. It has a 2 to 7 nm peptican layer covered by a 7 to 8 nm thick outer membrane. Because of the thick peptidoglycan layer, the cell walls of gram positive cells are more resistant to osmotic pressure than those of gram negative bacteria.

THE DIAGNOSIS OF INFECTIOUS DISEASE

It is the physician's responsibility to suspect infectious disease in patients and to initiate studies to confirm or reject this suspicion. Patients with infectious disease may present with a variety of signs and symptoms, some overt and easy to recognize, others observe and possibly misleading.

The diffuse redness and swelling of the throat or tonsils, purulent discharges from wounds or mucous membranes, and the accumulation of pus in abscesses or body cavities, often

resulting in pain, swelling, and increased heat to the area, are direct signs of infection calling for an immediate culture to establish the causative organism so that appropriate therapy may be started.

Cough, increased sputum production, burning on urination and dysentery are indirect signs that infection may involve deep organ systems. Fever, chills, flushing (i.e. vasodilation) and increase in pulse rate may be general.

Laboratory values suggesting the presence of infectious disease in patients with minimal or early symptoms include an elevation in the erythrocyte sedimentation rate, peripheral blood leucocytosis or monocytosis, and alterations such as elevations in gamma globulin or the presence of type specific antibodies.

CHARASTERISTICS OF INFECTIOUS DISEASES

The infectious diseases have characteristic signs and symptoms signs are objective changes in the body, for example fever. On these basis of a disease can be recognized. Symptoms are the subjective changes for example pain, loss of appetite, etc. which are felt by the patients. In a broad sense symptom is used for sign as well. In addition, a disease syndrome includes a set of signs and symptoms due to a particular disease; for example an AIDS patient experiences disease syndrome (*syndrome – a set of symptoms which occur together i.e. a symptom complex*).

Moreover, the characteristic symptoms of a disease develop during certain phases. The knowledge of the phases helps in the recognition of a disease. For example INCUBATION

PERIOD which refers to time required after infection to the appearance of signs/symptoms. Incubation period varies from organism to organism. Second is the PRODROMAL STAGE i.e. the period during which there is onset of signs and symptoms of a disease. Third, the PERIOD OF ILLNESS which is a phase during which the disease gets fully established and becomes most severe with characteristic signs and symptoms. The last characteristic phase is the period of decline when signs and symptoms disappear and the disease is recovered gradually. This stage is known as convalescence.

TRANSMISSION OF PATHODENS

For perpetration of disease and survival of the pathogen transmission from one host to the other occurs by any of the four main routes: air-borne, contact, vehicle and vector-borne.

1. **AIR BORNE TRANSMISSION:** The pathogens remain suspended in air and are transmitted through droplet which is small particles (1-4 m diameter) left from evaporation of large particles lies in the . The droplet mules remain in air for hours or days and carried to individuals because the pathogens cannot grow in air. Example of some air-borne diseases are chicken pox, flu, measles, mumps, viral pneumonia, diphtheria, pneumonia, tuberculosis, meningitis, etc.
2. **CONTACT TRANSMISSION:** Some of pathogens are spread when contact of the host is done with the reservoir of pathogen. In other words contact refers to person-to-person contact through touching, kissing or sexual contact. The diseases that spread through contact are hemps and boils (through contact of oral secretions or body lesions), infection of staphylococcus (by nursing mothers), and AIDS and syphilis (through placenta, or blood to blood contact).

3. **VEHICLE TRANSMISSION:** Vehicle refers to inanimate materials such as utensils, towels, beddings, surgical materials, needles, food, water, etc. Bacterial spreading through food and causing food poisoning are *Staphylococcus aureus*, *Bacillus cereus*, *Eschriedis coli*, *Vibrio cholera*, *Salmonella typhi*, *Clostridium difficile*, etc.

4. **VECTOR-BORNE TRANSMISSION:** A living organisms that transmit a pathogen is known as vector such as vertebrates (e.g. dogs, cat, bats, goat, sheep, etc.) or arthropods (e.g. flies, mites, insects, ticks, etc.). For example, flies carry flagella on their feed from faeces to food materials. Moreover, when pathogen does not undergo morphological and physiological changes within the vector is called harborage transmission e.g. the plague pathogen, *Yersinia pertis*. When the pathogen undergo morphological changes within the vector, it is called biological transmission e.g. *Plasmodium virae*.

CONTROL OF INFECTIOUS DISEASES

Since the infectious diseases are spread by several agents and cause epidemics, it can be controlled by one or several measures. This involves the breaking of the links of disease cycle, eliminating the reservoirs of the disease and making the individuals resistant (i.e. immunization).

1. **Breaking the links of disease cycle:** The pathogens survive on inmate or inanimate for some lines from where they are transmitted to suitable host. Therefore, if links between two stages of disease cycle are broken further spread of the pathogen does not occur. This includes general sanitation methods.

   a) pasteurization of milk (b) destruction of vectors by spraying insecticides

   c) chlorination of water supply (d) inspection of food and individuals handling it.

2. **Elimination of source of infections:** The source of infection can be eliminated by (a) adopting quarantine (legal prohibition of entry of goods, animals, etc. from one country to other or one state to other within a country) and isolating the carriers (b) destruction of animal reservoir (e.g. the cattle infected with foot and mouth disease virus are killed in other countries) (c) treatment of sewage (to check water-borne transmission of pathogens) and (d) use of chemicals by individuals to eliminate the pathogens.
3. **Immunization of Individuals:** For increasing the level of immunity (vaccination) mass immunization programmes are very good means of controlling infectious diseases such as polio, whooping cough, pertuency or tetanus (DPT), etc.

PROOF OF THE ETIOLOGY OF A DISEASE

The isolation of a particular organism from an infected person does not establish proof that it is the causative agent of the disease. It may exist in or near a lesion merely as normal flora or as a transient contaminant. In the early days of bacteriology some rules were laid down to establish whether or not an isolated organism was indeed the pathogen. These rules, called koch's postulate, can be summarized as follows:

1. The same organism must be found in all cases of a given disease.
2. The organism must be isolated and grown in pure culture from the infected person.
3. The organism from the pure culture must reproduce the disease when inoculated in to a susceptible animal.
4. The organism then must be isolated in pure culture from the experimentally infected animal.

Although these postulates are effective in determining the causative agents of most bacterial diseases, there are a few exceptions. For example, *Treponemapallidum* has been established as the causative agent of syphilis, but the organism has never been grown on artificial media. Similarly, *Mycobacterium leprae* has never been grown in the laboratory and, therefore, fits only the first postulate. Some vital diseases also fall into a category in which the last three postulates not always fulfilled.

HOW PATHOGEN ENTER AND LEAVE THE BODY

Each organism capable of producing disease has its own portal or portals of entry as well as a means of escaping from the host. The infectious agent in discharges from infected areas must be destroyed to prevent the transmission of the agent to a new host.

Microorganisms have been found to enter the body through the following areas:

1. Gastrointestinal trait via mouth. Examples include agent responsible for diseases such as typhoid fever, paratyphoid fever, dysentery, cholera, poliomyelitis, and infectious hepatitis, as well as food illnesses.
2. Respiratory trait via nose and mouth. This is the portal of entrance of all microbes causing respiratory diseases such as the common cold, measles, pneumonia and tuberculosis.
3. Skin and mucous membranes. Although the skin provides an effective protective barriers, minor breaks are undoubtedly always present that may allow the entrance of certain organisms. The staphylococcus that causes boil (furuncles) is one the more frequent organisms entering in this way, however, the streptococci also may cause spreading skin infections.

4. Gantourinary system. The mucous membranes of the genital tract are the common site for invasion by venereal disease agents such as those causing syphilis and gonorrhea. In addition, the urinary tract may be infected by microorganisms in the blood, or by their infection into the bladder during catheterization.

5. Blood. Those organisms that must be introduced directly into the blood in order to cause disease usually are transmitted from one individual to another by insect bites. The best known examples of diseases in this category are malaria and yellow fever – both transmitted by the mosquito. Other include the rickettsial diseases. Inadequate sterilization of needles and syringes can cause direct blood inoculation of hepatitis (particularly Hepatitis B) and Human immune deficiency virus (HIV).

The portals of exist for a disease agent are usually the same as their portal of entry. Thus, diseases of the respiratory tract are spread by way of secretion and excretions of the respiratory tract and mouth. In like manner, enteric infections (typhoid fever, polio myelitis, dysentery, etc.) leave the body by the intestinal tract and the spread through faecal contamination. Blood infections, which are spread by insects or contaminated needles and syringes, usually leave the individual in a similar manner, i.e. through direct contamination of a needle or syringe during the withdrawal of blood or the ingestion of the microorganism by a biting insect.

FUSOBACTERIUM

The fusiform bacilli are spindle shaped gram negative bacilli which may be curved or straight rods, usually with tapered ends. These organisms are fastidifus in their growth requirements most species are strict anaerobes. They grow under the same general

environmental conditions as the Bacteroides species and are found as normal flora in the mouth and intestinal tract.

The pathogenity of the fusobaria is somewhat limited and questionable although they have been found in association with necrolic and gangrenous conditions.

The one species *Fusobacteriumfusiforme* is of interest to the medical laboratory because of its appearance in increased numbers in the fuso-spirochetal infection known as Vincents angina/Trench mouth).

SPIROCHAETES

Spirochaetes differ from bacteria mainly in that they do not have a rigid cell wall. Most spirochaetes are so thin that they cannot be seen microscopically without staining.

The spirtochaetes of medical importance are contained in the following three genera:

1. **Borrelia-**may vary in thickness and the coils are irregular and more open than the other forms. They display a "lashing" motility and live strictly parasitic existence.

2. **Treponema-**show more regularity in the coils and the spirals are more tightly wounded. They display a more constant graceful motility than *Borrelia*. Treponema live a strictly parasitic existence.

3. **Leptospira**-the coils are uniform in size, very close together and hooked on one or both ends. Their motility is mainly rotary with some lashing and gliding movement taking place intermittently. They are found free-living in contaminated water.

Borrelia vincentii is the spirochaete partly responsible for fuso-spirochaetal infection i.e. Vincents angina-infection of the gums and pulmonary abscesses. Diagnosis is made by demonstrating the spinochaetes in blood films stained by Gemba's or Leishman's method, by examining fresh preparations by dark-background illumination (Darkfield exam) mice and rats and detectingthe organisms in their blood after 48 hour.

Treponema pallidum is the etiological agent of syphilis. Their spirochete does not stain with Gemsa's or Leishman's stain. It is necessary therefore to utilize special staining techniques such as the silver impregnation method in which a precipitate is built up on the organisms which renders it visible. The organism is also visible under darkfiled examination. Teponema for many of the diagnostic laboratory tests are grown in rabbit testicles.

Laboratory diagnosis of syphilis is based on serologic tests using lipid antigen. The particular antibody concerned, often referred to as "regain" can be measured by Wassermann reaction, which is a complement fixation reaction, or by one of several very similar precipation (flocculation) reactions including the Kahn test and the UDRL (Venereal Disease Research Laboratory) test.

Leptospira icterohemorrhagiae and other species of Leptospira cause weils disease. Various sains of Leptospira may be cultured on flechers medium for primary isolation laboratory diagnosis is based on the serologic tests by agglutination method and Darkfield examination of blood and urine after 14-36 days of infection in which Leptospira are looked for.

Weil's disease classically takes the form of an acute illness with fever, conjunctivitis albuminuria, haemorrhages and jaundice. Some patients developed meningitis.

Spirillum minus

Spirillum minus is a rigid cork screw like organism usually display two or three undulations and bipolar flagella. In the process of motion, which is darklike, it rotates around its long axis. It is gram negative, but Giemsa stain is preferred for smears.

The organism causes sodoku or rat-bite fever, which may follow the bite of a rat, mouse or other rodent. An ulcer forms at the site of the bite followed by generalized symptoms such as lymphadenitis, fever and rash.

Laboratory diagnosis is based on demonstration of the organism in the exudates of the primary and secondary skin lesion, in blood and in smear of regional lymph modes are blood smears are stained with Giemsa stain.

The darkfield technique is excellent for the examination of wet preparations.

BIOCHEMICAL TESTS AND REACTIONS

Detection of bacteria species is based primarily on the determination of the presence or absence of different enzymes coded by the genetic material of the bacterial chromosome. These enzymes direct the metabolism of bacteria along one of several pathways that can be detected by special media used in culture techniques. Substitutes upon which these enzmes can react are incorporated into the culture medium, together with an indicator system that can detect either the decay of the substrate or the presence of specific metabolic products. By selecting a series of media that measure different metabolic characteristics of the microorganism to be tested, a biochemical fingerprint can be determined for making a species identification.

CATALASE TEST: Add a drop of 3% H_2O_2 to a microscope slide. Touch a loopful of the organism to the drop of H_2O_2 foaming or bubbling indicates a positive test – Evolution of H_2O and O_2. The Reaction:

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

CITRATE UTILIZATION (CHRISTENSEN): The slant is inoculated with the organism and incubated at 37°C for 18-24 hours. Of the organism utilizes citrate as its source of energy in the presence of organic nitrogen, a deep red color will occur in the slanted portion of the tube. Strong reactions may be obtained even after 4-8 hours. This is a good test for separating *Shigella* species (Christensen positive from the Christensen citrate negative *E. coli*.

COAGULASE TEST (SLIDE): Using an inoculating loop make a heavy milky suspension of the staphylococcus organism in a drop of distilled water on a microscope slide. The suspension must be homogenous. Flame the loop and add a loopful of rabbit plasma to the suspension mix. Coagulase production is denoted by almost immediate chumping of the suspension. This test differentiates coagulase positive *Staphylococcus aures* from coagulase negative *Staphylococcus epidermidis*.

COAGULASE TEST (TUBE): Aseptically pipette 0.5mL of rabbit plasma into a sterile small tube and inoculate heavily with a 24-hour culture of the organism. Incubate at 37°C in a water bath. Complete or partial coagulation in 1 to 4 hours is interpreted as a positive test for free coagulase and identifies the organism as *Staphylococcus aureus*.

CELATIN LIQUIFICATION (TUBE): Heavily inoculate the organism into 15% gelatin tubes and incubate for 48 hours or longer at 37°C. To read the results place the tubes in a refrigerator for approximately 15 minutes. If gelatinase production has occurred the

gelatin will be liquid after removal from the refrigerator. If the organism does not produce gelatinase the gelatin in the tubes will be completely solid after removal from the refrigerator.

H_2S Production: Blackening along the line of inoculation or some part of the medium indicates that hydrogen sulfide has been produced and has reacted with iron in the medium to give the black iron sulfide precipitate. Triple sugar iron (TSI) or SIM (sulfide, indol, motility) medium can be used. The utilization if lead acetate paper strips is a more sensitive method for detection of H_2S production. These strips can be placed into TSI or SIM medium just above the agar surface. The H_2S reacts with lead acetate to give lead sulfide and the strip turns black.

IMVIC Reaction: This strands for indol. methyl red, voges – proskaner and citrate.

This pattern of biochemical tests is most often used for the differentiation of *E. coli* from the Klebsiella – Enterobacter groups of organisms.

	I	M	Vi	C
E. coli	+	+	-	-
Klebsiella-Enterobacter	-	-	+	+

Indol Production: (Sulfide Indol, Motility), SIM medium is inoculated with an inoculating needle to the bottom of the tube and incubated at 37^{o}C for 18-24 hours. Add a dropperful of chloroform to the tube to extract the indol if present. Next add equal amount of KOVAC;s reagent. The presence of a red color indicates the production of indol from the amino acid tryptophane. A yellow color indicates that the organism does not produce typtophane deaminase and there is no splitting off of indol from tryptophane.

LITMUS Milk Test: Litmus milk medium containing litmus blue indicator is inoculated and incubated at 37^{o}C for 48 hours or longer. A number of reactions can be obtained:

(a) A pink color indicates an acid reaction due to the fermentation of lactose.

(b) A purple or blue color, which is an alkaline reaction, indicates no fermentation of lactose.

(c) Coagulation or Clot formation is caused by the precipitation of casein due to the acid produced from the lactose fermentation. *Clostroidium perfringes*, an etiological of gas gangrene, will produce tremendous amount of gas in the medium thus literally causing the clot to explode. This is referred to as "storming fermentation".

Lysine Decarboxylase Test (Lysine Iron Agar Slant): Inoculate the slant and stab the butt to the bottom of the tube. After 24 hours incubation at 37^{o}C a lysine decarboxylase positive organism will give alkaline conditions (purple) in both the slant and butt. A lysine decarboxylase negative organism will give a yellow butt and alkaline slant. The formation of a black precipitate in the medium is indicative of H_2S production. So any intestinal gram negative rod is lysine decarboxylase positive and H_2S positive may be a *Salmonella sp*. Or Arizona and further biochemical and serological studies are warranted.

Note: The majority of Arizona and *Salmonella sp*. are H_2S positive. *Salmonella paratyphi A* is lysine decarboxylase negative and H_2S negative.

Methyl Red Test: Inoculate a tube of MRVP (methyl) red Voge-Proskaver) broth and incubate for 2-4 days at 37^{o}C. If upon the addition of 5 drops methyl red indicator the broth turns red, the test is considered MR positive and indicates a mixed acid type of fermentation from glucose. A negative methyl red test is denoted by a yellow color. *E. coli* is MR positive and *Klebsiella-Enterobacted* Group of organisms are MR negative.

ONPG-Test: A tablet of ortho-Nitrophenyl beta-galactopyranoside (ONPG) is dissolved is distilled water and inoculated with a heavy suspension of the organism to be tested followed by incubationa t 37^{o}C for 6 hours. Hydrolysis of ONPG is detected by the liberation of ortho-Nitrophenyl, with characteritistic yellow color, often within 30 minutes. Thus a positive ONPG test indicates that the organism contains lactose-fermenting enzymes and may be classified as lactose fermenter.

The ability of certain gram negative bacilli to ferment lactose is a useful criterion for the identification of certain members of the family Enterobacteriaceae.

Examples**:** *E. coli* is lactose positive while shigella sp. and salmonella sp. are lactose negative.

Oxidase Test (Plate): Place a drop of oxidase reagent (1% N, N-Dimethyl – P-phenylenediamine Monohydrochloride) on a group of colonies. A positive indophenols oxidase test is indicate by the development of a pinkish color in the colonies. Oxidase reagent is unstable and should not be used if it is black and contains a precipitate. The plate oxidase test is most useful for screening colonies of *Neisseria gonorrheal* from other colonies that constitute the normal, flora of vaginal cultures.

Oxidative-Fermentative Test (OF): Inoculate two tubes of OF Dextrose semisolid medium by stabbing the medium. One of the inoculated tubes is covered with 2mL of sterile mineral oil to provide anaerobic conditions and the other is left uncovered. The tubes are then incubated at 37^{o}C for 48 hours or longer. Fermentative organisms will produce and reaction (yellow) in both the covered and uncovered media. Oxidase organisms will reduce an acid reaction in the uncovered medium and yield slight to no growth without change (green) in the covered medium. A non-oxidative, non-fermentative organism will

produce no change in the covered tube and no change or slightly alkaline reaction (blue) in the open tube.

Sugar fermentations: This test consists of a basal broth which contains a 1% concentration of the carbohydrate being-tested, i.e. glucose, maltose, lactose, etc. Broncresol purple is the indicator employed. The medium is inoculated using an inoculating loop and incubated at 37^{o}C for 18-24 hours. If the sugar is not fermented the medium will remain neutral or turn slightly alkaline which is indicated by a purple color. If the sugar is fermented by the organism the acid end products will cause the indicator to turn yellow. Sugar fermentation patterns are useful in the differentiation and identification of many microorganisms especially the enteric bacteria.

Urea hydrolysis: Using an inoculating loop inoculate the organism onto the slant of the urea agar. Incubate at 37^{o}C for 24-28 hours. If the medium turns red, urea has been hydrolyzed by the bacterial enzyme urease resulting in the formation of ammonia. This test is most useful in differentiating the urea positive *proteus sp*. from the urea negative *providence group*.

$$NH_2 - CO - NH_2 + H_2O \longrightarrow 2NH_3 + CO_2$$