PRACTICAL NOTE BOOK ON BACTERIOLOGY (MIPA-220)

Registration no.....

Group.....

Level.....

Semester.....

Session.....

Microbiology and Parasitology Department
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Experiment no…..

Selection, collection and transportation of sample for laboratory diagnosis of bacterial infections

The term 'clinical specimen' denotes those materials, e.g. tissues, blood, urine, skin scrapings, body fluids, taken from animals for diagnostic purposes. Such materials must reach to the diagnostic laboratory with as little change as possible from their original state.

A recurring problem in Clinical Veterinary Microbiology results from the submission of unsatisfactory specimens with little or no history or no clinicians' comments. Veterinarians should know the appropriate techniques on the selection and shipment of specimens.

Just prior to death and shortly thereafter a number of intestinal bacteria may invade the host's tissues. Live sick animal presented for necropsy are usually the best source of specimens.

Preservation and Shipment

1. ***Tissues and organs***
    a. Asepsis should be practiced as much as possible in collecting and handling materials for culture.
    b. Tissues should be placed in individual plastic bags or leak proof jars.
    c. Portions of intestines should be packed separately at last. During collection the two ends of the intestine should be ligated.
    d. Specimen should be conveniently shipped in an ice-chased containing a generous amount of ice. Ice with plenty of insulation is preferred for longer preservation. Formalin should not be used to preserve sample for bacteriological investigation as these sorts of chemicals can kill the microorganisms.
    e. Brain send for examination should be halved longitudinally. Half is refrigerated or frozen over ice and the other is placed in 10% formalin for histopathological examination. Tissues in formalin should not be frozen.
    f. An open rib from a small animal or a 4 to 5 in aseptically cut piece of rib from a large animal will often yield the causative bacterium in pure or nearly pure culture. Muscle or periosteal tissues should be removed from the ribs before submission.
2. ***Swabs***

    Swabs are of value in many instances for the transportation of infectious materials to the laboratory. However, because many bacteria are susceptible to desiccation during shipment, it

is advisable to place the swabs in non-nutritional transport medium, as for example, Stuart's transport media.

3. ***Urine sample***

   Urine should be collected aseptically by mid stream, catheter or bladder tap. After collection, urine should be refrigerated immediately as it can support the growth of bacteria.

4. ***Blood sample***

   Blood samples are taken when there is a reason to suspect a clinically significant bacteraemia. Because only a small number of bacteria may present in the blood of animal with bacteraemia, 3-10 ml of blood depending upon the size of the animal is taken aseptically. If there is no anticoagulant in the media an anticoagulant should be added when the blood is taken.

   The vacutainer culture tube is particularly convenient for small animals as it contains an anticoagulant and support the growth of microorganisms and because the blood is inoculated directly from the animals the chances for contamination are reduced.

5. ***Feces***

   Fecal samples should be obtained directly from the rectum. Because of contamination, "ground droppings" should be avoided.

6. ***Milk***

   Milk should be collected from animals aseptically in sterile screw-capped or stoppered vials. Examinations may be negative if samples are taken during treatment.

Fig. 1: Etiologic agents logo that must be affixed to the outside of any package containing potentially hazardous and infectious biologic materials

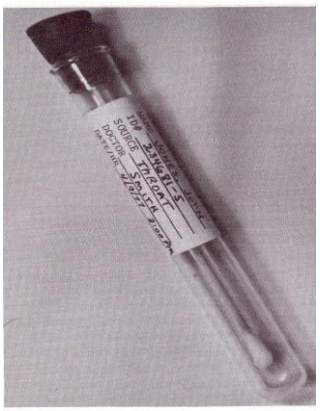


Fig. 2: A culture transport tube with a properly written identification label

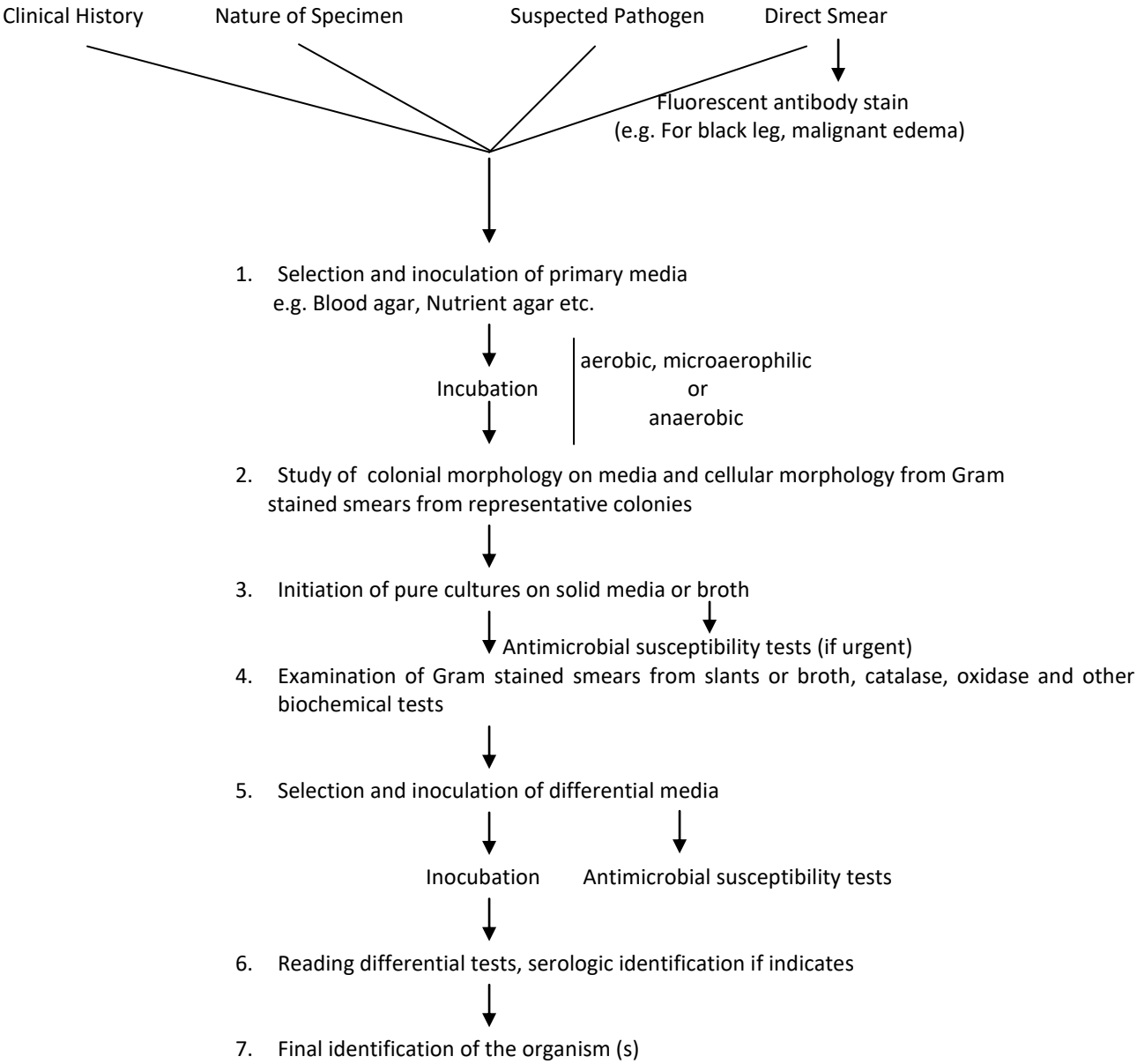
Information to be sent with the Samples

1. Name and address of the owner
2. Disease(s) suspected
3. Samples submitted, tests required and date of sampling
4. Description of the spread of infection in the herd or flock
5. Number of animals dead, the number showing clinical signs
6. A list and description of the samples examined during post-mortem examination and observed findings
7. Any medication and vaccination already applied to the animals and when given
8. Name and address of the sender

Experiment no…..

Isolation and identification of bacteria

Steps usually followed in the isolation and identification of bacteria from clinical specimens:



Primary Inoculation of Media

1. ***Inoculation from tissues and organs***

   Sear the surface of the specimen with a hot spatula, then incise with a sterile scalpel. From this incision, material is transferred to media with an inoculation loop or Pasteur pipette.

2. ***For small specimens***

   The external surface of the specimen is sterilized by holding it with sterile forceps and passing it through a Bunsen flame several times. It is then sectioned with sterile scissors and the exposed surface is impressed on the agar surface. The inoculum is then spread with an inoculating loop.

The two goals of primary inoculation are-

i) To cultivate organisms and

ii) To obtain discrete colonies. From the colonies pure cultures are obtained

Experiment no…..

Preservation of bacteria

Objectives

- To produce modified live vaccines
- To maintain stock cultures of the bacteria for teaching and research purposes.

Methods of Preservation of Bacteria

1. **Lyophilization**

   Although somewhat laborious, lyophilization or freeze drying is very useful process for the preservation of almost all bacteria and mycoplasmas. Several apparatus are available commercially including 'Belco' all glass ampoule freeze drying apparatus. Prior to be dispensed in ampoules, organisms are suspended in a protective medium such as the well known 'Mist Dessicans'. One volume of nutrient broth with 30% glucose is mixed with three volumes sterile inactivated serum. After lyophilization, the ampoules are sealed, and then kept at 2 to 8°C.

2. **Maintenance Media**

   Many gram negative organisms and some gram positive organisms can be maintained in a viable state in Stock Culture Agar (SCA, Difco). The medium is dispensed in screw cap or rubber stopper tubes. The unslanted medium is stabbed several times, incubated, and then stored in the dark at room temperature. Many aerobes and anaerobes including *Clostridia* remain viable for many months in tubes of sealed cooked meat medium at room temperature.

3. **Deep Freezing**

   A. i. Grow the organisms on a blood plate or other suitable medium.
   ii. Place 0.5 ml of defibrinated blood in a small, sterile tube.
   iii. Suspend loopfuls of bacteria in the blood, store in deep freeze, preferably at -70°C.
   iv. To recover, remove the tube and allow the edge of the blood to thaw. Remove loopfuls of the melted blood
   from between the frozen plug of blood and the wall of the glass tube.
   v. Plate a suitable medium, and then return the incompletely thawed tube to the freezer.

   B. i. Prepare lawn of pure culture of bacteria on blood agar plate and incubate 48 hours.
   ii. Add 1 ml of sterile 2% glycerol water solution to the surface of the plate.

iii. Pour 20 to 30 sterile glass beads (4mm) on to the plate and swirl the beads to coat with the bacteria.

iv. Store in deep freeze, preferably at -70°C.

v. A frozen bead can be removed and placed on a blood agar plate when a fresh culture is desired.

4. Liquid Nitrogen

Freezing in liquid nitrogen (-196°C) is the preferred method of long term storage.

Experiment no…..

Cultural procedures employed for clinical specimens

Summary of Some Routine Cultural Procedures

Sl. No.	Specimens	Organisms	Media	Atmosphere	Incubation
01	Organs, Tissues, Pus, Urine	Aerobes (Not Enterobacteria)	Blood Agar	Aerobic	37°C
02	Feces, Fecal Swabs, Intestine	Enterobacteria	Mac Conkey Selinite Brilliant green Endolevina	Aerobic	37°C
03	Milk	Aerobes	Blood agar	Aerobic	37°C
04	Organs, Tissues, Pus, Swabs, Intestinal contents etc.	Anaerobes(*Clostridia*)	Thioglycollate Cooked meat media Blood agar	Anaerobic	37°C
05	Organ, tissues	Microaerophilic *Brucella*, *Campylobacter*	Blood agar	5-10% CO_2	37°C
06	Intestinal content, Suspected enteroxemia	-	Mouse/Rabbit inoculation	-	-
07	Organs, Tissues, Pus, Swabs	Fungi in general	Sabouraud agar	Aerobic	22°-25°C

Biochemical tests

Biochemical reaction

Microorganisms act on a wide variety of chemical substances with the help of a large number of individual enzymes they possess. During the process of biochemical reactions, they produce a lot of energy for doing work and also utilize some metabolites for their growth and reproduction. To find out the metabolic activities of a cell, either the disappearance of some substances from the medium or the appearance of some metabolic products or end products can be detected. The pattern of metabolic activity helps in the differentiation of microorganisms from one another.

Experiment no…..

Carbohydrate fermentation test

Purpose

This test is used to determine the ability of an organism to ferment various simple carbohydrates (sugars). The fermentation characteristics are used in identification of the bacteria.

Principle

Fermentation is a metabolic process. Each medium has a single fermentable carbohydrate added to a peptone medium. Phenol red is also added as a p^H indicator. A small tube (Durham's tube) is inverted and placed in each larger test tube of liquid medium. The inverted tube is able to trap any gas products.

The indicator, phenol red will turn yellow below p^H 6.8 and a darker pinkish-red above p^H 7.4. If the organism metabolizes the carbohydrate, subsequent acid production will result in lowered p^H. If the organism does not ferment the carbohydrate, the p^H may remain neutral. If the organism does not ferment the carbohydrate and also utilizes the peptone, accumulation of the ammonia as a degradation product will raise the p^H.

Materials

Bacterial culture, Durham's tube, Sugars (glucose, sucrose, lactose, maltose etc.)

Procedure

1. Label sugar tubes properly in a rack.
2. With the help of a sterile Pasteur pipette, inoculate one tube of each sugar with 2 drops of bacterial culture. Keep second tube of each sugar as uninoculated control.
3. Incubate at 37°C for 48 hours.

Interpretation

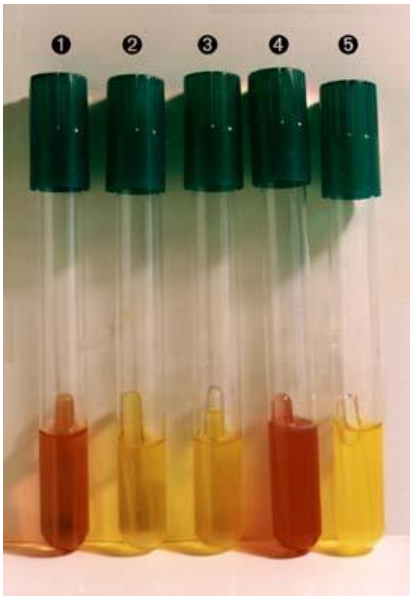
Acid (yellow): Acid production produces a color change from red to yellow, indicating that the organism is capable of metabolizing the sugar in the tube.

Acid, Gas (yellow plus gas bubble): Fermentation of the sugar is indicated by a color change to yellow. Gas is trapped in the Durham's tube, replacing the medium in the tube. A bubble indicates gas production.

Negative: Negative fermentation can be indicated by two ways:

1. No color change in the tube means that the sugar was not utilized by the organism.

2. Color change to a dark, pinkish-red: this darker color indicates an alkaline or basic metabolic product which is due to the utilization of the peptone, rather than the sugar. If the tube is read within 48 hours, the darker red color would be an indication of negative fermentation; although the result is usually recorded as alkaline.



Glucose Fermentation

Organism	Test Result
1. Control	Negative
2. *S. aureus*	Acid
3. *P. vulgaris*	Acid, Gas
4. *P. aeruginosa*	Negative
5. *E. coli*	Acid, Gas

Experiment no…..

Coagulase Test

Purpose

This test is used to detect the ability of certain *Staphylococcus* species to clot plasma. Since coagulase production is a characteristic of the potentially pathogenic *Staphylococcus aureus*, it is a useful test for identifying these gram positive, catalase-positive cocci and differentiates it from the other species of staphylococci.

Principle

Coagulase is a protein having a prothrombin like activity capable of converting fibrinogen into fibrin, which results in the formation of a visible clot in a suitable test system. Coagulase is believed to function in vivo by producing a fibrin barrier at the site of staphylococcal infection. This probably plays a role in localizing the abscesses (e.g. carbuncles and furuncles).

Coagulase is present in two forms, bound and free.

Bound coagulase (slide test): Bound coagulase, also known as clumping factor, is attached to the bacterial cell wall and is not present in the culture filtrates. Fibrin strands are formed between the bacterial cells when suspended in plasma (fibrinogen), causing them to clump into visible aggregates.

Free coagulase (tube test): Free coagulase is a thrombin-like substance present in culture filtrates. When a suspension of coagulase-producing organisms is prepared in plasma in a test tube, a visible clot forms as the result of coagulase reacting with a serum substance form a complex which, in turn, reacts with fibrinogen to produce the fibrin clot.

Media and Reagents

Rabbit plasma with EDTA (commercially available in lyophilized form).

Procedure

i. Slide test

1. Sufficient bacteria are emulsified in a drop of water on a microscope slide to yield a thick suspension.
2. The suspension is stirred with a straight wire that has been dipped in suitable plasma.

Interpretation

A positive reaction is indicated by clumping within 5 sec.

ii. Tube test

1. To two drops of an overnight broth culture in a tube (One loopful of organisms from a solid medium is also suitable) add 0.5 ml plasma diluted 1:5 with sterile physiological saline and mix.
2. Bring to 37°C in the incubator or place in a 37°C water bath for 2 hours.

Interpretation

If the strain is coagulase positive, the plasma will clot usually within 4 hr. Observe again at 24 hr. Partial clotting is considered a positive test.

Fig: Coagulase test

Experiment no…..

Methyl red test

Purpose

This test is used to identify bacteria that produce stable acid end products by means of mixed acid fermentation of glucose.

Principle

Methyl red is a p^H indicator with a range between 6.0 (yellow) and 4.4 (red). The methyl red test is a quantitative test for acid production, requiring positive organisms to produce strong acids (lactic, acetic, formic) from glucose. The color of the medium will be distinctly red after the addition of methyl red indicator. Some organisms may produce sufficient acidity to lower the p^H to 4.2 in the beginning but subsequently metabolize the acids and give rise to various neutral products (ethyl alcohol, acetyl carbinol, diacetyl etc.). The duration of incubation period of the culture is therefore very important to conduct the test.

Media and Reagents

a. MR/VP broth

Polypeptone	7 g
Glucose	5 g
Dipotassium phosphate	5 g
Distilled water to	1 L

Final p^H = 6.9

b. Methyl red p^H indicator

Methyl red, 0.1 g, in 300 mL of 95% ethyl alcohol

Distilled water, 200 mL

Procedure

1. Inoculate the MR/VP broth with a pure culture of the test organism.
2. Incubate the broth at 35°C for 48 to 72 hours.
3. At the end of this time, add 5 drops of the methyl red reagent directly to the broth.

Interpretation

The development of a stable red color in the surface of the medium indicates sufficient acid production to lower the p^H to 4.4 and constitutes a positive test. Because other organisms may produce smaller quantities of acid from the test substrate, an intermediate orange color between yellow and red may develop. This does not indicate a positive test.

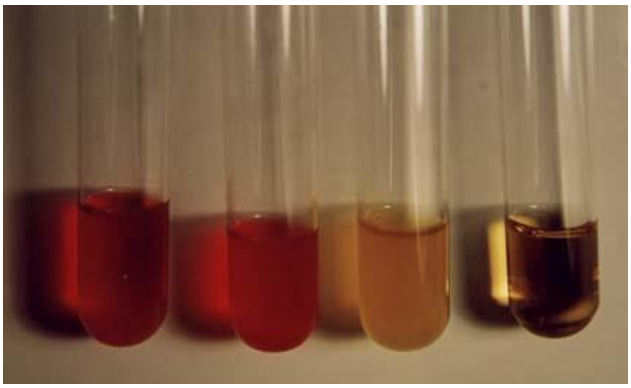


Fig.1: Above are tubes of MR-VP broth that have been inoculated, incubated for 48 hours, and the Methyl Red reagent has been added after 48 hrs. of growth.
Left to Right: positive, positive, negative, control.

Fig. 2: The methyl red reagent

Experiment no…..

Voges Proskauer Test

Purpose

This test is used to identify organisms able to produce acetoin from the degradation of glucose during a 2,3-butanediol fermentation. Both the MR and VP tests are especially useful in differentiating between members of the Enterobacteriaceae.

Principle

This test depends upon the ability of the organisms to produce acid from glucose and subsequently to convert them to a neutral product acetyl methyl carbinol. By the addition of alkali followed by vigorous shaking of the tube, acetyl methyl carbinol is oxidized to diacetyl. Diacetyl reacts with guanidine group of arginine which is present in the medium. This reaction produces a pink color.

Reagents

Solution 1:

5% α-naphthol in absolute ethyl alcohol.

Solution 2:

40% KOH containing 0.3% creatine.

Procedure

1. Transfer 1 ml of a 48 hr culture (37°C) grown in MR-VP broth or glucose phosphate broth.
2. Add 0.6 ml of solution1, then 0.2 ml of solution2.
3. Shake well and leave 5-10 min.

Interpretation

A bright orange-red color develops and gradually extends throughout the broth if acetyl methyl carbinol has been produced.

Alternative Procedure

1. Add 5 ml of 10% KOH to 5 ml of culture.
2. Mix well and allow standing exposed to air.
3. Observe at intervals of 2, 12 and 24 hr.

Interpretation

A positive test is indicated by the development of an eosin pink color.

Fig. 1: VP test
Right: positive (red color)
Left: uninoculated control

Fig. 2: VP reagents
Left is reagent A (alpha-napthol) and
Right is reagentB (KOH--potassium hydroxide)

Experiment no…..

Urease Test

Purpose

This test is used to differentiate organisms based on their ability to hydrolyze urea with the enzyme urease. This test is particularly useful in distinguishing the genus Proteus from other enteric bacteria.

Principle

Urease is an enzyme possessed by many species of microorganisms that can hydrolyze urea to form ammonia and carbon dioxide. The ammonia reacts in solution to form ammonium carbonate, resulting in alkalinization and an increase in the p^H of the medium.

$$\text{Urea} \xrightarrow[H_2O]{\text{Urease}} \text{Ammonia} + CO_2$$

$$\underset{p^H < 8.1}{\text{Phenolphthalein (colorless)}} \xrightarrow{\text{ammonia}} \underset{p^H > 8.1}{\text{Phenolphthalein (pink-red)}}$$

Media and Reagents

Stuart's urea broth and Christensen's urea agar are the two media most commonly used in clinical laboratories for the detection of urease activity.

Stuart's urea broth	
Yeast extracts	0.1 g
Monopotassium phosphate	9.1 g
Disodium phosphate	9.5 g
Urea	20 g
Phenol red	0.01 g
Distilled water to	1 L
Final p^H = 6.8	

Christensen's urea agar	
Peptone	1 g
Glucose	1 g
Sodium chloride	5 g
Monopotassium phosphate	2 g
Urea	20 g
Phenol red	0.012 g
Agar	15 g
Distilled water to	1 L
Final p^H = 6.8	

Procedure

1. The broth medium is inoculated with a loopful of a pure culture of the test organism.
2. The surface of the agar slant is streaked with the test organism.
3. Both media are incubated at 35°C for 18 to 24 hours.

Interpretation

A positive reaction is indicated by a pink or red color as urea hydrolysis increases alkalinity. If negative there is no color change.

Spot Urease Test

Procedure

1. Prepare a solution of urea reagent and store in the refrigerator.
2. A circular filter paper is placed in a petri dish then moistened with several drops of the reagent.
3. A portion of a colony is rubbed onto the moist filter paper with a wooden stick.

Interpretation

A color change to pink or red, usually within 2 minutes, indicates urease activity.

Fig: In this row of urea broth, each of which has been inoculated with a different G^{-ve} organism, the only organism which is positive for urea hydrolysis is *Proteus vulgaris* --the tube which has turned pink, third from the left.

Experiment no…..

Oxidase Test

Purpose

This test is used to identify bacteria containing the respiratory enzyme cytochrome oxidase. This is especially useful in distinguishing the oxidase negative Enterobacteriacae from Pseudomonadaceae, that are oxidase positive.

Principle

Cytochrome oxidase is an enzyme found in some bacteria that transfers electrons to oxygen, the final electron acceptor in some electron transport chains. Thus, the enzyme oxidizes reduced cytochrome C to make this transfer of energy. If the enzyme is present, the colorless dye will turn a purple to blue color. No color change is a negative test.

Reagent

Prepare a 1% solution of (0.1 gm in 10 ml distilled water) of *p*-aminodimethylaniline monohydrochloride. The dye is added to the distilled water and allowed to stand for 15 min prior to using. The solution should be kept in a brown bottle and refrigerated. The solution is satisfactory for approximately a week.

Procedure

The dye solution is added to portions of plate cultures containing suspicious colonies.

Interpretation

Colonies producing cytochrome oxidase become pink, changing to red indicates positive test.

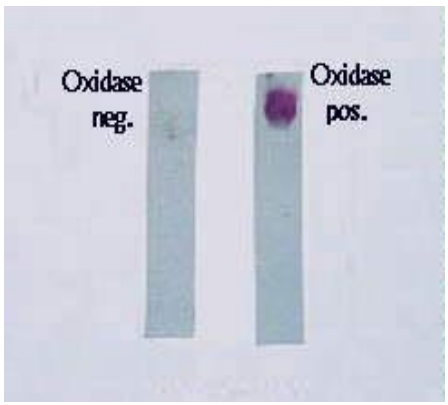


Fig: Oxidase test

Experiment no…..

Catalase Test

Purpose

This test is used to determine those organisms that produce catalase enzyme. It is used to distinguish the catalase positive Micrococcaceae such as *Staphylococcus*, from the catalase negative Streptococcaceae, which include the *Streptococcus* and *Enterococcus* genera.

Principle

Catalase is an enzyme that decomposes hydrogen peroxide into water and oxygen. Hydrogen peroxide forms as one of the oxidative end products of aerobic carbohydrate metabolism. If allowed to accumulate, it is lethal to bacterial cells. Catalase converts hydrogen peroxide into oxygen and water as shown by the following reaction:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Procedure

1. A slant culture is used. 1 ml of 3% solution of hydrogen peroxide is poured over the growth.
2. The slant is tilted so that the solution covers the growth.

Interpretation

A rapid ebullition of gas indicates a positive reaction.

Alternatively,

The test can also be performed transferring a small amount of growth, preferably a single colony, from solid medium to a microscope slide. A drop of fresh hydrogen peroxide (3%) added, and then a cover slip is applied. The production of gas bubbles constitutes a positive reaction.

Experiment no…..
Gelatin Liquefaction

Purpose

This test is used to determine the ability of a microbe to produce hydrolytic exoenzymes called gelatinases that digest and liquefy gelatin.

Principle

The exoenzyme gelatinase hydrolyzes gelatin, a protein derived from collagen. The enzymes first hydrolyze the gelatin into polypeptides, and then further break down the polypeptides into the smaller amino acid molecules. These can then be easily transported into the bacterial cells.

Procedure

1. The prepared gelatin medium is inoculated heavily with the test organism.
2. It is incubated up to in the incubator at 37°C.
3. The media is tested after incubation period.

Interpretation

If the test organism produces gelatinase, the medium will remain liquid after being kept in cold water.

Fig: Hydrolysis of Gelatin
***Escherichia coli*: negative (Gelatin is solid)**
***Pseudomonas aeruginosa* (Gelatin has been liquefied)**

Experiment no…..

Hydrogen Sulphide Production

Production of hydrogen sulphide by the microorganisms is tested in the medium containing sulphur compounds like cystine, methionine and glutathione. Hydrogen sulphide produced by the breakdown of sulphur compounds can be detected by the incorporation of a heavy metal salt (e.g. lead, iron etc.) into the medium. Hydrogen sulphide reacts with the metal to form black metal sulphides. The production of hydrogen sulphide is usually detected by hanging a lead acetate filter paper strip in the culture tube.

Lead acetate + Hydrogen sulphide → Lead sulphide + Acetic acid

Procedure

1. Inoculate a slant and insert a strip of lead acetate filter paper in between the side of the tube and cotton plug so that it hangs freely into the tube up to the height of about an inch from the top of the medium.
2. Incubate the tube at 37°C for 48 hours.

Interpretation

Hydrogen sulphide production will turn the paper brown or black by converting lead acetate to lead sulphide.

Experiment no…..

Indole Test

Purpose

This test is used to identify the bacteria that possess the enzyme tryptophanase, capable of hydrolyzing and deaminating tryptophan with the production of indole.

Principle

Bacteria utilize various amino acids as their food. Tryptophane, one of the amino acids, may be utilized by certain bacteria producing indole as its by-product. Indole can be detected in the medium by the colorimetric test on addition of suitable reagents. To carry out this test, it is essential to have tryptophane in the medium. This is normally present in most of the proteins.

Indole production of a test culture can be determined by any of the following three methods-

A. By indole test reagent
B. Spot indole test by strip of filter paper saturated by indole reagent
C. Oxalic acid test paper for indole

A. By indole test reagent

Reagent

1. *p* dimethylaminobenzaldehyde	2 gm
2. 95% Ethyl alcohol	190 ml
3. Conc. HCl	40 ml

Procedure

1. 1 ml of ether or xylene is added to 5 ml of 48 hours culture.
2. After shaking well, it is allowed to stand until the ether or xylene rises to the top.
3. Gently run 0.5 ml of the reagent down the side of the tube.

Interpretation

If indole has been accumulated by the ether or xylene, a brilliant red ring will develop just below the ether or xylene layer. If there is no indole, no color will develop.

B. Spot indole test by strip of filter paper saturated by indole reagent

Reagent

1. *p* dimethylaminobenzaldehyde	2 gm
2. 95% Ethyl alcohol	190 ml
3. Conc. HCl	40 ml

Procedure

1. For each day a strip of filter paper is saturated with the indole reagent.
2. With an inoculating loop, colonial growth is rubbed on the filter paper.

Interpretation

A positive test is indicated by a blue color which usually appears within 30 seconds.

C. Oxalic acid test paper for indole

Procedure

A piece of filter paper is soaked in saturated oxalic acid solution. Dry and cut into strips approximately 10 mm to 50 mm. These are suspended over the medium and held in place by the cotton plug.

Interpretation

Indole is indicated by the presence of pale pink color at the lower end of the test paper.

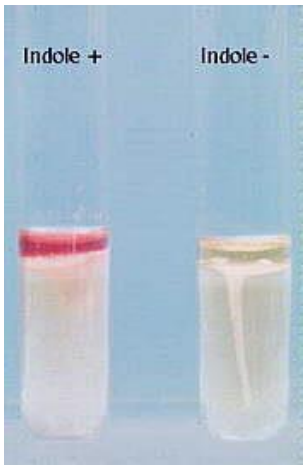


Fig: Indole test

Experiment no…..
Nitrate Reduction Test

Purpose

This test detects the ability of an organism to reduce nitrate (NO_3) to nitrite (NO_2) or some other nitrogenous compound, such as molecular nitrogen (N_2), using the enzyme nitrate reductase.

Principle

Nitrate (NO_3) may be reduced to several different compounds, either by anaerobic respiration or by denitrification. This test is used to detect whether or not the reduction has taken place. The nitrate medium contains potassium nitrate as the substrate. If the organism reduces the nitrate to nitrite, the nitrite will react with added reagents sulfanilic acid and α-naphthylamine to produce a red color. If no color is produced, this can indicate either of two reactions: (1) the nitrate was not reduced (2) the nitrate was reduced even further to compounds other than nitrite.

(To distinguish between the negative reaction, or the complete reduction, zinc dust is added. If nitrate remains in the medium, zinc will reduce it to nitrate, and a pink color is observed. This is a negative reaction. No color change after zinc is added means that nitrate has been reduced to compounds other than nitrite. This is interpreted as positive and is often calling positive complete to distinguish it from the first positive test discussed.)

In this test nitrate present in the medium disappears in the form of its reduction products, i.e. the nitrite, ammonia or free nitrogen. In the test the presence of nitrite in the culture medium is considered positive for the nitrate reduction test.

Reagents

Solution 1:

α –Naphthylamine	5.0 g
5 *N* acetic acid (sp. gr 1.041)	1.0 liter

Filter through clean absorbent cotton.

Solution 2:

Sulfanilic acid	8.0 g
5 *N* acetic acid (sp. gr 1.041)	1.0 liter

Procedure

Add to 5 ml of trypticase nitrate broth culture 1 ml of solution 2, followed by 1 ml of solution 1 added drop by drop.

Interpretation

If nitrite is present, a red, pink or maroon color develops.

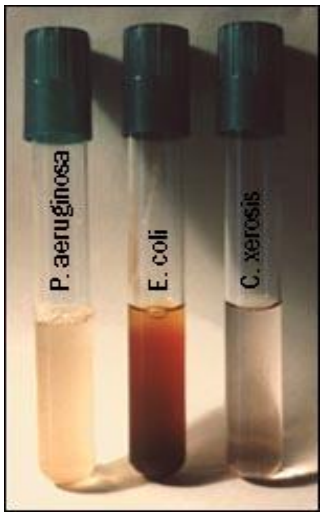


Fig: Nitrate reduction test

***P. aeruginosa*: Negative; *E. coli*: Positive; *C. xerosis*: Negative**

Experiment no…..

Citrate Utilization Test

Purpose

The citrate utilization test is used to determine the ability of an organism, using the enzyme citrase, to use citrate as its sole carbon source.

Principle

Sodium citrate is a salt of citric acid found as one of the metabolites in the TCA cycle. Some microorganisms use citrate as a sole source of carbon and energy. The utilization of citrate by a test bacterium is detected in citrate medium by the production of alkaline by-products. Simmon's citrate agar is a defined medium containing sodium citrate as the sole carbon source and the ammonium ion as the sole nitrogen source. Bacteria that can use citrate can also extract nitrogen from the ammonium salt, with the production of ammonia, leading to alkalinization of the medium. Bromthymol blue – yellow below p^H 6.0 and blue above p^H 7.6 – is the indicator.

Media and Reagents

The citrate medium most commonly used is the formula of Simmons. The medium is poured into a tube on a slant.

Procedure

1. A well-isolated colony is picked from the surface of a primarily isolation medium and inoculated as a single streak on the slant surface of the citrate agar tube.
2. The tube is incubated at 35°C for 24 to 48 hours.

Interpretation

A positive test is represented by the development of a deep blue color within 24 to 48 hours, indicating that the test organism has been able to utilize the citrate contained in the medium, with the production of alkaline products.

Fig: Citrate Utilization test

Experiment no…..

H_2S, Indole Production and Motility Detection in SIM Media

Procedure

1. Inoculate the medium with a straight stab to a depth of two inches.
2. To test for indole, an oxalic acid test paper is suspended over the medium at the time of inoculation and held in place by cotton plug. Indole production is recorded as pink color on the paper.
3. Motility is evidenced by diffuse growth producing turbidity throughout the medium.
4. H_2S production is indicated by blackening of the medium

Fig: *Staph. aureus* (Non motile, negative for H_2S production)

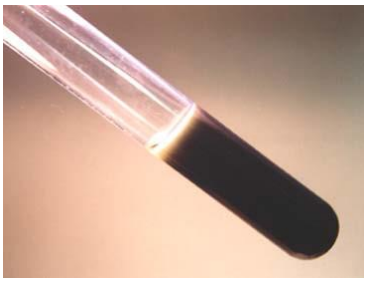


Fig: *Sal. typhimurium* (Motile, positive for H_2S production)

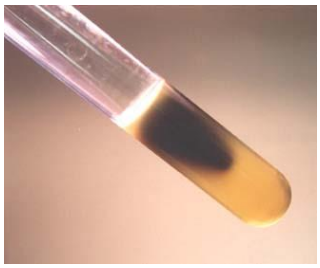


Fig: *Proteus vulgaris* (Motile, positive for H_2S production)

Differentiation of *Bacillus anthracis* and *Bacillus cereus*

Characteristics	*Bacillus anthracis*	*Bacillus cereus*
Motility	-	Almost always positive
Capsulation	+ve	-ve
Penicillin	Susceptible	Resistant
Hemolysis	-ve or very weak	Often markedly hemolytic
Growth at 45°C	Slow	Rapid
Gelatin hydrolysis	Slow (3 – 7 days)	Rapid

Some Differential Characteristics of Important Species of *Clostridium*

Species	Nitrate	Indole	Acid Production					Urease
			Glucose	Maltose	Lactose	Salicin	Sucrose	
Cl. perfringens	+	-	+	+	+	Variable	+	-
Cl. septicum	+	-	+	+	+	+	-	-
Cl. chauvoei	+	-	+	+	+	+	+	-
Cl. novyi A	-	-	+	+	-	-	-	-
Cl. haemolyticum	-	+	+	-	-	-	-	-
Cl. botulinum	-	-	+	Variable	-	Variable	Variable	-
Cl. tetani	-	-	+	+	-	+	+	-

Appearance of Important Enterobacteria on Selective Media

Organisms	Brilliant Green Agar	Salmonella-Shigella (S.S) Agar	MacConkey Agar
E. coli *Enterobacter* *Klebsiella*	Inhibited. If present are yellowish-green.	Inhibited. If present are red.	Grow and are red. *Enterobacter* and *Klebsiella* may be larger and mucoid.
Salmonella	Grow. Red due to peptone hydrolysis.	Grow; colorless. H_2S producers: dark centers.	Grow; colorless.
Proteus	Grow; don't spread; yellowish-green. Sucrose negative: strains are colorless.	Grow; don't spread, colorless. H_2S producers: dark centers.	Grow and spread. Colorless.

Reaction Noted on TST Agar Slants

Appearance	Reactions
Slant – Red; Butt – Yellow	Glucose +ve but lactose –ve
Butt and slant – Yellow (acid through out the medium)	Glucose, lactose and/or sucrose are fermented.
Gas bubbles in butt and medium frequently split	Gas production
Butt shows blackening	H_2S +ve
Unchanged or Red butt and slant	None of the three sugars fermented.

Biochemical Differentiation of *Salmonella pullorum* and *Salmonella gallinarum*

Test	*Salmonella pullorum*	*Salmonella gallinarum*
Glucose	+	-
Dulcitol	-	+
Maltose	-	+
Ornithine	+	-
Rhamnose	+	-

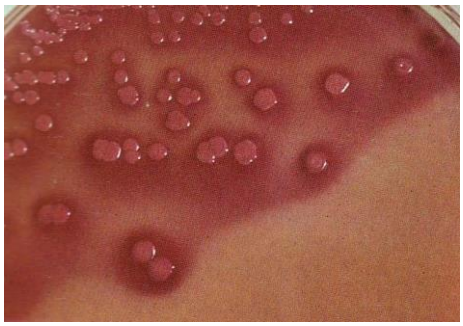


Fig. 1: Growth of *E. coli* on MacConkey agar

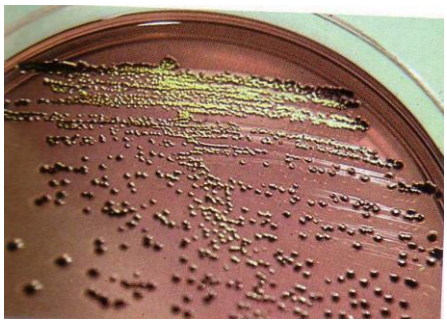


Fig. 2: Growth of *E. coli* on EMB agar

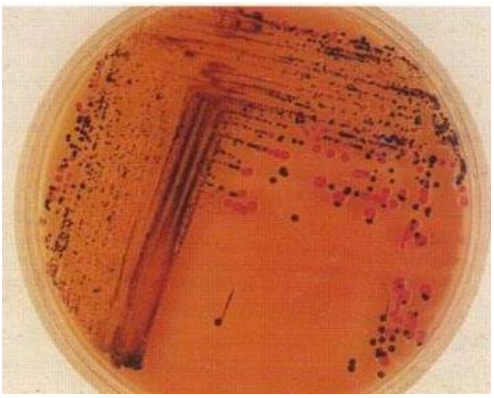


Fig.3: Colonies of *Salmonella* (black color) on SS agar

Differentiation of important *Brucella* species

Species	CO_2 Requirement	Urease	H_2S Production	Agglutination		Growth in presence of	
				A	M	Basic Fuchsin 1:100,000	Thionin 1: 100,000
Brucella melitensis	-	+ (slow)	-	-	+	+	+
Brucella abortus	+	+ (slow)	Moderate (2-3 days)	+	-	+ (except biotype 2)	- (except biotypes 1, 2 & 4)
Brucella suis	-	+ (rapid)	Heavy (4-5 days)	+	-	- (except biotypes 3 & 4)	+
Brucella canis	-	+ (rapid)	-	-	-	-	+

Experiment no…..
Antimicrobial sensitivity test

Principle

A thin uniform inoculum of the test strain is exposed to a disk of known concentration of antimicrobial agent. The antimicrobial agent from the disk gradually diffuses into the agar and creates a concentration gradient of the drug. The susceptibility of the organism to the agent is indicated by a clear zone of inhibition around the disk. The diameter of the zone of inhibition is directly proportional to the susceptibility of the organism tested. Absence of a zone of inhibition around the drug reservoir indicates complete resistance.

Procedure

1. Petri dishes are prepared with Mueller-Hinton agar 5 to 6 mm in depth. (For fastidious organisms add 5 % blood).
2. Plates are allowed to dry for a minimum of 30 minutes before inoculation.
3. A suspension of the test organism is prepared by either of the following methods:
    a. Select a few colonies from the original culture plate and place in a tube containing suitable broth medium (e.g. Tryptose phosphate or tryptose soy broth). Incubate the tube at 37°C for 2 to 5 hours. The suspension is then diluted with sterile water of saline to a density visually equivalent to a standard prepared by adding 0.5 ml of a 1.175 % barium chloride to 99.5 ml of 1 % sulfuric acid (or 0.5 MacFarland Nephelometer standard turbidity tube which is commercially available).
    b. Make a direct saline or broth suspension of colonies from a nutrient non-selective agar plate that has been incubated for 18 to 24 hourS and immediately adjust the inoculum to the standard density.
4. Streak the suspension of the test organism evenly in three planes onto the surface of the medium with a cotton swab (rotating the plate approximately 60° to ensure an even distribution of the inoculum). Surplus suspension is removed from the swab by gently rotating the swab against the sides of the tubes prior to inoculation of the plate.
5. Permit the inoculum to dry for 5 to 30 minutes, and place the discs on the agar with flamed forceps or a disc applicator. Space the discs so that there is no overlapping of the zones. Discs should be located 10 to 15 mm from the edge of the plate.
6. Gently press each disc with sterile forceps to ensure contact with the agar surface.
7. Incubate the plates overnight at 37^0C.

Results

- Measure zone diameters on the underside of the plate using a metric ruler or with calipers held near the surface of the medium (16 to 18 hours is the standard time at which the zone diameters are measured, although it can be measured as early as 4 hours). The end point is taken as complete inhibition as determined by the naked eye. If several individual colonies develop within the zone of inhibition, the culture should be checked for purity and retested. If such colonies are still present, they should be regarded as significant growth. With sulfonamides, the organisms will grow through several generations before inhibition takes place; therefore, slight growth is ignored and the margin of heavy growth is read to determine zone size.
- Record the zone diameters and interpret them according to table.

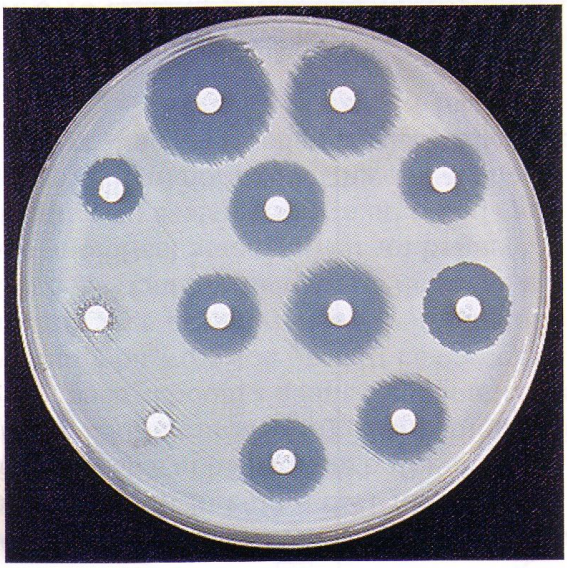


Fig: An antibiogram. Following the application of antimicrobial discs, the inoculated plate is incubated at 37°C for 18 hours. The diameter of zone of inhibition are measured and compared to internationally accepted measurements to determine the susceptibility or resistance of the isolate