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Expt. No. 1 **Date: -**

i) Isolation and identification of *Staphylococcus aureus*

Aim: - To isolate and identify the *Staphylococcus aureus* from the sample provided.

Theory / Approach: -

Staphylococci are Gram positive, cocci shaped, occur in grape like cluster, about 1 µ in diameter and nonmotile, nonsporeforming bacteria. Their ability to develop resistance to penicillin and other antibiotics is important in human pathogenicity.

They produce deep wound infections leading to pus formation, Osteomyelitis, arthritis, tonsilitis, pharyngitis, meningitis, endocarditis and some strains cause food poisoning.

Most strains of *Staphylococcus aureus* produce golden yellow pigment, though some may be white, orange or yellow. The pigment production occurs optimally at 22 ^{0} C and in aerobic cultures.

On sheep blood agar most strains produce Beta type of hemolysis (zone of clearing or complete lysis without a marked colour change).

Several selective media have been deviced which include mainly 8 to 10 % NaCl.

They produce Coagulase, Catalase, Urease, Phosphatase and Gelatinase enzymes. Ferment mannitol. MR and VP positive.

Requirements: -

1. Specimen / Sample
2. Mannitol Salt Agar

D. Water	100 ml
Mannitol	1 gm
Peptone	1 gm
NaCl	7 gm
Meat extract	0.1 gm
Phenol red	2.5 mg
Agar Agar	2.5 gm
pH	7.4

Sterilization: - At 10 lbs/inch^2 (115 ^{0} C) for 30 minutes.

3. Sterile Sheep blood agar plate
4. Sterile Mannitol sugar tube
5. Human plasma for coagulase test
6. 3 % Hydrogen peroxide solution for catalase test

Procedure: -

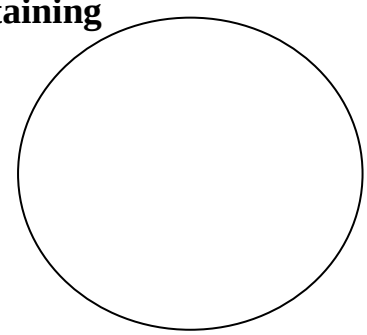
1. The given specimen is streaked on sterile Mannitol Salt agar and incubated at 37 ^{0} C for 24 hours.
2. Golden yellow coloured colony is studied for colony characters.
3. Suspension from well-isolated colony is prepared and Gram staining is done.
4. A loopful of suspension is inoculated in sterile mannitol tube and incubated at 37 ^{0} C for 24 hours.
5. One loopful is streaked on sterile sheep blood agar plate and plate is incubated at 37 ^{0} C for 24 hours.
6. Coagulase test is done by adding loopful of suspension in a drop of plasma on the slide and observed for coagulation.
7. Catalase test is done by immersing part of colony with the help of nichrome wire loop into 3% Hydrogen peroxide solution and observed for effervescence of oxygen bubbles.

Observation: -

1. Colony characters

Size	
Shape	
Colour	
Margin	
Elevation	
Opacity	
Consistency	

2. Gram staining



3. Beta hemolysis observed / not observed

4. Pink colour observed / not observed in mannitol tube.

5. Clumping is observed / not observed in coagulase test.

6. Effervescence of oxygen bubbles observed / not observed in catalase test.

Result: -

From the cultural, colony, morphological and biochemical characteristics, the isolated bacterium is *Staphylococcus aureus.*

Conclusion: -

The cultural, colony, morphological and biochemical characteristics of isolated species agree with characteristics of *Staphylococcus aureus* therefore it is concluded that the isolated bacterium is *Staphylococcus aureus.*

Expt. No. 1 **Date: -**

ii) Isolation and identification of *Salmonella species*

Aim: To study the morphological, cultural and biochemical study of ***Salmonella*** species.

Introduction: The genus ***Salmonella*** consists of Gram negative bacilli that parasitise the intestine of man (and large number of vertebrate species) leading to gastroenteritis, enteric fever, septicemia and the carrier state. The important member of the genus is ***Salmonella typhi***, which causes typhoid fever. Salmonellae comprise over 1800 species. All are potentially pathogenic. They may be divided into (1) Enteric fever group (2) The food poisoning group. ***S.typhi, S. paratyphi*** **A** and usually ***S. paratyphi*** **B** are confined to man. ***Salmonella typhimurium*** is common food poisoning salmonellae.

Clinical significance

● ***Salmonella typhi***

This species causes typhoid fever. The reticuloendothelial system, gallbladder and kidneys become infected when the bacteria pass from the small intestine into blood through lymphatic system. These organisms invade the intestine causing inflammationa and ulceration. Symptoms of the infection include fever, headache, toxemia, enlargement of spleen and low pulse rate.

S. tphi and S. paratyphi B produce black coloured colonies due to production of H_2S, while S.typhi produce green coloured colonies due to non ability to produce H_2S.

Requirements:

1) Blood or stool sample
2) Sterile Wilson and Blair's medium plate

Composition of the medium: -

Solution A: - Bismuth sulphite Glucose Phosphate mixture

D. Water	100 ml
Bismuth Ammonio citrate	3 gm
Sodium sulphite	10 gm
Na_2HPO_4 . 12 H_2O	10 gm
Glucose	5 gm

Dissolve Bismuth Ammonio citrate in 25 ml boiling water and Sodium sulphite in 50 ml boiling water. Mix two solutions and boil. Add the Disodium phosphate crystals while boiling the mixture. Cool. Add the glucose dissolved in 25 ml boiling water and cooled.

Solution B: - Iron Citrate Brilliant Green Mixture

Ferric Citrate solution (1% in sterile D/W)	200 ml
Brilliant Green solution (1% in sterile D/W)	25 ml
Mix together	

Complete Medium: -

Sterile Nutrient Agar	100 ml
Solution A	20 ml
Solution B	4.5 ml

5. Biochemical media

a. Glucose Phosphate Broth

d. Tryptone water

e. Koser’s citrate medium

f. Christensen’s Urea medium

g. Sugars: Xylose, Glucose, Lactose, Mannitol, Sucrose, Maltose,

6. Reagents:

a. Oxidase reagent

b. Hydrogen peroxide

c. Methyl Red

d. Kovac's reagent

e. α-Naphthol reagent

f. Sulphanilic acid

Procedure:

1) Streak a loop-full of the sample on Wilson and Blair's medium Agar.

4) Keep in the incubator at 35^{o}C for 24 hrs.

5) Observe for colony characters form the plates.

6) Perform Gram staining and motility.

7) Inoculate various biochemical media from a single colony of the organisms.

7) Keep in the incubator at 35^{o}C for 24 hrs.

8) Read the results.

Expected observations

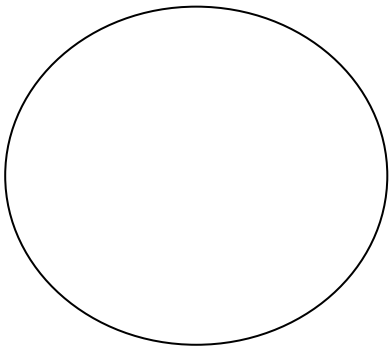
(1) Gram staining : Gram negative bacilli

(2) Motility : Motile organisms

(3) Cultural characters (colony characters)

● **Organisms**

Size		
Shape		
Colour		
Margin		
Elevation		
Opacity		
Consistancy		
Gram nature		



Biochemical reactions:

	S. typhi reactions	**S. paratyphi A reactions**	**S. paratyphi B. reactions**
Oxidase	-	-	-
Urease	-	-	-
TSI	Acid butt and alkaline slant and little H_2S	Acid butt and alkaline slant and no H_2S	Acid butt and alkaline slant and excessive H_2S
Indol	-	-	-
Methyl Red	+	+	+

Vogus Prauskauer	-	-	-
Citrate	-	-	Different stains gives different result
Nitrate	+	+	+
Gelatin	-	-	-

Sugar fermentation test:

	S. typhi reactions	S. paratyphi A reactions	S. paratyphi B. reactions
Xylose	+ Acid	-	+Acid, Gas
Glucose	+ Acid	+Acid, +Gas	+Acid, Gas
Sucrose	-	-	-
Maltose	-	-	-
Lactose	-	-	-
Mannitol	+	+ Acid	+Acid, Gas

Result & Conclusion: -

From the cultural, morphological and biochemical characters the isolated bacterium is Salmonella typhi / Salmonella paratyphi A / Salmonella paratyphi B.

Expt. 2 **Date**

Isolation & Identification of *Candida albicans*

Aim- To isolate and identify *Candida albicans.*

Theory / Approach

Yeasts are oval, unicellular fungi that reproduce by budding. Most are dimorphic (grow as both molds and yeasts). The parasitic form occurs in host tissues and on enriched media at 37°C and generally appears as **yeast-like** cells. The saprobic form occurs in nature and on special media at room temperature (25°C), and appears as **hyphae**. The parasitic form can be converted to the saprobic form by changing the environmental conditions. The genus Candida comprises several dozen species of which at least eight have been reported to cause diseases in man. *Candida albicans* is the most important pathogen in the group. While Candida species are common members of the normal flora of the nasopharynx and gastrointestinal tract, they may cause severe cutaneous, mucocutaneous (e.g., oral thrush) and systemic infections. Conditions favoring infection are long-term antibiotic therapy, and suppression of cell-mediated immunity by chemotherapy or radiation. The organism is dimorphic; the yeast form occurs in the upper respiratory and intestinal tracts and in the vagina, while the hyphal or invasive form is found in mucosal and cutaneous lesions and in deeper tissues. Infection is initiated when a significant alteration in the mucosal or cutaneous tissue occurs, allowing the yeasts to produce hyphae and pseudohyphae that penetrates the epithelium.

Isolation of yeast from oral swabs or blood specimen can be done on Corn meal agar. Identification of *C. albicans* is based upon germ tube production, or production of terminal chlamydospores on hyphae and pseudohyphae grown on special media at 25°C.

i. It is small, oval and yeast like oval fungus
ii. It shows pseudomycelial growth & multilateral budding. The cell elongates & develops into pseudomycelium
iii. The genus comprises of imperfect forms of ascomycetous & basidiomycetous yeasts of various genera.

iv. *C. albicans* is the imperfect state of *Syringospora,* which is a member of basidiomycotina.

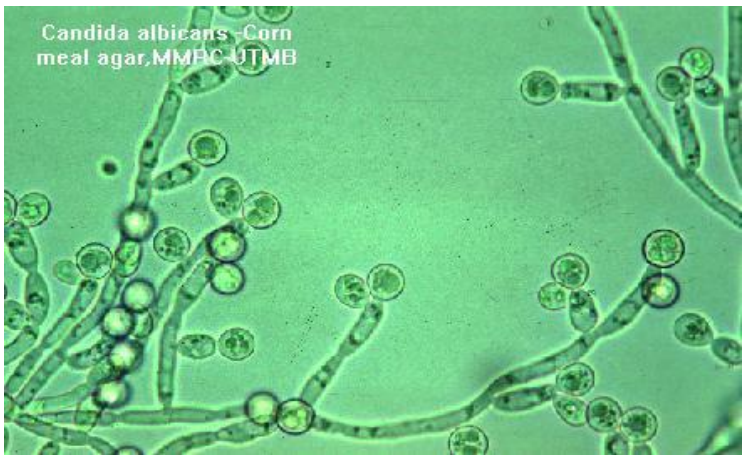
v. They form chlamydospores & produce spherical clusters of blastospores

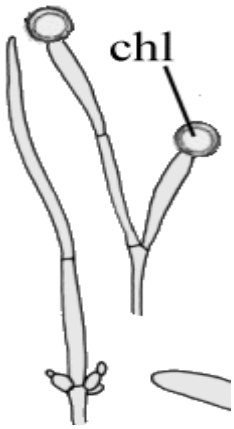
vi. The colonies are moderate in size, smooth & pasty. The older colonies have honey comb like appearance in the centre & develop radial furrows.

vii. They possess a capsular form of polysaccharide that shows pyrogenic activity.

Clasification

Division- Deuteromycotina
Class- Blastomycetes
Order- Cryptococcales
Family- Cryptococcaceae
Genus- *Candida*
Species- *albicans*





Requirements

1. Corn Meal agar
2. Given sample from patient
3. Gram staining set
4. Serum
5. India ink

Composition of corn meal agar

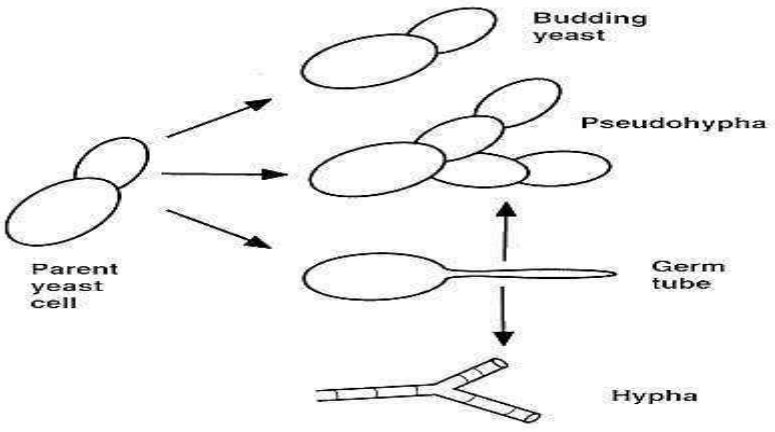
Water	100 ml
Corn meal	4 gm
Agar	2 gm
pH	6.8

Procedure:

1. Streak the given sample on Corn Meal agar plate and incubate at 25^0 C for 24 hours.
2. Examine the agar plate cultures, and observe the colonial morphology of these organisms.
3. Prepare a Gram stain and observe and compare their microscopic morphologies. They are distinctly different.
4. Demonstrations at the front table:

GERM TUBE FORMATION:

A small amount of each yeast was suspended in individual tubes containing serum. The test tubes were incubated at 37°C for 2 to 3 hours. One drop of each yeast-serum suspension was placed on a glass slide along with India ink, to be observed as a wet preparation.



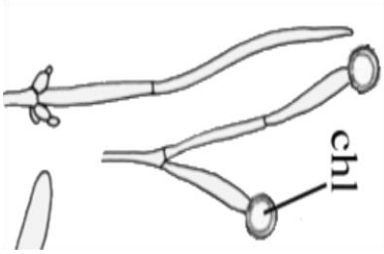
Examine each slide under the microscope for the presence of germ tubes.

Organism	Germ Tube Formation
Candida albicans	+
Other Candida species	-

CHLAMYDOSPORE PRODUCTION:

Examine the stained microscope slide and photographs for the presence of large, round thick-walled chlamydospores.

Organism	Chlamydospores Present
Candida albicans	+
Other Candida species	-



Observation: - Germ tube and Chlamydospores are observed.
Result: - ***Candida albicans*** is isolated from patient's sample

Expt. No. 3 **Date: -**

DEMONSTRATION OF HAEMOLYSIN AND PLASMA COAGULASE TEST

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a. DEMONSRATION OF HAEMOLYSIN

The blood agar plate cultures show zones of haemolysis on the growth of haemolytic bacteria such as Staphylococci, Streptococci, etc. This test is used to study the ability of the pathogen to show the haemolysis. It is classified as alpha and beta-haemolysis.

Principle:

The lysis of RBC with reduction of haemoglobin to methaemoglobin by the bacteria results in a greenish halo around the bacterial growth indicates alpha-haemolysis while complete destruction of RBC results in a clear zone surrounding the colonies is due to beta-haemolysis.

Requirements:

1. Blood agar plate
2. Test organism such as Staphylococci

Procedure:

1. Prepare the blood agar plate
2. Spot inoculate at the centre of the plate by using the test bacterial sample.
3. Incubate the plate at 37 ^{o}C for 24 hours.
4. On incubation observe the plate for haemolysin.

Observation: -

Clear zone of haemolysis is observed / not observed.

Result: -

Haemolysin is produced / not produced

Conclusion: - The given bacterial culture has ability / no ability to produce haemolysin.

Expt. 3b **Date**

Demonsration of plasma coagulase test

Aim: - To demonstrate coagulase test

This test is used to study the ability of a pathogen to synthesize the coagulase. This test is helpful in the identification procedure of the bacteria like ***Staphylococcus aureus*.** It is used to differentiate ***Staphylococcus aureus*** from ***Staphylococcus epidermis*** and ***Staphylococcus saphrophyticus.***

Principle:

Staphylococcus aureus produces the enzyme coagulase which causes plasma to clot by converting soluble fibrinogen to insoluble fibrin.

Requirements:

1. Oxalate or citrated plasma
2. Bacterial test sample-*S**taphylococcus aureus***.
3. Glass slide
4. Normal saline

Procedure:

1. Place a drop of physiological saline at the center of a slide.
2. Make thick suspensions of the organisms in the drop of the saline.
3. Add a drop of plasma to the suspension. Mix gently. Look for clumping of the organisms within 10 seconds.
4. Observe for the coagulation of the plasma within two minutes.

Interpretation of Result:

1. Clumping within 10 seconds : ***Staphylococcus aureus***
2. No clumping within 10 seconds : No production of coagulase

Observation

Clumping is observed / not observed

Result

Coagulase is produced / not produced by *S. aureus*

Expt. No. 4 **Date: -**

Determination of antibiotic resistance of bacteria

Aim :-

To determine the resistance of bacteria to different antibiotics

Theory:

Antibiotic is the organic compound produced by one type of microorganism, which inhibits or kills the other kinds of microorganisms, even when it is present in very low concentration.

Alexander Fleming discovered antibiotic penicillin in 1929. Since then number of antibiotics are discovered. Narrow spectrum antibiotics are effective only against a limited variety of pathogens. Broad-spectrum antibiotics are effective against many different kinds of pathogens.

Antibiotics are used for the treatment of various diseases caused by microorganism. Some pathogens are sensitive to some antibiotics while some show resistance to that antibiotic.

Antibiotic resistance is becoming an extremely serious problem nowadays. Much of the difficulty arises from misuse and frequent overuse of antibiotics. Bacteria become resistant to antibiotics in several different ways—

→ By preventing entrance of antibiotic

→ By inactivating it through chemical modification

→ Because of modification of target enzyme or organelle

→ Use of an alternate pathway to bypass the sequence inhibited by the antibiotic or increases

the production of target metabolite.

→ Presence of genes for antibiotic resistance on both bacterial chromosomes and plasmids.

There are various methods of determination of antibiotic resistance. Generally paper disc method is used. In this method different antibiotic discs

containing known concentration of antibiotics are placed on lawn culture of test bacterium on a nutrient agar plate. After incubation, the antibiotic discs without any inhibition zone are noted. Thus it is concluded that the given pathogen is resistant to these antibiotics.

Requirements:

1. Sterile nutrient agar plate
2. Sterile melted and cooled to 45 ^{0}C nutrient agar in test tube.
3. Test culture of pathogen
4. Discs of different antibiotics

Procedure:

1. 0.1 ml of thick suspension of given bacterium is added in sterile melted and cooled to

45 ^{0}C nutrient agar tube.

2. Mixed well and poured on sterile nutrient agar plate.

3. After solidifications different antibiotic discs are placed with the help of sterile forceps on

the surface of it.

4. Plate is kept in freeze at 4 ^{0}C for 30 minutes for diffusion of antibiotics.

5. Then plate is incubated at 37 ^{0}C for 24 hours. Do not incubate plate in inverted position.

6. After incubation, the antibiotic discs without any inhibition zone are noted.

Observations:

Inhibition zone is not observed around the discs of following antibiotics

1

2

3

4

Result:

The given bacterial pathogen is resistant to above antibiotics.

Conclusion:

Zone of inhibition is not observed around the discs of

Antibiotics. Thus this bacterium is resistant to these antibiotics and they should not be used for the treatment of disease caused by this bacterium.

Expt. No. 05 **Date**

DETECTION OF SPECIFIC ANTIGEN BY ELISA

Aim: To detect specific antigen by using Enzyme-Linked-Immuno-Sorbant Assay (ELISA.)

Introduction:

The ELISA is an immunological technique which is used to measure either antigen or antibodies present in serum. For the measurement of antigens, the ELISA of antibody-coated wells is used and *vice versa*. In the former case, the test serum containing antigens is used in wells that combines with the specific antibodies immobilized on wells which are detected after adding the second labeled enzyme and substrate.

Requirements:

1. The test sample
2. Enzyme conjugate (antibody according to specific antigen is conjugated to enzyme.
3. Horse radish peroxidase is the commonly used enzyme. The commonly used cross-
4. linking agents are the glutaraldehyde and dimaleimide)
5. Substrate: Orthophenyldiamine (OPD) OR 5-amino salicylic acid)
6. Antibody microtest plate (96 well containing plates)
7. Diluent (phosphate buffer saline (PBS); 0.1 M, pH 7.2 or carbonate buffer pH 9.5 or normal saline with 0.1 % sodium azide.

(For preparation of PBS, take 18 ml of Na_2HPO_4 (14.32 g/200ml) solution and add to it 7 ml of K_2HPO_4 (5.44 g/200ml). Now add 4.38 g of NaCl and make the volume up to 500ml)

Procedure:

1. Dilute the antibody to 1:500 in PBS at pH 7.2 and add 75 mm to the bottom of each well.
2. Incubate the plates at 4^{o}C in a moist chamber overnight for 18-20 hours.
3. Wash the plates three times with PBS supplemented with 0.05 % Tween 20 (PBS-T) for 5 minutes each.

4. Add 200 ml of 1 % bovine serum albumin in each well and incubate the plates overnight at 4 ^{o}C in a moist chamber.

5. Add 25 ml of each test sample into the test plates coated with antibody.

6. Incubate the plates for 2 hours at room temperature.

7. Wash three times with PBS-T and add 50 ml of peroxidase conjugated antibody to each well.

8. Again incubate the plates at room temperature for 2 hours followed by five times washing with PBS-T.

9. Now, add 100 ml of freshly prepared substrate i.e. OPD in citrate buffer pH 5.0 and hydrogen peroxide.

10. After 30 minutes of incubation at room temperature in a dark box, add 75 ml of 2 M H_2SO_4 to stop the reaction.

11. Add 75 ml of PBS to make final volume of reaction mixture as 250 ml.

Recording of results:

Production of yellow colour as result of product of enzyme reaction shows a positive result. This is observed with naked eyes against a white background or by measuring the optical density at 492 nm by using a micro ELISA reader by using the following formula.

$$\text{O.D. of sample} = \frac{\text{O.D. of test sample} - \text{O.D. of blank II}}{\text{O.D. of Blank I} - \text{O.D. of blank II}}$$

A sample is labeled positive (i) if the yellow color is stronger than that of negative control, and (ii) if the optical density measured as the ratio at test to negative control is more than 2:1.

O.D. of blank I = It is average optical density of 5-7 negative controls

O.D. of blank II= It is the optical density of 100 ml substrate, 75 ml of 75 M H_2SO_4 add 75 ml of PBS.