CHAPTER 5

Conventional Identification Techniques

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Conventional detection of pathogenic bacteria is mainly based on cultivation procedures, which use enrichment broths followed by the isolation of colonies on selective media, biochemical identification and confirmation of pathogenicity. This culture method is selective for the search of one type of pathogen at a time.

Conventional techniques for identification of bacterial cultures includes:

1. Gram's staining and microscopy
2. Motility
3. Test for catalase
4. Penicillin sensitivity
5. H&L glucose O/F reaction
6. Cytochrome oxidase test
7. Fermentation of glucose
8. Pigmentation
9. Growth at zero NaCl level

a).Catalase test

Place a speck of young culture on a clean glass slide and flood with 2 drops of 3% H_2O_2 (H2O2 bottle is usually stored in a refrigerator). Bring the temperature of H_2O_2 to room temperature before the test). Evolution of gas from the culture indicates positive test for catalase. Note: Catalase is an enzyme, which decomposes H2O2.

b).Cytochrome oxidase test

Smear a little of the young culture on the test paper (already impregnated with Kovac's cytochrome oxidase reagent). Development of a blue colour in a few seconds indicates a positive test.

c).Penicillin sensitivity

Prepare pre-set antibiotic agar plates and dry the surface at 56°C for 45 min. Cool to room temperature. Divide each plate into 4 quarters by drawing lines on the bottom so that we can use one plate for 4 cultures. A little of the culture is smeared over about 4 cm2 area in each quarter.

A filter paper disk impregnated with penicillin (each disk contains 2.5 IU penicillin) is placed on the surface of each smear. Plates are incubated without inverting for 18-24 h. Examine for clear zones of inhibition around the discs. Cultures showing clear zones of inhibition are sensitive to 2.5 IU penicillin.

d).H&L glucose O/F reaction test.(Hugh &Leifson's oxidative verses fermentative reaction test using glucose as substrate)

Using a platinum wire (needle), a little of the culture is stab-inoculated into the H&L glucose O/F medium, in such a way that at least 2 cm long column of the medium at the bottom of the tube remains uninoculated. Incubate for 18-24 h and observe the changes. A colour change into yellow indicates acid production from glucose. A deepening of red colour of the medium indicates an increase in pH to alkaline level.

Growth of bacteria along the line of inoculation and an yellow colour throughout the medium indicate fermentative reaction (Fermentative with acid but no gas; FANG). If gas bubbles are also seen trapped in medium, reaction is fermentative with gas production (Fermentative with acid and gas; FAG).

If yellow colour appears only at the top part of the medium, the reaction is oxidative. Sometimes, a deep pink colour develops near the top surface, indicating change of pH to alkaline side (Alkaline top). In both these cases, the reaction is nonfermentative (NF).

e). Fermentation of glucose (Durham tube method)

Inoculate the culture into glucose fermentation broth and incubate. Note acid and gas production after 24-48 h. A deep pink colour shows acid production. Gas bubble in the Durham's tube shows fermentation of glucose with gas production.

f).Pigmentation

Note the colour of the bacterial culture on TGA slants after 72-96 h. Some bacterial cultures are pigmented yellow, red, pink, violet, brown or red.

g).Growth at zero NaCl level

Inoculate the culture to peptone medium without NaCl (P1No) medium and incubate. Note growth after 48 h. Turbidity indicates bacterial growth.

Gram's staining. Gram's staining, originally devised by Christian Gram (1884) is a differential staining. By this method of staining, bacteria are divided to two groups: Gram Positive and Gram Negative.

1. Gram's Staining

Staining solutions used are:

2. Gram's Crystal violet
1. Gram's iodine
2. Safranine
3. Ethyl alcohol for destaining

a) Preparation of smear

Only young cultures shall be used for staining. Usually 16-24 h old cultures are considered young. Take a dust free, dirt free and oil free microscopic glass slide. A speck of young culture is emulsified with a drop of sterile water in the middle of the slide and spread uniformly. Dry in the air. Fix by passing the slide 3-4 times through the blue flame of bunsen burner (Care not to char the smear).

- Staining
- Place the slide on a staining bridge.
- Flood the smear with Gram's crystal violet for 1 min.
- Wash with water.
- Flood with Gram's iodine for 1 min.
- Wash with water.
- Destain with dropwise addition of alcohol until washings are free from violet colour(Note: collect alcohol washings in a bottle for recovery of alcohol).
- Wash with water.
- Counterstain with Safraninefor 1 min.
- Dry in air.
- Microscopy

Observe the slides under the microscope using oil immersion objective (95X or 100X). Cells stained violet, bluish violet or bluish purple are Gram +ve. Cells stained red are Gram -ve. Note the shape, size and arrangement of cells. Also examine whether the culture is pure or mixed. In case the cells are G+ve rods, examine whether there is spore formation (Note: All G+ve rods are not spore formers).

- Motility of bacteria

Some bacteria exhibit the property of movement in a liquid phase, with the help of their flagella. This movement is referred as motility. Motility is observed under a microscope using high power objective (40X, 45X or 50X). Two methods are usually used to observe bacterial motility.

- Hanging drop method

A small drop of DW is placed on the middle of a cover slip, a speck of young culture from agar slant is emulsified with it. A cavity slide is taken, the margin of the cavity is smeared with a little paraffin jelly. The slide is inverted on the cover slip in such a way, that the cover slip gets attached to the slide and on turning upside down, the culture drop hangs into the cavity. Observe under the microscope.

2) Plain slide method

Make an emulsion of the culture in a drop of DW on an ordinary microscopic slide, place a cover slip on it and observe under microscope.

An actual movement of the cells (individually) indicates motility. Depending on the intensity and speed of movement, motility is referred as weakly motile, moderately motile and actively motile.

After conventional identification of bacterial cultures upto genus level, species level idenfication can be carried out by employing DNA based PCR assay.