UNIT 7 DETECTION OF PATHOGENS IN FOODS

7.0 OBJECTIVES

After going through this unit you should be able to:

- explain major bacterial pathogens found in foods;
- identify the common source of contamination of these pathogens;
- specify types of media used; and
- describe the detection protocol of various pathogens.

7.1 INTRODUCTION

Foods serve as important source of nutrition providing a range of valuable nutrients including essential amino acids, proteins, fats, carbohydrates, vitamins and minerals. However, prepared foods on contamination also act as carriers of harmful pathogenic bacteria such as *Staphylococcus aureus, E. coli, Vibrio, Listeria, Shigella* etc., which affect health and create symptoms ranging from minor food poisoning to deadly illness on consumption of such foods. In this context the ability to cause disease is known as **pathogenicity** and **virulence** is the notion applied to the degree of pathogenicity. The pathogens may exhibit pathogenicity towards both human as well as animals and hence pose a public health hazard. They cause diseases in their host either by invasion of tissues such as mucous membrane or/ and skin (infection) or by producing poisonous metabolites known as 'toxins'. Toxins may be termed as exotoxin or endotoxin on the basis of site of their production in cell. **Exotoxins** are secreted outside the cell (mostly in gram positive bacteria) while **Endotoxins** are part of outer cell wall of gram negative bacteria. On the basis of their site of action toxins may be classified as **Neurotoxins** (interfere with normal nerve impulses), **Cytotoxins** (kill cells) and **Enterotoxins** (infect the cells lining the gastro-intestinal tract). Detection of these organisms is very important for the food industry from the safety point of view as they can even pose serious threat to life and are part of regulatory requirement such as Prevention of Food Adulteration Act. This task is carried out by a separate quality control unit of any food industry. It is important therefore, to have easy and effective methods of detection of these harmful bacteria with good detection limits, which can be carried out with ease in a microbiological laboratory. Hence this unit is dedicated to provide the available microbiological detection methods of important food pathogens including *Staphylococcus aureus, Salmonella,* Coliforms, *Bacillus cereus, Listeria, Campylobacter and Clostridium* in an easily comprehensible way. The detection methods for viral pathogens are also discussed.

In the following sections the protocols of detection of various bacterial pathogens especially important from regulatory point of view have been described.

7.2 DETECTION OF *BACILLUS CEREUS*

Bacillus cereus is a common cause of food poisoning. It is an aerobic gram positive rod shaped spore forming bacillus and is widely distributed in soil, vegetables and several raw and processed foods. Consumption of foods containing greater than 10^6 *B. cereus* cells may lead to food poisoning. It causes food poisoning by enterotoxin production and its excessive growth in heat treated foods especially, dairy products e.g. pasteurized milk, pasteurized cream, Ultra High Temperature (UHT) milk, dried milk results in defects such as 'Bitty Cream' and 'Sweet Curdling'. Sterilization of food products either by autoclaving or ultra high heat treatment can reduce the risk of proliferation of this organism.

A. REQUIREMENTS

Butterfields phosphate buffered dilution water, mannitol-egg yolk-polymyxin (MYP) agar plates, trypticase soy-polymyxin broth, phenol red glucose broth, nitrate broth, reagent A, reagent B, MR-VP broth, α naphthol, potassium hydroxide, lysozyme

B. METHODS OF DETECTION

7.2.1 Plate count of *B. cereus*

1. Weigh 50 gms of sample in a sterile blender jar. Add 450 ml of sterile Butterfields phosphate buffered dilution water and blend for 2 min. at high speed. This is the 10^{-1} dilution.
2. Prepare serial dilutions by adding 10 ml of 10^{-1} dilution to 90 ml of diluent to give 10^{-2} dilution and so on and so forth so as to attain a final dilution of 10^{-6}.
3. Plate 0.1 ml of each dilution on the surface of MYP agar in duplicate and incubate the plates at 30°C for 24 h.
4. Observe for typical *B. cereus* colonies which will be of pink colour surrounded by a precipitate zone indicating the production of lecithinase.
5. Count the number of typical *B. cereus* colonies from the plates on which the colony numbers range from 15-150 colonies. Pick 5 or more representative colonies and transfer them to nutrient agar slants and incubate at 30°C for 24h. Carry out confirmatory tests from the growth obtained on nutrient agar slants, as described in the following sections.
6. Calculate the numbers of *B. cereus* cells per gm of sample, based on the percentage of colonies tested that are confirmed as *B. cereus* e.g. if average counts obtained for 10^{-5} dilution is 52 and all 5 out of 5 colonies tested were confirmed as *B. cereus* then the counts will be (52x5/5) x 10^6 = 52,000,000.

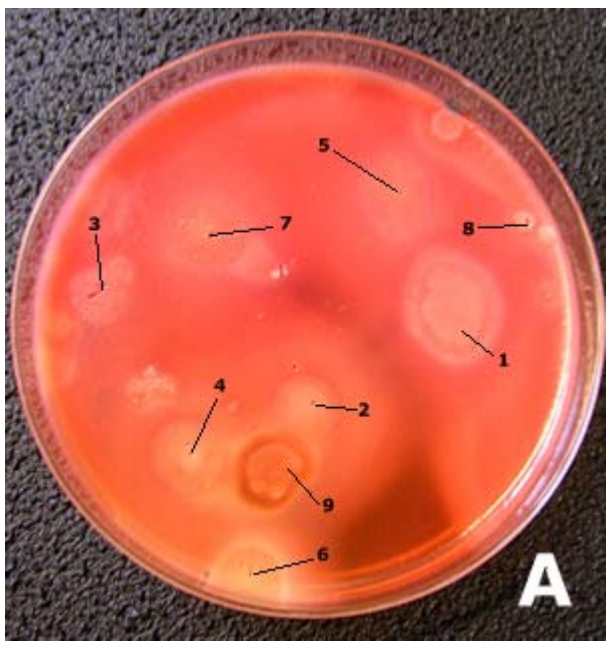

Fig 5.1: Growth of B. cereus on MYP agar

7.2.2 MPN for *B. cereus*

Most probable number (MPN) is carried out for the foods where the suspected count of *B. cereus* is fewer than 10 cells / g of food sample.

1. Inoculate trypticase soy-polymyxin broth tubes with 1 ml of 10^{-1}, 10^{-2} and 10^{-3} dilutions. Three tubes should be inoculated for each dilution.
2. Incubate tubes at 30°C for 48 h. Observe for dense growth.
3. Streak MYP agar plates from the tubes showing heavy growth. Incubate the plates at 30°C for 24 h and look for typical *B. cereus* colonies.

4. Confirm the isolates as *B. cereus* and calculate MPN of *B. cereus* per gram of sample (Appendix 3) based on the number of tubes that were positive for *B. cereus* isolates.

7.2.3 Confirmatory tests for *B. cereus*

1. Use the growth obtained on the nutrient agar for confirmatory tests.
2. Carry out gram and spore staining of the culture. *B. cereus* will appear as large gram positive bacilli in chains. The spores will be ellipsoidal, central to sub-terminal and will not swell the sporangium.
3. Prepare the suspension of bacterial cells from the slant in 0.5 ml of sterile phosphate buffered dilution water. Use this suspension to perform various tests as described.

(i) Glucose utilization in anaerobic conditions: Inoculate 3 ml phenol red glucose broth and incubate anaerobically (Gas Pack system) at 35°C. Observe for colour change to yellow that indicates a positive test for *B. cereus*.

(ii) Nitrate Reduction: The medium is evaluated for nitrate reduction by the addition of two reagents, Nitrate A Reagent (0.8% sulfanilic acid in 5N acetic acid) and Nitrate B Reagent (0.6% N, N-dimethyl-alpha-naphthylamine in 5N acetic acid), which detect the presence of a catabolic end product, and by the addition of Nitrate C Reagent, zinc dust, which detects the absence of remaining nitrate in the medium. Inoculate 5 ml of nitrate broth with a loopful of culture and incubate tubes at 35°C for 24 h. Add nitrite test reagents A and C and look for development of orange color. This indicates a positive test for nitrate reduction.

(iii) VP test: Inoculate 5 ml of VP broth and incubate at 35°C for 48 h. To 1ml of this culture add 0.6 ml of α naphthol solution and 0.2 ml of 40% potassium hydroxide. Mix vigorously and add a few crystals of creatine. Observe after keeping at room temperature for 1hr. Consider the appearance of pink colour as positive test.

(iv) Tyrosine decomposition: Streak the slant of tyrosine agar and incubate it at 35°C for 24 h. Observe for clearing of medium near growth, which indicates the degradation of tyrosine.

(v) Growth in presence of lysozyme: Inoculate 2.5 ml of nutrient broth containing 0.001% lysozyme with a loopful of culture. Keep inoculated nutrient broth tube without addition of lysozyme as a control. Incubate tubes for 24 h at 35°C. Observe for turbidity.

Tentatively identify the cultures by comparing the results produced by standard culture as given in the Table 7.1. Based on these tests it is difficult to differentiate *B. cereus* from other *Bacillus* species and some other tests such as motility, hemolysis and protein crystal formation may be carried out, but these tests too are not confirmatory for *B. cereus* as some strains of *B. cereus* are non-motile or weakly hemolytic. However, except *B. thuringenesis* other *Bacillus* species are not frequently encountered in food products so one may rely on these cultural methods as till date no other method is available for specific detection of *B. cereus*.

Bacillus cereus	
Extraction:	Sterile Butterfields phosphate buffer
	MYP agar (Positive: Pink coloured colonies)
	NA slant
MPN:	TSP broth (Positive: dense growth)
	Streak on MYP
Confirmatory:	Growth from NA slants
	Gram staining
	Spore staining
	Anaerobic glucose utilization
	Nitrate reduction
	VP
	Tyrosine decomposition
	Lysozyme resistance
Others:	Motility
	Hemolysis
	Protein crystal formation


Fig 7.2: Positive reactions of B. cereus for nitrate reduction and Catalase

Check your Progress Exercise 1

Note: a) Use the space below for your answer.

b) Compare your answers with those provided at the end of this unit.

Q 1. The colour of *B. cereus* colonies on MYP agar will be …….

Q 2. What is the principle of precipitate zone formation around *B. cereus* colonies on MYP agar?

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Q3. Describe the role of polymyxin in MYP agar?

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Q 4. What is the role of phenol red in phenol red glucose broth?

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7.3 Detection of *Campylobacter*

The genus *Campylobacter* includes gram negative bacteria that exhibit rod shaped morphology and darting motility. *Campylobacter* species contaminating foods include *C. jejuni, C. coli* and *C. lari*. They are the most frequently identified agents of acute infective diarrheoa in developing countries. Detection of these bacteria starts with the enrichment at lower incubation temperatures followed by isolation on selective agars and identification by biochemical and physiological tests.

A. REQUIREMENTS

Boulton broth, sodium hydroxide, Abeyta-Hunt-Bark (AHB) agar, hydrogen peroxide, oxidase reagent, carbol fuchsin, hippurate reagent, ninhydrin reagent, triple sugar iron (TSI) agar, 0.1% peptone water, nalidixic

acid, cephalothin, MacConkey Agar, glycine, sodium chloride, cysteine medium, nitrate broth, reagent A, reagent B.

B. METHODS OF DETECTION

7.3.1 Procedure

1. Weigh 25 g of sample in a sterile bag and add 100 ml Boulton broth to it. Shake it gently for 5 min.
2. Filter the contents of bag through a sterile cheese cloth in a sterile bag or flask. Adjust the pH of broth obtained after rinsing to 7.4 by 2N NaOH.
3. Keep the bag for pre-enrichment at 37°C for 4h under micro-aerophilic conditions (Campy gas generating system).
4. Further incubate the samples for enrichment, at 42°C for 24-48 h, using Campy gas envelope.
5. For isolation streak the enriched broth on AHB agar and incubate at 42°C for 24-48 h, anaerobically. Observe for typical colonies. *Campylobacter* produce thick translucent white growth to spreading, film-like transparent growth.
6. Check for motility as described in section 3.5.2. Cells picked from agar often demonstrate only "wiggly" motility, whereas those from broth swim rapidly in corkscrew motion.
7. If organism appear typical then re-streak on AHB agar without antibiotics and incubate at 42°C for 24-48 h.
8. Perform catalase test as described below (*Campylobacter* are positive for catalase):

   The enzyme catalase acts upon hydrogen peroxide and converts it to water and molecular oxygen. Hydrogen peroxide is toxic to bacterial cells and catalase serves as a mechanism for removal of this toxic compound.

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

   (i) Take a drop of H_2O_2 on a clean dry slide.

   (ii) Transfer growth from the TSA slant to the drop of H_2O_2.

   (iii) Observe the slide for the production of gas bubbles.

   (iv) *Camylobacter* is positive for catalase test.
9. Perform oxidase test by rubbing a loopful of culture on the surface of a filter paper dampened with oxidase reagent. The oxidase test identifies organisms that produce the enzyme cytochrome oxidase. Cytochrome oxidase participates in the electron transport chain by transferring electrons from a donor molecule to oxygen. In this test, an artificial final electron acceptor (N, N, N', N'-tetra-methyl phenylene-di-amine di-hydrochloride) is used in the place of oxygen. This acceptor is a chemical that changes color to a dark blue/purple when it takes the electron from the last element (cytochrome oxidase) in the electron transport chain. If the test organism produces cytochrome oxidase, the oxidase reagent will turn blue or purple within 15 seconds. All *Campylobacter* are positive for oxidase.
10. Carry out gram staining as described in the section 1.5.3 but with a variation. Use 0.5% carbol fuchsin as counter stain. *Campylobacter* are Gram negative.
11. Perform hippurate test by emulsifying a loopful of culture to 0.4 ml hippurate solution in a test tube. Incubate for 2 h at 37°C and add 0.2 ml of ninhydrin reagent. Agitate the tubes and re-incubate for 10 min. Appearance of violet colour is considered positive reaction. The test is positive only for *C. jejuni*.
12. Inoculate the TSI slants by stab and streak procedure. Incubate for 5 days at 37°C under micro-aerobic conditions. All *Campylobacter* spp. produce alkaline slant and alkaline butt. *C. jejuni* do not produce H_2S but strains belonging to *C. coli* and *C. lari* may produce H_2S. Blackening of the slant indicates H_2S production.
13. Inoculate oxidation-fermentation media for glucose utilization and incubate at 37°C for 4 days under micro-aerobic conditions. Observe for colour change. *Campylobacter* species do not utilize glucose.

7.3.2 Biochemical tests

Emulsify a colony into 5 ml of 0.1% peptone water. Use this as inocula for performing other tests such as:

(i) Antibiotic sensitivity: Swab the surface of AHB agar and place antibiotic disks of nalidixic acid and cephalothin. Incubate micro-aerobically at 37 °C for 24-48 h. Observe for inhibition zones. Presence of these zones indicates the sensitivity of the culture towards the above mentioned antibiotics.

(ii) Growth temperature tolerance: Streak a line of culture on the surface of 3 AHB agar plates. Four cultures may be streaked on a single plate followed by incubation of these plates at different growth temperatures (25°C, 35-37°C and 42°C) for 3 days under micro-aerobic conditions. More growth than the initial inoculum is considered as positive test.

(iii) Growth on MacConkey agar: Perform this test as described in the step above, but incubate only at 37°C for 3 days.

(iv) Growth in modified semi-solid media: Inoculate surfaces of the glycine, sodium chloride, cysteine and nitrate media with 0.1 ml diluted culture. Incubate all these tubes at 35-37 °C under micro-aerobic conditions for 3 days except nitrate media which is to be incubated for 5 days. Observe for growth after the end of incubation period. Growth will be in a narrow band pattern just under the surface.

- 1% glycine. Record ± growth.
- 3.5% NaCl. Record ± growth.
- H_2S from cysteine. Inoculate cysteine medium and hang a lead acetate strip from top, keeping cap loose. Do not let strip touch medium. Blackening of strip, even slightly, is positive reaction.
- Nitrate reduction. After 5 days, add nitrate reagents (Sulfanilic acid) A and (Napthylamine) B. Red colour is positive reaction.

Match the results of the above tests with the standard reactions exhibited by *Campylobacter* spp. as shown in Table 3.2 and identify the isolated bacteria.

Campylobacter	
Extraction:	Sterile Boulton broth pH 7.4
Enrichment:	In micro-aerophilic conditions
Culturing:	AHB agar/ 42°C (Positive: thick white growth to spreading film like transparent growth)
Others:	Motility Catalase test positive Oxidase positive Gram negative Hippurate test: positive violet colour TSI slant: no H_2S; alkaline slant Oxidation fermentation: under microaerophilic conditions (no glucose utilization) McConkey agar (37°C): positive

Check Your Progress Exercise 2

Note: a) Use the space below for your answer.
b) Compare your answers with those given at the end of the unit.

1) The nitrate test includes reagent A, B and C, what does it represent and how these reagents are involved to interpret the result?

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2) Is Campylobacter positive for catalase and oxidase test? What is the principle of catalase test?

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3) What is the name of oxidase reagent and how does it help to interpret the result in oxidase test?

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4) Describe the identification of *C. jejuni* by hippurate test?

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5) How H_2S will be identified in TSI slants?

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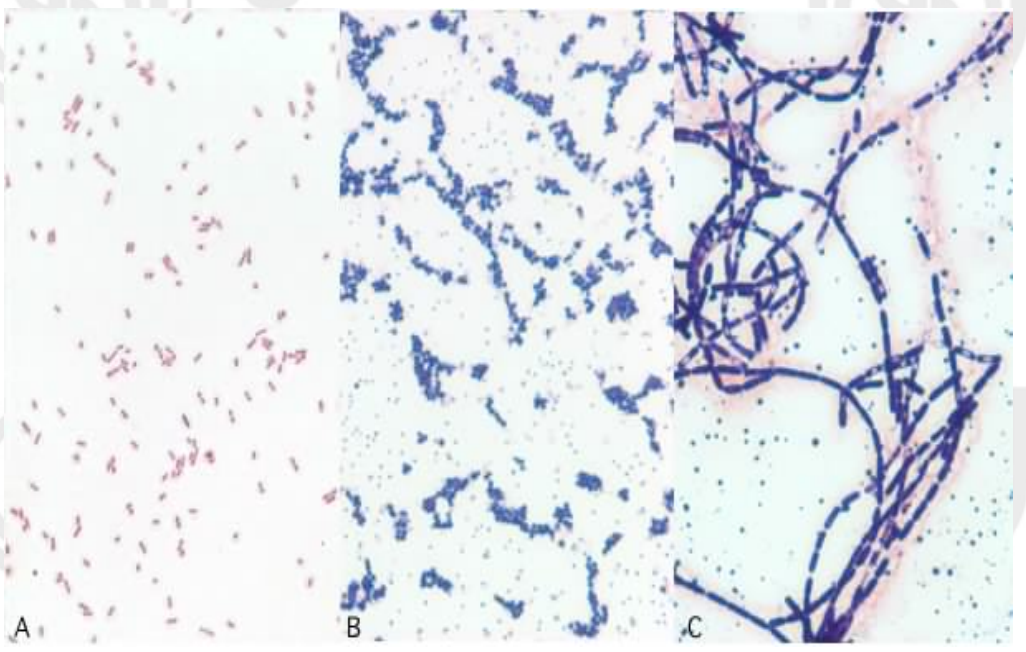

Fig 7.3: The Gram stain reactions of common microbes. *E. coli*, Gram negative (A), *Staphylococcus epidermidis*, Gram positive (B) and *Bacillus cereus*, Gram positive

7.4 DETECTION OF ESCHERICHIA COLI AND COLIFORMS

E. coli is a member of family Enterobacteriaceae that also includes other bacterial genera such as *Salmonella*, *Shigella* and *Yersinia*. Although most of the strains of *E. coli* are non-pathogenic they may act as opportunistic pathogens and cause disease. Pathogenic strains of *E. coli* including enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC) and enteroaggregative *E. coli* (EAEC) are also known. *E. coli* has also found its use as the indicator of fecal contamination of food and water. It is found in the human and animal gastro-intestinal tract and is easily identified due to its capacity to ferment lactose. However, the other genera such as *Klebsiella*, *Citrobacter* and *Enterobacter* are also known to ferment lactose and are difficult to distinguish from *E. coli* based on their phenotypic characteristics, all these genera are categorized as 'coliforms', which included the Gram-negative, facultative anaerobic rod-shaped bacteria that ferment lactose to produce acid and gas within 48 h at 35°C. The problem of *E. coli* as indicator organisms resurfaces as the other members of Coliform group are found in environments other than the gastro-intestinal tract, so by testing the lactose fermentation one cannot be sure of the contamination by *E. coli* or fecaes. This leads to the emergence of other related group called 'fecal coliforms'. These included all the coliforms that ferment lactose to produce acid and gas at elevated temperatures of incubation and hence are also referred as thermo-tolerant coliforms. These coliforms include *E. coli* as a major group but *Klebsiella* is also known to ferment lactose in these conditions. Their detection is carried by lactose fermentation tests at temperatures of around 45°C. The tests for coliforms and *E. coli* are based on the same principle but have several variations depending upon the type of the food in which the detection has to be carried out or the format of the test. There are several formats of these tests as discussed in the coming sections.

A. REQUIREMENTS

Butterfield's phosphate-buffered water, lauryl sulphate tryptose (LST) broth, brilliant green lactose bile (BGLB) broth, EC broth, levine-eosine methylene blue (L-EMB) agar, plate count agar (PCA), tryptone broth, MR-VP broth, Kovacs reagent, α-naphthol, potassium hydroxide, Koser's citrate broth, creatine, 4-methylumbelliferyl β-D-glucuronide (MUG), M-endo medium, LES endo agar, ColiComplete (CC) discs, Universal pre-enrichment broth (UPEB)

B. METHODS OF DETECTION

7.4.1 Most Probable Number (MPN) method

a) Presumptive test for detection of coliforms, fecal coliforms and *E. coli*

1. Weigh 50 g of sample in a sterile jar and add 450 ml of Butterfield's phosphate-buffered water. Blend it for 2 min. This gives a 10^{-1} dilution.
2. Prepare decimal dilutions from first dilution.
3. Mix the dilutions by shaking the bottles or by vortexing.
4. Transfer 1ml of dilution to 3 tubes of LST broth each for 3 consecutive dilutions. Transfer 1 ml dilution to 5 LST tubes if analyzing shellfish and shellfish harvest waters.
5. Incubate all tubes at 35°C for 48 h and look for gas production after 24 h and 48 h of incubation.
6. Use tubes showing positive reaction for gas production for MPN confirmed tests.

b) Confirmed test for coliforms

Inoculate a loopful of inoculum from positive (Gassing) LST tubes to BGLB broth and incubate them at 35°C for 48 h. Examine the tubes for gas production and calculate MPN of coliforms based on proportion of confirmed gassing LST tubes for 3 consecutive dilutions.

c) Confirmed test for *E. coli and* fecal coliforms

Inoculate a loopful from the positive LST tubes (presumptive test) to EC broth. Incubate these tubes at 45.5°C (44.5°C if analyzing shellfish) for 48 h. Observe for gas production and calculate MPN for fecal coliforms.

d) MPN – Completed test for *E. coli*

1. Streak a loopful of culture from EC tube (positive for gas) on L-EMB agar and incubate the plates at 35°C for 24 h.
2. Observe for dark centered flat colonies with or without green metallic sheen. Only gram-negative bacteria grow on EMB agar as Gram-positive bacteria are inhibited by the dyes eosin and methylene blue added to the agar. Based on its rate of lactose fermentation, *Escherichia coli* produces dark, blue-black colonies with a metallic green sheen on EMB agar. The metallic sheen is due to the large amounts of acid the E. coli produces on the EMB agar. The acid causes an amide bond to form between the two dyes (eosin and methylene blue), giving rise to a sheen.
3. Transfer the suspected colonies (5 colonies) to plate count agar (PCA) slants and incubate at 35°C for 24 h.
4. Use the growth obtained on PCA for further tests such as gram staining.
5. Select gram negative and bacilli shape cultures for further testing. Confirm the reaction (gas) in LST tube from the PCA cultures.
6. Indole test: This test is positive for bacteria producing tryptophanase that leads to formation of indole from tryptophan. Indole can be detected by reaction with Kovac's reagent (para-methylaminobenzaldehyde in alcohol) to produce a red colour. Inoculate tryptone brothfrom suspected colonies and incubate at 35°C for 24 h. Add 0.3 ml Kovac's reagent and consider the appearance of red color in upper layer as positive.

7. Methyl red test: This test determines the capability of the bacterial culture to utilize glucose and produce acidic end products. Inoculate MR-VP broth with suspected culture and incubate at 35°C for 48 h. Add a few drops of methyl red indicator to this culture. Consider the appearance of red color as positive reaction.
8. Voges-Proskauer (VP test): This test determines the capability of bacterial culture to produce neutral end products by fermenting glucose. Inoculate MR-VP broth with suspected culture and incubate at 35°C for 48 h. Add 0.6 ml α-naphthol solution and 0.2 ml 40% KOH. Shake the tube vigorously and then add a few crystals of creatine. Shake and let stand for 2 h. Consider the development of deep rose color as positive VP test.
9. Citrate Test: This test is used to detect citrate utilization by the bacterial culture. Inoculate Koser's citrate broth and incubate at 35°C for 96 h. Consider the development of turbidity as positive reaction for citrate utilization.

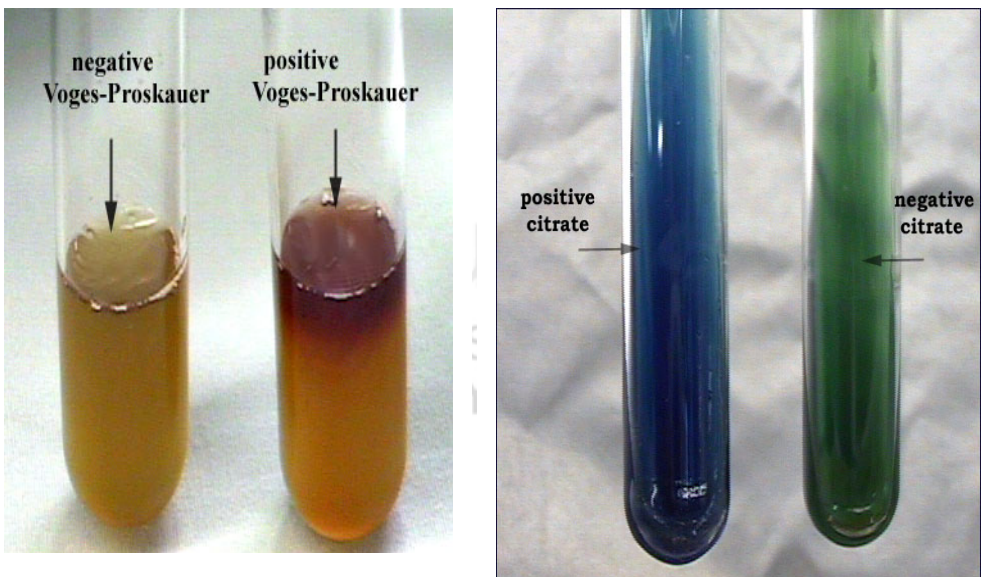

Fig 7.4: VP and Citrate test

Interpretation

All gram negative rods that ferment lactose with gas production and give typical IMViC reaction as ++-- and -+-- are categorized as *E. coli* biotype 1 and biotype 2, respectively. Commercially available kits may also be used to perform these biochemical tests.

7.4.2 LST-MUG method for *E. coli* detection

This method for detection of *E. coli* is based on the detection of enzyme β glucuronidase (GUD) that cleaves the substrate 4-methylumbelliferyl β-D-glucuronide (MUG), to release 4-methylumbelliferone (MU). On exposure to 365 nm UV, MU produces bluish fluorescence. This substrate can be added to any media and fluorescence observed due to the production of GUD, which indicates the presence of *E. coli*. However, some other bacteria such as *Shigella* and *Salmonella* produce GUD and *E. coli* strain O157:H7 do not produce it.

A. PROCEDURE

1. Inoculate LST-MUG broth as done in the MPN-presumptive test.
2. Incubate it at 35°C for 48 h.
3. Observe for bluish fluorescence under UV exposure. Compare with the proper negative and positive controls.
4. Streak L-EMB plates from the tubes giving positive reaction and continue with the confirmed and complete tests as indicated in the above sections.

A.1. Analysis of bottled water by membrane filter method for Coliforms

1. Filter 100 ml of water sample.
2. Place the filter on the surface of M-Endo medium or LES Endo agar.
3. Incubate the plates at 35°C for 24 h.
4. Observe for the presence of typical colonies that are pink to dark red in color with a green metallic surface sheen.
5. Count the typical colonies. If they are in range of 5-10 in numbers inoculate all the colonies in LST medium and incubate at 35°C for 48 h. If the colony number is more than 10 randomly pick 10 colonies exhibiting the typical morphology.
6. Look for gas production in LST tubes and subculture from the tubes that are gas positive to BGLB. Incubate the tubes at 35°C for 48 h and again observe for gas production.
7. Gas evolution in BGLB gives a completed test. Report the result as number of coliform colonies per 100 ml of sample.

7.4.3 Analysis of *E. coli* in Citrus juices

Detection of *E. coli* in citrus juices cannot be done by standard methods because the pH of these juices is acidic and interferes with the standard tests. The method for detection of *E. coli* in citrus juices is based on the same principles of lactose fermentation, glucuronidase and β-galactosidase, however it involves an additional step of pre-enrichment.

A. Procedure

1. Add 10 ml juice to 90 ml of UPEB and incubate at 35°C for 24 h.
2. Transfer 1 ml of enriched sample to 9 ml of EC broth containing ColiComplete (CC) discs. Incubate these tubes at 44.5°C for 24h.
3. Observe the tubes in long wave length UV light. Appearance of bluish fluorescence indicates the presence of *E. coli*. Appearance of blue colour on the surface of discs also indicates the presence of coliforms.

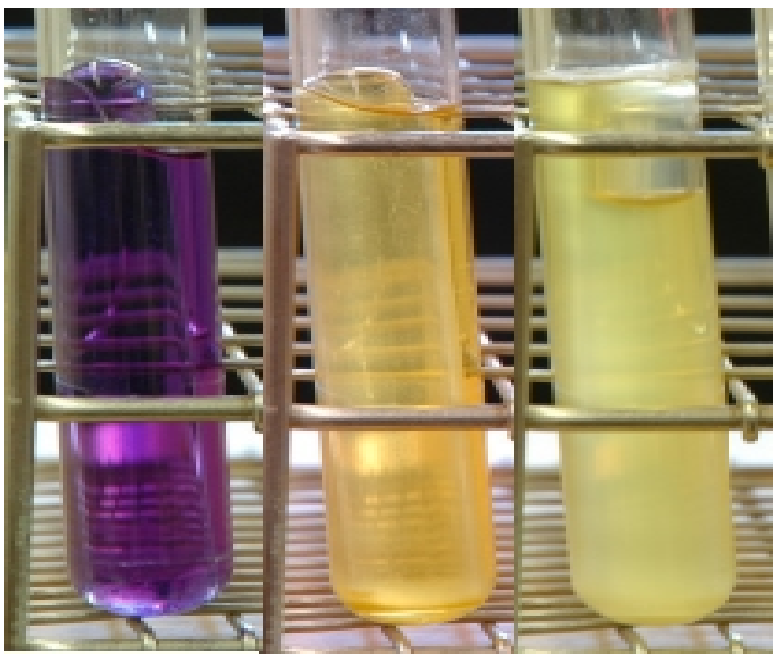

Fig. 5.5: Example reactions of typical strains in fermentation broth. If a microbe does not ferment the test sugar, the indicator dye remains purple and no gas is produced (left). If fermentation does take place, acid is most often produced, lowering the pH and changing the color of the broth to yellow (middle). Some microbes produce hydrogen gas during fermentation and this will be trapped inside the inverted tube, called a Durham tube, and is observed as a bubble (right).

Check your Progress: Exercise 3

Note: a) Use the space below for your answer.
b) Compare your answers with those provided at the end of this unit.

1) Which color layer is formed in VP broth during VP test?

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2) Which coloured colonies of *E.coli* are formed on EMB plate and what is the reason behind it?

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3) Which chemical represents the Kovac's reagent?

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4) Write the name of indicator in MR test and how does it help to interpret the result?

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5) Which broth is used in citrate test?

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6) What is the principle of LST-MUG method for the detection of *E. coli*?

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7.5 *Listeria monocytogenes*

Listeria (named after J. Lister) *monocytogenes* is a gram positive flagellated bacterium, which is a dangerous pathogen. Its infection may lead to septicemia, meningitis, encephalitis and intrauterine or cervical complications in women. Its common habitats include intestinal tracts of domestic and wild animals, fecal matter, soil, silage, sewage, house flies, ticks and human carriers and has been detected in raw milk, supposedly pasteurized fluid milk, cheeses (particularly, soft-ripened varieties), ice cream, raw vegetables, raw and cooked poultry, raw meats, raw and smoked fish. It produces haemolysin (haemolysis) and an endotoxin which causes meningoencephalitis. Listeria is killed in 3 min by boiling and in 20 minutes at a temperature of 70^{o} C. This organism can remain viable for 7 years in dry state. Besides being able to survive high temperature and desiccation, it is also able to grow at refrigeration temperatures and fewer than thousand bacterial cells can cause disease in a susceptible person. Detection of *Listeria* in food samples is carried after enrichment and if the sample is positive then enumeration is carried out.

A. REQUIREMENTS

Buffered *Listeria* enrichment broth (BLEB), primaricin, Oxford agar (OXA), trypticase soy agar with yeast extract (TSAye), 0.85% saline, hydrogen peroxide, sheep blood agar (5%), nitrate broth, reagent A, reagent B, purple carbohydrate broth, dextrose, esculin, maltose, rhamnose, mannitol, xylose, Difco fluorescent antibody (FA) buffer, Tryptose broth.

B. METHOD OF DETECTION

7.5.1 Plate count

1. Weigh 25 g of food sample in a sterilized jar. Add 225 ml of BLEB.
2. Blend the sample at high speeds and incubate at 30^{o}C for 4 h.
3. Add primaricin (natamycin) at a concentration of 25 mg/l and further incubate at 30^{o}C for 48 hours.
4. Streak this sample on OXA and incubate these plates at 35^{o}C for 24 – 48 h. Typical *Listeria* colonies will be black in colour surrounded by black halo.
5. Pick 5 or more typical colonies and streak on Trypticase soy agar with yeast extract (TSAye) and incubate at 30^{o}C.
6. Pick a typical colony from TSAye medium and prepare a thick emulsion in 0.85% saline. Put a drop of this emulsion on a coverslip and flip it on a concavity slide. Observe this preparation under phase contrast microscope and look for motile cells. *Listeria* spp. are thin, short rods with slight rotating or tumbling motility.

7.5.2 Biochemical tests

7. Test for catalase (Section 3.2.2.2). *Listeria* are positive for catalase test.
8. Carry out Grams staining of the suspected colonies. *Listeria* are Gram positive rods and may appear as stack of cells (palisade arrangement) in thick smears.
9. Inoculate isolates on sheep blood agar (5%) by stabbing the medium. Stab as near as possible without touching the bottom of plate. Incubate at 35^{o}C for 24-48 h. Examine the plates with bright light and observe for haemolysis. *L. monocytogenes* and *L. seeligeri* produce a slightly cleared zone around the stab. *L. innocua* shows no zone of haemolysis, whereas *L. ivanovii* produces a well-defined clear zone around the stab.
10. Carry out nitrate reduction test for the isolates as described in section 3.2.1.2.3. Only *L. grayi* ssp. *murrayi* reduces nitrates.
11. Inoculate SIM or MTM from TSBye. Incubate for 7 days at room temperature. Observe daily. *Listeria* spp. are motile, giving a typical umbrella-like growth pattern.
12. Test the carbohydrate utilization pattern of the isolates by inoculating purple carbohydrate broth (having 0.5% dextrose, esculin, maltose, rhamnose, mannitol, and xylose). Incubate the tubes at 35^{o}C for 7 days. Consider the appearance of yellow colour as a positive test. Compare the carbohydrate utilization profile with that of standard cultures as given in Table 3.3.
13. Perform Christie-Atkins-Munch-Peterson (CAMP) test. This test detects the enhanced haemolysis by *Listeria* sp. in the presence of *S. aureus* and *Rhodococcus equi* Streak weakly haemolytic *S. aureus* (ATCC 49444) and *R. equi* (ATCC 6939) on sheep blood agar . Streak the suspected cultures of *Listeria* in parallel lines between the two vertical streaks of *S. aureus* and *R. equi* Incubate the plates at 35^{o}C for 48 h. Observe after 24 and 48 h. Haemolysis of *L. monocytogenes* and *L. seeligeri* is enhanced near the *S. aureus* streak; *L. ivanovii* haemolysis is enhanced near the *R. equi* streak. Other species are non-haemolytic and do not react in this test.
14. Characterize the *Listeria* isolates into type 1, type 4 or not type 1 or type 4. Inoculate tryptose broth and incubate at 35^{o}C for 24 h. Inoculate tryptose agar slants (2 per culture) from this culture and incubate at 35^{o}C for 24 h. Wash both slants in a total of 3 ml Difco fluorescent antibody (FA) buffer and transfer to a sterile 16 x 125-mm screw-cap tube. Heat in a water bath at 80°C for 1 h. Sediment cells by centrifugation at 1600 g for 30 min. Remove 2.2-2.3 ml of supernatant fluid and resuspend the pellet in the remainder of buffer. Follow manufacturer's recommendations for sera dilution and agglutination procedure. This is the serological characterization and is important for epidemiological considerations. More than 90% isolates can be typed using commercially available sera.
15. *Listeria* can also be detected by commercially available kits (Table 7.4 A & B).

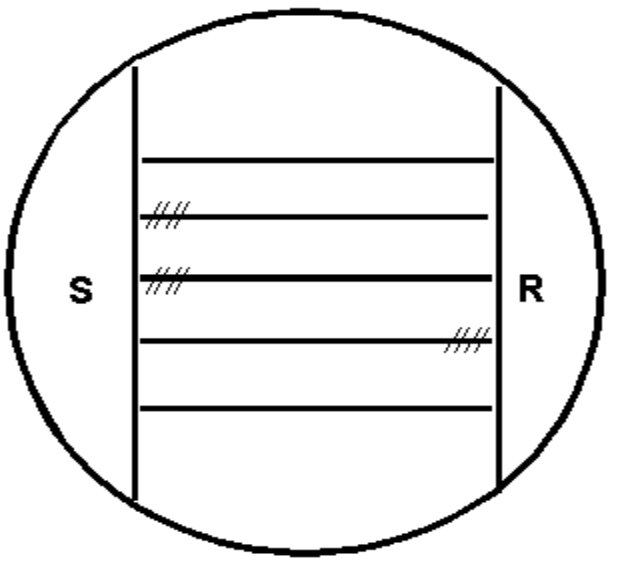

Fig 7.6: CAMP test for *Listeria monocytogenes*: Inoculation pattern of the sheep blood agar plate. Horizontal lines represent streak inoculations of 5 test strains. Vertical lines represent streak inoculations of *Staphylococcus aureus* (S) and *Rhodococcus equi* (R). Hatched lines indicate (diagrammatically only) the locations of hemolysis enhancement regions

7.6 *Salmonella*

Salmonella include Gram negative bacilli that are usually found in poultry, eggs, unprocessed milk, meat and water. These bacteria have been implicated in several food poisoning instances. Members of genus *Salmonella* are motile and possess two antigens that play an important role in the identification of these bacteria. These antigens are flagellar H antigen and somatic O antigen.

A. REQUIREMENTS

Selenite cystine (SC) broth, tetrathionate (TT) broth, Rappaport-Vassiliadis (RV), bismuth sulfite (BS) agar, xylose lysine desoxycholate (XLD) agar, Hektoen enteric (HE) agar, triple sugar Iron (TSI) agar, lysine iron (LI) agar, urea broth, physiological saline, Spicer-Edwards flagellar (H) antisera, phenol red dulcitol broth, tryptone broth, polyvalent somatic (O) antisera, MR-VP broth, Koser citrate broth, α-naphthol, Potassium hydroxide

B. METHODS OF DETECTION

7.6.1 Plate Count

1. Sample preparation is carried out by dissolving a representative quantity of food product in the medium for pre-enrichment/enrichment purposes. The exact procedure differs for the different kinds of foods, but overall it involves adding 25 g of food product to the enrichment broth so as to have a ratio of 1:9. This step is followed by the homogenization of food sample and incubation at 35°C for 24 h. The enrichment medium and its components depend on the type of food from which the isolation of *Salmonella* is desired (Table 3.5). Not only the media for enrichment varies but the procedure for sample preparation also varies e.g. cellulose is added during the sample preparation of guar gum while for other foods surfactants such as triton X100 may be added.
2. Gently shake the samples after incubation.
3. Add 1ml of prepared sample to SC broth (10ml) and TT broth (10 ml). This step is to be taken for foods where the presence of *S. typhi* is suspected.
4. For all other foods add 0.1 ml of sample to RV medium (10 ml) and 1 ml to TT broth (10 ml).
5. Incubate the tubes of RV medium at 42°C for 24 h and tubes of TT broth at 43°C. This step is to be taken for foods with high microbial load.
6. For foods with low microbial load incubate RV tubes as in step 5 while incubate TT at 35°C for 24 h.
7. For guar gum and foods suspected for the presence of *S. typhi* incubate the SC and the TT tubes at 35°C for 24 h.
8. Vortex the tubes and streak 10 µl of the culture from all the tubes on BS agar, XLD agar and HE agar. Incubate the plates at 35°C for 24 h.
9. Examine the plates for the colonies that may be *Salmonella* i.e., yellow colonies with or without black centers (Table 3.6).

7.6.2 Biochemical tests

10. Pick at least two suspected *Salmonella* colonies from each agar and carry out Triple sugar iron (TSI) agar and Lysine iron (LI) agar tests.
11. Inoculate the TSI slant by stab and streak method. Without heating the loop stab the butt twice and streak the slant of LI agar.
12. Incubate the tubes at 35°C for 24 h.
13. Examine the results obtained in the TSI and LI tests. *Salmonella* produces an acidic butt (yellow) and alkaline slant (red). The blackening of butt may or may not occur depending on the H_2S production. In LI test *Salmonella* produce an alkaline butt (purple) with H_2S production. All the cultures giving alkaline reaction (purple butt) for LI agar are used for confirmation. Cultures giving an acidic reaction (yellow butt) in LI agar but an acidic butt and alkaline slant in TSI are also used.
14. Examine a minimum of 6 TSI cultures for 25 g of sample, for identification of *Salmonella*.
15. Inoculate urea broth with the presumptive *Salmonella* isolates and incubate the tubes at 35°C for 24 h. Keep an uninoculated tube as a control. This broth contains the pH indicator phenol red. The phenol red is peach color at a pH of 6.8 and turns a hot pink at a pH of 8.4. Discard the cultures if the colour of medium changes to purple red indicating a positive test for urease production because *Salmonella* is known to be negative for urease test.
16. Carry out the serological polyvalent flagellar (H) test with the urease negative cultures. Inoculate BHI broth with the cultures from TSI slant and incubate at 35°C until visible growth occurs. Add 2.5 ml of formalinized physiological saline solution to 5 ml of BHI culture. Add 0.5 ml of polyvalent flagellar (H) antisera to 10X75 mm serological test tube. To this add 0.5 ml of formalinized broth. Prepare control by adding 0.5 ml of formalinized physiological saline to the formalinized antigen. Incubate the tubes at 48 – 50°C for 1 hr. Observe for agglutination. The test is positive if agglutination is observed and negative if there is no agglutination. In some cases the test may be nonspecific where agglutination is observed both in the test mixture and in control. Repeat this test with Spicer-Edwards flagellar (H) antisera for the cultures giving a negative result.
17. Perform the additional biochemical tests on the urease negative isolates by inoculating them in Phenol red dulcitol broth in Durhams tube. Development of yellow color and gas production indicates positive reaction. *Salmonella* is positive for this test.
18. Inoculate the growth from TSI slants into tryptone broth tube and incubate at 35°C for 24 h. Further inoculate potassium cyanide broth and malonate broth from the tube containing tryptone broth. Keep these tubes at 35°C for 48 h. Observe for growth in potassium cyanide broth. *Salmonella* is not able to grow in this broth. *Salmonella* also give a negative test for malonate which is indicated by the green or unchanged color of the broth.
19. Transfer 5 ml of tryptone broth culture to an empty test tube and add 0.3 ml of Kovacs reagent. Development of deep red color is positive test. *Salmonella* is negative for this test.
20. Perform the polyvalent somatic (O) test for *Salmonella* by preparing an emulsion of growth obtained from TSI slant in 2 ml of 0.85% saline. Take a drop of this suspension on a clean slide and add a drop of polyvalent somatic (O) antisera. Mix them and tilt the slide back and forth for 1 min. For control add a drop of saline to the drop of culture suspension and mix as described above. Observe for agglutination. If agglutination is positive for test and negative for culture the result is taken as positive for *Salmonella*. If both are negative the test is also negative for *Salmonella* and if agglutination is positive then the test is nonspecific.

21. Perform additional biochemical tests including lactose fermentation, sucrose fermentation, MR-VP and citrate utilization.

22. Compare the results obtained in the above biochemical and serological tests with the standard reactions exhibited by *Salmonella* as shown in Table 3.7.

Biochemical tests can also be carried out using commercially available kits such as API 20E, Enterotube II, *Enterobacteriaceae* II, MICRO-ID, or Vitek GNI for presumptive identification of food borne *Salmonella*.

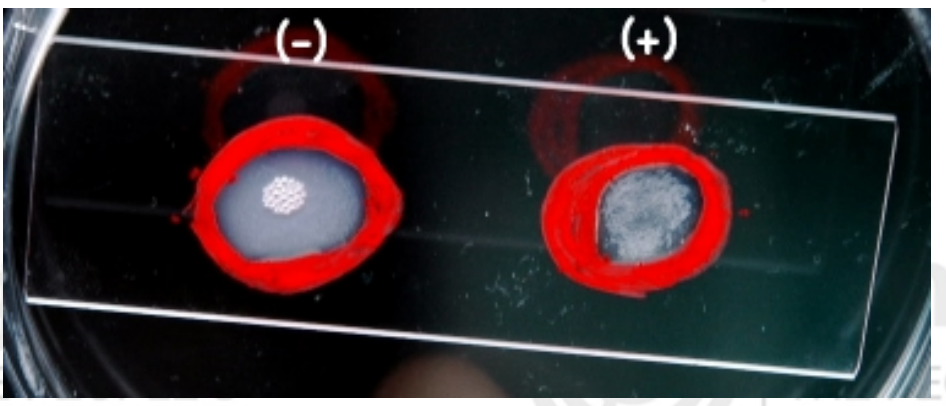

Fig 7.7: Antibody reactions of salmonella. Note the grainy appearance of the positive reaction

Salmonella	
Extraction:	Lactose broth
Enrichment:	SC broth (in case S. typhi is suspected) or RV broth (42^{o}C) Tetrathionate broth
Culturing:	BS agar XLD agar HE agar
	TSI slant and stab [Acidic butt (yellow) and alkaline slant (red)] Lysine iron slant [Acidic butt and H_2S production (black colour)]
	Urea broth (Salmonella if present yields no purple colour)
Others:	Serological polyvalent flegellar (H) test BHI broth inoculated from TSI + slant TSI – slant: Spicer-Edwards flagellar antisera
	Phenol red dulcitol (positive: yellow) Tryptone broth KCN broth Malonate broth Kovac's reagent Polyvalent somatic (O) test
Biochemical tests:	Lactose fermentation Sucrose fermentation MRVP and Citrate utilization

7.8 *Staphylococcus aureus*

S. aureus is spherical shaped, facultative anaerobe, non spore forming, coagulase positive, gram positive bacterium. Some strains are capable of producing a highly heat-stable protein toxin that causes illness in humans. *Staphylococcus aureus* is responsible for pyogenic infections. It is carried in the nasopharynx region of 50-75% of healthy individuals. *S. aureus* is known to produce several toxins including haemolysins, fibrinolysin, coagulase, leucocidin, hyaluronidase, DNAase, epidermolytic toxins, enterotoxins and toxin shock syndrome toxin (TSST-1). These toxins play an important role in the pathogenesis of these bacteria. *S. aureus* have been identified as the causative agent in various food poisoning outbreaks and their count of the magnitude 10^2 cfu/g or more in the ready to eat foods deems them unsatisfactory for consumption. Staphylococcal food poisoning (staphyloenterotoxicosis; staphyloenterotoxemia) is the name of the condition caused by the enterotoxins which some strains of *S. aureus* produce. *Staphylococcus aureus* is a common cause of mastitis in dairy cattle and are frequent contaminants of raw milk and are found in dairy products. They are present in nasopharyngeal cavity and on skin and there are chances of contamination with staphylococci every time the food is handled. They cause staphylococcal food poisoning. The presence of these bacteria in the foods points towards the poor sanitary conditions of the processing plant. As these bacteria are readily inactivated by heat and sanitizer treatments, their presence indicates post processing contamination of the food product. Pasteurisation, low temperature storage, proper hygiene and sanitation are some of the control measures.

A. REQUIREMENTS

Baird Parker medium, trypticase soy agar (TSA), brain heart infusion (BHI) broth, carbohydrate utilization medium, rabbit plasma with EDTA, toluidine blue DNA agar, 0.02 M phosphate-saline buffer with 1% NaCl, Butterfield's phosphate-buffered dilution water, sterile paraffin oil, Lysostaphin, hydrogen peroxide.

B. METHOD OF DETECTION

7.8.1 Isolation, identification and enumeration of *S. aureus*

Isolation of *S. aureus* from food products is carried out by direct plate count method as follows:

1. Weigh 50 g of food sample in a sterile jar and add 450 ml of Butterfield's phosphate-buffered dilution water to it. Blend it at high speed. This is 10^{-1} dilution of the food sample.

2. Further prepare the decimal dilutions by adding 10 ml of previous dilution to 90 ml of sterile diluent. Mix the dilutions properly.

3. Transfer 1ml of each dilution aseptically on 3 plates of Baird Parker agar. 1 ml of inoculum should be equitably distributed on three plates.

4. Spread the inoculum evenly on the surface of the media by sterile glass spreader.

5. Incubate these plates at 35^{o}C for 45-48 h in inverted position.

6. Consider the plates having typical *S. aureus* colonies (circular, smooth, convex, moist, 2-3 mm in diameter, gray to jet-black, frequently with light-coloured (off-white) margin, surrounded by opaque zone and frequently with an outer clear zone; colonies have buttery to gummy consistency (when touched with inoculating needle). Some other bacteria may also form colonies similar to that of *S. aureus* but these colonies lack opaque and clear zones as formed by *S. aureus* colonies. Rough colonies with dry texture may also be produced by *S. aureus* in certain cases. Plates having typical *S. aureus* colonies and counting in the range of 20 – 200 colonies (including other bacterial colonies) are used for determining cfu, however, when typical colonies are present at lower dilutions only, then plates with > 200 colonies may be used. In this case, colonies showing typical morphology of *S. aureus* are counted for determining cfu. Select >1 colony of each counted colony type and test for coagulase production.

7. Count all the colonies of particular types that give positive coagulase test. Add all such colonies obtained on triplicate plates and multiply with the sample dilution factor to report the cfu of *S. aureus* / g of sample.

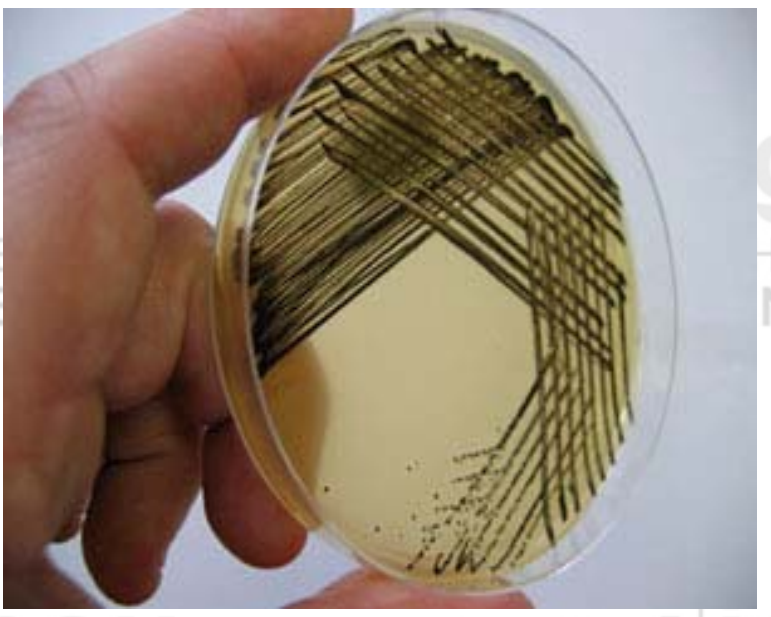

Fig 7.8: *Staphylococcus aureus* on Baird parker agar

7.8.2 Coagulase Test

1. Transfer the suspect colonies to narrow tubes containing 0.3 ml of BHI broth and emulsify. Inoculate TSA medium with a loopful of BHI suspension and incubate them (BHI tubes and TSA slants) at 35°C for 24 h.
2. Add 0.5 ml of reconstituted plasma with EDTA to BHI suspension and incubate at 35°C for 6h. Examine periodically for clot formation during the incubation period.
3. Consider the tubes exhibiting firm clot as positive for *S. aureus*.
4. Keep the positive and negative controls for the coagulase test.
5. Carry out gram staining for all the suspect cultures and observe microscopically for presence of gram positive cocci in bunches.

Staphylococcus aureus

Extraction:	Sterile Butterfields phosphate buffer
Direct plate count:	Baird Parker agar [circular, smooth, convex, moist, 2-3 mm in diameter, gray to jet-black, frequently with light-coloured (off-white) margin, surrounded by opaque zone and frequently with an outer clear zone; colonies have buttery to gummy consistency (when touched with inoculating needle]
Coagulase test:	positive
	(Incubation on TSA medium in presence of BHI broth + Plasma with EDTA)
Gram staining:	positive; cocci in bunches
Supportive tests:	Thermostable nuclease test
	Catalase test
	Lysostaphin sensitivity
	Anaerobic utilization of glucose and mannitol (positive: yellow colour)

7.8.3 Supportive Tests

These tests are also carried out for identification of *S. aureus* and its differentiation from *S. epidermidis* and *Micrococci*.

a) Thermostable Nuclease Test

S. aureus produces a heat stable DNase that hydrolyzes DNA to oligonucleotides. This test is as specific as coagulase test and is often used as a supportive test for strains giving weak coagulase test (where firm clot is not formed).

1. Prepare micro-slides by adding toluidine blue – deoxyribonucleic acid agar on the surface of microscopic slide.
2. Allow agar to solidify and cut wells (2mm diameter) on its surface.
3. Take the broth culture and heat it for 15 min. in the boiling water bath.
4. Add 10 µl of heated broth sample.
5. Prepare a moist chamber by adding a layer of wet cotton to the Petri plate.
6. Incubate the slide in this chamber at 35°C for 4 h.
7. Appearance of bright pink halo around the wells indicate the positive reaction.

b) Catalase test (as per Section 7.3.1.B)

c) Lysostaphin sensitivity

Lysostaphin is an endopeptidase which cleaves the pentaglycine cross linkages occurring in the *Staphylococcus* peptidoglycan. Sensitivity of Staphylococcal strains against Lysostaphin can be used to differentiate it from micrococci.

1. Transfer and emulsify an isolated bacterial colony from agar plate to 0.2 ml of phosphate saline buffer.
2. Transfer 0.1 ml of this suspension to another tube.
3. Add 0.1 ml lysostaphin solution (25 µg/ ml of lysostaphin dissolved in 0.02 M phosphate saline buffer containing 1% NaCl) to one tube (test) and 0.1 ml of phosphate saline buffer to second tube (control).
4. Incubate both the tubes at 35°C for 2 h.
5. Test is considered positive if the turbidity clears (due to cell lysis) within 2 h.

d) Anaerobic utilization of glucose and mannitol

1. Inoculate the tubes of carbohydrate fermentation medium containing glucose (0.5%). Add a layer of sterile paraffin oil on the surface of the fermentation medium. Incubate the tubes at 37°C for 5 days.
2. Repeat step 1 with media containing 0.5 % mannitol.
3. Observe for appearance of yellow colour throughout the tube and note it as positive test for *S. aureus.*

Biochemical activities of *S. aureus* and its comparison to *S. epidermidis* and micrococci are depicted in Table 3.8.

7.9 *Clostridium perfringens*

Clostridium perfringens continues to be a major food safety concern to the food industry. This organism has been implicated as the cause of food-borne illness in roast beef, turkey, meat-containing foods, and other meat dishes. Foods contaminated with large numbers of vegetative cells of *Clostridium perfringens* can give rise to illness characterised by diarrhoea and abdominal pain which may be accompanied by vomiting. *C. perfringens* produces spores which can survive normal cooking processes and cause a hazard in meat products that are left at ambient temperatures for a long time after cooking. *C. perfringens* spores may survive cooking and receive sufficient heat activation to germinate and produce cells that may subsequently multiply in cooked foods if the rate and extent of cooling are not sufficient. *C. perfringens* generation time is as rapid as 7.4 min in autoclaved ground beef with an optimal growth range of 37 to 45 °C, and growth has been reported at temperatures as low as 68 °C. Other sulphite-reducing clostridia are implicated in food spoilage, especially of poorly processed canned food.

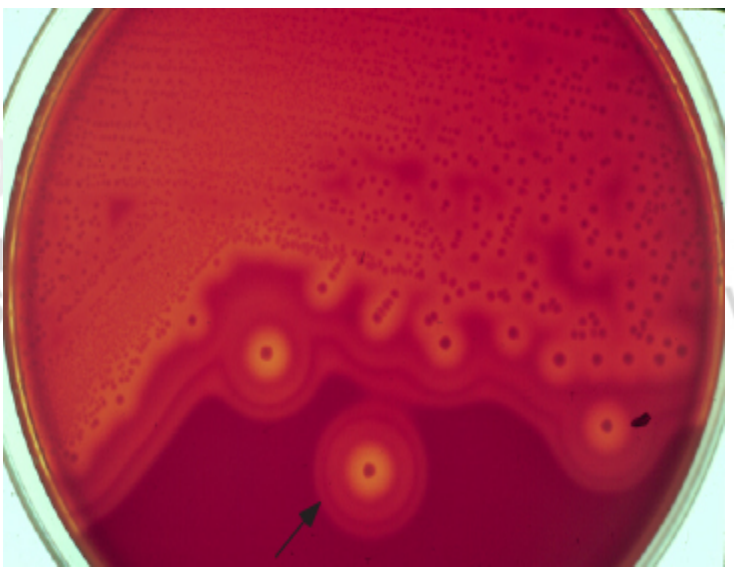

Fig 7.9: Anaerobic culture of Clostridium perfringens on blood agar. The characteristic double zone of clear beta-hemolysis around a colony is clearly seen (arrow)

7.9.1 Cultural Methods for Enumeration and Identification of *Clostridium perfringens* in Foods

Specified volumes of dilutions of the sample are mixed with a tempered molten selective culture medium in a sterile Petridish. An overlay of the same medium is added and the plates are incubated anaerobically at 37^{o}C for 20 h. The number of black colonies is counted and the number of *C. perfringens* colonies determined following results of confirmation tests. *C. perfringens* is provisionally identified as a non-motile, Gram-positive bacillus which produces black colonies in TSC agar, reduces nitrates to nitrites, produces acid and gas from lactose, and liquefies gelatin within 48 h. Some strains of *C. perfringens* exhibit poor sporulation in sporulation medium or weak lecithinase reactions on TSC agar containing egg yolk. For dilution, a 10-1 homogenate is prepared in either peptone saline diluent (PSD) or buffered peptone water (BPW) and further decimal dilutions as required in PSD. Starting with the highest dilution 1 mL of each dilution is transferred to a sterile Petri dish and 15 to 20mL of tryptose sulphite cycloserine agar (TSCA), tempered to 45 ^{o}C, is poured into each petri dish. After proper mixing (rotating each dish five times clockwise, anti-clockwise and sideways in each direction), plates are allowed to set and further a 10 mL of TSCA is added as an overlay and allowed to solidify. Plates are incubated under anaerobic conditions in an incubator at 37C for 20 ± 2 h. On completion of incubation, the plates are examined for presence of black colonies and typical colonies are enumerated and recorded.

7.9.2 Confirmatory Tests

5-10 black colonies are transferred to two blood agar (BA) plates. Incubate one plate aerobically and the other anaerobically in an incubator at 37C for 18 - 24 h. Examine the plates for the presence or absence of growth and for purity. Perform confirmatory tests for *C. perfringens* on colonies that fail to grow aerobically. Anaerobic cultures with a diffuse spreading morphology are considered motile, and therefore confirmatory tests are not carried out. Using pure non spreading cultures from the anaerobic blood agar subculture plates following media are incubated:

1. Motility-nitrate medium

Immediately prior to use, heat the medium in boiling water for 15 minutes and then cool rapidly to set. Inoculate by stabbing into the medium and incubate under anaerobic conditions at 37±1 ^{o}C for 20 - 24 h. After incubation, examine the medium for growth along the stab-line. Motility is evident as diffuse growth out into the medium away from the stab line.

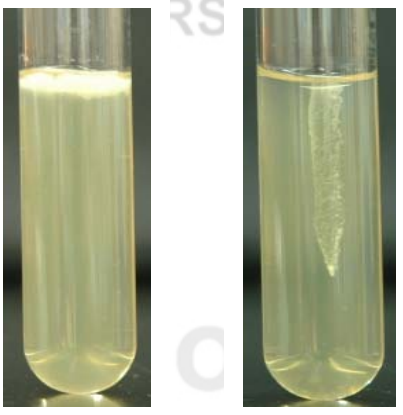

Fig 7.10: Motlity tests: (a) Positive for *B. cereus* (b) negative for *Staphylococcus epidermidis*

Mix equal volumes of nitrite reagent A and B just before use and test for the presence of nitrite by adding 0.2 to 0.5 mL of this mixture to each tube of motility-nitrate medium. The formation of a red colour confirms the reduction of nitrate to nitrite. If a red colour does not develop within 15 minutes add a small amount of zinc dust and allow standing for 10 minutes. If a red colour develops after the addition of zinc dust no reduction of nitrate has taken place.

2. Lactose gelatine medium

Immediately prior to use heat the medium in boiling water for 15 minutes and then cool rapidly. Inoculate the medium and incubate anaerobically at 37 ^{o}C for 20 - 24 h. Examine the tubes for the presence of gas and for a yellow colour indicating the production of acid. Chill the tubes for 1 hour at 2 to 8^{o}C and check for gelatine liquefaction. If the medium has solidified re-incubate for an additional 24 h and again check for liquefaction of the gelatine.

lostridium perfringens	
xtraction:	Peptone saline diluent/ peptone diluent buffer
rect plate count:	Tryptose Sulphite Cycloserine agar (black colonies)
thers:	Nitrate reduction: positive Lactose fermentation: with acid and gas Gelatin liquefication: positive
onfirmatory tests:	Blood agar incubated aerobically and anaerobically (non spreading culture used for further tests)
otility-nitrate medium:	diffuse growth and red colour (if not initially present add zinc dust and wait for 10 min)
ctose gelatine medium:	(under anaerobic conditions) gas and yellow colouration; Chilling used to check for gelatin liquefication

7.10 VIRAL PATHOGENS

Detection of viruses in food is most likely to be undertaken when an outbreak has occurred. It would be helpful, however, if some routine testing methods were available to apply to foods, such as shellfish, that often serve as vehicles for viruses. Outbreak investigation and routine monitoring present very different sets of priorities. When people have already been made ill, the relatively high costs of attempting to detect viruses in food samples may be acceptable, especially if litigation is anticipated. However, as was mentioned earlier, an outbreak of hepatitis A affecting several people is likely to be recognized only 4 weeks or more after the contaminated food was consumed, so pertinent food samples most likely will be unavailable for testing. With other viral diseases that have shorter incubation periods, clinical histories and diagnostic samples of feces and blood serum may afford some indication of which virus is to be sought in the food, which could prove very helpful.

In contrast, testing foods for virus contamination in the hope of preventing human illness presents special problems. Costs are likely to outweigh demonstrable benefits. In addition, since almost all foodborne viruses are transmitted by fecal-oral cycles, one virus is as likely as another to occur in food, subject to fecal contamination. As ingenious as the methods are that have been devised for detecting food-borne viruses, none could be considered routine. A fundamental problem is that the viruses of greatest concern, hepatitis A viruses and the Norwalk like gastroenteritis viruses, replicate slowly and in apparently or not at all in laboratory cell cultures. If food-borne viruses cannot be detected on the basis of their infectivity, alternate basis include their morphology (as seen by electron microscopy), their antigenic specificity (as demonstrated by reactions with homologous antibody), their genetic specificity (as demonstrated with complementary probes or polymerase chain reaction [PCR] primers), or combinations of these. These methods may be less sensitive than tests based on infectivity, and by their nature all carry some risk of yielding a positive result with virus that has been inactivated (no longer infectious). The final detection method to be used must be considered when the food sample is being processed for testing. An additional problem

encountered with the hepatitis A virus is that the incubation period of the illness is so long (average 4 weeks) that pertinent food samples are unlikely to be available once the disease has been recognized.

Most foods other than milk, water, and a few others require liquefaction as a first processing step. Addition of liquid for this purpose is usually kept to a minimum because virus that is present in food will be diluted, to the detriment of the sensitivity of the method. Solid food samples are shaken, comminuted, or otherwise dispersed in a diluent that has been selected to encourage dissociation of virus from the food solids, which will be removed by centrifugation, filtration, or other means. Additives may be used to encourage separation of the food solids from the liquid suspension containing the virus. Because foods suspected of viral contamination are likely to contain bacteria from both the food and feces, a step to remove or kill bacteria in the suspension is usually included. Because the detection methods available can usually accommodate only very small volumes of sample, as much water as possible is removed from the food sample extract before testing begins. Applicable concentration methods include adsorption-elution, differential precipitation, ultra-centrifugation, and ultra-filtration.

METHODS FOR DETECTING VIRUSES EXTRACTED FROM FOODS

The detection methods are based on the morphology, antigenic specificity, or genetic specificity of the viral particle.

7.10.1 Electron Microscopy

Morphology of viral particles affords an important basis for their classification. However, many viruses not of human origin are indistinguishable by simple electron microscopy from viruses suspected as food contaminants. One way to demonstrate that the viruses seen in the electron micrograph are indeed the suspected type is to attach them to the grid or to each other with homologous antibody (immune electron microscopy.), which is a combination of serologic and morphologic criteria. When the serological type of the virus is unknown, an alternative is to compare immune electron micrographs done with acute and convalescent serum from a patient, assuming that paired samples are available. If the convalescent phase serum reacts with the virus and the acute phase serum does not, this is likely to have been the agent that caused the illness. Unfortunately, the method will not detect the small numbers of viral particles that are capable of causing disease.

7.10.2 Immunoassay

Antigenic specificity of the viral coat protein can serve directly as a means of detection by enzyme immunoassay or by radioimmunoassay. These methods have been of considerable use in diagnostic virology, where they are applied to fecal samples containing levels of virus often exceeding a million particles per gram. Because contaminated foods are likely to contain only imperceptible levels of feces, the quantities of virus present in food samples are likely to be much smaller, and immunoassay tests are seldom adequately sensitive. Therefore, serologic reactions between virus and antibody are most often applied in combination with electron microscopy or with nucleic acid-based tests.

7.10.3 PCR Methods

Nucleic acid tests are generally based on the specific interactions of portions of the viral genome with complementary probes, PCR primers, or both. PCR and other genetic amplification methods afford highly sensitive means of detecting small quantities of virus. Because almost all known food-borne viruses are RNA agents, the viral nucleic acid must be extracted and reverse-transcribed to complementary DNA before amplification by PCR can begin. Short complementary probes bracketing a selected region of the viral genome are introduced with a polymerase that functions at high temperature, and many successive cycles of replication, denaturation, and annealing are carried out by a programmed thermal cycler. The result is millions of short copies of the selected region of the viral genome. The specificity of these copies is demonstrated on the basis of their appropriate length (in terms of number of nucleotide bases), their reaction with a complementary probe that is directed at a portion of the amplified segment, or both. Probes used for this purpose are labeled with a radionuclide, an enzyme, or some other means of expressing their presence in association with the PCR-amplified product. In addition to the challenges of performing the demanding PCR and probe-specificity tests themselves, testing food samples has been found to present some special problems. Certain food components interfere with reverse transcription or with PCR amplification.

7.10.4 Combined Methods

Combined methods consist typically of a serologic method followed by another detection procedure, as with the immune electron microscopy approach described above. Virus has also been captured from sample extracts with homologous antibody for later detection by PCR. This method seems to obviate some of the problems with food inhibitors of reverse transcription and PCR, and it allows RNA to be released from the viral particle simply by heating. It seems that detection methods combining nucleic acid tests and morphologic tests have not been devised.

Check Your Progress Exercise 4

Note: a) Use the space below for your answer.
b) Compare your answers with those given at the end of the unit.

1) What coloured colonies of *Listeria monocytogenes* are formed on oxford agar?

……

……

2) What is CAMP test?

……

……

……

3) How *Salmonella* will be identified by TSI slant and LF slant test?

……

……

……

4) Which coloured colonies of *Salmonella* are formed on BSA, XLD, and HE agar?

……

……

……

5) What is the name of indicator in urea broth?

……

……

……

6) What coloured colonies of *Staphylococcus aureus* are forms on Braid Parker medium?

……

……

……

7) Name the substrate used for coagulation by *staphylococcus aureus* in coagulase test?

……

……

……

8) What is the name of the media used in thermostable nuclease test and which indicator is used in this test?

……

……

……

9) Why we are heating *Staph ylococcus aureus* sample for 15 min during this TNase test?

……

……

……

10) What colour zone is formed in TNase test by *staphylococcus aureus*?

……

……

11) Is *staphylococcus aureus* positive for mannitol fermentation?

……

……

……

12) Is *staphylococcus aureus* sensitive to lysostaphin?

……

……

……..

7.11 LET US SUM UP

Owing to their high nutritive value, foods both raw and processed are ideal medium for growth of microorganisms including pathogens. Pathogens manifest pathogenecity either through invasion of host tissues or by production of toxins. The pathogens found in foods broadly belong to bacteria, fungi and viruses. Bacterial pathogens are the predominant group and include *Bacillus cereus, Campylobacter, Escherichia coli, Listeria monocytogenes, Salmonella species, Staphylococcus aureus* etc. Most of the pathogens are heat labile and are destroyed by heat treatment. However, most of the toxins produced by them being heat resistant survive processing and can pose public health hazards. Detection of such pathogens is mandatory from public health and regulatory point of view. They have specific requirement for growth media and cultivation and are identified on the basis of their typical morphological and biochemical characteristics. Specific growth media, detection protocol and interpretation of results with respect to above mentioned food pathogens have been discussed in this unit. The information rendered in Table 3.9 can be practically used as a kind of ready reckoner for enumeration and detection of pathogens in different foods.

7.12 KEYWORDS

Pathogenicity : The ability to cause disease

Virulence : The degree of pathogenicity

Exotoxins : Toxins secreted outside the cell

Endotoxins : Toxins as part of outer cell wall of bacterial cell

Neurotoxins : Toxins which interfere with normal nerve impulses

Cytotoxins : Toxins which kill cells

Enterotoxins : Toxins infecting the cells lining of Gastrointenstinal tract

EEC E. coli : Enterovirulent *E. coli* that cause gastroenteritis

MYP : Agar Mannitol-egg yolk-polymyxin agar

Salmonellosis : Gastroenteritis caused by *Salmonella* spp.

Campylobacteriosis : Gastroenteritis caused by *Campylobacter* spp

Staphyloenterotoxicosis : Staphylococcal food poisoning

Staphyloenterotoxemia : Staphylococcal food poisoning

7.13 SUGGESTED FURTHER READING

American Public Health Association (APHA). (2001). Compendium of Methods for the Microbiological Examination of Foods. 4th edition. F.P. Downes and K. Ito (Editors), American Public Health Association, Washington, D.C.

American Public Health Association (1998). Standard Methods for the Examination of Water and Wastewater, 20th edition, APHA, Washington, DC.

AOAC INTERNATIONAL (1995). Official Methods of Analysis, 15th ed., sec. 987.09, AOAC INTERNATIONAL, Arlington, VA.

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AOAC INTERNATIONAL (2000). Official Methods of Analysis, 17th edition, Sec. 967.25-967.28, 978.24, 989.12, 991.13, 994.04, and 995.20. AOAC INTERNATIONAL, Gaithersburg, MD.

AOAC Official Method 993.09 (2000). *Listeria* in dairy products, seafoods, and meats. Colorimetric deoxyribonucleic acid hybridization method (GENE-TRAK *Listeria A*ssay). Chapter 17.10.04, pp. 147-150 In: Official Methods of Analysis of AOAC International. 17th edition. W. Horwitz (Editor). Volume 1. Agricultural Chemicals, Contaminants and Drugs. AOAC International, Gaithersburg, MD.

AOAC Official Method 994.03 (2000). *Listeria monocytogenes* in dairy products, seafoods, and meats. Colorimetric monoclonal enzyme-linked immunosorbent assay method (*Listeria* -Tek). Chapter 17.10.05, pp. 150-152 In: Official Methods of Analysis of AOAC International. 17th edition. W. Horwitz (ed.). Volume 1. Agricultural Chemicals, Contaminants and Drugs. AOAC International, Gaithersburg, MD.

AOAC Official Method 995.22 (2000). *Listeria* in foods. Colorimetric polyclonal enzyme immunoassay screening method (TECRA *Listeria* Visual Immunoassay [TLVIA]). Chapter 17.10.06, pp. 152-155 In: Official Methods of Analysis of AOAC International. 17th edition. W. Horwitz (Editor). Volume 1. Agricultural Chemicals, Contaminants and Drugs. AOAC International, Gaithersburg, MD.

AOAC Official Method 996.14 (2000). Assurance Polyclonal Enzyme Immunoassay Method. Chapter 17.10.07, pp. 155-158 In: Official Methods of Analysis of AOAC International. 17th Edition. W. Horwitz (Editor). Volume 1. Agricultural Chemicals, Contaminants and Drugs. AOAC International, Gaithersburg, MD.

AOAC Official Method 997.03 (2000). Visual Immunoprecipitate Assay (VIP). Chapter 17.10.08, pp. 158-160 In: Official Methods of Analysis of AOAC International. 17th edition. W. Horwitz (Editor). Volume 1. Agricultural Chemicals, Contaminants and Drugs. AOAC International, Gaithersburg, MD

Bacteriological Analytical Manual Online (www.cfsan.fda.gov) http://www.bact.wisc.edu:

7.14 ANSWERS TO CHECK YOUR PROGRESS EXERCISES

Check Your Progress Exercise 1

Your answer should include following points:

1) Pink colour surrounded by a precipitate zone indicating the production of lecithinase.

2) The presence of precipitate zone around colonies of B. cereus indicates that lecithinase is produced. *B. cereus* colonies are usually a pink color which becomes more intense after additional incubation.

3) Polymyxin B is added in MYP agar to suppress the growth of other bacteria able to grow at such a high salt concentration, e.g. *Proteus* spp.

4) Phenol red is *an acid-base indicator*. Phenol red changes from red to yellow when the medium has a pH < 6.8 (more acidic) and from red to fuchsia when the medium has a pH >7.4 (less acidic).

Check Your Progress Exercise 2

Your answer should include following points:

1) The medium is evaluated for nitrate reduction by the addition of two reagents, Nitrate A Reagent (0.8% sulfanilic acid in 5N acetic acid) and Nitrate B Reagent (0.6% N, N-dimethyl-alpha-naphthylamine in 5N acetic acid), which detect the presence of a catabolic end product, and by the addition of Nitrate C Reagent, zinc dust, which detects the absence of remaining nitrate in the medium.

2) Yes, the enzyme catalase acts upon hydrogen peroxide and converts it to water and molecular oxygen. Hydrogen peroxide is toxic to bacterial cells and catalase serves as a mechanism for removal of this toxic compound.

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

The production of gas bubbles indicates a positive catalase test.

3) The oxidase test identifies organisms that produce the enzyme cytochrome oxidase. Cytochrome oxidase participates in the electron transport chain by transferring electrons from a donor molecule to oxygen. In this test, an artificial final electron acceptor (N,N,N',N'-tetramethyl phenylenediamine dihydrochloride) is used in the place of oxygen. This acceptor is a chemical that changes color to a dark blue/purple when it takes the electron from the last element (cytochrome oxidase) in the electron transport chain. If the test organism produces cytochrome oxidase, the oxidase reagent will turn blue or purple within 15 seconds.

4) Perform hippurate test by emulsifying a loopful of culture to 0.4 ml hippurate solution in a test tube. Incubate for 2 h at 37^{o}C and add 0.2 ml of ninhydrin reagent. Agitate the tubes and re-incubate for 10 min. Appearance of violet colour is considered positive reaction.

5) H_2S production is indicated by blackening of slant.

Check Your Progress Exercise 3

Your answer should include following points:

1) The appearance of pink colour indicates positive results for test.

2) Only gram-negative bacteria grow on EMB agar as Gram-positive bacteria are inhibited by the dyes eosin and methylene blue added to the agar. Based on its rate of lactose fermentation, *Escherichia coli* produces dark, blue-black colonies with a metallic green sheen on EMB agar. The metallic sheen is due to the large amounts of acid the E. coli produces on the EMB agar. The acid causes an amide bond to form between the two dyes (eosin and methylene blue), giving rise to a sheen.

3) Kovac's reagent is para-methylaminobenzaldehyde in alcohol.

4) Methyl Red. It is an acid base indicator. Its colour changes from red (at pH 4.4) yellow at pH > 6.2.

5) Koser's citrate broth

6) Enzyme β glucuronidase (GUD) cleaves the substrate 4-methylumbelliferyl 3-D-glucuronide (MUG), to release 4-methylumbelliferone (MU). On exposure to 365 nm UV, MU produces bluish fluorescence. This substrate can be added to any media and fluorescence observed due to the production of GUD, which indicates the presence of *E. coli*.

Check Your Progress Exercise 4

Your answer should include following points:

1) Typical *Listeria* colonies will be black in colour surrounded by black halo.

2) This test detects the enhanced haemolysis by *Listeria* sp. in the presence of *S. aureus* and *Rhodococcus equi*. Haemolysis of *L. monocytogenes* and *L. seeligeri* is enhanced near the *S. aureus* streak; *L. ivanovii* haemolysis is enhanced near the *R. equi* streak. Other species are non-haemolytic and do not react in this test.

3) *Salmonella* produces an acidic butt (yellow) and alkaline slant (red). The blackening of butt may or may not occur depending on the H_2S production. In LI test *Salmonella* produce an alkaline butt (purple) with H_2S production. All the cultures giving alkaline reaction (purple butt) for LI agar are used for confirmation. Cultures giving an acidic reaction (yellow butt) in LI agar but an acidic butt and alkaline slant in TSI are also used.

4) BSA: Green colonies with little or no darkening of the surrounding medium; XLD: Yellow colonies with or without black centers; HE: Yellow colonies with or without black centers

5) This broth contains the pH indicator phenol red. The phenol red is peach color at a pH of 6.8 and turns a hot pink at a pH of 8.4.

6) circular, smooth, convex, moist, 2-3 mm in diameter, gray to jet-black, frequently with light-coloured (off-white) margin, surrounded by opaque zone and frequently with an outer clear zone; colonies have buttery to gummy consistency.

7) reconstituted plasma with EDTA to BHI suspension

8) The medium used is toluidine blue - DNA agar (TDA).

9) *S. aureus* produces a heat stable DNase that hydrolyzes DNA to oligonucleotides

10) Appearance of bright pink halo around the wells indicate the positive reaction.

11) yes

12) Yes

able 7.1 Differential characteristics of large-celled group I *Bacillus* species

Feature	***B. cereus***	***B. thuringiensis***	***B. mycoides***	***B. anthracis***	***B. megaterium***
Gram reaction	+[(a)]	+	+	+	+
Catalase	+	+	+	+	+
Motility	+/-[(b)]	+/-	-[(c)]	-	+/-
Reduction of nitrate	+	+/	+	+	-[(d)]
Tyrosine decomposed	+	+	+/-	-[(d)]	+/-
Lysozyme-resistant	+	+	+	+	-
Egg yolk reaction	+	+	+	+	-
Anaerobic utilization of glucose	+	+	+	+	-
VP reaction	+	+	+	+	-
Acid produced from mannitol	-	-	-	-	+
Haemolysis (Sheep RBC)	+	+	+	-[(d)]	-
Known pathogenicity[(e)]/ characteristic	produces enterotoxins	endotoxin crystals pathogenic to insects	rhizoidal growth	pathogenic to animals and humans	
[a] +, 90-100% of strains are positive.					
[b] +/-, 50% of strains are positive.					
[c] -, 90-100% of strains are negative.					
[d] -, Most strains are negative.					

Table 7.2 Biochemical tests of *Campylobacter* spp

Characteristics	*C. jejuni*	*C. jejuni* subsp. *doylei*	*C. coli*	*C. lari*	*C. fetus* subsp. *fetus*	*C. hyo-intestinalis*	*"C. upsali*
Growth at 25°C	-	±	-	-	+	D	-0
Growth at 35-37°C	+	+	+	+	+	+	+
Growth at 42°C	+	±	+	+	D	+	+
Nitrate reduction	+	-	+	+	+	+	+
3.5% NaCl	-	-	-	-	-	-	-
H_2S, lead acetate strip	+	+	+	+	+	+	+
H_2S, TSI	-	-	D	-	-	+[(c)]	-
Catalase	+	+	+	+	+	+	-
Oxidase	+	+	+	+	+	+	+
MacConkey's agar	+	+	+	+	+	+	-
Motility (wet mount)	+(81%)	+	+	+	+	+	+
Growth in 1% glycine	+	+	+	+	+	+	+
Glucose utilization	-	-	-	-	-	-	-
Hippurate hydrolysis	+	+	-	-	-	-	-
Resistance to naladixic acid	S[(d)]	S	S	R	R	R	S
Resistance to cephalothin	R	R	R	R	S[(e)]	S	S

[a] Symbols: +, 90% or more of strains are positive; -, 90% or more of strains are negative; D, 11-89% of strains are pos
resistant; S, susceptible.
[b] Proposed species name.
[c] Small amount of H_2S on fresh (<3 days) TSI slants.
[d] Nalidixic acid-resistant *C. jejuni* have been reported.
[e] Cephalothin-resistant *C. fetus* subsp. *fetus* strains have been reported.

Table 7.3 Differentiation of *Listeria* species

Species	Acid produced from			
	L-Haemolysis[a]	Mannitol	Rhamnose	Xylose
L. monocytogenes	+	-	+	-
L. ivanovii[b]	+	-	-	+
L. innocua	-	-	V[c]	-
L. welshimeri	-	-	V[c]	+
L. seeligeri	+	-	-	+
L. grayi[d]	-	+	V[c]	-

[a] Sheep blood agar stab.

[b] Ribose fermenting strains are classified as *L. ivanovii* subsp. *ivanovii* and ribose non-fermenters as *L. ivanovii* subsp. *londiniensis*.

[c] V, variable biotypes

[d] Includes two subspecies - *L. grayi* subsp. *murrayi* reduces nitrate; *L. grayi* subsp. *grayi* does not reduce nitrate.

Table 7.4 A. *Listeria* genus detection test kits prescribed for regulatory screening

1. AOAC Official Method 993.09. 2000. *Listeria* in dairy products, seafoods, and meats. Colorimetric deoxyribonucleic acid hybridization method (GENE-TRAK *Listeria* Assay).
2. AOAC Official Method 994.03. 2000. *Listeria monocytogenes* in dairy products, seafoods, and meats. Colorimetric monoclonal enzyme-linked immunosorbent assay method (*Listeria* Tek).
3. AOAC Official Method 995.22. 2000. *Listeria* in foods. Colourimetric polyclonal enzyme immunoassay screening method (TECRA *Listeria* Visual Immunoassay [TLVIA]).
4. AOAC Official Method 996.14. 2000. Assurance (Polyclonal Enzyme Immunoassay Method).
5. AOAC Official Method 997.03. 2000. Visual Immunoprecipitate Assay (VIP).
6. AOAC Official Method 999.06. 2000. Enzyme Linked Immunofluorescent Assay (ELFA) VIDAS LIS Assay Screening Method.

Table 7.4 B. Test kits useful in confirming *Listeria* isolates as *Listeria monocytogenes* or not*

1. AccuProbe™ *Listeria monocytogenes* culture confirmation test (Gen-Probe, Inc, San Diego, CA).
2. GeneTrak *Listeria monocytogenes* test kit (Neogen, Lansing, MI).
3. Probelia *Listeria monocytogenes* test kit (BioControl, Seattle, WA).
4. VIDAS *Listeria monocytogenes* test kit (BioMerieux).
5. Transia Plate *Listeria monocytogenes* (Diffchamb SA, Lyon, France)
6. FDA, SRL application of Niederhauser *et al*. method for PCR detection and identification of *L. monocytogenes*
7. BAX *Listeria monocytogenes* test. (Qualicon, Inc., Wilmington, DE)

***** These kits are in various stages of validation and when suitably validated can also be used to screen enrichments for *L. monocytogenes*. Presently, FDA only prescribes validated kits that screen for all *Listeria* species.

Table 7.5 Enrichment of *Salmonella*

Product	Media
k, dried egg whites, dried whole eggs, liquid lk, 2% fat milk, whole, and buttermilk), sodium net casein, soy flour, egg-containing products rolls, macaroni, spaghetti), cheese, dough, ls (ham, egg, chicken, tuna, turkey), fresh, d fruits and vegetables, nut meats, crustaceans crayfish, langostinos, lobster), and fish, meats, es, meat by-products, animal substances, lucts, and meals (fish, meat, bone), gelatin, guar	Lactose broth
quid whole eggs(Homogenised)	Trypticase soy broth supplemented with ferrous sulfate
ggs (chicken, duck, and others), dried yeast active yeast, black pepper, white pepper, celery , chili powder, cumin, paprika, parsley flakes, ame seed, thyme, and vegetable flakes	Trypticase soy broth
stant, dry whole milk	Brilliant green water
orange juice (pasteurized and unpasteurized), asteurized and unpasteurized), and apple juice	Universal pre-enrichment broth
opping mixes	Nutrient broth
onion powder, garlic flakes	Trypticase soy broth with potassium sulfite
ndy coating (including chocolate)	Reconstituted non fat dry milk
d food coloring substances	Tetrathionate broth
	Buffered peptone water

Table 7.6 Colony morphology of *Salmonella* on various selective media

Culture media	Typical *Salmonella* colonies	Atypical *Salmonella* colonies
Bismuth sulfite (BS) agar	Brown, gray, or black colonies; sometimes they have a metallic sheen. Surrounding medium is usually brown at first, but may turn black in time with increased incubation.	Green colonies with little or no darkening of the surrounding medium
Xylose lysine desoxycholate (XLD) agar	Pink colonies with or without black centers. Colonies may be glossy with black centers or may appear as almost completely black.	Yellow colonies with or without black centers
Hektoen enteric (HE) agar	Blue-green to blue colonies with or without black centers. Colonies may be glossy with black centers or may appear as almost completely black	Yellow colonies with or without black centers

Table 7.7 Biochemical and serological reactions of *Salmonella*

r substrate	Result		*Salmonella* species reaction[a]
	Positive	Negative	
	yellow butt	red butt	+
ecarboxylase	purple butt	yellow butt	+
LIA)	blackening	no blackening	+
	purple-red colour	no color change	-
ecarboxylase	purple colour	yellow colour	+
ed dulcitol	yellow colour and/or gas	no gas; no colour change	+[b]
oth	growth	no growth	-
e broth	blue colour	no colour change	-[c]
st	violet colour at surface	yellow colour at surface	-
ent flagellar test	agglutination	no agglutination	+
ent somatic	agglutination	no agglutination	+
red lactose	yellow colour and/or gas	no gas; no colour change	-[c]
red sucrose	yellow colour and/or gas	no gas; no colour change	-
Proskauer	pink-to-red colour	no colour change	-
red test	diffuse red colour	diffuse yellow colour	+
ns citrate	growth; blue colour	no growth; no colour change	v

more positive in 1 or 2 days; -, 90% or more negative in 1 or 2 days; v, variable.

of *S. arizonae* cultures are negative.

of *S. arizonae* cultures are positive.

Table 7.8 Typical characteristics of *S. aureus*, *S. epidermidis* and micrococci[(a)]

Characteristic	*S. aureus*	*S. epidermidis*	Micrococci
Catalase activity	+	+	+
Coagulase production	+	-	-
Thermonuclease production	+	-	-
Lysostaphin sensitivity	+	+	-
Anaerobic utilization of			
glucose	+	+	-
mannitol	+	-	-
[a] +, Most (90% or more) strains are positive; -, most (90% or more) strains are negative.			

Table 7.9: Evaluation of microbiological quality of foods with special reference to pathogens

Food	Microbiological test
• Cereal products • Nuts and nuts products	• Enumeration of micro-organisms at 30ºC • Enumeration of micro-organisms by spiral plating at 30ºC • Enumeration of *Staphylococcus aureus* • Enumeration of *E. coli* at 44ºC • Enumeration of *Bacillus cereus* • Enumeration of *Clostridium perfringens* • Detection of *Salmonella* species • Detection of *Listeria monocytogenes* • Enumeration of *Listeria monocytogenes* and *Listeria* species
• Dairy products	• Enumeration of micro-organisms at 30ºC • Enumeration of micro-organisms at 37ºC • Enumeration of micro-organisms by spiral plating at 30ºC and 37ºC • Enumeration of *Staphylococcus aureus* • Enumeration of *E. coli* at 44ºC • Enumeration of *Bacillus cereus* • Detection of *Salmonella* species • Detection of *Listeria monocytogenes* • Enumeration of *Listeria monocytogenes* and *Listeria* species • Detection of thermo-tolerant *Campylobacter* species and identification of *C.jejuni*, *C.coli* • Enumeration of Enterobacteriacae • Enumeration of coliforms at 30ºC • Enumeration of coliforms at 37ºC • Detection and enumeration of Enterobacteriaceae by MPN
• Meat and meat products • Poultry and poultry products	• Enumeration of micro-organisms at 30ºC • Enumeration of micro-organisms at 37ºC • Enumeration of micro-organisms by spiral plating at 30ºC and 37ºC • Enumeration of *Staphylococcus aureus* • Enumeration of *E. coli* at 44ºC • Enumeration of *Bacillus cereus* • Detection of *Salmonella* species • Detection of *Listeria monocytogenes* • Enumeration of *Listeria monocytogenes* and *Listeria* species • Detection of thermotolerant *Campylobacter* species and identification of *C.jejuni*, *C.coli* • Enumeration of Enterobacteriacae • Enumeration of coliforms at 30ºC • Enumeration of coliforms at 37ºC • Detection and enumeration of Enterobacteriaceae by MPN

• Eggs and egg products	• Enumeration of microorganisms at 30ºC • Enumeration of microorganisms by spiral plating at 30ºC and 37ºC • Enumeration of *Staphylococcus aureus* • Enumeration of *E. coli* at 44ºC • Enumeration of *Bacillus cereus* • Detection of *Salmonella* species • Detection of *Listeria monocytogenes* • Enumeration of *Listeria monocytogenes* and *Listeria* species • Detection of thermo-tolerant *Campylobacter* species and identification of *C.jejuni*, *C.coli* • Enumeration of Enterobacteriacae • Detection and enumeration of Enterobacteriaceae by MPN
• Fish crustaceans and molluscs	• Enumeration of micro-organisms at 30ºC • Enumeration of micro-organisms at 22ºC • Enumeration of micro-organisms by spiral plating at 30ºC and 22ºC • Enumeration of Staphylococcus aureus • Enumeration of *E.coli* in shellfish using the MPN technique and detection of Salmonella • Enumeration of *Bacillus cereus* • Detection of *Salmonella* species • Detection of *Listeria monocytogenes* • Enumeration of *Listeria monocytogenes* and *Listeria* species
• Edible fats/oils	• Enumeration of microorganisms at 30ºC • Enumeration of microorganisms by spiral plating at 30ºC • Enumeration of *E.coli* at 44ºC • Enumeration of Staphylococcus aureus • Enumeration of *Clostridium perfringens* • Detection of *Salmonella* species • Detection of *Listeria monocytogenes* • Enumeration of *Listeria monocytogenes* and *Listeria* species
• Heat processed foods in sealed containers	• Enumeration of microorganisms at 37ºC • Enumeration of microorganisms by spiral plating at 37ºC • Enumeration of *Staphylococcus aureus* • Enumeration of *E. coli* at 44ºC • Enumeration of *Bacillus cereus* • Detection of *Salmonella* species • Enumeration of *Clostridium perfringens* • Detection of *Listeria monocytogenes* • Enumeration of *Listeria monocytogenes* and *Listeria* species • Detection of thermo-tolerant *Campylobacter* species and identification of *C.jejuni*, *C.coli* • Enumeration of Enterobacteriacae • Enumeration of coliforms at 30ºC • Enumeration of coliforms at 37ºC
• Sugar products honey and confectionery	• Enumeration of microorganisms at 30ºC • Enumeration of microorganisms by spiral plating at 30ºC • Enumeration of *E.coli* at 44ºC using membranes • Enumeration of *Staphylococcus aureus* • Detection of *Salmonella* species • Detection of *Listeria monocytogenes* • Enumeration of *Listeria monocytogenes* and *Listeria* species • Enumeration of coliforms at 30ºC • Enumeration of coliforms at 37ºC • Enumeration of *Bacillus cereus* • Detection of thermotolerant *Campylobacter* species and identification of *C. jejuni*, *C. coli*
• Beverages • Fruit juice and concentrates • Alcoholic beverages	• Enumeration of microorganisms at 30ºC • Enumeration of microorganisms by spiral plating at 30ºC • Enumeration of *E.coli* at 44ºC • Enumeration of *Staphylococcus aureus* • Detection of *Salmonella* species Enumeration of coliforms at 37ºC
• Bottled waters	• Enumeration of coliforms and *E. coli* • Enumeration of Enterococci • Colony count by pour plate method • Enumeration of *Clostridium perfringens by* • Enumeration of *Pseudomonas aeruginosa* • Enumeration of coliforms and *E. coli*
• Surfaces (factory hygiene)	• Enumeration of microorganisms at 30ºC • Enumeration of microorganisms by spiral plating at 30ºC • Enumeration of *E.coli* at 44ºC using membranes • Enumeration of *Staphylococcus aureus* • Detection of *Salmonella* species • Detection of *Listeria monocytogenes* • Enumeration of *Listeria monocytogenes* and *Listeria* species • Enumeration of coliforms at 37ºC • Detection of thermo-tolerant *Campylobacter* species and identification of *C.jejuni*, *C.coli* • Enumeration of *Clostridium perfringens*
• Potable water • Environmental water	• Enumeration of coliforms and *E. coli* • Enumeration of Enterococci • Colony count by pour plate method • Enumeration of *Clostridium perfringens* • Detection of *Salmonella* • Enumeration of coliforms and *E. coli*