MANUAL ON METHOD OF MICROBIOLOGICAL TESTING

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Note: *The test methods given in the manuals are validated/ standardized test methods. However, it would be the responsibility of the respective testing laboratory to confirm that the above methods are validated in its laboratory and gives proper result in their laboratory.*

MICROBIOLOGICAL METHODS FOR ANALYSIS OF FOODS, BEVERAGES AND ADJUNCTS

1. 1 Aerobic Mesophilic Plate count

Indicates microbial counts for quality assessment of foods

1.2 Equipment:

Refer to Chapter 3 (Equipment, Materials & Glassware).

1.3 Medium:

(i) Plate count agar;

(ii) Peptone water 0.1%,

(iii) (Chapter 2 for composition of medium)

1.4 Procedure:

1.4.1 Preparation of food homogenate

Make a 1:10 dilution of the well mixed sample, by aseptically transferring sample to the desired volume of diluent.

Measure non-viscous liquid samples (i.e., viscosity not greater than milk) volumetrically and mix thoroughly with the appropriate volume of diluent (11 mL into 99 mL, or 10 mL into 90 mL or 50mL into 450 mL).

Weigh viscous liquid sample and mix thoroughly with the appropriate volume of diluent (11 $\pm$ 0.1gm into 99mL; 10$\pm$ 0.1gm into 90mL or 50$\pm$0.1gm into 450mL).

Weigh as required of solid or semi-solid sample into a sterile blender jar or into a stomacher bag. Add 450 mL of diluents. Blend for 2 minutes at low speed (approximately 8000 rpm) or mix in the Stomacher for 30-60 seconds. Powdered samples may be weighed and

directly mixed with the diluent. Shake vigorously (50 times through 30 cm arc).

In most of the food samples particulate matter floats in the dilution water. In such cases allow the particles to settle for two to three minutes and then draw the diluent from that portion of dilution where food particles are minimum and proceed.

1.4.2 Dilution:

If the count is expected to be more than 2.5 $x10^3$ per mL or gm, prepare decimal dilutions as follows. Shake each dilution 25 times in 30cm arc. For each dilution use fresh sterile pipette. Alternately use auto pipette. Pipette 1 mL of food homogenate into a tube containing 9mL of the diluent. From the first dilution transfer 1mL to second dilution tube containing 9mL of the diluents. Cyclomixer can also be used.

Repeat using a third, fourth or more tubes until the desired dilution is obtained.

1.4.3 Pour plating:

Label all petriplates with the sample number, dilution, date and any other desired information. Pipette 1mL of the food homogenate and of such dilutions which have been selected for plating into a petri dish in duplicate. Pour into each petri dish 10 to 12mL of the molten PCA (cooled to 42-45°C) within 15 min from the time of preparation of original dilution. Mix the media and dilutions by swirling gently clockwise, anti-clockwise, to and fro thrice and taking care that the contents do not touch the lid. Allow to set.

1.5 Incubation:

Incubate the prepared dishes, inverted at 35°C ±2°C for 48hours. (or the desired temperature as per food regulation e.g. in case of packaged drinking water).

1.6 Counting Colonies:

Following incubation count all colonies on dishes containing 30-300 colonies and record the results per dilution counted.

1.7 Calculation

In dishes which contain 30-300 (95 to 100 mm – size of petriplate) or 20 to 200 (90 to 95mm – size of petriplate) colonies count the actual number in both plates of a dilution and as per the formula given below, when the colony count is 100 or above

$$N = \frac{\sum C}{(N1 + 0.1N2)\, D}$$

$\sum C$ is the sum of colonies counted on all the dishes retained

N1 is the no. of dishes retained in the first dilution

N2 is the no of dishes retained in the second dilution

D is the dilution factor corresponding to first dilution

E.g

At the first dilution retained (10^{-2}):165 & 218 colonies

At the second dilution retained (10^{-3}) 15 & 24

$$N = \frac{165+218+15+24}{[2 + (0.1x2) \times 10x\text{-}2]} = \frac{422}{0.022} = 19182$$

Rounding the result to first two digits gives 19000 or 1.9 x 10^4 CFU/g or mL.

$$\mathbf{N=} \frac{\sum C}{V \times 1.1 \times d}$$

$\sum$ C is the sum of colonies counted on all the dishes retained

V is the volume of inoculum in each dish, in milliliters.

d is the dilution corresponding to the first dilution retained [d = 1 when the undiluted liquid product (test sample) is retained].

e.g.

At the first dilution retained (10^{-2}):168 colonies

At the second dilution retained (10^{-3}) 14 colonies

$$N= \frac{168+14}{1 \times 1.1 \times 10^{-2}} = \frac{180}{0.011} = 16545$$

Rounding the result to first two digits gives 17000 or 1.7 x 10^4 CFU/g or mL.

1.8 Expression of Result

- Aerobic (Mesophilic) Plate Count = 19000 CFU/g or 1.9x10^4 CFU/g or ml
- Aerobic (Mesophilic) Plate Count = 17000 CFU/g or 1.7x10^4 CFU/g or mL

or

If plates from all dilutions have no colonies and inhibitory substances have not been detected, the result is expressed as less than 1×10^1 CFU per g or mL.

If plates from the lowest dilutions contain less than 30 colonies, record the actual number and calculate as above but express results as CFU per g or mL.

Note: - *This method, as all other methods, has some limitations. Microbial cells often occur as clumps, clusters, chains or pairs in foods, and may not be well distributed irrespective of the mixing and dilution of the sample. Moreover the single agar medium used, the conditions of incubation, aeration etc., are not conducive to the growth of various populations of bacteria that may be present in a food sample.*

For statistical reasons alone, in 95% of cases the confidence limits of this test vary from ± 12% to ± 37%. In practice even greater variation may be found especially among results obtained by different microbiologists. (Corvell and Morsettle, J. Sci. Fd. Agric., 1969, vol. 20 p 573)

References:

1. Official Methods of Analysis of AOAC International (1995). 16th Edition. Edited by Patricia Cuniff. Published by AOAC International. Virginia. USA. Test 17.2.01 p.3-4.
2. Compendium of Methods for the Microbiological Examination of Foods. (1992) Carl Vanderzant and Don F. Splittstoesser Eds. Washington D.C. p. 75-87
3. Bacteriological Analytical Manual (1992) 6th Edn. Arlington, V.A. Association of Official Analytical Chemists for FDA, Washington, D.C. p. 17-21.
4. Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations - ISO 7218:2013
5. Microbiology- General Guidance for the enumeration of Microorganisms-Colony count technique at 35°C (first revision) IS5402-2002, ISO4833:1991. Bureau of Indian Standards, Manak Bhavan, 9 Bhadur Shah Zafar Marg, New Delhi110002.

2. Detection and Determination of *Bacillus cereus* in Foods, and Beverages or Determination of aerobic mesophillic spores in Foods and Beverages

2.1 Equipment:

Refer to Chapter 3

2.2 Culture media and reagents

(i) Mannitol-egg yolk-polymyxin (MYP) agar

(ii) Trypticase-soy-polymyxin broth

(iii) Phenol red dextrose broth

(iv) Nitrate broth

(v) Nutrient agar slants and plates

(vi) Nutrient agar with L-tyrosine

(vii) Nutrient broth with lysozyme

(viii) Modified Voges- Proskauer medium (VP)

(ix) Motility medium

(x) Nitrate test reagents

(xi) Voges- Proskauer test reagents

2.3 Procedure

2.3.1 Preparation of food homogenate

Prepare as directed in 1.4.1

2.3.2 For aerobic spore count pasteurize 10^{-1} dilution of sample at 80°C for 10 minutes and proceed as given below:

2.3.3 Dilution

Prepare decimal dilutions by pouring 1mL in 9 mL of dilution water.

2.3.A. Most Probable Number Method

This procedure is suitable for the examination of foods which are expected to contain fewer than 1000 *B. cereus* per gm.

i. Inoculate each of three tubes of trypticase-soy-polymyxin broth with 1 mL food homogenate and its dilutions.

ii. Incubate at 30°C for 48 hours.

iii. Examine for dense growth typical of *B. cereus*

iv. Vortex-mix and using a 3mm loop transfer one loopful from each growth positive tube to dried MYP medium plates. Streak to obtain isolated colonies.

v. Incubate at 30°C for 48 hours

vi. Pick one or more eosin pink (mannitol fermentation positive) colonies surrounded by precipitate zone (due to lecithinase activity) from each plate and transfer to nutrient agar slants for confirmation tests.

vii. The confirmed *B. cereus* count is determined using the MPN Table 4.1 of Test No. 10 for coliform count. On the basis of the number of tubes at each dilution in which *B.cereus* was detected and reported as MPN of *B.cereus* per gram.

2.3.B. Plate Count Techniques

This procedure is suitable for the examination of foods expected to contain more than 1000 *B. cereus* per gram.

Inoculate duplicate MYP agar plates with the homogenate and each dilution of homogenate by spreading 0.1 mL evenly on to each plate in duplicate with sterile bent glass streaking rods (hockey sticks). Incubate plates 24 hours at 30°C.

2.3.B.I. Counting Colonies

The numbers of eosin pink colonies surrounded by lecithinase zone are counted. If reactions are not clear, incubate plates for another 24 hours before counting. Plates must ideally have 15-150 colonies.

Five or more colonies of presumptive *B. cereus* are picked from plates and transferred to nutrient agar slants for confirmation (2.4).

2.4 Confirmation Techniques

Gram Stain

Incubate the streaked nutrient agar slant either from for confirmation for 24 hours at 30°C. Make Gram stain and examine under microscope. *B. cereus* will appear as large Gram positive bacilli in short to long chains; spores are ellipsoidal, central to sub-terminal and do not swell sporangium.

Biochemical tests

Transfer 3 mm loopful of this culture to a tube containing 0.5mL sterile diluent. Vortex mix. Inoculate (or streak) the suspended culture into the following media and read the biochemical reaction.

Table 3: Biochemical tests for Bacillus cereus

Media	Incubation at 35°C	Typical Reaction
Phenol red dextrose broth	Incubate anaerobically for 24	Acid produced (color changes from red to

	hours	yellow).
Nitrate broth	For 24 hours	Reduces nitrates to nitrites
Modified VP Medium	For 48 hours	Positive
Nutrient agar with tyrosine	For 48 hours	Positive
Nutrient broth with lysozyme	For 24 hours	Growth positive

2.5 Calculations:

$$N = \frac{\sum C}{(N1+0.1N2)\ D}$$

Refer 1.6 and 1.7 for calculation of case studies

2.6 Expression of Results:

Bacillus cereus= Present/Absent

In case of count Bacillus cereus = X no. /gm

2.7 References:

1. Official Methods of Analysis of AOAC International (1995). 16th Edition. Edited by Patricia Cuniff. Published by AOAC International. Virginia. USA Test 17.8.01 p.52-54

2. Compendium of Methods for the Microbiological Examination of Foods. (1992) Carl Vanderzant and Don F. Splittstoesser Eds. Washington. D.C. p.593 – 603

3. Bacteriological Analytical Manual (1992) 6th Edn. Arlingon. V.A. Published by Association of Official Analytical Chemists for FDA, Washington.D.C.p.191-198

3. Detection and Determination of Anaerobic Mesophilic Spore formers *(Clostridium perfringens)*

3.1 Equipment and media

3.1.1 Equipment: Refer to Chapter 3. (Equipment, Materials and Glassware)

3.1.2 Media: Tryptone sulfite cycloserine agar (TSC),
Cooked meat medium
Willis and Hobbs Medium
DRCM Medium
Clostridial Agar

3.2 Procedure

Inoculate 2 gm of food sample into 15 to 20 mL of cooked meat medium in duplicates. Incubate at 35°C for 24 h. Before incubation heat the inoculated medium in boiling water bath (100°C) for one hrs.

Positive tubes showing turbidity and gas production are streaked on to TSC agar plates. Overlay with TSC agar. Incubate plates upright, anaerobically for 18 to 20 h at 35°C.

Count all colonies that are black in color surrounded by a zone of precipitate.

3.3 Confirmation

Nagler Reaction- *In vitro test for Alpha Toxin* (This test confirms presence of Toxins producing *C. perfringens)*

Plate of egg yolk medium is taken. In half of the palte two drops of standards *C. perfringens* antitoxins is spread and dried the area of antitoxin is highlighted. Entire plate is inoculated with the suspect strain and incubated at 37°C anaerobically. Lecithinase activity is shown around colonies by precipitation in the area of the plate which is not spread with antitoxin this reaction is inhibited in the other half with specific antitoxin. The production of enzyme Lecthinase C, as demonstrated in the Nagler reaction by all types of *C. perfringens* is used to distinguish *Clostridium perfringens* from other species of clostridia. However, *C. bifermentes* also produce lecithinase and may be differentiated from *C. perfringens* by *C. bifermentes* showing proteolytic activity, ready sporulation and non-fermentation of lactose. Lactose fermentation is carried out in 1 percent peptone water sugar medium incubated anaerobically at 37°C.

Inoculate a portion of the selected black colony from TSC agar on to motility nitrate agar and lactose gelatin agar by stabbing. Also inoculate a tube of fluid thioglycollate medium. Incubate at 35°C for 24 h.

Observe microscopically the culture growing in thioglycollate media for the presence of large gram-positive rods. The culture is non-motile and growth therefore occurs only along the line of inoculum in motility nitrate agar, and they are positive for reduction of nitrate to nitrite which is indicated by the development of red or orange color of the medium. On lactose gelatin medium, the culture shows positive reaction for fermentation of lactose as indicated by gas bubbles and change in color of medium from red to yellow. Gelatin is liquified by *C.perfringens.*

3.4 Calculation

NA

3.5 Expression of Result

Clostridium perfringens = present/absent in 25gm or mL

3.6 Reference:

1. Official Methods of Analysis of AOAC International (1995). 16th Edition. Edited by Patricia Cuniff. Published by AOAC International. Virginia. USA. Test No. 17.7.02 p. 48 – 50

2. Compendium of Methods for the Microbiological Examination of Foods. (1992) Carl Vanderzant and Don F. Splittstoesser. Eds. Washington D.C. p. 623 – 635

3. Bacteriological Analytical Manual (1992) 6th Edn. Arlington.V.A. Association of Official Analytical Chemists for FDA, WashingtonDC.p.209–214

4. Detection, Determination and Confirmation of *Coliforms* and *Escherichia coli* in Foods and Beverages.

4.1 Equipment:

Refer to Chapter 3. (Equipment, Media and Glassware)

4.2 Culture media and reagents

Refer to test 5

4.3 Procedure

4.3.1 Test for Coliforms

Coliforms in foods may be enumerated by the solid medium method or by the Most Probable Number (MPN) method.

4.3.1.A Solid medium method

Preparation of food homogenate

Prepare as directed under 1.4.1

4.3.1.A.1 Dilutions

Prepare as directed under 1.4.2

4.3.1.A.2 Pour Plating

Pipette 1mL of the food homogenate (prepared sample) and of each dilution into each of the appropriately marked duplicate petri dishes.

Pour into each petri-dish 10-12 mL of VRBA (tempered to 48°C) and swirl plates to mix. Allow to solidify. Overlay with 3 to 5 mL VRBA and allow to solidify.

Incubate the dishes, inverted at 37°C for 18 to 24 hours.

4.3.1.A.3 Counting the colonies

Following incubation, count all colonies that are purple red in colour, 0.5 mm in diameter or larger and are surrounded by a zone of precipitated bile acids. Optimally the plates should have 30 to 100 colonies (diameter of plates 95- 100 mm). In case of pre-sterilized plastic petriplates the count should be between 15-150 (Plate diameter of 90 to 95 mm).

4.3.1.A.4. Calculation

Follow the procedure as described at 1.7

4.3.1.B. Most Probable Number method

This method is valuable in those samples where coliform density is low because higher quantity of sample can be used for examination. It is based on probability statistics wherein aliquots of decimal volumes/dilutions of the sample are transferred to several (1 to 5) tubes of specific medium. Positive tubes are scored and the MPN estimate is directly made using the Table 4.1

4.3.1.B.1 Preparation of food homogenate

Prepare as directed under 1.4.1

4.3.B.1.2 Dilutions:

Prepare as directed under 1.4.2

4. 3.B.1.3 Inoculation

Inoculate each of 3 tubes of LST broth (containing inverted Durham tubes) with 1mL of food homogenate (1:10).

Carry out the same operation from the first (1 in 100) and the second (1 in 1000) dilution tubes. Using a fresh sterile pipette for each dilution.

4. 3.B.1.4 Incubation

Incubate the LST tubes at 37±0.5°C for 24 and 48 hours in an incubator

4.4 Presumptive test for coliforms

Record tubes showing gas production after 24 hours and reincubate negative tubes for further 24 hours. Then record tubes showing gas production as positive.

4.5 Confirmed test for coliforms

Transfer a loopful from each gas positive tube of LST to a separate tube of BGLB broth.

Incubate the BGLB broth tubes at 35±0.5°C for 48±2h.

The formation of gas confirms the presence of coliform bacteria. Record the number of positive tubes that were confirmed as positive for coliform.

4.6 Calculation

Note the MPN appropriate to the number of positive tubes from the table 4.1

For example:

3 in 1:10; 1 in 1:100 and 0 in 1:1000. The table shows that MPN=43 coliforms per gm or mL.

Expression of Result: Coliforms= present/absent per gm or mL

Table 4.1 Most Probable Number (MPN) per 1 gm of sample, using 3 tubes with each of 0.1, 0.01, and 0.001 gm portions

Positive tubes				Positive Tubes				Positive tubes				Positive tubes			
0.1	**0.01**	**0.001**	**MPN**	**0.1**	**0.01**	**0.001**	**MPN**	**0.1**	**0.01**	**0.001**	**MPN**	**0.1**	**0.01**	**0.001**	**MPN**
0	0	0	<3	1	0	0	3.6	2	0	0	9.1	3	0	0	23
0	0	1	3	1	0	1	7.2	2	0	1	14	3	0	1	39
0	0	2	6	1	0	2	11	2	0	2	20	3	0	2	64
0	0	3	9	1	0	3	15	2	0	3	26	3	0	3	95
0	1	0	3	1	1	0	7.3	2	1	0	15	3	1	0	43
0	1	1	6.1	1	1	1	11	2	1	1	20	3	1	1	75
0	1	2	9.2	1	1	2	15	2	1	2	27	3	1	2	120
0	1	3	12	1	1	3	19	2	1	3	34	3	1	3	160
0	2	0	6.2	1	2	0	11	2	2	0	21	3	2	0	93
0	2	1	9.3	1	2	1	15	2	2	1	28	3	2	1	150
0	2	2	12	1	2	2	20	2	2	2	35	3	2	2	210
0	2	3	16	1	2	3	24	2	2	3	42	3	2	3	290
0	3	0	9.4	1	3	0	16	2	3	0	29	3	3	0	240

0	3	1	13	1	3	1	20	2	3	1	36	3	3	1	460
0	3	2	16	1	3	2	24	2	3	2	44	3	3	2	1100
0	3	3	19	1	3	3	29	2	3	3	53	3	3	3	>1100

4.7 Test for *Escherichia col*

(i) Proceed as directed under test for faecal coliforms

Streak one plate L-EMB from each positive BGLB tube in a way to obtain discrete colonies.

Incubate inverted plates at 37 ±0.5° C for 24±2 hours.

Examine plates for typical nucleated dark centered colonies with or without sheen. If typical colonies are present pick two from each EMB plate by touching needle to the center of the colony and transfer to a PCA slant.

Incubate slants at 37±0.5° C for 18 to 24 hours

Transfer growth from PCA slants to the following broth for biochemical tests (vide Chapter 4 under Biochemical Tests)

Tryptone broth: Incubate 24±2 hours at 37±0.5°C and test for indole.

MR-VP Medium: Incubate 48±2 hours at 37±0.5°C. Aseptically transfer 1ml of culture to a 13x100 mm tube and perform the Voges Proskauer test. Incubate the remainder of MR-VP culture an additional 48h and test for methyl red reaction.

Koser citrate broth: Inoculate koser citrate broth and Incubate 96 hours at 37±0.5°C and record as + or – for growth.

LST broth: Incubate 48±2 hours at 37±0.5°C and examine for gas formation.

Gram stain: Perform the Gram stain in a smear prepared from 18 hours PCA slant. Presence of small red coloured rods confirms *Escherichia coli*.

Compute MPN of *E.coli* per gm or mL considering gram negative, nonspore forming rods producing gas in lactose and classify biochemical types as follows (IMViC)(Table 5.1).

Table 5.1: Micro-organism & IMViC

Indole	MR	VP	Citrate	Type
+	+	-	-	Typical *E. coli*.
-	+	-	-	Atypical *E. coli*.
+	+	-	+	**Typical intermediate**
-	+	-	+	Atypical Intermediate
-	-	+	+	Typical *Enterobacter aerogenes*
+	-	+	+	Atypical *Enterobacter aerogenes*

4.8 Calculations

As per MPN table

4.9 Interpretation

Escherichia coli= x MPN/g or mL

4.10 Reference:

1. Official Methods of Analysis of AOAC International (1995). 16th Edition. Edited by Patricia Cuniff. Published by AOAC International. Virginia. USA. Test No. 17.2.02, p. 4-5.

2. Compendium of Methods for the Microbiological Examination of Foods. (1992) Carl Vanderzant and Don F. Splittstoesser. Eds. Washington D.C. p.325-341.

3. Bacteriological Analytical Manual (1992) 6th Edn. Arlington. V.A. Published by Association of Official Analytical Chemists for FDA, WashingtonD.C.p.27–31.

5. Direct Microscopic Count in Tomato Puree, Sauce, Paste, Chutney.

1. Howard Mold Count
2. Yeast and Bacterial Spore Count

5.1Equipment:

Refer to Chapter 3.

5.2 Special Equipment

5.2.1 Howard mould counting slide

The Howard mould counting slide is a thick glass slide with a flat plane of rectangle of 20x15 mm in the middle of the slide, surrounded by a moat flanked on each side by shoulders 0.1mm higher than flat plane surface. The cover glass when placed is supported on the shoulders and leaves a depth of 0.1mm between underside of cover glass and plane surface.

5.2.2 Haemocytometer

In the case of Haemocytometer the flat plane surface is ruled in the form of a square with sides measuring 1mm each. This square is divided into 25 medium size squares and 400 small size squares, surrounded by a moat flanked on each side by shoulders 0.1mm higher than flat plane surface. The cover glass when placed is supported on the shoulders and leaves a depth of 0.1mm between underside of cover glass and plane surface.

5.3 Procedure:

5.3.1 Preparation of sample

Tomato juice: Use juice as it comes from container

Catsup (Ketchup) or sauce: Place 50mL stabilizer solution in 100 mL graduated cylinder, add 50mL well mixed sample by displacement and mix thoroughly.

Stabilizer solution: 0.5% Sodium Carboxy Methyl Cellulose (NaCMC) – place 500mL boiling water in high speed blender. With blender running add 2.5gm NaCMC and 10mL formalin and blend for 1 minute. Keep in a stoppered bottle (handle the blender carefully because hot materials in the blender create pressure on closure with blender lid).

Puree and Paste: Dilute the sample with stabilizer solution and mix thoroughly so that the refractive index of 1.3448 to 1.3454 at 20°C (or 1.3442 to 1.3448 at 25°C) is obtained.

5.3.2 Preparation of slide

Clean Howard slide so that Newton's rings are produced between slide and cover glass. Remove cover and with knife, blade or scalpel, place portion of well mixed sample upon central disk. Spread evenly over disk and cover with cover glass to give uniform distribution. Discard any mount showing uneven distribution or absence of Newton's ring or spillage of liquid into moat.

5.3.3 Mould count

Place slide under microscope and examine with such adjustments that each field of view covers 1.5 sq.mm obtained by so adjusting draw-tube that diameter of field becomes 1.382 mm^2. When such adjustment is not possible make use of accessory drop in ocular diaphragm with aperture accurately cut to necessary size. Diameter of area of field of view can be determined by use of stage micrometer. When instrument is properly adjusted, volume of liquid examined per field is 0.15 mm^3. Use magnification of 90-125X. Use approximately 200X magnification to confirm identity of mould filament.

Prepare two mounts and count only 25 fields from each, observing in such a manner as to be representative of all sections of mount. Observe each field noting presence or absence of mould filament and recording results as positive when aggregate length of not less than 3 filaments present exceeds 1/6 of diameter of field. In case a single filament is traversing several fields of microscope it is counted as one positive field. For calculations refer 5.4.

5.4 Calculations

5.4.1 Calculation for Mould Count

Calculate proportions of positive fields from results of examination of all observed field and report as percent fields containing mould filaments.

$$\text{Percent positive fields} = \frac{\text{No. of positive fields}}{\text{No. of fields observed}} \times 100$$

5.4.2 Calculation for yeast and spores

Calculate number of yeasts/bacterial spores per 1/60 mm^3 as follows:

No. of yeasts/spores in 200 small squares = Y

No. of yeast/spores in 400 small squares = (400 x Y)/200 or = 2Y

Or 0.1 mm^3 contains = 2Y yeast

1.0 mm^3 contains = 2 x 10 Y yeasts

1/60 mm3 = (2 x 10 x 1 Y yeast)/60

Or 1/3 Y yeasts

If diluted 5 times then 5 x 1/3Y; or

5/3 Y yeasts/bacterial spores per 1/60 mm^3 of the sample.

5.5 Expression of Results

Mould Hyphae positive fields = %

Yeast and Bacterial spores = 1/60 mm^3

5.6 Reference:

1. Official Methods of Analysis of AOAC International (1995). 16th Edition. Edited by Patricia Cuniff. Published by AOAC International. Virginia. USA.

2. Compendium of Methods for the Microbiological Examination of Foods. (1992) Carl Vanderzant and Don F. Splittstoesser. Eds. Washington D.C. p. 97-104.

6. Fermentation Test (Incubation test for Cans, Tetra packs, Standy pouches).

To determine commercial sterility of processed canned and aseptically packed foods.

6.1 Equipment

Refer to Chapter 3. (Equipment, Materials and Glassware)

6.2 Media

(i) Tryptone broth
(ii) Cooked meat medium
(iii) Orange serum broth
(iv) Potato dextrose agar
(v) APT broth

6.3 Procedure

The most reliable test for determining commercial sterility of a container of a product is to incubate that container in an appropriate temperature, long enough to allow any significant microorganisms

contained therein to grow and to manifest their presence. This is the incubation or fermentation test.

6.4 Routine Production Monitoring

For low acid products destined for storage at temperatures above 40°C, containers from each sampling period or retort load should be incubated at 55°C for 5 to 7 days.

For all other low-acid products incubate at 30°C to 35°C for 10 days.

For acid or acidified foods incubate at 25°C to 30°C for 10 days.

6.5 Examination

Containers may be removed from the incubator whenever outward manifestations of microbial growth appear (e.g., swells or with transparent containers, noticeable product change). At the end of the incubation period, some containers should be opened to detect possible flat sour spoilage by measure of reduced pH as compared to good packs.

Weigh each suspect container to the nearest gram. Subtract the average tare weight of the empty container and determine net weight.

Before opening, the container must be cleaned with detergent and water, rinsed and wiped dry with clean paper towels.

Containers are opened employing aseptic techniques with extra precautions. Note abnormal odour, consistency changes, and frothiness. Measure pH electrometrically or colorimetrically.

6.6 Sub culturing of Product Samples

Transfer about 2gm of product from each container to media mentioned below. Tubes for anaerobes should be exhausted in flowing steam for an exposure of 20 min and cooled to 55°C prior to inoculation if not freshly prepared and autoclaved. For detection of molds in high acid foods, potato dextrose agar pour plates are prepared. Measure pH of the product and observe product odor and appearance.

6.6.A Low acid foods (pH > 4.5 or 4.5)

Medium	Incubation temperature and time	Organism
Tryptone broth	30 to 35°C for 5 days	Mesophilic aerobes
Tryptone broth	55°C for 5 days	Thermophilic aerobes
Cooked meat medium	30 to 35°C for 5 days	Mesophilic anaerobes
Cooked meat medium	55°C for 5 days	Thermophilic anaerobes

6.6.B High acid Foods (pH 4.5 or <4.5)

Medium	Incubation temperature and time	Organism
Orange serum broth	25 to 30°C for 5 days	For bacteria and yeasts
Potato dextrose agar	30°C for 5 days	For molds
APT broth	35°C for 5 days	For Lactobacilli, *B.coagulans* and other acid tolerant bacteria

6.7 Interpretation of Data

The development of swelled containers may indicate microbial activity. Growth must be confirmed by demonstrating excessive microorganisms by direct smear or by subculturing or abnormal product(pH, texture, odor, discolouration, evolution of gas).

Swelling may also be due to overfilling; low filling temperatures, improper vacuum closing procedures, incipient spoilage and chemical swells.

6.8 Expression of Results

Incubation test Negative/positive when incubated at 30°C/35°C for a period of 10 days.

6.9 References

1. Compendium of Methods for the Microbiological Examination of Foods. (1992) Carl Vanderzant and Don F. Splittstoesser. Eds. Washington D.C. p. 1037 – 1092.

7. Detection and Confirmation of *Salmonella species* in foods and beverages

7.1 Equipment: Refer to Chapter 3. (Equipment, Materials and Glassware)

7.2 Culture Media

(i) Lactose broth

(ii) Trypticase Soy Broth

(iii) Trypticase Soy Broth Containing Potassium Sulfite at a final concentration of 0.5%.

(iv) Reconstituted Non-Fat Dry Milk

(v) 1% aqueous Brilliant Green Dye Solution

(vi) R V Broth

(vii) Tetrathionate Broth

(viii) Xylose Lysine Deoxycholate (XLD) Agar

(ix) Hektoen Enteric Agar (HEA)

(x) Bismuth Sulphite Agar (BSA)

(xi) Brilliant Green Agar (BGA)

(xii) Triple Sugar Iron (TSI) Agar

(xiii) Lysine Iron Agar (LIA)

(xiv) Urea Broth

(xv) Phenol Red Dulcitol Broth

(xvi) Phenol Red Lactose Broth

(xvii) Tryptone Broth

(xviii) KCN Broth

(xix) Malonate Broth

(xx) Buffered Glucose (MR-VP) Medium

(xxi) Brain Heart Infusion (BHI) Broth

(xxii) Buffered Peptone Water

7.3 Procedure

7.3.1 Preparation of sample and pre-enrichment

Aseptically open the sample container and weigh 25gm sample into a sterile empty wide mouth container with screw cap or suitable closure or take 25 mL of liquid sample.

Add 225mL of sterile lactose broth to the sample or Buffered peptone water or Trypticase soy broth or nutrient broth can also be used for pre-enrichment. Make a uniform suspension by blending if necessary. Cap container and let stand at room temperature for 60 min. Instead of lactose broth the recommended pre-enrichment broth for the following food samples is as follows :

Non fat dry milk and dry whole milk – Sterile distilled water. Add 0.45 mL of 1% aqueous briliant green dye before incubation.
Dried active yeast – Trypticase soy broth
Onion-garlic powder – Trypticase soy broth containing potassium sulfite at a final concentration of 0.5%
Milk Chocolate **–** Reconstituted non fat dry milk.
Shake and adjust pH (if necessary) to 6.8±0.2 with sterile 1N NaOH or 1N HCl.

Incubate at 37°C for 24±2 hours

7.3.2 Selective enrichment

Gently shake incubated sample mixture and transfer 1 mL to 10mL of R V broth (Incubation Temp. 42°C and an additional 1 mL to tetrathionate broth. Incubate 24±2 hours at 37°C.

7.3.3 Selective media plating

Vortex – mix and streak 3 mm loopful of incubated R V medium at selenite cystine broth on selective media plates of XLD, HEA, BGA and BSA. Repeat with 3mm loopful of incubated tetrathionate broth.

Incubate plates at 37°C for 24±2 hours and 48±2 hours.

Observe plates for typical Salmonella colonies

On XLD (after 24h) - Pink colonies with or without black centres.

On HEA (after 24h) - Blue green to blue colonies with or without black centers.

On BGA (after 24 to 48h) – Small Pink colonies.

On BSA (after 24 to 48h) – Brown, grey or black colonies sometimes with metallic sheen. Surrounding medium is usually brown at first, turning black with increasing incubation time.

7.3.4 Treatment of typical or suspected colonies

Pick with needle typical or suspected five colonies (if present) from each XLD, HEA, BGA and BSA plates and streak over on Nutrient agar slants. Incubate at 37°C for 18 to 24 hrs. With help of a needle take

growth from the slants and inoculate a TSI agar slant, streaking slant and stabbing butt and then do the same into an LIA slant.

Incubate TSI and LIA slants at 37°C for 24±2 hours and 48±2h respectively. Cap tubes loosely to prevent blowing up of pluging due to excessive H_2S production.

Table 7.3.A Typical Salmonella reactions are :

	TSI	**LIA**
Slant	Alkaline (red)	Alkaline (Purple)
Butt	Acid (Yellow)	Alkaline (Purple)
H_2S production (blackening in butt)	+ or -	+ or -

A culture is treated as presumptive postive if the reactions are typical on either or both TSI and LIA slants.

7.4 Biochemical tests

Using sterile needle inoculate a portion of the presumptive positive culture on TSI slant into the following broths. Incubate at 37°C for the specified period of days and read for Salmonella typical reactions.

Table 7A: Biochemical tests

Broth/ Media	Time of incubation	Results
Urea broth	24±2h	Negative (no change in yellow colour of medium)
Phenol red lactose broth	48±2h	*Negative for gas and/or acid reaction
Phenol red sucrose broth	48±2h	*Negative for gas and/or acid reaction
Phenol red dulcitol broth	48±2h	*Postive for gas and/or acid reaction
Tryptone broth	24±2h	Negative for indole test
KCN broth	48±2h	Negative (no turbidity)
Malonate broth	48±2h	*Negative (green colour unchanged)
MR-VP medium	48±2h	Negative for VP test but positive for MR test.

***(Note : Majority of *S. arizonae* are atypical for these reactions).**

Table 7B: Criteria for discarding Non-Salmonella Cutlures

Test(s) or Substrate(s)	Results
Urease test	Postive (purple-red)
Indole test	Positive (red)
Flagellar test (Polyvalent or spicer-Edwards	Negative (no agglutination)
Lysine decarboxylase test	Negative (yellow)
KCN broth	Positive (growth)
Phenol red lactose broth*	Positive (acid and/or gas)**
Lysine decarboxylase test	Negative (yellow)
Phenol red sucrose broth	Positive (acid and/or gas)**
Lysine decarboxylase test	Negative (yellow)
KCN broth	Positive (growth)
Voges-Proskauer test	Positive (red)
Methyl red test	Negative (yellow)

* Malonate broth positive cultures are tested further to determine if they are *Salmonella arizonae*

** Do not discard positive broth cultures if corresponding LI agar cultures give typical Salmonella reactions; test further to determine if they are Salmonella sp. (vide 9).

7.5 Serological Tests

To reduce number of presumptive positive cultures (TSI positive and urease negative) carried through biochemical identification tests, the following serological flagellar (H) screening test may be carried out.

Transfer 3mm loopful of culture into 5mL of BHI broth and incubate at 37°C until visible growth occurs (About 4-6 hours).

Add about 2.5mL formalized physiological saline solution.

Test with Salmonella flagellar (H) antisera. Positive cultures show visible agglutination.

Further confirmation can be made by using Salmonella Polyvalent (O) antiserum.

7.6 Polyvalent somatic (O) test. Using wax pencil, mark off 2 sections about 1 × 2 cm each on inside of glass or plastic petri dish (15 × 100 mm). Commercially available sectioned slides may be used. Emulsify 3 mm loopful of culture from 24-48 h TSI slant or, preferably, tryptose blood agar base (without blood) with 2 mL 0.85% saline. Add 1 drop of culture suspension to upper portion of each rectangular crayon-marked section. Add 1 drop of saline solution to lower part of one section only. Add 1 drop of *Salmonella* polyvalent somatic (O) antiserum to other section only. With clean sterile transfer loop or needle, mix culture suspension with saline solution for one section and repeat for

other section containing antiserum. Tilt mixtures in back-and-forth motion for 1 min and observe against dark background in good illumination. Consider any degree of agglutination a positive reaction. Classify polyvalent somatic (O) test results as follows:

Positive — agglutination in test mixture; no agglutination in saline control.

Negative — no agglutination in test mixture; no agglutination in saline control.

Nonspecific — agglutination in test and in control mixtures. Perform further biochemical and serological tests as described in *Edwards and Ewing's Identification of Enterobacteriaceae* (2).

Somatic (O) group tests. Test as in above, using individual group somatic (O) antisera including Vi, if available, in place of *Salmonella* polyvalent somatic (O) antiserum. For special treatment of cultures giving positive Vi agglutination reaction, refer to sec. 967.28B in *Official Methods of Analysis* (1). Record cultures that give positive agglutination with individual somatic (O) antiserum as positive for that group. Record cultures that do not react with individual somatic (O) antiserum as negative for that group.

7.7 Calculation:

NA

7.8 Expression of Result:

Salmonella = Present/Absent per 25 gm or 25 mL

7.9 References:

1. Official Methods of Analysis of AOAC International (1995). 16th Edition. Edited by Patricia Cuniff. Published by AOAC International. Virginia. USA. Test. 17.9.01 p. 55 – 62.
2. Compendium of Methods for the Microbiological Examination of Foods. (1992) Carl Vanderzant and Don F. Splittstoesser. Eds. Washington D.C. p.371-422
3. Bacteriological Analytical Manual (1992) 6th Edn. Arlington. V.A. Association of Official Analytical Chemists for FDA, Washington, D.C.p.51–69

8. Detection and Confirmation of *Shigella* species foods and beverages.

8.1 Equipment:

Refer to Chapter 3. (Equipment, Materials and Glassware)

8.2 Culture media:

(i) Nutrient Broth (NB)
(ii) MacConkey agar
(iii) Deoxycholate citrate Agar (DCA)
(iv) Triple Sugar Iron (TSI) Agar slants
(v) Urea Broth
(vi) Acetate Agar Slants
(vii) Carbohydrate Fermentation Media

(viii) Tryptone Broth (for Indole test)

(ix) Buffered Glucose (MR-VP) Medium

(x) Koser's Citrate Broth

(xi) Decarboxylase Test Media with Lysine or Ornithine

(xii) Motility Test Medium

(xiii) Thornley's Semi-Solid Arginine Medium.

8.3 Procedure:

8.3.1 Enrichment:

Using aseptic techniques mix or blend if necessary 25 gm sample with 225 mL of Nurient Broth. Transfer to a sterile 500 mL bottle. Adjust pH (if necessary) to 6.0 - 7.0 with sterile 1N NaOH or 1N HCl. Incubate at 35-37°C for 18 hours.

8.3.2 Selective streaking:

Transfer a 5mm loopful of the Nutrient broth culture to the surface of MacConkey agar and DCA agar plates and streak to obtain isolated colonies.

Invert and incubate plates at 35-37°C for 24±2h. Typical Shigella colonies on XLD agar appear as red or pink colonies usually about 1mm in diameter and on Mac Conkey agar as opaque or transparent colonies.

Inoculate each suspected colony into TSI agar slant by streaking the slant and stabbing the butt. After overnight incubation at 35-37°C, typical Shigella reaction is alkaline (red) slant and acid (yellow) but with no H_2S or gas production.

8.4 Other Biochemical tests to confirm *Shigella*

Perform the following biochemical tests on a portion of the suspected culture on the TSI slant.

Table 8

Test	Reaction
Urease	-
Motility	-
Acetate utilization	-
Gas from glucose	-
IMVIC Reaction	+ + - - or - + - -
Lysine decarboxylase	-
Arginine dihydroloase	- or +
Ornithine decarboxylase	+ or -

8.5 Expression of Results

Shigella = Present / Absent per 25gm of sample

8.6 References:

1. Compendium of Methods for the Microbiological Examination of Foods. (1992) Carl Vanderzant and Don F. Splittstoesser. Eds. Washington D.C. p. 423 – 431

2. Bacteriological Analytical Manual (1992) 6th Edn. Arlingon. V.A. Association of Official Analytical Chemists for FDA, Washington D.C. p. 71 – 76

9. Detection, Determination and Confirmation of *Staphylococcus aureus* (Coagulase positive).

9.1 Equipment:

Refer to Chapter 3. (Equipment, Materials and Glassware).

9.2 Culture media:

(i) Tripticase (tryptic) soy broth with 10% sodium chloride and 1% sodium pyruvate.
(ii) Baird Parker (BP) Medium
(iii) Brain Heart Infusion (BHI) Broth
(iv) Desiccated Coagulase Plasma (rabbit) with EDTA
(v) Butterfields Buffered Phosphate Diluent
(vi) Plate Count Agar (PCA)

9.3 Procedure:

9.3.1 Preparation of food homogenate:

Aseptically weigh 50 gm food sample into the sterile blender jar. Add 450mL of diluent (1:10) and homogenize 2 min at high speed.

Alternately use stomacher for sample preparation.

In case of determination of staphylococci take 11 gm of sample 99mL of dilution water and pour on BPA 0.3 mL, 0.3 mL and 0.4 mL spread over on it. Incubate at 37°C for 24 to 48 hrs. (Proceed as 11.3 B)

9.3.2 Dilution:

Pipette 10mL of the food homogenate into 90mL of diluent (or 1mL to 9mL) to make a 1:100 dilution. Mix well using a vortex-mixer.

Transfer 1mL from this dilution to a fresh tube of 9mL to give a 1:1000 dilution. Repeat until the desired dilution is obtained.

9.3.3 Most probable number method:

This procedure is recommended for testing processed foods likely to contain a small number of *S. aureus.*

9.3.3.1 Inoculation:

Inoculate each of 3 tubes of tryptose soy broth (with 10% sodium chloride and 1% sodium pyruvate) with 1ml of food homogenate.

Carry out the same operation from the first and subsequent dilutions using a fresh sterile pipette each time. (In total three subsequent dilutions)

Maximum dilution of sample must be high enough to yield negative end point. Incubate at 35°C for 48h.

9.3.3.2 Surface Streaking:

Vortex mix the tubes from .A.1 and then using 3mm loop transfer one loopful from each growth positive tube to dried BP medium plates. Streak so as to obtain isolated colonies. Incubate at 35-37°C for 48

hours. Black colonies with halos zones observed indicates suspected staphylococcus colonies. Proceed further for coagulase test.

9.4 Interpretation:

Colonies of *S. aureus* are typically grey black to jet black, circular, smooth, convex, moist and 2-3 mm diameter on uncrowded plates. Frequently there is a light colored (off-white) margin, surrounded by opaque zone (precipitate) and frequently with outer opaque zone (precipitate) and frequently with outer clear zone; colonies have buttery to gummy consistency when touched with the inoculating needle.

9.5 Confirmation techniques:

Using a sterile needle, transfer (noting the dilution) at least one suspected colony from each plate to tubes containing 5mL BHI and to PCA slants.

Incubate BHI tubes and slants at 37°C for 18-24h.

Perform coagulase test on the BHI cultures by taking 0.3 mL of incubated BHI Broth in a serological tube add 0.3 mL of Reconstituted Rabbit Plasma. Incubate at 37°C in a water bath for five to six hrs. from time to time tilt the tube to see formation of Coagulum.

Retain slant cultures for repeat tests.

9.6 Reporting:

Coagulase positive cultures are considered to be *S. aureus*. Now record number of positive tubes (and the respective dilutions) of *S. aureus*. Report most probable number (MPN) of S. aureus per gram from Table 5 of MPN values.

9.7 Surface Plating method:

This method is applicable for general purpose use in testing foods expected to contain > 10 cells of *S. aureus* per gm.

Transfer 1mL of the food homogenate (1:10 dilution) and other dilutions to triplicate plates of BP medium and equitably distribute 1mL inoculum over the triplicate plates. Spread inoculum over agar surface using sterile bent glass streaking rods (hockey sticks).

Incubate plates in upright position in the 35-37°C incubator for about 1 hour or until inoculum is absorbed by medium. Then invert plates and incubate 45-48 hours.

9.8 Counting colonies:

Count colonies of typical *S. aureus* appearance (as described in 19.5.3.3). Test for coagulase production on suspected colonies. Add number of colonies on triplicate plates represented by colonies giving positive coagulate test. Multiply the count obtained by inverse of

corresponding sample dilution. Report as *S. aureus* per gm or mL of the sample.

9.9 Expression of result:

Staphylococcus aureus = x/gm

9.10 References:

1. Official Methods of Analysis of AOAC International (1995). 16th Edition. Edited by Patricia Cuniff. Published by AOAC International. Virginia. USA Test. 17.5.01 p.32 – 34
2. Compendium of Methods for the Microbiological Examination of Foods. (1992) Carl Vanderzant and Don F. Splittstoesser. Eds. Washington D.C. p. 533 – 550
3. Bacteriological Analytical Manual (1992) 6th Edn. Arlingon. V.A. Association of Official Analytical Chemists for FDA, Washington D.C. p. 161 – 165

10. Detection and Confirmation of Vibrio *cholera* in Foods and Beverages.

10.1 Equipment:

Refer to Chapter 3 (Equipment, Materials and Glassware).

10.2 Culture Medium:

(i) Alkaline peptone water (APW)

(ii) Gelatin Phosphate Salt Broth and Agar

(iii) Kligler Iron Agar

(iv) T1 N1 Agar

(v) TCBS Agar

10.3 Procedure:

10.3.1 Enrichment

Weigh 25gm sample and transfer to 225mL of GPS broth. Incubate at 35°C for 6 to 8 hr.

10.3.2 Plating:

Prepare dried plates of TCBS and GPS agar medium. Transfer a loopful of the surface growth of the broth culture to the surface of the two plating medium and streak in a manner that will yield isolated colonies.

Incubate plating medium for 18 to 24 hr at 35°C.

10.4 Interpretation:

Typical colonies of *V.cholerae* on TCBS agar are large (2 to 3 mm in diameter) smooth, yellow (occasional slow sucrose fermentors are green), and slightly flattened with opaque centers and translucent peripheries. On GPS agar the colonies have a cloudy zone around them that becomes more definite after a few minutes of refrigeration. In

oblique light, the colonies appear iridescent green to bronze colored and finely granular.

Typical colonies of *V. parahaemolyticus* on TCBS agar appear round, opaque, green or bluish colonies, 2 to 3 mm in diameter.

10.5 Confirmation:

Subculture all suspect colonies of *V. cholerae* on to T_1N_1 agar and incubate at 35°C for 24h. Stab streak a KIA slant with the culture and incubate the KIA slant overnight at 35°C. *V. cholerae* cultures have an alkaline (red) slant and an acid (yellow) butt, no gas and no blackening in the butt. Also perform the string test on suspect cultures as follows. Emulsify a large inoculum from the T_1 N_1 agar culture in a large drop of 0.5% sodium desoxycholate in 0.85% saline solution. Within 60 seconds, a mucoid mass forms and this material strings when a loopful is lifted (up to 2 to 3cm) from the slide. Further confirmation is by serological reactions.

Stab streak suspect colonies of *Vibrio* on the TSI slant and incubate overnight at 35°C. Typical reaction of *V. parahaemolyticus* is an alkaline slant and an acid butt but no gas or H_2S production

10.6 Results:

Test for *Vibrio cholera* = Positive or Negative/25gm

10.7 Reference:

1. Official Methods of Analysis of AOAC International (1995). 16th Edition. Edited by Patricia Cuniff. Published by AOAC International. Virginia. USA, Test No. 17.11.01 p. 108 – 110

2. Compendium of Methods for the Microbiological Examination of Foods. (1992) Carl Vanderzant and Don F. Splittstoesser. Eds. Washington D.C. p. 451 – 473

3. Bacteriological Analytical Manual (1992) 6th Edn. Arlington. V.A. Association of Official Analytical Chemists for FDA, Washington D.C.p.111–121

11 Detection and Confirmation of *Vibrio parahaemolyticus* in foods

11.1 Equipment:

Refer to Chapter 3. (Equipment, Materials and Glassware)

11.2 Culture Medium:

(i) Glucose salt-Teepol broth
(ii) TCBS Agar
(iii) Nutrient Agar for motility
(iv) Hugh-Leifson's Medium (additional 2-3% sodium chloride)
(v) Triple sugar iron agar
(vi) VP Medium

(vii) Tryptone broth with 8 and 10% NaCl

(viii) Media for Mannitol and Sucrose fermentation

(ix) Dihydrolase medium

(x) Decarboxylase medium

11.3 Procedure:

Take 25 gm of sample and 225 mL GSTB, blend with stomacher, and incubate at 37°C for 24 hrs.

A loopful from the incubated culture is streaked on to TCBS medium and incubated at 37°C for 18 hrs. The colonies of *V.parahaemolyticus* on TCBS medium are round, about 2-3 mm in diameter having green or blue centers. Such colonies shall be taken up further identification of V.parahaemolyticus. Pick up the typical colonies and streak over on NA slants. Proceeds with following tests:

11.3.1 Perform Gram staining. *V.parahaemolyticus* is Gram negative, comma shaped bacteria.

11.3.2 Test for Motility. Inoculate the motility medium by stabbing with straight wire in to the top the medium, the strain to be tested inside the glass tube to depth of about 5 millimeter. Do not contaminate the surface of medium outside the glass tube. Incubate at 37°C for 18-24 hrs. Motile stains can be found growing on the surface of the medium outside the 'inner glass tube'. If negative on the first day

continue the incubation at room temp. For a further 4-6 days before declaring the isolates as non-motile.

11.3.3 Test for Catalase - Take a nutrient agar slant of 18-24 hrs young culture and pour 1 mL of Hydrogen peroxide. Release of oxygen from hydrogen peroxide indicates presence of catalase.

11.3.4 Test of Oxidase - To the NA slants of culture add a few drops of freshly mix reagents (1% solution of alphanapthol in 95% ethanol and equal amount of 1% p-aminodimethylaniline hydrochloride in water. A positive reaction is indicated by the appearance of a blue colour within two minutes.

11.3.5 Hugh-Leifson's Test - The stain from fresh NA agar growth is stabbed in to two tube of medium, one of which is then layed over with a small amount of sterile liquid paraffin. Incubate both tube at 37°C and observed up to 4 days. Acid formation shown by yellow colour in the tube with liquid paraffin indicates fermentative utilization of glucose by *V.parahaemolyticus.*

11.3.6 Fermentation of carbohydrates - Inoculate each of peptone water medium with added sodium chloride containing respectively, Mannitol and sucrose. Incubate at 37°C for 4-5 days. Mannitol is fermented where as sucrose is not fermented by the *V.parahaemolyticus.*

11.3.7 Test for H_2S production – Inoculate TSI medium by stabbing the butt and streaking with a young culture isolates. Incubates at 37°C and observe daily for up to seven days for production of H_2S. *V.parahaemolyticus* does not produce H_2S.

11.3.8 Test for Growth in tryptone broth – Inoculate two tryptone broth tubes containing added sodium chloride (8% and 10%) and incubate for 24 hrs at 37°C. *V.parahaemolyticus* is capable of growing at in the presence of salt at 37°C and 42°C.

11.3.9 Test for VP Reaction – Inoculate MR-VP medium with added sodium chloride and incubate at 37°C for 48 hrs. Take 1 mL of broth in a tube and add 0.6 mL of 5% alpha napthol in ethanol. Shake and 0.2mL of 40% aqueous potassium hydroxide solution. Shake slope the tube and observe up to four hrs for appearance of a pink colour which indicate a positive reaction. VP test is negative for *V.parahaemolyticus.*

11.3.10 Test for Dihydrolase and Decarboxylase Activities - Inoculate using a straight wire, through the liquid paraffin each of four tubes of medium described at 14.2 (9 and 10) from a young culture on NA slant.

Incubate at 37°C and examine daily up to four days. The medium first becomes yellow due to acid production from the glucose, later, if Dihydrolation and decarboxylation of the respective amino acids occurs,

the medium violet in colour. *V.parahaemolyticus* is Lysine Decarboxylase positive and Arginine Dihydrolase negative.

11.4 Expression of Result:

V. parahaemolyticus = Positive or Negative / 25gm

12. Estimation of Yeasts and Molds in Foods and Beverages

12.1 Equipment:

Refer to Chapter 3 (Equipment, Materials and Glassware).

12.2 Media:

(i) Potato Dextrose Agar
(ii) Mycophilic Agar
(iii) Antibiotic Solution

12.3 Procedure:

Prepare food homogenate and decimal dilutions as directed under 1.4.1 and 1.4.2 respectively.

12.4 Pour plating:

Label all petri plates with the sample number, dilution, date and any other described information.

Pipette 1mL of the food homogenate of such dilutions which have been selected for plating into a petri dish in duplicate.

Acidify PDA or malt agar with sterile 10% tartaric acid to pH 3.5±0.1. Do not reheat medium once acid has been added. Pour 10-12mL of the agar medium (tempered to 45°C). Mix by swirling and allow to solidify.

(OR)

Add 2mL antibiotic solution to 100mL of plate count, mycophil or malt agar. Mix and pour 10-12mL of the agar medium tempered to 45°C. Mix by swirling and allow to solidify.

12.5 Incubation:

Invert plates and incubate at 20 or 25°C for 2 to 5 to 7 days. Discard plates after seven days of if growth is not observed, observe plates every day and mark the colonies because some time fungal growth spreads to entire plate and mask the colonies. Do not open the plates which are showing fungal sporangia.

12.6 Counting colonies:

Count colonies, multiply by the inverse of the corresponding dilution and report as yeast or mould count per gm or mL.

12.7 Reporting:

Yeast and Mould count = x/gm or mL

12.8 Reference:

1. Compendium of Methods for the Microbiological Examination of Foods. (1992) Carl Vanderzant and Don F. Splittstoesser. Eds. Washington D.C. p. 239 – 249

2. Bacteriological Analytical Manual (1992) 6th Edn. Arlington. V.A. Association of Official Analytical Chemists for FDA, Washington D.C. p.227-230

13. Detection and confirmation of *Listeria monocytogenes* in Food

Warning: while testing *L. monocytogene*s it is recommended that a properly equipped laboratory under supervision of skilled Microbiologist is done. The material used during testing is carefully disposed off after sterilization. Pregnant personnel may be asked to avoid handling of *L. monocytogenes* cultures and undertaking the tests.

13.1 Equipment:

Refer chapter 3

13.2 Culture media and reagents:

(i) Phosphate buffered peptone water
(ii) Listeria enrichment broth
(iii) Half Frazer broth
(iv) Frazer broth
(v) Modified Oxford Agar
(vi) PALCAM Agar
(vii) Tryptone Soya Yeast Extract Agar
(viii) Tryptone Soya Yeast Extract Broth
(ix) Sheep Blood Agar
(x) Carbohydrate utilization broth (Rhamnose and Xylose)
(xi) Motility Agar
(xii) CAMP Medium and test organisms
(xiii) Hydrogen peroxide solution

13.3 Preparation of test sample:

Take 25 gm of a well mixed sample in stomacher bag and use 225mL of Half Frazer broth. Stomach the sample for two minutes. Pour aseptically the contents in to a wide mouth bottle and incubate at 30°C for 24±2hr (a black coloration may develop).

Take one mL of the above culture and transfer to 9mL of Frazer broth. Incubate the inoculated tube at 37°C for 48± 2hr at 35-37°C.

From 24 hr culture of Half Frazer broth and 48hr Frazer broth streak out culture on Modified Oxford Agar and PALCAM agar so that well separated colonies are obtained.

Invert the plates and incubate at 35 or 37°C for 24 hr and if required an additional 18 hr if growth is slight or no colonies appear. Examine the plates for colonies presumed to be *L. monocytogenes*.

13.4 Appearance of colonies:

On M Ox agar the colonies are small (1mm) greyish surrounded by a black halo.

After 48 hr the colonies turn darker with a possible green luster and are about 2 mm in diameter with black halos and sunken centres.

On PALCAM agar after 24 hr the colonies appear 1.5 to 2 mm in diameter greyish green or olive green some times with black centre and always surrounded by a black halo and depressed centre.

13.5 Confirmation of Listeria species:

Select five typical colonies from one plate of each medium. If presumed colonies are less than five on a plate, take all of them.

Streak the selected colonies from each plate on to the surface of a well dried TSYEA for obtaining well separated colonies. Invert the plates

and incubate at 35°C or 37°C for 18 to 24 hr or until the growth is satisfactory.

Typical colonies are 1 mm to 2 mm in diameter, convex, colorless and opaque with an entire edge. Carry out the following tests from colonies of a pure culture on the TSYEA.

13.5.1 Catalase reaction:

With the help of loop pick up an isolated colony and place it in H_2O_2 solution on a glass slide. Immediate production of gas bubbles indicates catalase positive reaction.

13.5.2 Gram staining:

Perform Gram staining on a colony, Listeria are Gram positive slim short rods.

13.5.3 Motility Test:

Take colony from TSYEA plate and suspend it TSYE broth. Incubate at 25°C for 8 to 24 hr until cloudy medium is observed. Take a drop of culture and place it on a glass slide. Cover the top with a cover slip and observe under a microscope. *Listeria* is seen as slim rods with a tumbling motility (cultures grown above 25°C fail to show this motion. Compare them with a known culture – *cocci* or large rods with rapid motility are not *Listeria.*

As an alternative stab motility agar tube with an isolated colony from TSYEA and incubate at 25°C for 48 hr. typical umbrella like appearance around the stab indicate motility positive culture. If growth in not positive incubate up to five days and observe for the stab again.

13.5.4 Heamolysis test:

Take a colony from TSYEA and stab it on a well dried surface of sheep blood agar plate. Simultaneously stab positive (*L. monocytogenes*) and negative (*L. innocua*) control cultures. Inver the plates and incubate at 35°C or 37°C for 24±2 hr. examine the plates.

L. monocytogenes show clear light zones of beta haemolysis. *L. innocua* does not show any haemolysis. Examine the plates in a bright light to compare test cultures with the controls.

CAMP test

On a well dried surface of sheep blood agar streak each of the *Staphylococcus aureus* and *Rhodococcus equi* cultures in single lines and parallel to each other and diametrically opposite, a thin even innoculum is required.

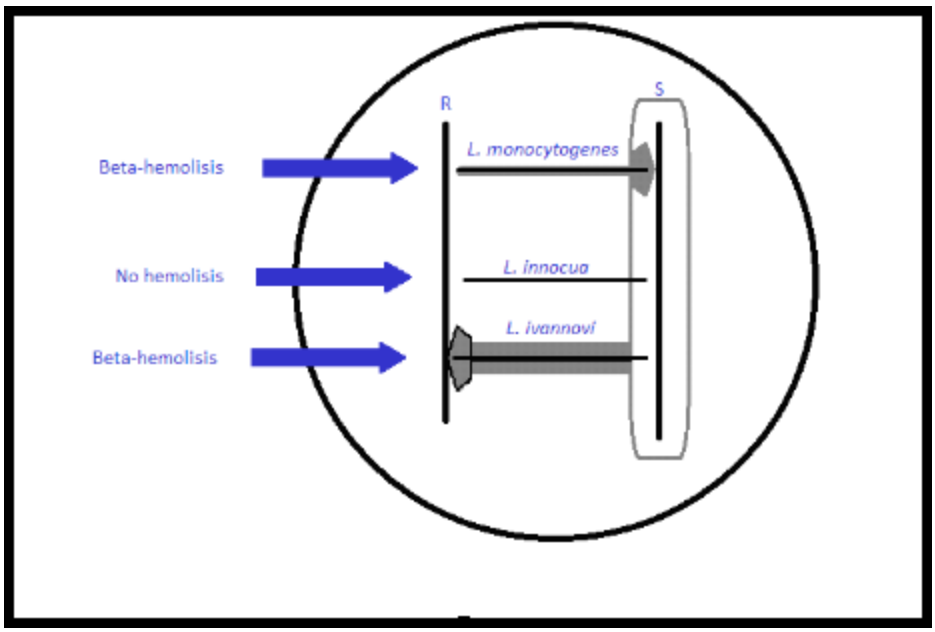
Streak the test strain separated in a similar manner at right angles to these cultures as that the test strain and *S. aureus* and *R.equi* cultures do not touch but their closest are about 1 mm or 2 mm apart. Several test strains can be streaked on the same plate. Simultaneously streak

control cultures of L *monocytogenes*, L innocua and *L. ivanovii.* Incubate plates at 35 to 37°C for 18 to 24 hr.

Observe plates against bright light. In *L. monocytogenes* case there is enhanced zone of beta haemolysis at the intersectiono of *S. aureus*.

L. innocua does not show any enhanced zone of haemolysis with *S. aureus* or *R. equi.*

In case of *L. ivanovii* enhamced beta zone of haemolysis is seen on *R. equi* side.



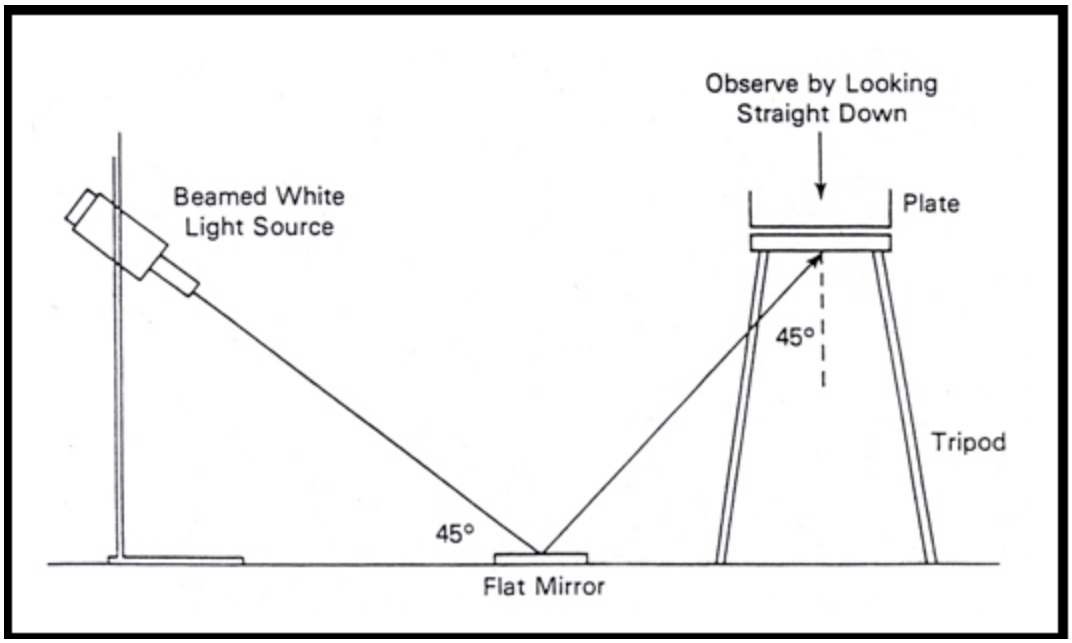
CAMP Test

13.5.5 Carbohydrate utilization:

Inoculate each of the carbohydrate utilization broths (rhamnose and xylose) with a culture from TSYE broth and incubate at 35°C or 37°C for upto 5 days. Appearance of yellow color indicates a positive reaction within24 to 48 hr.

13.5.6 Henry oblique transmitted illumination

Examine TSAYE plates for typical 1-3 mm diameter smooth convex white colonies. Observation with Henry oblique transmitted illumination can be helpful at this stage but is not mandatory.



13.6 Interpretation of results:

All Listeria species are small, Gram positive rods that demonstrate motility and catalase positive reaction. *L monocytogenes* are distinguished from other species by the characteristics listed in table given below.

Table 13.1

Species	Haemolysis	Production of acid with Rhamnose	Production of acid with Xylose	CAMP Test *S. aureus*	CAMP Test *R equi*
L.monocytogenes	+	+	-	+	-
L innocua	-	V	-	-	-

L. ivanovii	+	_	+	-	+
L. seeligeri	(+)	-	+	(+)	-
L welshmeri	-	V	+	-	-
L grayi sub species *grayi*	-	-	-	-	-
L..grayi subspecies *murrayi*	-	V	-	-	-

13.7 Expression of results:

Based on the observations and interpretation of the results report presence or absence of *L. monocytogenes* in test portion specifying the mass in grams or mililitres of the sample taken.

L. monocytogenes =present or absent/ gm or mL or 25/gm or mL.

14. Isolation, Identification and confirmation of *Campylobacter jejuni*

14.1 Equipment:

(i) Balances, 6000 gm capacity, accurate to 0.1 gm; and 200 gm capacity, accurate to 0.0001 gm

(ii) Sterile stomacher bags, 400 and 3500 mL bags and 400 mL filter bags (other bag types and sizes described in refs. 16, 21, and 22)

(iii) Whirl-pack bag racks and stainless steel baskets

(iv) Bench top shaker

(v) Centrifuge, refrigerated, capable of 20,000 x *g*

(vi) Polypropylene or stainless steel 250mL centrifuge bottles and 50mL centrifuge tubes, sterile

(vii) Large funnels with cheese cloth linings, sterile (for whole seafood and meat samples or if filter bags are unavailable)

(viii) White or orange grease pencils to mark blood-free agar plates

(ix) 50 mL sterile conical centrifuge tubes

(x) Plastic 5-10 mL tubes with screw cap lids, sterile

(xi) Cryotubes, 1 mL, sterile

(xii) Phase-contrast microscope, with 100X oil immersion objective or dark-field microscope with 63X objective or light microscope with 1000X objective

(xiii) Microscope slides, 1 cm sq cover slips and immersion oil

(xiv) Anærobe jars and bags

(xv) Water analysis apparatus

14.2 Chemicals/Media/Reagents:

(i) Camphylobacter enrichment broth

(ii) Camphylobacter isolation agar

(iii) Abeyta.hunt.bark agar

(iv) Heart infusion agar

(v) Peptone water

(vi) Triple sugar iron

(vii) MacConkey agar

(viii) Culture shipping media

(ix) Cary-blair medium

(x) Hippurate and ninhydrin reagents

(xi) Nalidixic acid and cephalothin antibiotic disk

(xii) Hydrogen peroxide

(xiii) Fetal bovine serum

(xiv) Oxidase reagent

(xv) Nitrate detection reagents

(xvi) Lead acetate strips

(xvii) Dryspot campy test

14.3 Sample preparation

14.3.1 Background information

Campylobacter spp. can survive, but not multiply, in food at refrigeration temperatures for 1-3 weeks, especially if foods (except raw milk) are in airtight containers. Their numbers decrease 2 logs upon freezing at -20°C, but the surviving organisms can be recovered ≥5 months. Samples should be analyzed for *Campylobacter* as soon as a sample package is opened; introduction of fresh oxygen adds significant stress to already weakened organisms.

Production of oxygen-neutralizing enzymes is decreased in microaerophiles, especially when cells are under stress. To combat this problem oxygen-quenching compounds, such as FBP, hemin, blood and/or charcoal, are added to the media. Prepared media absorb oxygen during storage; use freshly prepared media whenever possible. Alternatively, if prepared broth base is stored in tightly closed containers away from light (hemin is light sensitive), it can be used for

up to 2 months. Protect agar containing FPB from light and refrigerate when not in use.

Both the initial sample preparation and a 1:10 dilution are often needed for enrichment when high numbers of background flora (with broad species diversity) are present. With the sample dilution, antibiotics perform more effectively and campylobacter cells can utilize the low-oxygen atmosphere more efficiently. If heavy background contamination is suspected, add 1:10 dilution enrichment. The following instructions include mandatory dilution enrichments for shellfish and eggs.

14.3.2 Preparation of Samples

Add 2 rehydrated vials of Bolton antibiotic additives and 50 mL lysed horse blood to 1000 mL Bolton broth base. Alternatively, antibiotic additives can be prepared from individual components (G-1).

14.3.2.1 All sample types except those listed in following sect. 2(b-h)

Place filter bag in wire petri dish holder (type used in anaerobe jars). Hold bag lining in place with metal binder clip to prevent collapse during filling. Weigh 25 gm sample (50 gm if fruit or vegetables) into bag, and add 100 mL enrichment broth. Remove bag from holder, keeping clip attached and wrap twist-tie around top. Place bag(s) in basket or whirl-pak rack. Shake gently for 5 min. or place on a table-top shaker set at 25 rpm.

After the rinsing step, hold 5 min. Remove filter lining and allow it to drain a few seconds. If filter bag is not available, rinse sample in a sterile bag, and pour contents through a sterile, cheesecloth-lined funnel into the incubation bag or flask. When using metalized poly pouches for the gassed bag incubation, place filter liner from a stomacher bag into the pouch before weighing in the sample. **Note**: When analyzing acidic foods, such as chicken salad, adjust broth pH to 7.4 with 2N NaOH after the rinsing step.

14.3.2.2 Lobster tail or crab claws. Weigh 50-100 gm into a filter-lined bag and rinse as in a, above.

14.3.2.3 Whole meat carcass or sample that cannot be easily reduced to 25 gm (e.g., whole rabbit, lobster or larger piece of game meat)
Place sample into 3500mL stomacher or other sterile bag. Add 200mL 0.1% peptone water. Twist bag to seal, and swirl contents for 2-3 min. Tilt bag, and hold back food pieces to let rinse liquid flow to one corner. Sanitize a bottom bag corner with 1000 ppm hypochlorite solution or 70% ethanol; then rinse with sterile water. Aseptically cut corner of bag, and pour rinse through sterile cheesecloth-lined funnel into a 250 mL centrifuge bottle. Centrifuge at 16,000 × *g* for 15 min. Discard supernatant, and resuspend pellet in 10 mL 0.1% peptone water. Transfer 3 mL pellet mixture to 100 mL broth.

14.3.2.4 Liquid egg yolk or whole egg mixture

Divide sample into composites of two subsamples per composite, 25 gm per sub. Weigh 25 gm of each composite into 125 mL broth. After gently

mixing, transfer 25 mL to another 100 mL broth. Analyze both the 1:6 and 1:48 dilutions.

14.3.2.5 Shellfish, shucked

In general, a minimum of 12 shellfish shall be taken in order to obtain a representative sample (*APHA 1970, Recommended Procedures for the Examination of Sea Water and Shellfish*). Depending on the size of the species, this will yield an approximately 100 to 200 gm composite of shell liquor and meat. Collect the appropriate quantities of shell liquor and meats in a sterile blender or other suitable sterile container. Blend at low speed or stomach for 60 s. Remove 25 gm shellfish homogenate for sample analysis to a Stomacher bag or 500 mL flask.

Add 225 mL enrichment broth. Transfer 25 mL of the mixture to a second 225 mL enrichment broth. Analyze both the 1:10 and 1:100 enrichments.

(Several methods for enrichment and culturing are proposed for Campylobacter species requiring specific gas composition. These combinations of gases are not easily available in our country. There for anaerobic gas pack are suggested which bring down the oxygen concentration sufficiently to encourage the growth of Campylobacter species).

Incubate in anaerobe jars, reduce volume/flask or bag to 125 mL by dividing each enrichment into two parts. The gas does not penetrate into a larger volume sufficiently to provide proper growth of campylobacters.

14.3.2.6 Water

Request investigators to collect 2-4 liters for analysis. When collected, 5 mL of 1 M sodium thiosulfate should be added per liter of chlorinated water sample.

Filter smaller volume samples through 45 µm Zetapor filters, 47 mm diameter. These filters have a positive charge. The negatively charged Gram-negative organisms are more effectively retained in the filter. Filter larger volumes, especially those that are turbid, through 90 mm or larger diameter filters.

Place filter unit into autoclavable pan. If filter clogs, wear sterile gloves and open filter holder unit to aseptically remove filter with sterile forceps. Place filter into enrichment broth (see below). Place another sterile filter in unit, reassemble, and continue filtering. Use as many filters as needed per subsample. When analyzing sea or other salt water, flush excess salt off filter by running 100-1,000 mL (depending on filter size) sterile phosphate buffer through the filter as the last of the sample is going through the filter. Do this with every filter used for salt water analyses. Do not let filter become completely dry. Immediately transfer finished filter to broth(Campylobacters are very sensitive to drying and high salt concentrations).

Place filter(s) in broth in the enrichment container. When using large filters, fragment with a sterile pipet. Be sure the broth covers the filter(s).

Enrichments incubated in Campy gas in anaerobe jars should be 125 mL or less. Larger volumes should be divided into smaller amounts, aseptically dividing the filters.

14.3.2.7 Swabs

Pipet 10 mL enrichment broth into sterile 50 mL Erlenmeyer flasks with foil tops. Place one swab into each flask, aseptically breaking off the sticks below the top of the flask. Replace covers loosely. Place flasks in anaerobe jar. To fit two layers of flasks in jar, place a cardboard circle over bottom layer, leaving space around the cardboard's edge for gas circulation.

14.3.2.8 Milk, frozen dairy products

14.3.2.8.1 **Raw milk**. Instruct the investigator to test raw milk at the collection site by using a sterile pipette to place test portion onto pH test paper (pH 6-8 range). If the pH is below 7.6, add sterile 1-2 N NaOH and gently to adjust it to 7.5 ± 0.2. Immediately upon receipt in the laboratory, test the pH of the dairy sample with pH test paper and adjust to pH 7.5 ± 0.2 with sterile 1-2 N NaOH if necessary. Centrifuge a 50 gm portion at 20,000 × *g* for 40 minutes. Discard supernatant and dissolve pellet (not fat layer) in 10 mL enrichment broth. Transfer pellet to 90 mL enrichment broth.

14.3.2.8.2 **Other milk types and ice cream**. Adjust pH as in raw milk. Centrifuge a 50 gm portion at 20,000 x g for 40 minutes. Discard supernatant and dissolve pellet (not fat layer) in 10 mL enrichment broth. Transfer pellet to 90 mL enrichment broth.

Ice cream and other frozen dairy products: melt and aseptically remove any candy or other solids before weighing out.

14.3.2.8.3 **Cheese**. Weigh 50 gm into a filter bag and add 50 mL 0.1% peptone. Stomach 15-30 s. Remove lining, letting it drain 5 s, and discard. Centrifuge and remove pellet to broth as in raw milk (h,1).

14.3.2.8.4 **"Milk sock" or strainer (gauze piece used to filter out solids during milking)**. Place 50 gm piece in 100 mL broth.

14.4 Pre-enrichment and enrichment (modified Park and Humphrey methods)

14.4.1 Pre-enrichments

14.4..1.1 4 h pre-enrichment If the age of the sample is known to be within 10 days of production or time of contamination, or if the sample is a dairy product, pre-enrich at 37°C for 4 h. The pre-enrichment should be incubated under microaerobic conditions. (Anaerobic gas pack).

14.4.1.2 5 h pre-enrichment Use the 5 h method if any product has been refrigerated for ≥10 days. All water or shellfish samples are pre-enriched by the 5 h method.

Incubate at 30°C for 3 h, then at 37°C for 2 h. Perform the 37°C incubation under microaerobic conditions. This method yields greater recovery for severely stressed organisms.

14.4.2.1 Enrichment (microaerobic, D-3)

After pre-enrichment, raise the temperature in the water bath or move to a 42°C incubator. If analyzing for *C. fetus*, keep the temperature

at 37°C, even if a thermotolerant strain (growth at 42°C) was associated with the sample. Incubate **shaking** enrichments 23-24 hr, except shellfish samples, which are incubated an extra 4 hr. Dairy samples are incubated 48 hrs total. Incubate **non-shaking** enrichments 28-29 hr, except shellfish, which are incubated 48 h. Incubate samples for *C. fetus* at 48 hr (shaking) or 52 hr (non-shaking).

14.4.2.2 Incubation and atmosphere modification methods for enrichments. Incubate in anaerobe jars with a modified atmosphere with anaerobe gas pack.

14.4.2.2.1 **Gassed jar system**. Place stomacher bags with the tops loosely closed with a twist tie in a gas jar. Amount of broth in each bag should not be over 125 mL. When using the 5.5 liter rectangular jar, prepare a deep tray from foil and tape to contain the bottoms of the bags inside the jar.

14.4.2.2.2 **Gas pak envelopes.** Use 3 BBL Campy pak, Pack Plus or EM Anaerocult C gas-generating envelopes per 9.5 liter BBL jar and 1 per small jar. With the 3.4 litre Difco and Oxoid jars, use Difco or Oxoid gas pak envelopes, which are designed for use with a 3.4 litre jar. Gas paks requiring water need to be used with a catalyst. With a 2.5 litre rectangular jar use 1 gas pak **(type not used with water);** 3 for a 5.5 litre jar. **Or use 1 anaerobe** gas pak in a 9.5 litre BBL jar.

14.4.2.2.3 **Guidelines for storing and maintaining jars.** If a jar lid with gauges is knocked against a hard surface, a gauge can become misaligned. Mark new "0" place on gauge and adjust vacuum and gas readings accordingly.

Store jars with screw clamps placed in jars so that one end is lying over lip of jar bottom. Prop lid against clamp to allow free flow of air and prevent mold build-up from damp jar. Or clean jars between uses with 70% alcohol and dry before storing.

If a jar will not hold vacuum or gas pressure, check for the following: cracks in the jar bottom, cracked or missing rubber rings or seals in the lids or a faulty valve stem. Replacement valve stems and a Schrader extractor tool are available from the jars' distributors or bicycle shops. To replace stems, place prongs of extractor over valve stem and turn counter-clockwise until stem is removed. Drop new valve stem (pin-head side up) into valve and turn clockwise until meeting resistance.

14.4.2.3 Positive controls

Store *Campylobacter* cultures in freezing medium (G-) at -70°C. If cultures are used often, they can be kept at room temperature in semi-solid storage media (G-4). Control cultures can be ordered from ATCC. Labs should stock *C. jejuni*(ATCC 33560), *C. coli* (ATCC 33559), *C. lari* (ATCC 35211), and *C. fetus* (ATCC 27374).

Inoculate broth or agar positive controls from a frozen culture by rubbing a moistened sterile swab against the culture and breaking off the swab end into broth or swabbing agar plate. Incubate microaerobically.

To freeze a culture, grow it first on Abeyta-Hunt-Bark (AHB) agar without antibiotics. Inoculate plates generously and incubate under microaerobic conditions, 42°C, 24 h. Incubate *C. fetus* cultures at 37°C, 48 hr. Mix enough freezing media to allow 1 ml/plate. Wearing gloves, pipet 1.0 mL onto each plate. Use a sterile hockey stick to wash the

growth to one end of each plate. Transfer washings to a sterile test tube. Pipet 0.5 mL of culture washings to cryotubes or sterile polypropylene test tubes. Freeze at -70°C. Freezer shock can be reduced by freezing the cultures in an alcohol-dry ice bath. . Alcohol will remove most markers' identification, so mark tubes with tape labels on lids or use marker that will not be affected by alcohol.

When storing cultures in semi-solid medium, inoculate the medium **at the surface** and incubate loosely-capped tubes under microaerobic conditions, 24 hr. See previous paragraph for proper incubation temperatures. After incubation, tighten caps and place away from direct light. Cultures can be stored up to 2 months with subsequent transfer.

Note: To ship cultures, grow the culture on AHB agar plates w/o antibiotics and swab off growth. Place swab in a sterile polypropylene screw-cap shipping tube filled with Cary-Blair media. Aseptically break off the excess swab stem and tighten the tube cap. Alternatively, grow culture and ship on AHB agar slants (in shipping test tube) w/wo 5% lysed horse blood, w/o antibiotics.

15 Isolation, identification and confirmation

15.1 Isolation procedure

After 24 and 48 hr, streak enrichments onto **either** Abeyta-Hunt-Bark or modified CCDA agars. Make a 1:100 dilution (0.1mL to 9.9 mL 0.1% peptone) of each enrichment and streak undiluted and diluted portions. For shellfish, eggs, and other enrichments prepared as dilutions, streak from the broths only. Transfer two loopfuls of enrichment broth to each plate and then streak for isolation. Protect plates from light.

Place plates in anaerobe jars (1/2 full if possible) or air tight plastic bag (4 mil wt). Heat seal or roll close and tape the mouth of the bag. Do not delay bringing jars or bags to microaerobic conditions. For jars, use either the evacuation/gassing method, Campy gas paks or 1 anaerobe pak with a 9.5 litre BBL large jar (see D,3,c-gassed jar system). If using bags, attach a pipet to both the gassing and evacuation tubing. With the vacuum set very low, evacuate through a cut corner, then gas, repeat 2× and tape the corner to close. Bags can be used with Incubate at 42°C, 24-48 hr. Check for growth at 24 hr. If analyzing for *C. fetus*, incubate at 37°C for 48-72 hr.

The inoculated agars may be incubated at a range of 37-42°C, but thermophilic campylobacters show more rapid growth at higher temperature. **NOTE**: When preparing agar in plates, dry plates overnight on bench. If plates must be used the same day, place them in 42°C incubator for several hours. Do not dry in a hood with lids open. Even very brief drying of surface will inhibit campylobacter growth.

15.2 Identification

Campylobacter colonies on agar are round to irregular with smooth edges. They can show thick translucent white growth to spreading, film-like transparent growth. Pick one typical colony per plate and prepare wet mount slide. To prepare, emulsify pick in drop of saline or buffer on slide. Cover each with 22 × 22 mm cover slip and examine without oil under dark-field optics at 63× or with oil under phase-contrast at 1000×. Store plates to be picked at 4°C under microaerobic conditions if analysis is not begun quickly.

If neither type of scope is available, prepare wet mounts as follows: Emulsify a colony pick in 0.1 mL of contrast stain (50-50 mix of Gram's crystal violet to saline or buffer). After 3-5 min., prepare a wet mount and view under a 1000× oil-immersion light microscope. Compare with a positive control culture. *Campylobacter* cells are curved, 1.5-5 m long, usually in chains resembling zigzag shapes (any length). Cells picked from agar often demonstrate only "wiggly" motility, whereas those from broth swim rapidly in corkscrew motion. About 10% of strains are nonmotile. Older or stressed cells have decreased motility and may show coccoid forms. Wear gloves or wash hands and disinfect microscope stage and lens after completing wet mounts. An infective dose can be acquired from cell suspensions that leak from slides.

If organisms appear typical, restreak to Abeyta-Hunt-Bark agar without antibiotics, two colonies/sub. Confirm only one plate/sub. Choose the plate with the least background growth. Refrigerate isolation agar plates microaerobically in case repicking is necessary. Incubate restreaked picks at 42°C, 24-48 hr, microaerobically (37°C for *C. fetus*). Continue to restreak as necessary to obtain a pure culture. One or two plates can be incubated using the pouch-bag or pouch-jar systems. (A.15a)

15.3 Confirmation

Perform catalase and oxidase tests from growth on a restreaked AHB plate. Place a loopful of growth in a drop of 3% H_2O_2. Bubbles indicate positive catalase test. Rub a loopful of growth on filter dampened with oxidase reagent. If the reagent turns purple, it is oxidase-positive. All

Campylobacter spp. are oxidase-positive. Note: Colonies grown on charcoal agar plates can give a false-negative reaction.

15.4 Biochemical tests (Table 1)

All tests should include the following controls: *C. jejuni* (for hippurate and other tests) and *C. lari* (for antibiotic resistance and hippurate). If testing for *C. fetus*, also include *C. fetus* as a positive control.

Table1 Biochemical Tests

Characteristic	***C. jejuni***	***C. jejuni* subsp.*doylei***	***C. coli***	***C. lari***	***C. fetus* subsp.*fetus***	***C. hyo-intestinalis***	***"C. upsaliensis"*[b]**
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]	-

Characteristic	***C. jejuni***	***C. jejuni* subsp.*doylei***	***C. coli***	***C. lari***	***C. fetus* subsp.*fetus***	***C. hyo-intestinalis***	***"C. upsaliensis"*[b]**
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 positive; R, 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.

Characteristic	***C. jejuni***	***C. jejuni* subsp.*doylei***	***C. coli***	***C. lari***	***C. fetus* subsp.*fetus***	***C. hyo-intestinalis***	***"C. upsaliensis"*[b]**

[e] Cephalothin-resistant *C. fetus* subsp. *fetus* strains have been reported.

NOTE: *C. hyointestinalis* requires H_2 for vigorous growth and grows very poorly in O_2, CO_2, N_2 gas mixture. Use Campy Pak gas generating envelopes without catalyst for all incubations if analyzing samples for this species. "*C. upsaliensis*" does not grow in the FDA medium because of its sensitivity to antibiotics. Please call for more information.

Chart information adapted from T.J. Barret, C.M. Patton, and G.K. Morris (1988). Lab. Med. 19:96-102.

15.4.1 Gram stain. Use 0.5% carbol fuchsin as counterstain. *Campylobacter* spp. are Gram negative.

15.4.2 Hippurate hydrolysis. Emulsify generous 2 mm loopful of growth from the restreaked pick on the nonselective or antibiotic inhibition plate to 0.4 mL 1% hippurate solution in 13 × 100 mm tube. Incubate 2 hr in 37°C water bath. Add 0.2 mL ninhydrin reagent (R47), agitate, and reincubate 10 min. Violet (not medium or pale purple) color is positive reaction. Only *C. jejuni* is hippurate-positive. Refrigerate hippurate solution up to 1 month and ninhydrin solution up to 3 months.

15.4.3 TSI reaction. Generously inoculate slant and stab butt of TSI (M149) slant from blood plate. Incubate under microaerobic atmosphere at 35-37°C for 5 days. Eighty percent of *C. coli* and a few *C. lari* produce H_2S at stab; *C.jejuni* does not produce H_2S. All *Campylobacter* spp. produce alkaline/alkaline reactions. Prepare slants no more than 7 days before use.

15.4.4 Glucose utilization test. Stab 2 tubes of O-F media (M116), 3 times in each tube from blood plate. One tube contains glucose and one contains base alone. Incubate 4 days under microaerobic atmosphere at 35-37°C. *Campylobacter*spp. do not utilize glucose or other sugars and show no change in either tube.

15.4.5 Dryspot Campy Test or Alert for Campylobacter (see B, 2.i.). Follow manufacturer's instructions to test 1-2 colonies from an isolation agar plate (presumptive identification only), or a restreaked AHB plate. These kits produce a presumptive identification and are not a substitute for biochemical identification. They are not serotyping kits. If the kits do not produce a positive test, the culture might be another species of *Campylobacter* if other tests indicate*Campylobacter*.

15.4.6 Tests using diluted inoculum. Emulsify growth from colony into 5 mL 0.1% peptone and adjust turbidity to McFarland No. 1. Use this suspension to inoculate the following tests.

1. **Antibiotic inhibition**. Swab an Abeyta-Hunt-Bark agar plate without antibiotics with the suspension and drop nalidixic acid and cephalothin disks onto opposite sides of plate. Incubate microaerobically, 24-48 hr, 37°C. Any size zone indicates sensitivity.

2. **Growth temperature tolerance**. Using loopful of diluted culture, streak a line across each of 3 plates of Abeyta-Hunt-Bark agar. Inoculate up to 4 cultures or lines per plate. Incubate one plate at 25°C, one at 35-37°C, and one at 42°C under microaerophilic atmosphere for 3 days. More growth than the initial inoculum is a positive test.

3. **Growth on MacConkey agar (M91)**. This alternative test is not necessary to identify *C. jejuni*, *C. coli*, or *C. lari*, but is useful to identify other species. Streak loopful from diluted culture across MacConkey agar plate, 4 cultures per plate. Incubate under microaerophilic atmosphere, at 37°C for 3 days. Record positive or negative growth. Agar plates should be not more than 3 days old.

4. **Growth in modified semisolid media (G-5)**. Inoculate surfaces of the following biochemicals with 0.1 mL diluted culture. Incubate microaerobically all semisolid media at 35-37°C for 3 days, except nitrate media, which are incubated 5 days. **Growth will be in a narrow band pattern just under the surface.**

1% glycine. Record ± growth.

3.5% NaCl. Record ± growth.

15.4.7 **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.

15.4.8 **Nitrate reduction**. After 5 days, add nitrate reagents A and B (R48). Red color is positive reaction.

Chapter 2

Culture Media

Acetate agar

Sodium chloride	5.0 gm
Magnesium sulfate	0.1 gm
Monoammonium phosphate	1.0 gm
Dipotassium phosphate	1.0 gm
Sodium acetate	2.0 gm
Bromothymol blue	0.08 gm
Agar	20.0 gm
Distilled water	1.0 litre

Mix ingredients in distilled water and heat gently to dissolve. Dispense 7 mL portions into 16 x 150 mm tubes.

Sterilize at 121°C for 15 minutes, and slant the tubes to obtain a 1 inch butt and a 1.5 inch slant, pH 6.8±0.2.

Abeyta-Hunt Bark Agar

Heart infusion agar (Difco)	40 gm
Yeast extract	2 gm
Distilled water	950 mL

Autoclave 15 min at 121°C. Final pH, 7.4 ± 0.2. Cool and add sodium cefoperazone (**6.4 mL** if using broth preparation or **4 mL** of the agar preparation[below]), 4 mL rifampicin, 4 mL amphotericin B, and 50 mL lysed horse blood.

After pouring plates, dry plates overnight on bench.If plates must be used the same day, place them in 42°C incubator for several hours. Do not dry in a hood with lids open. Even very brief surface drying will inhibit campylobacter growth.

1. **Sodium cefoperazone**. Prepare as described for broth for final concentration of 32 mg/litre, adding 6.4 mL to agar or dissolve 0.8 gm in 100 mL water in a 100 mL volumetric flask, filter and add 4 mL to agar.

2. **Rifampicin**. Dissolve 0.25 gm slowly into 60-80 mL alcohol in a 100 volumetric flask, swirling repeatedly. When powder is dissolved completely, bring to the line with distilled water. Store up to 1 year at -20°C. Final concentration is 10 mg/litre.

3. **Amphotericin B, solubilized** (Sigma Cat. No. A9528). Dissolve 0.05 gm in water in a 100 mL volumetric flask and bring to the line. Filter sterilize and store at -20°C for 1 year. Final concentration is 2 mg/litre. Add 4 mL per liter.

4. **FBP**. Dissolve 6.25 gm Sodium pyruvate in 10-20 mL distilled water. Pour into a 100 mL volumetric. Add 6.25 gm Ferrous sulfate and 6.25 gm Sodium metabisulfite. Bring to the line with distilled water and filter sterilize. Use 4 mL/liter agar.

Note: FBP is light sensitive and absorbs oxygen rapidly. Prepare only the amount needed. 10-25 mL amounts can be filtered with a 0.22 µm syringe filter. Freeze unused portions in 5 mL amounts at -70°C as soon as possible after preparation. It can be stored at -70°C for 3 mos or -20°C for 1 mo.

Baird-Parker medium

Basal medium

Tryptone	10.0 gm
Beef extract	5.0 gm
Yeast extract	1.0 gm
Glycine	12.0 gm
Lithium chloride $6H_2O$	5.0 gm
Agar	20.0 gm

Suspend ingredients in 950 mL distilled water.

Boil to dissolve completely. Dispense 95.0 mL portions in screw capped bottles. Autoclave 15 minutes at 121°C. Final pH 6.8-7.2 at 25°C.

Bismuth Sulfite Agar

Peptone	10.0 gm
Beef extract	5.0 gm
Dextrose	5.0 gm
Disodium phosphate	4.0 gm
Ferrous sulfate	0.3 gm
Bismuth ammonium citrate	1.85 gm
Sodium sulfite	6.15 gm
Agar	20.0 gm
Brilliant green	0.025 gm
Distilled water	1.0 litre

Dissolve ingredients in distilled water by boiling approximately 1 minute. Adjust to pH 7.7$\pm$0.2, cool to 45 to 50°C, suspending precipitate with gentle agitation, and pour plates without sterilizing medium. Let plates dry with covers partially open. Caution: Plates lose selectivity after 72 hours.

Brain Heart Infusion Broth

Calf brain, infusion from	200.0 gm
Beef heart, infusion from	250.0 gm
Proteose peptone or polypeptone	10.0 gm
Dextrose	2.0 gm
Sodium chloride	5.0 gm
Disodium phosphate	2.5 gm
Distilled water	1.0 litre

Dissolve ingredients in distilled water by bringing to a boil. Dispense into tubes and autoclave for 15 minutes at 121°C. Final reaction should be pH 7.4±0.2.

Brilliant – Green Lactose Bile Broth 2%

Peptone	10.0 gm
Lactose	10.0 gm
Oxbile	20.0 gm
Brilliant-green	0.0133 gm
Distilled water	1.0 litre

Dissolve the peptone and lactose in 500mL of distilled water, add the ox bile dissolved in 200mL of water, mix and make up to 975 mL, and adjust pH to 7.4±0.1. Add 13.3 mL of 0.1% aqueous solution of brilliant green. Add distilled water to bring the total volume to 1 litre. Dispense in 10mL portions into 20 x 50 mm test tubes containing inverted Durham tubes. Sterilize for 15 minutes at 121°C.

Bromocresol Purple Carbohydrate Broth

Basal Medium	
Peptone	10.0 gm
Beef extract (optional)	3.0 gm
Sodium chloride	5.0 gm
Bromocresol purple	0.04 gm
Distilled water	1.0 litre

Dissolve the desired carbohydrate (5.0 gm or 10.0 gm glucose, 5.0gm adonitol, 5.0 gm arabinose, 5.0 gm mannitol 5.0 gm maltose, 5.0gm sucrose, 5.0 gm lactose, 5.0 gm sorbitol, 5.0 gm cellobiose, 5.0 gm salicin, 5.0 gm trehalose or raffinose) per litre of basal medium. Adjust pH to 7.0±0.2. Dispense 8 mL aliquots to 16 x 150 mm tubes containing inverted 12 x 75 mm tubes. Autoclave 10 minutes at 121°C. Allow autoclave temperature to drop slowly.

Buffered Peptone Water

Peptone	10.0 gm
Sodium chloride	5.0 gm
Disodium hydrogen phosphate	9.0 gm
Potassium dihydrogen phosphate	1.5 gm
Distilled water	1.0 litre

Adjust pH to 7.0, dispense in portions of 225mL into bottles of 500mL capacity and of 9mL in tubes. Sterilize for 20 min at 121°C.

Butterfield's Buffered Phosphate Diluent

Stock solution:

Monopotassium hydrogen phosphate	34.0 gm
Distilled water	500.0 mL

Adjust to pH 7.2 with about 175 mL sodium hydroxide solution dilute to one liter. Sterilize at 121°C for 15 minutes and store in refrigerator.

Diluent

Dilute 1.25 mL stock solution to 1.0 litre with distilled water. Prepare dilution blanks in suitable containers. Sterilize at 121°C for 15 minutes.

Cooked Meat Medium

Beef heart.....	454.0 gm
Proteose peptone	20.0 gm
NaCl	5.0 gm
Glucose.....	2.0 gm

Finely chop beef heart. Add approximately 1.5 gm of heart particles to test tubes. Add remaining components to distilled water and bring volume to 1.0 L. Mix thoroughly. Distribute into tubes in 10 mL volumes. Autoclave for 15 min at 121°C.

Czapek Yeast Autolysate (CYA) Agar

Sucrose	30.0 gm
Agar	15.0 gm
Yeast extracts	5.0 gm
$NaNO_3$	5.0 gm
K_2HPO_4	1.0 gm
KCL	0.5 gm
$MgSO_4.7H_2O$	0.5 gm
$FeSO_4.7H_2O$	0.01 gm

pH 7.3$\pm$0.2 at 25°C

Add sucrose to 100mL distilled water and autoclave for 15 min at 121°C. Cool to 50°C. Add the other components to 900 mL distilled water. Autoclave for 15 min at 121°C. Aseptically add the sterile sucrose solution after it has cooled to 50°C.

Cary- Blair Medium Base (Transport Medium w/o Charcoal)

Disodium phosphate……..	1.100
Sodium thioglycollate……..	1.500
Sodium chloride……	5.000
Agar……..	5.000

Final pH (at 25°C) 8.4±0.2 **Formula adjusted, standardized to suit performance parameters Directions Suspend 12.6 gm in 991 mL distilled water. Heat to boiling to dissolve the medium completely. Cool to 50°C and aseptically add 9 mL of 1% aqueous calcium chloride solution. Adjust pH to 8.4 if necessary. Distribute in 7 mL amounts in screw-capped tubes. Steam for 15 minutes. Cool and tighten the caps.

Compylobacter Enrichment Broth

Ingredients Gms/Litre	
Gelatin peptone #	10.000
Glucose monohydrate	5.000
Dehydrated bile ##	20.000
Disodium hydrogen phosphate, dihydrate	8.000
Potassium dihydrogen phosphate	2.000
Brilliant green	0.015
pH after heating (at 25°C)	7.2±0.2 **

Formula adjusted, standardized to suit performance parameters Directions Suspend 42.93 gm (the equivalent weight of dehydrated medium per litre) in 1000 mL purified/distilled water. Dispense 120 mL amounts in 250 mL flasks or 9 mL amounts in tubes. Stopper with cotton plugs or loose fitting caps. Heat in free for 30 flowing steam or boiling water (100°C) for 30 minutes and cool immediately. DO NOT AUTOCLAVE.

Compylobacter Isolation Agar

Proteose peptone……..	15.000
Liver digest……	2.500
Yeast extract …….	5.000
Sodium chloride ……..	5.000
Agar……	12.000

Final pH (at 25°C) 7.4±0.2

Suspend 19.75 gm in 500 mL distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C and aseptically add 5-7 %v/v sterile lysed horse blood or 10% sterile defibrinated sheep blood and rehydrated contents of 1 vial of Campylobacter Supplement-I (Blaser-Wang) (FD006) or Campylobacter Supplement-III (Skirrow) (FD008). Mix well and pour into sterile Petri plates.

Decarboxylase Test Media

Basal for use with Lysine, Arginine, Ornithine Moeller method (1954, 1955)

Basal medium

Peptone	5.0 litre
Beef extract	5.0 gm
Bromocresol purple (1.6%)	0.625 mL
Cresol red (0.2%)	2.5 mL
Glucose	0.5 gm
Pyridoxal	5.0 mg
Distilled water	1.0 litre

The basal medium is divided into four equal portions, one of which is tubed without the addition of any amino acids. These tubes of basal medium are used for control purposes. To one of the remaining portions of basal medium is added 1% of L-lysine dihydrochloride; to the second, 1% of L-arginine monohydrochloride and to the third portion, 1% of L-ornithine dihydrochloride. If DL amino acids are used, they should be incorporated into the medium in 2% concentration,

since the microorganisms apparently are active against the L forms only. The pH of the fraction to which ornithine is added should be readjusted after the addition and prior to sterilization. The amino acid medium may be tubed in 3 or 4 mL amount is small (13x100mm) screw capped tubes and sterilized at 121°C for 10 minutes. A small amount of floccular precipitate may be seen in the ornithine medium. This does not interfere with its use.

Inoculation: Inoculate lightly from a young agar slant culture. After inoculation, add a layer (about 10mm in thickness) of sterile mineral (paraffin) oil to each tube including the control. A control tube always should be inoculated with each culture under investigation. Incubate at 37°C; examine daily for 4 days. Positive reactions are indicated by alkalization of the medium with a color change from yellow to violet. Weakly positive reactions may be bluish gray.

Dextrose Tryptone Agar

Agar	15.0 gm
Pancreatic digest of casein	10.0 gm
Glucose	5.0 gm
Bromocresol purple	0.04 gm

pH: 6.9$\pm$0.2 at 25^{o}C.

Add components to distilled water and bring volume to 1.0 L. Mix thoroughly. Gently heat and bring to boiling. Autoclave at 121°C for 15min. Pour into sterile tubes or petri dishes.

Dihydrolase Broth Base

Peptic digest of animal tissue ……	5.000
Yeast extract……..	6.000
Dextrose……	2.000
Sodium chloride ……..	30.000
Bromo cresol purple ……..	0.032

Final pH (at 25°C) 6.8±0.2 **

Formula adjusted, standardized to suit performance parameters Directions Suspend 43.03 gm in 1000 mL distilled water. Heat, if necessary to dissolve the medium completely. Divide in 2 parts. Add 0.5% L-Arginine to first portion. Use second portion as control. Dissolve completely and dispense 3.0 mL into 13 mm x 100 mm screw cap tube. Sterilize by autoclaving at 115°C for 15 minutes

EC Broth

Trypticase or tryptone	20.0 gm
Bile salt No. 3.....	1.5 gm
Lactose	5.0 gm
Dipotassium hydrogen phosphate	4.0 gm
Potassium dihydrogen phosphate	1.5 gm
Sodium chloride	5.0 gm
Distilled water	1.0 litre

Adjust pH to 6.9±0.1; dispense 8mL portions into 16 x 150 mm test tubes containing 10 x 75 mm Durham tubes. Sterilize for 15 min at 121°C.

Egg yolk tellurite enrichment

Soak eggs in aqueous mercuric chloride 1:1000 for not less than one minute. Rinse in sterile water and dry with a sterile cloth.

Aseptically crack eggs and separate whites and yolks. Blend yolk and sterile physiological saline solution (3+7 v/v) in high speed sterile blender for 5 seconds. Mix 50.0 ml blended egg yolk to 10.0 mL of filter sterilized 1% potassium tellurite. Mix and store at 2 to 8°C.

Enterobacteriaceae Enrichment Broth

Ingredients	Gm/ Litre
Gelatin peptone #	10.000
Glucose monohydrate	5.000
Dehydrated bile ##	20.000
Disodium hydrogen phosphate, dehydrate	8.000
Potassium dihydrogen phosphate	2.000
Brilliant green	0.015
pH after heating (at 25°C)	7.2±0.2 **

Formula adjusted, standardized to suit performance parameters Directions Suspend 42.93 gm (the equivalent weight of dehydrated medium per litre) in 1000 mL purified/distilled water. Dispense 120 mL amounts in 250 mL flasks or 9 mL amounts in tubes. Stopper with cotton plugs or loose fitting caps. Heat in free for 30 flowing steam or

boiling water (100°C) for 30 minutes and cool immediately. DO NOT AUTOCLAVE.

Preparation of Plates

Add 5.0 mL pre-warmed (45 to 50°C) enrichment to 95 mL melted basal medium, which has been adjusted to 45to50°C. Mix well (avoiding bubbles), and pour 15.0 to 18.0 mL into sterile 15 x 100 mm Petri dishes. Plates can be stored at 2to8°C in plastic bags for 4 weeks. Immediately prior to use spread 0.5 mL per plate of 20% solution of Millipore filter sterilized sodium pyruvate and dry plates at 50°C for 2 hours or 4 hours at 35°C with agar surface uppermost.

If complete medium plates were prepared from commercial or laboratory prepared medium containing sodium pyruvate prior to adding Egg yolk tellurite. These plates must be used within 48 hours while being stored at 2 to 8°C. These plates should also be dried as indicated above prior to inoculating with sample.

Glucose Salt Teepol Broth (Twin Pack)

Glucose Salt Teepol Broth is used for enrichment of Vibrio parahaemolyticus and marine isolates. Composition**

Ingredients	Gm/Litre
Part A - Peptic digest of animal tissue	10.000
Beef extract…… .	3.000

Sodium chloride ……..	30.000
Glucose ……..	5.000
Methyl violet……..	0.002
Part B - Teepol ……..	4.000

Final pH (at 25°C) 8.8±0.2 **

Formula adjusted, standardized to suit performance parameters Directions Suspend 48 gm of Part A in 1000 mL distilled water containing 4.0 mL of Part B. Heat gently to dissolve the medium completely. Dispense in tubes as desired and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

Fraser Broth Base

Peptic digest of animal tissue…….	5.000
Casein enzymic hydrolysate ……..	5.000
Yeast extract …….	5.000
Meat extract…….	5.000
Sodium chloride…….	20.000
Disodium hydrogen phosphate.$2H_2O$ …….	12.000
Potassium dihydrogen phosphate ……..	1.350
Esculin …….	1.000
Lithium chloride……..	3.000

Final pH (at 25°C) 7.2±0.2 **

Formula adjusted, standardized to suit performance parameters Directions Suspend 54.92 gm (equivalent weight of dehydrated medium

per litre) in 1000 mL distilled water. Heat if necessary to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C and aseptically add rehydrated contents of 1 vial of Fraser Selective Supplement (FD125I) and 2 vials of Fraser Supplement (FD141) to 1000 mL medium for primary enrichment or 1 vial of each to 500 mL medium for secondary enrichment. Mix well and dispense as desired. Warning: Lithium chloride is harmful. Avoid bodily contact and inhalation of vapours. On contact with skin wash with plenty of water immediately.

L-EMB Agar

Peptone	10.0 gm
Lactose	10.0 gm
Disodium hydrogen phosphate	2.0 gm
Agar	15.0 gm
Distilled water	1.0 litre

Make a solution of (a), adjust pH to 7.1 to 7.2. Dispense in 100mL portions. Sterilize for 15 min at 121°C. Before use melt, and to each 100mL portion add 2.0 mL of aqueous 2% eosin Y solution and 1.3 mL of 0.5% aqueous methylene blue solution.

Gelatin Phosphate Salt Broth

Gelatin	10.0 gm
NaCl	10.0 gm
$K_2 HPO_4$	5.0 gm

PH 7.2+0.2 at 25 ^{o}C

Add components to distilled water (1 L). Antoclave at 121°C for 15 min.

Gram Negative (GN) Broth

Glucose	1.0 gm
D. mannitol	2.0 gm
Sodium citrate	5.0 gm
Sodium deoxycholate	0.5 gm
Dipotassium phosphate	4.0 gm
Monopotassium phosphate	1.5 gm
Sodium chloride	5.0 gm
Tryptose	20.0 gm
Distilled water	1.0 litre

Dissolve ingredients in distilled water by heat. Dispense in tubes in convenient amounts and sterilize at 116°C for 15 minutes. Final pH is 7.0±0.2. Avoid excessive heating.

Hektoen Enteric Agar

Proteose peptone	12.0 gm
Yeast extract	3.0 gm
Lactose	12.0 gm
Sucrose	12.0 gm
Salicin	2.0 gm
Bile complex	9.0 gm
Sodium chloride	5.0 gm
Sodium thiosulfate	5.0 gm
Ferric ammonium citrate	1.5 gm
Bromthymol blue	0.065 gm
Acid fucasin	0.1 gm
Agar	14.0 gm
Distilled water	1.0 litre

Suspend ingredients in distilled water. Boil with frequent stirring. Do not overheat or autoclave. When completely in solution, cool to 55 to 60°C and distribute into plates. Allow plates to solidify with lids ajar to provide a dry surface for inoculation. Plates may be refrigerated for future use. Final pH 7.5±0.2.

Hough and Liefson Medium

Peptone……	2gm
Sodium Chloride……	5gm
Dipotasium Hydrogen Sulphate……	0.3gm
Agar agar……..	3gm
Water……	1000mL
pH……..	7.1
Bromothymol blue 0.2% in alcohol……	15 mL

Boil to dissolve and before adding Bromothymol blue and distribute 3 to 4 mL in 14 x100 mm test tubes. Plug and autoclave at 115±1 ^{o}C for 20 minutes.

Add aseptically filter sterilized glucose solution to give a final concentration of 1% mix well.

Half Frasher Broth

Sodium Chloride…….. 20.00

Lithium Chloride…….. 3.00

Disodium Phosphate…….. 12.00

Monopotassium Phosphate…….. 1.30

Tryptone ……5.00

Esculin ……1.00

Meat Peptone ……..5.00

Acriflavine ……..0.0125

Beef Extract ……..5.00

Nalidixic Acid ……..0.01 Y

Yeast Extract ……..5.00

Suspend 28.7 gm of the medium in 500 mL. of distilled water. Mix well and dissolve by heating with frequent agitation. Boil for one minute until complete dissolution. Sterilize in autoclave at 121°C for 15 minutes. Cool to 45-50°C and aseptically add one vial of Ferric Ammonium Citrate Supplement (Cat. 6050), previously reconstituted in 5 mL of sterile distilled water. Homogenize gently and dispense into sterile containers. The prepared medium should be stored at 2-8°C. The color is amber. The dehydrated medium should be homogeneous, free-flowing and beige in color. If there are any physical changes, discard the medium. FERRIC AMMONIUM CITRATE SUPPLEMEN

Ferric Ammonium Citrate……0.25 gm

KF Streptococcus Agar

Proteose peptone #3 or polypeptone.....	10.0 gm
Yeast extract.....	10.0 gm
Sodium chloride.....	5.0 gm
Sodium glycerophosphate.....	10.0 gm
Maltose.....	20.0 gm
Lactose.....	1.0 gm
Sodium azide.....	0.4 gm

Bromocresol purple	0.015 gm
Agar	20.0 gm
Distilled water	1.0 litre

Dissolve ingredients in distilled water by boiling, and dispense in 100.0 mL portions. Autoclave at 121°C for 10 minutes. When ready to use, cool to 50°C and add 1.0 mL of 1% solution TTC (Triphenyl tetrazolium chloride) per 100.0 mL. Final pH should be 7.2. Do not overheat this medium.

Kligler Iron Agar

Peptone	20 gm
Agar	12 gm
Lactose	10 gm
NaCl	5 gm
Beef extract	3 gm
Yeast extract	3 gm
Glucose	1 gm
Ferric citrate	0.3 gm
$Na_2S_2O_3$	0.3 gm
Phenol red	0.05 gm

pH 7.4$\pm$0.2 at 25^{o}C.

Add components to 1 L of distilled water. Distribute into tubes and autoclave at 121°C for 15 min. Make slants with deep butts.

Koser's Citrate Broth

Sodium ammonium hydrogen phosphate	1.5 gm
Monopotassium hydrogen phosphate	1.0 gm
Magnesium sulphate	0.2 gm
Sodium citrate	3.0 gm

Distilled water ..... 1.0 litre

Adjust pH to 6.7±0.1, dispense in 10mL portions in test tubes. Sterilize for 15 min at 121°C.

Lactobacillus MRS Agar

Proteose peptone	10.0 gm
Beef extract	10.0 gm
Yeast Extract	5.0 gm
Dextrose	20.0 gm
Tween 80	1.0 gm
Ammonium citrate.....	2.0 gm
Sodium acetate	5.0 gm
Magnesium sulphate	0.1 gm
Manganese sulphate.....	0.05 gm
Dipotassium phosphate	2.0 gm
Agar	12.0 gm
Distilled water	1.0 litre

Suspend ingredients in water containing 10 mL glycerol. Boil to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Final pH 6.5 ± 0.2.

Lactose Broth

Beef extract	3.0 gm
Peptone	5.0 gm
Lactose	5.0 gm
Distilled water	1.0 litre

Adjust pH to 6.8, dispense into fermentation tubes. Sterilize for 15 min at 121°C. Allow temperature in autoclave to drop slowly below 75°C before opening.

Lactose Gelatin Medium

Gelatin	120 gm
Tryptone	15 gm
Lactose	10 gm
Yeast extract	10 gm
Phenol red	10 mL (of 0.5% solution)

pH 7.5+0.2 at 25°C.

Add gelatin to 590 mL distilled water. Gently heat while stirirng and bring to 50 to 60°C. Add phenol red. Add the rest of the components to 400 mL of distilled water and mix with gelatin solution. Dispense 10mL volumes in test tubes. Autoclave for 10 min at 121°C.

Lauryl Sulphate Tryptose Broth

Tryptose, tryptone or trypticase	20.0 gm
Lactose	5.0 gm
Dipotassium monohydrogen phosphate	2.75 gm
Sodium chloride	5.0 gm
Sodium lauryl sulphate	0.1 gm
Disti11ed water	1.0 litre
Potassium dihydrogen phosphate	2.75 gm

Adjust pH to 6.8±0.1, dispense in 10 mL portions in tubes with inverted Durham tubes. Sterilize for 15 min at 121°C.

Listeria Enrichment Broth

Tryptose…….. 10.000

Yeast extract…….. 5.000

Beef extract ……5.000

Sodium chloride…… 20.000

Disodium hydrogen phosphate ……..9.600

Monopotassium hydrogen phosphate……..1.350

Esculin ……..1.000

Nalidixic acid…….. 0.020

Acriflavin hydrochloride (Trypaflavin) ……..0.012

Final pH (at 25°C) 7.2±0.2 **Formula adjusted, standardized to suit performance parameters Directions Suspend 51.98 gm in 1000 mL distilled water. Heat if necessary to dissolve the medium completely. Dispense in tubes and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

Liver Broth

Fresh beef liver.....	500.0 gm
Distilled water	1.0 litre
Tryptone.....	10.0 gm
Soluble starch.....	1.0 gm
Dipotassium phosphate.....	1.0 gm

Remove the fat from 1 pound of fresh beef liver, grind, mix with 1000 mL of distilled water, and boil slowly for 1 hour. Adjust the pH to 7.6 and remove the liver particles by straining through cheesecloth. Make the volume of the broth back to 1000 mL with distilled water and

add the tryptone, Dipotassium phosphate, and soluble starch, and refilter. Dispense 15mL of the broth into 20 x150 mm tubes and add liver particles previously removed to a depth of one inch in each tube. Autoclave 20 minutes at 121°C.

Lysine Iron Agar (Edwards and Fife)

Peptone	5.0 gm
Yeast extract	3.0 gm
Glucose	1.0
L-lysine	10.0 gm
Ferric ammonium citrate	0.5 gm
Sodium thiosulfate	0.04 gm
Bromocresol purple	0.02 gm
Agar	15.0 gm
Distilled water	1.0 litre

Dissolve ingredients in distilled water and adjust to pH 6.7$\pm$0.2. Dispense in 14 mL amounts in 100 x 13 mm tubes and sterilize at 121°C for 12 minutes. Slant tubes to obtain a deep butt and a short slant.

Lysozyme Broth

Preparation A - Nutrient Broth: Prepare nutrient broth and dispense 99.0.mL amounts in bottles or flasks. Autoclave 15 minutes at 121°C

Preparation B - Lysozyme solution: Dissolve 0.1gm of lysozyme in 65 mL of sterile 0.0IN hydrochloric acid. Heat to boiling for 20 minutes and dilute to 100.0 mL with sterile 0.01N hydrochloric acid.

Alternatively dissolve 0.1 gm of lysozyme chloride in 100.0 mL of distilled water and sterilize by filtration. Test solution for sterility before use.

And 1.0 mL of sterile 0.1% lysozyme solution to each 99.0 mL of nutrient broth. Mix thoroughly and aseptically dispense 2.5 mL of complete medium into sterile 13 x 100 tubes.

MacConkey Agar

Peptone	20.0 gm
Lactose	10.0gm
Bile salts	1.5 gm
Sodium chloride	5.0
Agar	15.0 gm
Neutral red	0.03 gm
Crystal violet	0.001 gm
Distilled water	1.0 litre

Adjust pH to 7.1 sterilize for 15 min at 121°C. Pour in petri-dishes.

Malonate Broth

Yeast extract	1.0gm
Ammonium sulfate	2.0gm
Dipotassiurn phosphate	0.6gm
Monopotassiurn phosphate	0.4gm
Sodium chloride	2.0gm
Sodium malonate	3.0 gm
Glucose	0.25gm
Bromthymol blue	0.025 gm
Distilled water	1.0 litre

Dissolve ingredients in distilled water by heating, if necessary. Dispense into tubes and autoclave for 15 minutes at 121°C. Final pH 6.7±.0.1.

Malt Agar

Malt Extract.....	30.00 gm
Agar	15.00 gm
Distilled water	1.0 litre

Dissolve the ingredients in 1.0 litre distilled water with occasional agitation and boil gently for one minute. Dispense into suitable containers and sterilise at 121°C for 15 minutes.

Malt Agar (Acidified)

Malt agar acidified with 10% sterile tartaric acid to pH 3.5±0.2. Prepare acid solution by weighing 10.0 gm of tartaric acid into beaker and bringing up to 100.0 mL with water. Dissolve and sterilize at 121°C for 15 minutes. Acidify the sterile and tempered medium with a predetermined quantity of acid solution immediately before pouring plates. Do not attempt to reheat medium once acid has been added. Determine accuracy of adjusted pH by pouring an aliquot of the medium into a small beaker, cooling to temperature and placing a recently standardized pH directly into the solidified medium.

Malt Agar (With Antibiotic)

Solution A

Prepare malt agar.

Solution B

Add 500.0mg each, of chlorotetracycline HCl and chloramphenicol to 100.0 mL sterile buffered distilled water and mix. (Not all material dissolves. Therefore, the suspension must be evenly dispersed prior to pipetting into the medium).

To prepare mixture:

Melt medium (solution A above), temperature to 45±1°C and add 2.0mL of antibiotic solution per 100.0 mL medium.

Motility Test Medium (Motility Agar)
(Tittsler and Sandholzer)

Tryptose	10.0 gm
Sodium chloride	5.0 gm
Agar	5.0 gm
Distilled H_2O	1.0 litre

Suspend ingredients and heat to boiling to dissolve medium completely. Sterilize by autoclaving 15 minutes at 121°C. Final pH 7.2.

MR-VP Broth

Peptone	7.0 gm
Glucose	5.0 gm
Dispotassium hydrogen phosphate	5.0 gm

Distilled water ..... 1.0 litre

Adjust pH to 6.9±0.2 and dispense in 10 mL portions in tubes. Sterilize for 15 min at 121°C.

Mycological (Mycophil) Agar

Phytone or Soytone (papaic digest of soya meal)	10.0 gm
Dextrose	10.0 gm
Agar	18.0 gm
Distilled water	1.0 litre

Dissolve ingredients in distilled water with heat and autoclave 12 minutes at 118°C (12 Lb steam pressure for 10 minutes).

For yeast and mold counts of carbonated beverages, sugars, and other similar materials, adjust the pH to 4.5 to 4.7 by adding up to 15.0mL of sterile 10 percent lactic acid to each liter of melted medium prior to plating. Do not reheat after acidification.

Mycophil Agar + Antibiotic

Preparation of antibiotic solution: Add 500.0 mg each of chlortetracycline HCl and chloramphenicol to 100.0 mL sterile phosphate buffered distilled water and mix. (Not all material dissolves, therefore the suspension must be evenly dispersed before pipetting into the medium); Two mL of this solution is added per 100.0 mL of tempered agar giving a final concentration in the medium of 100 mg/L of each of the antibiotics. After swirling, the medium is ready for use.

MYP Agar (Mannitol Yolk Polymyxin)

Preparation A-

Meat extract	1.0 gm
Peptone	10.0 gm
D-mannitol	10.0 gm
Sodium chloride	10.0 gm
Phenol red	0.025 gm
Agar	15.0 gm
Distilled water	900.0 mL

Preparation B – Egg Yolk Emulsion: 50%: Wash fresh eggs with stiff brush and drain. Soak 1 hour in 70% alcohol. Aseptically remove yolk and mix (1+1) with sterile 0.85% sodium chloride solution. (Difco Egg Yolk Enrichment 50% is satisfactory).

Preparation C – Polymyxin B sulfate. This selective agent is obtainable in sterile powdered form (500,000 units, i.e., 50 mg per vial). To use, add aseptically, by syringe, 5.0 mL sterile distilled water. Mix to dissolve powder. Add 1.0 mL by syringe to a liter of final medium.

Mix ingredients of preparation A in distilled water. Adjust to pH 7.1$\pm$0.2. Sterilize at 121°C for 20 minutes, cool to 49$\pm$1°C and add 100.0 mL of preparation B and 1.0 mL of preparation C. Mix well, pour into petri dishes, allow to solidify and store in a manner to eliminate excess surface moisture. Plates may be stored at 4°C for 7 days.

MY-40 Agar (Malt, Yeast Extract 40% Sucrose)

Malt extract	20.0 gm
Yeast extract	5.0 gm
Agar	20.0 gm

Sucrose	400.0 gm
Distilled water	1.0 litre

Dissolve ingredients in distilled water heating gently. Sterilize 20 minutes at 121°C. pH is not adjusted. Do not overheat.

Nitrate Broth

Beef extract	3.0 gm
Peptone	5.0 gm
Potassium nitrate	15.0 gm
Distilled water	1.0 litre

Dissolve ingredients in distilled water. Distribute in tubes and sterilize for 15 minutes at 121°C. The final pH is 7.0.

Nutrient Broth

Beef extract	3.0 gm
Peptone	5.0 gm
Distilled water	1.0 litre

Suspend ingredients in distilled water and melt agar by gentle boiling. Dispense into suitable flasks or bottles and sterilize 15 minutes at 121°C. Final pH 7.3

Nutrient Agar

Beef extract	3.0 gm
Peptone	5.0 gm
Distilled water	1.0 litre

Modified Listeria Oxford Agar Base

Peptone special…….. 23.000

Corn starch ……1.000

Sodium chloride ……5.000

Aesculin ……..1.000

Iron (III) Ammonium citrate ……0.500

Lithium chloride…… 12.000

Agar ……10.000

Final pH (at 25°C) 7.2±0.2 **Formula adjusted, standardized to suit performance parameters. Directions: Suspend 52.5 gm in 1000 mL distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C and aseptically add the rehydrated contents of 1 vial of Modified Listeria Oxford Selective Supplement (FD306). Mix well before pouring into sterile Petri plates. Warning: Lithium chloride is harmful. Avoid bodily contact and inhalation of vapours. On contact with skin, wash with plenty of water immediately

Heat to dissolve, dispense into tubes or flasks, and autoclave 15 minutes at 121˚C. Final pH, 6.7±0.2

Listeria Identification Agar Base (PALCAM)

Peptic digest of animal tissue ……..23.000
Starch ……1.000
Sodium chloride ……..5.000
Mannitol ……10.000
Ammonium ferric citrate ……..0.500
Esculin ……..0.800
Dextrose ……..0.500
Lithium chloride……..15.000
Phenol red ……..0.080
Agar ……..13.000

Final pH (at 25°C) 7.0±0.2 **Formula adjusted, standardized to suit performance parameters Directions Suspend 34.44 gm in 500 mL distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to around 50°C and aseptically add rehydrated contents of 1 vial of Listeria Selective Supplement (PALCAM) (FD061). Mix well and pour into sterile Petri plates. Warning: Lithium chloride is harmful. Avoid bodily contact and inhalation of vapours. On contact with skin, wash with plenty of water immediately.

Peptone Water Diluent

Peptone.....	1.0gm
Distilled water	1.0 litre
pH.....	7.0gm

Sterilize for 15 min at 121°C

Plate Count Agar (PCA) (Standard Methods Agar) (TGE Agar)

Dehydrated yeast extract	2.5 gm
Pancreatic digest of casein (Tryptone)	5.0 gm
Glucose	1.0 gm
Agar	15-18 gm
Distilled water	1.0 litre

Adjust pH to 7.0±0.1 dispense in 15mL portrions in tubes or flasks. Sterilize for 15 min at 121°C. Before use melt the medium completely in boiling water and keep the tubes or flaks in water bath at 45 to 48°C.

Phenol Red Carbohydrate Broth

Trypticase	10.0 gm
Sodium chloride	5.0 gm
Beef extract (optional)	1.0 gm
Phenol red (0.25% solution)	7.2 mL
Distilled water	800.0 mL

Dissolve ingredients in distilled water. Dispense 2 mL portions into 13 x 100 mm test tubes containing inverted Durham tubes. Autoclave 15 min at 118°C and let cool.

Dissolve 5 gm dulcitol, 10gm lactose, or 10gm sucrose (as specified in title of test) in 200 mL of distilled water and sterilize by passing through bacteria retaining filter. Aseptically add 0.5 mL sterile

filtrate to each tube of sterilized broth after cooling to <45°C, shake gently to mix. Final pH 7.4±0.2.

Potassium Cyanide (KCN) Broth

Basal Broth:

Proteose peptone No. 3 or Polypeptone.....	3.0 gm
Disodium phosphate	5.64 gm
Monopotassium phosphate.....	0.225 gm
Sodium chloride	5.0 gm
Distilled water	1.0 litre

Dissolve ingredients in distilled water with stirring. Autoclave 100.0 mL portions 15 minutes at 121°C. Final pH should be 7.6. Prepare 0.5% potassium cyanide by weighing 0.5 gm into 100.0 mL sterile distilled water using pipette filter. Transfer 1.5 mL cold potassium cyanide solution to 100 mL basal broth (precooled). DO NO'T PIPETTE BY MOUTH. Mix. Distribute 1.0 mL portions to sterile 13 x 100 mm tubes and stopper immediately with No.2 corks impregnated with paraffin. (Prepare corks by boiling in paraffin for 5 minutes.) Store medium at 5 to 10°C. Storage life is two weeks. Exercise caution because potassium cyanide is lethal.

Potato Dextrose Agar (Acidified)

Infusion from white potatoes.....	200.0 mL
Dextrose	20.0 gm
Agar	15.0 gm
Distilled water	1.0 litre

Suspend ingredients in distilled water and heat mixture to boiling to dissolve. Distribute into tubes or flasks, and autoclave 15 minutes at 121°C (15 lb pressure). When used as plating medium for yeasts and molds, melt in flowing steam or boiling water, cool and acidify to pH 3.5 with sterile 10 percent tartartic acid solution. (For use in the cultivation of yeasts and molds, adjust to the desired pH if different from pH 3.5.) Mix thoroughly and pour into plates. To preserve solidifying properties of the agar do not heat medium after the addition of tartartic acid. For preparation of Potato Dextrose Agar with Antibiotic, add antibiotics as described under Mycophil agar with Antibiotic.

Pseudomonas presumptive test broth

	Single strength	Concentrated
DL Arginine……..	2gm	3.2gm
L Proline ……..	1gm	1.6gm
Anhydrous dipotasium hydrogen sulphate…	1gm	1.6gm
Magnesium sulphate heptahydrate ……... .	0.5gm	0.8gm
Anhydrous potassium sulphate……..	10gm	16.0gm
Water……..	1000mL	1000mL
Ethanol……..	25mL	40mL
pH……	7.2±0.2	

Sterilize ethanol by filtration and add required volume after sterilization of medium by autoclaving at 121±1°C for 15 minutes. Store at room temperature up to a maximum of three months.

Pseudomonas confirmation medium

Skim milk powder ……..	100gm
Yeast extract ……..	3gm

Peptone…… ..	10gm
Sodium chloride……..	5gm
Cetrimide……	0.3gm
Water ……..	1000mL
Agar agar……	15gm
pH……..	7.2± 0.2

Dissolve skim milk powder in 250 mL of warm water. Use magnetic stirrer if required.

Sterilize it at 121±1°C for five minutes (to avoid caramalisation). Dissolve rest of ingredients in 750 mL of water. Adjust pH and autoclave at 121±1°C for 15minutes. Cool the medium to 50 °C and add aseptically the skim milk powder solution.

Selenite Cystine Broth

Tryptone.....	5.0 gm
Lactose	4.0 gm
Disodium hydrogen phosphate	10.0 gm
Sodium selenite.....	4.0 gm
L.-cystine.....	0.01 gm
Distilled water	1.0 litre

Dissolve by boiling for 5 min. Dispense 10 ml portions into sterile 16 x 150 mm test tubes. Heat 10 min in flowing steam. Do not autoclave. Final pH,7.0$\pm$0.2. Medium is not sterile. Use same day as prepared.

Sulfite Agar

(For the Detection of Thermophilic Anaerobes Producing H_2S)

Tryptone or Trypticase.....	10.0gm
Sodium sulfite (anhydrous)	1.0 gm
Agar	20.0 gm
Distilled water	1.0 litre

Dissolve ingredients in distilled water, dispense into tubes in about 15.0 mL amounts, and into each tube place an iron nail or a small clean strip of iron or base plate. No adjustment of reaction is necessary. Autoclave 20 minutes at 121°C. Tubes should be used within a week after making.

As an alternate for the iron strip or nail, 10.0 mL of a 5% solution of iron citrate may be substituted in the sulfite medium formula. It is necessary to heat the citrate solution to completely dissolve ferric citrate scales or pearls.

Tetrathionate Broth

Basal medium:

Polypeptone or proteose peptone.....	5.0 gm
Bile salts	1.0 gm
Calcium carbonate	10.0 gm
Sodium thiosulfate.....	30.0 gm
Distilled water	1.0 litre
Iodine solution:	
Iodine.....	6.0 gm
Potassium iodide	5.0 gm
Distilled water	20.0 mL

Heat the ingredients of the basal medium in distilled water to boiling temperature, cool to less than 45°C, add 2.0 mL of iodine

solution to each 100.0 mL of base. Add 1.0 mL of 1:1000 solution of brilliant green per 100.0 mL of basal medium. The basal medium, with or without added brilliant green, may be tubed, sterilized at 121°C for 15 minutes, and stored. In this case, iodine solution is added (0.2 mL per 10 mL of medium) prior to use.

Sulfathiazole (0.125 mg per mL of medium) may be added to prevent excessive growth of Proteus.

Thiosulfate-Citrate-Bile Salts-Sucrose Agar (TCBS)

Yeast extract	5.0 gm
Polypeptone or Proteose Peptone No. 3	10.0 gm
Sucrose	20.0 gm
Sodium thiosulfate ($5H_2O$)	10.0 gm
Sodium citrate ($2H_2O$)	10.0 gm
Sodium cholate	3.0 gm
Oxgall	5.0 gm
Sodium chloride	10.0 gm
Ferric citrate	1.0 gm
Bromthymol blue	0.04 gm
Thymol blue	0.04 gm
Agar	15.0 gm
Distilled water	1.0 litre

Distilled ingredients in distilled water by bringing to boil. Adjust pH to 8.6+0.2. This medium should not be autoclaved.

T_1N_1 Agar

Trypticase (pancreatic digest of casein)	10.0 gm
Sodium chloride	10.0 gm
Agar	15.0 gm

Distilled water ..... 1.0 litre

Dissolve ingredients in distilled water by bringing to a boil. Dispense in tubes and sterilize at 121°C for 15 min. Allow to solidify in an inclined position (long slant). Final reaction should be pH 7.2±0.2. To prepare T_1N_1 broth, omit the agar.

Thioglycollate Agar

Pancreatic digest of casein USP	15.0gm
L-cystine	0.5 gm
Dextrose	5.0 gm
Yeast extract	5.0 gm
Sodium chloride	2.5 gm
Resazurin	0.001 gm
Agar	20.0 gm
Distilled water	1.0 litre

Suspend ingredients in distilled water and heat to boiling to dissolve completely. Distribute approximately 16.0 mL quantities to 20 x 150 mm screw capped tubes. Add to each tube with head down, an acid cleaned 6d nail. Sterilize at 121°C for 15 minutes. Final reaction should be 7.0 to 7.1. This medium should be used within one week of preparation.

This medium is the same formulation as Fluid Thioglycollate medium except for the addition of 20.0 gm of agar.

Tryptone Glucose Extract Agar

Beef extract ..... 3.0 gm

Tryptone	5.0 gm
Dextrose	1.0 gm
Agar	15.0 gm
Distilled water	1.0 litre

Dissolve ingredients in distilled water by boiling gently. Adjust to pH 7.0±0.2. Distribute in tubes or flasks. Sterilize 15 minutes at 121°C.

Thornley's Semi-Solid Arginine Medium

Peptone	0.1 gm
Sodium chloride	0.5 gm
Dipotassium hydrogen phosphate	0.03 gm
Arginine hydrochloride	1.0 gm
Phenol red	0.001 gm
Distilled water	1.0 litre

Adjust pH to 7.2. Dispense 15 ml in test tubes and sterilize for 15 min at 121°C.

Triple Sugar Iron Agar (TSI)

Meat extract	3.0 gm
Yeast extract	3.0 gm
Peptone	20.0 gm
Sodium chloride	5.0 gm
Lactose	10.0 gm
Sucrose	10.0 gm
Glucose	1.0 gm
Ferrous sulfate	0.3 gm
Sodium thiosulphate	0:3 gm
Phenol red	0.024 gm
Agar	12.0 gm
Distilled water	1.0 litre

Adjust pH to 7.4±0.2. Dispense in 10 mL portions into tubes. Sterilise for 12 minutes at 121°C. Allow to set in a sloping position to give a butt of 3 cms.

Tryptone (Tryptophane) Broth

Tryptone or trypticase	10.0 gm
Distilled water	1.0 litre

Dissolve with stirring. Autoclave 15 minutes at 121°C Final pH should be 6.9±0.2.

Tryptone Salt Broth

Casein enzymic hydrolysate…… 1.0

Sodium chloride…….. 8.0 or 10 gm as required

Final pH (at 25°C) 7.0±0.2

**Formula adjusted, standardized to suit performance parameters Directions Suspend 9.5 gm in 1000 mL distilled water. Heat if necessary to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well and dispense as desired.

Tryptone (Trypticase) Soy Broth

Tryptone or Trypticase	17.0 gm
Phytone or Soytone	3.0 gm
Sodium Chloride	5.0 gm
Dipotassiuin phosphate	2.5 gm

Dextrose	2.5 gm
Distilled water	1.0 litre

Dissolve ingredients in distilled water; warm slightly if necessary to complete solution. Dispense Into tubes or bottles, and sterilize by autoclaving 15 minutes at 121°C. Final reaction should be pH 7.3±0.2.

Trypticase Soy Polymyxin Broth

Preparation A

Trypticase peptone	17.0 gm
Phytone peptone	3.0 gm
Sodium Chloride	5.0 gm
Dipotassium phosphate	2.5 gm
Dextrose	2.5 gm
Distilled water	1.0 litre

Preparation B - Polymyxin B sulfate

This selective agent is available in sterile powdered form (500,000 units, i.e., 50 mg per vial). To use, dissolve.500,000 units of sterile powder in 33.3mL of sterile distilled water to give a 0.15% solution. Store solution at 4°C until used.

Suspend ingredients of preparation A in water. Mix thoroughly. Warm slightly if necessary to complete solution. Dispense 15 mL into 20 x 150 mm culture tubes and sterilize by autoclaving for 15 minutes at 121°C. Just before use, add 0.1 mL of sterile 0.15% polymyxin B sulfate solution to each tube and mix thoroughly.

Tryptose-Sulfite Cycloserine (TSC) Agar

Tryptose	15.0 gm
Soytone	5.0 gm
Yeast extract	5.0 gm
Sodium bisulfite (meta)	1.0 gm
Ferric ammonium citrate	1.0 gm
Agar	20.0 gm
Distilled water	1.0 litre

Dissolve ingredients in distilled water and adjust the pH to 7.6±0.2 and autoclave for 10 minutes at 121°C. To each liter of autoclaved medium cooled to 50°C, add 10.0 mL of a 4.0% filter sterilized solution of D-cycloserine to give a final concentration of approximately 400 μg per mL and add 40.0 mL of a sterile 50% egg yolk in saline emulsion per 500.0 mL of medium, with the exception of that used to overlay the plates. Egg Yolk enrichment 50% may be obtained from Difco Laboratories, Detroit, Michigan. Dispense the medium in standard Petri dishes for surface plating. Air dry at room temperature for 24 hours or until the surface of the agar is somewhat dry prior to use. Prepare plates fresh each time they are to be used.

Note: SFP agar base available commercially (Difco) is the same as the above basal medium.

Tyrosine Agar

Preparation A - Prepare nutrient agar and dispense 100.0 mL into bottles. Autoclave 15 minutes at 121°C. Cool to 48°C in water bath.

Preparation B - Add 0.5 gm of L-tyrosine to a 20 x 150 mm culture tube and suspend in 10.0 mL of distilled water using a Vortex mixer. Sterilize the suspension by autoclaving for 15 minutes at 121°C.

Mix Preparation A (100 mL) with sterile Preparation B (10 mL) and aseptically dispense 3.5 mL of complete medium into sterile 13 x 100 mm tubes. Slant tubes and cool rapidly to prevent separation of the tyrosine.

Urea Broth

Yeast extract	0.1 gm
Monobasic potassium phosphate	0.091 gm
Dibasic sodium phosphate	0.095 gm
Urea	20.0gm
Phenol red	0.01 gm
Distilled water	1.0 litre

Mix ingredients in the distilled water. This medium is filter-sterilized and tubed in sterile tubes in 3.0mL amounts. The basal medium (without urea) may be prepared in 900.0 mL of distilled water and sterilized at 121°C for 15 minutes. After cooling, 100.0 mL of 20% sterile urea solution are added and the medium dispensed in sterile tubes in 3.0 mL amounts.

Inoculation

Three loopful (2mm loop) from an agar slant culture are inoculated into a tube of medium and the tube is shaken to suspend the bacteria.

Incubation

Tubes are incubated in a water bath at 37°C, and the results are read after 10 minutes, 60 minutes, and 2 hours.

Violet Red Bile Agar (VRBA)

Yeast extract	3.0 gm
Peptone or Gelysate	7.0 gm
Sodium chloride	5.0 gm
Bile salts or Bile salts No. 3.	1.5 gm
Lactose	10.0 gm
Neutral red	0.03 gm
Crystal violet	0.002 gm
Agar	15.0 gm
Distilled water	1.0 litre

Suspend the ingredients in distilled water and allow to stand for a few minutes. Mix thoroughly and adjust to pH 7.4+0.2. Heat with agitation and boil for 2 minutes. Do not sterilize. Prior to use, cool to 45°C and use as a plating medium. After solidification, add a cover layer above the agar of approximately 3.0 to 4.0 mL to prevent surface growth and spreading of colonies.

Violet Red Bile Agar + Glucose

Prepare 1000 mL VRBA media. Add 10gm of glucose. Heat with agitation and boil for 2 min. Do not autoclave.

V-P Broth (Modified For *B. cereus*)

Proteose peptone	7.0 gm
Glucose	5.0 gm
NaCl	5.0 gm
Distilled water	1.0 litre

Dissolve ingredients in distilled water. Dispense 5.0 mL in 20 mm test tubes. Sterilize 15 minutes at 121°C.

Note: The medium is a modified medium and must be formulated in the laboratory.

Xylose Lysine Deoxycholate Agar (XLD)

Yeast extract	3.0 gm
L-lysine	5.0 gm
Xylose	3.5 gm
Lactose	7.5 gm
Sucrose	7.5 gm
Sodium chloride	5.0 gm
Phenol red	0.08 gm
Agar	13.5 gm
Distilled water	1.0 litre

Heat mixture in distilled water to boiling temperature to dissolve the ingredients. Sterilize at 121°C for 15 minutes, and then cool to 55 to 60°C. Aseptically add 20.0 mL of sterile solution containing.

Sodium thiosulfate	34.0 gm
Ferric ammonium citrate	4.0 gm
Distilled water	100.0 mL

Mix well to obtain a uniform suspension.

CHAPTER – 3

EQUIPMENT, MATERIALS AND GLASSWARES

1. Autoclave of sufficient size and number. Used for sterilization of media and for discarded plates / used media, etc (with calibrated thermometer and pressure gauge).
2. Anaerobic jars or incubators with equipment and material for obtaining anaerobic conditions.
3. Balance sensitive to 0.1 gm with 200 gm load.
4. Blenders with steel jar and lid / Stomacher.
5. Bunsen burners.
6. Colony Counter (Quebec or equivalent).
7. Dilution and media storage bottles. 120, 300, 600 and 1500 mL in capacity.
8. Durham's tubes
9. Glass test tubes 16 x150 mm. Rimless
10. Plastic caps for test tubes
11. Serological test tubes
12. Hot air ovens used for sterilization of glass and metal ware. They should have a thermostat range between 150-185°C.
13. Hockey sticks: Glass bent rods (or suitable plastic make) with fine polished edges, 3-4 mm diameter, 15-20 cms long with angled spreading surface 45-55 mm long or disposable plastic material.
14. Howard Mold Counting Chamber
15. Haemocytometer.
16. Incubators. At least 4 incubators are necessary, to be adjusted at 30°C, 37°C, 44.5°C and 55°C of proper size. B.O.D. incubator for temp. Less than ambient temperature.

17. Inoculating loops and wires. (3-5 mm dia of nichrome or platinum or plastic).
18. Magnetic stirrer
19. Membrane Filtration apparatus, for sterilizing fluids which are affected by heat, e.g. Seitz filter operationalzed membrane filters.
20. Microscope binocular with 900 x and higher magnification.
21. Microscopic slides and cover slips.
22. Non-adsorbant cotton.
23. Petri plate (glass or plastic)
24. Petri plate containers. (Stainless steel or aluminium, with covers) for hot air sterilization of glass petri plates.
25. Pipettes. (glass or plastic) Graduated, with 1, 5 and 10 mL total flow type / Automatic pipette with error < ±5 % with sterilisable or Autopipetor with Pre-sterilized plastic tips.
26. Pipette containers (Stainless steel plastic tip containers boxes)
27. pH meter. Electronic pH meter with accuracy of 0.1 pH unit shall be used.
28. Refrigerator and deep freezer.
29. Test tube racks and baskets to hold test tubes.
30. Thermometers.
31. Vortex – mixer.
32. Water bath for holding media at 44-46°C.
33. Serological water bath
34. Laminar flow chamber
35. Biological safety cabinet level II

CHAPTER – 4
BIOCHEMICAL TESTS

1. Carbohydrate fermentation

Inoculate one tube of each of the carbohydrate media (containing the specific sugar). Incubate at 35°C for 24 hours. Acid production is indicated by a change in color and gas production can be detected by observation of gas collection in the inverted Durham tube.

2. Catalase Test

Flood plates of the suspected culture with 3-5% hydrogen peroxide solution. Bubble formation is indicative of a positive reaction.

Or put a colony on glass slide with the help of a wire loop. Bubble formation is indicative of catalase reaction positive.

3. Citrate Test

Inoculate a tube of Koser citrate medium. Incubate at 35°C for 96 hours. Observe for turbidity due to growth.

4. Coagulase Test

4a. Dessicated coagulase plasma (rabbit) with EDTA; Reconstitute according to manufacture's directions.

4b. If plasma containing EDTA is not available, reconstitute desiccated rabbit plasma and add Na_2H_2 EDTA to a final concentration of 0.1% in

the reconstituted plasma. Do not store rehydrated plasma longer than 5days (at 2-8°C).

Transfer suspected *S. aureus* colonies into tubes containing 5 mL of Brain Heart infusion broth. Incubate 18-24 hours at 35-37°C.

Add 0.5 mL of the coagulase plasma with EDTA to 0.2 mL of broth culture. Incubate at 35-37°C and examine periodically during a 6 hours interval for clot formation. A 3+ or 4+ clot formation is considered a positive reaction for *S. aureus*.

5. Decarboxylase Tests

Inoculate the decarboxylase media (containing either lysine, arginine or ornithine) with a young slant culture. Use an oil seal and inoculate a control tube (no amino acid) with each culture under investigation. Incubate at 37°C. Examine daily for four days. The medium first becomes yellow because of acid production. Later if decarboxylation occurs, the medium becomes alkaline (purple). The control tubes remain acid (yellow).

6. FETAL BOVINE SERUM

: Refer sigma

7. Hydrogen Sulphide Production

Inoculate a tube of TSI agar by stabbing the butt and streaking the slope. Incubate for 24-48 hours. Observe for blackening due to H_2S production.

Hippurate Hydrolysis Test

Hippurate Hydrolysis Test is used for detection of hippurate hydrolyzing bacteria, mainly Streptococcal species. Directions Aseptically place hippurate disc in Brain Heart Infusion Broth (M210) inoculated with b haemolytic streptococci. Incubate at 35-37°C for 48 hours. Separate out the growth by centrifuging the broth. Add 2 mL of ferric chloride reagents to 2 mL of supernatant from the centrifuged culture tubes. Shake well and observe persistence of the precipitate formed even after 10 minutes. Preparation of ferric chloride reagent:

Ingredients: Grams/100mL

Ferric chloride:……..12.0 gm

Distilled water:…… 94.6 mL

Concentratedhydrochloricacid:…….5.4mL

8. Indole Production

Kovac's Reagent:

p-Dimethylaminobenzaldehyde	5.0 gm
Amyl alcohol.....	75.0 mL
Hydrochloric acid (concentrated).....	25.0 mL

Dissolve p-dimethylaminobenzaldehyde in the amyl alcohol, and then slowly add the hydrochloric acid. To test for indole, add 0.2 to 0.3 mL of reagent to 5.0 mL of a 24 hour culture of bacteria in tryptone broth. A dark red color in the surface layer constitutes a positive test for indole.

9. Growth in Potassium Cyanide Broth

Transfer a loopful of young culture to KCN broth. Incubate for up to 48±2 hours. Turbidity indicates growth and a positive test.

10. Methyl Red Reaction

Methyl Red indicator:

Methyl red	0.1 gm
Alcohol, 95% (ethanol)	300.0 mL

Dissolve methyl red in 300.0 mL of alcohol, and make up to 500.0 mL with distilled water. Incubate test cultures grown in MR-VP broth for 5 days at 30°C. Alternatively incubate at 37°C for 48 hours. Add 5 or 6 drops of reagent to cultures. Do not perform tests on cultures incubated less than 48 hours. If equivocal results are obtained, repeat tests on cultures incubated for 4 or 5 days. Duplicate tests should be incubated at 22 to 25°C.

11. Motility Test

Inoculate tubes of motility medium by stabbing the medium to a depth of about 5 mm. Incubate at the appropriate temperatures. Motile organisms migrate through the medium which becomes turbid; growth of non-motile organisms is confined to the stab.

12. Nitrate Reduction Test

Solution A:

Sulfanilic acid	0.5 gm
Glacial acetic acid	30.0 mL
Distilled water	120.0 mL

Solution B:

N-(1-naphthyl) ethylenediamine Dihydrochloride

(Marshal's reagent)	0.2 gm
Glacial acetic acid	30.0 mL
Distilled water	120.0 mL

Cleve's acid (5-amino-2 naphthylene sulfonic acid) may be substituted for Marshal's Reagent.

To 3.0 mL of an 18 hour culture in nitrate broth, add two drops of solution A and two drops of solution B. A red violet color which develops within 10 minutes indicates that nitrate has been reduced to nitrite. If the reaction is negative, one must examine for residual nitrate since conceivably the nitrate may have been reduced further. Add a few grains of powdered zinc. If a red violet color does not develop, nitrate has been reduced. Perform tests on uninoculated medium as a control.

13. Nilidixic acid and cephalothin antibiotic disc

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14. Oxidase (Cytochrome oxidase) Test (Kovac's method)

Oxidase testing Reagent:

Tetramethylparaphenylenediamine-2-HCl ……..... 0.25 gm
Distilled water ……..... 25.0 mL

Store at 4°C. A fresh solution should be made each week.

Soak small pieces of the filter paper in the reagent solution. Some filter paper gives a blue color and these must not be used. Dry or use wet. Scrape some of a fresh young culture with a glass rod and rub on the filter paper. A blue color within 1 minute is a postive oxidase test.

15. Urease test

Inoculate the urea media heavily with the culture being tested and incubate for 24 hours at 35°C. If urease is present, the urea is split to form ammonia, which changes the color of the indicator from yellow to pink.

16. Voges Proskauer (VP) Reaction:

Solution A:

Alpha-naphthol ..... 5.0 gm
Absolute ethanol ..... 100.0 mL

Solution B:

Potassium hydroxide..... 40.0 gm
Distilled water to make ..... 100.0 mL

Perform Voges-Proskauer (V-P) test at room temperature by transferring 1 mL of 48 hour culture to test tube and adding 0.6 mL of alpha-naphthol (Solution A) and 0.2 mL of 40% potassium hydroxide (Solution B); shake after addition of each solution. To intensify and speed reactions, add a few crystals of creatine to test medium. Read results 4 hours after adding reagents. Positive V-P test is the development of an eosin pink color.