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IS 5402(Part 1):2021

METHOD FOR ENUMERATION OF AEROBIC PLATE COUNT (PART 1- COLONY COUNT AT 30°C BY POUR PLATE TECHNIQUE)

INTRODUCTION

The Total Aerobic Mesophilic Plate Count, usually called Aerobic Plate Count (APC) or Standard Plate Count, is the most commonly used general indicator of bacterial populations in foods.

SCOPE

The horizontal method for the enumeration of microorganisms that are able to grow and form colonies in a solid medium after aerobic incubation at 30°C.

EQUIPMENT

- Laminar Air flow
- Autoclave
- Tubes and glass bottles
- Weighing Balance
- pH Meter
- Petri dishes (90-100mm diameter)
- Graduated pipettes
- Micropipette with tips
- Incubator (at 30 ± 1°C)
- Colony counter(Optional)
- Water Bath (at 44 °C to 47 °C)
- Refrigerator (at 2 °C – 8 °C)
- Cyclomixer

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MEDIUM

- 0.1% Peptone salt solution
- Plate count agar
- Agar medium

SAMPLE PREPARATION

Homogenous sample of processed food to be used.

ANALYTICAL PROCEDURE

- Prepare 225 ml of 0.1% peptone salt solution dilution fluid.
- Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes (Check the pH before sterilize, if necessary adjust pH using 0.1N Hcl / 0.1N NaOH)
- Take 25 gm sample in 225ml dilution fluid mix well minimum 25 times.
- Take two sterile Petri dish using a sterile pipette transfer the each dish 1 ml of test sample.
- If plates from more than one dilution are prepared, this may be reduced to one dish.
- Using a sterile pipette transfer to each dish 1 ml of the initial suspension (10^{-1}) to decimal dilution (10^{-2}) 9 ml of dilution fluid cyclometer mix well after take 1 ml transfer the two sterile Petri plate.
- If necessary, repeat the procedure with the further dilutions (10^{-3} , 10^{-4} , etc.), using a new sterile pipette for each decimal dilution.
- Pour about 12 ml to 15 ml of Plate count agar at 44°C to 47°C into each Petri dish. The time elapsing between the end of the preparation of the initial suspension and the moment when the medium is poured into the dishes shall not exceed 45 minutes.
- Carefully mix the inoculums with the medium by rotating the petri dishes and allow the mixture to solidify by leaving the Petri dishes standing on a cool horizontal surface, also prepare a control plate with 15 ml of the medium for checking its sterility.
- Invert the prepared dishes and place them in the incubator at 30°C $\pm$ 1°C for 72 hours $\pm$ 3 hours.
- In case there is possibility that the product contains microorganisms which will overgrow or swarm on the surface of the plate, pour an overlay of 4-5 ml of PCA and allow to solidify.

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COUNTING OF COLONIES

- After 72 ± 3 hours of incubation select the agar plates with, if possible, 10 or more colonies and fewer than 300 colonies. Count using the colony counting equipment, it is important that pinpoint colonies should be included in the count.
- Spreading colonies shall be considered as single colonies. If less than one –quarter of the dish is overgrown by spreading, count the colonies on the unaffected part of the dish and calculate the corresponding number of the entire dish. If more than one – quarter is overgrown by spreading colonies, discard the count.

METHOD OF CALCULATION

S. No	No of colonies	Formula	Explanation
1	10-150 Colonies	$N = \frac{\sum c}{v \times 1,1 \times d}$	$\sum c$ is the sum of colonies counted on the two dishes retained from two successive dilutions, at least one of which contain a minimum of 10 colonies v is the volume of inoculum placed in each dish, in milliliter's; d is the dilution corresponding to the first dilution retained
	Atypical colonies	$a = b / A * C$	b is the number of colonies complying with identification criteria among the identified colonies A. c is the total number presumptive colonies counted on the dish .
2	Less Than 10 Colonies	$\bar{c} = \bar{c} / v \times d$	$\bar{c}$ = Estimate the number Express as “Microorganisms are present but less than (Vx d) per gram or ml” C is the average of colonies on the petri dishes retained v is the volume of inoculums used in each dish d is the dilution corresponding to the dilution retained
3	No colonies	-	Express as less than 1/d microorganism per milliliter (liquid products) or per gram (other products)

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CAUTION

- Carry out the test under the control of skilled microbiologist and great care shall be taken in the disposal of all the incubated material.
- Follow safe and good laboratory practices to avoid cross contamination.

REFERENCE

- 1) **IS 5402 (Part 1):2021**- Microbiology of food chain- horizontal method for the enumeration of microorganisms – Part 1 colony count at 30°C by Pour plate technique
- 2) **ISO 7218:2007** – Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations.
- 3) **ISO 6887-1 (2017)** - Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions.

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IS 5401(Part 1):2012 (RA 2022)

METHOD FOR THE DETECTION AND ENUMERATION OF COLIFORMS

PART-1-COLONY COUNT TECHNIQUE

INTRODUCTION

Coliforms are a broad group of aerobic or facultative anaerobic, rod shaped, gram-negative, non-spore forming bacteria which can ferment lactose with the production of acid and gas when incubated at 30°C –37°C, Coliform are members of Enterobacteriaceae family. Most common genera are *E. coli*, *Salmonella*, *Citrobacter*, *Enterobacter*, and *Klebsiella*.

SCOPE

Enumeration of coliforms present in products intended for human consumption and for the feeding of animals by counting colonies after incubation on a solid medium at 30°C or at 37°C.

EQUIPMENT

- Laminar Air flow
- Biosafety cabinet
- Autoclave
- Tubes and glass bottles
- Weighing Balance
- pH Meter
- Petri dish
- Graduated pipettes
- Micropipette with tips
- Incubator
- Colony counting equipment
- Water Bath
- Refrigerator
- Durham Tube

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- Cyclomixer

MEDIUM

- 0.1% Peptone salt solution
- Brilliant Green Lactose Bile Broth
- Violet red bile agar

SAMPLE PREPARATION

Homogenous sample of processed food to be used.

ANALYTICAL PROCEDURE

- Prepare 225 ml of 0.1% peptone salt solution dilution fluid.
- Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes (Check the pH before sterilize, if necessary adjust pH using 0.1N Hcl / 0.1N NaOH)
- Take 25 gm sample in 225ml dilution fluid mix well minimum 20 times.
- Take two sterile petri dish using a sterile pipette transfer the each dish 1 ml of test sample.
- Using a sterile pipette transfer to each dish 1 ml of the initial suspension (10^{-1}) to decimal dilution (10^{-2}) 9 ml of dilution fluid cyclometer mix well after take 1 ml transfer the two sterile Petri dish.
- If necessary, repeat the procedure with the further dilutions (10^{-3} , 10^{-4} , etc.), using a new sterile pipette for each decimal dilution.
- Pour about 15 ml of VRBA medium at $44^{\circ}\text{C} \pm 47^{\circ}\text{C}$ each Petri dish. The time elapsing between the end of the preparation of the initial suspension and the moment when the medium is poured into the dishes shall not exceed 15 minutes.
- Carefully mix the inoculum with the medium and allow the mixture to solidify with the Petri dishes standing on a cool horizontal surface. Also prepare a control plate with 15 ml of the medium for checking its sterility.
- After complete solidification, pour about 4 ml of the VRBA medium at $44^{\circ}\text{C} \pm 47^{\circ}\text{C}$ on to the surface of the inoculated medium allow to solidify.
- Invert the prepared dishes and incubate them in the set at 30°C or 37°C for 24 hours $\pm$

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2 hour.

ENUMERATION

After 24 ±2 hours of incubation select the Petri dishes with, if possible, 10 or more colonies and fewer than 150 colonies. Count using the colony counting equipment, Purplish red colonies with a diameter of at least 0.5 mm (sometimes surrounded by a reddish zone of precipitated bile). Consider all these as typical colonies of coliform and do not require further confirmation. Count other atypical colonies (smaller size) also and all colonies derived from milk products that contain sugar other than lactose, immediately after the incubation and confirm.

CONFIRMATION

Select five colonies of each atypical type and into tubes of brilliant green lactose bile broth and incubate at 30°C or 37° for 24 hours ± 2 hours. Consider as coliforms colonies that show gas formation in the Durham tube.

METHOD OF CALCULATION

S. No	No of colonies	Formula	Explanation
1	10-150 Colonies	$N = \frac{\sum c}{v \times 1,1 \times d}$	$\sum c$ is the sum of colonies counted on the two dishes retained from two successive dilutions, at least one of which contain a minimum of 10 colonies v is the volume of inoculum placed in each dish, in milliliter's; d is the dilution corresponding to the first dilution retained
	Atypical colonies	$a = b / A * C$	b is the number of colonies complying with identification criteria among the identified colonies A. c is the total number presumptive colonies counted on the dish .
2	Less Than 10 Colonies	$2_n = 2 / 2 \times d$	2_n = Estimate the number Express as "Microorganisms are present but less than (Vx d) per gram or ml" C is the average of colonies on the petri dishes retained v is the volume of inoculums used in each dish d is the dilution corresponding to the dilution

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			retained
3	No colonies	-	Express as less than 1/d microorganism per milliliter (liquid products) or per gram (other products)

CAUTION

- Carry out the test under the control of skilled microbiologist and great care shall be taken in the disposal of all the incubated material.
- Follow safe and good laboratory practices to avoid cross contamination.

REFERENCE

1. **IS 5401 (Part-1):2012** - Microbiology of Food and Animal Feeding Stuffs – Horizontal Method for the Detection and Enumeration of Coliforms Part-1-Colony Count Technique.
2. **ISO 7218:2007** – Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations.
3. **ISO 6887-1 (2017)** - Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions.

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IS 5401 (Part 2): 2012

METHOD FOR THE DETECTION AND ENUMERATION OF COLIFORMS

PART 2 MOST PROBABLE NUMBER TECHNIQUE (MPN)

INTRODUCTION

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. Coliforms bacteria which, at the specified temperature (30 °C or 37 °C) cause fermentation of lactose with the production of gas under the specified conditions.

SCOPE

This method is applicable for isolation, Identification and enumeration of coliform bacteria in foods. Enumeration is carried out by calculation of the most probable number (MPN) after incubation in a liquid medium at 30 °C or 37 °C. When the number sought is expected to be in the range 1 to 100 per millilitre or per gram of test sample.

EQUIPMENT

- Laminar Air flow
- Biosafety cabinet
- Autoclave
- Tubes and glass bottles
- Weighing Balance
- pH Meter
- Petri dish
- Graduated pipettes
- Durham tubes
- Micropipette with tips

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- Incubator
- Microscope
- Colony counting equipment
- Water Bath
- Refrigerator
- Cyclomixer

MEDIUM

- Lauryl sulfate tryptose broth (Double-strength medium & single-strength medium)
- Brilliant green lactose bile broth

SAMPLE PREPARATION

Homogenous sample of processed food to be used.

DETECTION OF COLIFORMS

- 1 ml of the test sample if liquid, or 1 ml of the initial suspension in the case of other products, is transferred to a tube containing 10 ml of double-strength Lauryl sulfate tryptose broth.
- 1 ml < x < 10 ml, or to a tube containing 10 ml of single-strength selective enrichment medium when x ≥ 1 ml.
- Leave the tube of double-strength medium in the incubator set at 30 °C or 37 °C (as agreed) for 24 h ± 2 h.
- Leave the tube of single-strength medium in the incubator at 30 °C or 37 °C (as agreed) for 24 h ± 2 h or, if neither gas formation nor opacity preventing the detection of gas formation is observed at this stage, continue incubation for another 24 h ± 2 h.

CONFIRMATION

- Incubated tube of double-strength Lauryl sulfate tryptose broth Inoculate with a loop a tube of Brilliant green lactose bile broth.
- Incubate in the incubator set at 30 °C or 37 °C (as agreed) for 24 h ± 2 h or, if gas formation is not observed at this stage, for 48 h ± 2 h.
- Carry out the same procedure for the incubated tubes from single-strength medium showing gas formation, or opacity preventing the detection of gas formation, when either of these features is first observed (i.e. after 24 h ± 2 h or after 48 h ± 2 h).

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INTERPRETATION

- A tube from double-strength Lauryl sulfate tryptose broth..or Single -strength Lauryl sulfate tryptose broth..in which gas formation is observed after $24\text{ h} \pm 2\text{ h}$ or $48\text{ h} \pm 2\text{ h}$ is considered as a positive tube.

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Flowcharts for procedure

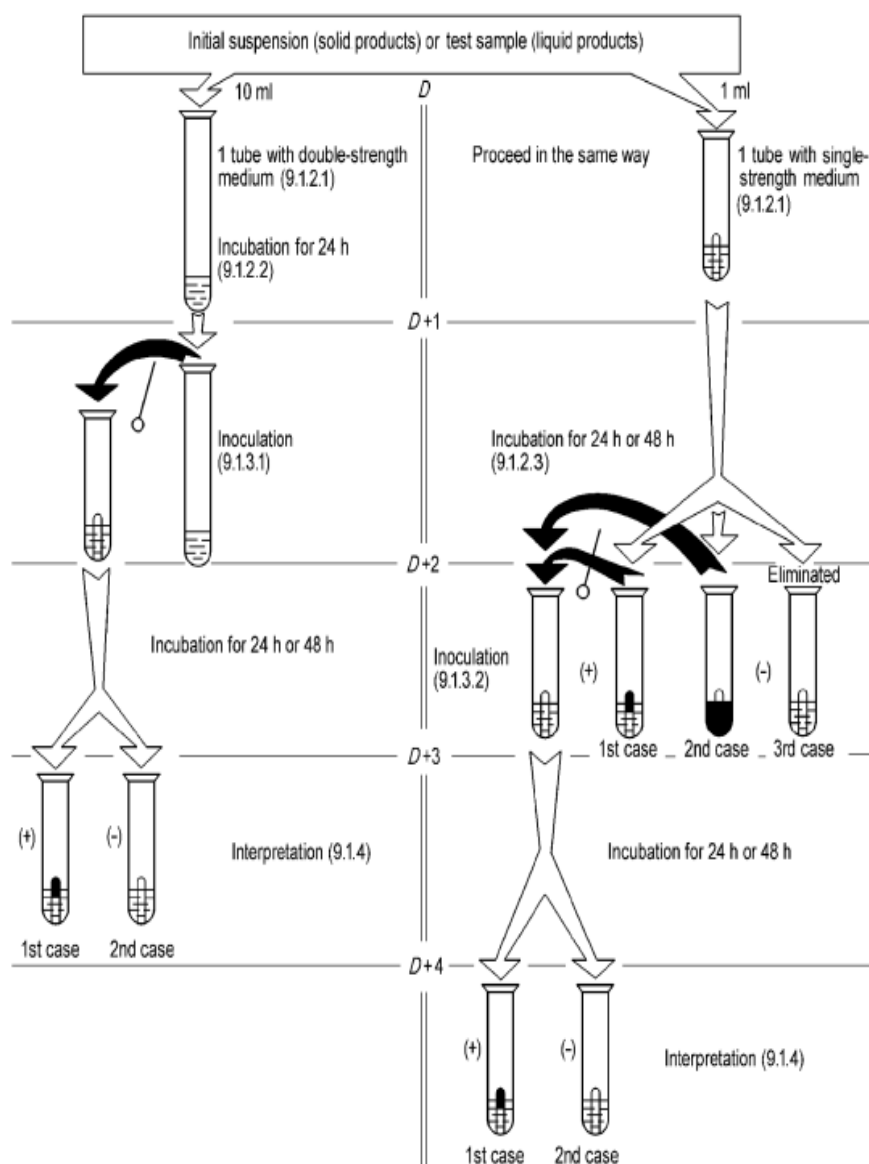


Figure A.1 — Detection method

ENUMERATION METHOD (MPN) METHOD

- This technique is recommended when the number sought is expected to be in the range 1 to 100 per millilitre or per gram of the test sample
- Take three tubes of Lauryl sulfate tryptose double-strength medium. Transfer to each

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of these tubes, using a pipette, 10 ml of the test sample if the product is liquid, or 10 ml of the initial suspension in the case of other products.

- Take three tubes of Lauryl sulfate tryptose single-strength medium. Transfer to each of these tubes, using another pipette 1 ml of the test sample if the product is liquid, or 1 ml of the initial suspension in the case of other products.
- Take three more tubes of single-strength medium. Transfer to each of these tubes, using another pipette 1 ml of the first decimal dilution (10^{-1}) in case of other products.
- Enrichment - Incubate the Lauryl sulfate tryptose DS & SS broth at 30 °C or 37 °C (as agreed) for 24 h $\pm$ 2 h or if neither gas formation nor opacity preventing of the detection of gas formation is observed at this stage, continue incubation for another 24 h $\pm$ 2 h.

CONFIRMATION

- Incubated tube of double-strength Lauryl sulfate tryptose broth Inoculate with a loop a tube of Brilliant green lactose bile broth.
- Incubate in the incubator set at 30 °C or 37 °C (as agreed) for 24 h $\pm$ 2 h or, if gas formation is not observed at this stage, for 48 h $\pm$ 2 h.
- Carry out the same procedure for the incubated tubes from single-strength medium showing gas formation, or opacity preventing the detection of gas formation, when either of these features is first observed (i.e. after 24 h $\pm$ 2 h or after 48 h $\pm$ 2 h).

INTERPRETATION

- A tube from double-strength Lauryl sulfate tryptose broth..or Single -strength Lauryl sulfate tryptose broth..in which gas formation is observed after 24 h $\pm$ 2 h or 48 h $\pm$ 2 h is considered as a positive tube.
- For each dilution, count the total number of tubes in which gas formation is observed in Brilliant green lactose bile broth (positive tubes) after 24 h $\pm$ 2 h or 48 h $\pm$ 2 h .



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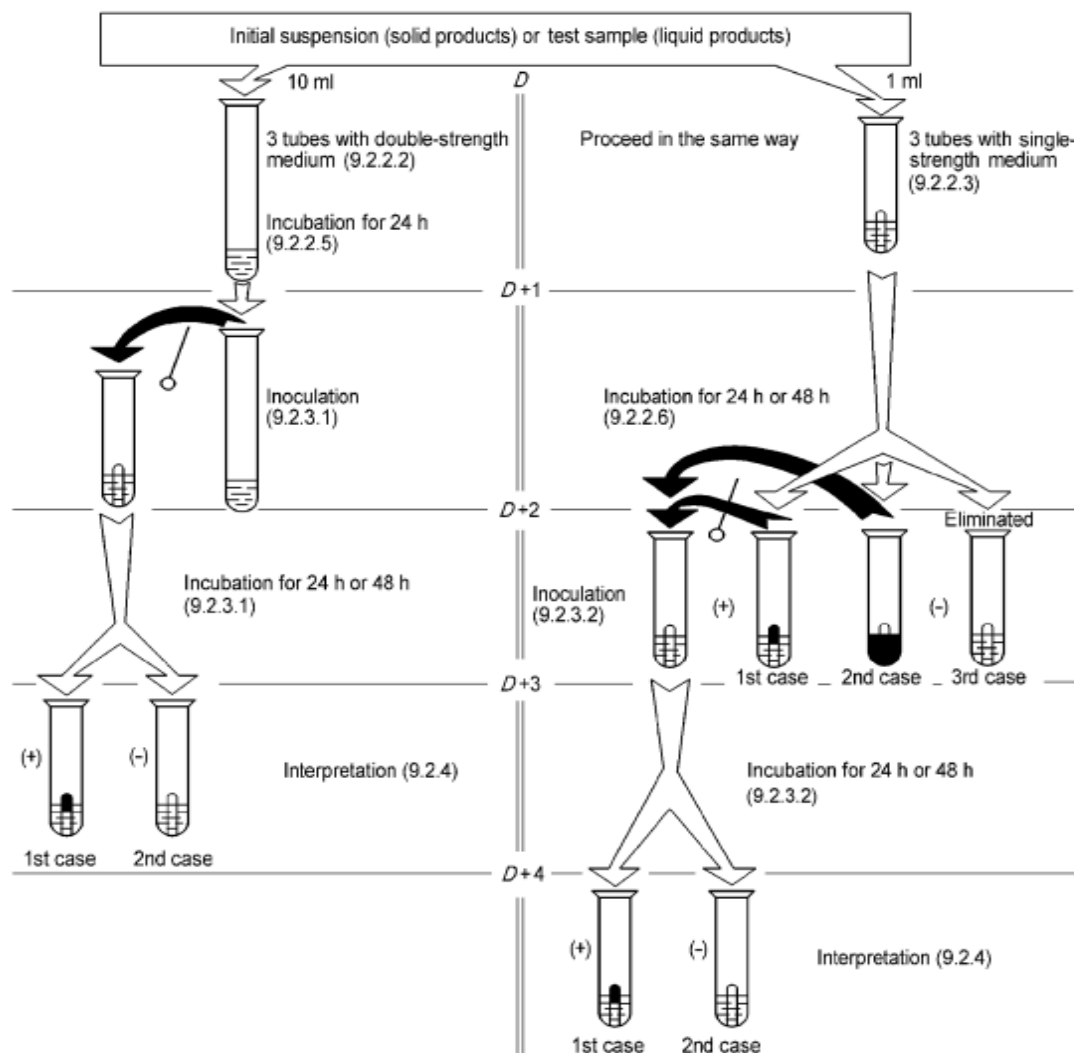
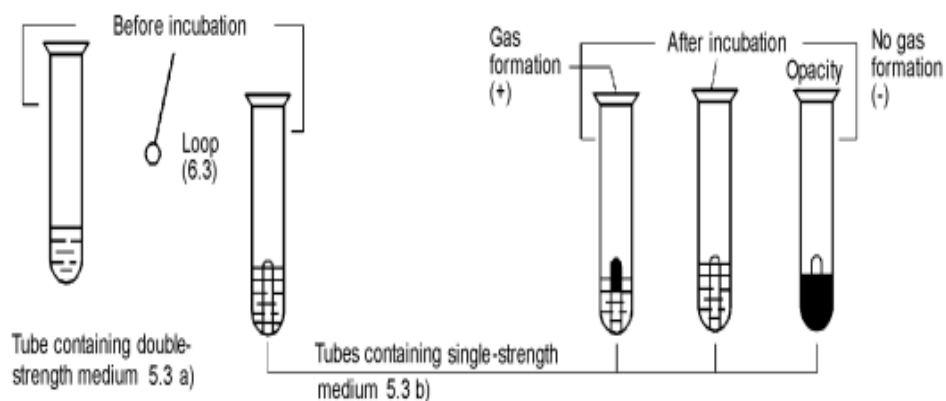


Figure A.2 — Enumeration method

CONFIRMATION STAGE

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EXPRESSION OF RESULTS

Results shall be expressed as a number between 1.0 and 9.9 multiplied by 10^x, where x is power of 10. In case of 0.1ml inoculum,

if plates of initial dilution have no colonies, the result is expressed as less than 10 cfu/ml or 100 cfu/g or ml.

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Table 1—MPN values per gram of sample and 95% confidence limits
 (when three test portions of 1g, three of 0.1 g and three of 0.01g are used)

Number positive results for inoculums volume, ml or g			MPN /ml or /g	95% Confidence limits	
1.00	0.10	0.01		Lower	Upper
0	0	0	0	0	1.1
0	1	0	0.30	0.04	2.3
1	0	0	0.36	0.05	2.7
1	0	1	0.72	0.17	3.0
1	1	0	0.74	0.18	3.1
1	2	0	1.1	0.35	3.7
2	0	0	0.92	0.21	4.0
2	0	1	1.4	0.42	4.8
2	1	0	1.5	0.43	5.0
2	1	1	2.0	0.69	6.0
2	2	0	2.1	0.71	6.2
3	0	0	2.3	0.55	9.7
3	0	1	3.8	0.93	16
3	1	0	4.3	0.95	19
3	1	1	7.5	1.9	30
3	1	2	12	3.6	37
3	2	0	9.3	2.2	40
3	2	1	15	4.4	51
3	2	2	21	7.2	64
3	3	0	24	5.6	100
3	3	1	46	9.6	220
3	3	2	110	25	480
3	3	3	∞	36	∞

REFERENCE

- **IS 5401 (Part 2): 2012-** Microbiology Of Food And Animal Feeding Stuffs — Horizontal Method For The Detection And Enumeration Of Coliforms. Part 2 Most Probable Number Technique
- **ISO 7218:2007** – Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations.

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IS 5403:1999 (RA: 2018)

METHOD FOR YEAST AND MOULD COUNT OF FOOD STUFF AND ANIMAL FEEDS

INTRODUCTION

Yeast and moulds are classified under the Kingdom Fungi. Yeast and mould are heterotrophic in nature and of great importance to the food industry. Yeast and mould can cause various degree of spoilage in food chain. Yeast colonies grow as smooth, moist, elevated or surface colonies. Mould colonies grow as fuzzy, thread like profuse growth of hyphae and may appear in different colour.

SCOPE

Enumeration of viable yeast and mould count in products intended for human consumption or feeding of animals by means of the colony count technique at 25°C.

EQUIPMENT

- Laminar Air flow
- Biosafety cabinet
- Autoclave
- Tubes and glass bottles
- Weighing Balance
- pH Meter
- Petri dish
- Graduated pipettes
- Micropipette with tips
- Incubator
- Colony counting equipment
- Water Bath
- Refrigerator
- Durham Tubes
- Cyclomixer

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MEDIUM

- 0.1% Peptone salt solution
- Yeast Extract Dextrose Chloramphenicol Agar
- Sodium Propionate

SAMPLE PREPARATION

- The sample should be handled aseptically and it should be truly representative of the lot. Homogenous sample of processed food to be used.

ANALYTICAL PROCEDURE

- Prepare 225 ml of 0.1% peptone salt solution dilution fluid.
- Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes (Check the pH before sterilize, if necessary adjust pH using 0.1N Hcl / 0.1N NaOH)
- Take 25 gm sample in 225ml dilution fluid mix well minimum 25 times.
- Take two sterile petri dish using a sterile pipette transfer the each dish 1 ml of test sample.
- Using a sterile pipette transfer to each dish 1 ml of the initial suspension (10^{-1}) to decimal dilution (10^{-2}) 9 ml of dilution fluid cyclometer mix well after take 1 ml transfer the two sterile Petri dish.
- If necessary, repeat the procedure with the further dilutions, using a new sterile pipette for each decimal dilution.
- Pour about 15 ml Yeast Extract Dextrose chloramphenicol Agar medium at $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ each Petri dish.
- The time elapsing between the end of the preparation of the initial suspension and the movement when the medium is poured into the dishes shall not exceed 15 minutes.
- Carefully mix the inoculum with the medium and allow the mixture to solidify with the Petri dishes standing on a cool horizontal surface.
- Prepare a control plate with 15 ml of the medium for checking its sterility.

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- Invert the plates and place them in the incubator set at $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$, for obtain 3,4 and 5 days of incubation.
- If only yeast counts are required, add 0.25 percent of sterile sodium propionate solution to the plate at the time of pouring to inhibit the growth of moulds.

OBSERVATION

- Count the colonies on each plate after 3, 4 and 5 days of incubation. Yeast colonies grow as smooth, moist, elevated or surface colonies.
- Mould colonies grow as fuzzy, thread like profuse growth of hyphae and may appear in different colour.

METHOD OF CALCULATION

S. No	No of colonies	Formula	Explanation
1	10-150 Colonies	$N = \frac{\sum c}{(n_1 + 0.1 n_2)d}$	N -Number of colonies per ml or g of product $\sum c$ is the sum of colonies counted on the two dishes retained from two successive dilutions, at least one of which contain a minimum of 10 colonies n1 - Number of dishes considered for the first dilution n2 - Number of dishes considered for the second dilution d is the dilution corresponding to the first dilution retained
2	Less Than 10 Colonies	$\bar{c} = \frac{C}{V} \times d$	$\bar{c}$ = Estimate the number Express as "Microorganisms are present but less than $(V \times d)$ per gram or ml" C is the average of colonies on the petri dishes retained V is the volume of inoculums used in each dish d is the dilution corresponding to the dilution retained
3	No colonies	-	Express as less than 1/d microorganism per milliliter (liquid products) or per gram (other products)

HAZARD ANALYSIS

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- Hazards: Chloramphenicol should be use carefully because it causes cancer.

CAUTION

- Carry out the test under the control of skilled microbiologist and great care shall be taken in the disposal of all the incubated material.
- Follow safe and good laboratory practices to avoid cross contamination.

REFERENCE

- **IS 5403: 1999 (RA 2018)** - Method for Yeast and Mould Count of Foodstuffs and animal feeds.
- **ISO 7218:2007** – Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations.
- **ISO 6887-1 (2017)** - Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions.

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IS: 5887 (Part 1) – 1976 (RA 2022)

DETECTION OF BACTERIA RESPONSIBLE FOR FOOD POISONING

PART 1 - ISOLATION, IDENTIFICATION AND ENUMERATION OF *ESCHERICHIA COLI*

INTRODUCTION

E. coli is included both in the group of total coliforms as in that of thermo tolerant coliforms. Its natural habitat is the intestinal tract of hot-blooded animals, although it may also be introduced into foods via non faecal sources.

SCOPE

This Standard (Part 1) Prescribes methods for isolation, identification and enumeration of *Escherichia coli* in foods

EQUIPMENT

- Screw cap bottle
- Autoclave
- Weighing Balance
- pH Meter
- Coat, Mask, Gloves
- Laminar Air flow
- Biosafety cabinet
- Petri plate
- Inoculation Loop
- Micropipette & Tips
- Water bath
- Incubator
- Colony counting equipment

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- Glass Slide & Glass Cavity Slide
- Microscope
- Refrigerator
- Durhams tube
- Cyclomixer

MEDIA

- 0.1% Peptone salt solution
- MacConkey agar
- Tergitol-7 agar
- Eosin Methylene Blue lactose agar medium
- MacConkey broth
- Motility Test Medium
- Gram Staining Kit
- TSI Medium
- Urea Agar
- Tryptone Broth
- MR-VP Medium
- Simmon Citrate Agar
- Nutrient Broth
- Nutrient Agar02

QUALITY OF REAGENTS

- Double distillation water are used

ANALYTICAL PROCEDURE

- Take 200 ml of 0.1% peptone solution for initial suspension.
- Sterilization at 121°C for 15 minutes.
- Blend the sample in a sterile blender jar for 2 minutes.
- Take approximately 200 ml of diluting fluid per 25g of the sample.
- Inoculate 1 ml of the sample into 10 ml of single strength MacConkey broth medium. If

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the number of organisms are assumed to be very small, inoculate 10 ml of double strength MacConkey broth medium.

- Also streak loop full on to MacConkey agar medium, Eosin methylene blue lactose agar and if available Tergitol 7 agar. Incubate all the inoculated media at 37°C overnight.
- If there is growth with fermentation of lactose in the MacConkey broth medium streak out a loopful on to each of the solid media and incubate at 37°C overnight.

TEST FOR IDENTIFICATION

Pick out and mark as many suspect colonies from the solid media as possible, but not less than 5 to investigate. The suspected colonies are smooth and are lactose fermenting on MacConkey agar and Purple violet black centred Green metallic sheen on Eosin Methylene Blue lactose agar and are yellow colonies surrounded by yellow zone on Targitol-7 agar medium.

BIOCHEMICAL IDENTIFICATION

1. Gram's Staining

The stain consists of:

- (1) 0.5 Percent Methyl violet or Crystal violet in water
- (2) Iodine solution (1 % Iodine and 2 % potassium iodide in water)
- (3) Counter stain

- Clean grease-free slide very light thin smear covering a small area, is made directly from the liquid culture and in clean tap water if from solid media.
- The smear is fixed by passing to and from over a flame and cooled.
- Cover the stain with the crystal violet For 30 sec pour off the stain and wash with iodine solution and then cover with iodine solution for 30 sec.
- Wash with ethanol until the dye cases stream out. Washing running tap water and apply for counter strain for 1 min
- Wash in tap water and dry for examination

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2. Test for Motility

- Inoculate by stabbing with a straight wire into top of the motility test medium in the strain to be tested, inside the glass tubing to a depth of about 5 mm.
- Take care that inoculation is not made on to the surface of the medium outside the glass tubing incubate at 37°C 18 to 24 hours.
- Motile strain strains shall be found to show growth on the surface of the medium outside the inner glass tube having travelled through the entire medium inside this inner tube.
- If negative on the first day, keep the inoculated tube at room temperature for a further 4 to 6 days to see if evidence of motility is present.

3. Test for H₂ S Production

- Inoculate TSI Medium by stabbing the strain into the butt and streaking the slope. Incubate at 37°C and observe daily for up to 7 days.
- The presence or absences of blackening in the butt of the medium shall be recorded.

4. Test for Urease

- Inoculate the organisms from the 24 hours incubated nutrient broth culture heavily over the entire slope surface Urease agar medium and incubate at 37°C for 18 to 24 hours.
- A positive urease is shown by the medium becoming pink or red on incubation.
- If negative, continue incubation for at least 4 days. **Proteus species** gives a positive result and may be used as control.

5. Test for Indole

- Inoculate Tryptone water medium with a loop full of 24 hours growth nutrient broth and incubate at 37°C for 48 hours.
- Add 0.5 ml of kovac's reagent. After adding kovac's reagent, Shake the tube gently, the appearance of a red colour indicates the presence of indole.

6. Test for Methyl Red

- Inoculate the MR-VP Medium and incubate 37°C for 2 days.
- Add 2 drops of methyl red solution. A positive reaction is indicated by red colour and a

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negative reaction by yellow colour.

7. Test for Voges-Proskauer Reaction

- Inoculate the MR-VP Medium and incubate at 37 °c for 2 days.
- Add 1 ml of Barritt Reagent A & Barritt Reagent B. Shake and slope the tube and observe for up to 4 hours for the appearance of a pink colour which indicated a positive reaction.

8. Test for Citrate utilization

- Inoculate the strain on to Simmons Citrate medium with a young nutrient agar slant culture using straight wire.
- Incubate at 37°C for up to 4 days for growth of the organism.

9. Test for Fermentation of carbohydrates

- Inoculate the peptone water medium and using 1 percent concentration for the lactose, and incubate at 37°C for 18 hours.
- Record the presence of acid from pink colour and that of gas in the Durham's Tube.

10. Test for Growth with Acid and Gas production in MacConkey Broth

- Inoculate the medium 1 ml and incubate at 44°C for 2 days.

ENUMERATION OF *Escherichia coli*

Preparation of Sample

Take 25 to 50 g of the sample in a, sterile blender jar and to this add diluting fluid to have dilution of 10-1. Blend at 8000 to 10000 rev/min for 2 minutes. Make serial ten-fold dilutions with the diluting fluid (0.1% peptone water), in duplicate series, up to 10-6 .

Spread out 0.1 ml from each dilutin tube, evenly n Tergitol-7 agar, and incubate at 37°C for 24 hours. Enumerae the colonies of E. coli which are of yellow clour surrounded by yellow zone and confirm these as being E. coli by the tests specified in this method. The number of viable colonies of E. coli per gram of sample shall be determined by multiplying by the dilution factor(s) and dividing by the mass of sample. Tergitol-7 agar plates/MacConkey agar plates or eosin methylene blue lactose agar plates may be used

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Determination of the Most Probable Number of E. coli

I. Obtain serial dilutions of the sample with a fresh sterile pipette, a measured volume of 1 ml of the homogenized mixture and of the five following serial-dilutions of both dilution series in triplicate to the tubes of 10 ml of single strength MacConkey broth containing Durham's tube for collection of gas.

II. Start with highest dilution and proceed to the lowest, filling and emptying the pipette three times before transferring the 1 ml portions to the tubes of medium.

III. When the number of E. coli is assumed to be very small, start by transferring 1.0 ml of the homogenized mixture in triplicate to 10-1 ml of double strength MacConkey broth medium containing Durham's tube for collection of gas, using a sterile 10 ml pipette.

IV. Incubate in a water-bath at 44°C for 48 hours.

V. Examine the tubes showing production of acid and gas, and using Table, obtain the most probable number (MPN) of E. coli per gram of the sample.

VI. Use for the calculation the results from three dilutions, selecting the highest dilution showing three positive tubes below which no sets with a smaller number of positive tubes occur, and the two following higher dilutions.

VII. The number obtained from Table 1 has to be multiplied by the lowest dilution factor, namely that of the first set of tubes, to obtain the most probable number of E. coli per gram of the sample. For example, when dilution 100 (= 10 ml of macerate), 10-1 and 10-2 are found to give the following numbers of positive tubes: 2, 2, 1, the MPN is 2.8 bacteria per gram, and when the dilutions 100, 10-1, 10-2, 10-3, 10-4 and 10-5 are found to give the following numbers of positive tubes: 3, 3, 3, 2, 0, 0, the MPN is 9.3 (3, 2, 0), multiplied by the dilution factor 102, that is, 9.3 X 102 bacteria per gram. The MPN is reported as the average of the results obtained from each of the duplicate dilution series.

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DISPOSAL PROCEDURE

- Put on the appropriate size protective gloves before handling any contaminated material.
- Place the bag into the autoclave, temperature should be at least 121 °C for 30 minutes at 15 lbs. Set the time for at least 30 minutes and run the sterilizer.
- Take out the bag and place in another larger sealed for disposal.

CAUTION

- Carry out the test under the control of skilled microbiologist and great care shall be taken in the disposal of all the incubated material.
- Follow safe and good laboratory practices to avoid cross contamination.

REFERENCE

- IS 5887 (Part-1):1976 - Detection of Bacteria Responsible for Food Poisoning - Part 1 - Isolation & Identification of *Escherichia coli*.
- ISO 7218:2007 – Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations

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IS 5887(Part II)-1976 (RA 2022)

ISOLATION, IDENTIFICATION AND ENUMERATION OF *STAPHYLOCOCCUS AUREUS*

INTRODUCTION

Staphylococci are aerobic Gram-positive bacteria, with diameters of 0.5 – 1.5 µm and characterized by individual cocci in grape-like clusters. They are non-motile, non-spore forming facultative anaerobes, Coagulase positive *Staphylococci* produce Enterotoxins, which is responsible for food-poisoning if contaminated food is consumed. *S. aureus* is the species with the more pathogenic of the genus *Staphylococcus*.

SCOPE

This method is applicable for isolation, Identification and enumeration of *staphylococcus aureus* in foods.

EQUIPMENT

- Laminar Air flow
- Biosafety cabinet
- Autoclave
- Tubes and glass bottles
- Weighing Balance
- pH Meter
- Bunsen Burner
- Petri dish
- Graduated pipettes
- Micropipette with tips
- Incubator
- Microscope
- Colony counting equipment

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- Water Bath
- Refrigerator

MEDIUM

- Nutrient Broth
- Nutrient Agar
- Blood Agar
- Cooked Salt Meat medium
- Baird parker medium
- Gram Stain kit
- Coagulase (Rabbit) Plasma

SAMPLE PREPARATION

Homogenous sample of processed food to be used.

ANALYTICAL PROCEDURE

- Prepare 225 ml of 0.1% peptone salt solution dilution fluid.
- Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes (Check the pH before sterilize, if necessary adjust pH using 0.1N Hcl / 0.1N NaOH)
- Take 25 gm sample in 225ml dilution fluid mix well minimum 20 times.
- Inoculate the sample on blood agar and in Cooked Salt Meat medium and Baird-parker agar.
- Incubated blood agar and in Cooked Salt Meat medium at 37°C at overnight and also inoculated on Baird-parker agar incubation at 37°C at least 30 hours.
- Cooked Salt Meat medium make subculture a blood agar and Baird-parker agar incubation at 37°C at least 30 hours.
- Examine not less than 5 of the suspect colonies of *staphylococcus* on the solid media and mark out as many suspect colonies as possible to investigate.
- A portion of each colony picked with a straight nichrome wire may be used for

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preliminary coagulase test using the slide technique.

- The remainder of the colony is streaked out on blood agar medium for incubation at 37°C to check purity and to proceed with identification.
- If preliminary slide testing is avoided, the suspect colony is checked for purity as just described. Pure colonies are stained by Gram's Method and tested for coagulase by slide and tube methods.

TEST FOR IDENTIFICATION

General Characteristics

- Aerobic, Gram-positive cocci in clusters usually, but not always producing a golden yellow coloured colonies on nutrient agar and blood agar, and shiny black colonies with or without narrow grey-white margin when grown on Baird-parker medium suspect colonies must show coagulase activity.

A) Gram stain

- On a clean grease-free slide very light and thin smear covering a small area, is made directly from liquid culture and in clean tap water from solid media.
- The smear fixed by passing to and fro over a flame and cooled.
- Cover the smear with the crystal violet for 30 seconds.
- Pour off the stain and wash with iodine solution and then cover with iodine solution and allow to remain for 30 seconds.
- Wash off with ethanol until the dye ceases to stream out.
- Wash in running tap water and apply counter stain for about one minute. Wash in tap water and dry for examination.

B) Coagulase Test

The test may be carried out by the following methods

(a) Slide Method: Emulsify a portion of the suspect colony in normal saline or water. Mix this with a straight wire dipped in human or rabbit plasma. Coagulase-positive staphylococci produce visible clumping immediately.

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(b) Tube Method: Emulsify a single suspect colony from a 24 hour growth of blood agar medium in 1 ml citrated rabbit plasma diluted 1 in 5 in 0.85 percent saline. The test is usually carried out in narrow tubes. Place in incubator preferably in water-bath at 37°C. Observe every hour to note clotting of plasma. Reading should be carried out for as long as possible, preferably avoiding overnight incubation. Positive control with a known coagulase-positive Strain of staphylococcus and a control of the diluted plasma plasma without inoculums should be included in the test. Tube method shall be preferred

EXPRESSION OF RESULTS

Presence/Absence of *S. aureus* given after confirmation of colonies in the sample examined. Test for *S. aureus*= Present/Absent per X mL sample

ENUMERATION

ANALYTICAL PROCEDURE

- Prepare the selective Media Baird parker Agar (Check the pH before sterilize, if necessary adjust pH using 0.1N Hcl / 0.1N NaOH)
- Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes sterilize, after sterilization pour the media into the Sterile Petri dish. Let it for solidification
- After the Solidification of Baird parker Agar, take 0.1 ml of inoculum using sterile pipette pour the inoculum on BPA Plate and spread the inoculum using L –Rod. (If need prepare the serial dilution up to 10⁻⁶)
- Carefully spread the inoculums as quickly as possible over the surface of agar medium, using the spreader, trying not to touch the sides of agar plate.
- Allow all the plates to dry with their lids on for 15 min at laboratory temperature.
- Incubate the BPA inoculated Plate at 37°C for 30 hours , After completion of incubation observe the characteristic colonies (Baird Parker Agar- Shiny Black colonies with or without Grey white margin, Blood Agar –Golden colour colonies)
- Incubate the BPA inoculated Plate at 37°C for 30 hours, After completion of incubation observe the characteristic colonies (Baird Parker Agar- Shiny Black colonies with or without Grey white margin, Blood Agar –Golden colour colonies)

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EXPRESSION OF RESULTS

Results shall be expressed as a number between 1.0 and 9.9 multiplied by 10^x, where x is power of 10. In case of 0.1ml inoculum, if plates of initial dilution have no colonies, the result is expressed as less than 10 cfu/ml or 100 cfu/g or mL (if primary suspension prepared)

CAUTION

- Carry out the test under the control of skilled microbiologist and great care shall be taken in the disposal of all the incubated material.
- Follow safe and good laboratory practices to avoid cross contamination.

REFERENCE

- **IS 5887 (Part-2):1976** - Method for Detection of Bacteria Responsible For Food Poisoning
Part-2 Isolation, Identification and Enumeration of Staphylococcus aureus.
- **ISO 7218:2007** – Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations.

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IS 5887(Part II)-1976 (RA 2022)

ISOLATION, IDENTIFICATION AND ENUMERATION OF FAECAL STREPTOCOCCI

INTRODUCTION

Aerobic, Gram-positive cocci usually in pairs or short chains, producing small pink colonies on Macconkey agar and colonies which are dark red or having red or pink centres when grown on ethyl violet agar. Growth obtained also at 44°C.

SCOPE

This method is applicable for isolation, Identification and enumeration of faecal streptococci in foods.

EQUIPMENT

- Laminar Air flow
- Biosafety cabinet
- Autoclave
- Tubes and glass bottles
- Weighing Balance
- pH Meter
- Bunsen Burner
- Petri dish
- Graduated pipettes
- Micropipette with tips
- Incubator
- Bunsen burner
- Microscope

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- Colony counting equipment
- Water Bath
- Refrigerator

MEDIUM

- Ethyl Violet Azide Dextrose Agar
- MacConkey Agar.
- Nutrient Agar

SAMPLE PREPARATION

Homogenous sample of processed food to be used.

ANALYTICAL PROCEDURE

- Prepare 225 ml of 0.1% peptone salt solution dilution fluid.
- Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes (Check the pH before sterilize, if necessary adjust pH using 0.1N Hcl / 0.1N NaOH)
- Take 25 gm sample in 225ml dilution fluid mix well minimum 20 times.
- Prepare the selective Media Ethyl Violet Azide Dextrose Agar, Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes sterilize, after sterilization pour the media into the Sterile Petri dish. Let it for solidification
- After the Solidification of Ethyl Violet Azide Dextrose Agar take 0.1 ml of inoculum using sterile pipette pour the inoculum on EVDA Plate and spread the inoculum using L –Rod. (If need prepare the serial dilution up to 10 -6)
- Carefully spread the inoculums as quickly as possible over the surface of agar medium, using the spreader, trying not to touch the sides of agar plate.
- Allow all the plates to dry with their lids on for 15 min at laboratory temperature.
- Incubate the EVDA inoculated Plate at 37 ° C for 48 hours , After completion of incubation observe the characteristic colonies (Baird Parker Agar- Shiny Black colonies with or without Grey white margin, Blood Agar –Golden colour colonies)

CONFIRMATION

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Enumerate the colonies which are as described in general characteristics and confirm these Gram's stain and growth at 44°C in Macconkey agar for small pink colonies.

The number of viable colonies per gram of sample is determined by multiplying by the dilution factor(s) and dividing by the mass of the sample.

TEST FOR IDENTIFICATION:

A) Gram stain:

- On a clean grease-free slide very light and thin smear covering a small area is made directly from liquid culture and in clean tap water from solid media.
- The smear is fixed by passing to and over a flame and cooled.
- Cover the smear with the stain crystal violet for 30 second
- Pour off the stain and wash with iodine solution and then cover with iodine solution and allow to remain for 30 second.
- Wash off with ethanol until the dye ceases to stream out.
- Wash in running tap water and apply counterstain for about 1 minute.
- Wash in tap water and dry for examination.

Enterococci Index:

- Obtain serial dilutions of the sample as in 0.1% peptone salt solution. Transfer with a fresh sterile pipette, a measured volume of 1ml of homogenized mixture and of the five following serial dilutions of both dilution series in triplicate to the tubes of 10 ml of ethyl violet azide dextrose broth
- Start with the highest dilution and proceed to the lowest, filling and emptying the pipette three times before transferring the 1 ml portions to the tubes of medium
- When the number of enterococci is assumed to be very small, start by transferring, using a sterile 10 ml pipette, 10 ml of homogenized mixture in triplicate to 10 ml of double strength broth which contains twice the amounts of the ingredient as in 1000ml of water incubate at 37°C for 48 hours.
- Record as positive the tube which have developed turbidity (Growth) and the growth having been confirmed as being faecal streptococci as described in

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- Using table 1, obtain the most probable number (MPN) of faecal streptococci per gram of the sample.
- Use for the calculation the results from three dilutions, Selecting the highest dilution showing three positive tube below which no seats with a smaller number of positive tube occur, and the two following higher dilutions.
- The number obtained from Table 1 has to be multiplied by the lower dilution factor, that is, that of the first set of tubes,
- To obtain the most probable number of streptococci per gram of the sample.
- For example, when dilution 10^0 (= 10 ml of macerate), 10^{-1} and 10^{-2} are found to give the following numbers of positive tubes: 2, 2, 1, MPN is 2.8 bacteria per gram, and when the dilutions $10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}$ and 10^{-5} are found to give the following numbers of positive tubes: 3, 3, 3, 2, 0, 0 the MPN is 9.3 (3, 2, 0), Multiplied by the dilution factor 10^2 , that is 9.3×10^2 bacteria per gram.
- Then MPN is reported as the average of the results obtained from each of the duplicate dilution series.

DISPOSAL PROCEDURE

- Put on the appropriate size protective gloves before handling any contaminated material
- Place the bag into the autoclave, temperature should be at least 121°C for 30 mins, 15 lbs
- Take out the bag and place in another larger sealed for disposal

EXPRESSION OF RESULTS

Results shall be expressed as a number between 1.0 and 9.9 multiplied by 10^x , where x is power of 10. In case of 0.1ml inoculum, if plates of initial dilution have no colonies, the result is expressed as less than 10 cfu/ml or 100 cfu/g or mL (if primary suspension prepared).

CAUTION

- Sodium Azide is highly toxic and mutagenic; precautions shall be taken to avoid contact with it, especially through the inhalation of fine dust during the preparation of media.

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- Azide containing media should not be mixed with strong inorganic acids, as toxic hydrogen azide (HN₃) may be produced.
- Solution containing azide can also form explosive compounds when in contact with metal pipework e.g. from sinks.

REFERENCE

- **IS 5887 (Part 2): 1976** - Methods for detection of bacteria responsible for food poisoning: Part 2 Isolation, identification and enumeration of staphylococcus aureus and faecal streptococci (First Revision)
- **ISO 7218:2007** – Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations.

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IS 5887 (Part 3): 1999

METHOD FOR DETECTION OF *SALMONELLA SPP*

INTRODUCTION

Salmonella is a genus belonging to the family Enterobacteriaceae. The family is defined as rod-shaped, Gram-negative, non-spore-forming, facultative anaerobic and oxidase-negative bacteria.

SCOPE

Horizontal method for the detection of salmonella. The detection of Salmonella requires 4 successive stages viz. pre-enrichment, enrichment, differential selective and confirmation.

EQUIPMENT

- Screw cap bottle
- Autoclave
- Weighing Balance
- pH Meter
- Laminar Air flow
- Biosafety cabinet
- Bunsen burner
- Petri plate
- Inoculation Loop
- Graduated pipettes & Tips
- Water bath
- Incubator

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- Colony counting equipment
- Glass Slide & Glass Cavity Slide
- Microscope
- Refrigerator
- Durhams tube

MEDIUM

- Buffered Peptone water
- Rappaport- Vassiliadis magnesium chloride/malachite green medium (RV medium)
- Selenite/cysteine Media
- Brilliant green agar
- Xylose lysine Deoxycholate citrate agar
- Nutrient agar
- Triple sugar iron agar
- Urea agar
- L-Lysine decarboxylation Medium
- β galactosidase reagent
- MR-VP Medium
- ONPG disc
- Tryptone water
- Kovacs reagent
- Salmonella antisera
- Saline solution

ANALYTICAL PROCEDURE

PREPARATION OF SAMPLE AND PRE-ENRICHMENT

- Aseptically add 25 g/ml of sample into a sterile empty wide mouth container with suitable closure. Add 225 ml of sterile Buffer peptone water for pre-enrichment.
- Incubate at 37°C for 16 hours to 20 hours.

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SELECTIVE ENRICHMENT

- Transfer 0.1 ml of the culture obtained from buffered peptone water to a tube containing 10 ml of the RV medium. Incubation at 42°C for 24 hours.
- Inoculated Selenite / Cystine medium at 35 °C or 37 °C (as agreed) for 24 h and a further 24 h.

SELECTIVE MEDIA PLATING

- Using the culture obtained in the RV medium & Selenite / Cystine medium Agar after incubation for 24 hours, inoculate by means of a loop, the surface of the XLD agar plate and BGA Plate.
- Invert the dishes and incubate at 37°C for 24 ± 3 hours.
- After incubation 24 hours examine the dishes BGA and XLD agar for presence of typical colonies of Salmonella grown on BGA agar cause the colour of the medium to change from pink to red and on XLD agar have a black centre and lightly transparent zone of reddish colour.
- If growth is slight or if no typical colonies of salmonella are present, re incubate at 37°C for further 18 hours to 24 hours.
- Re-examine the plates for the presence of typical colonies of Salmonella.

CONFIRMATION

- Take suspected five colonies (if present) from each of the selective plate.
- Streak the selected colonies onto the surface of pre-dried nutrient agar plate in a manner which will allow well-isolated colonies to develop.
- Incubate inoculated plates at 37°C for 18 hours to 24 hours.
- Use pure cultures for biochemical and serological confirmation.

BIOCHEMICAL IDENTIFICATION

1. TSI Agar

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- Streak the agar slope surface and stab the butt. Incubate at 34°C to 38°C for 24 ± 3 hours.
- Typical salmonella culture show alkaline (Red) Slants with gas formation and acid (yellow) butts. With (in about 90% of the cases) formation of hydrogen sulphide (H₂S) production (blackening of the sugar).
- When lactose-positive salmonella is isolated the TSI slant is yellow.

2. Test for Urease

- Streak the agar slope surface. Incubate at 34°C to 38°C for 24 ± 3 hours.
- If the reaction is positive, splitting of urea liberates ammonia which changes the colour of phenol red to rose pink and later to deep cerise. The reaction is often apparent after 2 hours to 4 hours.

3. L-Lysine Decarboxylation Medium

- Inoculate just below the surface of the liquid medium. Incubate at 34°C to 38°C for 24 hours.
- A purple colour after incubation indicates a positive reaction.
- A yellow colour indicates a negative reaction.

4. Detection of β-galactosidase

- Suspend a loopful of the suspected colony in a tube containing 0.25 ml of the saline solution. Add 1 drop of toluene and shake the tube.
- Put the tube in a water bath set at 35°C or 37°C and leave for several minutes.
- Add 0.25 ml of the reagent for detection of β-galactosidase and mix.
- Replace the tube in the water bath set 37°C, leave for 24 hours, examining the tube at intervals.
- A yellow colour indicates positive reaction. The reaction is often apparent after 20 min.
- If prepared paper disc are used for the detection of β-galactosidase, follow the manufacture's instruction.

5. Voges-Proskauer (VP) reaction

- Suspend a loopful of the suspected colony in a sterile tube containing 0,2 ml of the VP medium, Incubate at 35 °C or 37 °C for 24 h

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- After incubation, add two drops of the three drops of Barritt's Reagent A and then two drops of Barritt's Reagent B shake after the addition of each reagent.
- The formation of a pink to bright red colour within 15 min indicates a positive reaction.

. Indole Test

- Inoculate a tube containing 5 ml tryptone/tryptophan medium with the suspected colony. Incubate at 35 °C or 37°C for 24 ± 3 hours.
- After incubation, add 1 ml of the kovacs reagent. The formation of a red ring indicates positive reaction. A yellow-ring indicates a negative reaction

BIOCHEMICAL TESTS

Broth/ Media	Time of incubation	Results
Urea broth	34 °C or 38 °C 24 ± 3 h	Negative (no change in yellow colour of medium)
L-Lysine decarboxylation medium	34 °C or 38 °C 24 ± 3 h	Positive (Purple color)
β-galactosidase	34 °C or 38 °C 24 ± 3 h	Negative
Voges-Proskauer (VP)	35 °C or 37 °C for 24 h	Negative
Indole Test	35 °C or 37 °C for 24 h	Negative (Yellow brown reaction)

SEROLOGICAL CONFIRMATION

- The detection of presence of Salmonella O-, Vi- and H-antigens is tested by slide agglutination with the appropriate sera, from nutrient agar plate and after auto-agglutinable strains have been eliminated.

1. Elimination of auto-Agglutinable strains

- Place one drop of the saline solution onto a carefully cleaned glass slide.
- Using a loop, disperse in this drop part of the colony to be tested, So as to obtain a homogeneous and turbid suspension.

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- Rock the slide gently for 5 s to 60 s.
- Observe the result against a dark background, preferably with the aid of a magnifying glass.
- If the bacteria have formed granules in the suspension, the strain is considered auto-agglutinable.

2. Examination for O-antigens

- Using the pure colony recognized as non-auto-agglutinable, proceed according to the elimination of auto agglutinable stain test. Using one drop of the Polyvalent anti-O serum instead of the saline solution.
- If agglutination occurs, the reaction is considered positive.

3. Examination for Vi-antigens

- Proceed according to the elimination of auto agglutinable stain test. Using one drop of the anti-Vi serum instead of the saline solution.
- If agglutination occurs, the reaction is considered positive.

4. Examination for H-antigens

- Inoculate the semi-solid nutrient agar with a pure non-auto-agglutinable colony.
- Incubate the medium at 37°C for 18 h to 24 h.
- Use this culture for examination for the H-antigens, proceeding according to the elimination of auto agglutinable stain test. Using one drop of the anti-H serum instead of the saline solution.
- If agglutination occurs, the reaction is considered positive.

INTERPRETATION OF BIOCHEMICAL AND SEROLOGICAL REACTIONS

- Table 2 gives interpretation of confirmatory tests carried out on the colonies used.

Table 2

Biochemical Reactions	Auto-agglutination	Serological Reactions	Interpretation
Typical	No	O-, Vi or H-antigen positive	Strains considered to be Salmonella
Typical	No	O-, or H-antigen negative	

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Typical	Yes	Not Tested	Presumptive Salmonella
No typical reactions	-	All reactions negative	Not considered to be Salmonella

In accordance with the results of the interpretation, indicate the presence and absence of Salmonella in a test portion of x of product.

DEFINITIVE CONFIRMATION

- Strains which are considered to be Salmonella, or which may be Salmonella, shall be sent to a recognized Salmonella reference centre for definitive typing.

EXPRESSION OF RESULTS

- In accordance with the results of the interpretation, indicate the presence or absence of Salmonella in a test portion of x g of product.

DISPOSAL PROCEDURE

- Put on the appropriate size protective gloves before handling any contaminated material.
- Place the bag into the autoclave, temperature should be at least 121 °C for 30 minutes at 15 lbs. Set the time for at least 30 minutes and run the sterilizer.
- Take out the bag and place in another larger sealed for disposal

REFERENCE

- IS 5887 (Part 3) : 1999 - Methods For Detection Of Bacteria Responsible For Food Poisoning Part 3 General Guidance On Methods For the Detection Of *Salmonella spp.*
- ISO 7218:2007 – Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations.

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IS 5887 (Part-3/Sec 1):2020

METHOD FOR DETECTION OF *SALMONELLA SPP*

INTRODUCTION

Salmonella is a genus belonging to the family Enterobacteriaceae. The family is defined as rod-shaped, Gram-negative, non-spore-forming, facultative anaerobic and oxidase-negative bacteria.

SCOPE

Horizontal method for the detection of salmonella. The detection of Salmonella requires 4 successive stages viz. pre-enrichment, enrichment, differential selective and confirmation.

EQUIPMENT

- Screw cap bottle
- Autoclave
- Weighing Balance
- pH Meter
- Laminar Air flow
- Biosafety cabinet
- Bunsen burner
- Petri plate

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- Inoculation Loop
- Graduated pipettes & Tips
- Water bath
- Incubator
- Colony counting equipment
- Glass Slide & Glass Cavity Slide
- Microscope
- Refrigerator
- Durhams tube

MEDIA

- Buffered Peptone water
- Rappaport- vassiliadis medium with soya (RVS Broth)
- Modified semi-solid Rappaport –Vassiliadis (MSRV) agar
- Muller-Kauffmann tetrathionate –novobiocin (MKTn) broth
- Brilliant green agar
- Xylose lysine Deoxycholate citrate agar
- Nutrient agar
- Triple sugar iron agar
- Urea agar
- L-Lysine decarboxylation Medium
- β galactosidase reagent
- MR-VP Medium
- ONPG disc
- Tryptone water
- Kovacs reagent
- Salmonella antisera.

ANALYTICAL PROCEDURE

PREPARATION OF SAMPLE AND PRE-ENRICHMENT

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- Aseptically add 25 g/ml of sample into a sterile empty wide mouth container with suitable closure. Add 225 ml of sterile Buffer peptone water for pre-enrichment.
- Incubate at 37°C for 16 hours to 20 hours.

SELECTIVE ENRICHMENT

- Transfer 0.1 ml of the culture obtained from buffered peptone water to a tube containing 10 ml of the RVS broth. Incubation at 41.5°C for 24 ± 3 hours.
- Inoculate MSRV agar plate with one to three equally spaced spots on surface of the medium. Incubated the inoculated MSRV agar plates at 41.5°C for 24 ± 3 hours. **Do not invert the plates.**
- Transfer 1 ml of the culture obtained to a flask containing 10ml MKTTn. Incubation at 37°C for 24 ± 3 hours.

SELECTIVE MEDIA PLATING

- Using the culture obtained in the RVS medium or MSRV Agar after incubation for 24 hours, inoculate by means of a loop, the surface of the XLD agar plate and BGA Plate.
- Using the culture obtained in the MKTTn broth, after incubation for 48 hours, inoculate by means of a 10 µl loop, the surface of an XLD agar plate and BGA Plate.
- Invert the dishes and incubate at 37°C for 24 ± 3 hours.
- After incubation 24 hours examine the dishes BGA and XLD agar for presence of typical colonies of Salmonella grown on BGA agar cause the colour of the medium to change from pink to red and on XLD agar have a black centre and lightly transparent zone of reddish colour.
- If growth is slight or if no typical colonies of salmonella are present, re incubate at 37°C for further 18 hours to 24 hours.
- Re-examine the plates for the presence of typical colonies of Salmonella.

CONFIRMATION

Treatment of typical or suspected colonies

- Take suspected five colonies (if present) from each of the selective plates and streak onto Nutrient agar plates. Incubate at 35 °C or 37 °C for 18 to 24 h. Use this culture for

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biochemical and serological confirmation.

- Streak the selected colonies onto the surface of pre-dried nutrient agar plate in a manner which will allow well-isolated colonies to develop.
- Incubate inoculated plates at 37°C for 18 hours to 24 hours.
- Use pure cultures for biochemical and serological confirmation.

BIOCHEMICAL IDENTIFICATION

1. TSI Agar

- Streak the agar slope surface and stab the butt. Incubate at 34°C to 38°C for 24 ± 3 hours.
- Typical salmonella culture show alkaline (Red) Slants with gas formation and acid (yellow) butts. With (in about 90% of the cases) formation of hydrogen sulphide (H₂S) production (blackening of the sugar).
- When lactose-positive salmonella is isolated the TSI slant is yellow.

2. Test for Urease

- Streak the agar slope surface. Incubate at 34°C to 38°C for 24 ± 3 hours.
- If the reaction is positive, splitting of urea liberates ammonia which changes the colour of phenol red to rose pink and later to deep cerise. The reaction is often apparent after 2 hours to 4 hours.

3. L-Lysine Decarboxylation Medium

- Inoculate just below the surface of the liquid medium. Incubate at 34°C to 38°C for 24 hours.
- A purple colour after incubation indicates a positive reaction.
- A yellow colour indicates a negative reaction.

4. Detection of β-galactosidase

- Suspend a loopful of the suspected colony in a tube containing 0.25 ml of the saline solution. Add 1 drop of toluene and shake the tube.
- Put the tube in a water bath set at 35°C or 37°C and leave for several minutes.

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- Add 0.25 ml of the reagent for detection of β -galactosidase and mix.
- Replace the tube in the water bath set 37°C, leave for 24 hours, examining the tube at intervals.
- A yellow colour indicates positive reaction. The reaction is often apparent after 20 min.
- If prepared paper disc are used for the detection of β -galactosidase, follow the manufacturer's instruction.

5. Indole Test

- Inoculate a tube containing 5 ml tryptone/tryptophan medium with the suspected colony. Incubate at 35 °C or 37°C for 24 $\pm$ 3 hours.
- After incubation, add 1 ml of the kovacs reagent. The formation of a red ring indicates positive reaction. A yellow-ring indicates a negative reaction.

BIOCHEMICAL TESTS

BROTH/ MEDIA	TIME OF INCUBATION	RESULTS
Urea broth	34 °C or 38 °C 24 $\pm$ 3 h	Negative (no change in yellow colour of medium)
L-Lysine decarboxylation medium	34 °C or 38 °C 24 $\pm$ 3 h	Positive (Purple color)
β -galactosidase	34 °C or 38 °C 24 $\pm$ 3 h	Negative
Indole Test	35 °C or 37 °C for 24 h	Negative (Yellow brown reaction)

SEROLOGICAL CONFIRMATION

- The detection of presence of Salmonella O-, Vi- and H-antigens is tested by slide agglutination with the appropriate sera, from nutrient agar plate and after auto-agglutinable strains have been eliminated.

1. Elimination of auto-agglutinable strains

- Place one drop of the saline solution onto a carefully cleaned glass slide.
- Using a loop, disperse in this drop part of the colony to be tested, So as to obtain a

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homogeneous and turbid suspension.

- Rock the slide gently for 5 s to 60 s.
- Observe the result against a dark background, preferably with the aid of a magnifying glass.
- If the bacteria have formed granules in the suspension, the strain is considered auto-agglutinable.

2. Examination for O-antigens

- Using the pure colony recognized as non-auto-agglutinable, proceed according to the elimination of auto agglutinable stain test. Using one drop of the polyvalent anti-O serum instead of the saline solution.
- If agglutination occurs, the reaction is considered positive.

3. Examination for Vi-antigens

- Proceed according to the elimination of auto agglutinable stain test. Using one drop of the anti-Vi serum instead of the saline solution.
- If agglutination occurs, the reaction is considered positive.

4. Examination for H-antigens

- Inoculate the semi-solid nutrient agar with a pure non-auto-agglutinable colony.
- Incubate the medium at 37°C for 18 h to 24 h.
- Use this culture for examination for the H-antigens, proceeding according to the elimination of auto agglutinable stain test. Using one drop of the anti-H serum instead of the saline solution.
- If agglutination occurs, the reaction is considered positive.

INTERPRETATION OF BIOCHEMICAL AND SEROLOGICAL REACTIONS

- Table 2 gives interpretation of confirmatory tests carried out on the colonies used.

Table 2

Biochemical Reactions	Auto-agglutination	Serological Reactions	Interpretation
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Typical	No	O-, Vi or H-antigen positive	Strains considered to be Salmonella
Typical	No	O-, or H-antigen negative	Presumptive Salmonella
Typical	Yes	Not Tested	
No typical reactions	-	All reactions negative	Not considered to be Salmonella

In accordance with the results of the interpretation, indicate the presence and absence of Salmonella in a test portion of x of product.

DEFINITIVE CONFIRMATION

- Strains which are considered to be Salmonella, or which may be Salmonella, shall be sent to a recognized Salmonella reference centre for definitive typing.

EXPRESSION OF RESULTS

- In accordance with the results of the interpretation, indicate the presence or absence of Salmonella in a test portion of x g of product.

DISPOSAL PROCEDURE

- Put on the appropriate size protective gloves before handling any contaminated material.
- Place the bag into the autoclave, temperature should be at least 121 °C for 30 minutes at 15 lbs. Set the time for at least 30 minutes and run the sterilizer.
- Take out the bag and place in another larger sealed for disposal.

CAUTION

- In order to safeguard the health of laboratory personnel, it is essential that tests for detecting Salmonella spp. are only undertaken in properly equipped laboratories, under the control of a skilled microbiologist and that great care is taken in the disposal of all incubated materials.

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- It is also the responsibility of the user to establish appropriate safety and health practices in the laboratory.

REFERENCE

- **IS 5887 (Part-3/Sec 1):2020** - Method for Detection of Bacteria Responsible for Food Poisoning (Part-3) Horizontal Method for the Detection, Enumeration and serotyping of *salmonella spp.*
- **ISO 7218:2007** – Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations

IS 5887- PART 6: 2012(RA 2022)

ENUMERATION OF PRESUMPTIVE *BACILLUS CEREUS*

INTRODUCTION

Bacillus cereus is a pathogenic bacterium, which causes foodborne diseases classified by the International Commission on Microbiological Specifications for Foods (ICMSF, 2002) in Risk Group III: “diseases of moderate hazard usually not life threatening, normally of short duration without substantial sequel, causing symptoms that are self-limiting but can cause severe discomfort”. They are Gram-positive rods, spore forming, facultative anaerobic and each species is differentiated from *B. cereus* by basically for one single characteristic.

SCOPE

Enumeration of visible presumptive *Bacillus cereus* by means of the colony-count technique at 30°C.

EQUIPMENT

- Laminar Air flow
- Biosafety cabinet
- Autoclave
- Tubes and glass bottles

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- Weighing Balance
- pH Meter
- Bunsen Burner
- Petri dish
- Graduated pipettes
- Micropipette with tips
- Incubator
- Bunsen burner
- Microscope
- Colony counting equipment
- Water Bath
- Refrigerator
- Spreader (glass or plastic)
- Inoculation loops and straight wire
- Cyclomixer

MEDIUM

- Complete medium (MYP agar) having Basal Medium
- Polymyxin B solution
- Egg yolk emulsion
- Sheep blood agar

SAMPLE PREPARATION

Homogenous sample of processed food to be used.

ANALYTICAL PROCEDURE

- Prepare 225 ml of 0.1% peptone salt solution dilution fluid.
- Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes (Check the pH before sterilize, if necessary adjust pH using 0.1N Hcl / 0.1N NaOH)
- Take 25 gm sample in 225ml dilution fluid mix well minimum 20 times.
- Take two sterile Petri dish using a sterile pipette transfer the each dish 0.1 ml of test

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sample.

- Using a sterile pipette transfer to each dish 1 ml of the initial suspension (10^{-1}) to decimal dilution (10^{-2}) 9 ml of dilution fluid cyclometer mix well after take 0.1 ml transfer the two sterile Petri plate.
- If necessary, repeat the procedure with the further dilutions, using a new sterile pipette for each decimal dilution.
- Immediately spread the inoculum as quickly as possible over the surface of the agar plate without touching the sides of the dish with the spreader, also prepare a control plate with 15 ml of the medium for checking its sterility.
- Leave the plates with the lids on for about 15 min at ambient temperature for the inoculum to be absorbed into the agar
- Invert the prepared dishes and place them in the incubator at 30 °C for 18h to 24 hrs. If necessary re-incubate for 18h to 24 hrs.

COUNTING OF COLONIES

After the specified incubation period, Count the presumptive *B. cereus* colonies on each plate. The presumptive colonies are large, pink surrounded by a zone of precipitation.

CONFIRMATION

HAEMOLYSIS TEST ON SHEEP BLOOD AGAR

- Streak, stab or spot the selected colonies onto the surface of sheep blood agar in a manner which allows good interpretation of the haemolysis reaction.
- Incubate at 30 °C for 24 h $\pm$ 2 h and interpret the haemolysis reaction.

BIOCHEMICAL INTERPRETATION

Test	Result confirming presumptive <i>Bacillus cereus</i>
MYP agar	Formation of pink colonies surrounded by precipitate
Haemolysis	Positive reaction

METHOD OF CALCULATION

S. No	No of colonies	Formula	Explanation
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1	15-300 Colonies	$N = \frac{\sum c}{(n_1 + 0.1 n_2)d}$	N -Number of colonies per ml or g of product $\sum c$ is the sum of colonies counted on the two dishes retained from two successive dilutions, at least one of which contain a minimum of 10 colonies n1 - Number of dishes considered for the first dilution n2 - Number of dishes considered for the second dilution d is the dilution corresponding to the first dilution retained
2	Less Than 15 Colonies	$N_e = \frac{c}{2}$	Ne -Estimate the number c- sum of colonies of coagulase positive staphylococci counted on the two dishes retained from two successive dilutions
3	No colonies	-	Express as less than 1/d microorganism per milliliter (liquid products) or per gram (other products)

CAUTION

- Carry out the test under the control of skilled microbiologist and great care shall be taken in the disposal of all the incubated material.
- Follow safe and good laboratory practices to avoid cross contamination.

REFERENCE

- **IS 5887 (Part 6): 2012** Microbiology of food and animal feeding stuffs — horizontal method for the enumeration of presumptive bacillus cereus (part 6 colony-count technique at 30°C).
- **ISO 7218:2007** – Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations.

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(IS 5887-Part 7):1999 (RA 2022)

METHODS FOR ISOLATION AND IDENTIFICATION OF *SHIGELLA*

INTRODUCTION

Shigellosis is an infectious disease spread most commonly by person to person transmission. The genus consists of 4 species: *Shigella dysenteriae* (Subgroup A), *Shigella flexneri* (Subgroup B), *Shigella boydii* (Subgroup C) and *Shigella sonnei* (Subgroup D). These species are distinguishable by their biochemical and serological reactions

SCOPE

This standard prescribes the method for isolation and identification of *Shigella*.

EQUIPMENT

- Screw cap bottle
- Autoclave
- Weighing Balance
- pH Meter
- Coat, Mask, Gloves
- Laminar Air flow
- Biosafety cabinet
- Bunsen burner

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- Petri plate
- Inoculation Loop
- Micropipette & Tips
- Water bath
- Incubator
- Colony counting equipment
- Glass Slide & Glass Cavity Slide
- Microscope
- Refrigerator (at 2°C to 8°C)
- Durhams tube

MEDIUM

- Nutrient Broth
- Kauffmann-Muller's Tetrathionate Broth
- Selenite F Broth
- Deoxycholate citrate Agar (DCA) medium
- MacConkey agar
- Motility test medium
- Hugh leifson's medium
- Triple sugar iron agar
- Urea agar
- Simmon's citrate agar
- Tryptone water
- Malonate broth
- Gelatin liquification Medium
- Peptone water
- Decarboxylase Test Media with Lysine or Ornithine
- Carbohydrate Fermentation Media
- Motility Test Medium

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ANALYTICAL PROCEDURE

- All foods which have received dry heat treatment shall be pre enriched in a non-inhibitory medium such as Nutrient Broth.
- Raw food or food suspected to be grossly contaminated need no pre enrichment.
- Pre enrichment Using aseptic techniques mix or blend sample with Nutrient Broth.
- Pre-enrichment is not required, the sample is blended in a sterile blender jar for 2 minutes using approximately 200 ml selenite F broth per approximately 25 g of one portion of the sample, and 200 ml tetrathionate broth per approximately 25 g of another portion of the sample. Incubate at 37°C for 24 hours.
- Incubate the raw meat samples at 43 °C rather than at 37 °C.

ISOLATION

- From the incubated sample (incubated at 37°C) plate out loopfuls on to desoxycholate citrate agar medium, and incubate for 24 hours at 37°C.
- It is also advisable to plate out on to the solid medium loopfuls of each of the liquid medium after blending, but before incubating the plate at 37°C overnight as suspect *Shigella* colonies may be seen one day earlier.
- If there is no suspect growth on the solid medium after 24 hours, reincubate for a further 24 hours.
- *Shigella* colonies on desoxycholate citrate agar shall be opaque, with a ground-glass appearance and with even margins.

TEST FOR IDENTIFICATION

- Pick out suspect colonies (not less than 5) to investigate and plate out into MacConkey agar plates and incubate overnight at 37 °C.
- Pure colonies are transferred into NA plates /slants for further biochemical and serological testing.

BIOCHEMICAL CONFIRMATION

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Use pure cultures from the NA for biochemical and serological tests below.

1. Gram stain

- Take one loop full culture and spread evenly over the drop of normal saline on a clean glass slide to form a smooth smear.
- Heat fix the smear by gently passing the slide over the flame.
- Add crystal violet for 30 seconds.
- The stain was poured off and slide was gently washed under slightly running tap water.
- Add Gram's Iodine for 60 seconds. Iodine solution was decanted and slide was washed under slightly running tap water.
- Then slide was de-colourized with alcohol for 1 second and subsequently washed under slightly running tap water.
- Add Safranin as counter stain for 30 seconds, then wash with slightly running tap water and air dry. Examine stained slide under microscope at 100X.

2. Test for motility

- Stab the semi-solid nutrient agar with a colony using an inoculation needle. Inside the glass tubing to a depth of a 5mm.
- Incubate tube at 37°C for 18 to 24 hours.
- Motile strains shall be found to show growth on the surface of the medium outside the inner glass tube having travelled through the entire medium inside this inner tube.
- If negative on the first day, keep the inoculated tube at room temperature for a further 4 to 6 days to see if evidence of motility is present.

3. Test for catalase

- Grow the strain for 24 hours on nutrient agar slope and pour 1 ml hydrogen peroxide over the growth with the tube in a slanting position.
- Release of oxygen, shown as bubbles, from hydrogen peroxide indicates presence of catalase.

4. Test for Oxidase

- In freshly grown Nutrient Agar slant of culture, add few drops of freshly mixed test

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reagent,

- 1% solution of α -naphthol in 95% ethanol and equal amount of 1% solution of para-amino-dimethylaniline hydrochloride in water.
- A positive reaction is indicated by appearance of a blue colour within two minutes.

5. Hugh-leifson's Test

- The strain fresh nutrient agar growth is stabbed into two tubes of medium, one of which is then layered over with a small amount of sterile liquid paraffin. Incubate both tubes at 37°C and observe 4 days.
- Acid formation, yellow colour in the tube without paraffin indicates oxidative utilization of glucose.
- Acid in both tubes indicates fermentative reaction. Lack of acid in either tube indicates the strain as not being able to utilize glucose oxidatively or fermentatively.

6. Test for H₂S production

- Inoculate each suspect culture into TSI medium by stabbing the strain into the butt and streaking the slope.
- Incubate at 37°C and observe daily for up to 7 days.
- The presence or absence of blacking in the butt of the medium is to be recorded.

7. Test for Urease

- Inoculate the organisms from 24 hours incubated broth culture heavily over the entire slope surface of the medium as in Urease test medium and incubate at 37°C for 18 to 24 hours.
- If urea is hydrolysed, a rose-pink to deep cerise colour develops from the release of ammonia by the decomposition of the urea with a change in the colour of the pH indicator.
- If negative continue incubation for at least 4 days. *Proteus* species give a positive result and can be used as control.

8. Phenyl Pyruvic acid production

- Obtain an overnight heavy growth of the organisms on nutrient agar suspend growth in

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0.5ml of normal saline and transfer to a wide-bore test tube (diameter at least 1.5 cm).

- Add 0.5ml of 0.2 percent dl-phenylalanine in normal saline. Mix and keep lying horizontally for at least three hours at room temperature. Add few drops of half-saturated ferric chloride solution.
- A positive reaction is an immediate deep green colour which fades on keeping. *Proteus* strains give a positive reaction and serves as control.

9. Test for Citrate Utilization

- Inoculate the growth from nutrient agar slant on to medium using a straight wire.
- Incubate at 37°C for up to 4 days for growth of the organisms.

10. Test for Indole

- Inoculate a tube containing 5 ml of tryptone/tryptophan medium with the pure culture. Incubate at 37 °C for 48 h.
- After incubation, add 0.5ml kovac's reagent. After adding kovac's reagent shake the tube gently, the appearance of a red colour indicates the presence of indole and a yellow/brown colour indicates a negative reaction.

11. Tests for Fermentation carbohydrates

- Inoculate each of medium as in and the carbohydrates indicated in Table 1 using a 1 percent concentration for lactose, and incubate at 37°C for 18 to 48 hours.
- Record the presence of acid from pink colour and that gas in the Durham's tube.
- A positive reaction when carbohydrate is utilized gives a change in the pH indicator from purple to yellow.

12. Test for Dihydrolase and Decarboxylase Activities.

- Inoculate using straight wire, through the liquid paraffin each of the four tubes of the Dihydrolase and Decarboxylase medium from a freshly grown culture in nutrient agar.
- Incubate at 37°C and examine daily for up to 4 days only.
- The medium first becomes yellow due to acid production from the glucose; later if dihydrolation or decarboxylation of the respective amino acid occurs, the medium becomes violet in colour.

13. Test for utilization of Malonate

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- Inoculate the Malonate medium and incubate for 24 hours at 37°C.
- A positive Malonate reaction is indicated by a deep blue colour and a negative reaction by the unchanged greenish colour of the medium. With some negative strains. A yellow colour appears.

14. Gelatin liquifaction Test

- Inoculate the strain into Gelatin Liquifaction medium as a stab culture using a straight wire and incubate at 22°C for 4 days in an upright position.
- In warm temperatures, the medium is usually in a semi-solid state. Before inoculation and also for reading the result for liquifaction, the tubes should be kept in an ice bath or refrigerator to facilitate making a stab culture and also taking the reading.

SEROTYPING

Shigella strains are named on the basis of serotyping. Polyvalent *Shigella* sera, if available, will help in confirming strains as being shigellae. Strains conforming to the morphological characters and biochemical reactions as in Table 1 shall be sent to reference laboratories for confirmation and final identification. Polyvalent *Shigella* sera, if available, will help in confirming strains as being shigellae. Strains conforming to the morphological characters and biochemical reactions as in Table 1 shall be sent to reference laboratories for confirmation and final identification.



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Table 1 Useful characters of *Shigella* organisms

S.No.	Tests	Sub Gr. A	Sub Gr. B & C	Sub Gr. D
1	Gram-Negative rods	+	+	+
2.	Motility	-	-	-
3.	Catalase	+(a)	+	+
4.	Oxidase	-	-	-
5.	Hugh-leifson's Test	F	F	F
6.	TSI For H ₂ S	-	-	-
7.	Urease	-	-	-
8	Phenyl pyruvic acid	-	-	-
9.	Citrate	-	-	-
10	Indole	Varies(b)	Varies(b)	varies
11	Glucose	+	+	+
12	Lactose(1 percent)	-	-	(+)
13	Sucrose	-	-	(+)
14	Salicin	-	-	-
15	Dulcitol	-(c)	-(c)	-
16	Mannitol	-	+	+
17	Lysine	-	-	-
18	Arginine	- or +	- or +	- or +
19	Ornithine	-	-(d)	+
20	Malonate	-	-	-
21	Gelatin	-	-	-

NOTE-Some important exceptions to the above characters are as follow:

(a) *Shigella dysenteriae* 1(*Shigella*) is catalase – Positive; (b) *Shigella dysenteriae* 1 (*Sh.shiga*), *Sh.flexneri* 6 are always indole - Negative &

Sh. Dysenteriae 2 is always indole-positive; (c) Some strains of *Shigella* sub group A, *Sh.flexneri* 6 and subgroup C are dulcitol-Positive; (d) *Sh.boydii* 13 is ornithine-decarboxylase-positive.

+ = Present or positive

(+) = Late positive

- = Negative or no reaction

F = Fermentative Reaction

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EXPRESSION OF RESULTS

Shigella spp = Present or absent/ gm or ml.

DISPOSAL PROCEDURE

- Put on the appropriate size protective gloves before handling any contaminated material.
- Place the bag into the autoclave, temperature should be at least 121 °C for 30 minutes at 15 lbs. Set the time for at least 30 minutes and run the sterilizer.
- Take out the bag and place in another larger sealed for disposal

CAUTION

- In order to safeguard the health of laboratory personnel, it is essential that tests for detecting *Shigella spp.* are only undertaken in properly equipped laboratories, under the control of a skilled microbiologist, and that great care is taken in the disposal of all incubated materials.

REFERENCE

- IS 5887 (Part-7):1999 - Microbiology of food and animal feeding stuffs — Horizontal method for the detection of *Shigella spp.*
- ISO 7218:2007 – Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations.

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IS 5887- Part 8 / Sec 2:2002(RA 2022)

ENUMERATION OF COAGULASE POSITIVE STAPHYLOCOCCI

(IS 5887- Part 8 (Sec 2))

INTRODUCTION

Staphylococci are aerobic Gram-positive bacteria, with diameters of 0.5 – 1.5 µm and characterized by individual cocci, in grape-like clusters. They are non-motile, non-spore forming facultative anaerobes. Some of species of *Staphylococcus* genera have ability to produce coagulase enzyme i.e. *Staphylococcus aureus*, *Staphylococcus hyicus*, *Staphylococcus intermedius* etc. Coagulase positive *Staphylococci* produce Enterotoxins, which is responsible for food-poisoning if contaminated food is consumed.

SCOPE

Horizontal method for the enumeration of coagulase-positive staphylococci in products intended for human consumption or feeding of animals by counting of colonies obtained on a solid medium (rabbit plasma fibrinogen medium) after aerobic incubation at 35 °C or 37 °C.

EQUIPMENT

- Laminar Air flow
- Biosafety cabinet
- Autoclave
- Tubes and glass bottles
- Weighing Balance
- pH Meter
- Bunsen Burner
- Petri dish
- Graduated pipettes
- Micropipette with tips

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- Incubator
- Bunsen burner
- Microscope
- Colony counting equipment
- Water Bath
- Refrigerator
- Cyclomixer

MEDIUM

- 0.1% peptone salt solution
- Rabbit Plasma fibrinogen agar

SAMPLE PREPARATION

Homogenous sample of processed food to be used.

ANALYTICAL PROCEDURE

- Prepare 225 ml of 0.1% peptone salt solution dilution fluid.
- Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes (Check the pH before sterilize, if necessary adjust pH using 0.1N Hcl / 0.1N NaOH)
- Take 25 gm sample in 225ml dilution fluid mix well minimum 20 times.
- Take two sterile Petri dish using a sterile pipette transfer the each dish 1 ml of test sample.
- Using a sterile pipette transfer to each dish 1 ml of the initial suspension (10^{-1}) to decimal dilution (10^{-2}) 9 ml of dilution fluid cyclometer mix well after take 1 ml transfer the two sterile Petri plate.
- If necessary, repeat the procedure with the further dilutions, using a new sterile pipette for each decimal dilution.
- Immediately pour freshly prepared about 12 ml to 15 ml of Rabbit Plasma fibrinogen agar to a depth of approximately 3mm.
- Carefully mix the inoculums with the medium by rotating the petri dishes and allow the

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mixture to solidify by leaving the Petri dishes standing on a cool horizontal surface.

- Also prepare a control plate with 15 ml of the medium for checking its sterility.
- After complete solidification, invert the prepared dishes and place them in the incubator at 35 °C or 37 °C for 18h to 24 hrs. If necessary re-incubate for 18h to 24 hrs.

COUNTING OF COLONIES

After the specified incubation period, the staphylococci form black or grey or even white, small colonies surrounded by a halo of precipitation, indicating coagulase activity.

METHOD OF CALCULATION

S. No	No of colonies	Formula	Explanation
1	15-300 Colonies	$N = \frac{\sum c}{(n_1 + 0.1 n_2)d}$	N - Number of colonies per ml or g of product $\sum c$ is the sum of colonies counted on the two dishes retained from two successive dilutions, at least one of which contain a minimum of 10 colonies n1 - Number of dishes considered for the first dilution n2 - Number of dishes considered for the second dilution d is the dilution corresponding to the first dilution retained
2	Less Than 15 Colonies	$Ne = \frac{c}{2}$	Ne - Estimate the number c- sum of colonies of coagulase positive staphylococci counted on the two dishes retained from two successive dilutions
3	No colonies	-	Express as less than 1/d microorganism per milliliter (liquid products) or per gram (other products)

CAUTION

- Carry out the test under the control of skilled microbiologist and great care shall be taken in the disposal of all the incubated material.
- Follow safe and good laboratory practices to avoid cross contamination.

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REFERENCE

- **IS 5887-8-2 (2002)** Methods for Detection of Bacteria Responsible for Food Poisoning, Part 8: Horizontal Method for Enumeration of Coagulase-Positive Staphylococci (Staphylococcus aureus and other species), Section 2 Technique using rabbit plasma fibrinogen agar medium.
- **ISO 7218:2007** – Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations.

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IS: 14988-PART 1:2020

METHOD FOR DETECTION AND ENUMERATION OF *LISTERIA MONOCYTOGENES* AND OF *LISTERIA SPP*

INTRODUCTION

Listeria monocytogenes is an intracellular, foodborne pathogen potentially lethal for humans and animals.

SCOPE

Horizontal method for the detection of *Listeria*. The detection of *Listeria* requires 4 successive stages viz. pre-enrichment, enrichment, differential selective and confirmation.

Equipment

- Screw cap bottle
- Autoclave
- Weighing Balance
- pH Meter
- Laminar Air flow
- Biosafety cabinet
- Bunsen burner
- Petri plate
- Inoculation Loop
- Graduated pipettes & Tips
- Water bath
- Incubator
- Colony counting equipment
- Glass Slide & Glass Cavity Slide
- Microscope
- Refrigerator
- Durhams tube

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MEDIA

- Half Frazer broth
- Frazer broth
- Ottaviani & Agosti
- PALCAM Agar
- Tryptone Soya Yeast Extract Agar
- Tryptone Soya Yeast Extract Broth
- Sheep Blood Agar
- Carbohydrate utilization broth (Rhamnose and Xylose)
- Motility Agar
- CAMP Medium and test organism
- Hydrogen peroxide solution

ANALYTICAL PROCEDURE

- Aseptically open the sample container and weigh 25 gm sample into a sterile mixture bag/ empty wide mouth container with screw cap or suitable closure or take 25 ml of liquid sample.
- Add 225 ml of sterile half Fraser broth (pre warm at room temperature). Make a uniform suspension by blending if necessary and incubate at 30 °C ± for 25 ± 1 h.

SECONDARY ENRICHMENT

- Inoculate 0.1 ml of the Half Fraser Broth into 10 ml tubes of Fraser Broth.
- Incubate the tubes at 37 °C for 24 h ± 2 h for *Listeria monocytogenes*.

SELECTIVE DIFFERENTIAL PLATING

- Culture obtained in the primary enrichment (Half Fraser Broth after 25 ± 1 h at 30 °C) inoculate (streaking) the surface of the selective plating medium, Agar Listeria according to Ottaviani and Agosti & any second selective plating-out of PALCAM Agar .
- Culture obtained in the secondary enrichment (Fraser Broth after 24h ± 2 h at 37 °C) inoculate (streaking) the surface of the selective PALCAM agar plates.

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- Incubate the plates at 37 °C and observe for typical colonies after 24 h. If no suspect colonies are evident or if the growth is poor after 24 h, re-incubate the plates for an additional 24 h and read again

APPEARANCE OF COLONIES

After the incubation period, examine the plates for typical *Listeria monocytogenes* colonies.

- **Listeria Ottaviani and Agosti** -blue-green colonies surrounded by an opaque halo (typical colonies). Consider as presumptive *Listeria* spp all blue green colonies with and without halo.
- **PALCAM agar** - Greyish green or olive green sometimes with black centre and always surrounded by a black halo and depressed centre.

CONFIRMATION OF LISTERIA SPECIES

- Select at least one presumptive typical colony from one plate of each medium. Purify each culture by streaking 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 37 °C for 18 to 24 h or until the growth is satisfactory.
- Typical colonies are 1 mm to 2 mm in diameter, convex, colourless and opaque with an entire edge. Carry out the following tests from colonies of a pure culture on TSYEA.

BIOCHEMICAL IDENTIFICATION

1. CATALASE REACTION (OPTIONAL)

With the help of loop pick up an isolated colony and place it in H₂O₂ solution on a glass slide. Immediate production of gas bubbles indicates catalase positive reaction

2. GRAM STAINING

Perform Gram staining on a colony, *Listeria* are Gram positive slim short rods OR coccus bacilli.

3. MOTILITY TEST (OPTIONAL)

- Take colony from TSYEA plate and suspend it TSYE broth.
- Incubate at 25°C for 8 to 24 hr until cloudy medium is observed.

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- 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 h typical umbrella like appearance around the stab indicate motility positive culture.
- If growth is not positive incubate up to five days and observe for the stab again.

CONFIRMATION OF LISTERIA MONOCYTOGENES

4. HAEMOLYSIS TEST

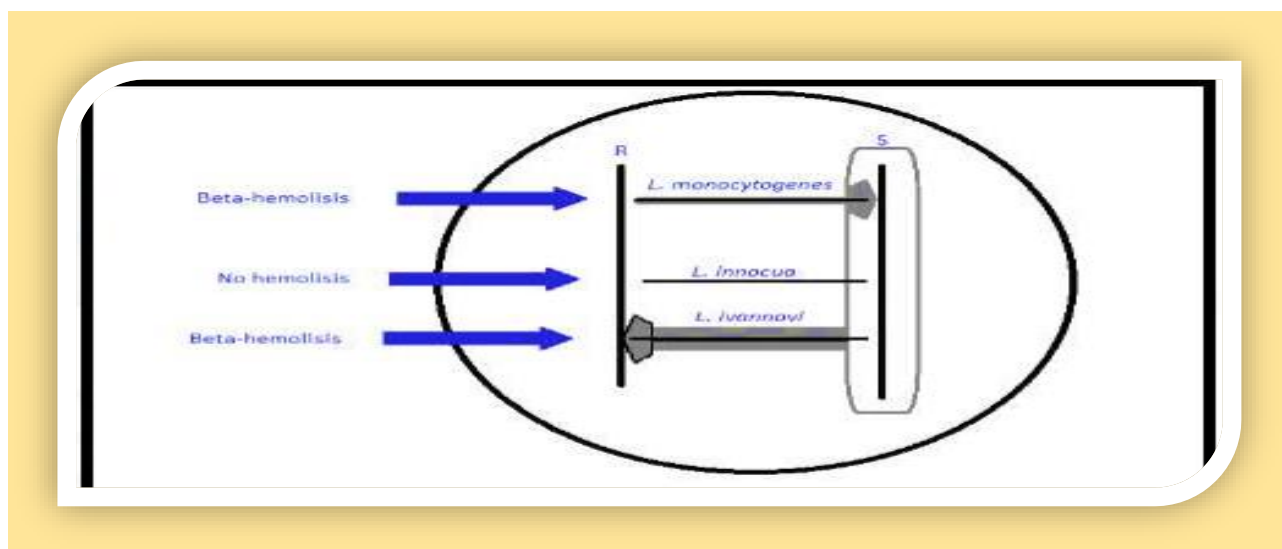
- Take a colony from TSYEA and stab it on a well dried surface of sheep blood agar plate. Simultaneously stab positive (*L. monocytogenes*) and negative (*L. innocua*) control cultures. Invert the plates and incubate at 37 °C for 24 ± 2 h.
- *L. monocytogenes* show clear light zones of beta haemolysis. *L. Innocua* does not show any haemolysis. Examine the plates in a bright light to compare test cultures with the controls.

5. CAMP TEST - Christie–Atkins–Munch-Peterson (OPTIONAL)

- CAMP test is recommended to demonstrate clearly that haemolysis is due to listeriolysin activity.
- On a well dried surface of 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 inoculum 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.

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- 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 intersection of *S. aureus*. *L. innocua* does not show any enhanced zone of haemolysis with *S. aureus* or *R. equi*. In case of *L. ivanovii* enhanced beta zone of haemolysis is seen on *R. equi* side
- Inoculate thin agar plates as in diagram. Vertical lines represent streaks of *S. aureus* (S) and *R. equi* (R). Horizontal lines represent streaks of test cultures. Hatched area indicates the location of enhanced haemolysis. The dotted area indicates the zone of influence of the *S. aureus* culture.



6. CARBOHYDRATE UTILIZATION:

- Inoculate each of the carbohydrate utilization broths (Rhamnose and Xylose) with a culture from TSYEA and incubate at 37°C for up to 5 days. Appearance of yellow colour indicates a positive reaction within 24 to 48 hr.

7. TEST FOR VOGES-PROSKAUER REACTION

- Inoculate a tube containing 3ml of the VP Medium and incubate at 37 °c for 24h ± 2 hrs.

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- Add 1 ml of Barritt Reagent A & Barritt Reagent B. Shake and slope the tube and observe for up to 4 hours for the appearance of a pink colour which indicated a positive reaction.

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.

DISPOSAL PROCEDURE

- Put on the appropriate size protective gloves before handling any contaminated material.
- Place the bag into the autoclave, temperature should be at least 121 °C for 30 minutes at 15 lbs. Set the time for at least 30 minutes and run the sterilizer.
- Take out the bag and place in another larger sealed for disposal.

CAUTION

- *L. monocytogenes* 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.

REFERENCES

IS 14988 (Pat 1:2020) Microbiology of the food chain — Horizontal method for the detection and enumeration of *Listeria monocytogenes* and of *Listeria* spp. — Part 1: Detection method.

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METHOD FOR DETECTION AND ENUMERATION OF *LISTERIA MONOCYTOGENES* AND OF *LISTERIA SPP*

INTRODUCTION

Listeria monocytogenes is an intracellular, foodborne pathogen potentially lethal for humans and animals.

SCOPE

Microorganisms which form typical colonies on solid selective media and which display the morphological, physiological and biochemical characteristics carried out in the enumeration methods.

EQUIPMENT

- Screw cap bottle
- Autoclave
- Weighing Balance
- pH Meter
- Laminar Air flow
- Biosafety cabinet
- Petri plate
- Inoculation Loop
- Graduated pipettes & Tips
- Water bath
- Incubator
- Colony counting equipment
- Glass Slide & Glass Cavity Slide

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- Microscope
- Refrigerator
- Durhams tube
- L- rod
- Spreader

MEDIA

- Half Frazer broth
- Frazer broth
- Ottaviani & Agosti
- PALCAM Agar
- Tryptone Soya Yeast Extract Agar
- Tryptone Soya Yeast Extract Broth
- Sheep Blood Agar
- Carbohydrate utilization broth (Rhamnose and Xylose)
- Motility Agar
- CAMP Medium and test organism
- Hydrogen peroxide solution

ANALYTICAL PROCEDURE

- Prepare 225 ml of half Fraser broth dilution fluid.
- Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes (Check the pH before sterilize, if necessary adjust pH using 0.1N Hcl / 0.1N NaOH)
- Take 25 gm sample in 225ml dilution fluid mix well minimum 20 times.
- Take two sterile Petri dish using a sterile pipette transfer the each dish 0.1 ml of test sample.
- If certain products, estimate low number of L.monocytogenes the limit may be raised by a factor of 10 by examining 1ml of test sample , Distribute the 1ml inoculum on the surface of large petridish 140mm
- Using a sterile pipette transfer to each dish 1 ml of the initial suspension (10^{-1}) to

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decimal dilution (10^{-2}) 9 ml of dilution fluid cyclometer mix well after take 0.1 ml transfer the two sterile Petri plate.

- If necessary, repeat the procedure with the further dilutions, using a new sterile pipette for each decimal dilution.
- Immediately spread the inoculum as quickly as possible over the surface of the agar plate without touching the sides of the dish with the spreader, also prepare a control plate with 15 ml of the medium for checking its sterility.
- Leave the plates with the lids on for about 15 min at ambient temperature for the inoculum to be absorbed into the agar
- Invert the prepared dishes and place them in the incubator at 37 °C for 24 ± 2 h. If necessary re-incubate for 24 ± 2 hrs.

COUNTING OF COLONIES

After Incubation for 24 ± 2 hrs incubation period (colonies development of colonies with large and overlapping opaque halos, which may make reading difficult). Additional 24 ± 2 h examine the petridishes for presence of presumptive colonies of *L.monocytogenes*

CONFIRMATION OF LISTERIA SPP.

- Select at least one presumptive typical colony from one plate of each medium. Purify each culture by streaking 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 37 °C for 18 to 24 h or until the growth is satisfactory.
- Typical colonies are 1 mm to 2 mm in diameter, convex, colourless and opaque with an entire edge. Carry out the following tests from colonies of a pure culture on TSYEA.

BIOCHEMICAL IDENTIFICATION

1. CATALASE REACTION (OPTIONAL)

With the help of loop pick up an isolated colony and place it in H₂O₂ solution on a glass slide. Immediate production of gas bubbles indicates catalase positive reaction

2. GRAM STAINING

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

3. MOTILITY TEST (OPTIONAL)

- 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 h typical umbrella like appearance around the stab indicate motility positive culture.
- If growth is not positive incubate up to five days and observe for the stab again.

CONFIRMATION OF LISTERIA MONOCYTOGENES

4. HAEMOLYSIS TEST

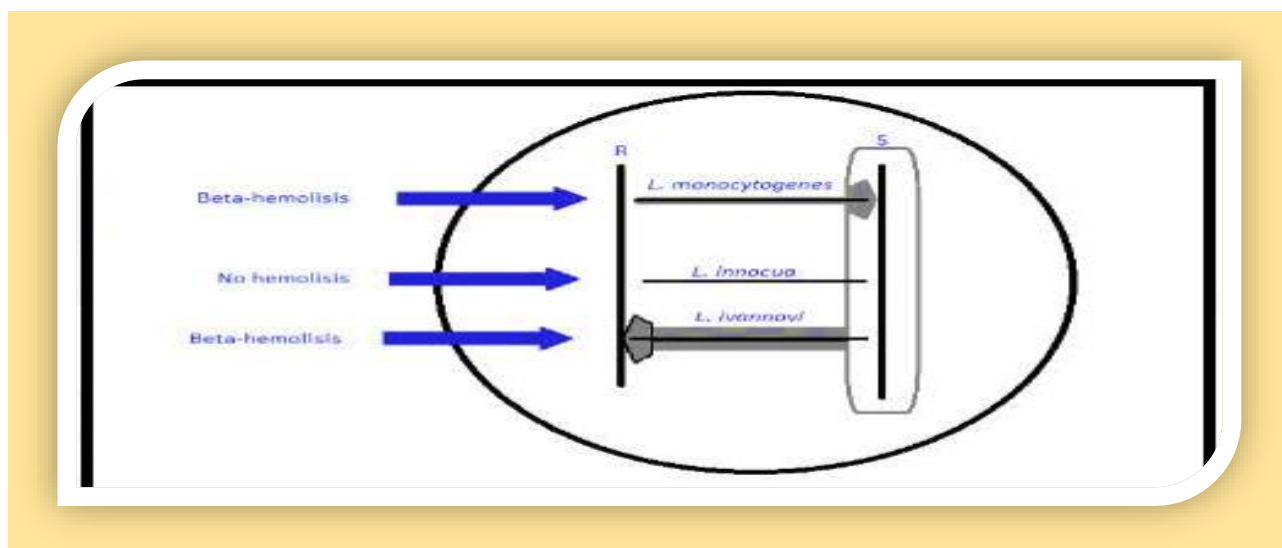
- Take a colony from TSYEA and stab it on a well dried surface of sheep blood agar plate. Simultaneously stab positive (*L. monocytogenes*) and negative (*L. innocua*) control cultures. Invert the plates and incubate at 37 °C for 24 ± 2 h.
- *L. monocytogenes* show clear light zones of beta haemolysis. *L. innocua* does not show any haemolysis. Examine the plates in a bright light to compare test cultures with the controls.

5. CAMP TEST - Christie–Atkins–Munch–Peterson (OPTIONAL)

- CAMP test is recommended to demonstrate clearly that haemolysis is due to listeriolysin activity.
- On a well dried surface of 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 inoculum is required.

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- 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 intersection of *S. aureus*. *L. innocua* does not show any enhanced zone of haemolysis with *S. aureus* or *R. equi*. In case of *L. ivanovii* enhanced beta zone of haemolysis is seen on *R. equi* side
- Inoculate thin agar plates as in diagram. Vertical lines represent streaks of *S. aureus* (S) and *R. equi* (R). Horizontal lines represent streaks of test cultures. Hatched area indicates the location of enhanced haemolysis. The dotted area indicates the zone of influence of the *S. aureus* culture.



6. CARBOHYDRATE UTILIZATION:

- Inoculate each of the carbohydrate utilization broths (Rhamnose and Xylose) with a culture from TSYEA and incubate at 37°C for up to 5 days. Appearance of yellow colour indicates a positive reaction within 24 to 48 hr.

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EXPRESSION OF RESULTS

METHOD OF CALCULATION

S. No	No of colonies	Formula	Explanation
1	10-150 Colonies	$N = \frac{\sum c}{v \times 1,1 \times d}$	$\sum c$ is the sum of colonies counted on the two dishes retained from two successive dilutions, at least one of which contain a minimum of 10 colonies v is the volume of inoculum placed in each dish, in milliliter's; d is the dilution corresponding to the first dilution retained
	Atypical colonies	$a = b / A * C$	b is the number of colonies complying with identification criteria among the identified colonies A. c is the total number presumptive colonies counted on the dish .
2	Less Than 10 Colonies	$\bar{c} = \bar{c} / v \times d$	$\bar{c}$ = Estimate the number Express as "Microorganisms are present but less than (Vx d) per gram or ml" C is the average of colonies on the petri dishes retained v is the volume of inoculums used in each dish d is the dilution corresponding to the dilution retained
3	No colonies	-	Express as less than 1/d microorganism per milliliter (liquid products) or per gram (other products)

DISPOSAL PROCEDURE

- Put on the appropriate size protective gloves before handling any contaminated material.
- Place the bag into the autoclave, temperature should be at least 121 °C for 30 minutes

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at 15 lbs. Set the time for at least 30 minutes and run the sterilizer.

- Take out the bag and place in another larger sealed for disposal.

CAUTION

- *L. monocytogenes* 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.

REFERENCES

IS 14988 (Pat 2:2020) Microbiology of the food chain — Horizontal method for the detection and enumeration of *Listeria monocytogenes* and of *Listeria* spp. — Part 1: Detection method.

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IS 1622:1981

METHOD FOR STANDARD PLATE COUNT

INTRODUCTION

The Standard plate Count is a direct measurement of the viable aerobic and facultative anaerobic in a water environment capable of growth on the selected plating medium.

EQUIPMENT

- Laminar Air flow
- Biosafety cabinet
- Autoclave
- Tubes and glass bottles
- Weighing Balance
- pH Meter
- Bunsen Burner
- Petri dishes
- Graduated pipettes
- Micropipette with tips
- Incubator
- Colony counting equipment
- Water Bath
- Refrigerator
- Cyclomixer

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MEDIUM

- Nutrient Agar
- Buffered dilution water
- Agar medium

SAMPLE PREPARATION

- Collect water samples aseptically in clean sterilized glass bottle of 250/500 mL capacity.
- If the water to be sampled contains or is likely to contain chlorine, sodium thiosulphate shall be added to the clean, dry sampling bottles before sterilization for neutralization. This can be done by adding 0.5 mL of 5 percent thiosulphate solution to a 250 mL bottle. Sterilize in an autoclave

ANALYTICAL PROCEDURE

- Prepare Buffered dilution water
- Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes (Check the pH before sterilize, if necessary adjust pH using 0.1N Hcl / 0.1N NaOH)
- Take Sterile Petri dish using a sterile pipette transfer the each dish 1 ml of test sample from the bottle.
- Using a sterile pipette transfer to each dish 1 ml of the initial suspension (10^0) to decimal dilution (10^{-1}) 9 ml of dilution fluid cyclometer mix well after take 1 ml transfer the two sterile Petri plate.
- If necessary, repeat the procedure with the further dilutions (10^{-3} , 10^{-4} , etc.), using a new sterile pipette for each decimal dilution.
- Pour about 10 ml to 15 ml of melted nutrient agar medium at 43°C to 45°C into Petri dish. The time elapsing between the end of the preparation of the initial suspension and the moment when the medium is poured into the dishes shall not exceed 45 minutes.
- Carefully mix the inoculums with the medium by rotating the petri dishes and allow the mixture to solidify by leaving the Petri dishes standing on a cool horizontal surface, also

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prepare a control plate with 15 ml of the medium for checking its sterility.

- Invert the prepared dishes and place them in the incubator at 37°C ± 1°C for 48 hours.
The incubation may be carried out for 72 hours where necessary.

COUNTING OF COLONIES

- After 48 hours of incubation select the agar plates with, if possible, 30 to 300 colonies. Count using the colony counting equipment, it is important that pinpoint colonies should be included in the count. If more than one quarter is overgrown by spreading colonies discard the plate.
- Report the result as the average of all plates falling within limits. Record the results only to two significant figures.

METHOD OF CALCULATION

S. No	No of colonies	Formula	Explanation
1	30-300 Colonies	$N = \frac{\sum c}{v \times 1,1 \times d}$	$\sum c$ is the sum of colonies counted on the two dishes retained from two successive dilutions, at least one of which contain a minimum of 10 colonies v is the volume of inoculum placed in each dish, in milliliter's; d is the dilution corresponding to the first dilution retained
2	No colonies	-	Express as Less than 1 cfu/ mL

CAUTION

- Carry out the test under the control of skilled microbiologist and great care shall be taken in the disposal of all the incubated material.
- Follow safe and good laboratory practices to avoid cross contamination.

REFERENCE

- 1) **IS 1622-1981 (RA 2019)** -Method of sampling and microbiological examination of water
- 2) **ISO 7218:2007** – Microbiology of food and animal feeding stuffs – General

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requirements and guidance for microbiological examinations.

- 3) ISO 6887-1 (2017) - Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions**

IS 1622:1981

METHOD FOR DETECTION OF ESCHERICHIA COLI

INTRODUCTION

E. coli is a member of the family *Enterobacteriaceae*, Most strains of *E. coli* are harmless and can even be beneficial to the digestive system, but some strains can cause illness if consumed through contaminated food or water.

EQUIPMENT

- Laminar Air flow
- Biosafety cabinet
- Autoclave
- Tubes and glass bottles
- Weighing Balance
- pH Meter
- Bunsen Burner
- Petri dishes
- Graduated pipettes
- Micropipette with tips
- Incubator
- Colony counting equipment
- Water Bath
- Refrigerator
- Cyclomixer

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MEDIUM

- Macconkey Broth
- Brilliant Green Bile Broth
- Tryptone water
-

SAMPLE PREPARATION

- Collect water samples aseptically in clean sterilized glass bottle of 250/500 mL capacity. (3)
- If the water to be sampled contains or is likely to contain chlorine, sodium thiosulphate shall be added to the clean, dry sampling bottles before sterilization for neutralization. This can be done by adding 0.5 mL of 5 percent thiosulphate solution to a 250 mL bottle. Sterilize in an autoclave

ANALYTICAL PROCEDURE

DETECTION OF COLIFORMS

- 1 ml of the test sample if liquid, or 1 ml of the initial suspension in the case of other products, is transferred to a tube containing 10 ml of double-strength Lauryl sulfate tryptose broth.
- 1 ml < x < 10 ml, or to a tube containing 10 ml of single-strength selective enrichment medium when x ≥ 1 ml.
- Leave the tube of double-strength medium in the incubator set at 44.5 °C (as agreed) for 24 h ± 2 h.
- Leave the tube of single-strength medium in the incubator at 30 °C or 37 °C (as agreed) for 24 h ± 2 h or, if neither gas formation nor opacity preventing the detection of gas formation is observed at this stage, continue incubation for another 24 h ± 2 h.

CONFIRMATION

- Incubated tube of double-strength Lauryl sulfate tryptose broth Inoculate with a loop a tube of Brilliant green lactose bile broth.

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- Incubate in the incubator set at 44.5 °C for 24 h ± 2 h or, if gas formation is not observed at this stage, for 48 h ± 2 h.
- Carry out the same procedure for the incubated tubes from single-strength medium showing gas formation, or opacity preventing the detection of gas formation, when either of these features is first observed (i.e. after 24 h ± 2 h or after 48 h ± 2 h).

Test for Indole

- Inoculate Tryptone water medium with a loop full of 24 hours growth nutrient broth and incubate at 37°C for 48 hours.
- Add 0.5 ml of kovac's reagent. After adding kovac's reagent, Shake the tube gently, the appearance of a red colour indicates the presence of indole.

INTERPRETATION

- A tube from double-strength Lauryl sulfate tryptose broth..or Single -strength Lauryl sulfate tryptose broth..in which gas formation is observed after 24 h ± 2 h or 48 h ± 2 h is considered as a positive tube.
- Presence/Absence of *E.coli* given in per unit of the sample examined.
 Test for *E.coli* = Present/absent per X mL of sample

CAUTION

- Carry out the test under the control of skilled microbiologist and great care shall be taken in the disposal of all the incubated material.
- Follow safe and good laboratory practices to avoid cross contamination.

REFERENCE

1. **IS 1622-1981 (RA 2019)** -Method of sampling and microbiological examination of water
2. **ISO 7218:2007** – Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations.
3. **ISO 6887-1 (2017)** - Microbiology of the food chain — Preparation of test samples,

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initial suspension and decimal dilutions for microbiological examination — Part 1:
General rules for the preparation of the initial suspension and decimal dilutions.

IS 1622:1981

METHOD FOR DETECTION OF TOTAL COLIFORM

INTRODUCTION

Coliforms are a broad group of aerobic or facultative anaerobic, rod shaped, gram-negative, non-spore forming bacteria which can ferment lactose with the production of acid and gas when incubated at 30°C –37°C, Coliform are members of Enterobacteriaceae family. Most common genera are *E. coli*, *Salmonella*, *Citrobacter*, *Enterobacter*, and *Klebsiella*.

EQUIPMENT

- Laminar Air flow
- Biosafety cabinet
- Autoclave
- Tubes and glass bottles
- Weighing Balance
- pH Meter
- Petri dishes
- Graduated pipettes
- Micropipette with tips
- Incubator
- Colony counting equipment
- Water Bath

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- Refrigerator
- Cyclomixer

MEDIUM

- Macconkey Broth
- Brilliant Green Bile Broth

SAMPLE PREPARATION

- Collect water samples aseptically in clean sterilized glass bottle of 250/500 mL capacity. (3)
- If the water to be sampled contains or is likely to contain chlorine, sodium thiosulphate shall be added to the clean, dry sampling bottles before sterilization for neutralization. This can be done by adding 0.5 mL of 5 percent thiosulphate solution to a 250 mL bottle. Sterilize in an autoclave

ANALYTICAL PROCEDURE

DETECTION OF COLIFORMS

- 1 ml of the test sample if liquid, or 1 ml of the initial suspension in the case of other products, is transferred to a tube containing 10 ml of Macconkey broth.
- 1 ml < x < 10 ml, or to a tube containing 10 ml of single-strength selective enrichment medium when x ≥ 1 ml.
- Leave the tube of double-strength medium in the incubator set at 44.5 °C (as agreed) for 24 h ± 2 h.
- Leave the tube of single-strength medium in the incubator at 30 °C or 37 °C (as agreed) for 24 h ± 2 h or, if neither gas formation nor opacity preventing the detection of gas formation is observed at this stage, continue incubation for another 24 h ± 2 h.

CONFIRMATION

- Incubated tube of Macconkey broth Inoculate with a loop a tube of Brilliant green lactose bile broth.

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- Incubate in the incubator set at 44.5 °C for 24 h $\pm$ 2 h or, if gas formation is not observed at this stage, for 48 h $\pm$ 2 h.
- Carry out the same procedure for the incubated tubes from single-strength medium showing gas formation, or opacity preventing the detection of gas formation, when either of these features is first observed (i.e. after 24 h $\pm$ 2 h or after 48 h $\pm$ 2 h).

INTERPRETATION

- A tube from Macconkey broth. In which gas formation is observed after 24 h $\pm$ 2 h or 48 h $\pm$ 2 h is considered as a positive tube.
- Presence/Absence of *Coliform* given in per unit of the sample examined.
 Test for *Coliform* = Present/absent per X mL of sample

CAUTION

- Carry out the test under the control of skilled microbiologist and great care shall be taken in the disposal of all the incubated material.
- Follow safe and good laboratory practices to avoid cross contamination.

REFERENCE

1. **IS 1622-1981 (RA 2019)** -Method of sampling and microbiological examination of water
2. **ISO 7218:2007** – Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations.

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METHOD FOR ENUMERATION OF AEROBIC PLATE COUNT
CHILLED, FROZEN, PRECOOKED OR PREPARED FOODS, AND NUT MEATS
(AOAC METHOD)

INTRODUCTION

The Total Aerobic Mesophilic Plate Count, usually called Aerobic Plate Count (APC) or Standard Plate Count, is the most commonly used general indicator of bacterial populations in foods.

SCOPE

The horizontal method for the enumeration of microorganisms that are able to grow and form colonies in a solid medium after aerobic incubation at 30°C.

EQUIPMENT

- Laminar Air flow
- Biosafety cabinet
- Autoclave
- Tubes and glass bottles
- Weighing Balance
- pH Meter
- Bunsen Burner
- Petri dishes

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- Graduated pipettes
- Micropipette with tips
- Incubator
- Colony counting equipment
- Water Bath
- Refrigerator
- Cyclomixer
-

MEDIUM

- Phosphate buffer diluent
- Plate count agar
- Agar medium

SAMPLE PREPARATION

Homogenous sample of processed food to be used.

ANALYTICAL PROCEDURE

- Prepare 225 ml of 0.1% Phosphate buffer diluent fluid.
- Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes (Check the pH before sterilize, if necessary adjust pH using 0.1N Hcl / 0.1N NaOH)
- Take 25 gm sample in 225ml dilution fluid mix well minimum 25 times.
- Take two sterile Petri dish using a sterile pipette transfer the each dish 1 ml of test sample.
- Using a sterile pipette transfer to each dish 1 ml of the initial suspension (10^{-1}) to decimal dilution (10^{-2}) 9 ml of dilution fluid cyclometer mix well after take 1 ml transfer the two sterile Petri plate.
- If necessary, repeat the procedure with the further dilutions (10^{-3} , 10^{-4} , etc.), using a new sterile pipette for each decimal dilution.
- Pour about 12 ml to 15 ml of Plate count agar at 44°C to 47°C into each Petri dish. The time elapsing between the end of the preparation of the initial suspension and the

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moment when the medium is poured into the dishes shall not exceed 45 minutes.

- Carefully mix the inoculums with the medium by rotating the petri dishes and allow the mixture to solidify by leaving the Petri dishes standing on a cool horizontal surface, also prepare a control plate with 15 ml of the medium for checking its sterility.
- Invert the prepared dishes and place them in the incubator at $35^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 48 hours $\pm$ 2 hours.

COUNTING OF COLONIES

- After 48 ± 2 hours of incubation select the agar plates with, if possible, 30 or more colonies and fewer than 300 colonies. Count using the colony counting equipment, it is important that pinpoint colonies should be included in the count.
- Spreading colonies shall be considered as single colonies. If less than one –quarter of the dish is overgrown by spreading, count the colonies on the unaffected part of the dish and calculate the corresponding number of the entire dish. If more than one – quarter is overgrown by spreading colonies, discard the count.

METHOD OF CALCULATION

S. No	No of colonies	Formula	Explanation
1	30-300 Colonies	$N = \frac{\sum c}{v \times 1,1 \times d}$	$\sum c$ is the sum of colonies counted on the two dishes retained from two successive dilutions, at least one of which contain a minimum of 10 colonies v is the volume of inoculum placed in each dish, in milliliter's; d is the dilution corresponding to the first dilution retained
2	No colonies	-	Express as less than $1/d$ microorganism per milliliter (liquid products) or per gram (other products)

CAUTION

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- Carry out the test under the control of skilled microbiologist and great care shall be taken in the disposal of all the incubated material.
- Follow safe and good laboratory practices to avoid cross contamination.

REFERENCE

- Official Methods of Analysis of **AOAC INTERNATIONAL 22nd** Edition, Volume 2 – 2023.

SURFACE PLATING METHOD FOR ISOLATION AND ENUMERATION OF **STAPHYLOCOCCUS AUREUS** **(AOAC METHOD)**

INTRODUCTION

Staphylococci are aerobic Gram-positive bacteria, with diameters of 0.5 – 1.5 µm and characterized by individual cocci in grape-like clusters. They are non-motile, non-spore forming facultative anaerobes, Coagulase positive *Staphylococci* produce Enterotoxins, which is responsible for food-poisoning if contaminated food is consumed. *S. aureus* is the species with the more pathogenic of the genus *Staphylococcus*.

SCOPE

This method is applicable for isolation and enumeration of *staphylococcus aureus* in foods.

EQUIPMENT

- Laminar Air flow
- Biosafety cabinet
- Autoclave
- Tubes and glass bottles
- Weighing Balance
- pH Meter

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- Bunsen Burner
- Petri dish
- Graduated pipettes
- Micropipette with tips
- Incubator
- Bunsen burner
- Microscope
- Colony counting equipment
- Water Bath
- Refrigerator
- L-Rod

MEDIUM

- Phosphate Buffer Solution
- Baird parker medium
- Coagulase (Rabbit) Plasma

SAMPLE PREPARATION

Homogenous sample of processed food to be used.

ANALYTICAL PROCEDURE

- Prepare 450 ml of Phosphate Buffered dilution fluid.
- Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes (Check the pH before sterilize, if necessary adjust pH using 0.1N Hcl / 0.1N NaOH)
- Take 50 gm sample in 450 ml dilution fluid mix well minimum 20 times.
- Prepare the selective Media Baird parker Agar & Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes sterilize, after sterilization pour the media into the Sterile Petri dish. Let it for solidification
- Add 1 ml of inoculum using sterile pipette pour in to triplicate plates (0.3, 0.3, 0.4 ml) of BPA medium and spread the inoculum using L –Rod. (If need prepare the serial dilution up to 10⁻⁶).

Carefully spread the inoculums as quickly as possible over the surface of agar medium, Avoid extreme edges of plates.

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- Allow all the plates to dry with their lids on for 15 min at laboratory temperature.
- Incubate the BPA inoculated Plate at 35-37 ° C for 45-48 Hours , After completion of incubation observe the characteristic colonies (Baird Parker Agar- Shiny Black colonies with or without Grey white margin, Blood Agar –Golden colour colonies)

EXPRESSION OF RESULTS

- Add Number of colonies on triplicate plates represented by colonies giving Positive coagulase test and multiply by test sample dilution factor
- Report this number of *s.aureus* /g of food.

CAUTION

- Carry out the test under the control of skilled microbiologist and great care shall be taken in the disposal of all the incubated material.
- Follow safe and good laboratory practices to avoid cross contamination.

REFERENCE

Official Methods of Analysis of **AOAC International 22nd Edition, 2023**