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METHODS FOR DETECTION OF BACTERIA RESPONSIBLE FOR FOOD POISONING

PART I ISOLATION, IDENTIFICATION AND ENUMERATION OF *ESCHERICHIA COLI*

(First Revision)

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Indian Standard

METHODS FOR DETECTION OF BACTERIA RESPONSIBLE FOR FOOD POISONING

PART I ISOLATION, IDENTIFICATION AND ENUMERATION OF *ESCHERICHIA COLI*

(First Revision)

0. FOREWORD

0.1 This Indian Standard (Part I) (First Revision) was adopted by the Indian Standards Institution on 13 December 1976, after the draft finalized by the Food Hygiene, Sampling and Analysis Sectional Committee had been approved by the Agricultural and Food Products Division Council.

0.2 Several micro-organisms contaminating food give rise to clinical symptoms. These are abdominal pain, nausea, vomitting, diarrhoea and sometimes pyrexia. A well-known exception is that of botulism where the symptoms are those of difficulty in swallowing, diplopia, aphonia and difficulty in respiration. Poisoning through food is characterized by the explosive nature with which the symptoms occur in otherwise healthy individuals. Often several persons after having consumed a particular item of food, develop symptoms that serve as important guide in suspecting food poisoning. Such explosive nature of food poisoning helps in differentiating conditions from those of out-breaks of food-borne infectious diseases which generally spread over a period of several days. The micro-organisms causing food poisoning belong to bacteria, protozoa and helminths, fungi and viruses. However, this standard covers the method for detection and estimation of important bacteria responsible for food poisoning food-borne diseases.

0.3 This standard was first published in 1970. It is being revised in parts covering methods of detection and estimation of various bacteria separately. This has been done with a view to making each part more comprehensive including various details of the method. It is expected that publication of these methods in parts will facilitate better implementation and adoption by the concerned organizations. This

will also make review and revision of the parts easier. The salient features of this revision are as follows:

a) Besides detection, estimation procedures for various organisms where applicable have been incorporated; and
b) Methods of identification have been updated.

0.4 In reporting the result of a test or analysis made in accordance with this standard, if the final value, observed or calculated, is to be rounded off, it shall be done in accordance with IS : 2-1960*.

1. SCOPE

1.1 This standard (Part I) prescribes methods for isolation, identification and enumeration of *Escherichia coli* in foods.

2. SAMPLING AND QUALITY OF REAGENTS

2.1 Sampling — For microbiological examination the samples should be handled carefully. For this purpose, IS : 5404-1969† shall be followed.

2.2 Quality of Reagents — Unless specified otherwise, pure chemicals shall be employed in tests and distilled water (*see* IS : 1070-1960‡) shall be used where use of water as a reagent is intended.

NOTE — ' Pure chemicals ' shall mean chemicals that do not contain impurities which affect the results of analysis.

3. GENERAL CHARACTERISTICS

3.1 The typical *Escherichia coli* is aerobic, Gram-negative rod, motile, fermenting lactose with the production of gas and usually produces smooth, non-mucoid colonies on solid media. However, there are non-lactose fermenting strains of *Esch. coli* and some strains produce mucoid colonies. The organism also exhibits the following characters:

a) Hydrogen sulphide (H_2S) production when done in TSI medium: Negative
b) Urease: Negative
c) Indole : Positive
d) Methyl red : Positive
e) Voges-Proskauer test : Negative
f) Simmon's citrate : Not utilized
g) Sucrose : Acid and gas production variable
h) Salicin : Acid and gas production variable, and
j) Growth at 44°C in MacConkey broth medium : Positive with acid and gas.

*Rules for rounding off numerical values (*revised*).
†Code of practice for handling of food samples for microbiological analysis.
‡Specification for water, distilled quality (*revised*).

4. MEDIA

4.1 Nutrient Broth — Mix and dissolve by heating 10 g peptone (*see* IS : 6853-1973*), 10 g meat extract (*see* IS : 6851-1973†), 5 g sodium chloride, in 1 000 ml water. When cool, adjust *p*H to 7·5 to 7·6. Remove precipitate by filtration through filter paper. Sterilize by autoclaving at 120°C for 15 minutes.

4.1.1 *Nutrient Agar* — To the medium as given in **4.1** add agar (*see* IS : 6850-1973‡) in such a concentration as will solidify and produce a sufficiently firm surface when poured in sterile petri dishes. The concentration of agar to be added varies from batch to batch and should be adjusted accordingly. Usual concentrations required vary from 1·5 to 3 percent. Dissolve the agar in the nutrient broth and sterilize by autoclaving at 120°C for 15 minutes. Prepare plates and slopes from sterile nutrient agar.

4.2 MacConkey Broth Medium

4.2.1 *Single Strength Medium* — Dissolve by steaming in 1 000 ml of water, 20 g peptone (*see* IS : 6853-1973*), 5 g sodium taurocholate or bile salts (*see* IS : 6852-1973§) and 5 g sodium chloride. Autoclave at 120°C for 15 minutes and filter while hot through a proper grade of filter paper, or a plug of cotton wrapped in gauge and placed in a funnel. Adjust *p*H of the filtrate to 7·3 at 50°C or to 7·5 at room temperature. Add 100 ml of 10 percent aqueous solution of lactose (or 10 g lactose) and 3·5 ml of 2 percent solution of neutral red in 50 percent ethanol. Mix thoroughly, distribute into sterilized flasks or tubes and sterilize by autoclaving at 120°C for 15 minutes.

4.2.2 *Double Strength Medium* — The procedure is same as in **4.2.1**, except that all the ingredients are double the amount mentioned in **4.2.1** dissolved in 1 000 ml water.

4.3 MacConkey Agar Medium — To ingredients as in single strength MacConkey broth medium (*see* **4.2.1**), add 15 to 30 g of agar (*see* IS : 6850-1973‡) and follow the procedure as in **4.2.1**, finally sterilizing by autoclaving at 120°C for 15 minutes. Pour into sterilized petri dishes and allow to set.

4.4 Eosin Methylene Blue Lactose Agar Medium — Dissolve by steaming in 1 000 ml of water, 10 g peptone (*see* IS : 6853-1973*), 2 g of dipotassium hydrogen phosphate (K_2HPO_4) and 15 to 30 g agar (*see* IS : 6850-1973‡). Make up to 1 000 ml by adding water. Dispense in 100 or 200 ml portions in suitable containers and sterilize at 120°C for 15 minutes. Final *p*H should be 7·1 ± 0·1.

*Specification for peptone, microbiological grade.
†Specification for meat extract, microbiological grade.
‡Specification for agar, microbiological grade.
§Specification for bile salts, microbiological grade.

Before use, melt, and to each 100 ml add 5 ml of sterile 20 percent lactose solution in water, 2 ml of aqueous, 2 percent eosin Y solution and 1·3 ml of 0·015 percent aqueous methylene blue solution.

4.5 Tergitol-7 Agar Medium — Dissolve by heating in 1 000 ml water, 5 g proteose peptone (*see* IS : 7128-1973*), 3 g yeast extract (*see* IS : 7004-1973†) 10 g lactose, 15 to 30 g agar (*see* IS : 6850-1973‡), 10 ml Tergitol-7 and 0·025 g bromthymol blue. Sterilize by autoclaving at 120°C for 15 minutes and pour into sterilized petri dishes. The final *p*H of medium should be 6·9.

4.6 Nutrient Agar Medium for Motility Test — Dissolve 3 g meat extract (*see* IS : 6851-1973§), 10 g peptone (*see* IS : 6853-1973‖), 5 g sodium chloride, and 4 to 5 g agar (*see* IS : 6850-1973‡) in 1 000 ml of water. Adjust *p*H between 7·5 and 7·6. Put the dissolved medium into test tubes to fill a part of the tube and place into this a glass tube open at both ends. One end of the glass tube shall project from above the surface of the agar for not less than 15 mm. Sterilize the tubes with the medium at 120°C for 15 minutes, and then cool. The consistency of the agar should be soft but not liquid. This should be achieved by altering the amount of agar used, if necessary.

4.7 TSI Medium for H_2S Test — Heat to dissolve in 1 000 ml water, 3 g meat extract (*see* IS : 6851-1973§), 3 g yeast extract (IS : 7004-1973†), 20 g peptone (*see* IS : 6853-1973‖) 1 g glucose, 10 g lactose, 10 g sucrose, 0·2 g ferrous sulphate ($Fe\ SO_4, 7H_2O$), 5 g sodium chloride, 0·3 g sodium thiosulphate ($Na_2\ S_2\ O_3, 5H_2O$) and 15 to 30 g agar (*see* IS : 6850-1973‡); add 12 ml of 0·2 percent phenol red solution mix and pour in tubes. Sterilize by autoclaving at 115°C for 20 minutes. Pour into sterile test tubes and cool to form a slope with deep butts.

4.8 Medium for Urease Test — Dissolve in 1 000 ml water, 1·5 g peptone (*see* IS : 7128-1973*), 5 g sodium chloride, 15 to 30 g agar (*see* IS : 6850-1973‡) 2 g potassium dihydrogen phosphate (KH_2PO_4) and add 6 ml of 1:500 aqueous solution of phenol red. Adjust *p*H between 6·8 and 6·9. Sterilize by autoclaving at 120°C for 15 minutes. When the solution has cooled to about 50°C, add a sterile solution of glucose to give a final concentration of 0·1 percent and add 100 ml of 20 percent solution of urea previously sterilized by seitz filtration. Distribute in sterile test tubes to form deep slopes.

*Specification for proteose peptone, microbiological grade.
†Specification for yeast extract, microbiological grade.
‡Specification for agar, microbiological grade.
§Specification for meat extract microbiological grade.
‖Specification for peptone, microbiological grade.

4.9 Medium for Indole Production — The medium consists of 20 g peptone (*see* IS : 6853-1973*) and 5 g of sodium chloride in 1 000 ml of water; adjust to *p*H 7·4. Place in tubes in 5 ml amounts and sterilize at 120°C for 15 minutes. The medium should be tested with a strain of bacterium known to produce indole.

4.10 Medium for Methyl Red and Voges-Proskauer Tests — Steam to dissolve in 1 000 ml water, 5 g peptone (IS : 6853-1973*) and 5 g dipotassium hydrogen phosphate (K_2HPO_4). Filter and adjust to *p*H 7·5. Add 5 g of glucose, mix to dissolve, distribute into tubes and sterilize at 115°C for 10 minutes.

4.11 Simmon's Citrate Agar — Dissolve in 1 000 ml water, 5 g sodium chloride, 0·2 g magnesium sulphate ($MgSO_4.7H_2O$), 1 g ammonium dihydrogen phosphate, 1 g dipotassium hydrogen phosphate, 2 g of sodium citrate, and 15 to 30 g agar (*see* IS : 6850-1973†). Add 40 ml of 0·2 percent bromothymol blue indicator solution. Adjust *p*H to 6·8 ± 0·1, and sterilize at 120°C for 15 minutes, tube and slant so that there is equal butt and slope.

4.12 Peptone Water Medium for Carbohydrate Fermentation Tests — Dissolve 10 g peptone (*see* IS : 6853-1973*) and 5 g sodium chloride in 900 ml of water and adjust the *p*H to 7·1 to 7·3 so that after addition of 10 ml of Andrade's indicator the *p*H shall be 7·5. (Andrade's indicator solution is prepared by adding 1 N sodium hydroxide solution to 0·5 percent aqueous solution of acid fuchsin until the colour of the indicator solution is just yellow). Sterilize at 115°C for 20 minutes. Dissolve 10 g of the requisite sugar in 90 ml of water and steam for 30 minutes or sterilize by filtration. With sterile precautions, add 90 ml of this sugar solution and 10 ml of Andrade's indicator solution to 900 ml of the sterile peptone water solution. Distribute into sterile test tubes containing inverted Durham's fermentation tubes, and steam for 30 minutes.

5. PROCEDURE FOR ISOLATION

5.1 Where necessary, blend the sample in a sterile blender jar for 2 minutes or macerate with sterile sand in a sterile mortar using approximately 200 ml of diluting fluid per 25 g of the sample. The diluting fluid for preparing the homogenate should be 0·1 precent peptone (*see* IS : 6853-1973*) solution in water, sterilized at 120°C for 20 minutes and final *p*H adjusted 6·8 ± 0·1, *or* 3·4 percent of potassium diyhdrogen phosphate (KH_2PO_4) in water, *p*H adjusted to 7·2 and sterilized at 120°C for 20 minutes. Inoculate 1 ml of the blended or macerated sample into 10 ml of single strength MacConkey broth medium (**4.2.1**). If the number of organisms are assumed to be very small, inoculate 10 ml of double strength

*Specification for peptone, microbiological grade.
†Specification for agar, microbiological grade.

MacConkey broth medium (**4.2.2**). Also streak loopfuls on to MacConkey agar medium (**4.3**), eosin methylene blue lactose agar (**4.4**), and if available Tergitol-7 agar (**4.5**). Incubate all the inoculated media at 37°C overnight. If there is growth with fermentation of lactose in the MacConkey broth medium (**4.2.1**) streak out a loopful on to each of the solid media (**4.3, 4.4** and **4.5**), and incubate at 37°C overnight.

6. TESTS FOR IDENTIFICATION

6.1 Pick out and mark as many suspect colonies from the solid media as possible, but not less than 5, to investigate. The suspect colonies are smooth and are lactose fermenting on MacConkey agar (**4.3**) and on eosin methylene blue lactose agar (**4.4**), and are yellow colonies surrounded by yellow zones on Tergitol-7 agar medium (**4.5**).

6.2 ***Escherichia coli*** — Suspect when conforming to the characters mentioned in **3** and tested as given in **6.2.1** to **6.2.10**.

6.2.1 *Gram's Stain* — The stain consists of: (a) 0·5 percent methyl violet or crystal violet in water, (b) iodine solution (1 percent iodine and 2 percent potassium iodide in water), and (c) counter-stain (0·1 g neutral red, 0·2 ml of 1 percent acetic acid and 100 ml water).

6.2.1.1 On a clean grease-free slide, very light and 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 fro over a flame and cooled. Cover the smear with the stain (a) for 30 seconds, pour off the stain and wash with (b) and then cover with (b) 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 (c) for about one minute. Wash in tap water and dry for examination.

6.2.2 *Test for Motility* — Inoculate by stabbing with a straight wire into the top of the medium as given in **4.6** 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 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.

6.2.3 *Test for H_2S Production* — Inoculate TSI medium (**4.7**) 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 blackening in the butt of the medium shall be recorded.

6.2.4 *Test for Urease* — Inoculate the organisms from the 24-hour incubated nutrient broth (**4.1**) culture heavily over the entire slope surface of medium as in **4.8** 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'.

6.2.5 *Test for Indole* — Inoculate medium as in **4.9** with a loopful of 24 hour growth in nutrient broth (**4.1**) and incubate at 37°C for 48 hours. Add 0·5 ml of Kovac's reagent, prepared by dissolving 10 g *p*-dimethyl-aminobenzaldehyde in 150 ml amyl alcohol or *iso*-amyl alcohol and to which 50 ml of concentrated hydrochloric acid is slowly added. Prepare the reagent in small quantities and store in refrigerator. After adding Kovac's reagent, shake the tube gently, the appearance of a red colour indicates the presence of indole.

6.2.6 *Test with Methyl Red* — Inoculate the medium as in **4.10** and incubate at 37°C for 2 days. Add 2 drops of methyl red solution prepared by dissolving 0·04 g of methyl red in 40 ml of absolute ethanol and diluting with water to make up to 100 ml. A positive reaction is indicated by red colour and a negative reaction by yellow colour.

6.2.7 *Test for Voges-Proskauer Reaction* — Inoculate the medium as in **4.10**, and incubate at 37°C for 2 days. To 1 ml of the growth add 0·6 ml of *alpha*-naphthol solution prepared as 5 percent solution in ethanol. Shake and add 0·2 ml of 40 percent aqueous solution of potassium hydroxide. Shake and slope the tube and observe for up to 4 hours for the appearance of a pink colour which indicates a positive reaction.

6.2.8 *Test for Citrate Utilization* — Inoculate the strain on to medium as in **4.11** with a young nutrient agar (**4.1.1**) slant culture using a straight wire. Incubate at 37°C for up to 4 days for growth of the organism.

6.2.9 *Test for Fermentation of Carbohydrates* — Inoculate the medium as in **4.12** and the carbohydrates indicated in **3.1**, 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.

6.2.10 *Test for Growth with Acid and Gas Production in MacConkey Broth* — Inoculate the medium as in **4.2.1** and incubate at 44°C for 2 days.

7. ENUMERATION OF *ESCHERICHIA COLI*

7.0 General — The procedure describes enumeration of *Esch. coli* and has been included in this standard as in many instances, the laboratory investigation is directed only towards enumeration of *Esch. coli* to assess the hygienic quality of a product. Since there is serious reservations regarding the use of the term 'coliforms', the name *Escherichia coli* is being used in this standard, and the procedure accordingly is for the enumeration of *Esch. coli*.

7.1 Preparation of Sample — Take 25 to 50 g of the sample in a sterile blender jar and to this add diluting fluid (*see* **5.1**) to have dilution of 10^{-1}. Blend at 8 000 to 10 000 rev/min for 2 minutes. Alternatively macerate with the diluting fluid in a sterile mortar with sterile sand. Make serial ten-fold dilutions with the diluting fluid, in duplicate series, up to 10^{-6}.

7.2 Plate Count — Spread out 0·1 ml from each dilution tube, as obtained in **7.1**, evenly on to Tergitol-7 agar (**4.5**), and incubate at 37°C for 24 hours. Enumerate the colonies of *Esch. coli*, which are yellow in colour surrounded by a yellow zone, and confirm these as being *Esch. coli* by the tests described in **6.2**. The number of viable colonies of *Esch. coli* per gram of sample shall be determined by multiplying by the dilution factor(s) and dividing by the mass of the sample. If Targitol-7 agar (**4.5**) is not in use, then MacConkey agar plates (**4.3**) or eosin methylene blue lactose agar plates (**4.4**) may be used.

7.3 Determination of the Most Probable Number of *Esch. coli* — Obtain serial dilutions of the sample as in **7.1**. Transfer, 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 medium (**4.2.1**) containing Durham's tube for collection of gas. 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 (**4.2.1**). When the number of *Esch. coli* is assumed to be very small, start by transferring 10 ml of the homogenized mixture in triplicate to 10 ml of double strength MacConkey broth medium (**4.2.2**) containing Durham's tube for collection of gas, using a sterile 10 ml pipette. Incubate in a water-bath at 44°C for 48 hours. Examine the tubes showing production of acid and gas, and using Table 1, obtain the most probable number (MPN) of *Esch. coli* per gram of the sample. 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. 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 *Esch. coli* 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, the 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, $9{\cdot}3 \times 10^{2}$ bacteria per gram. The MPN is reported as the average of the results obtained from each of the duplicate dilution series.

TABLE 1 MOST PROBABLE NUMBER (MPN) OF *ESCHERICHIA COLI*

(*Clause* 7.3)

Number of Positive Tubes per Dilution			MPN	Number of Positive Tubes per Dilution			MPN	Number of Positive Tubes per Dilution			MPN
10^{0}	10^{-1}	10^{-2}		10^{0}	10^{-1}	10^{-2}		10^{0}	10^{-1}	10^{-2}	
(1)	(2)	(3)	(4)	(1)	(2)	(3)	(4)	(1)	(2)	(3)	(4)
0	0	0	0·30	1	1	1	1·1	2	2	3	4·2
0	0	1	0·30	1	1	2	1·5	2	3	0	2·9
0	0	2	0·60	1	1	3	1·9	2	3	1	3·6
0	0	3	0·90	1	2	0	1·1	2	3	2	4·4
0	1	0	0·30	1	2	1	1·5	2	3	3	5·3
0	1	1	0·61	1	2	2	2·0	3	0	0	2·3
0	1	2	0·92	1	2	3	2·4	3	0	1	3·9
0	1	3	1·2	1	3	0	1·6	3	0	2	6·4
0	2	0	0·62	1	3	1	2·0	3	0	3	9·5
0	2	1	0·93	1	3	2	2·4	3	1	0	4·3
0	2	2	1·2	1	3	3	2·9	3	1	1	7·5
0	2	3	1·6	2	0	0	0·91	3	1	2	12·0
0	3	0	0·94	2	0	1	1·4	3	1	3	16·0
0	3	1	1·3	2	0	2	2·0	3	2	0	9·3
0	3	2	1·6	2	0	3	2·6	3	2	1	15·0
0	3	3	1·9	2	1	0	1·5	3	2	2	21·0
1	0	0	0·36	2	1	1	2·0	3	2	3	29·0
1	0	1	0·72	2	1	2	2·7	3	3	0	24·0
1	0	2	1·1	2	1	3	3·4	3	3	1	46·0
1	0	3	1·5	2	2	0	2·1	3	3	2	110·0
1	1	0	0·75	2	2	1	2·8	3	3	3	110·0
				2	2	2	3·5				

7.3.1 Before reporting the results as being the MPN of *Esch. coli*, confirm the growth in the tubes as being *Esch. coli* by plating out from the positive tubes on to MacConkey agar (**4.3**), or eosin methylene blue lactose agar (**4.4**), or Tergitol-7 agar (**4.5**), and confirming the typical colonies (**3.1** and **6.1**) as being *Esch. coli* by the tests described in **6.2**.

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