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METHODS OF SAMPLING AND TEST FOR SEWAGE EFFLUENTS

(First Revision)

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BUREAU OF INDIAN STANDARDS
MANAK BHAVAN, 9 BAHADUR SHAH ZAFAR MARG
NEW DELHI 110002

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Indian Standard

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(First Revision)

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Indian Standard

METHODS OF SAMPLING AND TEST FOR SEWAGE EFFLUENTS

(First Revision)

0. FOREWORD

0.1 This Indian Standard (First Revision) was adopted by the Indian Standards Institution on 18 March 1972, after the draft finalized by the Water Sectional Committee had been approved by the Chemical Division Council.

0.2 This standard was first published in 1968. Subsequently the Sectional Committee responsible for the preparation of this standard decided to revise it in order to incorporate the recent advances in the method for identification of *Salmonella*, *Shigella* and *Vibrio* organisms.

0.3 In the preparation of this standard considerable assistance has been derived from the following publications:

Manual on water, sewage and industrial wastes (Special report No. 47). 1964. Indian Council of Medical Research, New Delhi.

Standard methods for the examination of water, sewage and industrial wastes. Ed 12. 1965. American Public Health Association, American Water Works Association, and Federation of Sewage and Industrial Wastes Associations, New York.

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*.

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1. SCOPE

1.1 This standard prescribes the methods of sampling and test for sewage effluents.

2. SAMPLING

2.1 Point of Sampling — In those cases where the effluent at a specific point is to be tested, the question of choosing the point of sampling does not arise. However, where the composition of an effluent as finally discharged

*Rules for rounding off numerical values (*revised*).

by a sewage treament plant is to be ascertained, the point of sampling shall be the final outlet of the treatment plant.

2.2 Frequency of Sampling — When it is required to find out variations in the composition of the effluent during a specified period, such as that of peak discharge, the samples shall be taken at short and appropriate intervals, say, every 5, 10, 15 or 30 minutes and analysed. To study the average conditions over a cycle of operations or a period (usually 24 hours) or during the daily working period of the treatment plant, the collection of composite sample shall be adopted. The composite sample shall be made by collecting at appropriate intervals samples from the common channel or drain at a point where the flow of the effluent is likely to be most representative of the entire volume, and mixing. The volume of the individual samples shall be a fixed proportion of the volume of the effluent flowing at that time. The interval should depend upon the frequency of variation in the nature of the effluent and the volume of flow. Care shall be taken to take the samples in such a way as to maintain the true proportion of suspended solids. Samples shall not be taken by skimming the top or scraping the bottom. A point about one-third of the way from the bottom shall normally be selected. The samples shall be drawn gently without unnecessary aeration. In most cases, collection of samples every hour would be sufficient.

2.3 Sampling Instrument — Porcelain-lined or enamelled pails, in which the lining is unbroken, or glass vessels shall be used for taking samples. The vessels used for taking the sample shall be wide-mouthed and small enough for the contents to be transferred quickly to the sample container without leaving behind any deposit or scum. Automatic sampling devices, if available, may be used.

2.3.1 Each individual sample shall be deposited in a receptacle of sufficient size to hold the entire composite sample. Clean and dry carboys, other large glass containers or enamelled buckets with lids without chipping may be used for pooling the sample.

2.4 Sample Containers

2.4.1 The quantity of sample required for analysis shall be taken from the composite sample after thorough mixing in order to keep the solids in suspension.

2.4.2 The sample for analysis shall be drawn in clean glass stoppered bottles, which shall be rinsed with a portion of the sample. New bottles shall be washed with acid and thoroughly rinsed with distilled water before being brought into use. About 2 to 3 litres of the sample will be required for analysis. The bottle containing the final sample shall be filled so that a small air bubble is present after closure to prevent leakage or even breakage arising from any subsequent changes in temperature.

The stopper shall be firmly inserted and, if the sample is to be transported some distance, tied down to keep in position.

2.4.3 The label on the bottle shall bear the name of the sampling authority, details of the type of sample, place, date and time of sampling.

2.5 Preservation of Samples

2.5.1 The samples shall be kept at a low temperature (about 4°C) during collection and thereafter.

2.5.2 No single method of preservation is applicable for the sample for all the tests. The analysis shall be carried out, preferably, immediately after collection. Storage at 3 to 4°C in a well insulated ice box or refrigerator is the best way to preserve most samples till the next day. Where chemical preservatives are used as specified for individual tests, these shall be added to each portion of sample taken for the particular test and not to the entire sample.

3. GENERAL PRECAUTIONS AND DIRECTIONS FOR TESTS

3.1 Quality of Reagents — Unless specified otherwise, pure chemicals and distilled water (*see* IS: 1070-1960*) shall be used in tests.

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

3.2 It is important to obtain a representative sample. Appropriate methods of sampling are given in 2. However, in tests where specific sampling procedures are prescribed these shall be followed.

3.3 For determinations for which calibrated glass discs are available, these may be used for routine examination provided instructions of the manufacturer are followed. But in case of dispute, test methods as prescribed in this standard shall be followed.

4. TOTAL SUSPENDED SOLIDS

4.0 Outline of the Method — Suspended matter is determined by filtering the sample through an asbestos pad in a Gooch crucible.

4.1 Reagent

4.1.1 *Asbestos Cream* — Make a cream of acid-washed medium-fibre Gooch asbestos with water. Add one litre of water for every 15 g of asbestos. If the asbestos contains too much fine powder, remove the latter by repeated decantation.

4.2 Procedure

4.2.1 Make carefully an asbestos mat in the Gooch crucible by adding sufficient asbestos cream to produce a mat about 3 mm thick. In preparing

*Specification for water, distilled quality (*revised*).

the mat, first fill the crucible with well-mixed asbestos cream, let stand for about two minutes to allow the heavier particles to settle and then apply suction to the same extent as will be used for filtering the sample. Wash the mat with water with the suction on by filling and drawing through. Dry the crucible with the asbestos mat in an oven at 103 to 105°C for one hour, cool in a desiccator and weigh.

4.2.2 Filter the sample through the weighed Gooch crucible after moistening with a few drops of water. Add successive increments of 10 ml of the well-shaken sample for filtration using suction. Add each increment of sample before the mat becomes dry. The use of a pipette with an orifice wide enough to prevent clogging with suspended matter is recommended. Continue successive 10 ml additions of the sample until the filtration becomes inconveniently slow or until about 10 to 20 mg of suspended matter has been filtered. Carefully wash the mat two or three times, taking 10 ml of water each time, to remove soluble salts. Continue suction until draining is complete. Dry the crucible in an oven at 103 to 105°C for one hour, cool to room temperature in a desiccator and weigh.

4.3 Calculation

$$\text{Total suspended solids, mg/l} = \frac{1\,000\,W}{V}$$

where

W = weight in mg of the suspended matter, and

V = volume in ml of the sample taken for filtration.

4.3.1 Express the result to the nearest 5 mg/l.

5. DISSOLVED OXYGEN

5.0 Outline of the Method — All the methods for the determination of dissolved oxygen are based on the original Winkler procedure. The principle is that of precipitation of manganous hydroxide in a bottle filled with the sample brought about by the addition of a solution of manganous sulphate followed by alkaline potassium iodide solution. The oxygen present in the sample quickly reacts with the manganous hydroxide forming a brown precipitate of higher hydroxides. On subsequent acidification, iodine in an amount equivalent to the oxygen contained in the sample is liberated. The quantity of iodine liberated is estimated by titration against a standard solution of sodium thiosulphate, using starch as indicator.

5.0.1 The Winkler method is subject to interference due to various ions and compounds contained in the sample. Suitable modifications of the method are adopted to correct for these interferences. The choice of the

exact modified procedure will depend upon the nature of the sample and the interference present. The application of these procedures and the methods are given below:

Sl No.	*Procedure*	*Applicability*
i)	Winkler method	When no interfering substances are present
ii)	Alsterberg (sodium azide) modification	When not more than 0·1 mg/l of nitrite nitrogen and not more than 1 mg/l of ferrous iron are present and in the absence of other reducing or oxidizing agents. In the presence of 5 mg/l or more of ferric iron potassium fluoride is added. When potassium fluoride is added, the method is applicable in the presence of 100 to 200 mg/l of ferric iron
iii)	Rideal-Stewart (permanganate) modification	In the presence of ferrous iron only. If ferrous or ferric iron is more than 10 mg/l, potassium fluoride is added before acidifying
iv)	Alkali hypochlorite modification	In the presence of sulphite, thiosulphate, polythionate, free chlorine and hypochlorite. However, the results obtained with this method cannot be relied upon as this procedure gives somewhat low results
v)	Short-Theriault modification	In the presence of organic matter easily oxidized at the *p*H of the alkaline iodide treatment
vi)	Alum flocculation modification	In the presence of high amounts of suspended solids

5.1 Winkler Method

5.1.1 *Reagents*

5.1.1.1 *Manganous sulhpate solution*—Dissolve 480 g of manganous sulphate tetrahydrate ($MnSO_4 . 4H_2O$) in water, filter if not clear, and make up to 1 litre. The solution should not liberate more than a trace of iodine when added to an acidified potassium iodide solution.

5.1.1.2 *Alkaline iodide solution* — Dissolve 500 g of sodium hydroxide (or 700 g of potassium hydroxide) in its own weight of water and allow to cool. The caustic solution should be virtually free from carbonate. Dissolve separately 150 g of potassium iodide in a small quantity of freshly boiled and cooled water and add this solution to the caustic solution. Dilute the mixture to one litre.

5.1.1.3 *Concentrated sulphuric acid*

5.1.1.4 *Standard sodium thiosulphate working solution* — exactly 0·025 N, freshly standardized against potassium dichromate. One millilitre of this solution is equivalent to 0·2 mg of oxygen.

5.1.1.5 *Starch indicator solution* — Triturate 5 g of starch and 0·01 g of mercuric iodide with 30 ml of cold water and slowly pour it with stirring into 1 litre of boiling water. Boil for 3 minutes. Allow the solution to cool and decant off the supernatant clear liquid.

5.1.2 *Procedure* — Remove the stopper from a 250- to 300-ml bottle containing the sample and add 1·5 ml of manganous sulphate solution followed by 1·5 ml of alkaline iodide solution, keeping the tip of the pipette in each case well below the surface of the liquid. Carefully replace the stopper without the inclusion of air bubbles and thoroughly mix the contents by inverting and rotating the bottle several times; allow the precipitate formed to settle. When the precipitate settles leaving a clear supernatant above the manganese hydroxide floc, repeat mixing a second time and allow to settle. When further settling produces at least 100 ml of clear supernatant, carefully remove the stopper and immediately add 2 ml of concentrated sulphuric acid by running the acid down the neck of the bottle, restopper and mix well to ensure uniform distribution of iodine in the bottle. Titrate the solution immediately against standard sodium thiosulphate solution, adding 1 ml of starch indicator solution when the colour becomes pale yellow and completing the titration to the disappearance of the blue colour. The error due to the displacement of sample by reagents is insignificant.

5.1.3 *Calculation* — Dissolved oxygen in mg/l is equal to the volume in millilitres of standard sodium thiosulphate solution used in the titration.

5.2 Alsterberg (Sodium Azide) Modification

5.2.1 *Reagents* — Use all the reagents given in **5.1.1** except alkaline iodide solution, which is to be replaced by alkaline iodide-sodium azide solution, and potassium fluoride reagent.

5.2.1.1 *Alkaline iodide-sodium azide solution* — Dissolve 10 g of sodium azide in 40 ml of water. Add this with constant stirring to the cool alkaline iodide solution prepared as in **5.1.1.2** but made up to 950 ml.

5.2.1.2 *Potassium fluoride solution* — Dissolve 40 g of potassium fluoride ($KF.2H_2O$) in 100 ml of water.

5.2.2 *Procedure* — The procedure given in **5.1.2** shall be followed but, instead of alkaline iodide solution, alkaline iodide-sodium azide solution shall be used. When 5 mg/l or more of ferric iron is present, add 1 ml of potassium fluoride solution before acidifying the sample and titrate immediately after acid addition and mixing.

5.2.3 *Calculation* — Calculate as in **5.1.3**.

5.3 Rideal-Stewart (Permanganate) Modification

5.3.1 *Reagents* — Use all the reagents given in **5.1.1**, potassium fluoride solution given in **5.2.1.2** and, in addition, the following reagents.

5.3.1.1 *Potassium permanganate solution* — Dissolve 6·3 g of potassium permanganate in water and make up to 1 litre.

5.3.1.2 *Potassium oxalate solution* — Dissolve 2 g of potassium oxalate ($K_2C_2O_4.H_2O$) in 100 ml of water.

5.3.2 *Procedure* — Remove the stopper of the bottle containing the sample, add below the surface, with a 1-ml graduated pipette, 0·70 ml of concentrated sulphuric acid followed by sufficient potassium permanganate solution (about 1 ml) to produce a violet tinge which persists for 5 minutes. Avoid large excess of permanganate. Stopper and mix by inversion. After 5 minutes remove the excess permanganate by adding 0·5 ml portions of potassium oxalate solution and mixing. Allow 5 minutes interval after each addition of oxalate. Then proceed as in **5.1.2**, using 3 ml of alkaline iodide solution instead of 1·5 ml. When either ferrous or ferric iron is present in excess of 10 mg/l, add 1 ml of potassium fluoride solution immediately after permanganate addition. The titration after final acidification should be carried out without delay.

5.3.3 *Calculation* — Calculate as in **5.1.3**.

5.4 Alkali Hypochlorite Modification

5.4.1 *Reagents* — Use all the reagents given in **5.1.1**, substituting alkaline iodide solution with alkaline iodide-sodium azide solution (*see* **5.2.1.1**). In addition, use the following reagents.

5.4.1.1 *Alkali hypochlorite solution* — 2 N, prepared by passing chlorine gas through a 2·1 N sodium hydroxide solution, with cooling. One millilitre of this solution will require, on acidification in the presence of potassium iodide, about 20 ml of 0·1 N sodium thiosulphate solution for titration. Check the strength of the solution every week.

5.4.1.2 *Potassium iodide solution* — 1 N. Dissolve 17 g of potassium iodide in water and make up to 100 ml. Preserve by adding 1 ml of 1 N sodium hydroxide solution.

5.4.1.3 *Sulphuric acid* — 1 : 9 (*v/v*).

5.4.1.4 *Sodium sulphite solution* — 0·1 N. Dissolve 6·3 g of anhydrous sodium sulphite or 12·6 g of sodium sulphite heptahydrate in water and make up to 1 litre. Do not use the solution when its strength goes down to less than 80 percent of the original.

5.4.1.5 *Potassium biniodate solution* — 0·1 N. Dissolve 3·249 g of potassium biniodate [$KH(IO_3)_2$] in water and make up to 1 litre in a volumetric flask.

5.4.2 *Procedure* — Remove the stopper of the bottle containing the sample and add 0·2 ml or just sufficient quantity of alkali hypochlorite solution to oxidize the sulphite. Stopper and mix by inversion for 20 to 30 seconds. Add 1 ml of potassium iodide solution and acidify with 1 ml or more of sulphuric acid. Mix by inversion. Add 0·2 ml of starch indicator solution and destroy the iodine liberated with sodium sulphite solution. Restore the blue tinge with 0·1-ml portions of 0·1 N potassium biniodate solution. Proceed further as in **5.2.2** with the difference that 3 ml of alkali iodide-sodium azide solution has to be added instead of 1·5 ml.

5.4.3 *Calculation* — Calculate as in **5.1.3**.

5.5 Short-Theriault Modification

5.5.1 *Reagents* — Use all the reagents given in **5.1.1**, substituting alkaline iodide solution with alkaline iodide-sodium azide solution (*see* **5.2.1.1**).

5.5.2 *Procedure* — Remove the stopper of the bottle containing the sample, add 2 ml of manganous sulphate solution followed by 2 ml of alkali iodide-sodium azide solution, stopper and mix by inversion for 20 seconds. Immediately add 2 ml of concentrated sulphuric acid before the precipitate settles, remix and titrate as prescribed in **5.1.2**.

5.5.3 *Calculation* — Calculate as in **5.1.3**.

5.6 Alum Flocculation Modification

5.6.1 *Reagents* — Use all the reagents given in **5.2.1** and, in addition, the following reagents.

5.6.1.1 *Alum solution* — Dissolve 10 g of potassium aluminium sulphate in water and dilute to 100 ml.

5.6.1.2 *Ammonium hydroxide* — concentrated.

5.6.2 *Procedure* — Collect the sample in a glass-stoppered bottle of 500- to 1 000-ml capacity, using the same precautions as are necessary for the 300-ml sample. Add 10 ml of alum solution followed by 1 to 2 ml of ammonium hydroxide. Stopper and invert gently for about 1 minute. Allow it to settle for about 10 minutes and then siphon the clear supernatant into a

250 to 300 ml dissolved oxygen bottle until it overflows. Avoid aeration and keep the siphon submerged at least 20 cm. Then follow the procedure and calculate the result as given in **5.2, 5.3, 5.4** or **5.5** as appropriate.

6. BIOCHEMICAL OXYGEN DEMAND

6.0 Outline of the Method — Biochemical oxygen demand (BOD) is the quantity of oxygen required by a definite volume of the liquid effluent for oxidizing the organic matter contained in it by micro-organisms under specified conditions. For its determination, the dissolved oxygen content of the sample, with or without dilution, is measured before and after incubation at 20°C for 5 days.

6.1 Apparatus

6.1.1 *Glass-Stoppered Bottles* — narrow-neck bottles of about 200-ml capacity, with suitable water sealing.

6.2 Reagents

6.2.1 *Sodium Hydroxide Solution* — approximately 1 N.

6.2.2 *Hydrochloric Acid* — approximately 1 N.

6.2.3 *Sodium Sulphite Solution* — Dissolve 1·5 g of anhydrous sodium sulphite in 1 litre of water. Prepare fresh solution daily for use.

6.2.4 *Dilution Water* — distilled water of good quality, free from metals, particularly copper, and aerated.

6.2.5 *Phosphate Buffer Solution* — Dissolve 8·5 g of potassium dihydrogen phosphate (KH_2PO_4), 21·75 g of dipotassium hydrogen phosphate (K_2HPO_4), 33·4 g of disodium hydrogen phosphate ($Na_2HPO_4 . 7H_2O$) and 1·7 g of ammonium chloride in about 500 ml of water and dilute to 1 litre. The *p*H of this solution should be 7·2.

6.2.6 *Magnesium Sulphate Solution* — Dissolve 22·5 g of magnesium sulphate ($MgSO_4 . 7H_2O$) in water and dilute to 1 litre.

6.2.7 *Calcium Chloride Solution* — Dissolve 27·5 g of anhydrous calcium chloride in water and dilute to 1 litre.

6.2.8 *Ferric Chloride Solution* — Dissolve 0·25 g of ferric chloride ($FeCl_3 . 6H_2O$) in water and dilute to 1 litre.

6.2.9 *Seeding Material* — Supernatant liquor of domestic sewage stored for 24 to 36 hours at 20°C.

6.3 Procedure

6.3.1 Samples containing acidity or caustic alkalinity should be neutralized to *p*H about 7·0 with sodium hydroxide solution or hydrochloric acid respectively by adding a predetermined quantity.

6.3.2 Samples containing residual chlorine or chloramines should be dechlorinated if chlorine is not dissipated on standing for 2 hours. To dechlorinate, first determine the quantity of sodium sulphite solution required for a known aliquot of the sample by titration to starch-iodide end point after acidifying the sample with acetic acid (1 : 1) or sulphuric acid (1 : 50) followed by 10 ml of 10 percent potassium iodide solution. Then add to the requisite volume of the sample the predetermined quantity of sodium sulphite avoiding any excess, and check for the absence of chlorine after 20 minutes.

6.3.3 Samples containing toxic substances in large amounts would require special treatment. However, the effect of small amounts may be overcome by using the proper dilution so that toxicity is removed and the maximum BOD value is obtained. If increasing dilutions show increasing BOD, the dilution should be increased to a level where BOD levels off at a maximum.

6.3.4 To check the quality of the dilution water and the effectiveness of the seed, determine the BOD of a standard solution of 300 mg/l of either glucose or glutamic acid in the dilution water. Standard glucose solution should show a BOD of 224 ± 10 mg/l and glutamic acid 217 ± 10 mg/l.

6.3.5 Store the dilution water at 20°C and use when near that temperature. Take the desired volume of dilution water required for the test sample and add, for every 1 litre of water, 1 ml each of phosphate buffer solution, magnesium sulphate solution, calcium chloride solution and ferric chloride solution. Seed the dilution with seeding material. The quantity of seeding material (0·1 to 1 percent of settled sewage) added should be such that oxygen depletion in the dilution water control is between 0·2 and 0·8 mg/l after incubation at 20°C for 5 days.

6.3.6 Prepare as follows several dilutions of the sample (usually 5 to 25 percent) so as to obtain a depletion of at least 2 mg/l of dissolved oxygen after incubation for 5 days. In the case of dilutions greater than 1 : 100, prepare a 10 percent primary dilution in a volumetric flask and from this make the final dilutions.

6.3.7 Siphon carefully the prepared seeded dilution water into a graduated 1 000-ml measuring cylinder and fill to the 500-ml mark. Add the requisite quantity of the carefully well-mixed sample to make the particular dilution and fill with dilution water to 1 litre. Mix thoroughly but gently with a plunger type of rod without entraining air. Siphon the dilution into two glass-stoppered bottles, fill completely and stopper. Prepare succeeding dilutions of lower concentrations in the same manner. Determine the initial dissolved oxygen concentration in one of the two bottles of each dilution by the appropriate method given in 5. Water-seal the other bottles and incubate at 20°C for 5 days. At the same time, siphon the dilution water

alone into two glass-stoppered bottles and determine the blank in one and incubate the other at 20°C for 5 days. After incubation for 5 days determine the dissolved oxygen in the dilutions and the blank in the same manner as the initial dissolved oxygen content.

6.4 Calculation

$$\text{Biochemical oxygen demand (5 days at 20°C), mg/l} = \frac{(D_1 - D_2) - (C_1 - C_2)\,F}{P}$$

where

D_1 = initial dissolved oxygen content of the sample;

D_2 = dissolved oxygen content of the diluted sample after incubation;

C_1 = initial dissolved oxygen content of the seeded dilution water;

C_2 = dissolved oxygen content of the seeded dilution water after incubation;

F = ratio of the seed in the sample to that in the control, that is, percent seed in D_1 divided by percent seed in C_1; and

P = decimal fraction of the sample used.

6.4.1 Express the result to the nearest 0·2 mg/l.

7. PATHOGENIC BACTERIA

7.0 General

7.0.1 The common pathogenic bacteria that may be present in sewage are those which cause typhoid (*Salmonella typhi*), paratyphoid (*Salmonella paratyphi*), dysentery (*Shigella* species) and cholera (*Vibrio cholerae*). The *Salmonella* and *Shigella* groups of bacteria are grown in selective and enrichment media for subsequent isolation in pure culture. The organisms are examined by morphological and biochemical methods and finally identified using diagnostic sera.

7.0.2 As the number of pathogenic organisms in sewage may be small as compared with the non-pathogenic saprophytic organisms, the collection of a representative concentrate is important. The sewer-swab method affords a useful means for this purpose. From the swab collection the organisms are dispersed in nutrient broth and the isolation of the pathogenic organisms is done either by directly using the broth suspension or after filtering the broth suspension through kieselguhr filter and using the contents of the filter paper. Sampling and examination shall be repeated several times in a month.

7.1 Collection and Preparation of Sample

7.1.1 *Materials*

7.1.1.1 *Swab* — The swab consists of strips of surgical gauze, 30 cm × 15 cm, folded into a many-layered compact pad (approximately 6 × 4 cm). A few stones are attached to it for weighting and also a string of sufficient length. The whole is wrapped in kraft paper and sterilized in the autoclave at 1 kg/cm² pressure for 30 minutes.

7.1.1.2 *Nutrient broth* — Dissolve in 900 ml of hot water, 10 g of beef extract (lemco or equivalent), 5 g of sodium chloride, 10 g of peptone and bring to the boil. Cool, dilute to 1 litre with water and adjust *p*H to 7·5. Filter through a filter paper (Whatman No. 1 or equivalent). Distribute in 100 ml portions into 150-ml flasks and in 10 ml portions in tubes and autoclave at 1 kg/cm² for 15 minutes.

7.1.1.3 *Filter papers* — Whatman No. 1 or equivalent. Fold into pleats and place into glass filter funnels 10 cm in diameter. Place the filter funnel on the mouth of an all glass bottle, wrap the whole in brown paper (kraft paper) and secure it well. Sterilize by autoclaving at 1 kg/cm² for 15 minutes.

7.1.1.4 *Kieselguhr suspension* — Suspend 5·0 g of kieselguhr powder in 1 000 ml of water and distribute the suspension in all glass bottles in quantities of 500 ml. Sterilize by autoclaving at 1 kg/cm² for 15 minutes.

7.1.1.5 *Liquid medium for Vibrio cholerae** — Take the specified volumes of the solutions mentioned below and mix them in a sterile bottle along with 0·8 ml of absolute alcohol and 20 ml of water:

a) 17·6 ml of a 2·0 percent solution of peptone, with *p*H adjusted to 8·4, and which has been sterilized by autoclaving at 1 kg/cm² for 15 minutes;

b) 2·4 ml of a solution prepared by dissolving in 100 ml of water, 18·0 g of sodium chloride, 0·66 g of potassium chloride, 2·0 g of magnesium chloride ($MgCl_2.6H_2O$) and 1·16 g of magnesium sulphate ($MgSO_4.7H_2O$), previously sterilized by steaming for 30 minutes;

c) 4·0 ml of a 10 percent solution of sucrose which has been sterilized by steaming for 30 minutes;

d) 0·16 ml of bismuth ammonium citrate liquor prepared as given below:

Make a 500 ml mark in a ground glass stoppered bottle. Introduce into it 60 g of bismuth citrate through a funnel, followed

*The medium is double strength of a medium which may be referred to in the publication by Ahuja (ML.) *et al* in *Indian Journal Medical Research* 39; 1951; 135-139. Slight modifications have been made from the original formula.

by 50 ml of water. Stir with a glass rod and make the citrate into a smooth paste. Then add 20 ml of ammonium hydroxide (12·5 percent *w/w*) and stir with glass rod. Insert the stopper and shake the bottle. As soon as the bismuth citrate has entirely dissolved add water to the 500 ml mark. Prepare in small quantities as required for use;

e) 2·4 ml of 20 percent solution of sodium sulphite.

Add to the bottle containing the above mixture 1·6 ml of 0·01 percent solution of mercuric chloride. Adjust the *p*H with sodium hydroxide solution (1 N) to 9·2.

NOTE — The amount of medium prepared is required for one sample. Sterile precautions shall be taken at every stage of preparation.

7.1.2 *Procedure*

7.1.2.1 Suspend the swab (three swabs if filtration technique is to be adopted) in flowing sewage from a manhole cover or other suitable attachment and allow to remain in place for 48 hours. Then take them out and place each of them in a sterile wide mouth glass jar provided with a lid. Place in each jar enough sterile nutrient broth to soak the swab, then squeeze the swab with a sterile glass rod, and agitate the whole assembly thoroughly to ensure uniform dispersal of the contents from the swab into the broth.

7.1.2.2 When the filtration technique is adopted, pass the sterile kieselguhr suspension through a sterile filter paper so that the entire surface of the filter paper has been well covered with kieselguhr. Prepare three such filters. Then filter the nutrient broth in which the swab was dispersed through the coated filter paper. After filtration, use each filter paper as the specimen for isolation of different organisms.

7.1.2.3 For isolation of *Salmonella* and *Shigella* organisms, transfer one of the specimens to 50·0 ml of tetrathionate broth (**7.2.1.1**) and another specimen to 50·0 ml of selenite broth (**7.2.1.2**).

7.1.2.4 For isolation of *Vibrio cholerae*, transfer the third filter paper specimen to a bottle of liquid medium for *Vibrio cholerae* (**7.1.1.5**) and incubate at 37°C for 24 hours. Transfer a loopful on bile salt agar plate (**7.3.1.3**), spread it and then follow the procedure as under **7.3.2.**

7.2 *Salmonella* and *Shigella* Group

7.2.1 *Media*

7.2.1.1 *Kauffmann-Müller's tetrathionate broth* — To 90 ml of sterile nutrient broth, add with aseptic precautions, and with continuous agitation, 5 g of calcium carbonate which has previously been powdered and sterilized in the autoclave, 10 ml of 50 percent solution of sodium thiosulphate

(previously sterilized by steaming), 2 ml of potassium iodide-iodine solution (dissolve by grinding in a mortar 5·0 g of potassium iodide in 20 ml of water and then add slowly with constant grinding 4·0 g of iodine; keep the solution in dark glass stoppered bottle), 1 ml of 0·1 percent aqueous solution of brilliant green and 5 ml of sterile oxbile or 1·0 g of bile salt. Distribute in 10 ml quantities in sterile test tubes. The medium shall not be subjected to sterilization after preparation as the tetrathionate may decompose on heating.

7.2.1.2 *Selenite F broth* — Dissolve 4·0 g of sodium acid selenite, 5·0 g of peptone, 4·0 g of lactose, 9·5 g of disodium hydrogen phosphate, 0·5 g of sodium dihydrogen phosphate in 1 000 ml of sterile water (water autoclaved at 1 kg/cm^2 for 30 minutes) with sterile precautions and distribute the yellowish solution in 10 ml amounts in sterile screw capped bottles. Steam at 100°C for 30 minutes. The medium shall not be autoclaved. There may be a slight red precipitate in the medium, but this does not interfere with the action of the medium. The *p*H of the medium as prepared shall be 7·1 and the quantity of phosphates added may be varied slightly to achieve this.

7.2.1.3 *MacConkey agar medium* — Mix 5 g of sodium taurocholate, 20 g of peptone, 5 g of sodium chloride and 20 g of washed shredded or powdered agar with 1 000 ml of water. Steam until the solids are dissolved. Cool to about 50°C, and at this temperature adjust reaction to *p*H 7·6 to 7·8. Add egg white, if necessary, using the white of one egg for 3 litres of the medium. Autoclave at 0·7 kg/cm^2 pressure for 15 minutes and filter while hot through a good grade of filter paper, or a plug of cotton wrapped in gauze and placed in the funnel. Adjust reaction of the filtrate to *p*H 7·3 at 50°C or to *p*H 7·5 at room temperature. Add 100 ml of 10 percent aqueous solution of lactose and 3·5 ml of 2 percent solution of neutral red in 50 percent ethanol. Mix thoroughly, distribute into flasks and sterilize in the autoclave at 0·7 kg/cm^2 pressure for 15 minutes. For use, melt in the steamer, pour into sterile petri-dishes (12 to 15 ml in each) and allow to set. Dry the surface of the medium in the incubator before use.

7.2.1.4 *Desoxycholate citrate agar medium*

a) *Agar base* — Dissolve 20 g of beef extract (lemco or equivalent) in 200 ml of water over the flame, and make the solution just alkaline to phenolphthalein with 50 percent sodium hydroxide solution. Boil and filter through filter paper. Adjust the *p*H to 7·3, make up to 200 ml and add 20 g of proteose peptone. In another vessel, dissolve 90 g of washed and shredded or powdered agar in 3 700 ml of water by steaming for one hour. Filter the agar solution, add to it the beef extract-peptone solution and mix. Add 5 ml of 2 percent neutral red solution in 50 percent ethanol and 40 g of lactose. Make up to 4 litres with water.

Mix, bottle accurately in lots of 100 ml and sterilize in the autoclave by free steaming for one hour and then at 0·3 kg/cm² pressure for 10 minutes.

b) *Solution A* — Dissolve in 100 ml of sterile water, with heating, 17 g of sodium citrate, 17 g of sodium thiosulphate and 2 g of ferric ammonium citrate (green scales).

c) *Solution B* — Dissolve 10 g of sodium desoxycholate in 100 ml of sterile water.

d) Sterilize solution A and solution B at 60°C in a water bath for 1 hour. For preparing the desoxycholate citrate agar medium melt 100 ml of the agar base in a water bath and add 5 ml each of solution A and solution B in the order given, using separate pipettes. Mix well after each addition. Pour into petri-dishes (12 to 15 ml in each) and allow to set. Dry the surface of the medium in the incubator before use.

7.2.1.5 *Bismuth sulphite agar medium (Wilson and Blair medium)*

a) *Stock solution* — Dissolve in 50 ml of boiling water 6 g of bismuth ammonium citrate scales and separately in 100 ml of water 20 g of anhydrous sodium sulphite. Mix the two solutions and heat again. Add in the boiling solution 10 g of disodium hydrogen phosphate, cool and add 10 g of glucose previously dissolved in 50 ml of water.

b) *Agar base* — Dissolve by careful heating over a flame or by autoclaving, 30 g of agar in 1 000 ml of nutrient broth (**7.1.1.2**), cool to 50°C and adjust *p*H to 7·4 to 7·6. Distribute in flasks in 300 ml quantities, autoclave at 1 kg/cm² for 30 minutes and store in refrigerator.

c) For preparing the medium, add to 100 ml of molten agar base 20 ml of the stock solution, 1 ml of 8 percent aqueous solution of ferrous sulphate and 0·5 ml of 1 percent aqueous solution of brilliant green. Mix, pour into petri-dishes and allow to set. Use as freshly as possible. The plates should have a dry surface. Discard if the colour of the medium is not greenish.

7.2.1.6 *Sugar media* — To every 90 ml of peptone water prepared by dissolving 10 g of peptone and 5 g of sodium chloride in 1 litre of water and adjusting the *p*H to 7·5, add 10 ml of a 10 percent solution of the fermentable sugar* required for test and 1 ml of drades indicator (prepared by adding enough of 1N sodium hydroxide solution to 0·5 percent aqueous solution of acid fuchsin until the colour of the indicator solution is just yellow). Place in 3 ml quantities in 100 × 12 mm test tubes with

*These sugars are glucose, lactose, saccharose, dulcite, mannite and salicin. Mannitol and inositol may also be included for *V. cholerae*.

Durham's fermentation tube and sterilize in autoclave at 0·7 kg/cm² for 20 minutes or in free steam (100°C) for 20 minutes on three successive days.

7.2.1.7 *Medium for test for urease (Christensen's medium)* — Dissolve in 1 000 ml of water, 1·5 g of peptone, 5·0 g of sodium chloride, 20 g of agar, 2·0 g of dipotassium hydrogen phosphate and add 6·0 ml of 1 : 500 aqueous solution of phenol red. Adjust *p*H to 6·8 to 6·9 and 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.

7.2.1.8 *Medium for test for motility* — The medium consists of 3·0 g of beef extract, 10·0 g of peptone, 5·0 g of sodium chloride, and 4·0 to 5·0 g of agar dissolved in 1 000 ml of water. Adjust the *p*H to 7·5 to 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 tubing shall project from above the surface of the agar for not less than 1·5 cm. Sterilize the tubes with the medium at 120°C for 15 minutes. When cool, the consistency of the agar should be soft but not liquid. This is achieved by altering the amount of agar used.

7.2.1.9 *Medium for test for indole productiom* — The medium consists of peptone water [20·0 g of peptone and 5·0 g of sodium chloride in 1 000 ml of water (*p*H 7·4)]. Place in tubes in 5·0 ml amounts and sterilize at 120°C for 15 minutes. The peptone should be tested with a strain known to produce indole.

7.2.1.10 *Kovac's reagent* — Dissolve 10·0 g of *p*-dimethylaminobenzaldehyde in 150 ml of amyl or *iso*amyl alcohol. Add slowly 50 ml of concentrated hydrochloric acid. Prepare in small quantities and store in refrigerator.

7.2.1.11 *Reagent for gram staining* — The stains consist of two solutions, namely: (a) 0·5 percent solution of methyl violet or crystal violet in water, and (b) a solution containing 1·0 percent iodine and 2·0 percent potassium iodide in water. The counterstain consists of a solution in 1 000 ml of water of 0·1 g neutral red and 0·2 ml of acetic acid (1·0 percent).

7.2.1.12 *Nutrient agar* — Add agar, bacteriological quality, to nutrient broth (**7.1.1.2**) in such a concentration as will solidify and produce a sufficiently firm surface when poured in a sterile petri-dish. The concentration of agar to be added varies from batch to batch and needs to be adjusted accordingly. The concentration usually required varies from 1·5 to 3·0 percent. Dissolve the agar in the nutrient broth and sterilize by autoclaving at 120°C for 15 minutes. Prepare plates and slopes from the sterile nutrient agar.

7.2.1.13 *Medium for Hugh-Leifson's test* — Dissolve by heating in 1 000 ml of water, 2·0 g of peptone, 5·0 g of sodium chloride, 0·3 g of potassium phosphate (K_2HPO_4), and 3·0 g of agar. Adjust to *p*H 7·1, filter and add 15·0 ml of 0·2 percent alcoholic solution of bromothymol blue. Sterilize at 115°C for 20 minutes. Add sterile solution of glucose to give a final concentration of 1·0 percent. Mix and distribute in tubes, 12 × 100 mm adding 3·0 to 4·0 ml per tube.

7.2.1.14 *Triple sugar iron agar (TSI) medium* — Heat to dissolve in 1 000 ml of water 3·0 g of beef extract, 3·0 g of yeast extract, 20·0 g of peptone, 1·0 g of glucose, 10·0 g of lactose, 10·0 g of sucrose, 0·2 g of ferrous sulphate crystals ($Fe SO_4 . 7H_2O$), 5·0 g of sodium chloride, 0·3 g of sodium thiosulphate crystals ($Na_2 S_2O_3 . 5H_2O$) and 20·0 to 30·0 g of agar. Add 12·0 ml of 0·2 percent phenol red solution, mix and tube. Sterilise at 115°C for 20 minutes and cool to form a slope with deep butts.

7.2.1.15 *Simmons' citrate agar* — Dissolve in 1 000 ml of water 5·0 g of sodium chloride, 0·2 g of crystalline magnesium sulphate ($MgSO_4.7H_2O$), 1·0 g of ammonium phosphate ($NH_4H_2PO_4$), 1·0 g of potassium phosphate (K_2HPO_4), 2·0 g of sodium citrate and 20 to 30 g of agar. Add 40·0 ml of 1 in 500 bromothymol blue indicator solution. Sterilise at 120°C for 15 minutes, tube and slant so that there is equal butt and slope.

7.2.2 *Procedure*

7.2.2.1 Transfer with a sterile pipette 5·0 ml of the well mixed sample obtained from the swab (**7.1.2.1**) to one tube of tetrathionate broth and also the same amount to one tube of selenite F broth. Take also one large loopful of the sample and spread on desoxycholate citrate agar and on Wilson and Blair agar plates. Incubate all these at 37°C overnight for 18 to 24 hours. From the tetrathionate and selenite broths, plate out again on to fresh desoxycholate and Wilson and Blair agar plates and incubate at 37°C. The spreading on the plates should be aimed at obtaining discrete colonies. When the filtration procedure is followed, incubate the tetrathionate broth and the selenite broth to which the filter paper 'specimens' were transferred (**7.1.2.2**) at 37°C for 24 hours and spread a large loopful from them on desoxycholate citrate agar medium plate and Wilson and Blair agar medium plate. Suspect *Salmonella* or *Shigella* colonies are non-lactose fermenters on desoxycholate citrate agar, being round, small, pale, transparent, flat dew-drop in appearance. *Salmonella typhi* and *paratyphi* colonies on Wilson and Blair medium are black in colour. If desired, in addition to subculturing from liquid media to the two solid media mentioned, MacConkey agar medium may also be included. Colonies of *Salmonella* and *Shigella* are non-lactose fermenters and appear as pale or colourless, domed, round colonies on MacConkey agar. If growth on solid media is not obtained after overnight incubation,

incubate the plates for a further 24 hour period. Incubation of the liquid media for more than 18 to 24 hours is not likely to yield results and is to be avoided.

7.2.2.2 Pick out as many suspect colonies as are possible to investigate, but at least six from each plate, with a sterile needle and transfer to one tube each of nutrient broth (**7.1.1.2**) and incubate at 37°C for 18 to 24 hours. At the end of this incubation period, examine for staining characteristics and motility. Also spread one loopful of each of the cultures on MacConkey agar plate to confirm pure colonies of non-lactose fermenters. Incubate the media and the agar plates at 37°C for 24 hours and note the reactions. From the MacConkey agar plate, subculture the colonies on nutrient agar slants, incubate at 37°C for 24 hours and then keep in the refrigerator for confirmation by serological typing. *Salmonella* and *Shigella* organisms are gram negative and rod shaped bacteria. *Salmonella* and *Shigella* species are suspected when the organisms conform to the characteristics given in Table 1.

7.2.2.3 For confirmation it is essential that cultures in nutrient agar slopes be sent to the National Salmonella-Escherichia Centre at the Central Research Institute, Kasauli (Simla Hills), India. If diagnostic sera are available for indentification of *Salmonella typhi* and *Salmonella paratyphi A*, this may be undertaken. Identification and reporting for other *Salmonella* species and for *Shigella* species shall await report of a competent laboratory.

7.2.2.4 *Test for urease* — For urease test, inoculate the organisms from the 24 hour incubated nutrient broth culture (**7.2.2.2**) heavily over the entire medium slope surface (**7.2.1.7**) 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, commonly found in sewage, gives a positive result.

7.2.2.5 *Test for motility* — Inoculate by stabbing with a straight wire into the top of the medium (**7.2.1.8**) 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 will 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.

7.2.2.6 *Test for indole production* — Inoculate a loopful of the 24 hour incubated nutrient broth culture (**7.2.2.2**) into the medium (**7.2.1.9**) and incubate at 37°C for 48 hours. Add 0·5 ml of Kovac's reagent and shake the tube gently. A red colour develops in the presence of indole.

TABLE 1 CHARACTERISTICS OF *SALMONELLA* AND *SHIGELLA* ORGANISMS

(*Clause* 7.2.2.2)

Sl No.	Tests	Salmonella		Shigella		
		S. typhi	Others	Subgroup A	Subgroups B and C	Subgroup D
(1)	(2)	(3)	(4)	(5)	(6)	(7)
i)	Gram negative rods	Positive	Positive	Positive	Positive	Positive
ii)	Motility	Motile	Motile	Non-motile	Non-motile	Non-motile
iii)	Catalase test	Positive	Positive	Positive (exception is *Sh.Shiga*)	Positive	Positive
iv)	Hugh-Leifson's test	Fermentative	Fermentative	Fermentative	Fermentative	Fermentative
v)	Oxidase test	Negative	Negative	Negative	Negative	Negative
vi)	TSI test	Positive	Positive	do	do	do
vii)	Urease test	Negative	Negative	do	do	do
viii)	Phenyl pyruvic acid test	do	do	do	do	do
ix)	Citrate test	do	Positive	do	do	do
x)	Indole test	do	Negative	Positive or negative	Positive or negative	do
xi)	Glucose test	Acid only	Acid and gas	Acid only	Acid only*	Acid only
xii)	Lactose test	No acid and no gas	No acid and no gas	No acid and no gas	No acid and no gas	Late positive
xiii)	Sucrose test	do	do	do	do	do
xiv)	Salicin test	do	do	do	do	No acid and no gas
xv)	Dulcitol test	Usually no acid and no gas	Acid and gas	Reaction varies but most strains give no acid and no gas	Reaction varies but subgroup B gives no acid and no gas	do
xvi)	Mannite	Acid only	do	No acid and no gas	Acid only*	Acid only

NOTE — The characters indicated for 'other *Salmonellas*' are in many instances similar to those of species of *Arizona* and *Citrobacter*.

*Some strains of *Sh. flexneri* 6 produce gas in small amounts.

7.2.2.7 *Gram staining* — Make on a clean grease-free slide, very light and thin smear, covering a small area, directly from liquid culture and in clean water from solid media. Fix the smear by passing to and fro over a flame and cooling. Cover the smear with the methyl violet or crystal violet stain [**7.2.1.11**(a)] for 30 seconds, pour off the stain, wash with iodine solution [**7.2.1.11**(b)], then cover with the same solution and allow to remain for 30 seconds. Wash off with ethanol till a white background is obtained. Wash in running water and apply neutral red counterstain (**7.2.1.11**) for about one minute. Wash in water and dry for examination.

7.2.2.8 *Catalase test* — Grow strain for 24 hours on nutrient agar slope (**7.2.1.12**) and pour 1·0 ml of 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.

7.2.2.9 *Hugh-Leifsons test* — Inoculate by stabbing a strain from fresh agar growth (**7.2.1.12**) into two tubes containing the medium (**7.2.1.13**), one of which shall then be layered over with a small amount of sterile paraffin. Incubate both tubes at 37°C and observe daily up to 4 days. Acid formation shown by yellow colour in the tube without paraffin indicates oxidative utilisation 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.

7.2.2.10 *Oxidase test* — Add to nutrient agar (**7.2.1.12**) slant of the freshly grown culture, a few drops of freshly mixed test reagent consisting of equal volumes of 1·0 percent solution of alpha-naphthol in 95 percent ethanol and 1·0 percent solution of para-aminodimethylaniline hydrochloride in water. A positive reaction is indicated by the appearance of a blue colour within two minutes.

7.2.2.11 *Triple sugar iron agar test* — Inoculate a tube containing TSI medium (**7.2.1.14**) by stabbing into the butt the suspect strain and streaking the slope. Incubate at 37°C and observe daily for up to 7 days for changes in colour of medium to show acid production, disruption of medium along the line of inoculation, gas production, and blackening due to production of hydrogen sulphide.

7.2.2.12 *Phenyl pyruric acid test* — Obtain an overnight heavy growth of the organism on nutrient agar (**7.2.1.12**). Suspend the growth in 0·5 ml of normal saline and transfer to a wide bore test tube (diameter at least 1·5 cm). Add 0·5 ml of 0·2 percent *dl*-phenylalanine in normal saline. Mix and keep lying horizontally for at least 3 hours at room temperature. Add a 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 serve as a control.

7.2.2.13 *Citrate test* — Inoculate using a straight wire a fresh nutrient agar (**7.2.1.12**) slant culture suspected strain on to the citrate medium (**7.2.1.15**). Incubate at 37°C for up to 4 days for growth of the organisms.

7.2.2.14 *Tests on sugar* — Transfer a loopful of each of the cultures of the suspect colonies in nutrient broth (obtained in **7.2.2.2**) to each of the sugar media (**7.2.1.6**) given in Table 1. Incubate at 37°C for 24 hours and observe the reactions.

7.3 ***Vibrio Cholerae***

7.3.1 *Medium*

7.3.1.1 *Alkaline peptone water* — Dissolve 10 g of peptone and 5 g of sodium chloride in water and make up to 1 000 ml. Adjust *p*H to 8·2 and distribute in 50 ml quantities into suitable bottles or flasks and sterilize at 1 kg/cm² pressure for 30 minutes.

7.3.1.2 *Aronson's medium*

a) *Nutrient agar base* — Dissolve by heating 25 g of powdered or washed shredded agar in 100 ml of nutrient broth (**7.1.1.2**), cool to 50°C, adjust *p*H to 7·2 and steam for 10 minutes.

b) To 300 ml of melted nutrient agar base, add 20 ml of 10 percent solution of anhydrous sodium carbonate previously heated at 100°C for 30 minutes, and steam for 30 minutes. While still hot, add in this order 15 ml of 20 percent solution of sucrose, 15 ml of 20 percent solution of glucose, 1·2 ml of saturated alcoholic solution of basic fuchsin and 6·0 ml of 10 percent solution of sodium sulphite (in water) previously heated at 100°C for 30 minutes. Steam the mixture for 20 minutes. Use the clear supernatant liquid for pouring into petri-dishes and allow it to set. Store in dark and use within 3 days.

7.3.1.3 *Bile salt agar medium* — Dissolve by steaming in 1 000 ml of water, 10·0 g of peptone, 5·0 g of beef extract, 5·0 g of sodium chloride and 5·0 g of sodium taurocholate. Add 30 g of agar, mix and dissolve by heating. Adjust *p*H to 8·5 with sodium hydroxide solution. Cool and filter through a filter paper (Whatman No. 1 or equivalent) or absorbent cotton wool previously wetted with water. Distribute into sterile flasks in convenient amounts and sterilize by autoclaving at 1 kg/cm² for 30 minutes. Plates are made by melting the stock medium and pouring into sterile petri-dishes.

7.3.1.4 *Medium for gram staining* — same as **7.2.1.11**.

7.3.1.5 *Medium for motility test* — same as **7.2.1.8**.

7.3.1.6 *Medium for Hugh-Leifson's test* — same as **7.2.1.13**.

7.3.1.7 *Sugar media* — same as **7.2.1.6.**

7.3.1.8 *Arginine dehydrolase medium* (*see* also **7.3.1.11**) — Dissolve with heat 5·9 g of peptone (Orthana, Evans or equivalent), 5·0 g of beef extract, 5·0 mg of pyridoxal and 0·5 g of glucose, in 1 000 ml of water. Adjust to *p*H 6·0 and add 5·0 ml of 0·2 percent solution of bromocresol purple and 2·5 ml of 0·2 percent solution of cresol red. Sterilize at 115°C for 20 minutes. Add *l*-arginine hydrochloride to final concentration of 1·0 percent. If *dl*-amino acid is used, the concentration would be 2·0 percent. Distribute in 1·0 to 1·5 ml amounts into small tubes, 67 × 10 mm, and layer with sterile liquid paraffin to a height of about 5 mm. Sterilize at 115°C for 10 minutes.

7.3.1.9 *Lysine decarboxylase medium* (*see* also **7.3.1.11**) — Prepare the medium as in **7.3.1.8** substituting lysine hydrochloride for arginine.

7.3.1.10 *Ornithine decarboxylase medium* (*see* also **7.3.1.11**) — Prepare the medium as in **7.3.1.8** substituting ornithine hydrochloride for arginine.

7.3.1.11 *Alternative medium for decarboxylase and dehydrolase activity* — Dissolve in 1 000 ml of water 5·0 g of peptone (Evans, Orthana, Difco or equivalent), 3·0 g of yeast extract and 1·0 g of glucose, and adjust *p*H to 6·7. Add 10 ml of 0·2 percent bromocresol purple solution. Sterilize at 115°C for 20 minutes. To this base add the amino acid being tested (arginine hydrochloride or lysine hydrochloride or ornithine hydrochloride) to final concentration of 0·5 percent for *l*-salt and double the concentration, that is 1·0 percent, for *dl*-salt. Readjust *p*H to 6·7 if necessary. Tube in 2·0 ml amounts in tubes, 67 × 10 mm, and layer with sterile liquid paraffin to a height of about 5 mm. Sterilize at 115°C for 10 minutes.

7.3.2 *Procedure* — Transfer with a sterile pipette 5 ml of the well mixed sample (**7.1.2.1**) into one bottle of alkaline peptone water, and incubate for 18 to 24 hours at 37°C. Plate also a large loopful of the well mixed sample on bile salt agar plate and incubate at 37°C for 18 to 24 hours. From the inoculated alkaline peptone water liquid medium after incubation, pick out a large loopful of surface growth (the pellicle or flake) and spread on bile salt agar plates and Aronson's medium and incubate at 37°C for 18 to 24 hours. The suspicious colonies of *Vibrios* in Aronson's medium appear coloured like pomegranate seeds with a red central portion surrounded by a clear halo. On bile salt agar, the colonies have distinctive appearance which may be seen by growing a known strain of *Vibrio cholerae* on this medium and comparing the colony characters with, say, the growth of *Escherichia coli* on the same medium. Suspicious growths should be tested by slide agglutination test (**7.3.3**). Transfer the remaining portion of the colony to a tube of nutrient broth and incubate at 37°C for 4 to 6 hours. At the end of the period spread again a large loopful on another bile salt agar plate to obtain larger yield of pure colonies

and incubate at 37°C for 24 hours. Also carry out tests given in **7.3.2.1** to **7.3.2.9**. The characters of *Vibrios* are given below and these should be confirmed before sending strains to a reference laboratory for final confirmation:

a) Gram staining	Negative; rods
b) Motility	Positive
c) Catalase activity	Positive
d) Oxidase activity	Positive
e) Hugh-Leifson's test	Fermentative reaction
f) Glucose medium	Fermentation without gas production
g) Arginine dehydrolase activity	Negative
h) Lysine decarboxylase activity	Positive
j) Ornithine decarboxylase activity	Positive
k) Inositol medium	No fermentation
m) Mannitol medium	Fermentative reaction

The characters (a) to (f) above are shown by strains of *Aeromonas* and *Plesiomonas* also. These are differentiated from *Vibrios* in that *Aeromonas* strains are lysine decarboxylase negative, arginine dehydrolase positive, ferment mannitol and not inositol; *Plesiomonas* strains do not ferment mannitol, usually ferment inositol and are lysine decarboxylase positive.

NOTE—It is essential that all suspect Vibrio colonies, whether positive or negative by preliminary slide agglutination test, are sent to a competent laboratory for confirmation.

7.3.2.1 *Gram staining* — Carry out the test as in **7.2.2.7**.

7.3.2.2 *Test for motility* — Carry out the test as in **7.2.2.5**.

7.3.2.3 *Catalase test* — Carry out the test as in **7.2.2.8**.

7.3.2.4 *Oxidase test* — Carry out the test as in **7.2.2.10**.

7.3.2.5 *Hugh-Leifson's test* — Carry out the test as in **7.2.2.9**.

7.3.2.6 *Tests on sugar* — Transfer a loopful of the culture of the suspect colonies to each of the sugar media (**7.2.1.6**) given in **7.3.2**. Incubate at 37°C for 24 hours and observe for reactions.

7.3.2.7 *Arginine dehydrolase activity test* — Inoculate the medium (**7.3.1.8**) through the paraffin, using a straight wire, from a freshly grown culture in nutrient agar (**7.2.1.12**). 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 dehydrolation of arginine occurs, the medium becomes violet. Also include a tube of the medium inoculated with a known strain of *V. cholerae* which will show a yellow colour indicating negative reaction and another tube of medium with a known strain of *Aeromonas* or a strain of *Salm. typhi* which will show a positive reaction.

7.3.2.8 *Lysine decarboxylase activity test* — Carry out the test as in **7.3.2.7** using the corresponding medium (**7.3.1.9**).

7.3.2.9 *Ornithine decarboxylase activity test* — Carry out the test as in **7.3.2.7** using the corresponding medium (**7.3.1.10**).

7.3.3 *Rapid Slide Agglutination Test for Vibrio Cholerae* — Place on a clean glass slide a loopful of isotonic saline (0·87 percent of sodium chloride in water) and another loopful of the antiserum (high titre sera of combined Inaba and Ogawa sera types) by the side of the first. Suspend in the isotonic saline the growth picked from a nutrient agar culture or a culture grown in any other medium of the organisms to give a heavy suspension and mix this in the drop of the antiserum. A positive reaction is indicated by the development of a curdled appearance within half to one minute.

7.3.4 For further confirmation, send the strains on nutrient agar slants to the Cholera Research Centre, 3 Kyd Street, Calcutta 16. It should be remembered that stock strains of recently isolated strains of *Vibrio cholerae* after growth on nutrient agar slants shall not be kept in the refrigerator as the strains do not survive well at 4°C. *Vibrio cholerae* on nutrient agar slope will survive well at room temperature.

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