HS 1005 Coliform Bacteria Test

1. Purpose

The bacteria test is to determine the *Coliform bacteria* in the sample by using membrane filtration method. The main sources of the *Coliform bacteria* are soil, human and animal faeces.

The abundance of *Coliform bacteria* in test specimen is expressed as total *Coliform bacteria* per 100 mL of undiluted water sample prepared from test specimen.

2. Terminology

2.1 *Coliform bacteria*

It refers to all Gram-negative, non-spore-forming, facultative anaerobic, rod-shaped bacteria which develop red colonies with a metallic (golden) sheen within 24 hours at 35 °C on an Endo-type medium containing lactose.

3. Apparatus

3.1 Dilution bottles, preferably borosilicate glass, closed with glass stoppers or screw caps equipped with liners that do not produce toxic or bacteriostatic compounds on sterilisation.

3.2 Pipettes and graduated cylinders, sterilised in autoclave before use.

3.3 Containers for culture medium, any size or shape of flask which can provide adequate mixing of the medium contained and are convenient for storage may be used.

3.4 Petri dishes

3.5 Filtration units:

- 3.5.1 Filter holder.
- 3.5.2 Filtering flask.
- 3.5.3 Electric vacuum pump. Connect a flask of approximately the same capacity between filtering flask and vacuum pump to trap the carry-over water.
- 3.5.4 Funnel.
- 3.5.5 Filter with glass frit.

3.6 Membrane filter, with a rated pore diameter such that there is a complete retention of *Coliform* bacteria.

3.7 Absorbent pads, which consist of disks of filter paper or other suitable material certified for each lot by the manufacturer to be of high quality and free of sulphite or other substances of a concentration that could inhibit bacterial growth. Sterilise pads together with membrane filters available in resealable kraft envelopes.

3.8 Forceps, smooth flat forceps, without corrugations on the inner sides of the tips.

Sterilise before use by dipping in alcohol and flaming.

3.9 Alcohol burner.

3.10 Incubators, capable to provide a temperature of 35 °C ± 0.5 °C and maintain a humid environment of 60 % relative humidity.

3.11 Microscope or other suitable colony-counting equipment.

3.12 Autoclave for steam sterilisation, capable of operation at 121 °C and 100 kPa.

3.13 Scissors.

3.14 Ruler.

3.15 Analytical balance, capable of weighing to 0.1 g.

4. Reagents

4.1 Culture media

Use one of the following culture media either in solid form with agar or as a broth for saturating absorbent pads. Instructions for preparing the media are given in Annex A.1 and A.2.

4.1.1 LES Endo agar

4.1.2 M-Endo medium

4.2 Buffered dilution rinse water

The buffered water is used for diluting and rinsing samples. Instruction for preparing the buffered water is given in Annex A.3.

4.3 Reagent grade water

5. Operating procedures

5.1 Preparation of sample

5.1.1 The sample taken for testing should be in its ready-for-use state. The sample should be put into an aseptic container or bags instantly after collection. Obtain a sample in accordance with the requirements of the sampling programme and handle it with care to avoid contamination before testing. To avoid contamination, certain precautions are necessary which are common to all sampling procedures for bacteriological examination:

- Scrupulous care should be taken to avoid accidental contamination of the sample during collection and subsequent handling.
- The sample should be collected in a sufficient amount and tested as soon as possible after collection.

5.1.2 Under aseptic condition, open the container and use a sterile knife to cut the sample into pieces. Carefully open a sterile envelope and slip the test specimens into the inner envelope. Seal the outer envelope with an adhesive or pressure-sensitive tape. The specimens should be about 50 mm × 50 mm.

5.1.3 Place a closed petri dish on the pan of the balance and determine its tare mass. Cut the envelope holding the specimens along the top flap with a sterile knife or scissors. Open the envelope by pressing the sides, without touching the inner surface; remove the specimen with sterile tongs.

5.1.4 Hold the edge of the specimen with the tongs in one hand, trim, and discard the edges with sterile scissors. Make a series of cuts 10 to 20 mm-apart parallel to the side of specimen.

5.1.5 Partially remove the cover of the tared petri dish, but retain the cover on the balance pan. Cut squares of samples directly onto the petri dish by making a series of cuts perpendicular to those made previously.

5.1.6 Cut enough samples to give about 25.0 g of test pieces. Replace the cover of the petri dish. Weigh the petri dish plus test pieces and from the tare difference calculate the test piece mass.

5.1.7 The 25.0 g of test pieces are put in a sterilised conical flask with glass stopper containing 225 mL sterile rinse water. Shake for 30 minutes. The 1:10 homogenous diluted solution is obtained. Prepare a further dilution if it is suspected that the test sample has a high bacterial population. According to the quantity of bacteria in sample, choose the suitable dilution level for the test sample to yield 20 to 80 *coliform* colonies and not more than 200 colonies of all types on a membrane filter surface.

5.2 Preparation of petri dishes

5.2.1 If agar-based medium is used, dispense 5 to 7 mL quantities of selected medium into low section of glass or plastic petri dishes. If dishes of any other size are used, adjust quantity to give an equivalent depth of 4 to 5 mm.

5.2.2 If liquid medium is used, place an absorbent pad in the petri dish and saturate with at least 2 mL of medium solution and carefully remove excess medium by decanting the plate.

5.2.3 Close the petri dish and mark it appropriately for sample identification and set it aside. Do not expose poured plates to direct sunlight.

5.3 Filtration

5.3.1 Set up the sterile filtration units and place a sterile membrane filter (grid side up) over porous plate of filter holder. Carefully place matched funnel unit over filter holder and lock it in place. Connect a flask of approximately the same capacity between filtering flask and vacuum pump to trap the carry-over water.

5.3.2 Mix the sample prepared in Section 5.1.7 by vigorously shaking for several

seconds. Pipette 100 mL of sample into the funnel and apply the vacuum to filter the sample.

5.3.3 Rinse the interior surface of the funnel by filtering three 30 mL portions of sterile dilution water.

5.4 Incubation

5.4.1 After completion of final rinse, turn off the vacuum pump and remove the funnel. Use the sterile forceps to remove the membrane filter and place it on the previously prepared medium with a rolling motion to avoid entrapment of air.

5.4.2 Replace the cover tightly. Invert the dish and incubate for 22 to 24 hours at 35 ± 0.5 °C.

5.4.3 Re-sanitize the filter holder prior to insertion of a new membrane for the next sample by placing the holder into the ultraviolet steriliser for 2 minutes exposure. Or it can be sanitized by dipping or spraying with alcohol and then igniting or immersing in boiling water for 2 minutes.

5.4.4 Follow the procedure in Section 5.3 and filter 100 mL sterile rinse water sample to check for possible cross-contamination or contaminated rinse water. Incubate the rinse water control membrane filter under the same conditions as the sample.

5.5 Counting

5.5.1 After the required incubation period, turn the petri dish right side up and remove the lid of the dish. Place the dish in the microscope or other optical device to count the colony.

5.5.2 The typical *Coliform* colony has a pink to dark-red colour with a metallic surface sheen. The sheen area may vary in size from a small pinhead to complete coverage of the colony surface.

6. Report

The result is reported as total *coliform* per 100 g test samples. Compute the count, using membrane filer with 20 to 80 *coliform* colonies and not more than 200 colonies of all types per membrane by following equation:

Total Coliform count per 100 g sample = colonies count x dilution factor

Take the average colony number from samples of the same concentration.

Note: For example, if 100 mL of the 1:100 diluted solution of sample is used, after incubation of the plates, 2 colonies were counted on the plate, then the result is 2 × 100 = 200 colonies per 100 g of the undiluted sample.

7. Reference

1. APHA 9222B, Standard Total Coliform Membrane Filter Procedure, Standard Methods for the Examination of Water and Wastewater, 20th Edition, APHA, AWWA, WEF.

Annex A Culture medium, reagent and diluent

A.1 LES Endo agar

Yeast extract	1.2 g
Casitone or trypticase	3.7 g
Thiopetone or thiotone	3.7 g
Tryptose	7.5 g
Lactose	9.4 g
Dipotassium hydrogen phosphate, K_2HPO_4	3.3 g
Potassium dihydrogen phosphate, KH_2PO_4	1.0 g
Sodium chloride, NaCl	3.7 g
Sodium desoxycholate	0.1 g
Sodium lauryl sulphate	0.05 g
Sodium sulphite, $NaSO_3$	1.6 g
Basic fuchsin	0.8 g
Agar	15.0 g
Reagent grade water	1 L

Preparation:

Rehydrate product in 1 L water containing 20 mL 95 % ethanol. Do not use denatured ethanol, which reduces background growth and *Coliform* colony size. Bring to a near boil to dissolve agar, then promptly remove from heat and cool to 45 to 50 °C. Do not sterilise by autoclaving. Final pH should be 7.2 ± 0.2. Refrigerate it in the dark, preferably in sealed plastic bags or other containers to reduce moisture loss. Discard unused medium after 2 weeks or sooner if there is evidence of moisture loss, medium contamination, or medium deterioration (darkening of the medium).

A.2 M-Endo medium

Tryptose or polypetone	10.0 g
Thiopetone or thiotone	5.0 g
Casitone or trypticase	5.0 g
Yeast extract	1.5 g
Lactose	12.5 g
Sodium chloride, NaCl	5.0 g
Dipotassium hydrogen phosphate, K_2HPO_4	4.375 g
Potassium dihydrogen phosphate, KH_2PO_4	1.375 g
Sodium lauryl sulphate	0.05 g
Sodium desoxycholate	0.10 g
Sodium sulphite, $NaSO_3$	2.10 g
Basic fuchsin	1.05 g
Agar (optional)	15.0 g
Reagent grade water	1 L

(I) Agar preparation:

Rehydrate product in 1L water containing 20 mL 95 % ethanol. Heat to near boiling to dissolve agar, then promptly remove from heat and cool between 45 to 50 °C. Do not sterilise by autoclaving. Final pH should be 7.2 ± 0.2. A precipitate is normal in Endo-type medium.

(II) Broth preparation:

Prepare as above, omitting agar. Dispense liquid medium (at least 2.0 mL per plate) onto absorbent pads and carefully remove excess medium by decanting the plate. The broth may have a precipitate but this does not interfere with medium performance if pads are certified free of sulphite or other toxic agents at a concentration that could inhibit bacterial growth. Refrigerated broth may be stored for up to 4 days.

A.3 Buffered dilution rinsing water

Preparation:

Dissolve 34.0 g of potassium dihydrogen phosphate (KH_2PO_4) in 500 mL reagent grade water. Adjust the pH to 7.2 ± 0.5 with 1 N sodium hydroxide. And dilute to 1 litre with reagent grade water to prepare 1 litre stock phosphate buffer solution.

Dissolve 81.1 g of magnesium chloride $MgCl_2 \cdot 6H_2O$ in 1 litre reagent grade water to prepare the magnesium chloride solution. Add together 1.25 mL stock phosphate buffer solution and 5.0 mL magnesium chloride solution and dilute to 1 litre with reagent grade water.