

Numbers of *Enterobacteriaceae*

(Based on the ISO 21528-2:2004 Standard Protocol)

MATERIALS AND EQUIPMENT

1. Reagents

- 1.1 Maximum recovery diluent (MRD) in 9 ml and 90 ml volumes.
- 1.2 Violet red bile glucose agar (VRBG) 30 ml per plate.
- 1.3 Nutrient agar (NUT) pre-poured plates.
- 1.4 Glucose agar 15 ml per tube.
- 1.5 Oxidase reagent or test kit.

2. Apparatus

- 2.1 Sterile pipettes.
- 2.2 Balance with a resolution of 0.01g or better.
- 2.3 Vortex mixer.
- 2.4 Petri dishes.
- 2.5 Incubator running at $37 \pm 1^\circ\text{C}$.
- 2.6 Stomacher.

PROCEDURES

1. Initial preparation of inoculum

- 1.1 If the sample is liquid then directly plate 1 ml quantities into a labelled plate.
- 1.2 For solid or semi-solid samples, aseptically weigh 10 g (or a multiple of 10 g) of sample, add 90 g (or 9x the sample weight) of MRD and stomach for 2 minutes.
- 1.3 For swab samples, agitate by vigorous shaking or by use of a vortex mixer or a peristaltic homogeniser (stomacher) for 1 minute. Ensure the volume of the swab solution is taken into account for the initial dilution.

2. Perform decimal dilutions

Using a fresh sterile pipette transfer 1 ml of the initial inoculum into 9 ml of MRD, repeat the procedure for as many decimal dilutions as required. Mix the dilutions using a vortex mixer for 5 to 10 seconds.

3. Inoculation

Aseptically inoculate 1 ml of each required dilution into a labelled Petri dish.

4. Pour plate method

Add 15 ml of tempered ($46 \pm 2^\circ\text{C}$) VRBG to each Petri dish. Carefully mix and allow to set. The time elapsing between the addition of MRD and contact with VRBG should not exceed 15 minutes. When the agar is completely set add a further 10-15 ml of tempered ($46 \pm 2^\circ\text{C}$) VRBG onto the surface of the inoculated plate. Allow to set.

5. Incubation

Invert the Petri dishes and transfer to an incubator at $37 \pm 1^\circ\text{C}$ for 24 hours \pm 2 hours.

6. Counting of colonies

Select the plates containing <150 typical colonies and count them either manually or using correctly calibrated automatic equipment. Select at random 5 suspect colonies from each plate for biochemical confirmation.

7. Confirmation

7.1 Typical colonies are pink to red, with or without precipitation haloes or colourless mucoid colonies, with a diameter of 0.5 mm or more. Streak selected colonies onto pre-poured nutrient agar plates. Incubate at $37 \pm 1^\circ\text{C}$ for 24 hours. Select a well isolated colony from each plate for biochemical confirmation.

7.2 Biochemical confirmation: Perform an oxidase test and a glucose fermentation test on each selected colony.

7.3 Oxidase test

Streak a well-isolated colony onto filter paper moistened with oxidase reagent. Do not use a nickel/chromium loop. A positive detection should change colour within 10 seconds.

7.4 Glucose fermentation test

Using an inoculation needle, stab selected colonies into tubes of glucose agar. Incubate at 37°C for 24 h. If a yellow colour develops throughout the contents of the tube, the reaction is positive. Most strains also produce gas.

8. Calculation

If all of the selected typical colonies are oxidase negative, and glucose fermentation positive, then the number of *Enterobacteriaceae* will be the count obtained in 6.

In all other cases the number shall be calculated from the percentage of oxidase negative and glucose fermentation positive colonies in relation to the total number of selected colonies.

$$\text{Number of micro-organisms} = \frac{\Sigma c}{(n_1 + 0.1n_2) d}$$

Σc = The sum of characteristic colonies counted after identification

n_1 = The number of dishes retained in the 1st dilution

n_2 = The number of dishes retained in the 2nd dilution

d = The dilution factor corresponding to the 1st dilution

Round the result calculated to two significant figures.

If there are no confirmed colonies report the result as: <1 per ml or
<1 x the dilution factor per g or
<10 per swab.