

## Isolation and identification of *E. coli* from fish and fishery products by MPN and membrane filter methods

Coliforms are aerobic and facultatively anaerobic Gram-negative, non-spore forming rods that ferment lactose in MacConkey broth or Brilliant green Lactose Bile salt broth at  $37 \pm 1^{\circ}\text{C}$  within 48 h. They exhibit general morphological and biochemical similarities and are grouped together in the family of Enterobacteriaceae. The predominant aerobic bacterial flora of the large intestine of human beings and animals is composed of non-spore forming, Gram-negative bacteria. Members of the coliforms, including faecal coliforms are referred to as indicator organisms, since their presence may indicate the potential presence of pathogens in foods. *E. coli* is highly motile. An elevated temperature is used to differentiate coliforms of faecal and non-faecal origin. Faecal coliforms ferment lactose at  $44.5 \pm 0.5^{\circ}\text{C}$  within 24 h, whereas non-faecal coliforms cannot do so. Faecal coliforms normally grow in the gastro-intestinal tract of humans and other warm-blooded animals. They include members of the genera *Escherichia*, *Klebsiella* and *Enterobacter*. *E. coli* is normally present in faecal contaminated water and foods. They can not withstand the low and high temperature processing treatments. The maximum allowable number of *E. coli* in raw fish and fishery products is 20/g and it should be absent in cooked fishery products. Faecal coliforms in fish processing water should be less than 1/100ml by MPN technique.

Enumeration of total coliforms, faecal coliforms and *E. coli* is done by Most Probable Number (MPN) technique. MPN method makes use of a statistical technique for estimating the probable number of bacteria present per specified unit of material. In this method, specified volumes from at least three serial decimal dilutions of the sample are inoculated to the tubes containing specific media. The number of positive tubes in each set is referred to MacCready's Tables from which the most probable number of bacteria per unit sample (MPN index) can be calculated with a known degree of certainty. Three serial decimal dilutions are generally used and 5 tubes of medium are inoculated from each dilution. Usually the first three dilutions prepared are used but an expected high count may necessitate using higher dilutions.

Three sequential steps are generally followed for the detection of coliform bacteria in water or any other food sample. These include presumptive test for total coliforms, confirmed test for faecal coliforms, and completed test for confirming coliforms of faecal origin (*E. coli*).

### Materials required

1. Lauryl Sulphate Tryptose Broth (LSTB)
2. E.C. Broth
3. Eosine Methylene Blue (EMB) agar
4. Glucose
5. Citrate medium
6. Tryptone water
7. Kovac's reagent
8. 0.04% Methyl red solution
9. 6%  $\alpha$  - Naphthol
10. Potassium hydroxide solution
11. MacConkey broth
12. Physiological saline

### Part A : Presumptive test for the determination of the most probable number of total coliform bacteria

The presumptive test which is specific for the detection of total coliforms is performed by MPN method by adding measured aliquots of sample to lactose fermentation broth containing inverted Durham's tube. The positive test is indicated by the growth of bacteria in broth and collection of gas in Durham's tube. Only coliform bacteria ferment lactose while other enteric bacteria are not.

### Procedure

**Sample preparation:** 25g of seafood sample to be tested is weighed aseptically and homogenized with 225 ml of physiological saline and suitable serial dilutions prepared using saline solution.

To fifteen test tubes containing inverted durhams tubes, first 5 tubes are added with 10 ml double strength (2xLSTB) and the remaining with single strength Lauryl Sulphate Tryptose (1x LST) broth each. The tubes with the media are sterilized at 110°C for 10 minutes, and cooled and used for the inoculation of samples. Ten milliliter of appropriate dilution of the sample is inoculated in to each of the 5 tubes of double strength medium, 1 ml each to 5 tubes of single strength medium and 0.1 ml into the remaining 5 tubes of single strength medium. All the tubes are incubated at 37°C for 24 h. The tubes are observed for gas formation and acid production. This test is called as presumptive test, which answers for total coliforms. In the case of water samples, appropriate volumes may be added as described above without dilution. The development of gas in any of the tubes is considered presumptive evidence of presence of coliform bacteria in the sample. The MPN is estimated by determining the number of tubes positive in each group and referring to MPN table.

**Part B: Confirmed test for the determination of the most probable number of fecal coliform bacteria**

The purpose of the confirmed test is to confirm the presence of bacteria of faecal origin in a sample for which the presumptive test was positive. This is necessary since the positive presumptive may be result of organisms of noncoliform/ non-faecal origin. This is done by subjecting the tubes that are showing the positive result of acid and gas production in presumptive test to confirmatory test. 0.1 ml of the inoculum from each positive tube is transferred to 5 ml of the EC broth tubes containing durham tubes. The E.C. broth tubes are incubated at 44.5 ± 0.5°C for 24 h in the water bath. The tubes are observed for gas formation and acid production. This test is called as confirmatory test for faecal coliforms as only faecal coliforms such as *E. coli* are able to grow at elevated temperature. The MPN is estimated by determining the number of E.C. broth tubes positive in each group and referring to MPN table.

Further, the confirmed test requires streaking inoculums from the doubtful but positive EC broth onto Eosine Methylene Blue (EMB) agar plates, on which *E. coli* produces characteristic colonies. The plates are incubated at 37°C for 48 h and the colonies of *E. coli* on EMB agar plates appear as dark purple with metallic sheen. Suspected typical colonies of *E. coli* are counted, isolated and purified on Trypticase Soya Agar (TSA) plates and further subjected to biochemical tests for confirmation.

**Part C: Completed test for the confirmation of *E. coli***

The positive result obtained in the previous test is confirmed by subjecting the suspected colonies that developed on EMB agar to biochemical tests. The indole test, methyl red (MR) test, Voges – Proskauer (VP) test and citrate utilization tests differentiate *E. coli* from other coliform bacteria. *E. coli* is positive for indole and MR test while, negative for VP and citrate utilization test. The colonies showing typical reaction as *E. coli* are referred back to MPN tubes and MPN numbers expressed by referring to MPN table.

Indole test: \_\_\_\_\_  
 Methyl red test: \_\_\_\_\_  
 Voges-Proskauer test: \_\_\_\_\_  
 Citrate utilization test: \_\_\_\_\_

Biochemical reactions of *E. coli*

Tests	Reactions	Results
Indole	Formation of red ring	Positive
M.R.	Magenta red	Positive

V.P.	Pink colour	Negative
Citrate utilization	No growth (green colour)	Negative

### Media composition

#### ***Lauryl Sulphate Tryptose Broth (LSTB)***

Tryptone	- 20.0 g
Lactose	- 5.0 g
Sodium chloride	- 5.0 g
Dipotassium hydrogen phosphate	- 2.75 g
Potassium Dihydrogen phosphate	- 2.75 g
Sodium lauryl sulphate	- 0.1 g
Distilled water	- 1000 ml
pH	- 6.8 ± 0.2

Sterilize at 110<sup>0</sup>C for 10 minutes.

#### ***E. coli (EC) Broth***

Tryptone	- 20.0 g
Lactose	- 5.0 g
Bile salt Mixture	- 1.5 g
Dipotassium phosphate	- 4.0 g
Monopotassium phosphate	- 1.5 g
Sodium chloride	- 5.0 g
Distilled water	- 1000.0 ml
pH	- 6.9 ± 0.2

Sterilize at 110<sup>0</sup> C for 10 minutes.

#### ***Eosine Methylene Blue (EMB) Agar***

Peptone	- 10.0 g
Lactose	- 5.0 g
Sucrose	- 5.0 g
Dipotassium phosphate	- 2.0 g
Agar	- 13.5 g
Distilled water	- 1000.0 ml
Eosine Y	- 0.4 g
Methylene Blue	- 0.065 g
pH	- 7.2 ± 0.2

Sterilize at 121<sup>0</sup>C for 15 minutes. Cool, shake and then pour into plates.