
EXPERIMENT NO. 15 : MOST PROBABLE NUMBER (MPN)

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15.1 INTRODUCTION

The coliform group comprises all aerobic and facultative anaerobic, gram-negative, nonspore-forming, rod-shaped bacteria that ferment lactose with gas and acid formation within 48 h at 37°C.

The Standard test for the coliform group may be carried out either by the multiple tube fermentation technique (through the presumptive and confirmed phases) described herein or by the membrane filter (MF) technique. Each technique is applicable within the limitations specified and with due consideration of the purpose of the examination.

In multiple-tube fermentation method, the inherent property of bacteria to ferment nutrient broth (like lactose) to produce gas and acid within 48 hours at a temperature 37°C is utilized for detecting the presence coliform bacteria. Subsequently the presence of faecal coliform is differentiated when bacteria on fermenting lactose, produce gas in a relatively shorter period of time and at a relatively higher temperature (24 hours and 44°C). This method requires testing of 3 sets of dilutions; each set containing 5 samples of water of same strength and volume. Table 15.1 shows number of water samples of same strength required in each set together with the standardized quantity of water sample or its dilution per set.

For the multiple-tube fermentation technique, results of the examination of replicate tubes and dilutions are reported in terms of the Most Probable Number (MPN) of organisms present. This number, based on certain probability formulae, is an estimate of the mean density of coliforms in the sample. MPN is by definition related to a sample volume of 100 ml; hence, and MPN of 10 means 10 coliforms per 100 ml of water.

The precision of each test depends on the number of tubes used. The most satisfactory information will be obtained when the largest sample inoculum examined shows gas in some or all of the tubes and the smallest sample inoculum shows no gas in all or a majority of the tubes. Bacterial density can be estimated from table 15.2 using the number of positive tubes in the multiple dilutions. The number of sample portions selected will be governed by the desired precision of the result. However, if the sample is not adequately shaken before the portions are removed or if clumping of bacterial cells occurs, the MPN value will be an underestimate of the actual bacterial density.

**Table 15.1 : Volume of Water Sample Required in
Three Sets of 5 Tubes each**

Set No.	Volume of Water Sample				
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Set - I	10 ml	10 ml	10 ml	10 ml	10 ml
Set - II	1.0 ml	1.0 ml	1.0 ml	1.0 ml	1.0 ml
Set - III	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml

In set-I and II, water sample can be used directly and no dilution is required. However, in set-III, instead of taking 0.1 ml of water sample, in each of the five test tubes, it is desirable to prepare 1 : 10 dilution (using the phosphate dilution water) and then take 1 ml from this, which will be equivalent to 0.1 ml of the original sample.

The test is conducted in two stages – presumptive phase and confirmatory phase, and is summarised below.

(i) **Presumptive Phase**

Incubation of sample for 24 to 48 hours at 37°C using MacConkey broth.	Formation of gas indicates the possibility of presence of coliform bacteria in the water sample.
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(ii) **Confirmatory Phase**

Incubation of positive samples from presumptive test for 48 hours at 37°C using Brilliant Green Bile lactose broth (BGB).	Formation of gas confirms the presence of coliform bacteria in the water sample.
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Test for faecal coliforms can also be conducted by incubating positive samples from presumptive test for 24 hours at 44°C using BGB broth (or EC medium). Formation of gas confirms the presence of faecal coliforms in the water sample.

Objective

- To determine the most probable number (MPN) of coliform bacteria in the given water sample by Multiple-Tube Fermentation Technique.

15.2 PRESUMPTIVE PHASE

The purpose of this test is to determine the possibility of coliforms in the water sample.

15.2.1 Apparatus

- (i) Fermentation tubes (150 ml long, 15 mm ϕ)
- (ii) Durham tubes (25 mm long, 5 mm ϕ)
- (iii) Pipette (calibrated in 0.1 ml increments)
- (iv) Rack (having three rows to hold 5 fermentation tubes in each row)
- (v) Autoclave
- (vi) Incubator
- (vii) Laminar Flow Chamber : It is a working chamber which provides bacteria free-environment.

15.2.2 Culture Medium

MacConkey Broth

It is available in the market with the composition as given below.

	g / litre
Peptic digest of animal tissue	20.0
Lactose	10.0
Bile salts	5.0
Sodium chloride	5.0
Neutral red	0.75

Suspended 40 g of broth in 1000 ml of distilled water, mix thoroughly and heat (if necessary) to dissolve the medium completely. Such prepared medium is called as Single Strength Medium and is used for testing 1.0 ml and 0.1 ml volumes of the water sample.

Dispense 10 ml of the broth and put a Durham tube (in inverted position) in each of the 10 fermentation tubes. One set of five tubes will be used for 1 ml water

sample and another set of five tubes will be used for 0.1 ml sample. Plug the open end of the tubes with cotton and sterilize the tubes for 15 minutes at 121°C in autoclave. Cool the tubes before inoculation.

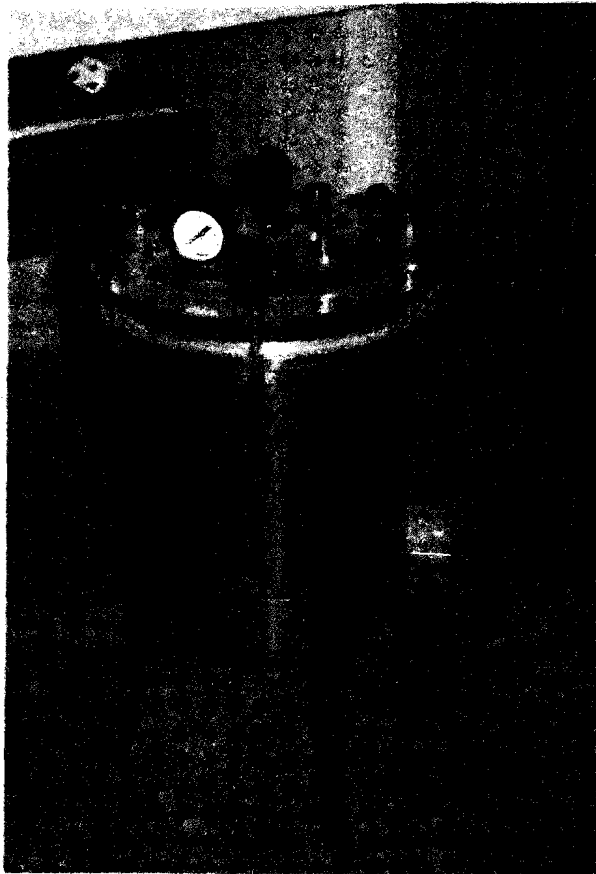


Figure 15.1 : Autoclave

For testing of 10 ml water samples double strength medium is required, and can be prepared by dissolving 80 g of MacConkey Broth in 1000 ml of distilled water. Dispense 10 ml volume of this medium in a set of 5 fermentation tubes and keep a Durham tube (in inverted position) in each tube. Sterilize the tube by autoclaving as mentioned above.

15.2.3 Procedure

- (i) Arrange fermentation tubes in three rows in a test tube rack; each row containing five tubes

Shake sample and dilutions vigorously about 25 times. Inoculate each tube of the set of five with replicate sample volumes as per the scheme shown below. Mix test portions in the medium by gentle agitation. Plug the tubes with cotton.

Set of Fermentation Tubes	Volume of MacConkey Medium	Volume of Water Samples
Set-I (containing 5 fermentation tubes)	10 ml (double strength medium)	10 ml
Set-II (containing 5 fermentation tubes)	10 ml (single strength medium)	1 ml
Set-III (containing 5 fermentation tubes)	10 ml (single strength medium)	0.1 ml (or 1 ml of 1 : 10 dilution)

- (ii) Incubate inoculated tubes at 37°C. After 24 ± 2 h, shake each tube gently and examine it for gas or acidic growth (distinctive yellow colour). The tubes in which gas is formed (in the Durham tube), are considered positive and submitted for performing the confirmatory test. Remaining test tubes (having no

gas formation) are then reincubated for another 24 hours at 37°C and at the end of which, presence of gas is again examined. Those test tube which show the presence of gas are also considered positive. In context to the gas formation, this is pointed out that even if gas is not formed at the end of 24 or 48 hours, it is important to shake the tubes gently to see whether streams of tiny bubbles are coming out of the medium, if so such types of tubes also constitute a positive presumptive test. Schematic diagram showing the details of presumptive test (together with an example) is given below :

Set of Test-tubes	Volume of Sample in Tubes	Position after Incubation for 24 h at 37°C	Position after Reincubation	Decision
Set - I	10 ml 10 ml	+ ve } + ve }		Tubes to be submitted for confirmatory phase
	10 ml 10 ml 10 ml	- ve - ve - ve	- ve } - ve } - ve }	Tubes to be discarded
Set - II	1 ml	+ ve		Tube to be submitted for confirmatory phase
	1 ml 1 ml 1 ml 1 ml	- ve - ve - ve - ve	- ve } - ve } - ve } - ve }	Tubes to be discarded
Set - III	0.1 ml	+ ve		Tube to be submitted for confirmatory phase
	0.1 ml 0.1 ml 0.1 ml 0.1 ml	- ve - ve - ve - ve	- ve } - ve } - ve } - ve }	Tubes to be discarded

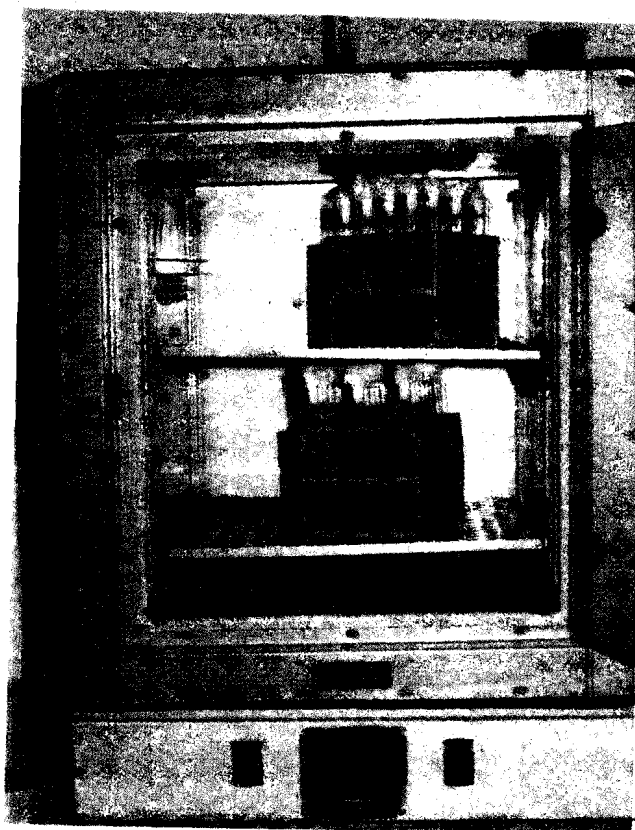


Figure 15.2 : Microbiological Incubator

15.3 CONFIRMATORY PHASE

The purpose of confirmatory test is to confirm the presence of coliform bacteria in the water sample.

15.3.1 Culture Medium

Brilliant green lactose bile broth fermentation tubes are used for the confirmed phase.

Brilliant green lactose bile broth (BGB)

Peptone	10.0 g
Lactose	10.0 g
Bile salt	20.0 g
Brilliant green.	0.0133 g
Distilled water	1 L

Add dehydrated ingredients to water, mix thoroughly, and heat to dissolve. pH should be 7.2 ± 0.2 after sterilisation. Before sterilisation, dispense 10 ml of BGB broth, in 5 fermentation tubes each having an inverted Durham tube. Plug the tubes with cotton and sterilize for 15 minutes at 121°C in autoclave. Cool the tubes before inoculation.

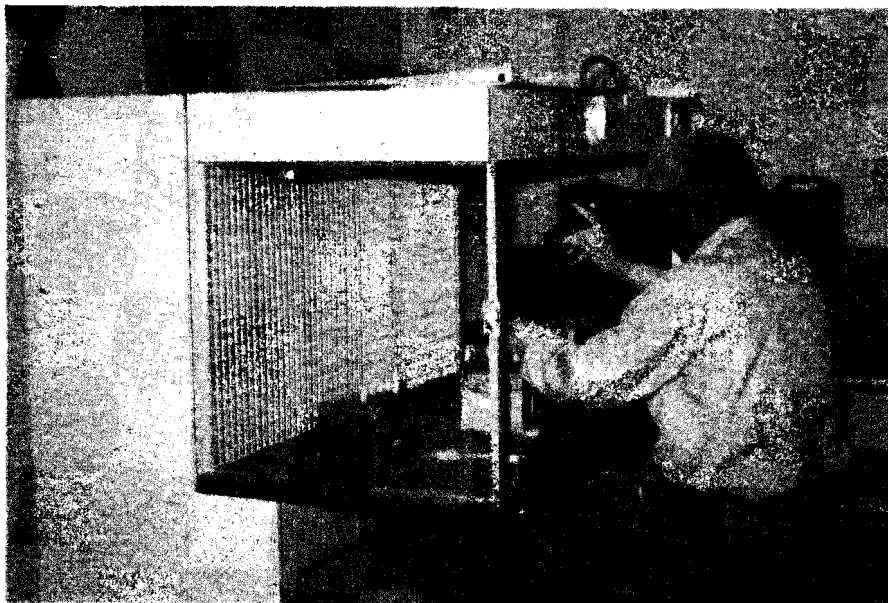


Figure 15.3 : Laminar Flow Chamber (Inoculation in Progress)

15.3.2 Procedure

Submit all primary tubes showing any amount of gas or acidic growth within 24 h of incubation to the confirmed phase. If active fermentation or acidic growth appears in the primary tube earlier than 24-h, transfer to the confirmatory medium, preferably without waiting for the full 24-h period to elapse. If additional primary tubes show acidic growth at the end of a 48-h incubation period, submit these to the confirmed phase.

Gently shake or rotate primary tube showing gas or acidic growth to resuspend the organisms. With a sterile metal loop 3 mm in diameter, transfer one loopful (equivalent to one / two drops) of culture to a fermentation tube containing brilliant green lactose bile broth. Gently shake the tube to mix the culture with BGB broth. Repeat for all other positive presumptive tubes.

Incubate the inoculated brilliant green lactose bile broth tube for 48 hours at 37°C . Formation of gas in any amount in the inverted Durham tube of the brilliant green lactose bile broth fermentation tube at any time within 48 hours constitutes a positive confirmed phase. Calculate the MPN value from the number of positive brilliant green lactose bile tubes as described below.

15.4 RESULT

For example 4 test tubes (as shown below) appeared positive at the end of presumptive test. If in confirmatory test all the four tubes appear + ve, then the result can be summarised as :

Sample Portion (ml)	No. of Positive Tubes out of Five Tubes	
	Presumptive Test	Confirmatory Test
10 ml	2	2
1 ml	1	1
0.1 ml	1	1

MPN for coliform bacteria for 2/1/1 is 9, (from table 15.2). It means 9 coliform bacteria per 100 ml of water sample are present.

Table 15.2 : MPN Index for Coliform Counts by the Multiple-Tube Fermentation Technique for various Combinations of Positive and Negative Results when Five 10-ml, Five 1-ml, and Five 0.1-ml Portions are Used

No. of Tubes Giving Positive Reaction out of			MPN Index	No. of Tubes Giving Positive Reaction out of			MPN Index
5 of 10 ml Each	5 of 1 ml Each	5 of 0.1 ml Each	Per 100 ml	5 of 10 ml Each	5 of 1 ml Each	5 of 0.1 ml Each	Per 100 ml
0	0	0	0	4	2	1	26
0	0	1	2	4	3	0	27
0	1	0	2	4	3	1	33
0	2	0	4	4	4	0	34
1	0	0	2	5	0	0	23
1	0	1	4	5	0	1	31
1	1	0	4	5	0	2	43
1	1	1	6	5	1	0	33
1	2	0	6	5	1	1	46
				5	1	2	63
2	0	0	5	5	2	0	49
2	0	1	7	5	2	1	70
2	1	0	7	5	2	2	94
2	1	1	9	5	3	0	79
2	2	0	9	5	3	1	110
2	3	0	12	5	3	2	140
3	0	0	8				
3	0	1	11	5	3	3	180
3	1	0	11	5	4	0	130
3	1	1	14	5	4	1	170
3	2	0	14	5	4	2	220
3	2	1	17	5	4	3	280
3	3	0	17	5	4	4	350
4	0	0	13	5	5	0	240
4	0	1	17	5	5	1	350
4	1	0	17	5	5	2	540
4	1	1	21	5	5	3	920
4	1	2	26	5	5	4	1600
4	2	0	22	5	5	5	2400