LAB #2
BACTERIOLOGICAL EXAMINATION OF WATER: MOST PROBABLE NUMBER TEST AND MEMBRANE FILTRATION

Routine examination of water for the presence of intestinal pathogens would be a tedious and difficult task. It is much easier to demonstrate the presence of some non-pathogenic intestinal organisms, such as *Escherichia coli* and *Streptococcus faecalis*. These organisms are the normal inhabitants of the human intestine. Therefore, their presence in soil or water may be an indication of fecal pollution. The U.S. Public Health Service selected the coliform group as indicators of fecal contamination in drinking water in 1914. Since that time, coliforms have been used as an official standard for drinking water in the U.S. and have been adopted in many other countries as well.

Coliform organisms can be defined as all aerobic and facultative anaerobic, gram-negative, non-spore-forming, rod shaped bacteria that produce gas upon lactose fermentation. Coliforms (e.g., *E. coli*) are typically nonpathogenic bacteria that may be associated with enteric pathogenic organisms and have been shown to be useful indicators of the presence of fecal contamination. Coliform bacteria occur normally in the intestines of humans and other warm-blooded animals and are discharged in great numbers in human and animal waste. When members of the coliform group are present, other kinds of microorganisms capable of causing disease may also be present.

Coliform bacteria are hardier than disease causing non-spore forming bacteria; therefore, their absence from water is an indication that the water is bacteriologically safe for human consumption. However, they are less sensitive than viruses and protozoan cysts to environmental factors and disinfection. Furthermore, coliform bacteria have been observed to grow or recover in distribution systems, which may provide a false indication of fecal contamination. In spite of its limitations, the coliform group remains a standard indicator of fecal contamination, and in general, experience has demonstrated that bacterial waterborne disease outbreaks do not occur when 100 ml of drinking water tests negative for coliforms.

The two methods most commonly used to detect coliforms in water are the most probable number (MPN) method and the membrane filtration (MF) method.

MOST PROBABLE NUMBER (MPN) TEST

Introduction

The most probable number (MPN) technique is sometimes used in place of standard plate count methods to estimate microbial counts in the environment. The MPN test for coliforms consists of three steps: the presumptive test, the confirmative test, and the complete test. All three tests are necessary to prove that an organism in a water sample is actually a coliform. *In actual practice, when the presumptive and confirmed tests give the same results, then the completed test is generally not performed because of the time it takes.*

For the **presumptive test**, the sample to be assayed is dispersed in an extracting solution and successively diluted, as in the plate count technique. The MPN method relies on the dilution of the population to extinction. The dilutions are used to inoculate 5 – 10 replicate tubes containing a specific liquid media. After incubation, the tubes are scored as positive (+) or negative (-) for growth on the basis of factors such as turbidity, gas production, and appearance or disappearance of substrate. Scoring a tube (+) for growth means that at least one culturable organism was present in the dilution used for its inoculation. The number of positive and negative tubes at each dilution is used to calculate the number of organisms present in the original sample through the use of published statistical MPN tables or computer programs designed to simplify the analysis.

In the **confirmed test**, the contents of the positive presumptive test tubes are used to inoculate a streak plate. The streak plate may contain Levine's EMB agar or mEndo agar. Levine's EMB agar contains methylene blue, which inhibits gram-positive bacteria. Gram-negative lactose fermenters (coliforms) that grow on this medium produce "nucleated colonies" (dark centers). *E. coli* and *Enterobacter aerogenes* can be differentiated on the basis of size and the presence of a greenish sheen. *E. coli* colonies are small and have a metallic sheen, whereas *E. aerogenes* colonies are larger and usually lack the sheen. mEndo agar contains fuchsin sulfate indicator, which makes the identification of lactose fermentation relatively easy. After 24 hr at 35°C, coliform colonies appear green with a golden sheen on mEndo agar, whereas non-lactose fermenting, non-coliforms appear colorless. Other media that can be used for the confirmed test is brilliant green lactose broth (BGLB), Eijkman's medium, and *E. coli* (EC) medium. BGLB broth contains a dye called brilliant green, which is inhibitory to the gram-positive bacteria.

In the **complete test**, colonies that were isolated on the agar are inoculated back into LST broth and observed for the production of gas and acid after 24 – 48 hours.

Reference:

Maier, R.M.; Pepper, I.L.; Gerba, C.P. 2000. *Environmental Microbiology*. Academic Press, San Diego, CA.

MPN Procedure

First Period (Presumptive Test)

Materials:

3 Durham tubes of DSLB (double-strength lactose broth)
6 Durham tubes of SSLB (single-strength lactose broth)
Incubator at 35°C

Methods:

1. Set up three DSLB and six SSLB tubes. Lable each tube according to the amount of water that is to be dispensed to it:10 mL, 1.0mL, and 0.1 mL, respectively.
2. Mix the bottle of water to be tested by shaking 25 times.
3. With a 10-mL pipette, transfer 10 mL of water to the DSLB tubes.
4. With a 1.0-mL pipette, transfer 1 mL of water to each of the middle set of SSLB tubes and 0.1 mL to each of the last three SSLB tubes.Incubate the tubes at 35°C for 24 – 48 hours.

Second Period (Confirmed Test)

Materials:

Levine's EMB agar
mEndo agar
Inoculation loop
Gas burner

Methods:

1. Record the number of tubes with 10% or more gas.
2. Determine MPN using the MPN table. *Remember that the MPN table gives numbers of coliforms per **100 ml**.*
3. Select one positive lactose broth tube from the presumptive test and streak one plate of Levine's EMB agar.
4. Incubate the plate at 35°C for 24 hours.

Third Period (Confirmed Test cont.)

1. Look for typical coliform colonies.
2. If no coliform colonies are present, the water is considered safe to drink.

Questions

1. Why are coliforms used instead of directly testing for pathogens? 3 pts
2. List four criteria for an ideal indicator organism. Do coliforms satisfy these criteria? 2 pts
3. Name the four genera of bacteria designated as coliforms. Name the two genera of bacteria designated as fecal coliforms. 2 pts
4. Name four waterborne diseases and the microbe associated with each. 2 pts

MEMBRANE FILTRATION TECHNIQUE

Introduction

The membrane filtration method is used for the detection and quantification of bacteria in water. It serves as an alternative to the Most Probable Number (MPN) procedure for microbiological analysis of water samples. The concentration of larger samples on a membrane filter is a key benefit of the technique over the MPN procedure, as well as over the pour plate and spread plate techniques. Many industry and U.S. EPA test procedures require samples of 100 ml of water or more to be analyzed for the presence of bacteria. Membrane filtration also requires fewer test tubes and less labor compared to the MPN procedure.

A water sample is passed through a membrane with a pore size of about 0.45 μm, which traps bacteria on its surface. The membrane is then placed on a thin layer of nutrient medium required for the growth of the desired bacterium. The success of the method depends on the use of effective differential or selective media, several examples of which are listed below.

- Endo medium can be used for coliforms. Within 24 hours of incubation at 35°C, coliforms form red colored colonies with a golden metallic sheen. Generally, red, pink, blue, white, or colorless colonies lacking the sheen are considered to be non-coliforms.
- Modified Endo medium (mEndo) can be used for coliforms. Within 24 hours of incubation at 35°C, coliforms form bright green colonies with a golden/green sheen. Any colony that is not green with a golden sheen is considered to be non-coliform bacteria.
- Kenner fecal (KF) streptococcus broth can be used for fecal *streptococci*. Within 48 hours of incubation at 35°C, *streptococci* form red colonies. Other colors are non-coliforms.
- Membrane fecal coliform (mFC) media can be used for fecal coliforms. Within 24 hours of incubation at 44.5°C, coliforms form blue colonies. Any shade of blue is considered to be a fecal coliform, while colonies of any other color are considered to be non-coliforms.

Advantages of using the membrane filtration technique include:

1. Permits the use of large sample volumes.
2. Reduces preparation time, as compared to many traditional methods.
3. Allows for the isolation and enumeration of discrete colonies of bacteria.
4. Provides presence/absence information within 24 hours.
5. It is an effective and accepted laboratory technique used to monitor drinking water in government laboratories.
6. It is useful for bacterial monitoring in the pharmaceutical, cosmetics, electronics, and food and beverage industries.
7. Allows for the removal of bacteriostatic or bactericidal agents that would not be removed in the pour plate, spread plate, or MPN techniques.

A disadvantage of this technique is that it cannot be used with highly turbid waters since the filter may clog.

Reference:

Maier, R.M.; Pepper, I.L.; Gerba, C.P. 2000. *Environmental Microbiology*. Academic Press, San Diego, CA.

MF Procedure

First Period

Materials:

Forceps
Ethyl alcohol
Sterile agar plates (mFC and mEndo agar)
Sterile 0.45 μm pore, 47-mm diameter membrane filters
1-Liter flask
Filter unit, graduated with top
Nanopure water
Vacuum hoses for the filter
Vacuum source

Methods:

1. Disinfect the filtration apparatus by flaming the surface.
2. With alcohol flamed forceps, place a sterile membrane filter in the center with the grid side up.
3. Place the funnel on top, being careful not to tear the filter.
4. Carefully pour about 10 ml of nanopure water on top of the filter.
5. Pipette 10 ml of the water sample into the funnel.
6. Gently apply the vacuum. Just as the liquid level approaches the filter, rinse the sides with a small amount of nanopure water and let the vacuum draw all of the water through the filter.
7. Unclamp the funnel top with the vacuum still applied. With sterile forceps, remove the filter and carefully roll onto the mEndo / mFC agar. Avoid trapping bubbles of air. Label the plate "10 ml."
8. Repeat steps 1 – 7, this time using 100 ml of water sample instead of 10 ml, and label accordingly.
9. Incubate the mEndo agar plates at 35°C for 24 hrs.
10. Incubate the mFC plates at 44.5°C for 24 hrs.

Second Period

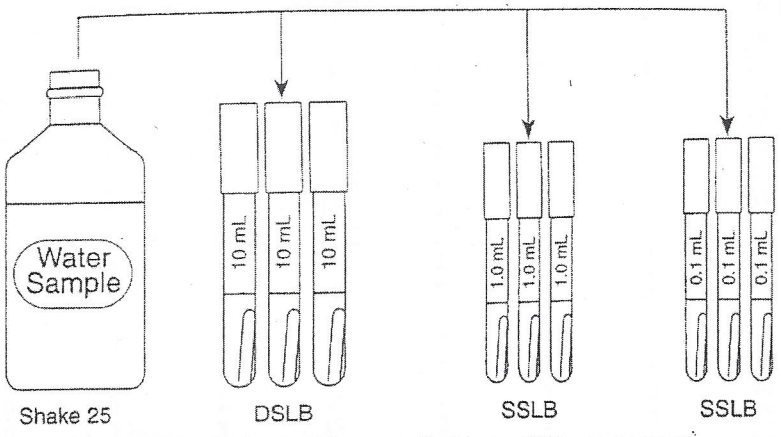
1. Examine the mEndo agar plates. Coliform colonies are green with a bright golden metallic sheen. Colonies without the sheen are non-coliforms. Count the coliform colonies.
2. Examine the mFC plates in the same fashion. Fecal coliform colonies are blue, regardless of shade. All others are not coliforms. Count and record the results.
3. Coliform results are usually reported as colony forming units **(CFU)/100 ml**.

Questions

5. List an advantage and a disadvantage of the membrane filtration technique. 2 pts
6. Do coliforms, even fecal coliforms, always mean that there is fecal pollution? Why or why not? 3 pts
7. Discuss the differences between coliforms and fecal coliforms. 3 pts
8. Describe the U.S. EPA coliform standards for drinking water. 3 pts

Presumptive Test

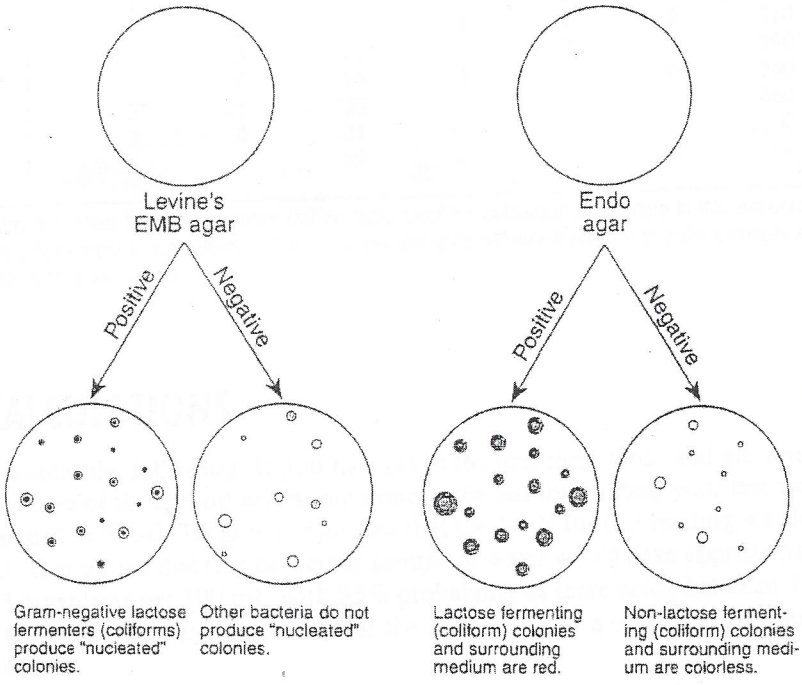
1 Transfer the specified volumes of sample to each tube.
Incubate 24 h at 35°C. Go to 2.



2 Examine the tubes and record the ones that have 10% gas or more.
Report your results. Go to 3.

Confirmed Test

3 Choose any positive tube from above, as indicated by the presence of gas trapped in the inner tube, and streak a plate of Levine's EMB agar and Endo agar.
Incubate 24 h at 35°C. Go to 4.



4 Examine the plates for typical coliform colonies as described beneath each illustration above. Report your results.

Figure 9-1 Procedure for performing an MPN test (presumptive and confirmed tests) for coliforms o water samples as described in this experiment.

Number of Positive Tubes in Dilutions				Number of Positive Tubes in Dilutions			
10 mL	1 mL	0.1 mL	MPN per 100 mL	10 mL	1 mL	0.1 mL	MPN per 100 mL
0	0	0	3	2	0	0	9.1
0	1	0	3	2	0	1	14
0	0	2	6	2	0	2	20
0	0	3	9	2	0	3	26
0	1	0	3	2	1	0	15
0	1	1	6.1	2	1	1	20
0	1	2	9.2	2	1	2	27
0	1	3	12	2	1	3	34
0	2	0	6.2	2	2	0	21
0	2	1	9.3	2	2	1	28
0	2	2	12	2	2	2	35
0	2	3	16	2	2	3	42
0	3	0	9.4	2	3	0	29
0	3	1	13	2	3	1	36
0	3	2	16	2	3	2	44
0	3	3	19	2	3	3	53
1	0	0	3.6	3	0	0	23
1	0	1	7.2	3	0	1	39
1	0	2	11	3	0	2	64
1	0	3	15	3	0	3	95
1	1	0	7.3	3	1	0	**43**
1	1	1	11	3	1	1	75
1	1	2	15	3	1	2	120
1	1	3	19	3	1	3	160
1	2	0	11	3	2	0	93
1	2	1	15	3	2	1	150
1	2	2	20	3	2	2	210
1	2	3	24	3	2	3	290
1	3	0	16	3	3	0	240
1	3	1	20	3	3	1	460
1	3	2	24	3	3	2	1100
1	3	3	29	—	—	—	—

Table 9-1 Most Probable Number (MPN) table used for evaluation of the data in this experiment, using three tubes in each dilution. The value printed white on black is referred to in the example in the Calculations section on.

CALCULATIONS

Consider the following: If you had gas in the first three tubes and gas only in one tube of the second series, but none in the last three tubes, your test would be read as 3-1-0. Table 9-1 indicates that the MPN for this reading would be 43. This means that this particular sample of water would have approximately 43 organisms per 100 mL with 95% probability of there being between 7 and 210 organisms. Keep in mind that the MPN of 43 is a statistical probability number.