ENV H 433 LABORATORY EXERCISE 1

Multiple tube fermentation and presence/absence methods to detect total coliforms, fecal coliforms and *Escherichia coli*

I. LABORATORY GOAL

To determine concentration of total coliform, fecal coliform and *E. coli* in samples of surface water and drinking water by using selective media with multiple tube fermentation techniques and presence/absence techniques.

II. ASSIGNED READINGS

Standard Methods (SM) 9221) (see readings on EH433 website)
USEPA Method 1104 (see readings on EH433 website)
Difco™ & BBL™ Manual (DBM) for BGLB (on-line)

III. LABORATORY SCHEDULE

Table 1. Tasks by day of experiment.

Day	MPN	P/A
1 Mon	Dilute samples Inoculate MPN tubes	Inoculate P/A tubes
2 Tue TA	Refrigerate	Refrigerate
3 Wed	Read MPN tubes @ 48 h Inoculate BGLB tubes	Read P/A tubes
4 Thru TA	Refrigerator BGLB	
5 Fri	Read BGLB broth	

IV. BACKGROUND

A. Indicator organisms

Indicator organisms (aka indicator bacteria) are non-pathogenic organisms that inhabit the intestines of warm-blooded animals (such as humans) and are present in feces. While they are not themselves pathogenic, they are naturally present in all feces at much higher concentrations than pathogenic organisms, and are therefore easier to detect. The presence of indicator organisms reveals that fecal contamination has occurred and pathogens associated with feces may also be present. Present standards and regulations for sanitary water quality are generally expressed in terms of concentrations of indicator organisms.

Three common indicator organisms will be studied in this laboratory: total coliform, fecal coliform, and *Escherichia coli*. All three of these indicator organisms are commonly found in the feces of humans and higher animals. The term 'coliform' is taxonomically meaningless, yet provides a methodologically useful category because coliforms may be monitored rapidly and easily due to their biochemical properties. "coliform" species include all of the lactose fermenting species of the family Enterobacteriaceae. Those species that do not ferment lactose or ferment slowly are not included even though some are enteric pathogens in humans and other animals and many of which exist naturally in the environment. Thus, the ability to ferment lactose is a characteristic of considerable diagnostic importance in distinguishing among the various groups of enteric bacteria.

A further distinction is made based on optimal temperatures for growth. Coliform originating from the intestinal track are "thermotolerant", and this trait is used to differentiate between total coliform and fecal coliform, as outlined in Standard Method 9221 E. The fecal coliform test is

now widely applied to surface and ground water, sewage treatment systems and general monitoring of natural waters for sanitary quality, including recreational and shellfish waters, and water quality standards have been developed based on fecal coliforms concentrations. However, the total coliform test is still used in the examination of potable waters because coliform bacteria of any kind are considered undesirable and cannot be tolerated in finished drinking water. Today, both the total and fecal coliform tests are used in the examination of drinking water.

As for the third indicator organism, *E. coli* is a taxonomically defined species. However, one must remember when interpreting results that the species *E. coli* includes both enteric pathogenic and non-pathogenic strains. Thus, positive results must still be communicated only as a potential indicator of feces contamination, and not as a positive indication of pathogenic presence.

B. Methods

Using growth media and conditions targeting specific organisms, the indicator organisms can be detected and/or quantified. We will be using some of these methods in this laboratory.

1. Multiple Tube Fermentation

In the multiple tube fermentation method (Standard Method 9221B), the coliform bacteria are quantified by their ability to grow and produce gas in lactose-containing liquid medium under specified incubation conditions. This technique consists of three successive steps: Presumptive, Confirmed and Completed. For the Presumptive Test, replicate dilutions of the sample are inoculated into fermentation tubes of lactose or lauryl tryptose broth (LTB) and incubated at 35°C for 48 hours. By recording the dilution tubes that resulted in positive growth (indicated by gas production), a concentration of organisms can be calculated that has the highest probability of being correct. This concentration is called the "Most Probable Number" or "MPN". Due to the complex nature in solving the MPN equation, tables have been generated to interpret the results. However, in this age of advanced computing, it is now possible to solve the MPN equation directly – should this be desired.

By challenging, organisms from all positive MPN tubes (those with growth plus gas) with a new media and growth conditions, further confirmation of their identity can be established. This is done in the "Confirmed Phase" using fermentation tubes with brilliant green lactose bile broth (brilliant green lactose bile 2% broth – BGLB broth). Tubes showing both growth and gas are considered positive Confirmed tubes.

For the Completed Test, organisms from positive Confirmed tubes are isolated in pure culture on agar plates of differential/selective media (mEndo or eosin methylene blue agar) and then tested for: (1) growth and gas production in fermentation tubes of lactose or LTB incubated at 35ºC for 48 hours; and a negative reaction in the Gram stain. For a positive Completed Test, the organisms must show growth plus gas production in the fermentation tubes and be Gram negative. The Completed test is rarely done in practice due to its complexity and the time required to complete the test.

2. Presence-Absence Tests for Indicator Organisms

For drinking water, where coliforms and *E. coli* should be largely or always absent, respectively, testing for these organisms is often done in a "presence-absence" (P/A) format using a larger sample volume. Because regulations state that coliforms and *E. coli* must have concentration of less the 1 organism per 100-ml volumes of drinking water, a single 100-ml volume is examined for the presence or absence of the organisms. The test is repeated on multiple 100-ml samples on a regular basis in order to determine if coliforms and *E. coli* are regularly absent.

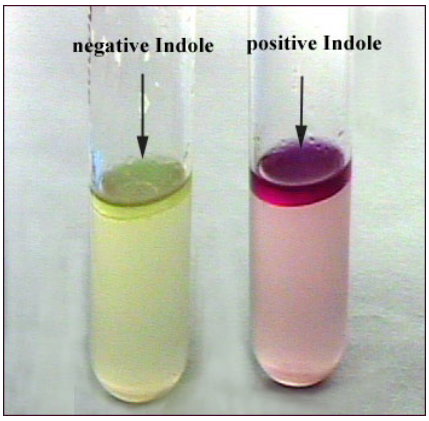
3. *Escherichia coli* tests

In recent years there has been an increased interest in specific detection of *E. coli*. An early approach to this effort was the use of four biochemical tests to separate *E. coli* from other lactose fermenting.

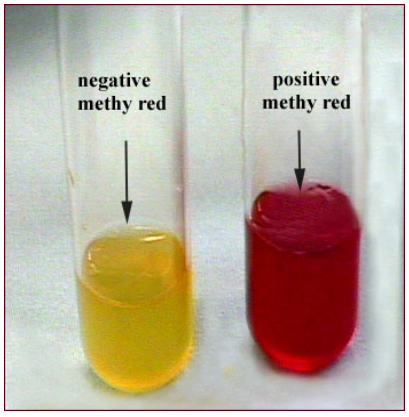
Enterobacteriaceae:

Indole test – detects indole production from tryptophane.

E. coli is positive (+); many other coliforms are negative (-).



Methyl Red test – detects acid production in the medium; intended to distinguish between the type of fermentation (mixed acid versus butylene glycol).

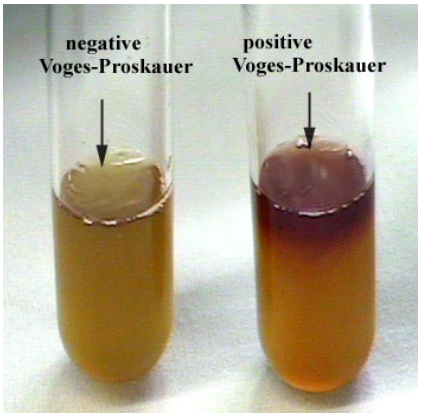


E. coli is (+) and some other coliforms are (-).

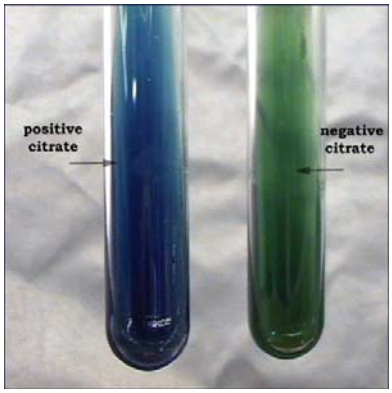
Voges-Proskauer test – detects acetoin, an intermediate in the butylene glycol pathway.

Acetoin is oxidized to diacetyl under alkaline conditions in the presence of air, and when reacted with creatine, it forms a pink color.

E. coli is (-) and some other coliforms are (+).



Citrate utilization as sole carbon source.
E. coli is (-) and many other coliforms are (+).



In interpreting this series of four tests, called the IMViC tests, *E. coli* is typically ++-- and *Enterobacter aerogenes* is typically --++. Other coliforms give different reaction patterns and are designated "intermediates". However, the IMViC reactions have been found to be imperfect in speciation of *E. coli*, and subsequently other approaches to *E. coli* and coliform speciation have been developed.

One additional approach to coliform speciation is the use of rapid, commercial, biochemical test kits designed to carry out several biochemical tests simultaneously in incubation periods of 4 to 24 hours. The results of each test are scored as (+) or (-) and assigned a number based on the relative reliability of the test. These results constitute a "code" which is compared against a database of accumulated reaction codes for nearly all of the medically important *Enterobacteriaceae*. The code that is identical or closest to the organism tested is taken as its identity at the genus and species level.

An alternative approach to the simultaneous detection and quantification of coliforms and *E. coli* in water and other samples is the use of a single property or biochemical test in addition to lactose fermentation to definitively identify *E. coli* among other coliforms. One such property is the presence of the enzyme β-glucuronidase, which is found only in *E. coli* and some *Salmonella* and *Shigella*. A rapid test for this enzyme has been developed in which the β-glucuronidase substrate MUG (4-methylumbelliferyl-beta-D-glucoronide) is incorporated into a coliform medium. If *E. coli* is present, the MUG is hydrolyzed to yield a fluorogenic product (4-methylumbelliferone) whose fluorescence can be readily seen by shining a long wavelength UV light onto the culture. One commercially available medium and test system for *E. coli* and coliforms uses defined substrates (ONPG) for β-galactosidase and MUG for β-glucuronidase in a mineral salts solution to simultaneously detect and/or quantify coliforms and *E. coli*, respectively, by characteristic color changes resulting from hydrolysis of the defined substrates. This commercial test is available in several formats, including multiple tube or multiple well and is known as Colilert®. It is now widely used in bacteriological analysis of drinking water.

General Laboratory comments

Sample Dilution (general)

1. Mix the original sample by shaking 25 times before diluting.
2. To make 10-fold dilutions, pipette 1.0 ml of sample into a 9.0 ml dilution blank. Place the cap on the dilution tube and mix on the vortex mixer for at least 15 seconds.
3. Repeat this operation, using a new pipette, until the desired dilutions are complete (as indicated by instructor)
4. Always use aseptic technique throughout.
5. **You must have on your lab coat, gloves, and full-face shield before turning on UV lamp**

V. MATERIALS

Multiple Tube Fermentation

Presumptive Phase (Day 1)

1. Lake Washington water, primary clarifier effluent, or treated wastewater effluent samples. Each group will be assigned one of the water samples for evaluation. (1 liter sample per group)
2. Dilution tubes: 5 dilution tubes, 9.0 mL of pH 7.2 sterile phosphate buffer in each tube
3. MPN tubes with 10 mL of LTB for Presumptive tests: 5 tubes per dilution, 25 tubes total + 2 tubes for Positive Control and 2 tubes for Negative Control
4. 10-mL serological pipettes (sterile, individually wrapped), and 10-mL pipette pump (green) or pipette bulb (4 pipettes per group)
5. 2-mL serological pipettes (sterile, individually wrapped), and 2-mL pipette pump (blue) or pipette bulb (10 pipettes per group)
6. Air incubator – set at 35 ºC.
7. Controls
8. 500 mL Nalgene bottle containing Positive Control (1 per class)
9. 100 mL Nalgene bottle containing Negative Control (sterile phosphate buffer solution) (1 per group)
10. Test tube rack

Confirmation Phase (Day 3)

1. MPN tubes containing BGLB broth (20 tubes of BGLB broth per group)
2. Metal inoculation loops
3. Water bath set at 44.5 ºC
4. Test tube rack

Presence/Absence (Day 1)

1. Drinking water sample from tap in the building provide
2. Prepackaged tubes of Colilert dry media
3. Pre-sterilized 120 ml polystyrene bottles with sodium thiosulfate to quench chlorine residual in sample
4. Sterile 100 ml graduated cylinders (4/class)

1. Drinking water sample: obtain from a tap in the building.
2. Pre-packaged tubes of Colilert dry media
3. Pre-sterilized 120-mL polystyrene bottles containing sodium thiosulfate (to quench chlorine residual in sample)
4. Sterile 100-mL graduated cylinders (4 per class)

VI. PROCEDURE 2 experiments to do this week

1) Multiple Tube Fermentation Technique

Part 1 – Presumptive Phase

Mon Day 1

1. Label your tube rack with your group number.
2. Arrange fermentation tubes (MPN tubes) of LTB in a test tube rack in 5 rows. These will be your 5 replicate series.
3. Label the tubes with their dilution.
4. Mix the original sample by shaking 25 times before diluting.
4. Using a 2-ml pipette, inoculate 1-ml volumes of each sample dilution to be tested into the 5 replicate tubes designated for that dilution.

5. To make 10-fold dilutions, pipette 1.0 ml of sample into the next 9.0 ml dilution blank IN THE SAME ROW. Place the cap on the dilution tube and mix on the vortex mixer for at least 15 seconds.
6. Repeat this operation, using a new pipette, until the desired dilutions are complete (will vary based on the sample source, check with the instructor).
7. Inoculate 2 additional MPN tubes using the Positive Control sample and 2 additional MPN tubes using the Negative Control sample.
8. Incubate the tubes in a 35ºC air incubator.

2) Presence/Absence

Day 1

1. Label two P/A bottles with your assigned sample number. type and group

2. Using aseptic techniques, break the seal on a sterile sample bottle.

3. Using the 100-mL graduated cylinder designated for your add 100 mL of sample bottle

4. Carefully remove on package of Colilert^{R} media, do not open other packages in the group
5. Remove lid from sample bottle while holding media package over the bottle, break open the media package at score line and empty into bottle. **Take care not to touch the media or bottle to prevent contamination**

6. Cap bottle and mix by inverting until media is nearly dissolved

7. Place the bottle in 35 ^{0}C air incubator

Tue TA refrigerates tubes

Wed Day 2

1. Gently shake the rack of tubes back and forth several times to release gas in positive tubes.
2. Record the presence of growth (turbidity or cloudiness) and gas (look in the small inverted tube) for all tubes
3. Score tubes showing both as Presumptive positive.
4. In a rack, arrange and label enough fermentation tubes with brilliant green lactose bile broth (BGLB broth) and for all Presumptive positive tubes.

5. For each MPN tubes that are Presumptive positive, insert a sterile loop into the broth of the positive tubes to a depth of >1 inch to wet the end. Transfer the organisms on the applicator to a fermentation tube with BGLB broth.
6. Ethanol rinse and flame loop. Allow loop to cool before re-using.
7. Repeat starting at step 2 for each positive Presumptive tube, using a flame sterilized loop for each.
8. Incubate the BGLB broth tubes in a 35ºC air incubator

Fri Day 3

1. Examine the BGLB broth tubes at 48 +/- 3 hr for growth plus gas.
2. Tubes showing both are scored as Confirmed positive for total coliforms.
3. Calculate the Most Probable Number of total coliform, fecal coliform, and *E. coli* according to the procedures described in Standard Method 9221C.
4. Discuss and share your results with the class before you leave.

Tue TA refrigerate Colilert

Day 2

1. Remove bottles from refrigerator

2. Compare color of your bottle to the Colilert^{R} color comparator bottle and score your sample
3. **You must have on lb coat, gloves, and full-face Shield before you turn on UV lamp**.
   Hold long wave length UV lamp ~ 6 inches from your sample bottle. If the bottle fluoresces blue then it is positive for *E. coli.*

VII. WRITTEN LAB EXERCISE

Instructions. Use a laboratory notebook.

- Each student turns in a separate assignment (i.e. not to be completed with your lab partner(s))
- Details laboratory reports are not required. Number your answers to the questions below.
- For short-answer questions, answer questions in full sentences so that the question is understood.
- Answer all questions using your own words. Direct copying, even of a few sentences, constitutes plagiarism.
- Brevity is a learned skill that is critical to communication of public health hazards.

Questions can generally be answered in one or two sentences. Excessively long answers will be returned (un-graded) with editing suggestions to reduce wordiness.

1. Prepare a table summarizing the P/A results for all groups.
2. Prepare a bar graph showing your lab group's MPN data; show the 95% confidence intervals as error bars on the graph. (Remember to include axis labels with units, a legend (if necessary), and a title (e.g. "Lab 1 – Group 3 MPN of indictor organisms in Lake Washington samples").
3. Consider the results of replicates (e.g. are they identical or does one replicate fall within the 95% confidence limit of the other replicate)? Is this what you would expect? Explain, in terms of expected variation within the experimental design.