

## **Lab Activity #14 - Bacteriological Examination Of Water and Milk**

(Adapted from Lab manual by Dr. Diehl)

Some of the diseases that humans can contract from drinking polluted water include typhoid, dysentery, cholera and hepatitis. Ideally, the sanitary examination of water for human consumption would be to search for the pathogens transmitted from host to host via polluted water. This is impractical since the pathogens may be few in number, they may have a short life span outside the host's body and, if present, they may be overgrown by other bacteria during laboratory cultivation.

It's been demonstrated that human intestinal pathogens are present in water that has been contaminated with feces and water that has not been polluted with intestinal discharges is generally considered safe for consumption. Based on the above facts, no effort is made to detect pathogens in the routine examination of water, but instead the routine test checks for the presence of normal intestinal flora which indicates that the water has been polluted and is potentially hazardous.

Various groups of bacteria are always found in the intestine and feces. These are the fecal streptococci (enterococci); spore-forming, H<sub>2</sub>S-producing anaerobes; and the coliforms. Among these, the coliform bacteria are the most widely accepted indicators of water pollution. More important, the absence of coliforms in water indicates that intestinal pathogens are probably not present and the water is considered safe for consumption.

Under certain circumstances, a water supply may contain coliforms without containing pathogens. However, the contamination from combined sewage of many persons will eventually contain the feces of one infected person. In sewage from large cities, one pathogen usually exists for every 10 million coliforms.

When harvesting water for bacteriological analysis, the sample must be collected in sterile containers and must be representative of the supply from which it is taken. Contamination of the sample must be avoided before and after collection and it must be tested as promptly as possible. If there is delay in testing the sample, it should be stored between 0°C and 10°C.

The standard method for the bacteriological examination of water involves three tests which will result in the identification of coliforms if they are present in the water sample. The three tests are (1) presumptive test, (2) confirmed tests, and (3) completed test. The identification of coliforms by these tests is based on the ability of these bacteria to ferment lactose with the production of acids and gas.

The above are qualitative tests in that the results indicate what kind of bacteria are present, but reveal nothing concerning the number of bacteria present. The number of bacteria in a water sample is generally determined by a plate count technique. Although the sanitary quality of the water is generally judged on the basis of the coliform content, satisfactory water usually contains no more than 100 bacteria per ml.

A self-contained, pocket-sized water tester for detecting total coliform populations in water containing more than 10-15 coliforms per ml is commercially available from Millipore Corporation, Bedford, Mass. 01730. The pocket tester is composed of a rectangular culture surface measuring approximately 2X4 cm. The culture surface is contained in a plastic reservoir which will hold 18 mls of water. The rectangular culture surface will absorb one ml of water, thus causing the number of bacteria in one ml of water to adhere to the surface. The surface contains a differential and selective medium for coliforms, so when incubated the number of colonies can be counted and the number of coliforms per ml of water tested can be determined. Since statistically valid results occur only with water samples containing 10 or more coliforms per ml, potable water cannot be assayed with this device. Also, this tester is not an approved substitute for the standard method for bacterial examination of water. The device is convenient for field-testing waterways, lakes and ponds, as well as sewage outflows in rivers and streams.

WORK FOR THIS EXERCISE:

### **I. Qualitative Examination of Water**

#### **A. Presumptive test for coliform group:**

1. The water sample must be shaken thoroughly to insure a homogenous suspension of microbes. A standardized procedure is described as shaking the sample 25 times, in the space of one foot, in an arc of 90° in seven seconds.
2. With a sterile pipet, aseptically inoculate a tube with 2x double strength lactose fermentation broth with 2 ml of the water sample.
3. Incubate these tubes at 37°C for 24 hours. If at the end of this time, acid and gas have formed, streak on Endo or E.M.B. agar from any tube showing gas (10% in Durham fermentation tube). If the presumptive test is negative after 24 hours at 37°C, re-incubate for an additional 24 hours. If no acid or gas is formed after this second incubation period, it is assumed that no coliforms were present and the test ends here. The water is considered safe for consumption.

#### **B. The confirmed test:**

1. Incubate the Endo or E.M.B. streak plates at 37°C for 24 hours.
2. After incubation, examine plates for typical colonies suggestive of coliform bacteria (brick red colonies on Endo or dark colonies with metallic gold-green sheen on E.M.B.).
3. If no typical colonies are present, the test ends here and the water is considered safe. This also indicates the presumptive test was a "false positive" and the lactose fermentation was due to the presence of some non-coliform bacteria. If typical colonies are present, you must perform the completed test.

C. The completed test:

1. Select a typical colony on the Endo or E.M.B. plate and transfer it to a tube of lactose and a nutrient agar slant and incubate both at 37°C for 24 hours.
2. After incubation, prepare a Gram stain from the agar slant and examine the lactose broth for evidence of fermentation. A gram-negative rod which ferments lactose with acid and gas production is confirmed evidence that the organism isolated is a member of the coli-enterobacter group.

**II. Quantitative Examination of Water**

- A. Label two TSA plates with water-50 µL and water 500µL.
- B. Pipet the appropriate amount of water onto the two plates, and spread with a flamed glass spreader. Incubate the plates at 32°C for 48 hours.
- C. After incubation, count the number of colonies on the plates and determine the number of viable bacteria per mL of original sample.

**III. Qualitative and quantitative examination of water by the membrane filter technique**

The instructor will demonstrate this technique.

**IV. Total Coliform Determination Using the Millipore Coli-Count Water Tester**

A. Testing still waters:

1. The Coli-Counter is contained inside a sterile plastic envelope. Tear the envelope at the handle end of the Coli-Counter, but do not remove it.
2. Grip the envelope and case tightly and withdraw the rectangular culture surface from the case.
3. Completely submerge the rectangular culture surface in the water for 30 seconds, but do not let the water contact the skin (contamination from you skin may be revealed in the analysis if water got on your skin).
4. Withdraw, give one good shake and return the culture surface into its case.
5. Discard the plastic envelope, label the case and incubate for 18-20 hours at 35°C.
6. Interpretation and analysis of results are presented below in Part D.

B. Testing flowing waters:

1. Remove the Coli-Count from the plastic envelope, then remove the culture surface.
2. Obtain a representative sample of water in the clear plastic case. Pour off excess water until the meniscus is at the upper mark of the case.
3. Insert the culture surface and agitate for 30 seconds. Remove the culture surface, discard the water and return the surface to the case.
4. Incubate as described above.

C. Dilution techniques:

Often bacterial population densities are so great that one must dilute the sample to be analyzed. A rapid 1:10 dilution can be achieved by adding water to be analyzed (test water) to the clear plastic Coli-Counter case to the lower mark (an approximate volume of 1.8 ml), then add sterile water to the top mark (approximately 18 ml total volume). Put the rectangular culture surface in the diluted water for 30 seconds. Remove culture surface, discard water, shake both surface and case once, restore surface to case and incubate at 37°C for 24 hours.

D. Analyzing results:

The optimum range of colonies for counting should be from 20 to 200. Coliform colonies appear as varying shades of blue ranging from light blue-green to dark blue. Count as coliform only those colonies in this color range. The number of colonies counted correspond to the number of coliforms per ml of water tested.

#### **IV. Agar Plate Count Of Bacteria In Milk**

The most widely used method for determining the bacterial content in milk is the standard plate count technique. Dilutions of the milk sample are prepared in sterile distilled water and aliquots of these dilutions are plated in tryptone-glucose-yeast extract agar also known as Plate count agar. The plates are incubated between 32°C-35°C for 48 hours. Plates containing between 30 or 300 colonies are counted and the number of bacteria per unit volume of milk is calculated. It should be realized that this method gives only a close approximation of the actual number of bacteria since many forms exist in chains or clusters of cells. The expression, standard plate count, is used in preference to bacterial count since a single colony can arise from a group of cells rather than from a single cell.

The various grades of milk are determined by the bacterial content at the time of delivery to the consumer. For example, grade A pasteurized milk cannot have a total plate count in excess of 30,000 per ml and the coliform count cannot exceed 10 per ml. Grade B pasteurized milk can have a total count up to 50,000 but again the coliform count must not exceed 10 per ml. Certified milk is produced under the supervision of a medical milk commission and the total count cannot exceed 500 for pasteurized milk and the coliform count must be less than 1 per ml.

Several other techniques are available for estimating the number of microbes in milk. One is the reductase test and is based on the fact that microbes growing in milk consume oxygen and produce reducing substances (such as NADH and NADPH). In the presence of redox indicators like methylene blue or resazurin, the change in the redox potential of the milk can be monitored and can be related to the number of bacteria present. Methylene blue in the oxidized form is blue, but is colorless in the reduced state, while resazurin is slate blue in the oxidized form, pink when partially reduced and colorless when completely reduced. The indicators are added to the milk and the time required for the color to disappear is recorded. In general, the more bacteria there are present in the milk, the less time required for color change to occur.

One can determine if milk has been properly Pasteurized by performing a phosphatase test. Phosphatase is an enzyme in milk which will catalyze the hydrolysis of organic phosphates. If milk has been properly Pasteurized, the heat will have denatured the phosphatase enzyme and it will no longer be active. One can test a milk sample for phosphatase activity and if the enzyme is inactive, the milk is assumed to have been properly Pasteurized. A positive phosphatase test indicates that either the milk has not been properly Pasteurized, or that it has been adulterated with raw milk after Pasteurization. The test is performed by adding the substrate disodiumphenyl phosphate to the milk. If active, the enzyme will catalyze the hydrolysis of this structure to give the products phenol and phosphate. The phenol will turn blue in the presence of 2,6-dichloroquinone-chloroimide and  $\text{CuSO}_4$ . Absence of a blue color in this indicator is interpreted as a negative phosphatase test.

#### **WORK FOR THIS EXERCISE:**

##### **I. Quantitative Plating of Pasteurized Milk**

1. Prepare a  $10^{-2}$  dilution of milk by adding 30  $\mu\text{L}$  of milk to 3 mL sterile water in a 13 x 100 mm tube, vortex.
2. Label 3 TSA plates as follows: milk-50  $\mu\text{L}$ , milk-500  $\mu\text{L}$  of  $10^{-2}$ , milk-50 $\mu\text{L}$  of  $10^{-2}$ . Pipette the appropriate amount of the appropriate samples onto the plates. Spread the samples with a flamed glass spreader. Incubate plates at  $32^\circ\text{C}$  for 48 hours.
3. After incubation, select a plate containing between 30 and 300 colonies, count the colonies on such plates and determine the number of bacteria per mL in the original sample.