NAME _____

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Carolina™ Bacterial Pollution of Water Kit

Background

In 2010 a massive earthquake in Haiti killed over 200,000 people, left many more homeless, and completely disrupted the infrastructure in an area including the densely populated capital, Port-au-Prince. The resulting displacement of people and lack of clean water facilitated a cholera outbreak that rapidly spread. Cholera is caused by the bacterium *Vibrio cholerae*, which is usually spread through contaminated water, or sometimes food. Cholera may be mild to severe; only about 5% of infected people experience the severe form of the disease—severe watery diarrhea and leg cramps, often accompanied by vomiting. These symptoms often lead to severe dehydration and shock. Death from the disease can occur within a few hours. Patients with severe cholera are given large volumes of a solution of water, salts, and sugar to drink for rehydration. Those with especially serious cases are given the fluids intravenously.

Vibrio cholerae is a member of the coliform group, and spreads more easily in areas with inadequate sanitation, a lack of clean water, and displacement of a large number of people. From the time the 2010 outbreak in Haiti began through October of 2012, at least 600,000 people were infected and at least 7,400 people died. Haiti's epidemic was a large one, but outbreaks of cholera are not unusual on a global scale. In 2004 over 100,000 cases of cholera were reported worldwide to the World Health Organization; in 2011 over 580,000 cases were reported. It is estimated that a huge number of cholera cases and deaths go unreported.

The cholera outbreak in Haiti is just one example highlighting the importance of clean water in maintaining a community's health. The water that a community uses for drinking, food preparation, crop irrigation, bathing, or swimming needs to be clean. In the United States, as well as in many other countries, water quality is regulated. Even in countries where water destined for human use is tested and much of it treated to eliminate pathogenic microbes, there are occasional outbreaks of waterborne disease. These outbreaks usually result from a breakdown in the water purification process or in the water delivery system. Some of the more common organisms that cause waterborne diseases in the United States include the parasites *Cryptosporidium* and *Giardia*, the viruses Norovirus and hepatitis A, and the bacteria *Shigella*, *Legionella*, *Campylobacter*, and *Salmonella*. The source for many of these organisms is fecal material that has contaminated the water.

Because a large number of the organisms that cause waterborne illness are present in fecal material, the most commonly used water purity test assays for the number of coliform bacteria present in the water. Lack of coliform bacteria are an indication that the water is safe for human use. Coliforms are a group of bacteria found mainly in the intestines of warm-blooded animals and are present in feces. They are Gram-negative, rod-shaped bacteria that ferment lactose. Over the years, scientists working to determine the best method for testing the safety of water for human use have determined that specific numbers of coliform bacteria present in water are a likely indication of unsafe contamination of the water by fecal material. Contaminated water is likely to contain not just the coliforms detected by the assay (which may be pathogenic or nonpathogenic), but also other potential pathogens. Debate exists about how accurate the coliform test is in assessing the safety of water for human use, but at this time the method is still the most commonly used. Standards for how many coliform bacteria are allowed per 100 µL of water are set either as guidelines or as legally binding rules. The standard for the number of coliforms allowed varies according to how the water is used (e.g., drinking water typically has the most stringent standards).

A widespread method for treating water against microbial contamination is to add chlorine. Another method is to have chloramine in the water. Chloramines are chemicals that result when chlorine and ammonia combine. Chloramines persist longer in the water than chlorine does. Chloramines may be added to water by mixing chlorine and ammonia and then adding the mixture to the water, or they may be created by separately adding both chlorine and ammonia to the water. Because they persist longer in the treated water than free chlorine does, chloramines are better than chlorine at preventing microbial recontamination that may occur as the water flows through pipes in the community. During water treatment with chlorine or with chloramines, the concentrations of the chemicals used are carefully monitored and kept within an appropriate range. If the concentrations are too high, the levels of some of the harmful by-products that can result from chlorine or chloramine treatment will be present at unsafe levels. If the concentrations are too low, not enough microbes will be eliminated.

In this lab, you will use test strips that indicate the concentration of chlorine and chloramine in the water to determine if your group's water sample contains any chlorine or chloramines. The strip will measure free chlorine and total chlorine. Free chlorine is the amount of hypochlorous acid and hypochlorite ion in the water. Hypochlorous acid and hypochlorite ion are two chemicals that result when chlorine is added to the water. Combined chlorine refers to chloramines, the chemicals that result when free chlorine combines with ammonia or organic nitrogen present in the water. Total chlorine includes both free chlorine and combined chlorine. Think about what you would expect to find, given the source of your water sample.

You will also use nutrient agar plates to detect total numbers of bacteria in your sample, and MacConkey agar plates to detect any coliform bacteria. Nutrient agar is a general medium that supports the growth of a wide variety of bacteria. MacConkey agar selects against the growth of most Gram-positive bacteria and supports the growth of most Gram-negative bacteria. Bacteria that ferment lactose will turn a shade of pink or red or possibly purple on the MacConkey agar plate. Remember that bacteria in the coliform group are Gram negative and ferment lactose; thus, if the bacteria are part of the coliform group of bacteria they will grow on the MacConkey plates and the colonies they form will be a shade of pink, red, or purple.

Each colony on a plate represents a single bacterium. When the water is plated, single bacterial cells are spread across the plate. When the plate is incubated, if the growth conditions are favorable for that bacterium, it will divide multiple times and produce a visible colony.

References

World Health Organization Fact Sheet on Cholera
http://www.who.int/mediacentre/factsheets/fs107/en/index.html

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CDC's Web page on cholera. http://www.cdc.gov/cholera/general/

Water-related Disease and Contaminants in Public Water Systems
http://www.cdc.gov/healthywater/drinking/public/water_diseases.html

Part 1. Collecting the Water Sample and Testing for Chlorine and Chloramines

Collect a sample as close as possible to the time you will plate it. Plate the sample within 16 hours of collection. Test for chlorine and chloramines at the time of collection.

Materials

1 sterile collection tube
1 small, sealable, plastic bag for trash
1 sterile bulb pipet
1 free chlorine/total chlorine test strip and chart
1 permanent marker
1 container for measuring and holding 250 mL of water

Collecting the Water Sample

1. As a group and with input from your instructor, decide where your group will collect your water sample. Remember, the water sample needs to be plated within 16 hours of being collected.
2. Label your collecting tube with the source of the water sample, the time and date at which the sample was collected, and your group name or number.
3. Collect your sample using the following procedure. You may find it easier to have one person handle the pipet and the other handle the tube.
   a. To prevent the water sample from becoming contaminated, keep the cap on your collection tube and the pipet in the wrapper until immediately before the sample is pipetted into the tube.
   b. Peel open the wrapper on the bulb end of the sterile 1-mL bulb pipet, and draw the pipet out. Handle the pipet only on the bulb end and do not let the stem come into contact with anything except the water you are collecting.
   c. Hold the pipet in one hand and the collecting tube in the other.
   d. Use one or more free fingers to remove the cap of the tube. Keep the open side of the cap facing down, and continue to hold the cap in your hand. Do not put the cap down or touch the inside surface of the cap or the rim of the tube.
   e. Use the pipet to place approximately 2 mL of water in the tube. Work quickly, and do not touch the pipet to the side of the tube as you pipet the water into the tube.
   f. As soon as you finish pipetting water into the tube, place the cap back on the tube, and screw it on tightly for transport. Place the water sample back in the plastic bag.
   g. Place the bulb pipet in the plastic bag designated for trash and seal the bag.
   h. Store the sample at room temperature (do not refrigerate!) and with the cap loosened when possible. Once you are out of the field, clean the 250-mL container and prop the tube up to keep the water from leaking out when the cap is loose.

Testing the Water Sample for Chlorine and Chloramines

1. Fill a container with a minimum of 250 mL (1 cup) of water from the same source that you took your water sample.

2. Dip the test strip into the cup of water, while moving the strip back and forth. Continue to move the test strip back and forth for 10 seconds.
3. Remove the strip and shake it briskly to remove any excess water.
4. Wait 15 seconds. Match the colors seen through the windows on the strip to the appropriate color square on the chart to determine the levels of free chlorine and total chlorine in the water sample. The window at the end of the strip shows the reading for free chlorine; the window closer to the handle shows the reading for total chlorine.
5. Enter your readings on the lines below. Give your answers in milligrams per liter. On the chart, both parts per million (ppm) and milligrams per liter (mg/L) are given. In this case, they are equivalent measures.
6. Determine the value for combined chlorine by subtracting the value for free chlorine from the value for total chlorine.

total chlorine _____

free chlorine _____

combined chlorine _____

Part 2. Plating the Water Sample

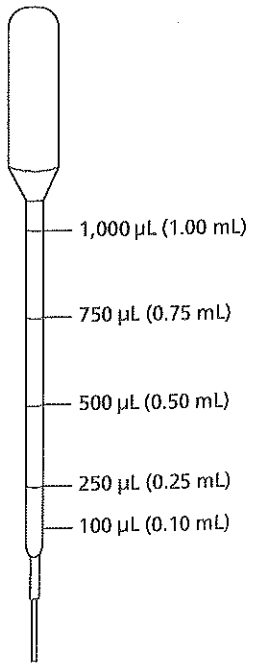
Materials

1 nutrient agar plate
1 MacConkey agar plate
1 permanent marker
1 small, sealable, plastic bag for trash
2 sterile bulb pipets
2 disposable spreaders (at shared station)
clear tape or Parafilm (at shared station)

Procedure

Use the following procedure to plate 250 µL of your water sample onto the nutrient agar plate and 250 µL onto the MacConkey agar plate. As before, you may find it easier to have one person handle the pipet and another handle your water sample tube.

1. Label both plates with the source of your water sample, the date, and the name or number of your group. Label the bottom of the plate along the edge, so that the label does not obscure any colonies.
2. Peel open the wrapper on the bulb end of a sterile 1-mL bulb pipet, and draw the pipet out. As before, handle the pipet only by the bulb. Do not let the stem touch anything except the water you are plating.
3. Hold the pipet in one hand and the water sample tube in the other.
4. Use one or more free fingers to remove the cap on the water sample tube. Keep the open side of the cap facing down. Do not touch the inside surface of the cap or the rim of the tube, and do not put the cap down.
5. Fill the pipet with 250 µL of your water sample (see the figure). Lift the lid on the nutrient agar plate and gently expel the 250 µL onto the plate. Replace the lid of the petri dish immediately.
6. Place the bulb pipet in the plastic trash bag and seal the bag.



1-mL bulb pipet showing 250-µL level

7. Repeat steps 2–6 to place 250 µL of your water sample onto the MacConkey agar plate. **Be sure to use a new pipet.**
8. Immediately carry both plates to the shared station where the packages of sterile disposable spreaders are located.
9. Use the following procedure to spread the 250 µL of the water sample on each plate. **Make sure that you use a fresh spreader for each plate.**
   a. Open the pack of spreaders and quickly remove one spreader. To maintain as much sterility as possible, handle the spreader only by the handle end, do not touch the other spreaders, and quickly close the pack once you have removed your spreader.
   b. Keeping the plate covered as much as possible and the inside surface of the lid facing down, lift the lid of the nutrient agar plate. To spread the 250 µL, move the spreader back and forth across the plate while turning the plate in a circular motion with your other hand. If the water sample is spread unevenly, colonies may be difficult to count.
   c. After spreading, immediately replace the lid. Put the spreader into the trash bag.
   d. Repeat steps a–c with the MacConkey agar plate. Make sure that you use a new spreader.
10. Seal the plates with tape or Parafilm and incubate them as directed by your instructor. Since pathogenic organisms may grow on the plates, from this point on do not open the plates.

Part 3. Analyzing the Results

1. **Do not open the lid.** Looking down through the lid, count the total number of colonies on the nutrient agar plate and on the MacConkey agar plate. Use the permanent marker to place a mark on the bottom of the plate over each colony as you count it. Enter the data in the Culture Results table. If the colonies are too dense to distinguish for counting, write "TNTC" for "too numerous to count." If the colonies are so numerous that they have merged to form a lawn write "lawn."
2. Count the number of red, pink, or purple colonies on the MacConkey agar plate and record the data in the table. These are the coliform colonies.
3. Determine the total number of colonies and the number of coliform colonies per 100 µL of water. (Remember, you plated 250 µL.)

Culture Results		
Number of Colonies Per Plate		
	Total Colonies	Red/Pink/Purple Colonies
Nutrient Agar		
MacConkey Agar		

Total number of bacterial colonies/100 µL on nutrient agar: _____

Total number of bacterial colonies/100 µL on MacConkey agar: _____

Total number of coliform colonies/100 µL: _____

4. Find out the legal standards or guidelines in your state for the coliform bacteria counts allowed in the water used for various purposes. Fill in the chart below.

Water Use:	# Coliform Colonies/Unit
drinking	
swimming	
recreation	
irrigation	

5. Compare the results of your test to your state's standards for that type of water. Share information with your classmates who tested other types.

Laboratory Questions

1. Do the levels of chlorine and chloramines found in your sample match what you expected to find? Why or why not?

2. Did you find coliform colonies in your water sample? If so, what percentage of the total number of colonies were coliform colonies.

3. In the analysis, you reported your number of colonies as number of colonies on the plate and as number of colonies per 100 μL. Although reporting things such as measurements indicating water quality is usually done in a standard format, sometimes different groups may report findings in different ways. Suppose that you have been asked to report the number of colonies in your water sample as colonies per milliliter. What numbers would you report? Show how you got the numbers.

4. If either of your water sample plates had a lawn or too many colonies to count, what might you have done, without reducing the volume of liquid you put on the plate, so that you had ended with a countable number of colonies?

5. What does the presence, or absence, of coliforms indicate about your water sample?

6. If you see coliforms on your plate, do you know with certainty that the coliforms are disease-causing? Why or why not? For the purpose of this type of water quality test, is it important to know if the coliform bacteria present cause disease?

7. If the water sample you collected is a type with a suggested or required standard for the number of coliform colonies allowed (e.g., public drinking water), was the number of coliform colonies in your sample within the limits set by the standard? Include in your answer what type of water you were testing, what the standard is for that type, and how many coliform colonies you found in your sample. State the number of colonies in your sample, using the same units as the standard.

8. What steps would you take to reduce the coliform count of drinking water whose coliform count exceeded the standard?