

Investigation of *Escherichia coli* in Freshwater Sources Using Membrane Filtration and Rep-PCR DNA Fingerprinting with Introductory Biology Students

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Escherichia coli is a common indicator bacterium for fecal contamination and testing for *E. coli* levels is a measure of recreational freshwater quality. In this multi-component laboratory activity for detection and identification of *E. coli*, students use a membrane filtration protocol adapted from the U.S. Environmental Protection Agency for detection and enumeration of *E. coli* from freshwater sources. Students then use standard biochemical methods to confirm isolates as *E. coli*. Finally, students use Rep-PCR DNA fingerprinting to determine the relatedness of *E. coli* isolates.

Keywords: *E. coli*, membrane filtration, Rep-PCR, DNA fingerprinting

Introduction

This paper presents a multi-component laboratory activity for detection and identification of *E. coli* from freshwater sources. Students use a membrane filtration protocol adapted from the EPA for detection and enumeration of *E. coli* from a water sample. Students then use standard biochemical methods to confirm isolates as *E. coli*. Finally, students use Rep-PCR DNA fingerprinting to determine the relatedness of *E. coli* isolates. Beginning with sample collection and proceeding to molecular analysis, students learn necessary skills and are exposed to current methodology used by government, industry and academic researchers for the analysis of microbial contamination of water, food and pharmaceuticals.

This series of experiments can be used with any level of student, ranging from high school to upper level undergraduate. It is appropriate for a microbiology course or a general biology course. It does require several consecutive class or lab periods to complete all of the experiments, however not all of the activities require a full class period, and depending on the level of the student and previous course work, some of the steps can be completed by the students outside of class time.

Day 1: Sample Collection and Membrane Filtration

Depending on the number of students and equipment availability, this can be completed in as little as 15-30 minutes.

Day 2: Enumeration of *E. coli* using Urea Substrate Medium

This can be completed in 30 minutes

Day 3-5: Verification of *E. coli* colonies using MacConkey agar, IMViC and Oxidase tests (optional)

Students inoculate media and perform the oxidase test on day 3, which may require 15-30 minutes. Students read the results of the tests on days 4 and 5. At least fifteen minutes should be allowed each day. If students have performed the tests previously in the course, these steps can be completed by the students on their own outside of class time.

Day 6: DNA fingerprinting of *E. coli* isolates by rep-PCR

At least 30 minutes should be allowed for students to set up PCR reactions

Day 7: Gel Electrophoresis of PCR products

The gels should be run over a full lab period to allow greatest band separation.

I have used this series of experiments in several contexts. It was used as part of a case study in an introductory microbiology summer enrichment course for high school and college students. This course met for 4 hours daily for two weeks. In this course, I led students through a fictional case study of a child who is in the hospital with an *E. coli* infection. I had the students assume the role of a microbiologist, first trying to first diagnose his illness. Laboratory exercises

involving obtaining a pure culture and microscopy were done. They then investigated the source of his illness and whether it was from a recent trip to the beach by doing the series of labs presented in this paper.

It was also used in a similar general biology summer enrichment course. In this course, I had students adopt a local lake. They researched the overall water quality of the lake including factors such as environmental quality (clarity, chlorophyll A, total phosphorous), lake aesthetics (color, odor, garbage, debris), recreational interference (aquatic plants) and public health (*E. coli*). The series of experiments presented in this paper were used for the public health aspect of the project. In this project, students saw where microbiology fits

into the overall assessment of the quality of freshwater. The end product was a written report summarizing their research. This can easily have a service learning aspect if students present their report to interested constituents such as local residents or government agents.

Finally, I have had students perform this series of experiments as an independent research project. Students monitored a lake for *E. coli* levels on a weekly basis for the entire summer using the EPA membrane filtration protocol presented in this paper. At the end of the season, they summarized their findings in a written report, prepared a poster, and during the school year came into my microbiology course to help teach this unit to the next batch of students.

Student Outline

Background: Testing Recreational Water for *E. coli*

Human disease can be caused by waterborne microbes that can contaminate recreational water (Madigan, 2006). One common disease associated with recreational water in the US is gastroenteritis, which can be caused by bacteria (*Salmonella* or *E. coli* O157:H7), parasites (*Cryptosporidium*) or viruses (Norwalk-like viruses). For the sake of public health, it is important that recreational water be routinely monitored for the presence of pathogens. Since fecal material is a source of these pathogens, it is standard to test for indicators of fecal contamination, rather than testing for each individual pathogen. *Escherichia coli* is a common inhabitant of the intestinal tract of warm-blood animals, and unlike O157:H7, most strains of *E. coli* are actually non-pathogenic. Therefore, it is a common indicator bacterium for fecal contamination. The presence of *E. coli* in water samples indicates that the water has been polluted by feces and may contain other enteric pathogens.

Governmental agencies including park boards, cities, counties, states and the federal government are typically involved in testing recreational water for pathogens. The U.S. Environmental Protection Agency (EPA) has published several methods for measuring recreational water quality and you will be using their method 1103.1 to test a freshwater source for *E. coli* levels (EPA, 2002).

In this protocol, bacteria from a water sample are filtered through a membrane with a pore size of 0.45 μM . Bacteria become trapped on the filter and can grow into visible colonies on the surface of the membrane when the membrane is transferred to an appropriate growth medium. The color of the colonies is an initial indicator of the type of bacteria present. Membrane thermo-tolerant *E. coli* (mTEC) agar is a selective and differential medium. The presence of sodium lauryl sulfate and sodium desoxycholate in this medium selects against growth of Gram-positive organisms. mTEC also contains lactose and the pH indicators bromcresol purple and bromphenol red. These cause bacteria to be yellow/orange at a pH less than 8.4 and red/pink at a pH of greater than 8.4. Bacteria such as *E. coli* that are able to ferment lactose will produce an acid byproduct causing the colony to be yellow/orange. Since other bacteria might also have this same phenotype on mTEC agar, bacterial colonies are subsequently exposed to urea substrate medium, which contains urea and phenol red. Urea is the substrate on which the enzyme urease acts to produce ammonia. Production of ammonia causes the pH of the colony to become more basic, which is detected by a color change to red/pink. Since *E. coli* does not have the urease enzyme, *E. coli* colonies will not change color and will be yellow, yellow-green, or yellow-brown.

The combination of growth on mTEC agar and testing with urease substrate medium allows for the presumptive identification and enumeration of the number of *E. coli* cells in a water sample. The EPA has set standards for *E. coli* in recreational water such that the content shall not exceed 235 *E. coli* per 100 mL of water and the geometric mean of not less than 5 samples, equally spaced over a 30 day period, should not exceed 126 *E. coli* per 100 mL of water. If *E. coli* levels exceed these levels, then it is recommended that individuals should not enter the water until *E. coli* levels drop.

Procedure

Day 1 – Sample Collection and Membrane Filtration

1. Immediately before class, aseptically obtain a 50 mL water sample from a fresh water source.
 - a. Use a sterile disposable 50 mL sterile screw cap tube
 - b. Remove the cap, invert the tube and place underwater to a depth of about 12 inches below the surface.
 - c. Turn the tube right side up to fill the tube.
 - d. Replace the cap, place the water sample on ice and transport it immediately to the lab.
 - e. Record the location where your sample was taken here:
2. Aseptically filter the water sample through a 0.45 μm membrane.
 - a. Attach the filter funnel to the vacuum pump.
 - b. Pour 50 mL of water into the filter funnel
 - c. Turn on the vacuum pump until all water is filtered.

3. Using ethanol flamed forceps, aseptically transfer the filter to a Petri plate containing mTEC agar.
 - a. Separate the funnel from the filter base.
 - b. Dip the forceps into 95% ethanol, let the excess ethanol drip off and burn off the excess ethanol very carefully, taking care that burning ethanol does not drip onto your hand, into the beaker of ethanol or onto other flammable material.
 - c. Carefully and firmly grab the filter membrane with the sterile forceps. Be careful to just get the membrane and not the paper pad supporting the membrane.
 - d. Roll the membrane onto the surface of an mTEC agar plate, avoiding the formation of bubbles between the membrane and agar. The grid of the membrane should be facing up.

4. Incubate mTEC plate overnight
 - a. Label the plate with the date and your initials.
 - b. Incubate the plate agar side up at 37°C for 2 hours
 - c. Transfer the plate to 45°C and incubate 22-24 hours

Day 2 – Enumeration of E. coli using Urea Substrate Medium

1. Remove your mTEC plate from the incubator and record your results below for question #1.
 - a. Precautionary statement: Use care and proper aseptic technique when working with cultured microorganisms. While bacteria isolated from the lake are unlikely to be pathogenic, some *E. coli* can cause disease.

2. Transfer the membrane to urea substrate medium
 - a. Obtain a different Petri plate containing a paper pad.
 - b. Use a transfer pipette to add 2 mL Urea Substrate Medium to the pad.
 - c. Using flamed forceps, aseptically transfer the membrane (containing the bacteria) to the pad.
 - d. Incubate for 15-20 minutes at room temperature and record your results below for questions #2.

3. Streak 4 colonies that you presume to be *E. coli* onto MacConkey agar to obtain isolated colonies.
4. Label the MacConkey agar plates and incubate agar side up at 37°C overnight.
5. Record your results and answer the following questions.

Results/Questions

1. Record the number and characteristics (especially color) of colonies on the membrane before transferring to urea substrate medium.
2. Record the characteristics of colonies on the membrane after transferring to urea substrate medium.
3. How many *E. coli* are present on your membrane?
4. Calculate the number of *E. coli* per 100 mL of water.
5. According to standards for recreational waters, is it advisable to swim in the water based on your results?

Day 3 – Verification of *E. coli* colonies using MacConkey Agar, IMViC and Oxidase Tests

Your MacConkey agar plates have isolated unknown bacteria from Lake Johanna that are presumed to be *E. coli*. Today you will verify that these unknown colonies are *E. coli* by interpreting the MacConkey agar plates and also performing the oxidase test and IMViC series of tests.

Bacteria can be classified and identified based on the types of **enzymes** they possess. Enzymes are proteins that speed up chemical reactions. Enzymes are necessary for life and every organism has a different set of enzymes, depending on the chemical reactions performed.

For example, some bacteria can use the sugar lactose to get energy and some cannot use lactose. Those that can use lactose have a special enzyme called lactase that breaks down lactose. By testing an unknown bacterium for the presence of the lactase enzyme, one can begin to identify the bacterium.

There are many biochemical tests that can be used to determine what enzymes a microbe has. Most of these tests are done by growing the bacteria in a certain type of medium and making observations about its growth characteristics or reactions with chemicals that are added after incubation. You will perform 6 tests.

MacConkey Agar Plates

MacConkey agar is a selective and differential medium used to culture gut bacteria. Only Gram-negative organisms grow on MacConkey agar due to the presence of bile salts and crystal violet. MacConkey agar also contains lactose and the pH indicator neutral red, which is red at a pH less than 6.8 and colorless above pH 6.8. If organisms grown on MacConkey agar can ferment the sugar lactose and accumulate acid, colonies will appear pink (positive result). If they cannot ferment lactose, colonies will be white. Thus, much like mTEC agar, MacConkey agar tests for the presence of enzymes involved in lactose fermentation. *E. coli* are Gram-negative lactose fermenters so will be red on MacConkey plates.

Oxidase Test

This is a test for the presence of the cytochrome oxidase enzyme used in cellular respiration. Bacteria are exposed to an artificial electron donor (oxidase reagent) and if oxidase is present, it will oxidize the reagent causing it to turn dark blue, a positive result. A negative reaction is typically observed for *E. coli*.

IMViC Test

This is actually a set of four tests.

- The **Indole** test identifies bacteria able to produce the byproduct indole using the enzyme tryptophanase. Bacteria are grown in T-Soy broth and the presence of indole is detected by the addition of Kovac's reagent. The development of a red color at the top of the tube is a positive reaction. A positive reaction is typically observed for *E. coli*.
- The **Methyl Red** test is done to identify bacteria that perform mixed-acid fermentation of glucose. Bacteria are grown in MR-VP broth and the accumulation of acidic end products of this type of fermentation is detected by addition of methyl red. Methyl red is a pH indicator that turns red in extremely acidic solutions (pH 4-5) and yellow in less acidic environments. Methyl red is rather toxic to bacteria, so it is added to the MR-VP broth after growth has occurred. A red color indicates the presence of high concentrations of acid (positive) and a yellow color indicates a negative reaction. A positive reaction is typically observed for *E. coli*.
- The **Voges-Proskauer** tests for the presence of the chemical acetoin, which is a byproduct of glucose fermentation (2,3 butanediol pathway). Bacteria are grown in MR-VP broth and the presence of acetoin is detected by the addition of α -naphthol and KOH. The development of a red color indicates the presence of acetoin (positive). *E. coli* typically displays a negative reaction.
- The **Citrate** test determines whether citrate can be used as the sole carbon source for growth due to the presence of the citrase enzyme. Alkaline byproducts of citrate catabolism cause the bromthymol blue pH indicator to change from green to blue (pH > 7.6), a positive result. *E. coli* is typically negative.

Procedure

1. Fill out the first two columns of Table 1 with the results you expect for *E. coli*. Describe the expected outcome (ex. color of medium) and whether this outcome is considered a positive (+) or negative (-) result.
2. Observe the MacConkey agar plates on which you streaked your unknown bacteria (that are presumed to be *E. coli*). Record your results in Table 1.
 - a. Choose one of the 4 plates that has isolated colonies of the expected phenotype to test in steps 2 through 5.
3. Perform the Oxidase test
 - a. Obtain a piece of paper towel.
 - b. Put a few drops of oxidase reagent on the paper towel.
 - c. Use a sterile toothpick to transfer an isolated colony from your MacConkey agar plate to the moistened paper towel.
 - d. Dispose of the paper towel in the biohazardous waste.
 - e. Record your results in Table 1.
4. Inoculate media to perform the Indole test.
 - a. Obtain a tube of T-Soy broth.
 - b. Label the tubes with the type of broth and your initials
 - c. Inoculate the broth with an isolated colony from your MacConkey agar plate using a sterile inoculating loop.
5. Inoculate media to perform the Methyl-Red and Voges-Proskauer tests:
 - a. Both tests use MR-VP broth so obtain two tubes of MR-VP broth.
 - b. Label one tube MR and one VP. Label each tube with your initials.
 - c. Inoculate broths with an isolated colony from your MacConkey agar plate using a sterile inoculating loop.
6. Inoculate media to perform the Citrate test
 - a. Obtain a citrate slant.
 - b. Label the tube with your initials.
 - c. Inoculate the surface of the slant with an isolated colony from your MacConkey agar plate using a sterile loop.
7. Incubate all tubes at 37°C.

Day 4 – Reading the Indole and Citrate Tests

1. The Indole and citrate tests can be read after overnight growth.
2. Obtain the T-Soy tubes and Citrate slants from the incubator.
3. Read the results of the Indole test:
 - a. Verify that there is bacterial growth in the T-Soy tube.
 - b. If there is no bacterial growth, then this test will by default be negative.
 - c. Working in the fume hood and wearing gloves, add several drops of Kovac's reagent to your tube.
 - d. Observe and record what happens in Table 1.
The appearance of a red ring is a positive result.
4. Read the results of the citrate test:
 - a. Verify that there is bacterial growth on the citrate agar, but be aware that there is often very scant growth.
 - b. Observe and record the color of the media in Table 1.
 - c. A change in color from green to blue is a positive result.

Day 5 – Reading the Methyl Red and Voges-Proskauer Tests

1. The Methyl Red and Voges-Proskauer tests should be incubated 48 hours before being read.
2. Obtain the MR and VP tubes from the incubator.
3. For the MR test add 5 drops of methyl red to the tube.
 - a. Record your results in Table 1.
 - b. A red color is a positive reaction and a yellow color indicates a negative reaction.
4. For the VP test, add 1 mL of α -naphthol (VP-A) and 0.5 mL of KOH (VP-B) to the broth.
 - a. Record your results in Table 1. It may take several minutes for color to develop.
 - b. A red color is a positive reaction.

Table 1. Biochemical Test Results.

	<i>E. coli</i>		Bacterial Unknown	
	Expected Reaction	Expected Result (+ or –)	Observed Reaction	Result (+ or –)
MacConkey Agar				
Oxidase				
Indole				
Methyl Red				
Voges-Proskauer				
Citrate				

Day 6 - DNA fingerprinting of E. coli Isolates by Rep-PCR

High levels of *E. coli* in water sample indicate that there is likely fecal contamination of the water. Sources of fecal contamination vary. Feces from mammals and birds, either wild or domesticated, can be carried into the water by storm water run off. Fecal material from children swimming is often not sufficiently contained by diapers. Untreated sewage released into the water can also be a problem. It is often desirable to determine the source of the fecal contamination to remedy the situation. This process is called **microbial source tracking**. DNA fingerprinting is a rapid, inexpensive and reliable method for source tracking. DNA fingerprints from contaminating *E. coli* are compared to fingerprints of *E. coli* from known sources present in a prepared database. As we don't have access to a database of fingerprints from known sources, you will be performing DNA fingerprinting to observe the relatedness of the *E. coli* unknown strains you isolated from the lake.

To prepare the DNA fingerprint, you will do PCR using the BOXA1R PCR primer. This primer anneals to a repetitive sequence element of *E. coli*. The element is found in either orientation throughout the genome and its location and frequency differs between strains. Thus, a unique pattern of PCR products is generated from every strain. Following PCR amplification, the PCR products are separated by agarose gel electrophoresis to make a DNA fingerprint.

Procedure

1. Obtain 5 sterile microtubes. Label them 1-5.
2. Add 100 μL of sterile water to each tube.
3. Use a sterile toothpick to resuspend your unknown *E. coli* colonies into tubes 1-4.
 - a. Choose one well isolated red colony from each of your MacConkey agar plates.
 - b. Pick the colony with a sterile toothpick
 - c. Place the toothpick with the bacteria into the tube of water and twist it around with your fingers to transfer the cells to the water.
4. In the same way, resuspend a known *E. coli* colony provided by your instructor in tube 5.
5. Record below which cells are in which tube.
6. Obtain 6 PCR tubes and label them 1-6
7. Obtain a tube of PCR cocktail
8. Aliquot 23 μL of the cocktail to each PCR tube
9. Add 2 μL of your resuspended cells to the appropriate tube.
10. To tube 6 add 2 μL of sterile water.
11. Place your tubes in the PCR machine that has been set for the following cycling conditions:
 - 1 cycle: 95°C for 2 minutes
 - 30 cycles: 94°C for 3 sec., 92°C for 30 sec., 50°C for 1 min., 65°C for 8 min.
 - 1 cycle: 65°C for 8 minutes

Day 7 - Gel Electrophoresis of PCR Products

Today you will be separating the DNA in your PCR reactions by agarose gel electrophoresis. Agarose gel electrophoresis separates DNA molecules based on their size. Because DNA is negatively charged, it will migrate toward the positive pole when placed in an electrical field. Agarose is a semisolid substance (made from seaweed) that provides a network through which the DNA must move. The result is that large DNA fragments will migrate more slowly through the agarose, whereas smaller DNA fragments will migrate more rapidly. These different rates allow for the separation of a mixture of DNA fragments by size.

1. Work in groups of two to prepare a gel and run your PCR reactions. You will be running your PCR products on a 1.5% agarose gel. This is prepared by dissolving an appropriate amount of powdered agarose in 50 ml of 1x TAE buffer.
 - a. Measure 0.75 g agarose and put it in a bottle
 - b. Measure 50 mL of 1 x TAE and add to the bottle with agarose
 - c. Microwave the bottle in short bursts of 15-20 seconds to boil the solution and dissolve the agarose.
 - d. Be careful to prevent the solution from boiling over.
 - e. After each burst, remove the bottle (wearing gloves), swirl it and visually inspect it to see if the agarose has dissolved.
 - f. Once it is dissolved, let the agarose cool to the point where you can pick the bottle up with your bare hands. Be careful not to let it cool too much, or the solution will solidify.
2. While the gel solution is cooling, prepare a casting chamber with a 12-well comb.

3. While the gel is cooling, prepare your PCR samples to be loaded.
 - a. Obtain your PCR reactions.
 - b. To each PCR tube, add 5 μ L of 6x loading dye.
 - The loading dye contains glycerol, which will associate with the DNA, increasing its density and causing it to sink to the bottom of the well. It also contains three dyes that allow one to approximate how far the DNA has run on the gel. (Note: The dye does not stain your DNA. Your DNA is invisible at this point. The dye simply has a molecular weight that causes it to migrate at a rate similar to DNA fragments. Xylene cyanol (green) migrates at approximately 4 kb, bromophenol blue at approximately 300 bp and orange G at approximately 50 bp.)
 - c. Obtain a tube with 1 kb ladder. The 1 kb ladder contains a series of fragments of known size. By comparing the size of your fragments to this ladder, you can determine the approximate size of each of your fragments. (Sizes of bands (bp) in 1 kb ladder: 10000, 8000, 6000, 5000, 4000, 3000, 2500, 2000, 1500, 1000, 750, 500, 253, 250).
4. While your gel is cooling, ask the instructor to come and add 2 μ L of ethidium bromide (EtBr) to your gel solution.
 - a. EtBr is a dye that intercalates into DNA (inserts into the double helix). When exposed to ultraviolet (UV) light, EtBr will fluoresce and cause the DNA to be visible. **CAUTION:** EtBr is a **carcinogen** – use gloves when handling and from this point on, don't touch your gel without gloves and don't touch anything with gloved hands after handling the gel.
5. Pour the cooled gel solution poured into the casting chamber and allow it to solidify.
6. When the gel is solid, remove the comb and lower the sides of the casting chamber. Place the gel in a gel box with 1 x TAE buffer. The buffer should cover the gel and fill the wells.
 - a. The red wire indicates the positive electrode and the black electrode indicates the negative electrode. Given this information, how should you orient the wells on your gel relative to the red and black electrodes to properly separate your DNA?
7. Load 10 μ L of your PCR reactions on your gel and 5 μ L of 1 kb ladder in one well.
 - a. Since there are only 12 wells and each group has 6 wells and you also need to run the 1 kb ladder, one group should **not** run their PCR tube in which only water was added.
 - b. Record what lanes you load your reactions in here: _____
8. Put the lid on the gel box, plug the electrodes into the power supply and run at 110 V for about 1 hour.
9. Carefully remove the casting chamber with your gel, observe it under UV light and take a photo.
10. Summarize your results.
 - a. How many bands are in each lane?
 - b. Do any lanes have the same banding pattern?
 - c. How many strains of *E. coli* do you think are present on you gel?

Materials

Day 1 – Membrane Filtration

Per student:

- 1 50 mL sterile screw cap tube
- 1 Nalgene Analytical Test filter funnel, 100 mL, 0.45 mm
- One 60 mm mTEC agar plate
- Bunsen burner, ethanol, forceps

General Laboratory Equipment:

- Vacuum pump and collection flask
- Incubator that can be set to 37°C and 45°C

Day 2 – Enumeration of *E. coli* using Urea Substrate Medium

Per student:

- One 60 mm Petri plate with sterile filter pad
- 2 mL urea substrate
- 4 - 100 mm MacConkey agar plates
- Loop and Bunsen burner or sterile toothpicks
- Bunsen burner, ethanol, forceps
- Transfer pipette

General Laboratory Equipment:

- 37°C incubator

Day 3 – Verification of *E. coli* Colonies using MacConkey Agar, IMViC and Oxidase Tests

Per Student:

- One tube of T-Soy broth
- One citrate slant
- Two tubes of MR-VP broth
- Oxidase reagent
- Loop and Bunsen burner
- sterile toothpicks
- paper towel

General Laboratory Equipment:

- 37°C incubator

Day 4 – Reading the Indole and Citrate Tests

General Laboratory Supplies and Equipment:

- Kovac's reagent
- Fume hood
- Gloves

Day 5 – Reading the Methyl Red and Voges- Proskauer Tests

Per Student:

- 1 mL of α -naphthol (VP-A) and 0.5 mL of KOH (VP-B)
- Several drops Methyl Red

Day 6 - DNA Fingerprinting of *E. coli* Isolates by Rep-PCR

Per student:

- p-20, p-200 micropipettes and sterile tips
- 5 sterile microcentrifuge tubes
- 6 sterile PCR tubes
- PCR cocktail
- Sterile toothpicks

General Laboratory Equipment:

- Thermocycler

Day 7 - Gel Electrophoresis of PCR products

Per student pair:

- One 1.5% agarose gel with 12 wells, 2 μ L ethidium bromide
- 1 electrophoresis running chamber
- 1 power supply
- 1 x TAE
- 60 μ L 6 x loading dye
- 5 μ L 1 kb ladder

General Laboratory Equipment:

- Imaging system for EtBr stained gels

Notes for the Instructor

This is a series of laboratory experiments that takes place over several class periods. Some of the activities do not take that long and we typically do additional activities on those days. To shorten the laboratory activity, it is possible to omit the biochemical characterization of the isolates using the IM-ViC series of tests and the oxidase test. This step is not absolutely necessary, as colonies that are yellow on mTEC agar and pink on MacConkey agar, are presumed to be *E.coli*. However, the EPA protocol (2) and the paper from which the rep-PCR protocol was adapted (3) both utilize a biochemical characterization step for quality control to confirm that colonies are *E.coli*. If included, and depending on the context of the course you use this in, it will either introduce or reinforce the principle of biochemical testing for bacterial identification, which is an important student outcome in microbiology. I use the IMViC series of tests because they reinforce mate-

rial I have covered previously and are standard tests that are described in any microbiology laboratory manual. The EPA and rep-PCR paper use slightly different tests. The time required for the experiment could be further shortened by preparing the agarose gels in advance.

It is important to note that the activities on Day 1 and Day 2 need to be done on consecutive days. Leaving the mTEC agar plates for longer (regardless of the temperature) will cause the colonies to change color due to pH changes not related to lactose fermentation.

It is generally a good idea to play close attention to the colonies during the 15 minute incubation in Urea Substrate Medium to watch for any color changes in the colonies. Have students pay particular attention to colonies that were a nice, bright yellow on mTEC before transferring to Urea Substrate. These colonies will generally darken in color, but if they are *E. coli*, they should turn yellow-green or yellow-brown, not turn a pink/red color. Have students look for colonies that do change to a pink/red color to compare to the presumptive *E. coli* colonies.

Once incubated with the urea substrate medium, the colonies on the membrane tend to become mucoid in texture and start to run into one another. For this reason, do not incubate too long, and try to maintain a reasonable number of colonies on the plate.

The expected results of the membrane filtration can vary significantly, depending mainly on the water source that was sampled, and you may want to do a trial run in advance. Some water sources will have more bacteria than others. You don't want too many bacteria on the plate – aim for plates with 20-80 colonies. The plates are small! If you filter 50 mL of water and find that the bacterial density is too high, then filter a smaller volume of water and use the dilution factor to calculate the number of *E. coli* per 100 ml of water. Additionally, if the water sample is quite turbid with other aquatic life or debris, the filter may become clogged or it may be difficult to distinguish the bacteria. In this case, let the debris settle out before filtering or filter a smaller sample volume.

Students are sometimes disappointed when they do not have high levels of *E. coli* on their plates. Discuss with them that this is a good thing; high levels of *E. coli* indicate fecal contamination. If students don't obtain any *E. coli*, have them use *E. coli* from another student's plate so that they can continue with the experiments.

For PCR amplification, a bacterial suspension in water is used as the template, with the initial heating step in the PCR sufficient to lyse the cells and release the DNA. As a positive control, students use a laboratory strain of *E. coli* (DH5 α) to compare their unknown strains to. It is also important to include a no-template control by adding water to one tube instead of a bacterial suspension. PCR amplification using the BOXA1R primers has been observed from laboratory strains of *Pseudomonas fluorescens* and *P. aeruginosa*, but the fingerprints look quite different from those of *E. coli* (data not shown). Amplification was not observed from *Serratia marscesens* or *Bacillus cereus*. In running the PCR reac-

tions on an agarose gel, it is recommended that gels be run as slowly as time allows, providing for the best and most even separation.

For further discussion or background information, references by the EPA (2002) and Dombek (2000) are highly recommended, as they are the sources from which this exercise was adapted. Details about laboratory prep are provided in the Appendix.

There are several safety concerns that the instructor should be aware of. Many of these are general safety concerns regarding work in the microbiology laboratory. Students should be taught general rules regarding the microbiology laboratory. Aspects that are especially relevant to this lab are work with Bunsen burners, burning ethanol, biohazardous waste disposal, and working with hazardous chemicals. All biological waste should be disposed of properly after autoclaving. Instructors should check with their campus chemical waste management for proper disposal procedures of chemicals. Students and instructors should also be especially aware of the chemical hazard of ethidium bromide, which is a carcinogen. Students should wear gloves when touching the gels or running buffer (as EtBr can leach out of the gel into the buffer). I prefer to add EtBr directly to the gel to avoid the time involved in staining/destaining baths. To limit student exposure to high concentrations of EtBr, I add the EtBr to their agarose gels. Instructors may also consider using alternative staining techniques such as GelRed, which is less toxic.

Acknowledgments

This laboratory exercise was developed in part due to funding through the Roberta Williams Laboratory Teaching Initiative Grant awarded by the Association for Biology Laboratory Education.

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About the Author

Joanna Klein is an associate professor of Biology at Northwestern College in St. Paul, MN where she teaches genetics, microbiology, biotechnology and a non-majors biology course. She received a B.S. in biology and a Ph.D. degree in genetics, both from the University of Iowa. Her postdoctoral training was at the University of Minnesota and she currently has an active undergraduate research program studying bacterial genetics. She is the co-director of a local research-based science enrichment program (the Science Research Institute) that partners college students with high school students who are typically underrepresented in the sciences.

Appendix: Instructor Laboratory Prep

Ordering

Dehydrated media, reagents and supplies for filtering can be obtained from Fisher Scientific (www.fishersci.com)

Media	Cat. #
mTEC	DF 0334-15-0
MacConkey Agar	B 12360
MR-VP	B11383
T-Soy	DF0370-17-3
Citrate Agar	B11620

Reagents	Cat. #
Oxidase Reagent	14-910-16
Voges-Proskauer A (KOH)	14-910-20
Voges-Proskauer B (α -Naphthol)	14-910-22
Methyl Red	R21236
Kovac's Reagent	23-294-950

Supplies	Cat. #
Nalgene Analytical Test filter funnel, 100 mL, 0.45 μ m	145-0045
Filter pads, 47 mm , pack of 100	FP10 404 700
Disposable borosilicate glass tubes, 13x100 mm	14-961-27
Tube caps	14-57-91

PCR supplies were obtained from Promega (www.promega.com)

Item	Cat #
GoTaq Flexi DNA Polymerase	M8295
PCR nucleotide mix	C1141
1 kb ladder	G5711

Primer was ordered from Integrated DNA Technologies (www.idtdna.com)

Box A1R Primer:

5' CTA CGG CAA GGC GAC GCT GAC G 3'

Preparation of Media and Solutions

Urea Substrate Media

2 g Urea
0.01 g Phenol Red
100 mL reagent grade distilled water.

Add dry ingredients to water, stir to dissolve. Adjust to pH 3-4 with 1 N HCl. The solution should be yellow in color. Store at 4°C. Remake the solution if it changes color after prolonged storage.

mTEC Agar Plates

Following package directions, prepare 250 ml of mTEC agar in distilled water. Autoclave at 121°C for 15 min and cool in a 50°C water bath. Pour media into 60 mm Petri plates to a depth of 5 mm. Allow to solidify and store at 4°C until used.

MacConkey Agar Plates

Dehydrated MacConkey agar is manufactured by Difco. Following package directions, add an appropriate volume of MacConkey agar to distilled water. Autoclave at 121°C for 15 min and cool in a 50°C water bath. Pour media into 100 mm Petri plates to a depth of 5 mm. Allow to solidify and store at 4°C until used. To calculate the volume needed, assume 15-20 ml medium per plate.

MR—VP Broth

Following package directions dissolve an appropriate volume of MR—VP broth in distilled water. Aliquot 5 mL broth to a 13x100 mm disposable glass test tube and cap. Autoclave at 121°C for 15 min. Store at 4°C until used.

T-Soy Broth

Following package directions dissolve an appropriate volume of T-Soy Broth in distilled water. Aliquot 5 mL broth to a 13x100 mm disposable glass test tube and cap. Autoclave at 121°C for 15 min. Store at 4°C until used.

Citrate Slants

Following package directions, add an appropriate volume of citrate agar to distilled water. Heat to boiling to dissolve agar and aliquot 4 ml to 13x100 mm disposable glass test tubes and cap. Autoclave at 121°C for 15 min. Immediately lay the tubes down to a nearly horizontal position and allow to solidify.

Guidelines for PCR Reaction

- 5 μ L 5 x Colorless GoTaq Flexi PCR buffer (Promega)
- 0.5 μ L PCR nucleotide mix (10 mM each)
- 6.7 μ L $MgCl_2$ (25 mM)
- 0.5 μ L primer (100 μ M, Box A1R,
5' CTA CGG CAA GGC GAC GCT GAC G 3')
- 0.25 GoTaq Flexi DNA Polymerase (Promega)
- 10.05 μ L H_2O

Prepare one cocktail of this reaction mix (times 8) for each student pair in advance of the lab. Each group sets up 6 PCR reactions, and making enough for 8 reactions covers pipetting errors.

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Citing This Article

Klein, J.R. 2011. Investigation of *Escherichia coli* in Freshwater Sources Using Membrane Filtration and Rep-PCR DNA Fingerprinting with Introductory Biology Students. Pages 104-116, in *Tested Studies for Laboratory Teaching*, Volume 32 (K. McMahon, Editor). Proceedings of the 32nd Conference of the Association for Biology Laboratory Education (ABLE), 445 pages. <http://www.ableweb.org/volumes/vol-32/?art=9>

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