Science in the Real World
Microbes in Action

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At a Glance

Description:
A scientific method, outlined as Koch's Postulate, is used in this laboratory exercise to demonstrate that a bacterium called *Erwinia carotovora* is the organism that causes a plant disease called "soft rot". This laboratory is a model for the study of infectious disease; however, this organism is harmless to humans.

Instructional Objectives:
At the end of this unit of activities the student should be able to:
1. demonstrate the methods of scientific inquiry by
    a. stating a problem
    b. writing a hypothesis
    c. performing an experiment
    d. gathering and organizing data
    e. analyzing data
    f. developing further investigations

2. demonstrate the following laboratory skills
    a. use of sterile technique
    b. comparing and contrasting
    c. transferring bacteria using an inoculating loop
    d. streaking an agar plate

3. demonstrate the following concepts
    a. listing the steps of Koch's Postulates
    b. applying the steps of Koch's Postulates to a new example

Time requirements:
This laboratory takes place over four consecutive days. Day one activities will require 50 minutes. Procedures for days two, three and four each require only 20-30 minutes. Students should be familiar with basic sterile technique or it should be included at the beginning of this unit.

Curriculum Placement:
This unit can be used to teach: the use of models in scientific analysis, an introduction to infectious disease, or as an introduction to microorganisms and microbiology techniques.

Equipment:
autoclave or pressure cooker
forceps
incubator (optional)
scissors
Bunsen burners (optional)
plastic knives
inoculating loop or toothpicks

Materials: (For a class of 30 working in pairs)
45 petri plates (15 x 3)
tap water
30 nutrient agar plates (15 x 2)
5 carrots
1 master culture of *Erwinia carotovora*
soap
10% bleach solution
15 marking pens
paper towel or filter paper
95% ethanol

Something Rotten in the Vegetable Bin

Background

As long ago as the 16th century, it was thought that something unseen could cause disease. Even in ancient times, it was recognized that diseases could be contagious (individuals with leprosy were segregated from healthy people). Ignaz Semmelweis and Joseph Lister recognized the importance of bacteria in causing disease, but added little data in support of the "germ theory". In 1876 Robert Koch was studying a disease of cattle called anthrax, and found convincing evidence that the disease was caused by a bacterium that was named *Bacillus anthracis.* In studying this disease he established criteria and a method for determining whether a microorganism actually causes a disease. This method is called Koch's Postulates.

In 1876 Koch observed that cows infected with a disease called anthrax had millions of bacteria in their blood. He hypothesized that the bacteria found in the blood of the sick cow caused the disease. To test his hypothesis he isolated bacteria from the host (the organism harboring the microorganism) and grew it in the laboratory. He then inoculated a healthy cow with these suspected bacteria that had been grown in the lab. After some time the new host showed signs of the disease. He isolated the bacteria found in his new host and then compared it with the original bacteria and identified them to be the same. He concluded after many trials that specific bacteria did indeed cause the disease.

Koch's Postulates

1. The microorganism is found in the diseased host and in other individuals suffering from the same disease. This organism is not found in healthy individuals.

2. Microorganisms must be isolated from the infected individual and cultivated in the laboratory away from the host.

3. The pure culture grown in the laboratory, when inoculated into a healthy individual, should initiate the characteristic symptoms of the disease being studied.

4. The suspected microorganism should again be isolated from the experimental animal and cultured. It must then be identified as the original microorganism isolated in the First postulate.

Microorganisms that cause diseases are called pathogens. These pathogens can be isolated today, much in the same way as Robert Koch established many years ago. In this lab you will be the scientist responsible for testing a specific bacterium using Koch's Postulates. It is important to note that the bacterium with which you will be working is not pathogenic to humans; it cannot make you sick. This bacterium is a pathogen only to plant roots such as carrots and potatoes. It causes the roots to rot and the plants to die.

Purpose

1. To understand the importance of a scientific approach in learning the cause of disease.
2. To learn how to grow and identify a microorganism that causes an infectious disease, root rot, in plants.

Materials per group of 2 students:

1 "diseased" carrot
1 "healthy" carrot
10% bleach solution
3 petri plates (disposable sterile polystyrene)
1 inoculating loop
1 marking pen
paper towels or filter paper
scissors for cutting paper towels or filter papers
plastic knife for peeling and cutting the carrot
forceps
water - tap or distilled
water - sterile
alcohol
2 sterile nutrient agar plates
metric ruler

Procedure

Day 1

1. Observe the diseased and the healthy carrot slices. Make close observations and record these on your student worksheet, p. 10.

2. Koch's First Postulate: Test the hypothesis that the diseased carrot has an infectious disease. Prepare a petri plate by cutting paper towels or filter paper to fit the inside of the petri plate. Use 2-3 layers of paper. Place the paper layers in the bottom of the plate. Add water to wet the paper thoroughly. Pour off all the excess water.

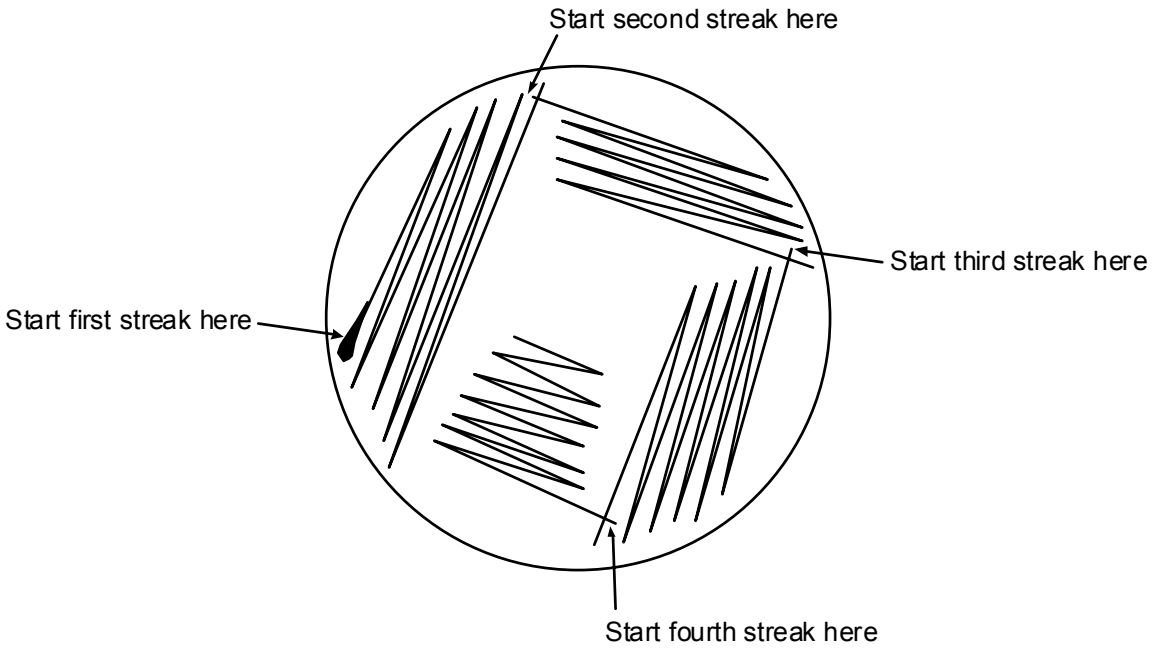
3. Using sterile forceps, place a diseased carrot slice on the wet paper in the petri plate. Sterilize the forceps in alcohol and use them to place a healthy carrot slice on top of the diseased carrot slice. Make sure that the healthy carrot touches the rotting portion of the diseased carrot slice. Move the healthy carrot slice next to the diseased carrot so that the sides are touching. Label the plate with your initials, class period and indicate that this is the experimental sample.

4. Sterilize the forceps in alcohol.

5. Using a different petri plate, repeat the steps described above using two healthy carrot slices to act as a control. Label the plate with your initials, class period and indicate that this is the control sample.

6. Koch's Second Postulate. Transfer some of the infected carrot to a sterile nutrient agar plate. To do this, gently rub a sterile inoculating loop or toothpick over the diseases part of the carrot slice. Transfer the material on the loop or toothpick to one edge of the agar surface. Using the diagram below as a guide, make a streak plate. Gently move the inoculating loop or the toothpick over the surface of the agar in the pattern shown. Be careful not to gouge the agar by pressing too hard into the agar. Label the plate with your initials and class period.

Streaking an agar plate



7. Incubate all the plates at room temperature or in a 25 degrees C incubator as directed by your instructor.

8. Disinfect your work area and wash your hands.

Day Two:

1. Observe your carrot slices in the plates. Did the healthy carrot get sick? Did the "control carrot" show any signs of disease? Do you think that what you observed is an infectious disease? Record your observations and analysis on the student worksheet.

2. Observe the nutrient agar plate you prepared from the diseased carrot. Do you observe any bacterial growth? Record your observations on #4 of the student worksheet and answer questions #5 and #6.

3. Prepare a petri plate by cutting paper towels or filter paper to fit the inside of the petri plate. Use 2-3 layers of paper. Place the paper layers in the bottom of the plate. Add water to wet the paper thoroughly. Pour off all the excess water.

4. Mark the bottom of the plate with your initials and class period.

5. Wash a fresh carrot in dishwashing detergent and rinse with clean tap water. Disinfect a plastic knife with alcohol. Use the sterile knife to peel the outer layer of skin from the carrot.

6. Wash the peeled carrot in the disinfectant (10% bleach solution) and immediately rinse in sterile water. Sterilize your knife with alcohol and carefully cut the carrot into four slices that are 5 to 8 mm thick.

7. Koch's Third Postulate: Sterilize the forceps in alcohol and use them to place the four slices of carrot on top of the filter paper in the plate.
8. Use the nutrient agar plate from Day 1 that has bacterial growth from the original diseased carrot slice. Using a sterile toothpick or inoculating loop, transfer a small amount of the bacteria from the region of heavy bacterial growth from the nutrient agar plate to the center of three slices of carrot. Do not add it to the fourth slice. Mark the bottom outside of the plate for the slices inoculated with bacteria. Why do you not inoculate all four of them with bacteria?

9. Return the cover to the plate. Answer question #7 on the student worksheet.

10. Incubate the plates right side up at room temperature or in an incubator set at 25 degrees C for 24 to 48 hours.

Day Three:
1. Observe the carrots that you inoculated with the isolated bacterial culture. Look, smell, and touch the carrot slices with a sterile toothpick. Compare the 3 inoculated carrot slices to the one that was not inoculated. What do you notice? Record your results in question #8 of the student worksheet.

2. Koch's Fourth Postulate: After your carrot slices show signs of the disease, verify the next Koch's Postulate. Transfer a culture from a "diseased" carrot slice to a new sterile nutrient agar plate. To do this, gently rub a sterile inoculating loop or toothpick over the diseases part of the carrot slice. Transfer the material on the loop or toothpick to one edge of the agar surface. Using the diagram from the previous page as a guide, make a streak plate. Gently move the inoculating loop or the toothpick over the surface of the agar in the pattern shown. Be careful not to gouge the agar by pressing too hard into the agar. Label the plate with your initials and class period.

3. Label the bottom of the plate with your initials, class period and the number.

4. Incubate the plates right side up at room temperature or in an incubator set at 25 degrees C for 24 to 48 hours.

Day Four:

1. Observe the nutrient agar plates you prepared from the "diseased" carrot slices. Do you observe any bacterial growth? Describe your observations in question #9 of the student worksheet.

2. In a research lab, the isolated culture, once again, would be used to infect a host organism. Koch did this on experimental animals as many as 20 times before he was convinced of his findings.

Name _____

Date _____

Results and Analysis

1. List in your own words Koch's four Postulates.

_____

_____

_____

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2. Carefully describe the characteristics of the diseased carrot on Day One of the laboratory.

_____

_____

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3. Record your observations on Day Two. Did the healthy carrot get sick? Did the "control" carrot show any signs of disease? Do you think what you observed is an infectious disease?

_____

_____

_____

4. On Day Two, you observed your agar plate prepared from the diseased carrot. Did you observe any growth? Describe it

_____

_____

_____

5. Which of Koch's Postulates would this represent?

_____

_____

6. What would be the next Postulate to verify?

7. Describe your observations of the healthy carrot slices before being inoculated with bacteria. On the diagram below show which carrots were inoculated with the bacteria and which were not. Show any identifying marks, shapes and relative sizes of the carrot slices.

8. Describe and draw your observations of the carrots after 24-48 hours of incubation.

9. Describe your observations of the nutrient agar plates from Day Three of the Laboratory. Does the bacterial growth appear the same or different than the plates prepared on Day One of the laboratory?

10. Describe your observations of the isolated culture made on Day Four.

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11. Were Koch's Postulates verified in this laboratory procedure? Explain.

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12. Can scientists today use Koch's Postulates to test deadly new pathogens such as the AIDS virus or the Ebola virus? Explain.

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Teacher pages

Background

As long ago as the 16th century, it was thought that something unseen could cause disease. Even in ancient times, it was recognized that diseases could be contagious (individuals with leprosy were segregated from healthy people). Ignaz Semmelweis and Joseph Lister recognized the importance of bacteria in causing disease, but added little data in support of the "germ theory". In 1876 Robert Koch was studying a disease of cattle called anthrax, and found convincing evidence that the disease was caused by a bacterium that was named Bacillus anthracis. In studying this disease he established criteria and a method for determining whether a microorganism actually caused a disease. This method is called Koch's Postulates. By 1883 he had isolated many microorganisms from air, water and soil and found that they had little significance in disease. In fact, of the tens of thousands of different microorganisms that inhabit every ecological niche on the earth, only a few hundred or so can cause disease. In addition to establishing criteria for disease, Koch also established techniques that we still use today for the isolation of bacteria in pure culture.

Microorganisms that cause disease are called pathogens. Diseases that are readily transferred from an infected individual to a non-infected individual are called communicable diseases. One route of transmission of disease is through contact, either directly with an infected individual, with aerosols produced by coughs or sneezes, or sometimes with a contaminated object, such as a handkerchief used by someone with a disease. Another mechanism of disease transfer is via a vector such as an insect that may bite first an infected individual and then an uninfected individual. Malaria is transmitted by mosquitoes. Some rare diseases are transmitted directly through animal bites (e.g., rabies virus). Many diseases are transmitted through food and water and some through air. Only a few diseases are both deadly and easily transmitted. Good hygiene and a healthy body protect most people from diseases caused by microorganisms.

Our understanding of microorganisms and how to grow and identify them was significantly advanced by the work of Robert Koch and by Louis Pasteur. The development of sewage and water treatment facilities removed a major source of pathogenic organisms in daily life. Good sterile technique and improved hygiene in hospitals decreased deaths from childbirth and surgical procedures. The identification and manufacture of antibiotics and the development of vaccines have significantly reduced communicable diseases in the 20th century. Significantly, however, communicable diseases are still a major problem in third world countries where sanitation is poor and drugs are not readily available.

Time Line

Day One:
Students will examine their prior knowledge with a worksheet and discussions. Then the lab begins with a "diseased" carrot and a "healthy" carrot. Students will verify Koch's First Postulate as they bring the diseased carrot in contact with the healthy one. Next, they confirm Koch's Second Postulate as they isolate a culture from the diseased carrot.
Day Two:
Students observe the plate and carrot slices. Next, they verify Koch's Third Postulate as they inoculate "healthy" carrot slices with bacteria from plate.
Day Three:
Students observe the carrot slices from Day Two. Next, they isolate a culture from the "Day Two diseased" carrot onto a nutrient agar plate to confirm the fourth postulate.
Day Four:
Students observe the isolated cultures and analyze their results.

Preparations

- At least a week before the lab, obtain a culture of *Erwinia carotovora.* Store culture capped in the refrigerator.
- Prepare 10% bleach solution in advance. (Warn the students that bleach can discolor their clothes.)
- Prepare nutrient agar plates. One liter of medium will provide about 40-50 plates. Dissolve 23 grams of nutrient agar powder into 1 liter of distilled water (tap water will work). Prepare enough to use approximately 20 ml per plate. Prepare the agar in batches that only fill the flask half way to prevent boilover. Once you have prepared the nutrient agar, cover the flasks with aluminum foil and place them in the autoclave or pressure cooker. Follow the instructions on your sterilizer to complete the sterilization process. Generally, you need 15 lbs. of pressure and a temperature of 121 degrees C for 15 minutes to achieve sterility.
  Once the materials have been sterilized, the nutrient agar must be poured into the plates before it solidifies. It is best to pour the agar when it has cooled enough to be held comfortably in your hand (45-50 degrees C). Spread the plates out on the lab tables, lift the lid straight up and pour the agar into the plate. You may want to pour 20 ml of water into an empty plate to give you an idea of how much agar to pour into each plate. Once all of the plates have been poured, let them set until they have solidified. Store the plates upside down until you are going to use them. If it is going to be several days until you use the plates, put them back into the plastic sleeve in which they were shipped and store them upside down in a refrigerator or a cool place.
- A day or two before the laboratory, prepare several (1 per student group) "diseased" carrot slices. Place a carrot slice in a sterile plate. Using a sterile toothpick or loop, add a visible amount of the *Erwinia* culture to the carrot slice. Incubate at room temperature until the carrot looks soft and rotten.

Sources of Supplies

Carolina
2700 York Road
Burlington, NC 27215
(800) 334-5551

Description	Stock number	Quantity	Cost
petri dishes	F6-70-3033	500	$95.00
disposable pipettes	F6-73-6898	10	$1.75
culture of Erwinia carotovora	F6-15-5045	1 tube	$7.00

Sigma Chemical
PO Box 14508
St. Louis, MO 63178
(800) 521-0851

Description	Stock number	Quantity	Cost
Nutrient Agar	N 0394	250 g	$29.00

Teacher Hints & Troubleshooting

1. This organism does not require an incubator. It will grow at room temperature (20-22 degrees C). If you have an incubator, 25 degrees C is an ideal growth temperature and will give the best results.

2. If visible results are not seen within 24 hours, allow additional time for the incubation. Results appear to depend on the concentration of the *Erwinia* culture.

3. Testing the hypothesis that the diseased carrot has an infectious disease, (steps 2 and 3, page 12) may be done as a classroom demonstration to save on materials and preparation time.

4. Cultures and plates can be soaked in 10% bleach and discarded in the trash.

Dear Parents,

Have you ever reached into your vegetable crisper and found carrots that weren't so crisp? Why does that happen? Is it simply age? Is it rot? Or is it something else? This week in biology class, your child will be looking for the answers to those questions.

In the process, students will learn more about the history of microbiology and the spread of disease. By the end of this week, they should be able to explain why, if they get a cold then you may get a cold. But don't worry, even though carrot diseases spread in the same manner as human diseases, it won't spread to people.

If you want to get to the "root" of the problem, come to biology class this week.