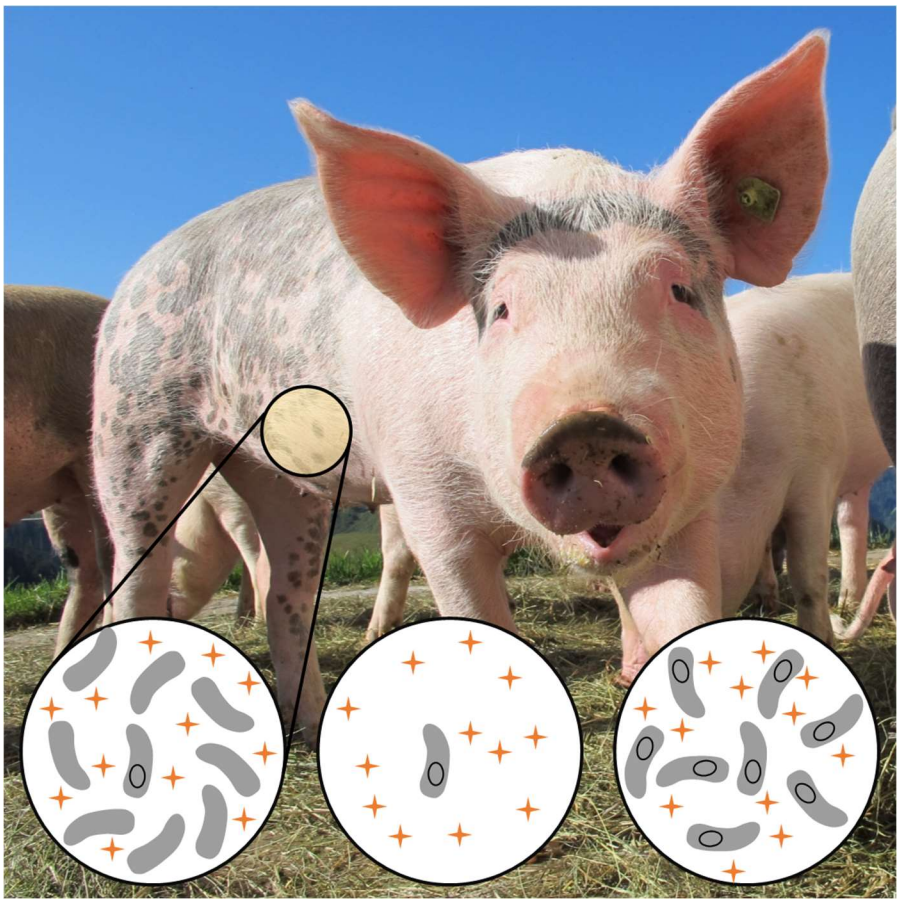
miniPCR™ Antibiotic Resistance Lab: Monitoring resistant organisms in the environment.

Produced in collaboration with

PARE – Prevalence of Antibiotic Resistance in the Environment



Instructor's Guide Contents

1. Synopsis

This lab represents a fictional case study of a very real problem. Using PCR and gel electrophoresis, students will investigate the spread of carbapenem resistant *E. coli* in the environment. *No potentially harmful environmental samples are used in this lab.*

- ***Techniques utilized***: PCR, gel electrophoresis, and DNA visualization
- ***Time required***: One 90 min. period or two 45 min. periods
- ***Reagents needed***: 'PARE' reagents kit (available from miniPCR), gel electrophoresis reagents (See sections 6 and 9)
- ***Suggested skill level***: Familiarity with DNA amplification concepts, basic familiarity with micropipetting techniques

2. Learning goals and skills developed

Student Learning Goals:

- Use PCR as a technique to amplify and identify specific genes
- Define and describe the risk of antibiotic resistance in the environment
- Relate the process of natural selection to the emergence of antibiotic resistance
- Analyze and interpret results of a molecular diagnostic test

Scientific Inquiry Skills:

- Students will create hypotheses and predict results
- Students will compare results to their predictions and to a real-world example
- Students will use experimental results to make conclusions based on hypotheses
- Students will follow laboratory safety protocols

Molecular Biology Skills:

- Micropipetting skills
- Principles and practice of PCR
- Preparation of agarose gels
- Agarose gel DNA electrophoresis
- Staining, visualization, and molecular weight analysis of DNA fragments

3. Standards alignment

Next Generation Science Standards - Students will be able to…

HS-LS1-1 Construct an explanation based on evidence for how the structure of DNA determines the structure of proteins which carry out the essential functions of life through systems of specialized cells.

HS-LS3-1 Ask questions to clarify relationships about the role of DNA and chromosomes in coding the instructions for characteristic traits passed from parents to offspring.

HS-LS3-2 Make and defend a claim based on evidence that inheritable genetic variations may result from: (1) new genetic combinations through meiosis, (2) viable errors occurring during replication, and/or (3) mutations caused by environmental factors.

HS-LS4-1 Communicate scientific information that common ancestry and biological evolution are supported by multiple lines of empirical evidence.

HS-LS4-4 Construct an explanation based on evidence for how natural selection leads to adaptation of populations.

Common Core English Language Arts Standards - Students will be able to…

RST.11-12.1 Cite specific textual evidence to support analysis of science and technical texts, attending to important distinctions the author makes and to any gaps or inconsistencies in the account.

WHST.9-12.2 Write informative/explanatory texts, including the narration of historical events, scientific procedures/ experiments, or technical processes.

WHST.9-12.9 Draw evidence from informational texts to support analysis, reflection, and research.

SL.11-12.5 Make strategic use of digital media (e.g., textual, graphical, audio, visual, and interactive elements) in presentations to enhance understanding of findings, reasoning, and evidence and to add interest.

4. Background information

Teacher's note. This case study is fictional, but is based on a synthesis of data representing several different actual incidences:

1) an outbreak in Denmark of Cipro-resistant *Salmonella* traced to consumption of pork that originated from one farm;
2) the first patient isolate ever reported containing the NDM-1 gene;
3) subsequent sampling of water samples from around New Delhi for presence of the NDM-1 gene;
4) a multi-state outbreak of *E. coli* traced to lettuce from the Yuma, Arizona region and, ultimately, to water from an irrigation canal;
5) identification of carbapenem-resistant bacteria in agricultural settings.

This lab uses primers that simulate testing for the carbapenem resistance gene bla_{NDM-1}. This lab is designed to introduce students to the problem of antibiotic resistance in the environment and to techniques that could be used to monitor the problem.

A) Antibiotic resistance in the environment

Bacteria are all around us. They live on you; they live inside you. They also live in the soil, water, and pretty much anywhere available carbon can be obtained. By some estimates, a single teaspoon of productive soil will have about as many living bacteria as there are people living in the United States[1]. We normally think of bacteria as "germs" that make us sick, but the vast majority of bacteria living in the environment would be about as successful living inside of you as you would living with them under the ground.

But we all know that some rare bacteria do make us sick. And when we get sick with these, we can take an antibiotic drug for a few days that will typically get rid of the infection. This hasn't always been the case. Widespread use of antibiotics has only been around for less than 100 years. Alexander Fleming's discovery and characterization of penicillin in 1928 is widely seen as ushering in the modern world of antibiotic medicine. For the first time in human history, infections that would regularly kill were easily and routinely cleared up within a few days of beginning treatment. Within a few decades, several dozen varieties of antibiotics were introduced and available, and their use is thought to be responsible for saving the lives of hundreds of millions of people.

[1] https://www.nrcs.usda.gov/wps/portal/nrcs/detailfull/soils/health/biology/?cid=nrcs142p2_053862

But as the use of antibiotics has spread, so has bacteria's resistance to them. It is fairly common today for routine infections to be resistant to antibiotics that were once used to treat them. And as bacteria gain resistance to more and more different drugs, a future where antibiotics can no longer treat some routine infections is a serious possibility that we may have to face sooner rather than later. In hospital settings, the fear of antibiotic resistant infections is a very real one. Hospitals regularly see sick patients and treat them with antibiotics. Because so many infections are brought to hospitals and resistant infections are so difficult to kill, hospitals become enriched environments for antibiotic resistance. In fact, hospitals are one of the places where people are most likely to become infected with resistant bacteria.

But not all resistant bacteria live in hospitals. More and more, antibiotic resistant bacteria are being found simply in the environment – living in the soil and water. Nobody knows for sure exactly how widespread resistant bacteria in the environment are, but as it is becoming more and more clear that environmental pathogens are an important source of infections in humans, the concern that we could be infected with resistant pathogens from our environment is a real one. In 2018, about 200 people across 36 states were sickened by a pathogenic strain of *E. coli* that had contaminated romaine lettuce; five people died. The source of the contamination was environmental; the bacteria had spread through an irrigation canal. Luckily, in this case, the bacteria were not resistant to antibiotics. But what if they had been?

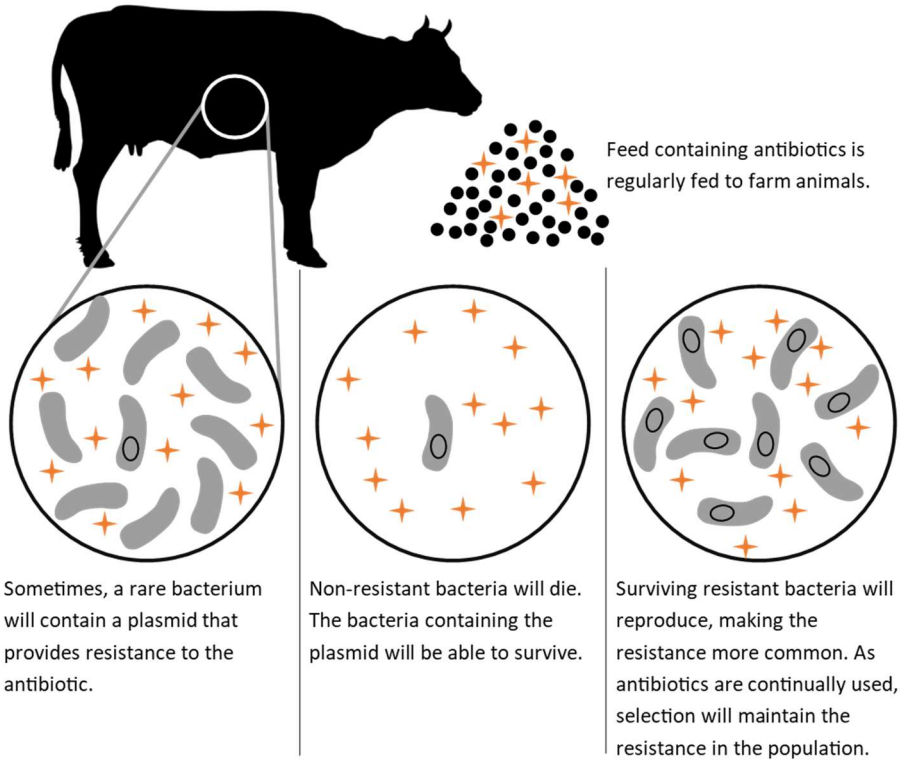
B) Where does resistance come from?

Antibiotic resistance is one of the clearest examples of evolution by natural selection that humans have been able to view in real time. In under 100 years, we have gone from virtually no widespread antibiotic resistance to a world where antibiotic resistance is so widespread that it is viewed as a global health crisis. How did this happen so fast?

When we take an antibiotic, the goal is to kill all of the bacteria that are making us sick. But treatments often don't kill one hundred percent of the bacteria. The few surviving organisms after an antibiotic treatment were likely able to survive because they were more resistant to the antibiotics than all their counterparts that died. If their ability to survive treatment was due to a genetic variation, when these remaining bacteria reproduce, they will pass on this resistance to their offspring. When another dose is taken, the cycle will repeat itself. Over time, after successive antibiotic treatments, the only bacteria left in the population will be ones that were able to survive because they inherited the set of genes that made them resistant. This is why antibiotic resistance is now such a problem in hospital settings – all the non-resistant bacteria

are routinely eliminated. The populations that remain are the ones that evolved to survive in a world where they regularly must overcome antibiotic treatments.

Humans, of course, are not the only animals that can get sick from bacteria. Farm animals are also susceptible to bacterial infections, and so farmers regularly treat their animals with antibiotics. In fact, over 70% of medically important antibiotics sold in the United States are used on animals.[2] This has led to some of the problems with antibiotic resistance in the environment that we see today. For a long time, a standard practice has been to include low levels of antibiotics in animal feed. This constant low-level use reduces the incidence of infections in farm animals and also, for mainly unknown reasons, often increases the growth rate of the animals. But the constant low-level use means that bacteria are under constant selective pressure. Every time antibiotics are administered, antibiotics will kill much, but not all, of the bacterial population. Those bacteria that survive to reproduce will do so because they possess a resistance to the antibiotic being used. As the same antibiotics are used in the feed, over and over, year after year, the bacteria that are able to survive can do so because they inherit genes that provide resistance.



Of course, bacteria don't stay inside animals forever. Animals (and people) constantly spread the bacteria living inside them, for example, whenever they cough, sneeze, and, perhaps most importantly, through feces. For humans, this is often how bacterial infections spread, but the problem is largely mitigated by using modern sewage disposal and treatment systems. For farm animals, where there is no sewage treatment, this can be a major problem. The biota living

[2] http://www.cidrap.umn.edu/news-perspective/2017/01/farm-antibiotics-does-new-fda-policy-go-far-enough

inside farm animals are regularly enriched for antibiotic resistance, and then, those surviving resistant organisms are released into the environment through the significant amounts of fecal matter produced on farms. These bacteria can then spread considerable distances by being carried in water run-off from rain or other sources.

This is all made even more troublesome because, in bacteria, these resistance genes can spread in a way that genes in you cannot. You get your DNA from your mom and your dad, and nowhere else. Bacteria, on the other hand, can be a little looser with where they get DNA from. Bacteria will often pick up DNA from the environment or exchange DNA from neighboring bacteria in the form of a plasmid. A plasmid is a small circular segment of DNA that contains an origin of replication and a few genes. Where most bacterial DNA is passed asexually directly from parent to offspring in a single circular genome (what we call *vertical* transmission), plasmids can be passed both vertically and *horizontally*, from one unrelated organism to another, often even across different species. This means that once resistance evolves in one species, if that resistance gene ends up as part of a plasmid, it can spread relatively quickly to many different species. Genes from plasmids can even be integrated into a bacterium's chromosome leading to more stable vertical transmission of the resistance. Today, many plasmids are passed in the environment (sometimes referred to as eDNA, or environmental DNA) that contain not one, but several resistance genes.

Ultimately, we can expect populations of antibiotic-resistant bacteria to emerge and become more common any time the selective pressure of antibiotics is regularly applied. This, indeed, is a powerful example of evolution in action. This, however, does not mean that bacteria somehow change when antibiotics are applied; it just means that the bacteria that are susceptible to antibiotics die while bacteria that are resistant can expand. This also doesn't mean that resistance to antibiotics is a new thing. Even though antibiotics have only been used by humans for under a century, most antibiotics that we use were discovered already existing in natural sources. Penicillin, for example, is a molecule that is naturally produced by the fungus, *Penicillium chrysogenum*. Because certain types of bacteria have always lived in the specific environment where *Penicillium chrysogenum* occurs, selection for resistance to penicillin has been occurring in this environment for a very, very long time. Those bacteria that originally carried the penicillin resistance likely do not cause human infections, but as described above, horizontal gene transfer can pass resistance genes to bacteria that never had them before. Now, with humans using penicillin regularly, when a plasmid containing a resistance gene is passed into a human pathogen, natural selection leads to that now resistant strain of bacteria becoming more and more common.

So how does resistance work? There are a few ways that bacteria can become resistant to antibiotics. An enzyme can break down or change the antibiotic into a harmless molecule. A molecular pump can pump the molecule out of the cell, or other changes can make it more difficult for the antibiotic to enter the cell in the first place. Or, a change in the physiology of the bacteria or the binding site of the antibiotic can make the antibiotic lose efficacy. Molecular pumps and enzymes that inactivate the antibiotic are encoded on large chunks of DNA which can be passed around bacterial populations horizontally on plasmids and also through vertical transmission. Alternatively, changes that affect the binding site for the antibiotic often result from point mutations on the bacterial genome which can only be transmitted vertically.

Whenever an organism reproduces, it must copy its DNA. Occasionally, rare copying mistakes, or mutations, are made. Even more rarely, these mutations may lead to a random change in a gene that happens to provide an increase in resistance to an antibiotic. The important thing to remember is that these genes already existed, performing a function for the cell closely related to how it works with antibiotics. When an antibiotic is administered, the very rare bacterium that possesses this unique sequence of DNA is more likely to survive and spread that resistance trait on in the population. As the antibiotic is continually used, the bacteria that possess these genetic sequences survive to reproduce, while other bacteria die. Over time, under the selective pressure of continued antibiotic use, resistance will become more and more common in the population until all the non-resistant bacteria have died off and only bacteria that contain the resistance DNA sequences remain.

That the genetic code is universal and that DNA can spread between bacterial species on plasmids means that a single case of one of these DNA sequences in a single bacterium can lead to eventual worldwide antibiotic resistance in many different bacterial species. Today, many plasmids are circulating in the environment that contain several resistance genes linked together, allowing bacteria to become resistant to many different antibiotics by taking up a single piece of DNA from the environment. These resistant bacteria can be identified in different ways. Scientists can try to grow them, plating environmental samples on agar plates that contain antibiotics and looking for growth. They can also use molecular techniques such as PCR, amplifying DNA from environmental samples to try to identify the resistance genes being passed on plasmids.

C) What to do?

The problem with antibiotic resistance is a bit of a paradox. Using antibiotics leads to resistance spreading within a population of bacteria, which in turn makes our antibiotics no longer useful. If we want to stop new resistant strains from spreading, we need to stop using that antibiotic, but that isn't a great option for a person who is sick and in need of treatment.

Still, virtually all experts generally agree that the problem of spreading antibiotic resistance could be slowed significantly if antibiotics were used much less often and much more judiciously. When antibiotics are used too widely, bacteria are under constant selective pressure to develop and maintain resistance. But when antibiotics are not present, we know that non-resistant bacteria will regularly outcompete the resistant strains and populations will tend to become less resistant over time. The general consensus is therefore that antibiotics should be used much more sparingly than they generally are, and that specific "last line of defense" antibiotics should only be used when absolutely necessary. This is true for human use, but also especially true for animal health use. Already, use of antibiotics to promote growth of livestock has been banned in Europe and a directive from the FDA banned the practice in the United States in 2017. But many people think this does not go far enough. The drugs can still be used under the supervision of veterinarians to treat and even prevent diseases in animals, and in many countries around the world there are no restrictions at all. In preventing the spread of antibiotic resistance, we may know some important steps to take; actually, taking them is much more difficult.

5. Case study:

Note: *The following case study represents a fictional outbreak of a real and spreading antibiotic resistance threat. It is a presented as a possible scenario for students to investigate how antibiotic resistance in the environment can be seen as a real and growing problem.*

Facts of the case

An outbreak of *E. coli* has infected 42 people across 12 different states. The individuals hospitalized showed severe symptoms of food poisoning including hemorrhagic diarrhea and some cases of kidney failure. Doctors treating the patients immediately administered the antibiotic imipenem, a powerful drug from the carbapenem class of antibiotics. Patients did not respond to the treatment. Doctors suspect that the *E. coli* were resistant to carbapenems and switched to another antibiotic, colistin. Luckily, most of the patients responded to the new treatment. Still, 8 of the 42 patients died.

Subsequent testing confirmed that the *E. coli* possessed a plasmid that contained the gene bla_{NMD-1}, a relatively new but spreading carbapenem resistance gene.

Public health officials have tracked the source of the infection to tainted pork that originated from a single farm. Hog farming produces considerable amounts of manure waste, and neighboring farms are now concerned that that waste may be spreading the genes responsible for carbapenem resistance into the soil and water. Two farms in particular, Apple Point Farms and Barrow Creek Farm have reached out to public health officials to try to assess their possible risk. Soil samples have been collected from these farms in order to test for the presence of the bla_{NDM-1} gene.

Information about carbapenems

Carbapenems are antibiotics that are generally used only in extreme, last resort settings and are considered one of our last lines of defense against antibiotic resistant bacteria. In 2007, however, a man was infected in India with a strain of bacteria that showed resistance to treatment with carbapenems. Subsequent testing identified that resistance was provided for by a gene coding for a protein that breaks down carbapenems. The protein was named NDM-1 (New Delhi metallo-beta-lactamase 1), a carbapenemase that hydrolyzes the carbapenems. The gene that codes for NDM-1, bla_{NMD-1}, was located on a plasmid that can be spread through horizontal gene transfer. The bla_{NMD-1} gene has since spread worldwide and has been found in settings ranging New Delhi drinking water to United States hospitals. The spread of this resistance gene

has been particularly alarming; in just ten years, it has gone from being completely unknown to being identified in samples worldwide.

Carbapenems are restricted to hospital use, so the identification of resistance in environmental settings is especially concerning. But while NDM-1 provides resistance to carbapenems, it also provides resistance to several other antibiotics[3], some of which are used regularly in agriculture. It is possible that widespread use of these other more commonly used antibiotics is fueling the spread of bla_{NMD-1} in environmental settings.

Today's lab

You will be provided with DNA extracted from the soil from two farms, Apple Point and Barrow Creek, and will use PCR to identify if *bla_{NMD-1}* is present in one, both, or neither of the environmental samples. You will use primers that are specific to *bla_{NMD-1}* to test for the resistance gene. When *bla_{NMD-1}* is present in a sample, these primers will amplify a 700 base pair fragment of DNA. A second set of primers will be used to amplify a 400 base pair region of the 16S ribosomal RNA gene. This second set of primers will be used as a PCR control, to make sure that DNA was present in the sample and that DNA amplification in the PCR was successful. You will also be provided a sample of DNA extracted from an *E. coli* isolate known to contain *bla_{NMD-1}* and a second sample of DNA from an *E. coli* isolate known to be susceptible to carbapenem. These samples of DNA will serve as positive and negative controls for your experiment.

Your job: Determine if the *bla_{NMD-1}* gene is spreading resistance on either Apple Point or Barrow Creek farms.

[3] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1932750/

6. Laboratory set-up manual

Reagent	Volume needed per lab group	Storage	Teacher's checklist	
EZ PCR Master Mix, Load Ready™ • 5x Mix with *Taq* DNA polymerase • dNTPs (included) • PCR buffer with Mg^{2+} (included) • Gel Loading Dye (included)	25 µL	-20°C freezer		*Supplied in Kit*
PARE Primer Mix • *'PARE'* primers mix	50 µL	-20°C freezer		
Template DNA • *Negative Control* DNA sample • *Positive Control* sample • *Apple Point* DNA sample • *Barrow Creek* DNA sample	15 µL each	-20°C freezer		
100 bp DNA Ladder, Load Ready™ • DNA molecular weight marker	12 µL	-20°C freezer		
DNA staining agent • Gel Green DNA Stain (for Blue light transilluminators)	Follow supplier instructions	Room temp., dark		*Available at miniPCR.com*
1.6% agarose gels • Electrophoresis grade agarose	5 lanes per lab group	Room temp.		
Electrophoresis buffer • e.g., 1X TBE	Depending on gel apparatus	Room temp.		

Equipment and Supplies	Teacher's checklist
PCR Thermal cycler: e.g. miniPCR™ machine	
Micropipettes • 2-20 µL: one per lab group • 20-200 µL: one for the teacher (to dispense reagents)	
Disposable micropipette tips	
PCR tubes: 4 x 200 µL microtubes per lab group	
Plastic tubes: 6 x 1.5 or 1.7 mL tubes (to aliquot reagents for each lab group)	
Horizontal gel electrophoresis apparatus: e.g., blueGel™	
DC power supply for electrophoresis apparatus (included with blueGel™)	
Transilluminator: UV or Blue light (included with blueGel™)	
Scale for weighing agarose	
250ml flasks or beakers to dissolve agarose	
Microwave or hot plate to dissolve agarose	
Microcentrifuge (optional; only needed to collect liquid at tube bottom)	
Gel documentation system (optional, or use cell phone camera instead)	
Other supplies: • UV safety goggles (if using UV transilluminator) • Disposable laboratory gloves • Permanent marker	

Available at miniPCR.com

Planning your time

This experiment has 4 stages:

A. PCR reaction set up
B. PCR programming and monitoring
C. Separation of PCR products by DNA electrophoresis
D. Size determination of PCR products and interpretation

An overview of the 90-minute experimental plan is represented below:

Preparatory activity	Experimental stage
Dispense reagents and prepare equipment • *20 min*	**A PCR set up** • 15 min
	B PCR programming & monitoring • PCR programming • 45 min PCR monitoring and discussion
STOP	*Possible stopping point* *Store PCR product in fridge (up to 1 week) or freezer (longer term)*
Pour agarose gels (e.g. during stage B) • *20 min*	**C Gel electrophoresis** • 20 min
	D Size determination & interpretation • 5 min visualization • 5 min discussion

Quick guide: Preparatory activities

A. PCR set up

- Thaw tubes containing the primers and DNA samples by placing them on a rack or water bath at room temperature
- For each lab group, label and dispense six tubes:
  - EZ PCR Master Mix 25 µL
  - PARE Primer Mix 50 µL
  - Negative Control DNA 15 µL
  - Positive Control DNA 15 µL
  - Apple Point DNA 15 µL
  - Barrow Creek DNA 15 µL
- Each lab group will additionally need:
  - Micropipettes *(we recommend 2-20µL range)*
  - Disposable micropipette tips and a small beaker or cup to dispose them
  - 4 PCR tubes (200 µL)
  - A permanent marker (fine-tipped)

B. PCR programming and monitoring

- Ensure each lab group's bench is set up with a miniPCR and power supply
- Ensure the miniPCR machines that are going to be monitored through the PCR reaction are connected to a computer or compatible tablet

C. Gel electrophoresis

- Gels can be poured in advance of the class (as described below)
- Pre-poured gels can be stored in the fridge, in a sealed container or wrapped in plastic wrap, and protected from light
- If doing the gel run on a different day than the PCR run, completed PCR reaction tubes can be stored in the fridge for up to one week until they are used, or in the freezer for longer-term storage

D. Size determination and interpretation

- Have the banding pattern of the 100bp DNA Ladder handy to help interpret the electrophoresis results

7. Instructor laboratory guide

A. PCR set up

1. **Label 4 PCR tubes** (200 µL tubes) per lab group. Label tubes on the **side wall**.
   - 1 tube labeled "N": 'Negative Control DNA' from non-resistant bacteria
   - 1 tube labeled "P": 'Positive Control DNA' from carbapenem resistant bacteria
   - 1 tube labeled "A": DNA from Apple Point Farm
   - 1 tube labeled "B": DNA from Barrow Creek Farm

   Also label each tube with the group's name on the side wall

2. **Add PCR reagents** to each 200 µL PCR tube

	Tube N	Tube P	Tube A	Tube B
Template DNA	Negative Control 'non-resistant' DNA 10 µL	Positive Control 'resistant' DNA 10 µL	DNA From Apple Point 10 µL	DNA From Barrow Creek 10 µL
PARE Primer Mix	10 µL	10 µL	10 µL	10 µL
5X EZ PCR Master Mix	5 µL	5 µL	5 µL	5 µL
TOTAL VOLUME	***25 µL***	***25 µL***	***25 µL***	***25 µL***

   Use a micropipette to add each of the reagents.
   Remember to change tips at each step!

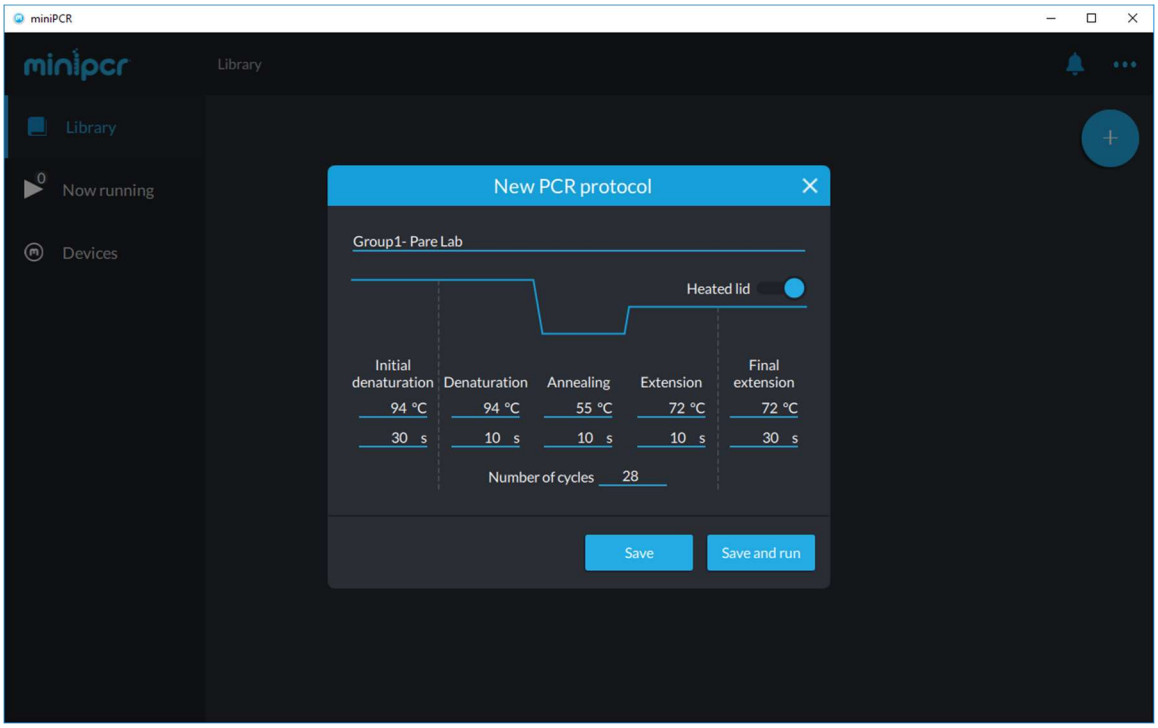
3. **Gently mix the reagents by pipetting up and down 3-4 times, cap the tubes**
   - Make sure all the liquid volume collects at the bottom of the tube (if necessary, spin the tubes briefly using a microcentrifuge)

4. **Place the tubes inside the PCR machine**
   - Press firmly on the tube caps to ensure a tight fit
   - Close the PCR machine lid and tighten it gently

B. ***PCR programming and monitoring*** *(illustrated using miniPCR® software)*

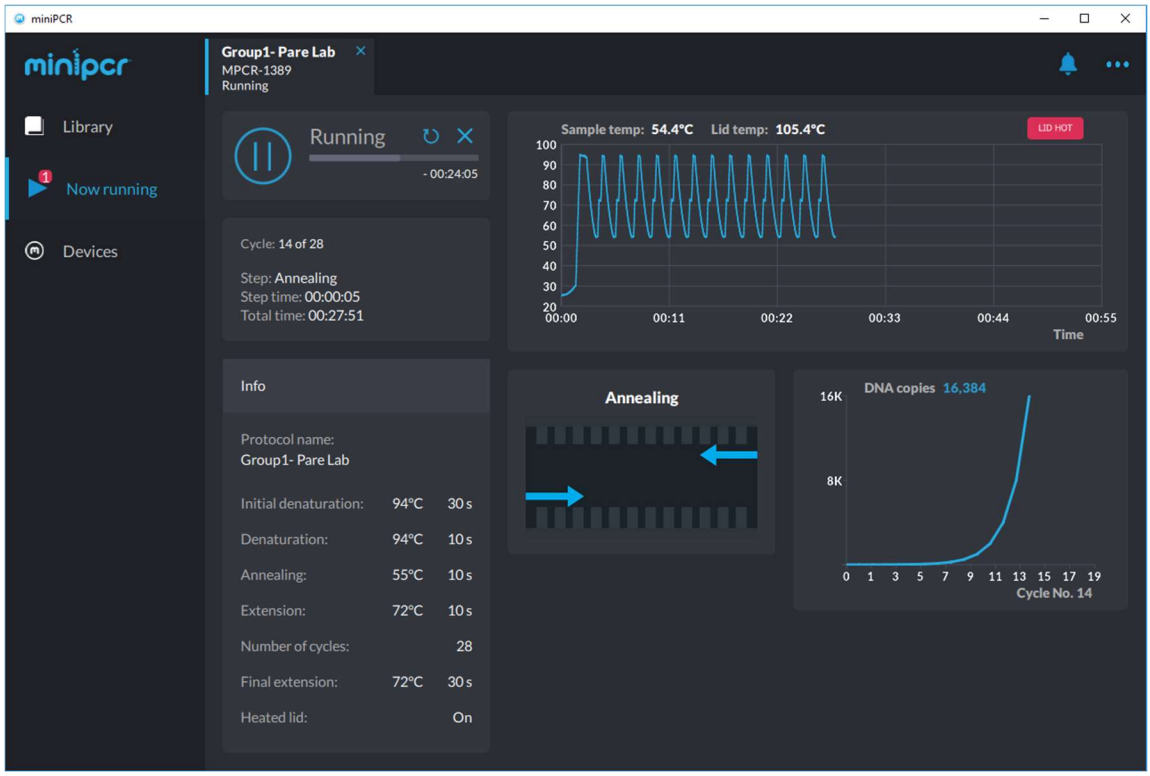
Note: depending on version of software used, programming procedures may vary slightly, but there are no changes to the temperatures, times, and numer of cycles used.

1. Open the miniPCR software app and remain on the **"Protocol Library"** tab
2. Click the **"New Protocol"** or + button
3. Select the PCR **"Protocol Type"** from the top drop-down menu
4. Enter a name for the Protocol; for example **"Group 1 – PARE Lab"**
5. Enter the PCR protocol parameters:
   - **Initial Denaturation** 94°C, 30 sec
   - **Denaturation** 94°C, 10 sec
   - **Annealing** 55°C, 10 sec
   - **Extension** 72°C, 10 sec
   - **Number of Cycles** 28
   - **Final Extension** 72°C, 30 sec
   - **Heated Lid** ON



6. Click **"Save"** to store the protocol or "**Save and Run"** to begin the protocol immediately.
    - Make sure that the power switch is in the ON position
    - For older software versions (v1.6) you will need to hit the **"Upload to miniPCR"** in the lower right-hand corner of the window.
    - If prompted, select the serial number of your miniPCR machine in the dialogue window. Serial numbers are located next to the USB port.

7. Click **"miniPCR [machine name]"** tab to begin monitoring the PCR reaction



The miniPCR™ software allows each lab group to monitor the reaction parameters in real time, and to export the reaction data for analysis as a spreadsheet.

Once the PCR run is completed (approximately 30-40 min), the screen will show "**Status: Completed" and all LEDs on your miniPCR machine will light up.**

You can now open the miniPCR lid and remove your PCR tubes.

⚠ ***Be very careful not to touch the metal lid which may still be hot***

The PCR product can now be stored for up to 1 week in the fridge or 1 year in a freezer.

TIME MANAGEMENT TIP

Gel electrophoresis – Pouring agarose gels (Preparatory activity)

If the lab is going to be completed in a single time block, agarose gels should be prepared during the PCR run to allow the gels to settle.

If the lab is going to be performed over two periods, gels can be prepared up to one day ahead of the second period and stored in a refrigerator, covered in plastic wrap and protected from light.

1. Prepare a clean and dry agarose gel casting tray
    - Seal off the ends of the tray as indicated for your apparatus
    - Place a well-forming comb at the top of the gel (4 lanes per group plus DNA ladder)

2. For each lab group, prepare a 1.6% agarose gel using 1X TBE buffer
    - Adjust volumes and weights according to the size of your gel tray
    - e.g., add 0.32 g of agarose to 20 ml of 1X TBE for blueGel™ system
    - Mix reagents in glass flask or beaker and swirl to mix
    - If using **blueGel Tabs**™
        - Use one tab per 25ml if making one gel
        - Two tabs in 50ml can make up to three gels

3. Heat the mixture using a microwave or hot plate
    - Until agarose powder is dissolved and the solution becomes clear
    - Use caution, as the mix tends to bubble over the top and is very hot

4. Let the agarose solution cool for about 2-3 min at room temperature.
    - Swirl the flask intermittently

5. Add gel staining dye (e.g. GelGreen™)
    - Follow dye manufacturer instructions
    - Typically, 1.0 µL of staining dye per 10 mL of agarose solution (2µl per 20ml gel)

    Note: Follow manufacturer's recommendations and state guidelines if handling and disposing of ethidium bromide

6. Pour the cooled agarose solution into the gel-casting tray with comb

7. Allow gel to completely solidify (until firm to the touch) and remove the comb
    - Typically, 15-20 minutes

8. Place the gel into the electrophoresis chamber and cover it with 1X TBE buffer

Gel electrophoresis – Running the gel

1. Make sure the gel is completely submerged in 1X TBE electrophoresis buffer
    - Ensure that there are no air bubbles in the wells (shake the gel gently if bubbles need to be dislodged)
    - Fill all reservoirs of the electrophoresis chamber and add just enough buffer to cover the gel and wells

2. Load PCR samples onto the gel in the following sequence
    - **Lane 1:** 10µL DNA ladder
    - **Lane 2**: 15µL **Negative Control (N)** PCR
    - **Lane 3**: 15µL **Positive Control (P)** PCR
    - **Lane 4**: 15µL **Farm A** PCR
    - **Lane 5**: 15µL **Farm B** PCR

    Note: there is no need to add gel loading dye to your samples. *miniPCR EZ PCR Master Mix* and *100 bp DNA Ladder* are Load-Ready™!

3. Place the cover on the gel electrophoresis box
    - Ensure the electrode terminals fit snugly into place

4. Insert the terminal leads into the power supply (not needed if using blueGel™)

5. If using blueGel™, simply press the "Run" button. Otherwise, set the voltage at 100-130V. Conduct electrophoresis for 15-20 minutes, or until the colored dye has progressed to about half the length of the gel
    - Check that small bubbles are forming near the terminals in the box
    - Longer electrophoresis times will result in better size resolution

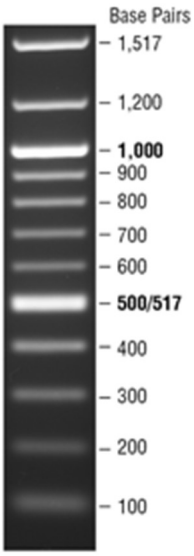
6. Once electrophoresis is completed, turn the power off and remove the gel from the box (not needed if using blueGel™ which has a built-in illuminator)

Size determination and interpretation

1. Place the gel on the transilluminator (or turn on the blueGel™ illuminator)
    - *Wear UV-protective goggles if using UV light* (not needed with blueGel)

2. Verify the presence of PCR product

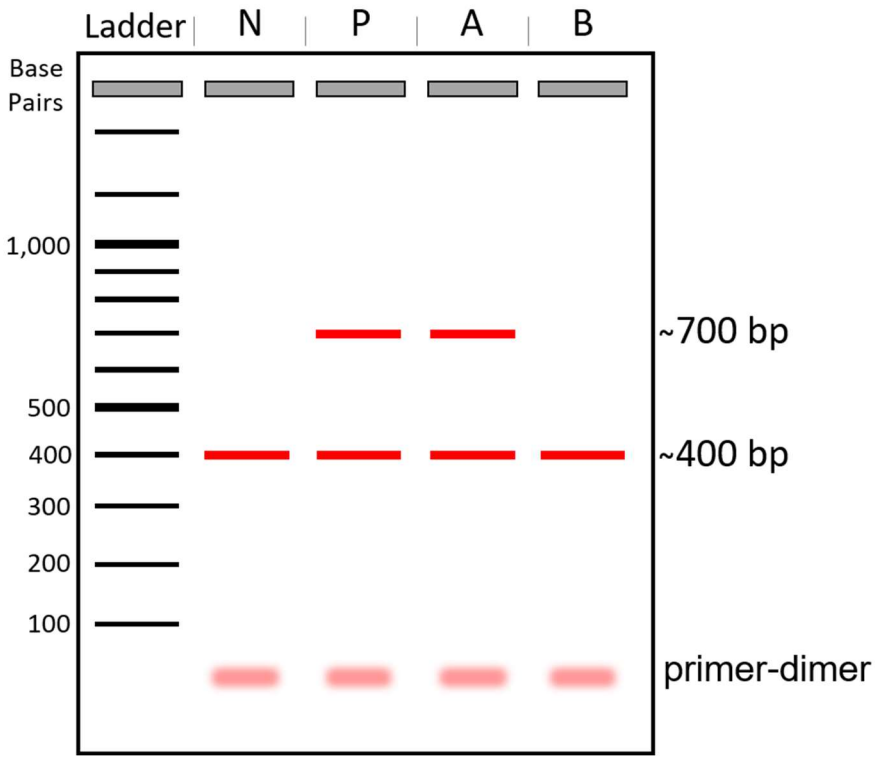
3. Ensure there is sufficient DNA band resolution in the 400-800 bp range of the 100bp DNA ladder
    - Run the gel longer if needed to increase resolution
    - DNA ladder should look approximately as shown



4. Document the size of the PCR amplified DNA fragments by comparing the PCR products to the molecular weight reference marker (100bp DNA ladder)
    - Capture an image with a smartphone camera
    - If available, use a Gel Documentation system

Expected experiment results

Expected results:



This schematic image shows the idealized experimental results

- Intensity of the bands will depend on
  - the efficiency of the PCR reaction
  - the efficiency of gel-loading
  - the quality of the detection reagents and system
- The migration patterns of the PCR product will vary with
  - the length of electrophoresis
  - the electrophoresis voltage

8. Study questions

Pre lab

1. When antibiotics were first introduced, there was virtually no human pathogen that had resistance. But resistance did exist in other non-pathogenic bacteria. Why may these other bacteria have possessed resistance?

2. If a particular type of bacteria is resistant to antibiotics, does that mean that it is bad for you?

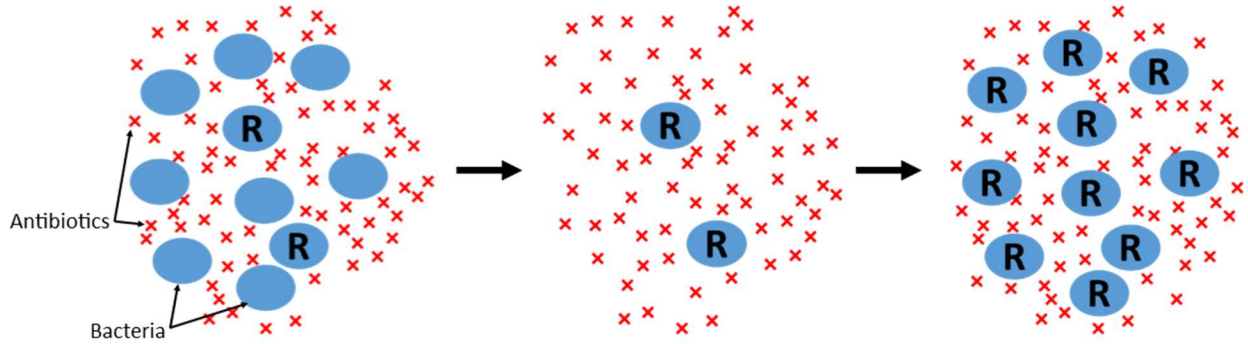
3. Evaluate this statement: "The use of antibiotics causes bacteria to become resistant."

4. Explain the difference between vertical and horizontal transmission of DNA. Why does horizontal transmission potentially make the problem of antibiotic resistance worse?

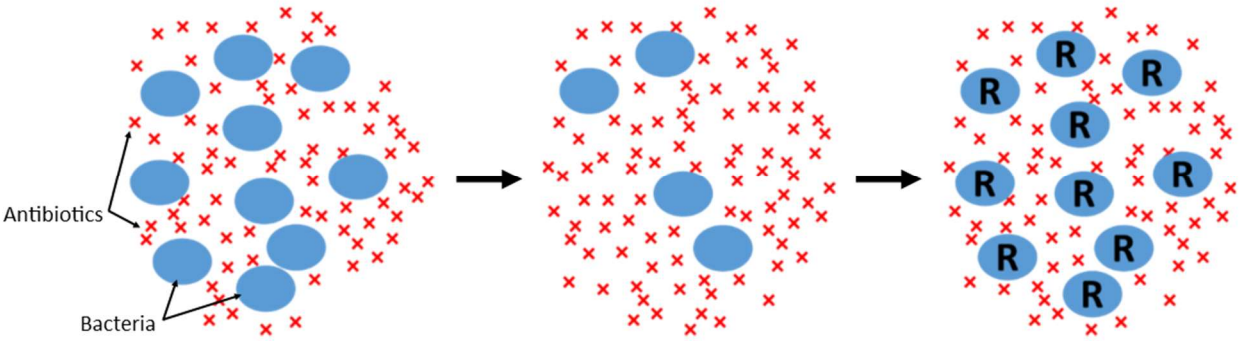
5. If you were to sample bacteria from a healthy human gut, do you think that you would find antibiotic resistance genes? Explain your answer.

6. Why would constant low-level usage of antibiotics on farms be more problematic for the development of antibiotic resistance than occasionally administering very high doses only when animals are sick?

7. Presented below are two models for the evolution of antibiotic resistance. Which model do you think is more accurate? Justify your answer with evidence from the text or other sources.

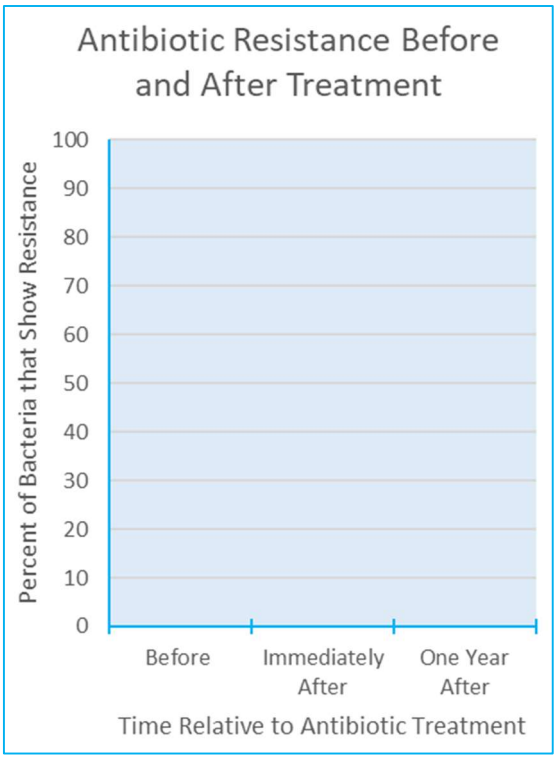


Model 1: Antibiotic resistance already exists in the population, but in low numbers. Use of antibiotics eliminates the non-resistant bacteria, allowing the resistant bacteria to proliferate.



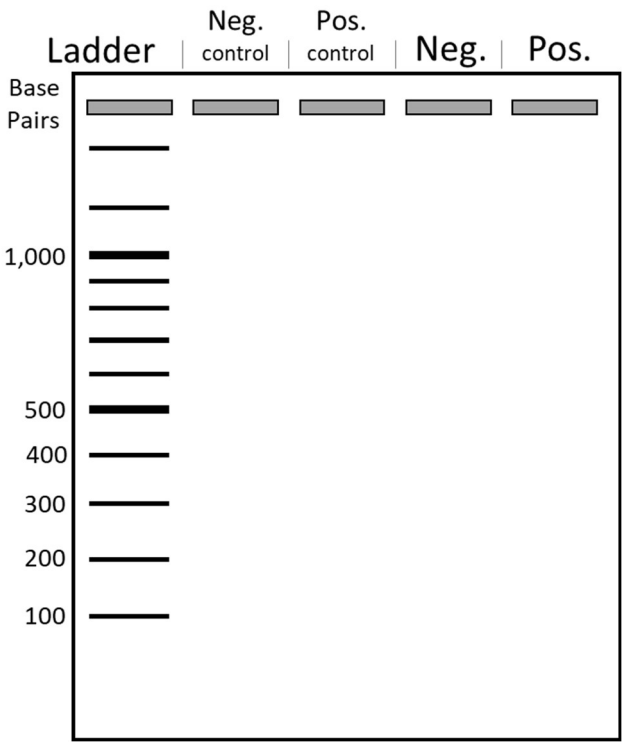
Model 2: Antibiotic resistance does not exist in the population. Use of antibiotics causes most bacteria to die. The ones that survive must adapt and change, making them resistant and allowing them to proliferate.

8. On the graph to the right, imagine that a farmer notices a possible disease developing in her herd. To combat the disease, she administers a course of antibiotics. If bacteria were sampled from the soil on this farm, predict what the percent of antibiotic resistant bacteria will be before, immediately after, and one year after treatment. You are not expected to know exact numbers, just try to predict generally when resistance will be high or low.



9. Explain why you drew what you did on the graph.

10. Before running your electrophoresis gel, predict your possible results. Draw bands on the gel where you expect to see them. A DNA ladder indicating DNA fragment size is provided in the first lane.



Post lab

1. Was either environmental sample positive for the bla_{NDM-1} gene?

2. In this lab, we are interested in whether carbapenem resistance is present in *two* different environmental samples. Explain then why you performed *four* PCR reactions.

3. The reason for performing this experiment was to test for the presence of a single gene bla_{NDM-1}. Why then do our positive results have two bands on the gel? What is the point of the second band?

4. Use of carbapenems are restricted to hospital settings in the United States. Why would carbapenem resistant bacteria be found in an environmental setting?

5. If you found the bla_{NDM-1} gene in the soil of one or both of the two farms, does this prove the food from this site is not safe to eat? Can you think of possibilities that could lead to finding the bla_{NDM-1} gene in the environment other than it coming from the pathogenic *E. coli*?

6. What is your recommendation to the farmers if antibiotic resistance genes are found on their property? What can they do to address the problem?

Discussion question: Imagine that you are a farmer raising hogs. You know that using antibiotics in your feed will increase the growth rate of your animals and reduce the frequency of infections spreading in your herd. But you also know that regularly using antibiotics in feed is a contributor to possible future health crises in the form of antibiotic resistance. Would you voluntarily stop adding antibiotics to your animals' feed? Why or why not? What would be the deciding factor that got you to change your mind?

CER Table

Fill in the table based on your results from the lab.

Question:

Can you determine if either environmental sample was positive for the bla_{NDM-1} gene?

Claim Make a clear statement that answers the above question.	
Evidence Provide data from the lab that supports your claim.	
Reasoning Explain clearly why the data you presented supports your claim. Include the underlying scientific principles that link your evidence to your claim.	

CER Table Rubric

For evaluating student CER tables.

	4	3	2	1
CLAIM *A statement that answers the original question/ problem.*	Makes a clear, accurate, and complete claim.	Makes an accurate and complete claim.	Makes an accurate but incomplete or vague claim.	Makes a claim that is inaccurate.
EVIDENCE *Data from the experiment that supports the claim.* *Data needs to be relevant and sufficient to support the claim.*	All of the evidence presented is highly relevant and clearly sufficient to support the claim.	Provides evidence that is relevant and sufficient to support the claim.	Provides relevant but insufficient evidence to support the claim. May include some non-relevant evidence.	Only provides evidence that does not support claim.
REASONING *Explain why your evidence supports your claim. This must include scientific principles/knowledge that you have about the topic to show why the data counts as evidence.*	Provides reasoning that clearly links the evidence to the claim. Relevant scientific principles are well integrated in the reasoning.	Provides reasoning that links the evidence to the claim. Relevant scientific principles are discussed.	Provides reasoning that links the evidence to the claim, but does not include relevant scientific principles or uses them incorrectly.	Provides reasoning that does not link the evidence to the claim. Does not include relevant scientific principles or uses them incorrectly.

Rubric Score	3	4	5	6	7	8	9	10	11	12
Equivalent Grade	55	60	65	70	75	80	85	90	95	100

We recommend that teachers use the following scale when assessing this assignment using the rubric.

Teachers should feel free to adjust this scale to their expectations.

9. Ordering information

To order miniPCR™ PARE Environmental Microbiology Lab reagent kits, you can:

- Call (781)-990-8PCR
- email us at orders@minipcr.com
- visit www.minipcr.com

miniPCR™ PARE Environmental Microbiology Lab kit (catalog no. KT-1010-01) contains the following reagents:

- 5X EZ PCR Master Mix, Load-Ready™
  - including *Taq* DNA polymerase, dNTPs, PCR buffer, and gel-loading dye
- PARE Primer Mix
- Negative Control DNA sample
- Positive Control DNA sample
- Apple Point DNA sample
- Barrow Creek DNA sample
- 100bp DNA ladder (50µg/ml)

Materials are sufficient for 8 lab groups, or 32 students

All components should be kept frozen at -20°C for long-term storage

Reagents must be used within 12 months of shipment

Other reagents needed (not included in the kit, available at miniPCR.com/store)

- Agarose (electrophoresis grade)
- DNA intercalating agent (e.g., Gel Green™)
- Gel electrophoresis buffer (e.g., 1X TBE)
- Distilled or deionized H_2O (to dilute 20X TBE buffer concentrate)

10. About PARE

The Prevalence of Antibiotic Resistance in the Environment (PARE) project is a citizen science project designed specifically for the classroom. By participating in PARE, students are able to contribute real data to a national research project.

The spread of antibiotic resistance in the environmental is a problem of growing world-wide concern. Soil and water can become contaminated with antibiotics from many sources, including both animal agriculture and farming of fish, where antibiotics are used intensively in both cases, as well as from human use and waste. Antibiotics present in soil and water can provide selective pressure for enrichment of antibiotic resistant bacteria, leading to concern that food or water contaminated with these bacteria may transfer the resistant organisms to humans. Indeed, the One-Health Initiative describes how excessive use of antibiotics in agricultural settings has led to clinically significant antimicrobial resistance in humans.

While surveillance of clinical infections for antibiotic-resistant microbes is common, there is no system in the U.S. for surveilling the environment. PARE aims to change this. PARE is a short-duration, low cost research project in which students sample soil in geographically diverse locations around the country for the levels of tetracycline-resistant bacteria. Student-generated data is then uploaded into a Global Database, where the goal is to use tetracycline-resistance as a "marker" for high antibiotic resistance levels. By identifying "hotspots"—regions with unusually high levels of antibiotic-resistant bacteria—we can take precautions before these organisms cause an infectious outbreak in humans. By participating in PARE, students not only gain an understanding and appreciation for the problem of environmental antibiotic resistance, they also become the scientists who are actively working to solve it!

This lab activity serves as an introduction to the PARE curriculum. For more information and to learn how to get involved, visit our website:
https://sites.tufts.edu/ctse/projects/pare/

PARE is partially funded by the National Science Foundation.

Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation.

11. About miniPCR Learning Labs™

This Learning Lab was developed by the miniPCR team in an effort to help more students understand concepts in molecular biology and to gain hands-on experience in real biology and biotechnology experimentation.

We believe, based on our direct involvement working in educational settings, that it is possible for these experiences to have a real impact in students' lives. Our goal is to increase everyone's love of DNA science, scientific inquiry, and STEM.

We develop Learning Labs to help achieve these goals, working closely with educators, students, academic researchers, and others committed to science education.

The guiding premise for this lab is that a 2-hour PCR-based experiment that recapitulates a real-life biotechnology application, provides the right balance between intellectual engagement, inquiry, and discussion. The design of this lab has simplified certain elements to achieve these goals. For example, we are not using actual environmental DNA isolates, and our PCR targets are not the bla_{NDM-1} or 16S ribosomal RNA genes.

We follow a proven model of experimental design[4] which has been incredibly effective for educational lab courses, and owe them for the inspiration.

Starting on a modest scale working with Massachusetts public schools, miniPCR Learning Labs™ have been received well, and their use is growing rapidly through academic and outreach collaborations. This lab represents one of those collaborations, and we are thankful to PARE for trusting us to help them develop innovative curriculum.

Authors: Bruce Bryan, MS, Sebastian Kraves, PhD, Carol Bascom-Slack, PhD, and Jennifer Larsen, PhD.

[4] See, for example: Bouakaze C, et al. "OpenLAB": A 2-hour PCR-based practical for high school students. Biochem Mol Biol Educ. 2010 Sep; 38(5):296-302. doi: 10.1002/bmb.20408. PubMed PMID: 21567848