Agricultural Monitoring Lab

A Case Study in Antibiotic Resistance

Produced in collaboration with the PARE project

Agricultural Monitoring Lab:
A Case Study in Antibiotic Resistance
KT-1010-01

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Contents

At a glance

Lab overview

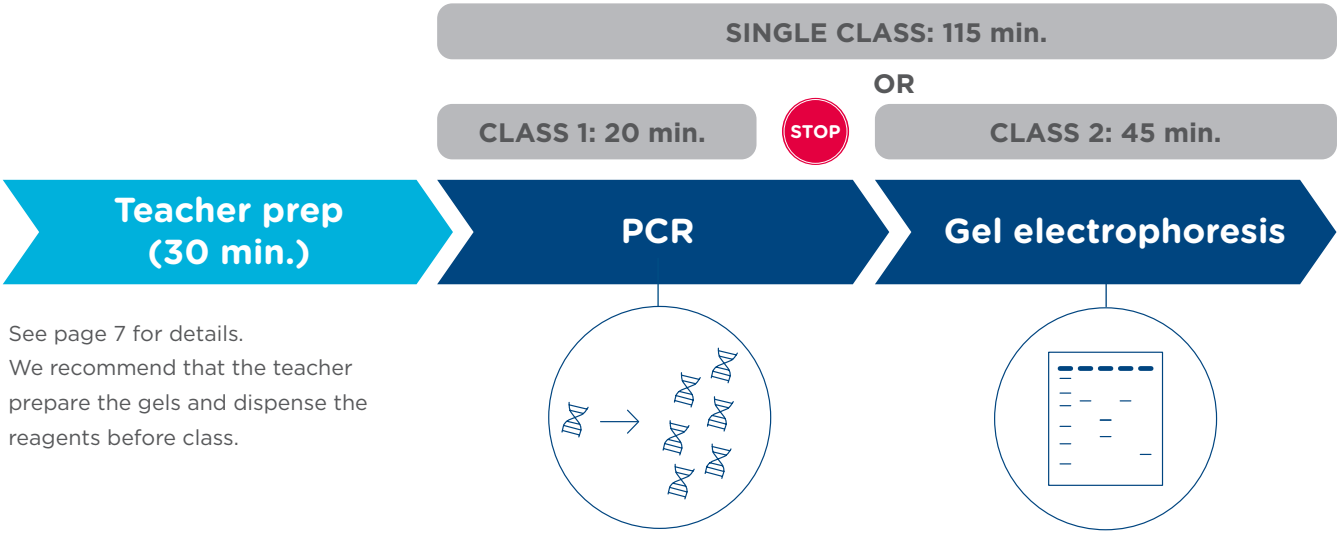
Antibiotic-resistant bacteria traced to one farm have raised concerns among neighbors. Students will use PCR to test soil from nearby farms for the presence of antibiotic-resistant bacteria, and learn how molecular tools aid environmental monitoring and surveillance. Produced in collaboration with the Tufts University PARE (Prevalence of Antibiotic Resistance in the Environment) project.
Disclaimer: no pathogenic materials are used.

TECHNIQUES	TOPICS	LEVEL
Micropipetting	Microbiology	General high school
PCR	Antibiotic resistance	Advanced high school
Gel electrophoresis	Environmental monitoring	College

Required lab skills

- Students must be proficient in accurately pipetting liquids in the 2-20 µl range.
- Instructional videos, worksheets, and free activities to help students build micropipetting skills can be found at https://www.minipcr.com/micropipetting/.

Planning your time



See page 7 for details.
We recommend that the teacher prepare the gels and dispense the reagents before class.

Technical support

If you have any questions about implementing this activity, contact support@minipcr.com.

Class time requirements

This protocol offers some flexibility to help you manage the class time needed.

Steps		Time required
1	**PCR**	
	A. Set up PCR samples and start the PCR program	20 minutes
	B. Run PCR	50 minutes The PCR program can be started during class and left to run without being monitored.
STOP	Optional stopping point: The PCR product is stable at room temperature for several days. For longer-term storage, place it in the freezer.	
2	**Gel electrophoresis**	
	Prep: Make gels	We recommend the teacher prepare the gels outside of class (see page 7). Allot 30 minutes of class time if you opt to have students prepare the gels.
	A. Load gel	10 minutes
	B. Run gel	15-25 minutes The gel does not need to be actively monitored during this time.
	C. Interpret results	5 minutes

Materials needed

Supplied in kit (KT-1010-01)

- Kit contains DNA samples and PCR reagents for eight lab groups.
- If kept in the freezer, reagents can be stored for 12 months after receipt. If kept in the refrigerator, reagents can be stored for 1 month after receipt.
- Reagents for preparing gels, plastic tubes for distributing reagents to individual groups, plastic tubes for PCR, and pipette tips are sold separately. See below for details.

Contents	Provided	Required	Storage
Simulated DNA samples • Negative Control DNA • Positive Control DNA • Apple Point DNA • Barrow Creek DNA	150 µl each	15 µl each	Freezer
5X EZ PCR Master Mix, Load-Ready™	240 µl	25 µl	Freezer
ABR Primer Mix	480 µl	50 µl	Freezer
Fast DNA Ladder 1	150 µl	15 µl	Freezer

Electrophoresis reagents and plastics sold separately

- This lab requires:
  - 2% agarose gels with a fluorescent DNA stain (*e.g.,* SeeGreen™ or GelGreen®).
  - Plastic tubes for distributing reagents to individual groups and 0.2 ml PCR tubes for running PCR.
- The Learning Lab Companion Kit (KT-1510-01) provides sufficient reagents to to make and run eight gels when using the blueGel™ or Bandit™ electrophoresis systems, as well as plastic tubes for distributing reagents to individual groups and plastic tubes for PCR.
- Alternatively, bulk electrophoresis reagents and plastics (tubes, pipette tips) are available for purchase from miniPCR bio.
- Gel electrophoresis reagents and plastics can also be purchased from other suppliers.

Required equipment

- This lab is compatible with any thermal cycler.
- This lab is compatible with any horizontal gel electrophoresis system in combination with:
  - A fluorescent DNA stain (e.g., SeeGreen™ or GelGreen®).
  - A transilluminator that is compatible with the DNA stain used. Fluorescent DNA stains typically require blue light (~470 nm) or UV (~260 nm) illumination.
- The table below outlines equipment from miniPCR bio that meets these requirements.

AVAILABLE AT MINIPCR.COM

Item	Recommended quantity
miniPCR thermal cycler	Each group will have 4 PCR samples Groups can share machines
Gel electrophoresis and visualization system Option 1: blueGel™ OR GELATO™ electrophoresis systems with integrated blue light transilluminator	1 blueGel can be shared by two groups 1 GELATO can be shared by four groups
Option 2: Bandit™ STEM electrophoresis kit paired with the Viewit™ Illumination Kit	1 Bandit + 1 Viewit per group
Option 3: Bandit™ STEM electrophoresis kit paired with a blueBox™ blue light transilluminator	1 Bandit per group + 1 blueBox for the class to share
Micropipettes and tips 2-20 µl adjustable micropipette 20-200 µl adjustable micropipette	1 pipette per group 1 pipette for teacher prep

Other materials supplied by user

- Distilled water
- Microwave or hot plate
- Heat-resistant flask or beaker
- Disposable laboratory gloves
- Protective eyewear
- Fine-tipped permanent marker

Teacher prep

Protective gloves and eyewear should be worn for the entirety of this experiment.

Overview

The table below provides an overview of the teacher prep, and the subsequent pages provide detailed instructions.

Prep	Time required	Timeline
Dispense reagents	10 minutes	Can be completed up to one week before use
Prepare electrophoresis buffer and agarose gels	20 minutes	Varies - If using gel reagents from miniPCR, gels can be prepared and stored for up to five days before use

Dispense reagents

- Reagents can be dispensed up to one week in advance and stored in the refrigerator until use.
- This kit provides sufficient reagents for eight lab groups.

Materials needed

From the lab kit (stored in the freezer):

- 5X EZ PCR Master Mix
- ABR Primer Mix
- Fast DNA Ladder 1
- Negative Control DNA
- Positive Control DNA
- Apple Point DNA
- Barrow Creek DNA

Supplied by user:

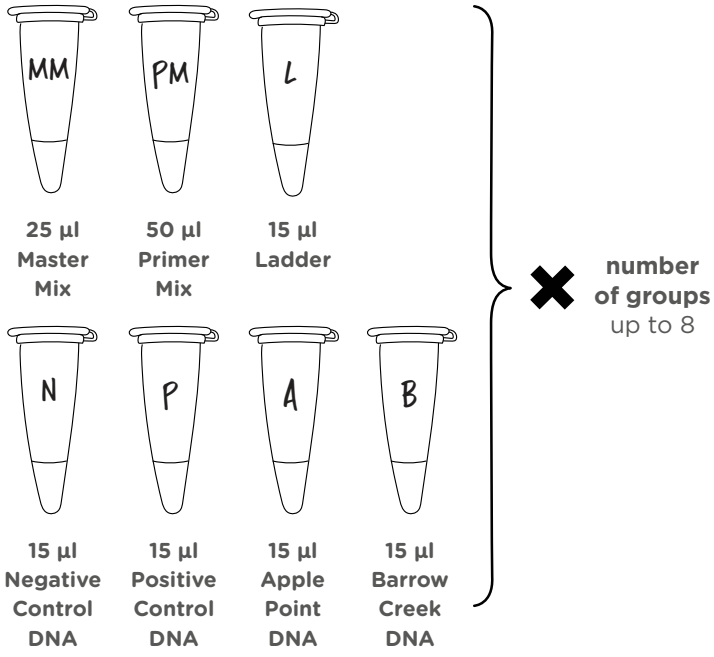
- Plastic tubes for dispensing reagents (1.5 ml or 0.2 ml tubes can be used)
- 2-20 µl and 20-200 µl micropipettes and tips
- Fine-tipped permanent marker

1. Thaw reagents by placing tubes at room temperature.

2. Collect the liquid at the bottom of each tube. Either spin briefly in a microcentrifuge or shake the liquid down with a flick of the wrist.

3. When you open each tube, check for liquid stuck inside the cap. If necessary, put the cap back on and repeat step 2.

4. For each lab group, dispense the following reagents into labeled plastic tubes. 1.5 ml or 0.2 ml plastic tubes can be used.
   - 5X PCR Master Mix (label tube as "MM") 25 µl
   - ABR Primer Mix (tube PM) 50 µl
   - Fast DNA Ladder 1 (tube L) 15 µl
   - Positive Control DNA (tube P) 15 µl
   - Negative Control DNA (tube N) 15 µl
   - Apple Point DNA (tube A) 15 µl
   - Barrow Creek DNA (tube B) 15 µl



5. If you are dispensing the reagents more than 24 hours before class, store the tubes in the refrigerator until use. Dispensed reagents can be stored in the refrigerator for up to one week.

Note: Ladder is not needed until the second day of the lab, but you can aliquot it now and store in the refrigerator until needed.

Prepare gel electrophoresis buffer and agarose gels

1. Prepare electrophoresis buffer.
   - Follow the manufacturer's instructions to prepare buffer solution.
   - The volume of buffer needed varies depending on the gel electrophoresis system.
     - For the blueGel and Bandit electrophoresis systems, 600 ml of TBE buffer is sufficient for at least eight gel runs.
     - For other systems, refer to the manufacturer's instructions for:
       (1) The buffer volume needed to prepare agarose gels.
       (2) The buffer volume needed for use as running buffer.

2. Prepare 2% agarose gels with fluorescent DNA stain.
   - You will need four sample lanes plus one lane for DNA ladder per group. If groups are sharing gels, a single lane for ladder per gel is sufficient.
   - This lab kit is compatible with any molecular grade agarose and fluorescent DNA stain (*e.g.,* SeeGreen™ or GelGreen®).
   - The volume of gel needed varies based on the gel electrophoresis system you are using. Refer to the manufacturer's instructions.
   - If using gel electrophoresis reagents from miniPCR bio, gels can be prepared up to five days in advance. Store prepared gels at room temperature in an airtight container protected from light. Do NOT soak the gels in buffer or wrap them in paper towels.

Detailed instructions for preparing buffer and gels for miniPCR electrophoresis systems

blueGel
https://links.minipcr.com/gelpouring

Bandit
https://links.minipcr.com/BanditDNAgel

Student workstation setup

Part 1: PCR

Every lab group should have:

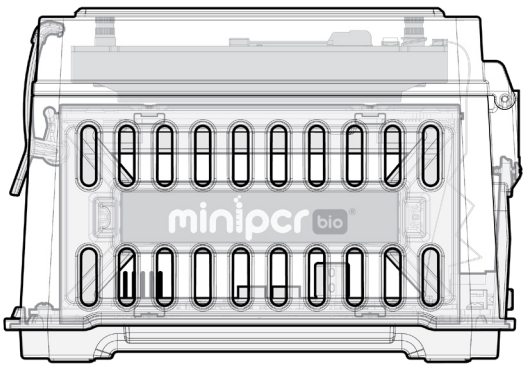
5X EZ PCR Master Mix (tube MM)	25 µl
ABR Primer Mix (tube PM)	50 µl
Simulated DNA samples • Negative Control DNA (tube N) • Positive Control DNA (tube P) • Apple Point DNA (tube A) • Barrow Creek DNA (tube B)	15 µl each
PCR tubes (0.2 ml)	4
2-20 µl micropipette and tips	
Space in a thermal cycler for 4 samples	

If using miniPCR thermal cyclers:

- Groups will need a miniPCR thermal cycler and power supply.
- Download the miniPCR app from the app store or at www.minipcr.com/downloads.
- Machines can be programmed ahead of time by the teacher or during class by the students.
- Once the program has started, the miniPCR will complete the program even if disconnected from the device running the app.
- If you want to monitor the reaction in real-time during the run, groups will need their miniPCR thermal cycler to remain connected to a computer or a compatible phone or tablet.

Detailed instructions for using a miniPCR thermal cycler

https://links.minipcr.com/minipcrRUN



Part 2: Gel electrophoresis

Every lab group should have:

PCR samples from previous class	25 µl each
Fast DNA Ladder 1 (tube L)	15 µl
Electrophoresis buffer *Volume depends on your electrophoresis system	30 ml TBE if using a blueGel or Bandit
2-20 µl micropipette and tips	
5 wells in a 2% agarose gel with fluorescent DNA stain	

Student guide

Background information

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. 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 year. 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.

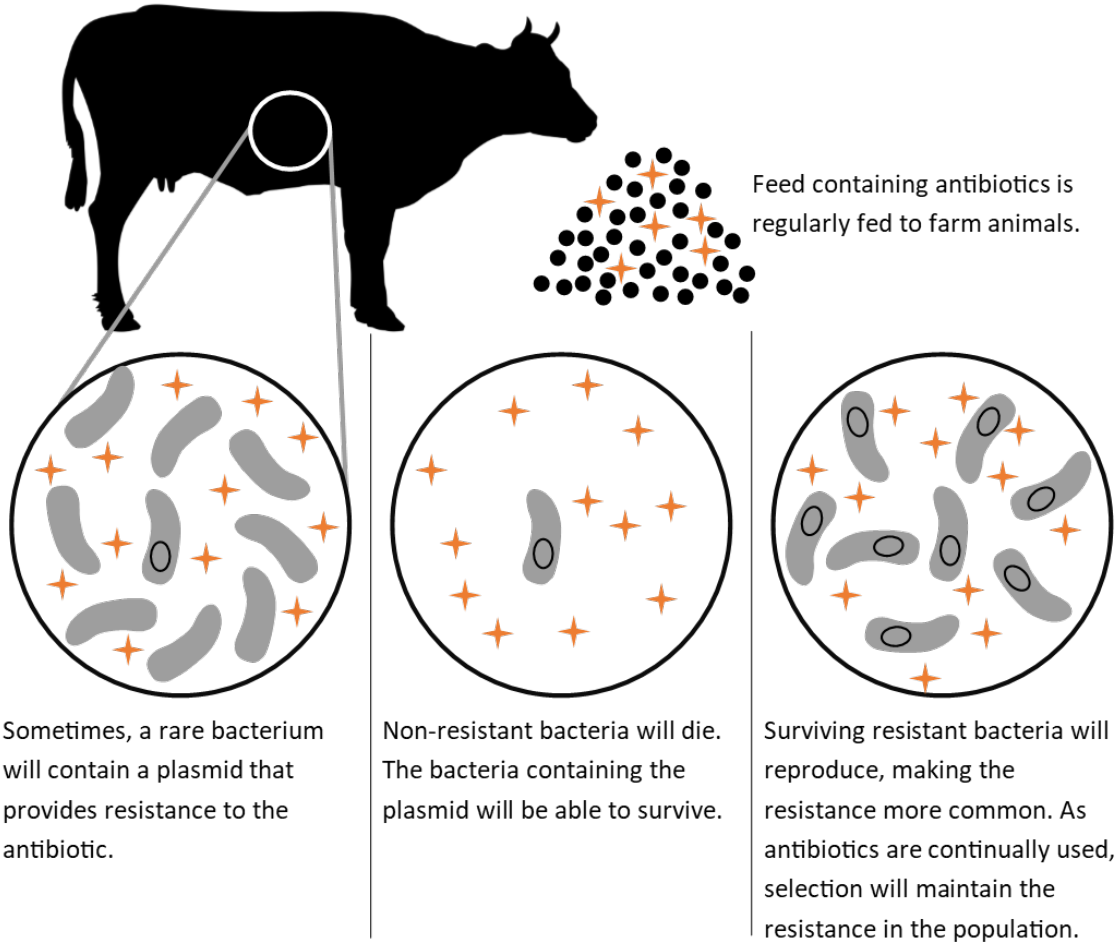
But as the use of antibiotics has spread, so has bacteria's resistance to them. It is fairly common today for routine infections to be resistant to antibiotics that were once used to treat them. And as bacteria gain resistance to more and more difficult drugs, a future where antibiotics can no longer 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 restistant 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 antibiotic. But what if they had been?

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. 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 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 biological parents, 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.

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 nonresistant 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.

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 Farm 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 *bla*$_{NMD-1}$ 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.

Student lab protocol

Set up PCR samples

Protective gloves and eyewear should be worn for the entirety of this experiment.

1. Label four PCR tubes 1, 2, 3, and 4. Write on the upper sidewall of the tube.

2. Add PCR reagents to the labeled tubes according to the table below. To prevent contamination, use a new tip for each addition.

	Tube 1	Tube 2	Tube 3	Tube 4
Master Mix (tube MM)	5 µl	5 µl	5 µl	5 µl
Primer Mix (tube PM)	10 µl	10 µl	10 µl	10 µl
DNA sample	Negative control DNA (tube N) 10 µl	Positive control DNA (tube P) 10 µl	Apple Point DNA (tube A) 10 µl	Barrow Creek DNA (tube B) 10 µl

3. Close the caps on the tubes. When they are closed correctly, you should feel the caps “click” into place.

4. Flick each tube to mix the contents. If available, a vortex mixer can be used.

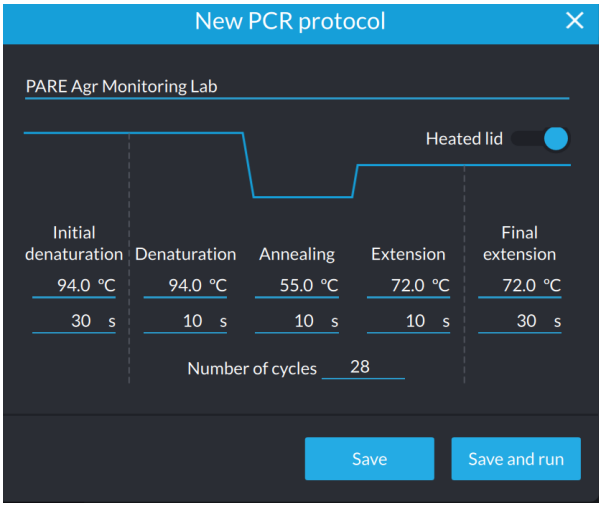
5. Make sure all the liquid is at the bottom of the tube. If there is liquid stuck on the sides of the tubes, shake it down with a flick of the wrist or a brief spin in a microcentrifuge.

6. Proceed immediately to the next section of the protocol.

Run PCR

- Program your thermal cycler with the following 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



- The PCR takes approximately 50 min when using a miniPCR® thermal cycler.
- Optional stopping point: PCR product is stable at room temperature for several days. For longer-term storage, move tubes to a freezer.

Detailed instructions for using a miniPCR thermal cycler

https://links.minipcr.com/minipcrRUN

Gel electrophoresis

Protective gloves and eyewear should be worn for the entirety of this experiment.

1. Place the prepared gel into the electrophoresis chamber.

2. Add enough electrophoresis buffer to fill the chamber and just cover the gel.
   - You will need 30 ml of TBE buffer for a blueGel™ or Bandit™ electrophoresis system. Do not overfill the chamber.
   - If using another electrophoresis system, refer to the manufacturer's instructions for the recommended buffer type and volume.

3. Use a micropipette to load samples in the following order. To prevent contamination, use a new tip for each sample.
   - Well 1: 10 µl Fast DNA Ladder 1 (tube L)
   - Well 2: 10 µl Sample 1 PCR product
   - Well 3: 10 µl Sample 2 PCR product
   - Well 4: 10 µl Sample 3 PCR product
   - Well 5: 10 µl Sample 4 PCR product

Detailed operating instructions for miniPCR electrophoresis systems

blueGel
https://links.minipcr.com/blueGelRun

bandit
https://links.minipcr.com/BanditViewit

4. Run the gel for 15-25 minutes.
   - The blueGel™ and Bandit™ electrophoresis systems run at a fixed voltage.
   - If using another gel electrophoresis system, set the voltage in the 70-90 V range.

5. To visualize the DNA samples, turn on the blue light in your electrophoresis system, or move the gel to a transilluminator.

6. If needed, continue to run the gel until there is sufficient separation between the 300-700 bp bands in the ladder to interpret the results.

7. If desired, take a photo to document the results.

8. Compare the bands from the DNA samples to the DNA ladder to obtain size estimates.

Pre-lab questions

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?

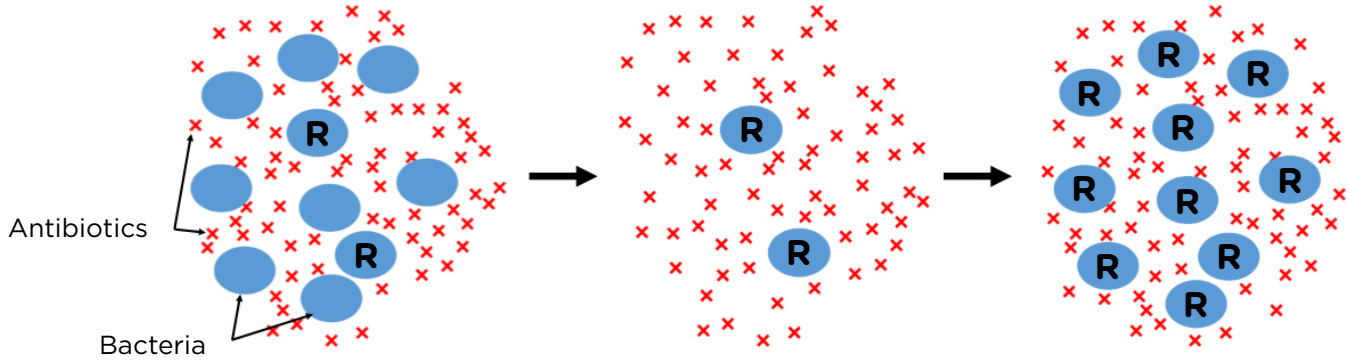
3. Evaluate this statement: “The use of antibiotics causes bacteria to become resistant.”

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

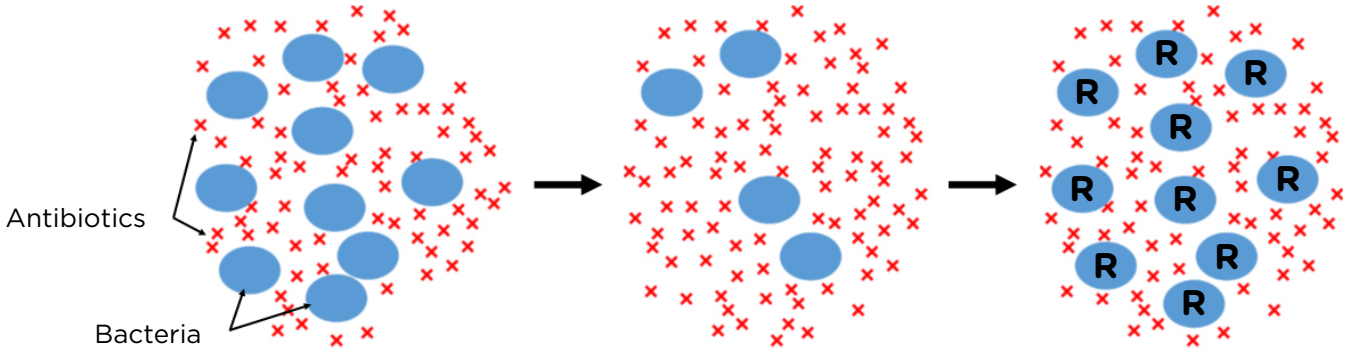
5. If you were to sample bacteria from a healthy human gut, do you think that you would find antibiotic resistance genes? Explain your answer.

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.

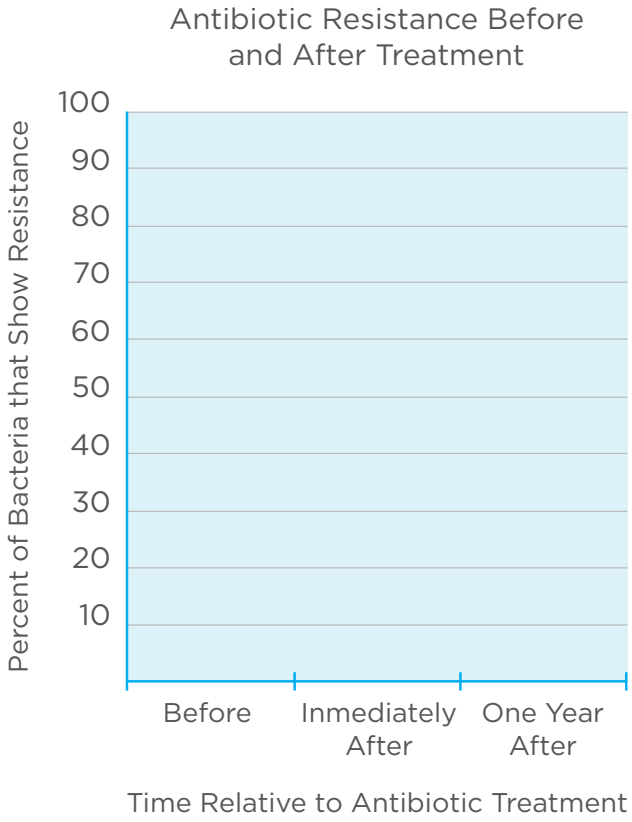


Model 1: Antibiotic resistance already exists in the population, but in low numbers. Use of antibiotics eliminates the non-resistant bacteria, allowing the resistant bacteria to proliferate.



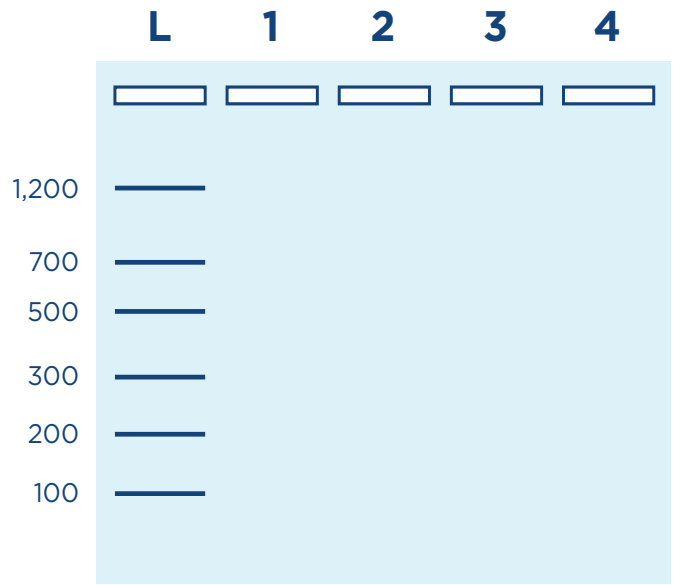
Model 2: Antibiotic resistance does not exist in the population. Use of antibiotics causes most bacteria to die. The ones that survive must adapt and change, making them resistant and allowing them to proliferate.

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.



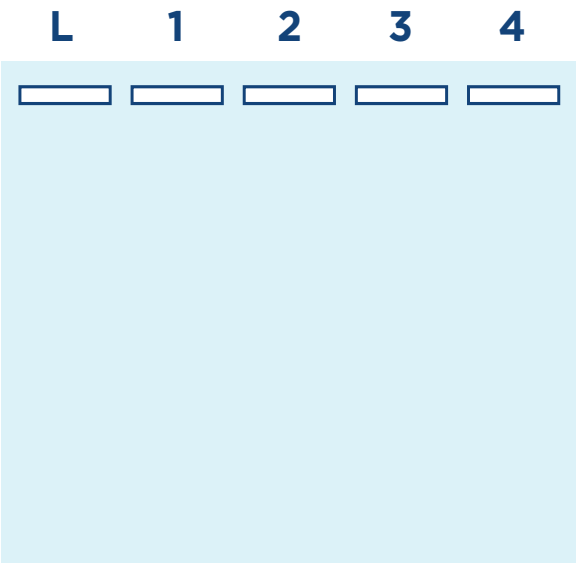
9. Explain why you drew what you did on the graph.

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 questions

Interpreting results



1. Use the schematic gel on the right to draw what your gel looks like. For each sample, draw the bands that you see on your actual gel.

2. Next to each band, write approximately how long (in base pairs) the DNA in that band is. Use the image of the ladder from page 22 to help you.

3. Use your gel electrophoresis results to complete the table below:
   a. Use checkmarks to record the gel electrophoresis results in the first two rows of the table
   b. Use the results to determine if antibiotic-resistant bacteria are present, and record that in the third row of the table.

	Tube 1: Negative control	Tube 2: Positive control	Tube 3: Apple Point Farm	Tube 4: Barrow Creek Farm
16S sequence (~400 bp)				
bla$_{NDM-1}$ gene (~700 bp)				
Antibiotic-resistant bacteria present? (Yes or No)				

Critical thinking

4. 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.

5. 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?

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

7. 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*?

8. 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. Refer to the rubric on the next page.

Question:

Based on your results, has the bla$_{NDM-1}$ antibiotic-resistance gene spread to Apple Point or Barrow Creek?

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.

Score	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 must 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.

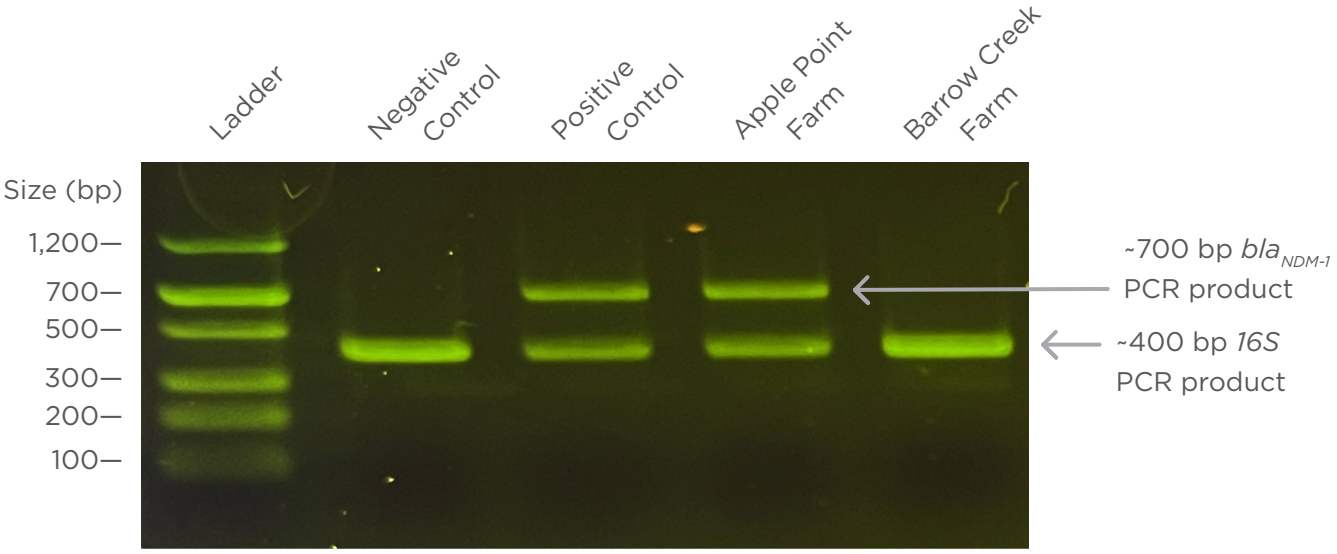
We recommend that teachers use the following scale when assessing this assignment using the rubric. Teachers should feel free to adjust this scale to their expectations.

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

Instructor guide

Expected results

Gel electrophoresis results are expected to resemble the gel image below:



This image represents results obtained after a 20 minute run using a blueGel™.

	Tube 1: Negative control	Tube 2: Positive control	Tube 3: Apple Point Farm	Tube 4: Barrow Creek Farm
16S sequence (~400 bp)	✓	✓	✓	✓
***bla*$_{NDM-1}$ gene (~700 bp)**		✓	✓	
Antibiotic-resistant bacteria present? (Yes or No)	No	Yes	Yes	No

For technical support, contact **support@minipcr.com**

For answers to the student questions, email **answers@minipcr.com**
Please include in the body of the email:

- The name of the lab
- Your name, school, and job title

Unexpected results and troubleshooting

If fluorescent DNA bands are faint or entirely absent from one or more student sample lanes, the following may have occurred:

- Suboptimal PCR amplification: Pipetting errors during PCR setup can lead to suboptimal amplification for individual student samples.

- Failure to load the DNA samples on the gel: Loading DNA samples for gel electrophoresis takes a little practice. The bands will appear faint if students do not successfully deposit the full sample volume into the well. Refer to https://www.minipcr.com/how-to-load-a-gel-electrophoresis/ for gel loading tips.

If fluorescent DNA bands are not visible on the gel, even for the DNA ladder, the following may have occurred:

- Failure to use a fluorescent DNA stain: This lab requires agarose gels made with a fluorescent DNA stain (e.g., SeeGreen™ or GelGreen®). DNA stains that reveal DNA with a visible blue compound are less sensitive and are not compatible with this lab kit.

- Incorrect visualization conditions: Fluorescent DNA stains (*e.g.,* SeeGreen™ or GelGreen®) must be viewed using a blue light or UV transilluminator. The blueGel system has an integrated blue light transilluminator. For DNA visualization, ensure that you have turned on the blueGel's blue light by pressing the light bulb button.

- Samples were run off the gel: If you run the gel too long, DNA samples may migrate off the gel. Monitor progress by occasionally checking the DNA samples under a transilluminator or tracking the loading dye, which is visible to the eye. Stop the run before the colored loading dye reaches the end of the gel.

- Reagents were stored improperly and/or are expired: The lab kit can be stored in the freezer for up to twelve months after receipt. Storage under different conditions or in excess of this guidance may impair performance.

For tips on picture-perfect gels, see https://www.minipcr.com/gel-electrophoresis-troubleshooting/.

For additional technical support, contact support@minipcr.com.

Notes on lab design

This lab serves as an introduction to the problem of antibiotic resistance in the environment and to techniques that could be used to monitor the problem. We believe this approach provides the right balance between intellectual engagement, inquiry, and accessibility. The design of this lab has simplified certain elements to achieve these goals. Some of these elements include:

- This lab uses prepared template DNA to simulate the carbapenem resistance gene *blaNDM-1*.
- This case study is fictional, but it is based on several real-life incidents.

About the PARE project

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 environment 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.

Additional student supports

E-worksheets: The student questions accompanying this lab are available for download here as editable text documents you can customize and upload to your LMS. E-worksheets can also be accessed from the Curriculum Downloads tab at https://www.minipcr.com/product/antibiotic-resistance-pare-lab/.

miniPCR tutorials: Access an extensive set of free resources to help your students succeed in molecular biology techniques. Visit https://www.minipcr.com/tutorials/. The resources most relevant to this lab are listed below.

- **Micropipetting:** Video, worksheet, and hands-on activity resources to train students in the basic use of a micropipette.
- **PCR:** Video and worksheet activity instructing students on the fundamentals and practice of PCR.
- **Gel electrophoresis:** Video and worksheet activity instructing students on the fundamentals and practice of agarose gel electrophoresis.

miniPCR Digital: Interactive tools for experiment-based learning with or without hands-on lab kits. Visit https://digital.minipcr.com/.

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:

- Identify or pose a testable question
- Formulate hypotheses
- Identify dependent and independent variables and appropriate experimental controls
- Follow detailed experimental protocols
- Create tables or graphs to present their results
- Interpret data presented in a chart or table
- Use data to evaluate a hypothesis
- Make a claim based in scientific evidence
- Use reasoning to justify a scientific claim

Molecular Biology Skills:

- Micropipetting
- PCR
- Agarose gel electrophoresis

Standards alignment

The standards alignment document for this activity is available for download here. This document can also be accessed from the Curriculum Downloads tab at https://www.minipcr.com/product/antibiotic-resistance-pare-lab/.

This activity is aligned to the following standards:

- Next Generation Science Standards: High School Life Science
- Advanced Placement Biology
- Advanced Placement Environmental Science
- Texas Essential Knowledge and Skills: Biology
- Texas Essential Knowledge and Skills: Biotechnology
- Biotechnician Assistant Credentialing Exam
- Common Core ELA/Literacy Standards (9-10)

For additional information on alignment to state standards, please contact support@minipcr.com.