



Agricultural Monitoring Lab

A Case Study in Antibiotic Resistance

**Produced in collaboration
with the PARE project**



Student guide



Background information	P. 13
Case study	P. 18
Today's lab	P. 19
Student lab protocol	P. 20
Pre-lab questions	P. 23
Post-lab questions	P. 26
CER table	P. 29



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 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.

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 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.

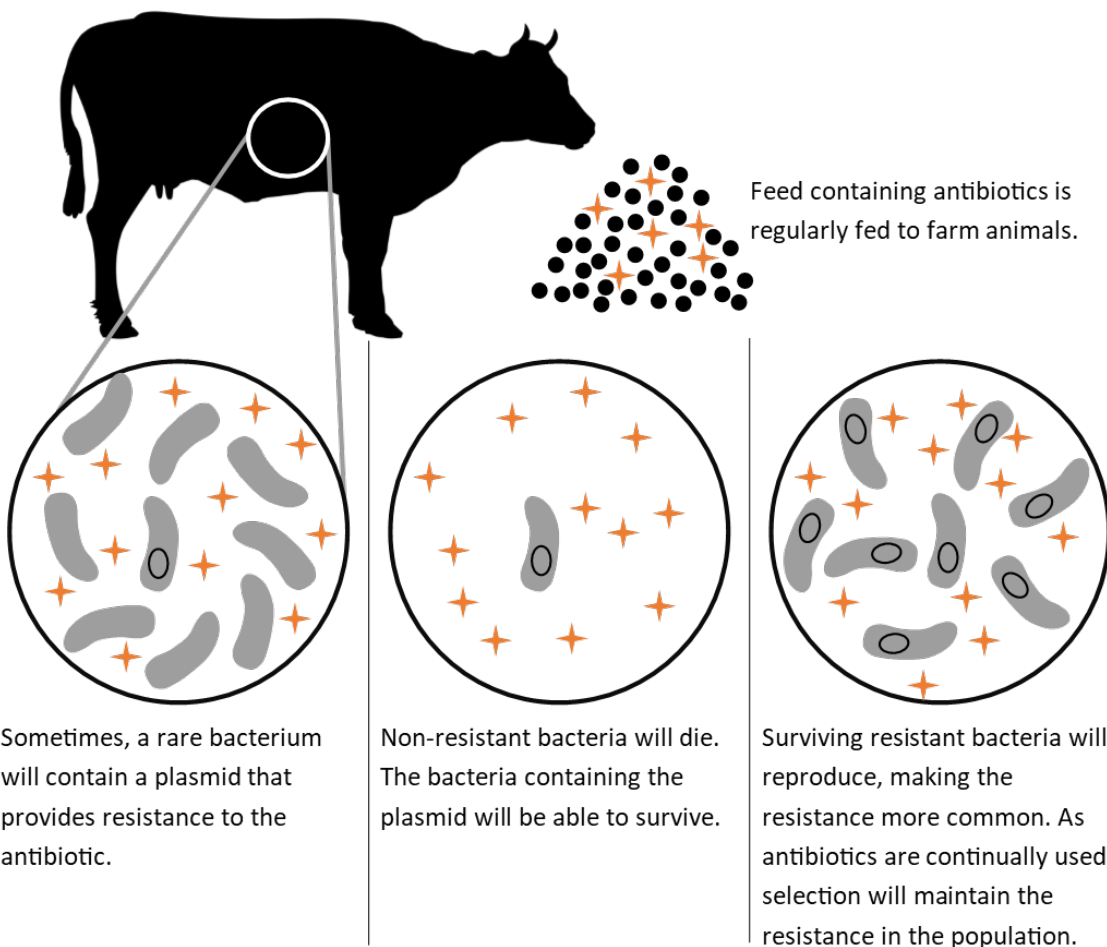
But not all resistant bacteria live in hospitals. More and more, antibiotic resistant bacteria are being found simply in the environment – living in the soil and water. Nobody knows for sure exactly how widespread resistant bacteria in the environment are, but as it is becoming more and more clear that 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 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 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*_{NMD-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 from 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 antibiotics³, 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 μ l	5 μ l	5 μ l	5 μ l
Primer Mix (tube PM)	10 μ l	10 μ l	10 μ l	10 μ l
DNA sample	Negative control DNA (tube N) 10 μ l	Positive control DNA (tube P) 10 μ l	Apple Point DNA (tube A) 10 μ l	Barrow Creek DNA (tube B) 10 μ l

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.



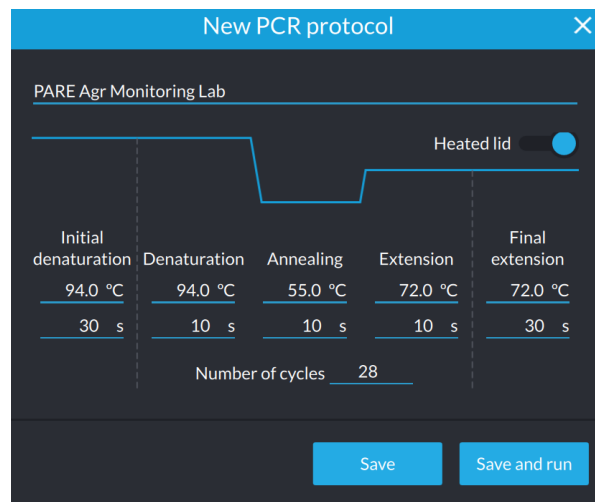
Run PCR

- 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.”

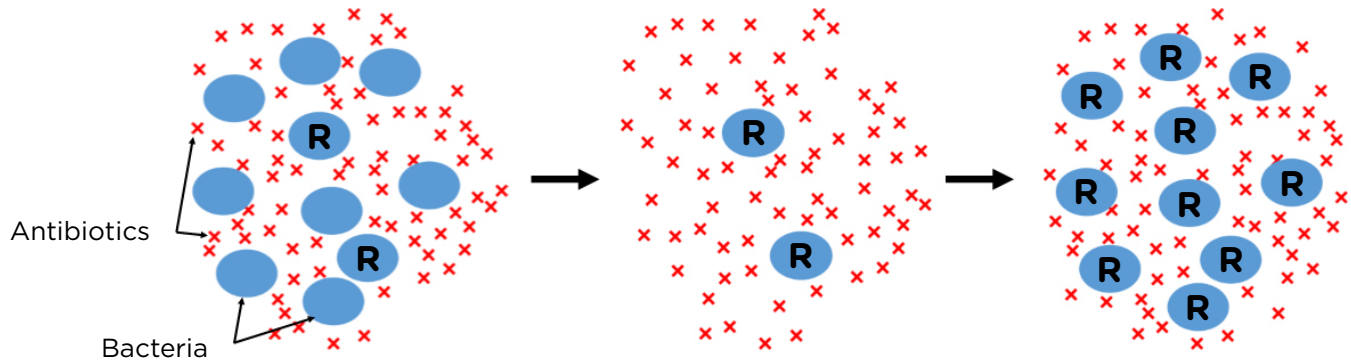
4. Explain the difference between vertical and horizontal transmission of DNA. Why does horizontal transmission potentially make the problem of antibiotic resistance worse?

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.

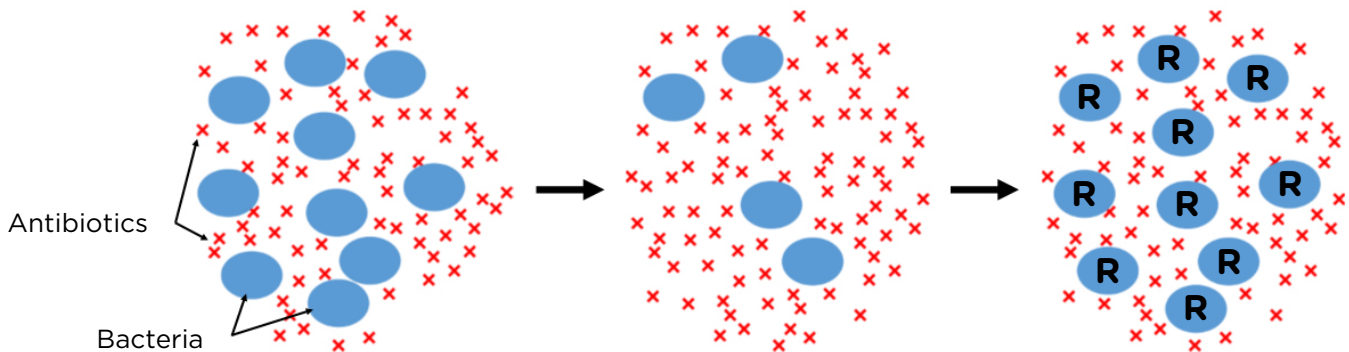
6. Why would constant low-level usage of antibiotics on farms be more problematic for the development of antibiotic resistance than occasionally administering very high doses only when animals are sick?



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.

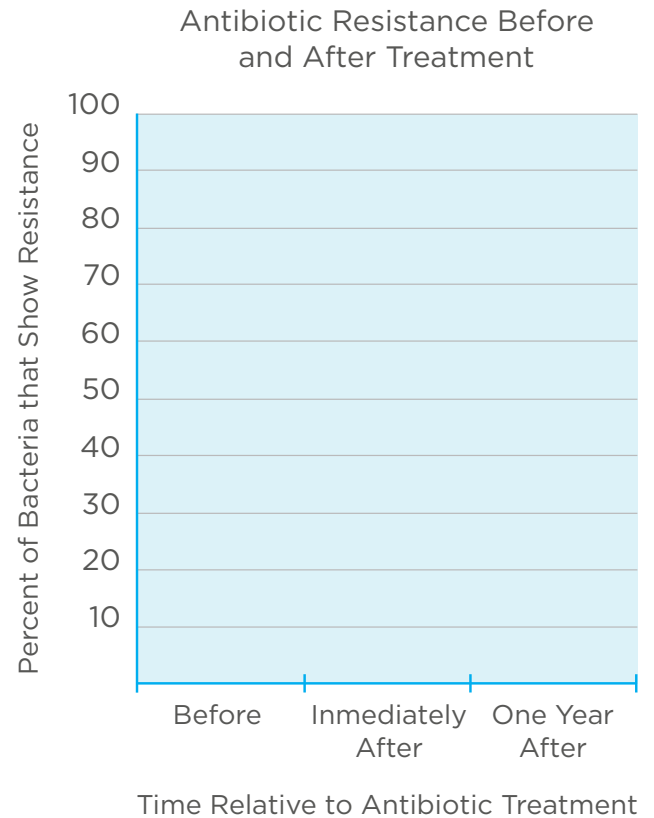


Model 2: Antibiotic resistance does not exist in the population. Use of antibiotics causes most bacteria to die. The ones that survive must adapt and change, making them resistant and allowing them to proliferate.

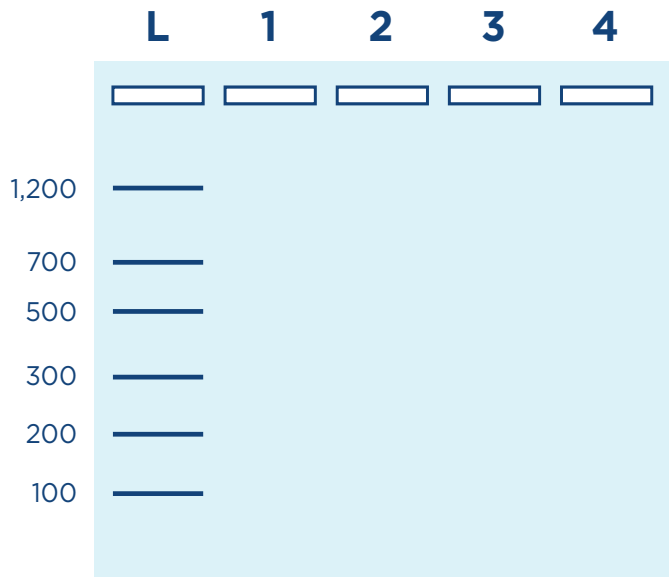


8. On the graph to the right, imagine that a farmer notices a possible disease developing in her herd. To combat the disease, she administers a course of antibiotics. If bacteria were sampled from the soil on this farm, predict what the percent of antibiotic resistant bacteria will be before, immediately 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.
- L

1

2

3

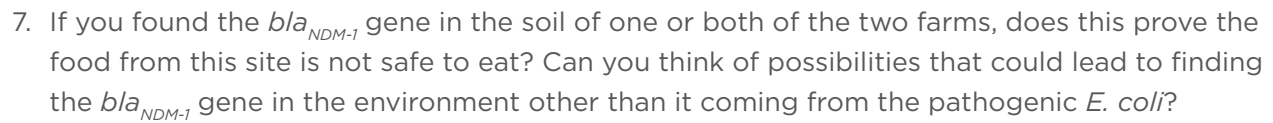
4
- | | Tube 1:
Negative control | Tube 2:
Positive control | Tube 3:
Apple Point Farm | Tube 4:
Barrow Creek Farm |
|--|-----------------------------|-----------------------------|-----------------------------|------------------------------|
| 16S sequence
(~400 bp) | | | | |
| <i>bla</i> _{NDM-1} gene
(~700 bp) | | | | |
| Antibiotic-resistant
bacteria present?
(Yes or No) | | | | |
- Agricultural Monitoring Lab: A Case Study in Antibiotic Resistance
Version: 1.5. Release: April 2025. © 2018-2025 by miniPCR bio™

P./26



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?



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 <u>relevant</u> and <u>sufficient</u> 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