An exploration of the resistance within bacteria to several antibiotics and their correct disposal methods.

To what extent is ampicillin affecting the resistance of bacteria (*E.coli*) to commonly used cephalosporins?

Biology

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Aim: The aim of this experiment is to determine whether ampicillin-resistant bacteria are also resistant to cephalosporins. The second aim of the experiment is to identify the most effective method for killing the drug-resistant bacteria in order to prevent further mutations amongst the bacteria.

Null Hypothesis: The ampicillin-resistant bacteria will not be resistant to commonly used cephalosporins and there is no difference between various disposal methods.

Hypothesis: The ampicillin-resistant bacteria will also be resistant to commonly used cephalosporins and various disposal methods can heavily impact the spread of bacteria.

Introduction:

Over the past several years, there has been an increased incidence of resistant bacteria amongst patients. Many strains of bacteria historically have been susceptible to most antibiotics, but now healthcare professionals are being challenged to find suitable antibiotics for the presenting infections. I have selected *Escherichia coli* given how ubiquitous it has been in the last couple of years and its high susceptibility to many antibiotics, including cephalosporins. Specifically, there has been a rise in the number of *E.coli* with ESBL (Extended Spectrum Beta Lactamase). Due to the frequent use of ampicillin and *E.coli* in school labs, there is a possibility that the bacteria contain the enzymes that break down the beta-lactam ring in the antibiotics. Moreover, the beta-lactamase enzyme found in resistant bacteria can be transferred to other bacteria through the process of horizontal gene transfer. When a resistant bacterium comes in contact with susceptible bacteria, they can transfer genes that code for antibiotic-resistance within the bacteria causing the susceptible bacterium to become transformed into a resistant form. If this happens multiple times, it will allow the bacteria to quickly multiply in the presence of various antibiotics and as time goes by become fully resistant to a wide spectrum of antibiotics

Given the fact that the bacteria are frequently disposed of incorrectly in various clinical and laboratory settings, there is significant concern that once in the environment, they perform horizontal gene transfer and further multiply (Sutcliffe J. G., 1978). This specific type of bacteria can become very lethal in situations where there is no present and effective antibiotic to fight off the infections.

Although there may be a primary benefit to the direct contact between antibiotics and bacteria, there may also be harmful disadvantages. It was also determined by several other experiments that the beta-lactamase enzyme is found in several bacteria which can break down antibiotics containing the beta-lactam ring, such as ampicillin, and could therefore be the main cause of the resistance. The beta-lactam ring, found in penicillin and other cephalosporins, attacks and diminishes the bacteria surrounding it by attacking the surrounding wall and terminating the production of proteins in the bacteria. This, in turn, tampers with the bacterial reproduction and diminishes the presence of the bacteria altogether (Healthline's Medical Network, 2018). However, scientists have discovered that the beta-lactamase enzyme causes resistance to the antibiotic and breaks down the beta-lactam ring (Briñas, 2002).

The more awareness there is to this issue, the more likely people in society can change their daily habits. I have been particularly interested to discover whether or not ampicillin, which is commonly used in scientific experiments, has influenced the *E.coli*'s resistance to common cephalosporins. Commonly used antibiotics, such as penicillin and ampicillin, are considered first line treatment for many infections including ear infections, Pharyngitis, Sinusitis, and skin infections. Due to the high rates of *E.coli* resistance in the past several years, there is some speculation of whether or not ampicillin is a possible contributor.

Certain types of *Escherichia coli,* commonly found in animal and human feces, can be extremely harmful to humans (Briñas, 2002). Antibiotics have proven over the years to help with killing such bacteria. In addition, the generation of the antibiotics can also help determine its effectiveness in killing bacteria. For example, third generation antibiotics, in comparison to first generation antibiotics, can eliminate bacteria more effectively. However, as a result of the bacteria's exposure to common cephalosporins amongst patients, there is an overwhelming increase in mutations that allow for resistant bacteria. In addition, ampicillin is prescribed to many patients, which can also be affecting the rate of resistant bacteria and could potentially permit horizontal gene transfer.

Ampicillin is part of the larger class of penicillin, a common cephalosporin, which are frequently utilized in order to treat various infections (Healthline's Medical Network, 2018). Due to the amount of ampicillin used by society, they can often come in contact with dangerous bacteria such as *E.coli.* Penicillin-binding proteins (bacterial proteins that attach to antibiotics that contain the beta-lactam ring) were first discovered by Alexander Fleming in 1928 through observations he made on bacterial colonies left out on the bench top (Goodsell, 2002). He initially found that "colonies of Penicillium mold growing in his bacterial cultures were able to stave off infection" (Goodsell, 2002). Although the penicillin was able to fight the bacteria initially, the bacterial population quickly adapted to its environment and developed a mutation that allows for it to break down the beta-lactam ring found in the antibiotics. Due to the bacteria's rapid reproduction, it developed many ways of resisting the penicillin. Some change the penicillin-binding proteins in subtle ways, so that they still perform their function but do not bind to the drugs. Some develop more effective ways to shield the sensitive enzymes from the drug or methods to pump drugs quickly away from the cell. But the most common method

occurs when a mutation gives rise to a special enzyme, such as beta-lactamase (also called penicillinase) that seeks out the drug and destroys it (Goodsell, 2002). As a result of these methods of fighting off penicillin, there has been an increase in the amount of resistance over the years due to the persistent contact between the two, as seen from previous observations. The observation also shows that "[c]ephalosporin, a drug similar to penicillin, is bound directly to the serine in the active site of the enzyme" (Goodsell, 2002), more specifically the beta-lactamase enzyme and it prevents it from breaking down the antibiotics. This supports the overall correlation between ampicillin and common cephalosporins. Because of their similarity, bacteria that is resistant to one antibiotic may also be resistant to other types. This is detrimental because if many common antibiotics have similar structures, over time bacteria will become immune to all of them as a result of mutations.

Table 1.0- Variables and explanations regarding the specifics of the experiment

	What?	**Why?**	**How?**
Independent Variable	Type of antibiotic	Bacteria have now become easily resistant to different antibiotics and this will be harmful if bacteria suddenly become resistant to all similarly structured antibiotics.	3 types of cephalosporins will be tested (Ceftriaxone, Ceftazidime, and Cefoxitin) in the presence of bacteria following their contact with the ampicillin in order to determine whether or not the ampicillin-resistant bacteria is also resistant to any of the 3 types of cephalosporins tested.
Dependent Variable	Size of inhibition zone	This comes as a result of the amount of time the bacteria has been	The amount of bacterial colonies surrounding the beta-lactam ring will be

		placed with the antibiotic discs. As more time passes, the amount of bacterial colonies that either become resistant or get killed off should grow.	counted following each day. These will be the resistant bacteria that contain the beta-lactamase enzyme on the surface of the plasmids.
Controlled Variables	Humidity	In order to prevent fluctuation in the amount of bacteria growing on each petri dish. More humidity could cause the bacteria to grow more rapidly.	Because the results will be collected from the same environment, the humidity levels should remain consistent for each trial.
	Luminosity	The amount of light each petri dish is receiving should remain consistent to avoid the possibilities of more bacterial growth on certain petri dishes.	There will be no additional light added to the trials and the environment will remain the same for the entirety of the experiment.
	Amount of cephalosporin and ampicillin discs utilized in each trial	The number of bacterial colonies will be counted after each day and the addition of antibiotic discs may cause a disruption in the resistance of the bacteria.	Four ampicillin and cephalosporin discs will be added to the petri dishes in each trial.
	Amount of bacteria applied to the antibiotics	With the addition of more bacteria to the petri dishes, it may influence the resistance of the *E.coli* to the antibiotics.	Each plate will contain an equal amount of bacteria that corresponds with the antibiotics.
	Room Temperature (23°C)	With the change in temperature, the	The experiment will take place in one area

		bacteria could grow more rapidly or not at all. Each plate will be kept at the same room temperature in order to prevent fluctuation in growth of the *E.coli.*	and an incubator will not be used for the agar plates.
	Time	In order to accurately and precisely figure out which bacteria grow or diminish in the presence of the differing antibiotics.	Each petri dish will remain in the lab, along with the others, for a total of 2 days.
Control	Agar plate with only *E.coli*	This will aid in the comparison between the number of bacteria killed from the experimental dishes and the number of bacteria initially added.	After making the agar plates, only the *E.coli* will be added and kept for the same amount of time as the experimental dishes.
	Agar plate left aside (resembling direct disposal in the trash)	This will aid in the comparison between the effectiveness of Clorox bleach/ autoclave to no attempt to kill the bacteria and it could directly correlate to why there is resistance within *E.coli* to certain antibiotics. The frequent contact between the two in the environment as a result of the ineffective disposal	This disposal method will be tested following the trials and the experiment. It will be left aside and compared to the petri dishes that were either soaked in the Clorox bleach or put in the autoclave. The number of bacteria will be compared thoroughly to see if there is any evident correlation between resistance and the disposal methods

		of the bacteria could cause more resistance in the future.	utilized in schools and labs.

Equipment/Materials:

1. 1 *E. coli* starter plate
2. 20 Poured nutrient agar plates (10 LB, 10 LB/amp, 5 LB/amp/ara)
3. 1 1000 ml Pyrex Erlenmeyer Flask (±5%)
4. 1 Transformation solution (1 ml)
5. 1 LB nutrient broth (1 ml)
6. 7 Inoculation loops (1 pk of 10)
7. 4 Disposable plastic transfer pipets (DPTPs)
8. 1 Foam microcentrifuge tube holder/float
9. 1 Container full of crushed ice (foam/paper cups)
10. 2 Microcentrifuge tubes
11. 1 Marking pen
12. 1 Rehydrated pGLO plasmid, vial
13. 1 42°C water bath and thermometer
14. 1 2–20 µl adjustable volume micropipet
15. 1 2–20 µl micropipet tips, box
16. Clorox Liquid Bleach

17. Medline Industries BBL Sensi-Disc Susceptibility Test Discs (Ceftriaxone, Ceftazidime, and Cefoxitin) (MedlinePlus, 2019)
18. Medline Industries Autoclave
19. Gloves
20. Masks
21. Lab Gown
22. Safety Goggles

Method/Procedure:

Safety note: This will be tested in the pathology lab at a hospital with trained professionals. Make sure to always have gloves, safety goggles, a lab gown, and a mask on in order to prevent any harmful situation. Any materials utilized must be correctly disposed in order to prevent future contamination and the spread of the bacteria into the open environment.

Part 1 (Biotechnology Explorer Team, 2019):

1. Prepare 20 grams of the nutrient agar and 500 ml of distilled water

2. Swirl the agar and water in a 1000 ml Erlenmeyer flask

3. Autoclave to sterilize

4. Prepare arabinose and ampicillin

Note: Excessive heat (>60°C) will destroy the ampicillin and the arabinose, but the nutrient agar solidifies at 27°C so one must carefully monitor the cooling of the agar and then pour the plates from start to finish without interruption. Keeping the flask w/molten agar in a water bath set to 50°C can help prevent the agar from cooling too quickly. Excess bubbles can be removed after

all the plates are poured by briefly flaming the surface of each plate with the flame of a Bunsen burner. After the plates are poured do not disturb them until the agar has solidified.

5. Label all 25 plates with permanent marker

Label 10 plates **LB**, 10 plates **LB/amp** and 5 plates **LB/amp/ara**.

6. Pour LB nutrient agar plates (LB, LB/amp, LB/amp/ara)

First, pour LB nutrient agar into the 10 plates that are labeled LB. Fill the plate about one-third to one-half (~12 ml) with agar, replace the lid and continue up the stack. **Second, add the hydrated ampicillin to the remaining LB nutrient agar.** Swirl briefly to mix. Pour into the 10 plates that are labeled as **LB/amp** using the technique utilized above.

Check the temperature to make sure it's less than 60°C and then add the ampicillin

Gently swirl pour into plates labelled **LB/amp**

Third, add the hydrated arabinose to the remaining LB nutrient agar containing ampicillin.

7. Plate storage

After the plates have cured for two days at room temperature, they are ready to use.

8. Rehydrate Bacteria

1. Using a sterile pipet, rehydrate the lyophilized *E. coli* HB101 by adding 250 µl of LB nutrient broth directly to the vial.
2. Recap the vial and shake it gently to ensure all bacteria are rehydrated. Incubate the vial at 37°C for 8–24 hr. If an incubator or water bath is not available, the vial can be incubated on the bench top at room temperature (23°C).

9. Streak starter plates to produce single bacterial colonies on agar plates

Using the rehydrated *E. coli* you prepared in the last step and five **LB** agar plates (prepared in step one), streak each of the plates separately. The purpose of streaking is to generate single colonies from a concentrated suspension of bacteria.

1. Gently shake the vial of *E. coli* HB101 to resuspend the bacteria. Insert a sterile loop into the rehydrated bacterial culture. Insert the loop straight into the vial. Remove the loop and ensure a film of bacteria is across the loop. Streaking takes place sequentially in four quadrants. The first streak is to just spread the cells out a little. Go back and forth with the loop about a dozen times in the small area. In subsequent quadrants the cells become more and more dilute, increasing the likelihood of producing single colonies.
2. Rotate the plate approximately 45° (so that the streaking motion is comfortable for your hand) and start the second streak. Do not dip into the rehydrated bacteria a second time. Go into the previous streak about two times and then back and forth for a total of about 10 times.
3. Rotate the plate again and repeat streaking.
4. Rotate the plate for the final time and make the final streak. Repeat steps 1-3 with the remaining **LB** plates. Use the same inoculation loop for all plates. When you are finished with each plate, cover it immediately to avoid contamination.
5. Place the plates upside down at room temperature for 2–3 days.
6. Use for transformation within 24–36 hours as bacteria must be actively growing to achieve high transformation efficiency. Delaying more than 36 hours will compromise transformation. **DO NOT REFRIGERATE BEFORE USE**.
7. After visible colonies have shown on the plates, start by swiping the *E.coli*

Part 2:

10. Start by swiping the *E.coli* onto the surface of the **LB/amp** plate and the **LB/amp/ara** plate with the sterile metal loop.
11. Make sure to spread the *E.coli* evenly across the surface of the agar.
12. Leave the plates upside down on the benchtop to incubate at room temperature (23°C) for 2 days.
13. Only the bacteria that have been transformed will grow on the plates that contain ampicillin (as prepared in part 1).
14. Any bacteria that have not been transformed will not contain the beta-lactamase enzyme and will not grow in the presence of ampicillin. The resistant bacteria will not be affected by the beta-lactam ring because they contain the enzyme that breaks it down.
15. Re-run the experiment (steps 10-14) three more times in order to compare each result and average the data. (Note: in the future repeat multiple times for accurate results/limited trials due to cost of the kit and hospital constraints).
16. In order to figure out which disposal method is the most effective in eliminating the possibility of horizontal gene transfer, first autoclave one of the agar plates filled with bacteria.
17. For the other agar plates containing bacteria, pour the Clorox bleach onto the surface until completely covered.
18. Use the third and final agar plate to test what would occur if the bacteria was directly disposed into the trash. For the sake of this experiment, set the agar petri dish aside in order to act as if the dish was regularly thrown away.

19. For the control, autoclave the agar plate and test for the presence of ampicillin. In order to accomplish this, add more of the susceptible bacteria to the agar plate and if there is still ampicillin, the bacteria will be killed. If the bacteria continue to grow, there is no presence of ampicillin following the use of the autoclave. This can be helpful in the establishment of the correct method of disposing bacteria and the harmful resistance genes. If the autoclave was successful in destroying both the ampicillin and the bacteria, utilizing the autoclave would be most effective moving forward in labs and hospitals.
20. The next step is to focus on transforming the resistant bacteria from the agar plates containing ampicillin onto the agar plates with the cephalosporin discs in order to determine if the beta-lactamase enzyme that resists ampicillin also resists the cephalosporins. This would make the bacteria multiple drug resistant.
21. Using a sterile loop, scrape off 2-4 colonies of the ampicillin-resistant bacteria.
22. Plate the resistant *E.coli* on a new agar plate.
23. Add 3 cephalosporin discs on the agar dish (Ceftriaxone, Ceftazidime, and Cefoxitin).
24. Grow the bacteria at room temperature (23°C) once again for 2 days. After the two days, the bacteria should either grow around the cephalosporin discs or be completely diminished.
25. If there are colonies surrounding the discs, the bacteria carry plasmids that code for enzymes that break down both cephalosporins and ampicillin.
26. If not, the genes that code for the enzymes are not present in the bacteria.
27. Repeat steps 28-33 (3 times) in order to accumulate an average of the data for further comparison.
28. Autoclave one of the used agar plates to eliminate the presence of any bacteria or cephalosporin discs.

29. Also, pour the Clorox bleach on one of the agar plates, until completely covered, in order to determine if that is as successful or possibly more effective than the autoclave.
30. To test the effectiveness of each disposal method, one of the agar plates must be set aside, in order to resemble being disposed into the trash, (left untouched) to become a control.
31. All petri dishes should be left at room temperature (23°C) for 2 days.
32. Following the 2 days, check for the presence of any bacterial colonies for each method of disposal and determine which process is the most effective in eliminating all the bacteria.

Results:

Table 2.1- Results of the three trials for each cephalosporin after 24 hours

	Size of Inhibition Zone (mm) (±1mm)-Trial 1	Size of Inhibition Zone (mm) (±1mm)-Trial 2	Size of Inhibition Zone (mm) (±1mm)-Trial 3
Cefoxitin (1 disc per plate)	18	18	19
Ceftazidime (1 disc per plate)	40	23	15
Ceftriaxone (1 disc per plate)	38	34	20

Table 2.2- Results of the three trials for each disposal method after 24 hours

	Number of Bacterial Colonies	Number of Bacterial Colonies	Number of Bacterial Colonies

Disposed in Trash	No change	No change	No change
Clorox Bleach	0	0	0
Autoclave	0	0	0

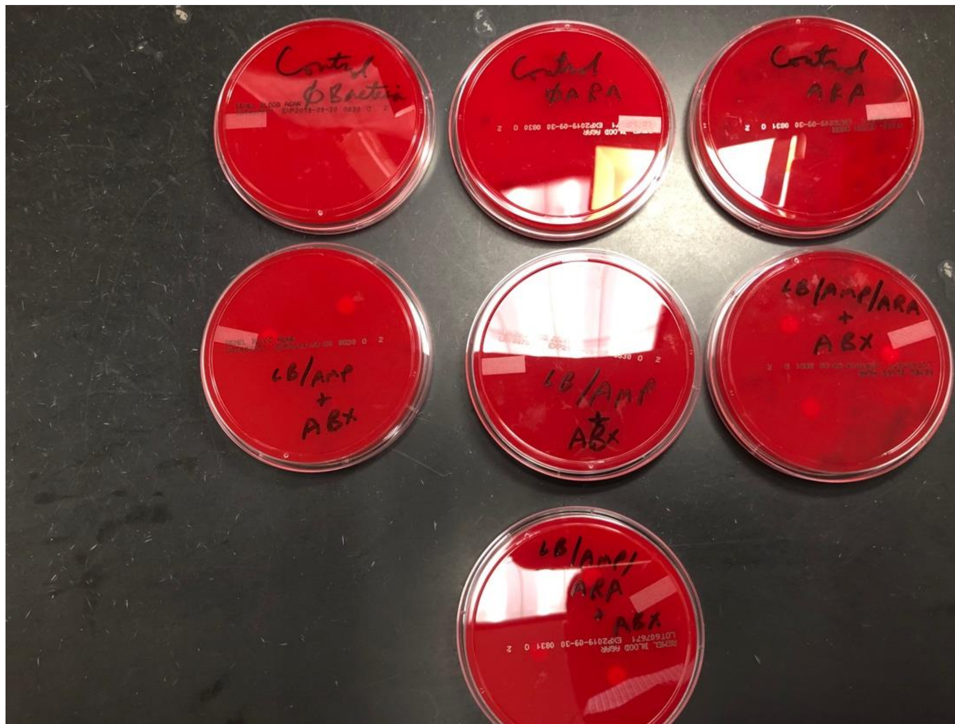

Figure 1-Trial #1 of the control and experimental agar plates
(Top Left-Control with no bacteria/ Top Middle-Control with bacteria and without arabinose / Top Right-Control with bacteria and arabinose/ Middle Left-LB/amp with bacteria and the antibiotic discs/ Middle-LB/amp with bacteria and the antibiotics discs/ Middle Right-LB/amp/ara with bacteria and the antibiotic discs/ Bottom- LB/amp/ara with bacteria and the antibiotic discs)

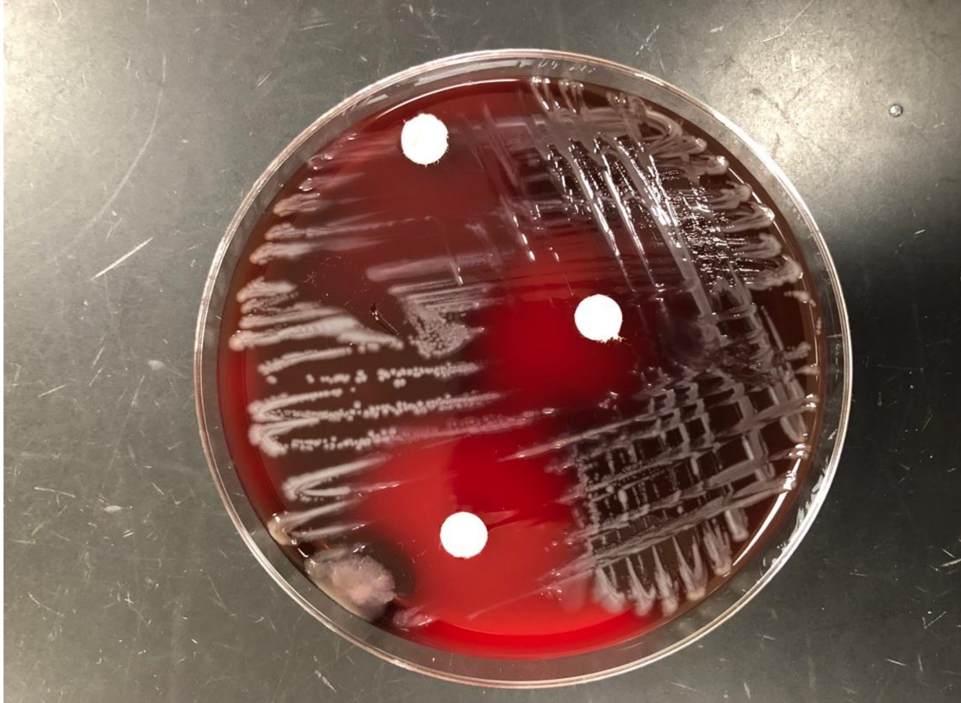

Figure 2-*E.coli* plated with Ceftriaxone, Cefazolin, and Cefalexin

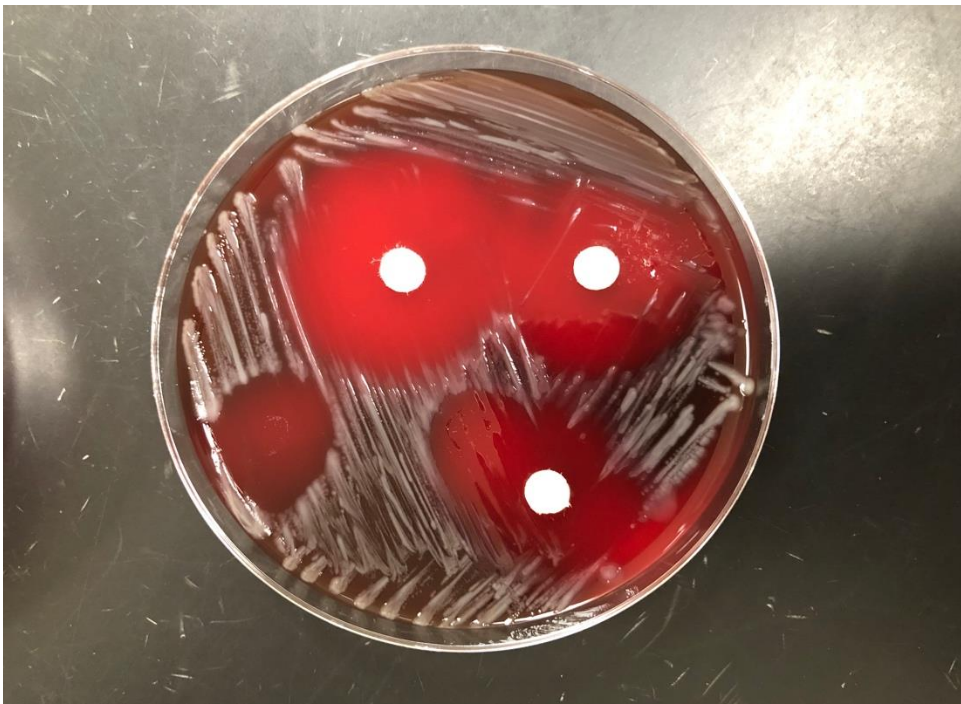

Figure 3-*E.coli* plated with Ceftriaxone, Cefazolin, and Cefalexin

Analysis and Evaluation:

Based on the susceptibility standards I used for this experiment (Weinstein, 2019), the results of the experiment show that the *E.*coli was sensitive to all 3 antibiotics tested in 2 out of the 3 trials. Only the last trial showed full resistance to Ceftazidime and intermediate resistance to Ceftriaxone. However, in all three trials, the Cefoxitin was effective in killing the bacteria due to the size of the inhibition zone remaining greater than or equal to 18 (Weinstein, 2019). Both the Ceftriaxone and the Ceftazidime are third generation cephalosporins which work more effectively on life-threatening bacteria such as gonorrhea and pseudomonas aeruginosa (Tulane University, 2016). In contrast, Cefoxitin is “similar to penicillin” and has “increased activity against Gram negative bacilli and greater stability against beta-lactamase inactivation” (Tulane University, 2016), which directly targets the *E.coli.* This can be beneficial for future research because it indicates the most effective antibiotics for killing specific types of harmful bacteria. Although the third-generation antibiotics impact fatal infections, in this case the second-generation cephalosporins produced better results (MedlinePlus, 2019). Nevertheless, it is important to note that the third trial could have been an outlier that does not signify the efficacy of the antibiotic class as a whole. Hence, *E. coli* that is resistant to Ampicillin (Penicillins) overall does not seem to be resistant to cephalosporins automatically. Of course, *E. coli* can be pan-resistant to Penicillins and cephalosporins among multiple other antibiotics (e.g. ESBL) but the question here was to what extent does ampicillin resistance to *E. coli* also affect resistance to cephalosporins.

In reference to the second part of the experiment, the autoclave and the bleach are, in fact, the most efficient methods for eliminating harmful bacteria such as *E.coli*. They both completely destroyed the bacteria and prevented the possibility of them spreading into the

environment. Additionally, the bleach is more adequate for daily use and can be accessed more conveniently by class teachers and lab professors due to its relatively low cost in comparison to the autoclave. The bacteria disposed directly into the trash will continue to grow and develop mutations over time which can further produce dangerous situations. This is essential for further research because it presents possible solutions to the rapid growth of resistant bacteria around the world. Some infections have not been able to be treated as a result of the abundance of the resistant bacteria to specific antibiotics. In the future, doctors and trained professionals will not be able to treat patients for certain diseases because the bacteria will have developed several mutations that will allow them to break down the beta-lactam ring found within the antibiotics. To avoid this hazard, schools and labs must always dispose bacteria by using bleach or, if available, an autoclave.

Conclusion:

The results ultimately fail to reject the main hypothesis that ampicillin-resistant bacteria is frequently resistant to cephalosporins. However, the second portion of the null hypothesis was successfully rejected because disposal methods are very significant for the safety of all humans. Some of the bacteria contained the beta-lactamase enzyme which broke down the beta-lactam ring in the antibiotics, however it is difficult to directly associate ampicillin-resistant bacteria to cephalosporin-resistant bacteria. The results can also explain the reasons as to why there has been an abundance of resistant bacteria in the last several years. Due to the inappropriate methods of disposal, many resistant bacteria, from labs, have been exposed to the environment in which they can reproduce and multiply their colonies. With more frequent contact between the

antibiotics and the bacteria, there are more mutations that result in resistant bacteria. This can result in several useless antibiotics because many of the bacteria will become resistant to the most commonly used antibiotics. This clearly identifies an issue which requires a solution in order to ensure the safety of the nation. With the proper precautions and disposal methods, the nation can avoid the production of resistant bacteria and therefore protect the people.

References

Ampicillin: MedlinePlus Drug Information. (2019). Retrieved February 10, 2019, from https://medlineplus.gov/druginfo/meds/a685002.html

Biotechnology Explorer Team. (2019). pGLO Bacterial Transformation Kit. *Biotechnology Explorer*, 1–69. Retrieved from pGlo_manual.pdf

Briñas, L., Zarazaga, M., Sáenz, Y., Ruiz-Larrea, F., & Torres, C. (2002, October). Beta-lactamases in ampicillin-resistant Escherichia coli isolates from foods, humans, and

healthy animals. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC128764/

Goodsell, A. (2002, October). β-Lactamases in Ampicillin-Resistant Escherichia coli Isolates from Foods, Humans, and Healthy Animals. Retrieved February 10, 2019, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC128764/

Healthline's Medical Network. (2018, May 4). Medical information and health advice you can trust. Retrieved October 3, 2019, from https://www.healthline.com/.

Sutcliffe J. G. (1978). Nucleotide sequence of the ampicillin resistance gene of *Escherichia coli* plasmid pBR322. *Proceedings of the National Academy of Sciences of the United States of America*, *75*(8), 3737-41.

Tulane University. (2016). Retrieved March 13, 2019, from http://tmedweb.tulane.edu/pharmwiki/

Weinstein, M. P., Patel, J. B., & Bobenchik, A. M. (2019). Performance Standards for Antimicrobial Susceptibility Testing. *Performance Standards for Antimicrobial Susceptibility Testing*, *39*(29), 34–37.