Lab Report One: (PARE) Antibiotic Resistance of CFUs from Collected Ground Soil

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Background and Purpose:

Antibiotic resistance in bacteria is a constant threat to humanity, as new and improved antibiotics must be developed to combat the rise of new mutations leading to resistance. Humanity is in a constant arms race with the inevitability of antibiotic-resistant antibiotic bacteria, as several factors lead to the rise of new resistance tactics (Doron, 2008). Plasmids are circular strands of DNA found exclusively in Prokaryotic cells that specialize in the survivability of the cell, which includes the bacterium these strands inhabit. These plasmids also allow cell-to-cell communication of various bacterial cells, allowing resistance to be passed down quickly (Orlek, 2017). Resistance can also arise from mutations, and errors in DNA replication that may rarely be beneficial to the host. Due to the rapid replication of bacteria, these mutations are much more likely to occur. Some of these mutations provide the bacterium with antibiotic resistance to prolong the survival of the cell (Watford, 2022).

These bacteria can occupy many surfaces, but ground soil is a common source of various forms of bacteria, including species unknown as of now (PARE, 2022). Exposure to bacteria of unknown origin can be potentially dangerous, as the degree of antibiotic resistance can be deadly when infections begin to arise. The goal of the PARE project is to document the accumulation of antibiotic resistance in a variety of mediums around the globe. These sites can be monitored to control the future spread of microbes, but a group effort is required as many individuals can select specific locations to report findings (PARE, 2020). The objective for the following procedure will focus on the soil medium, and the site will be The Lake Forest College Health and Wellness Center. This site is important, as antibiotic resistance can arise in the soil through animal feces, thrown out prescription medications, sources of water including filtration plants and rivers, and water runoff from sources of agriculture (Doron, 2008). The usage of antibiotic prescriptions leads to an increase in antibiotic resistance, even if indirectly applied to an environmental source. A study has shown that poultry treated with antibiotics leads to an increase in Salmonella cases of the consumers of the meat (Levy, 1976). The feces of poultry also make their way into the soil, which would further spread the presence of antibiotic-resistant bacteria treated with the initial antibiotic.

The Health and Wellness Center at Lake Forest College would pose as a beneficial site to study the soil, as the likelihood of antibiotic resistance could be high. The dumping of prescription medications through water runoff or garbage disposal leads to the rise of antibiotic-resistant bacteria in the medium (Lya et al. 2020). Additionally, countless students make their way in and out of the complex. Bacteria spreads through contact with shoes or clothing that encounter the soil (Doron, 2008). Students who take antibiotics may already spread the population of bacteria to the surrounding soil with contact, which would accumulate progressively in the soil over time.

Under the guidelines of the PARE project, the purpose of the following experiment is to study a collected soil sample from the specified location to provide a consensus of overall bacterial resistance visually and mathematically. It is hypothesized that the collected sample from the Health and Wellness Center with increased antibiotic resistance would differ in resistance compared to other local sites. A set of serial soil dilutions will be performed to prepare five individual bacterial mediums, in which growth will be observed on specified petri dishes. As the concentration through dilutions decreases, it is predicted that growth will slow down exponentially. For petri dishes with antibiotic treatment, fewer colonies are to be expected. However, a mathematical approach to calculate the percent of colony-forming units will be used to determine the relative percent of antibiotic resistant bacteria.

Procedure:

Safety:

For the series of wet labs, gloves, a lab coat, a mask, and safety goggles were prioritized. Unknown bacteria were likely to be encountered throughout the preparation. Contact with materials was handled with care, where standard sanitizer was used to remove any possible contaminants from personal belongings before proceeding to further steps of the experimental protocol. Pipettes used were to be placed flat on a surface if a tip was attached. If doubtful, a tip was removed and replaced with a fresh one. Any solid and liquid solutions or mediums used were always covered, except when following the experimental procedure.

Soil Collection:

An open area was located at the Lake Forest College Health and Wellness Center. The collection was authorized before commencing, as the site was the private property of campus grounds. The soil was collected while wearing sterile gloves, and a metal spatula was used to transfer an appropriate sample size into a test tube. The sample was roughly the size of a golf ball. The test tube with the sample was sealed tightly before being stored in a plastic zip-lock bag. After, the location of collection was marked on the PARE Project website, where resistance data could be implemented before bacterial incubation.

Serial Dilution:

The collected soil sample was retrieved, in which a rack of five 15 mL test tubes would be used. Each tube would be labeled with an appropriate dilution factor starting from 1*10^1 and proceeding towards 1*10^5. The dilution factor represents the serial dilution of the utilization of bacteria compared to water (a ratio), where the bacterial concentration will constantly increase by tenfold through each dilution. The first sample prepared would contain 9 mL of water and 1 gram of soil that has been measured out precisely and transferred with a metal micro-spatula. Ethanol was used to clean the spatula afterward. The soil added to the first test tube was then held for 1 min on a vortex on the setting high. After the first test tube was vortex, 1 mL of the bacterial solution would be transferred to the next tube with a higher dilution factor. A pipet would be used for all content transfers, in which a fresh tip would be used every time. The process would repeat until the final tube was vortexed.

Petri Dish Preparation:

After serial dilution, twenty-two individual petri dishes were collected. Ten plates would contain no antibiotic treatment on the agar, while the rest would have a varying applied concentration of a Tetracycline (TeT) antibiotic compound. Six plates would contain 3 µg/mL of the antibiotic while another six would contain 30 µg/mL of the antibiotic. All plates consisted of a MacConkey agar medium for growth conditions. Additionally, all plates would be equally divided to form two plate sets. Two replicate trials would be tested as a result. One plate set would contain five untreated plates, three would contain the 3 µg/mL Tetracycline treatment, and three more would contain 30 µg/mL Tetracycline treatment. Each petri dish is to remain covered to avoid contamination, and labeling is to occur before applying the bacterial solution. Labeling plates would be conducted as follows: Team name (WALI), treatment condition, dilution factor, replicate set (1 or 2), 1/30. Starting from the untreated plates, 200 µL of the bacterial-water solution would be pipetted directly to the petri dishes from the highest dilution factor to the lowest (1|10^5 to 1|10^1). While a new pipet tip was used every time to apply the bacterial solution, the plastic loops used to spread the solution on the dishes would be thrown out after completing the set. When moving on the petri dishes with antibiotic treatment, a new loop would be used, and the highest dilution factor would be 1|10^3 instead of 1|10^5. Application of the bacterial solution would continue to be applied from the highest dilution factor plates to the lowest. Once all plates in a set were completed, the second set would be prepared the same way. Once both sets were ready for incubation, the simple tape was used to seal each plate set part of the replicates. Plates were stored in incubation at 28° C for 48 hours, in which 7 days would elapse before collection.

Colony Observation:

Growth occurred after incubation. The two replicates were unsealed from the applied tape, and counting was possible with the formation of colonies. For labeling, colonies with an approximate amount over 300 were

marked with 300+ while those fewer than 30 were marked as 30-. An exception was set for plates with antibiotic treatment, as it is likely that fewer than 30 colonies would be observed for treated plates. Such plates were labeled with their respective number of colonies. A fine-point marker was used to count each colony for plates that had a large amount of growth. Additionally, a light source and magnifier were used to count colonies that were difficult to spot. For calculations, the plates featuring more than 30 but less than 300 were to be utilized for determining CFUs (colony-forming units). The volume for all plates would be consistent, as they were all the same size. However, the dilution factor was to be adjusted based on which respective plate was used. The untreated plate had its CFUs calculated, and the treated plates had the approximate resistant CFUs calculated to determine the percent of resistant units. All calculated percentages were corrected with an error checker, and data was implemented into the collection site of the PARE website. Local data was then used to compare the obtained resistance with that from the initial collection site.

Results:

Figure 1 presents the growth accumulation of the soil bacteria after incubation. Both replicates are featured respectively for plate set 1 and plate set 2. Growth on untreated plates is most with a dilution factor of 1|10^1. Growth on the untreated plates begins to steadily decrease until only a few colonies are present. For plates treated with their respective Tetracycline concentration, growth is substantially lower. Few colonies are present compared to the untreated plates, in which the 30 µg/mL TeT plates have the least growth compared to the 3 µg/mL TeT treatment group. Visually, the colony count exponentially decreases as the dilution factor decreases, like that of the untreated plates. For the highest concentration of TeT plate set 1 expresses zero colony growth when it comes to later dilution factors, 1|10^2 and above. Plate set 2 does express colony growth for the highest concentration, but it is very limited. Visually, plates with more growth express a slight tint of the color of the agar. Plates with more growth present an orange tint from afar, while those with the least growth retain the red color. Colonies with more growth also express a unique growth pattern compared to plates with less growth. An assortment of miniature and medium colonies also has a layer of sediment mixed with an array of clustered small colonies, which form a pool with a dark tint. The colonies with the least growth are slightly bigger with the greater room to grow, but some small colonies are also detected on other plates to a lesser extent.

Table 1 colonies have been carefully counted, in which a majority contain more than 300 for the untreated plates. Plate set 1 only has 43 countable colonies on the plate with a dilution factor of 1|10^4, in which the factor ahead has fewer than 30. Plate set 2 has 93 countable colonies for the same dilution factor. The 3ug/mL treatment groups are also in a countable range, in which plate set 1 has 94 at 1|10^2 and 98 at 1|10^2 for plate set 2. For Table 1, all values for both plate sets of the highest TeT concentration are below 30, which has been properly accounted for and adjusted. As a result, 10 colonies are counted at 1|10^1 for plate set 1 and 19 at 1|10^1 for plate set 2. Red values are to be utilized for calculations to determine CFUs per given area and for the percent of resistant CFUs.

Figure 1:

Plate Set 1:

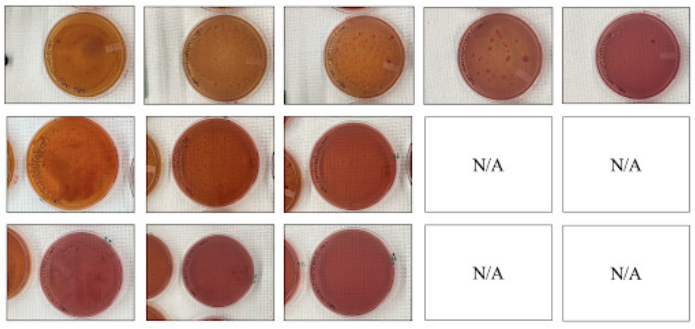
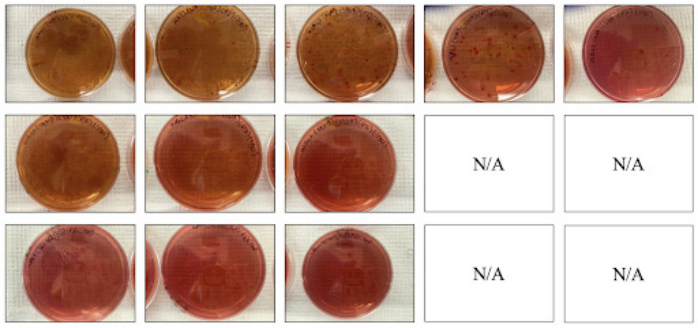


Plate Set 2:



Colony growth for plate sets 1 and 2 after incubation period. The top rows as untreated groups, the middle row as 3 µg/mL TeT treatment group, and the bottom row is 30 µg/mL TeT treatment group. N/A assigned groups indicate no treatment beyond a 1|10^4 dilution factor.

Table 1:

Plate Set 1:

Row	Treatment Type	1|10 DF	1|10^2 DF	1|10^3 DF	1|10^4 DF	1|10^5 DF
1	No Antibiotic	300+	300+	300+	43	30-
2	3ug/mL Tetracycline	300+	97	9	N/A	N/A
3	30ug/mL Tetracycline	10	0	0	N/A	N/A

Plate Set 2:

Row	Treatment Type	1|10 DF	1|10^2 DF	1|10^3 DF	1|10^4 DF	1|10^5 DF
1	No Antibiotic	300+	300+	300+	93	30-
2	3ug/mL Tetracycline	300+	98	5	N/A	N/A
3	30ug/mL Tetracycline	19	3	2	N/A	N/A

Colony counts for plate sets 1 and 2. Colonies individually counted and marked. Plates with over 300 and fewer than 30 labeled accordioning for being uncountable. Red numbers indicate values used for further calculations.

Table 2 utilizes the appropriate colony counts from table 1, where the CFUs are calculated accordingly. Both plates set 1 and 2 have their calculations conducted accordingly, where the volume plated, and dilution factor are the same. CFUs per gram of soil are conducted by multiplying the total colonies by the volume plated, before multiplying again by the corresponding dilution factor. The calculated numbers are both large and account for a great majority of bacterial accumulation for the untreated plates. Plate set 2 has the most growth out of the two sets with 4.65E6 CFUs per gram of soil.

Table 3 yields in the percent of resistant CFUs for plates treated with both concentrations of TeT. Corresponding TeT resistant CFUs per g soil are calculated the same way as the values in table 2. Calculations are adjusted according to dilution factors, as they are different based on the counted colonies. Corresponding percentages are obtained by dividing TeT resistant CFUs by the CFUs of the untreated groups before multiplying by 100 to result in a percent. For both plate sets, the percent of TeT resistant colonies at the highest concentration is 0.02%, while those at the lower concentration are different. Plate set 1 has a higher percent of resistant CFUs with 2.25% compared to 1.05% from plate 2. The percents are labeled in blue to better visualize the data part of the figures.

Table 2:
Plate Set 1:

Row		Values
1	# Colonies on Countable NA Plate	43
2	Volume Plated	5
3	Dilution Factor	1/10^4
4	Total CFU per gram soil	2.15E+06

Plate Set 2:

Row		Values
1	# Colonies on Countable NA Plate	93
2	Volume Plated	5
3	Dilution Factor	1/10^4
4	Total CFU per gram soil	4.65E+06

CFU calculations for plate sets 1 and 2. Values from rows 1, 2, and 3 are multiplied to obtain the total CFUs per gram of soil in row 4. Red values indicate counted colonies taken from table 1.

Table 3:
Plate Set 1:

Row		Values	Values
1	Tetracycline Concentration	3 µg/mL	30 µg/mL
2	# Colonies on Countable Plate	97	10
3	Volume Plated	5	5
4	Dilution Factor	100	10
5	Total TeTR CFUs per g Soil	4.85E+04	500
6	% TeTR CFUs	2.25%	0.02%

Plate Set 2:

Row		Values	Values
1	Tetracycline Concentration	3 µg/mL	30 µg/mL
2	# Colonies on Countable Plate	98	19
3	Volume Plated	5	5
4	Dilution Factor	100	10
5	Total TeTR CFUs per g Soil	4.90E+04	9.50E+02
6	% TeTR CFUs	1.05%	0.02%

Number of TeT resistant CFUs and percent resistant CFUs calculated for plate set 1 and 2. Red values from table 1, blue values as percent-based calculations using number of TeT resistant CFUs divided by number of untreated CFUs before multiplying by 100. Untreated CFUs from table 2.

Table 4 accumulates the data of the entire class where other students conducted the same procedure, except at different sites. The Percentages are all different from each other for both TeT concentration groups. Some locations present TeT resistance values higher than the blue values, which are the obtained resistance values from table 3. Hixon Hall, a site on the Lake Forest campus, appears to present more TeT resistance at 11.31% and 10.60% compared to all others at a concentration of 3 µg/mL for both plate sets. Lake Forest Hospital also reveals 0% resistance for a TeT concentration of 30 µg/mL for both plate sets, which is the lowest percentage. For groups 2 and 4, the difference in plate set resistance percentages is also variable while others are roughly close to being the same. These two groups, one of them being discussed here, have the highest variation of resistance compared to the others.

Table 4:
Plate Set 1:

Row		Group 1	Group 2	Group 3	Group 4
1	Team Name	DGMT221	WALI	Boomers	Biohaz
2	Location	LF Hospital	H&W Center	Hixon Hall	Townline Stables
3	% TeT3R CFUs	3.58%	2.26%	11.31%	5.63%
4	% TeT30R CFUs	0.00%	0.02%	0.13%	0.24%

Plate Set 2:

Row		Group 1	Group 2	Group 3	Group 4
1	Team Name	DGMT221	WALI	Boomers	Biohaz
2	Location	LF Hospital	H&W Center	Hixon Hall	Townline Stables
3	% TeT3R CFUs	3.81%	1.05%	10.60%	6.85%
4	% TeT30R CFUs	0.00%	0.02%	0.07%	0.11%

Percent resistance of CFUs for TeT at 3 µg/mL and 30 µg/mL for each unique group respectively. Each group featured with personal name and site of soil collection. Blue values of calculated percentages from table 3.

Discussion:

The initial expected results for the Health and Wellness sample are clearly visible when observing the colony growth in figure 1 and table 1. As the dilution factor increases for the bacterial mixture, the number of colonies decreases. With TeT treatment the presence of colonies decreases to an increasing amount as the concentration of the antibiotic increases. Mathematical analysis yields in percent of resistant CFUs while visual observation yields in observable colony growth. This analysis aligns with the purpose of the experiment and for the data acquisition required for the PARE project website. The hypothesis initially indicated that antibiotic resistance would be variable, where the possible environmental conditions that lead to the dispersion of antibiotics in the environment would result in the Health and Wellness Center having a large amount of antibiotic resistance in the soil. It is clearly visible from table 4 that the hypothesis is supported by the fact that there is a difference in local sites when it comes to antibiotic resistance. For a TeT concentration of 3 µg/mL, there is a greater distribution of resistant bacteria compared to the higher TeT concentration. The hypothesis is questioned when observing the resistance percentages for Hixon Hall, a residential site on the Lake Forest College campus. It was assumed that medical sites would have a greater number of resistant bacteria as a higher usage of prescription antibiotics are likely to be present. However, a residential site raises the risk factor of bacterial infection, as both 3 µg/mL TeT concentration plates reveal a resistance over 10%. Monitorization of the area is required to carefully monitor the likelihood of bacterial infections, and treatment in the overall reduction of antibiotic resistance is required to ensure the safety of students. The Health and Wellness Center and Lake Forest Hospital have the two lowest antibiotic resistance values out of the four locations, which suggests that they are well-maintained for being medical centers. Hixon Hall and the Townline Stables require more work to ensure that resistant bacteria are prevented from further growth.

The error arises when it comes to the possibility of contamination with the application of the bacterial solution on the plates. It is likely possible that a foreign, unwanted form of bacteria could have made contact with the plate during application, in which growth would have occurred. This would result in bacterial colonies from the Health and Wellness Center growing along with the foreign bacteria from the collection site. Colony counting would therefore interfere with calculations, and the percentage of antibiotic resistant CFUs would be incorrect. While this is a possibility for the plate sets from the collection site, the other three groups would have made the same mistake if there was poor management in bacterial application. One site could have conducted perfect application, while the other groups could have applied some contaminant. Additionally, using the loop to apply bacteria may have been done with lacking movement. When looping the plate with the bacterial solution pipetted, the entire surface of the plate is to be covered. If not, the growth of colonies would be disrupted to only occur at the center of the plate, rather than the entire available surface of the plate.

Future research with the PARE project could compare a variety of residential sites with the environmental dispersion of antibiotics. Hixon Hall has been observed to be a critical site when it comes to the dispersion of antibiotics. Potentially analyzing the soil would provide context as to how dispersion is controlled and provide future management to sites that have a high percent of resistant bacteria. Besides soil, the controlled medium could be adjusted to water. Bacteria can occupy a variety of various growth mediums, where water is another potential site of accumulation. Collecting water from the runoff site of a residential or commercial complex would provide context as in how antibiotic compounds are dumped through wastewater. Based on the collection of water runoff, would it be possible that residential sites with poorer management of antibiotics have a higher environmental percent of antibiotic resistance than those that are well maintained? Or would the percent of resistant bacteria remain the same?

References

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