QUANTIFICATION OF GENES THAT ENCODE FOR ANTIBIOTIC-RESISTANCE IN SOILS

An Undergraduate Research Scholars Thesis

by

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RESEARCH COMPLIANCE CERTIFICATION

Research activities involving the use of human subjects, vertebrate animals, and/or biohazards must be reviewed and approved by the appropriate Texas A&M University regulatory research committee (i.e., IRB, IACUC, IBC) before the activity can commence. This requirement applies to activities conducted at Texas A&M and to activities conducted at non-Texas A&M facilities or institutions. In both cases, students are responsible for working with the relevant Texas A&M research compliance program to ensure and document that all Texas A&M compliance obligations are met before the study begins.

I, Denise Pedraza, certify that all research compliance requirements related to this Undergraduate Research Scholars thesis have been addressed with my Research Faculty Advisor prior to the collection of any data used in this final thesis submission.

This project did require approval from the Texas A&M University Research Compliance & Biosafety office.

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ABSTRACT

Quantification of Genes that Encode for Antibiotic-resistance in Soils

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Antibiotic resistance decreases the effectiveness of antibiotics. With resistant bacteria, antibiotics will no longer stop an infection. With an increase exposure of antibiotics, bacteria become resistant to them. Antibiotic resistant bacteria multiply and their mechanism of resistance spreads. Antibiotics are introduced into the soil environment through animal waste and other source. We want to quantify specific antibiotic-resistant genes in soils and find threshold concentrations of antibiotics that will give rise to genes that encode for antibiotic resistance. Resistance genes to sulfonamides, tetracycline, and beta-lactam antibiotics were detected in experiments with varying conditions. Additionally, *sul*2 a gene resistant to sulfonamides was quantified. Preliminary results show that *sul*2 increased over time in samples exposed to sulfamethazine and was not detected in sterile samples with no antibiotic added. The preliminary data shows that the introduction of antibiotics in soils may influence antibiotic-resistance development.

DEDICATION

I dedicate this paper to my family, partner, and advisor who supported me throughout this research process. To my parents whose words of encouragement pushed me to pursue this project. My sister, Gladys who guided me through every step of my educational career and supported me emotionally while I typed this. Thank you, Dr. Mendoza, for presenting this opportunity to me and for being very patient and kind. Finally, thank you to my partner who listened to me ramble about my experiments and the many troubleshoots that I had to perform. I appreciate all of you for inspiring me.

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Contributors

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All other work conducted for the thesis was completed by the student independently.

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NOMENCLATURE

ARG	Antibiotic resistant gene
ddPCR	Digital droplet polymerase chain reaction
BlaCMY-2	Gene resistant to beta-lactam
*sul*2	Gene resistant to sulfonamide antibiotics
Tet	Gene resistant to tetracycline antibiotics
LOD	Limit of detection
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
SMZ	Sulfamethazine
TC	Tetracycline

1. INTRODUCTION

Antibiotic resistance has negative impacts since it decreases the effectiveness of antibiotics, and they no longer stop an infection caused by bacteria. Antibiotics are described as a substance that can inhibit the replication of growth of bacteria. They are different from antiseptics and disinfectants in that they target bacterial infections in a body. They are used for life-threatening infections in both humans and animals. Different families of antibiotics act through different mechanisms to affect bacteria cells which include inhibiting cell wall synthesis, protein synthesis, and nuclei acid synthesis. Antibiotic resistance is when bacteria or fungi can overcome the mechanism by which the antibiotic work. Antibiotic resistance bacteria multiply and their mechanism of resistance spreads. The resistance mechanism is determined by the DNA which instructs the bacteria to make specific proteins. The antibiotic-resistance genes are usually transferred from one species to another through horizontal gene transfer. The genetic material is transferred amongst organism; this genetic change is a factor to the evolution of organisms. This type of transfer is more common in prokaryotes than eukaryotes and can take form of bacterial transformation, transduction, and conjugation. Resistance in the environment is concerning because non-pathogenic bacteria that is naturally in the environment could transfer resistance genes to pathogens.

Antibiotics are introduced into the soil environment through animal waste and other sources, which give rise to antibiotic resistant bacteria strains[1]. Since many animals are treated with antibiotics, their waste containing antibiotics enter the environment. With a given exposure of antibiotics comes a resistance to them which is how the bacteria in the soil that receives waste will develop antibiotic resistance. Although soils contain a natural background level of

antibiotic-resistant bacteria, it has been observed that when the levels of antibiotics increase, the antibiotic-resistant bacteria and genes increase as well. Contaminated soils may lead to a greater risk of exposure to these bacteria when people are consistently exposed to contaminated soils[2]. Human contact with antibiotic-resistant bacteria in the environment can lead to infection[3]. Soils contaminated with antibiotics have been recognized as a threat for those working in the agricultural sector and the neighboring communities because of the potential exposure to the antibiotics and the antibiotic-resistant bacteria in the soils. Several studies have isolated the same antibiotic-resistant strains from livestock and infected humans[4,5]. A route of infection for farmworkers is dust since there was positive association between nasal carriage in farmworkers, community members, and antibiotic-resistance bacteria in airborne soil samples[6]. The proximity to manure-applied crops and high-density livestock facilities has been associated with antibiotic-resistant infections in humans[7]. In the agricultural environment high concentrations of antibiotics have been linked to high levels of antibiotic-resistant bacteria and genes in soils and dust[8]. Although there has been some research in antibiotic resistant genes there are few studies on soils, especially those using quantitative polymerase chain reaction (qPCR). We hypothesized that with a defined concentration of antibiotics introduced to the soil there will be an increase in the resistance genes observed throughout a period, which can be determined through the quantification of specific antibiotic-resistant genes (ARG) in soils.

2. METHODS

Soils were collected from the Howdy Farm, placed in sterile containers and stored at 4°C. The experimental conditions consisted of soils exposed to the antibiotics Tetracycline (TC) and Sulfamethazine (SMZ) in experimental soil microcosms. Each microcosm, a small version of the original environment, consisted of glass vials containing soil plus the antibiotic. SMZ and TC were selected for the exposure experiments because they are commonly used in livestock facilities. Microcosms were incubated at room temperature and soil samples were obtained from the microcosms at day 0, 4, 6, 16, 35 and 56. A total of 120 DNA soil samples were tested namely: 48 samples from the experiment with added TC at high and low concentrations, 48 samples with added SMZ at high and low concentrations, and 24 samples with no antibiotic added over the 56 days as seen in **Figure 2.1**. DNA was extracted from the samples and the concentration of *sul*2, a gene resistant to the antibiotic sulfonamide, was measured. It has been established that long-term use of an antibiotic will cause bacteria to develop resistance to not only that antibiotic, but to other antibiotics not associated with it[9], which is why we tested for *sul*2 in samples that were treated with TC (not related) and SMZ (related). Additionally, the presence of genes that confer resistance to tetracycline (*Tet)* and beta-lactam (*BlaCMY-2)* were tested in microcosms treated with SMZ and TC[10].

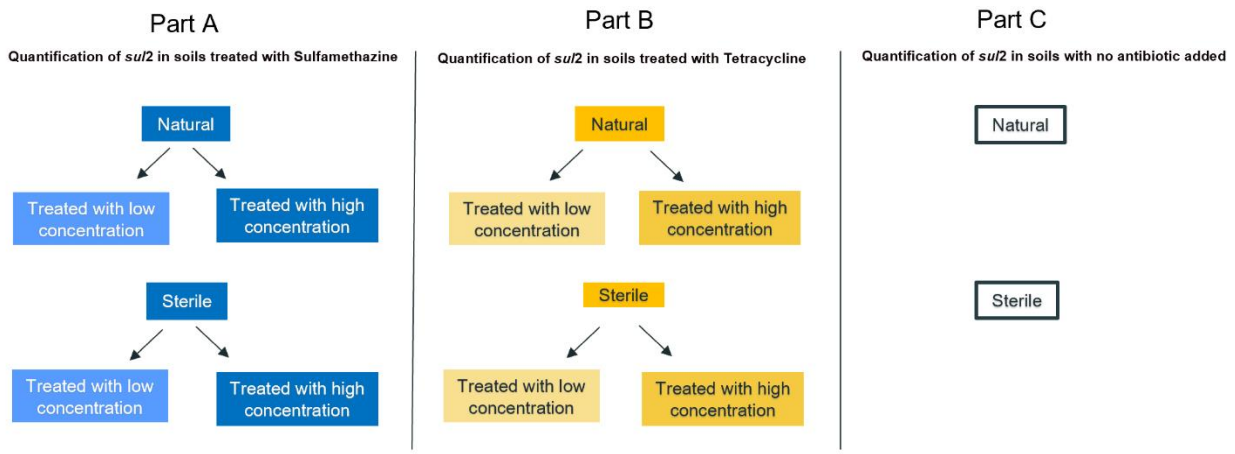

Figure 2.1*: Experimental Conditions. Low concentrations are 21 ng/gram of soil and high concentrations are 21 ng/gram of soils. On the left of the figure, Part A, is the first condition where soils were treated with the antibiotic Sulfamethazine. Half of this group was sterilized using an autoclave and the other half was not. With these two groups half of them were treated with a high concentration of antibiotic and the other half with a low concentration. In Part B, the difference is that the soil was treated with the antibiotic Tetracycline. In Part C, this soil was not treated with any antibiotic and half of the group was sterilized.*

2.1 Real-time q-PCR of ARG in Soils

The main difference between polymerase chain reaction (PCR) and quantitative polymerase chain reaction (qPCR) is in the quantification of the resistance gene. PCR is used to amplify DNA segments. Conventional PCR includes three temperature stages. The first is denaturing, where the DNA separates into two single strands due to the heat, which is 98°C for 2 minutes. The second is annealing where the primers attach to the DNA due to the decrease in temperature, 60°C for 0.50 minutes. The third is extension, where the Taq polymerase enzyme makes new DNA strands due to the increase in temperature to 72°C for 2 minutes. The three stages are repeated for several cycles to achieve observable amplification of the DNA segment. The presence of the DNA segment is measured at the end[11]. In qPCR the fluorescence is measured at each amplification cycle. The fluorescence depends on how many copies of the gene are obtained. Since the dye binds to the DNA, the fluorescence will be detected proportionally to the amount of amplified DNA. There are a total of 7 temperature changes within the three stages

of preincubation, 3-step amplification (which is repeated for 40 cycles) and melting. The experiment run parameters have this temperature profile for the heating and cooling cycles to achieve the 3-step-amplification of denaturing, annealing, and extension to amplify DNA segments. qPCR helps determine the concentration of the DNA segment specific to the antibiotic resistance gene *sul*2 by setting up controls (or standards) with a range of known *sul*2, determining the cycle at which those concentrations produce fluorescence and comparing the cycle at which unknown samples produces fluoresce to the standards seen in **Figure 2.2**. Since the standards are *E.coli* with the *sul*2 amplicon (191bp) clones we already know their concentration (copy gene/µL). Quantification of the target resistant genes was done by qPCR in a LightCycler®96 System, the qPCR machine where reactions take place. The overview of the experiment can be found in **Figure 2.2** where in part A of the figure are the sterile containers with the soils that were collected. In part B the DNA was extracted at different times from each container. In part C two samples were taken from the extractions of one time. For each DNA soil sample in the qPCR reaction triplicates were pipetted in the well plate as well as standards and non-template controls as seen in part D. After the qPCR reaction, we obtain amplification curves as seen in part E where we are then able to determine the concentration of the genes.

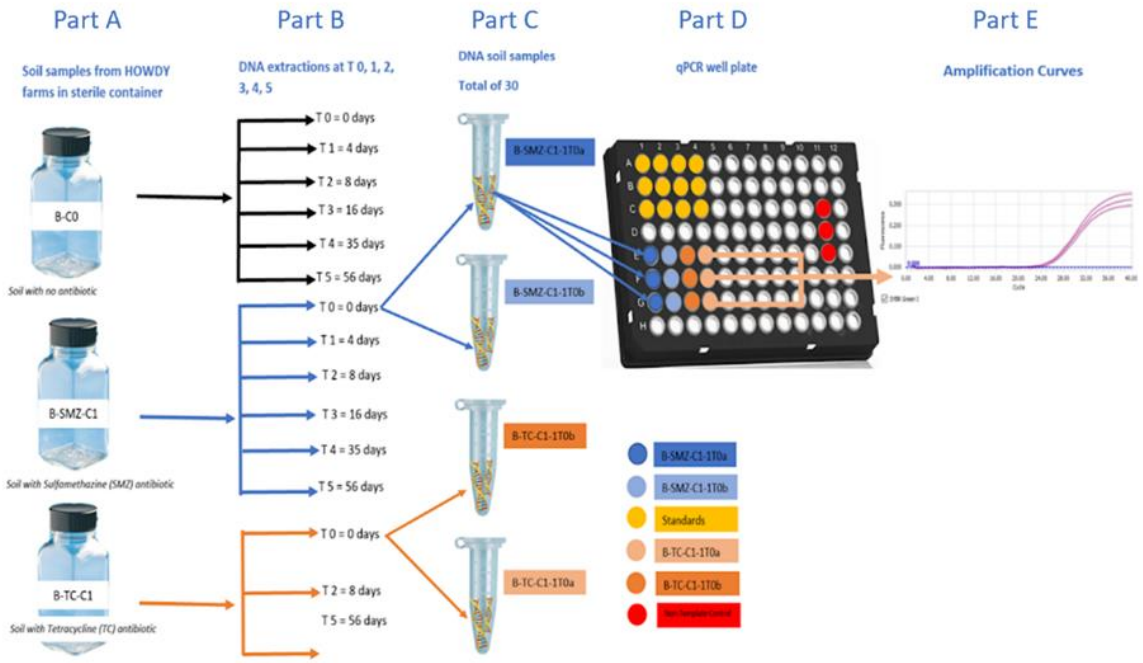

***Figure 2.2**: Overview of experiment. Image of well plate by Brooks Life Science[12]. Image of microfuge tube by ThermoFisher Scientific[13]. Image of sterile container by Kimble Kontes[14]. In Part A, are the microcosms where the soils were kept and treated. In Part B, is the DNA extraction phase which occurred on different day intervals. In Part C, is a representation of the reaction tubes of the soil samples that were quantified. In Part D, is the qPCR well plate and the outline of how the triplicate of the samples were placed. In Part E, is an example of the amplification curves that are analyzed after the qPCR reaction.*

2.2 Preparation of Positive Template and Standards for ARG Quantification

The positive and standard controls were cultured from strains that contain E. coli with plasmids that have the DNA fragments of the targeted ARG. This plasmid was obtained by first culturing the cells on LB agar plates as explained in **Appendix A: Cell Culturing**. This was done under a hood and the plates were inverted and incubated at 37℃ overnight. The growth on the plates appeared to be circular, raised, and cloudy along the streaks. A single bacterial colony was acquired from the plate and transferred to a LB liquid broth. Once the broth was inoculated it was incubated at 37℃ on a shaker overnight. After 24 hours there appeared to be growth in the tubes. The color change was from a golden yellow to a cloudy yellow throughout the tube. The

bacteria was then purified using the PureLink^{TM}Quick Plasmid Miniprep Kits. This kit is used to clean the DNA of contaminating genomic DNA. The purified plasmid that was extracted from the culture is about ~ 4 kilobases and was suitable enough to use for the positive controls for the detection of only *Tet* and *blaCMY-2*CMY-2. This was because we only tested for the presence and not the quantification of these genes. This was not suitable for the experiments with *sul2* since we had to do quantification. The DNA fragment from the plasmid which size is ~100 bases was amplified with PCR. This was to test that the template had enough amplification to use as a positive control and to amplify the DNA fragment of the plasmid. Then, this PCR reaction was purified using the PureLink®PCR Purification Kit to extract the DNA fragment from the plasmid. This kit removes unwanted primers, enzymes, and salts that are present in PCR products. The purified PCR product from the kit is then able to be used for automated fluorescent DNA sequencing. The concentration of the purified amplicon was determined using the Qubit®dsDNA HS Assay Kit. The assay used was highly selective for double-stranded DNA and the concentration was read using the Qubit® fluorometer. This quantified concentration was used to make serial dilutions and were the standards used to measure the concentration in the soil samples for the quantification of *sul*2.

2.3 q-PCR for Quantification

Quantification of the target resistance gene to the sulfonamide antibiotic was done using real time qPCR amplification. The probe and primers used are in **Table 2.1**. Standard curves were generated with known gene copy number of PCR amplicons for each targeted gene. The same standards and nuclease-free water were used for every experiment.

Each qPCR reaction was composed of iTaq™ Universal Probes Supermix, 200 x 20 µl rxns, 2 ml (2 x 1 ml) (biorad), DNA template, and 10 pmol per µL of forward, reverse primers

and probe in a final volume of 12.5 µL as seen in **Table B.1**. Triplicate reactions of the DNA template, negative template, and standards were included in all qPCR experiments as seen in **Appendix B: QPCR**. The cycling parameters applied in the LightCycler®96 Application software are included in **Table B.2**.

Table 2.1*: Primers and Probes for Sul2 and 16s.*

Name of Primes and Probes	**Sequence**	**Amplicon Size (bp)**
Sul 2 fw	GCCTATCTCAATGATATTCGC	191
Sul 2 rv	GACGAGTTTGGCAGATGA	
Sul 2 Probe	AGACGCTGCGTTCTATCCG	

The log copy number of genes per µL DNA template solution for the calibration curve and the copy of genes/gram of sediment were calculated using the previously published equation[15]. In this equation, when b is Avogadro's constant ($6.022x10^{23}$ mol), c is the concentration of the purified target DNA ($0.000322\ \frac{\mu g}{\mu L}$), L is the length of the DNA fragment containing the target gene (0.191kb), a is the weight of kb DNA per pmol ($0.66\frac{\mu g}{pmol}$). The results of **Equation 2.1** with the data filled in from the experiment results in the copy gene/ µL DNA which is used for the calibration curve of the samples. The copy number was calculated with the following equation below.

$$\frac{(copy\ gene)}{\mu L\ DNA} = \frac{b \cdot c}{L \cdot a \cdot 10^{12}} = \frac{(6.022x10^{23} mol)(0.000322\ \frac{\mu g}{\mu L})}{(0.19 kb)(0.66\ \frac{\mu g}{pmol})(10^{12})} = 9.18701 \rightarrow 10^{9.1870} = 1.53x10^{9} \quad \textbf{(2.1)}$$

2.4 Testing for the Presence of Tet and blaCMY-2 Using PCR

The target resistance genes *Tet* and *BlaCMY-2* were detected using PCR amplification[16]. This was followed by capillary electrophoresis detection. Each PCR reaction was composed of Platinum^{TM} Hot Start PCR Master Mix (2×), 200–300 ng DNA template and 10 pmol per μL of forward and reverse primer in a final volume of 12.5 μL. Reactions with positive and negative templates were included in all PCR sets of detection. All the time 0 days samples were tested from the different experimental conditions. The time 8 days and 56 days samples need to be tested to see if there is any detection of these genes. This is because the soil samples could have a low concentration at time 0 days and later have an increase that is detectable at 56 days. Details of primers are included in **Table 2.2**. The experimental volumes used are listed in **Table B.3**. The QIAxcel instrument was the automated capillary electrophoresis device used for the analysis of the samples. The reagents required are the gel cartridge, intensity calibration marker, DNA separation buffer, wash buffer, dilution buffer, mineral oil, alignment marker, and DNA size marker. The DNA size marker used ranged from 25-500 bp and the alignment marker ranged from 15 bp/600 bp. A volume of 10 mL of the QX wash buffer was added with 2 mL of the mineral oil into the first part of the reservoir. The other wells had 8 mL of the DNA wash buffer, 18 mL of the DNA separation buffer, and mineral oil on top of both to prevent evaporation. A total of 15 μL of the QX alignment marker and intensity calibration marker were added to the corresponding 12-tube strip along with one drop of mineral oil. A total of 10 μL of the DNA size marker and of each sample was used.

***Table 2.2**: Primers for Tet.*

Name of Primers	**Sequence**	**Amplicon Size (bp)**
tetA_fw_fp	CATTTCGCTTGCCGCATTT	96
tetA_rv_fp	ATGAGTGCCCGCCTTTC	
tetB_fw_fp	AACGGTGTGGGTGCTATTT	82
tetB_rv_fp	AAGACCAAGACCCGCTAATG	
tetC_fw_fp	CAACCCAGTCAGCTCCTTC	91
tetC_rv_fp	CTGTCCTACGAGTTGCATGATAA	
tetD_fw_fp	CTGGGCAGATGGTCAGATAAG	111
tetD_rv_fp	CAACATCCACAGCACATTGG	
tetE_fw_fp	AGGACACATTGGTTTGGTATGA	100
tetE_rv_fp	GTGCCTGTACCGAAAGTTGA	
tetM_fw_fp	CACGCCAGGACATATGGATT	105
tetM_rv_fp	GTTTGTGCTTGTACGCCATC	
tetO_fw_fp	CTATCCAGACAGCAGTGACATC	130
tetO_rv_fp	GAACGGTATACTTCCGCCAA	
tetX_fw_fp	GAAAGAGACAACGACCGAGAG	130
tetX_rv_fp	CACCCATTGGTAAGGCTAAGT	
tetW_fw_fp	AGCGGAGCCATTTCAGAAC	128
tetW_rv_fp	TGGGATTACCATTCAAGCGGCAGT	
blaCMY-2CMY_2fw	ACTCCAGCATTGGTCTGTTT	122
blaCMY-2_CMY_2rv	CGGAACCGTAATCCAGGTATG	

3. RESULTS

The DNA concentration (copy gene/ µL DNA) found for the samples are shown in **Figure 3.1**. The detection limit of each gene was determined by the standard that was most diluted and a C_t value was still defined. For some samples although fluorescence occurred, meaning that the sample has a concentration of ARG, it was too low to quantify and were defined as being negative since they were below the limit of detection (LOD). In **Figure 3.1**, the orange graph bars (right side) correspond to the samples with the antibiotic TC added to the soil. The blue bar graphs (left side) were those with SMZ added to the soil, and the grey (bottom) are those samples with no antibiotics added. Resistance to sulfonamides (*sul*2) was detected in most of the samples except for the sterile conditions at times 4 and 8 days for the soils treated with Sulfamethazine and times 0, 4, and 8 days for those treated with Tetracycline. The error bars in the bar graphs were calculated by the difference between the average of the triplicates minus the lowest concentration for the negative error and the difference between the maximum concentration and the average of the triplicates for the positive error.

Furthermore, as seen in **Table 3.1, Table 3.2, and Table 3.3** the presence of Tet A, B, D, E, M, O and blaCMY-2 were not detected in most of the samples except for two. Tet A was detected in the natural sample treated with a high concentration of sulfamethazine at time 0 days and blaCMY-2 was detected in the natural sample treated with a high concentration of tetracycline at time 0 days.

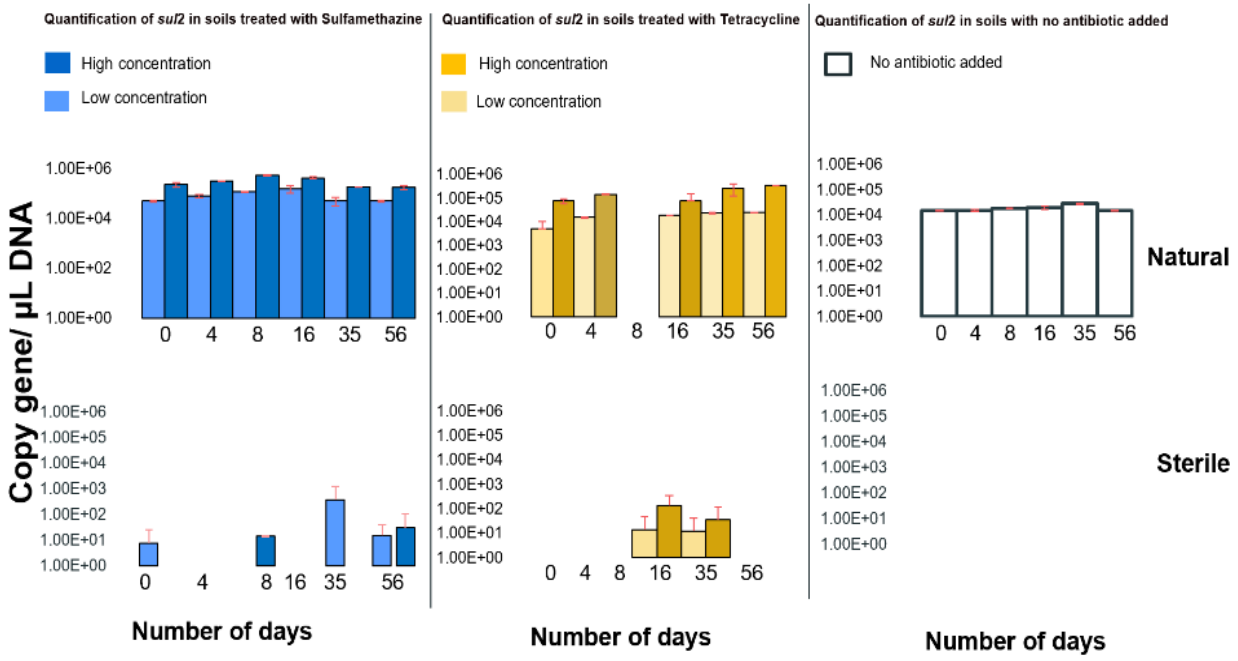

***Figure 3.1**: Results of microcosms using Howdy Farm soil.*

***Table 3.1**: PCR detection of Tet and blaCMY-2 genes in soil samples treated with Tetracycline at 0 days.*

Sample Name	Tet A	Tet B	Tet D	Tet E	Tet M	Tet O	blaCMY-2
Natural – Low Concentration	0	0	0	0	0	0	0
Natural – High Concentration	0	0	0	0	0	0	1
Sterile – Low Concentration	0	0	0	0	0	0	0
Sterile – High Concentration	0	0	0	0	0	0	0

***Table 3.2**: PCR detection of Tet and blaCMY-2 genes in soil samples treated with Sulfamethazine at 0 days.*

Sample Name	**Tet A**	**Tet B**	**Tet D**	**Tet E**	**Tet M**	**Tet O**	**blaCMY-2**
Natural – Low Concentration	0	0	0	0	0	0	0
Natural – High Concentration	1	0	0	0	0	0	0
Sterile – Low Concentration	0	0	0	0	0	0	0
Sterile – High Concentration	0	0	0	0	0	0	0

***Table 3.3**: PCR detection of Tet and blaCMY-2 in soil samples treated with no antibiotics at 0 days.*

Sample Name	**Tet A**	**Tet B**	**Tet D**	**Tet E**	**Tet M**	**Tet O**	**blaCMY-2**
Natural	0	0	0	0	0	0	0
Sterile	0	0	0	0	0	0	0

4. CONCLUSION

4.1 Sulfonamide Resistance

In conclusion, although we hypothesized that with a defined concentration of antibiotics introduced to the soil there would be an increase in the resistance gene observed throughout a period, we were not able to conclude an increase from our results rather only saw a slight shift within the samples. From the experiments conducted we were able to see a difference in the quantification of *sul*2 observed in the different experimental conditions. There was a significant decrease in the samples that were sterilized with no quantifiable data in those treated with no antibiotic added. The trend observed in the experiments with a natural experimental condition were similar in that there was a quantifiable presence of *sul*2 throughout the samples treated with antibiotics and no antibiotic added. The was no significant difference in the samples treated with sulfamethazine and those treated with tetracycline. Though the findings are preliminary, results show that the introduction of antibiotics in soils may influence antibiotic-resistance development. However, we must conduct more tests under differing conditions. If a positive correlation is observed in further studies, results would be especially concerning for agricultural workers who work with soils. There are some precautions that can be taken to further spread antibiotic resistance for instance slowing the development of resistance through improved use of antibiotics in both animals and humans. Resistance is not only found in soils but also in the air, contaminated water, animals, humans, and other contaminated surfaces.

Furthermore, the detection of resistance to tetracycline and beta-lactam antibiotics will show that with the long-term use of an antibiotic bacteria will develop resistance to not only that antibiotic, but to other antibiotics not associated with it. For detection, the capillary

electrophoresis process was faster and simpler than doing gel electrophoresis. Both techniques separate the DNA fragments according to the molecular weight; however, we were able to process up to 96 samples per run opposed to the 7 samples in the gel. The antibiotic resistance gene conferring resistance to beta-lactam, *bla*, was selected based on it being a clinically relevant gene having human risk, it confers resistance to frequently used antibiotics such as penicillin and aminoglycosides, and it has been previously reported in mobile genetic elements[16]. In some studies, with antibiotic resistant genes the samples analyzed had a relative abundance of *Tet* O and *Tet* W genes. These studies implied that with contamination there will be a detection of these genes in the samples since they have an ability to spread among and across population.

4.2 Future Directions

Future directions for the progress of this experiment are to test the samples against more *Tet* genes and quantify those that are detected. Although the time 0 days have been tested using PCR, the samples of the other times should also be tested. This will help stay consistent with the detection of the genes in all the soil samples. Another limitation of this experiment is the number of antibiotic resistant genes being used. In the continuation of this experiment more ARG's such as *Tet* C, X, and W will be tested against the soil samples. After the antibiotic resistant genes present in the samples are established and quantified using PCR and qPCR, then the samples could be tested in a digital droplet PCR. This way we can compare the quantification of the antibiotic-resistance gene *sul*2 in the soil samples using two different technologies. For improvements of this experiment getting the cross contamination to a minimum would help in determining a more accurate limit of detection. In all the qPCR experiments there was usually some fluorescence in the negative template control which could be due to environmental contamination. There can be a reduction in the amount of contamination if a PCR

decontamination kit is used before the quantification. There should also be a normalization of samples to account for changes in the growth of bacteria in the microcosms by testing and quantifying for 16s rRNA and comparing it to the concertation of the *sul*2 gene. This will help us determine that the increase of ARG's is not due to an overall increase in the bacterial population. Since there were two samples in which *Tet* A and *bla* were present, then future directions would be to quantify *Tet* A and *bla* for all the samples using qPCR and the same parameters as *sul*2. By quantifying *Tet and bla* genes in the soil samples treated with sulfamethazine and tetracycline, we will be able to confirm again that the establishment of long-term use of an antibiotic causes bacterium to develop resistance to not only that antibiotic, but to other antibiotics not associated with it. Another improvement to this experiment would be to increase the number of samples being quantified. This will help with variability and will give us a more accurate representation of the concentration of antibiotic-resistance genes present. In the replication of this experiment there should be an increase in the number of microcosms with a couple of them being a replication of the environment and experimental conditions. In the extraction phase there would be an increase in the number of samples since there will be two different sets one from the first microcosm and the second set from the replicated microcosm. All these samples should be quantified and analyzed using the same procedures and parameters. Along with the replication of microcosm a variety of sources of soils should be considered. With multiple locations and multiple samples, the results of the experiments will have a generalized appeal to those in different locations.

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APPENDIX A: CELL CULTURING

Step One: Preparing LB Agar, LB Broth, and Plates

Materials

- Glass ware
- LB Agar
- LB Broth Base
- Plates
- Antibiotic (ampicillin)

Procedure

1. Dissolve 6.4g of the LB Agar in 200 mL of water in a sterile vial. The dilution factor used was 32g/1L.
2. Dissolve 2g of the LB Broth Base in 100 mL of water in a sterile vial. The dilution factor used was 20g/1L.
3. Autoclave the mediums and store in room temperature.
4. When plates are ready to be poured, then warm the solidified LB Agar and place in water bath at 55℃ until it is ready to be used. It will solidify if it is at a lower temperature.
5. Label the bottom of plates and pour 25 mL of the LB Agar and 25 µL of the ampicillin 100 mg/mL. The dilution factor used is 1/1000.
6. Allow the plates to set and incubate inverted at 4℃.

Step Two: Bacterial Culture Inoculation and Incubation

Materials

- Agar plates
- ARG strains
- Inoculator
- Antibiotic (ampicillin 100 mg/mL)
- LB Broth Base
- Conical tubes

Procedure

1. Streak agar plates with appropriate ARG strain using the inoculator and incubate inverted at 37℃.
2. After 24 hours growth should appear on the plate and single colonies should be present. If no growth is observed, then incubate for longer period.
3. Add 100 µL of the antibiotic to the 100 mL of the prepared broth.
4. Pour 3 mL of the LB Broth base with ampicillin to each of the conical tubes (one per strain).
5. Using the inoculator select a single colony and transfer to the liquid.
6. Incubate in shaking incubator for 24 hours at 37℃.

APPENDIX B: QPCR

Materials

- 96-well plate
- Removable caps for 96-well plates
- Film
- Reaction components

Procedure

1. Begin by pipetting 9 µL of the master mix - water, master mix, assay, dsDNase, and DTT into each experimental well. (The dsDNase and DTT are part of the decontamination kit used only when testing 16s).
2. Cover the 96-well plate with removable caps and proceed with the decontamination process with the conditions shown below.
3. Once the cycles are complete pipette 1µL of the DNA into appropriate well and cover with film. We found that using the caps and then the film was more efficient, since the caps would get worn out if used too many times and the film was difficult to take of once it was sealed.
4. For each reaction include triplicates of the standards as well as each sample. This is important to determine if experiment can be replicated in the same environment and obtain similar results.
5. Each run should consist of at least four different standards (enough to measure the highest and lowest concertation of the samples), a negative sample without DNA, and the DNA extracted from the soil samples.

***Table B.1**: Volume for the components in a qPCR reaction for a volume of 10 µL.*

Component	**Volume (10µL/well)**
Nuclease-Free Water	3
Master Mix	5
Probe	1
DNA Template	1

Note: These volumes were used only in reactions for the quantification of sul2. The mix volume is 9 µL for a subsequent sample input of 1µL/reaction. These components are needed for the DNA amplification to take place during the 3-step-amplification.

***Table B.2**: Profile for LightCycler®96 Application software used for soil samples.*

Programs	Steps			
Name	Number of Cycles	Duration(s)	Target (°C)	Acquisition Mode
Preincubation	1	120	50	None
Preincubation	1	120	95	None
3-step Amplification	40	15	95	None
		15	60	Single
		60	72	None
Melting	1	15	95	None
		60	60	None
		15	95	Continuous 7 Readings/°C

Note: During the denaturing cycle the temperature needs to increase to separate the DNA into two single strands. Then, the temperature decreases during the annealing cycle for the primers to attach and finally for the extension cycle the Taq polymerase enzyme makes new DNA strands due to the increase in temperature.

***Table B.3**: Volume for the components in a PCR reaction for a volume of 12.5 µL.*

Component	**Volume (12.5µL/reaction)**
Nuclease-Free Water	4.75
Master Mix	6.25
Forward Primer	0.25
Reverse Primer	0.25
DNA Template	1

Note: These volumes were used only in reactions for the detection of ARG. The mix volume is 11.5 µL for a subsequent sample input of 1µL/reaction.