Drug Recycling: Quantifying the positive impacts of the Wyoming Medication Donation Program on bacterial antibiotic resistance in wastewater by county

Emily Armitage, Katie Moncrief, Natasha Radosevich

Project Summary

Bacterial antibiotic resistance is a rising problem in medicine, leaving many infections untreatable. The Centers for Disease Control estimates that 23,000 deaths have resulted from this growing issue (Centers 2017). Disposing of antibiotics incorrectly, such as through water systems, only amplifies the problem. A 2015 study in the Berglund laboratories detailed that it is possible that many antibiotic resistance genes originate and are spread through wastewater due to the commingling of environmental bacteria, pathogenic bacteria and antibiotic waste. This is a widespread problem as the associated press estimates that several tons of antibiotics are flushed down the drain annually (Donn 2008).

In response to improper drug disposal, Wyoming implemented the Wyoming Medication Donation Program (WMDP). This program was established to recycle unused prescription drugs and redistribute them to low-income people at little to no cost. While it seems obvious that this program would reduce the level of antibiotic resistant bacteria in Wyoming's wastewater, we can find no data to support this positive impact. Preliminary baselines made available to us indicate that some Wyoming counties have redistributed a notable amount of antimicrobials, keeping them out of the wastewater, and some counties have not recycled any drugs. At the University of Wyoming, we propose to assay for antibiotic resistance in bacteria isolated from the wastewater of two counties that participate in the program and two that do not in order to elucidate the impacts of the WMDP on the extent of antibiotic resistance in Wyoming's wastewater. We hypothesize that counties in Wyoming that donate fewer pounds of antibiotics will have a higher rate of antibiotic resistant bacteria in wastewater quantified genotypically by PCR and phenotypically by the Kirby-Bauer assay. If this research is successful, the Wyoming Medication Donation Program could use this information to further expand their services across the state.

Statement of Problem Significance

Medical waste has the potential to cause environmental and health problems around the world. A critical consequence of pharmaceutical pollution is that it contributes to the already growing concern of antibiotic resistance. Bacteria that do not respond to antibiotic treatment can be life threatening. The Centers for Disease Control estimates that antibiotic resistant bacteria have caused more than two million illnesses (Centers 2017). The Wyoming Medication Donation Program serves the state by promoting the incineration and recycling of drugs, including antibiotics. Twenty counties in Wyoming participate in the WMDP in which unused medication is donated, processed, and either safely destroyed or distributed to disadvantaged people who could not afford these life-saving drugs otherwise. The program supplied 433,950 units of drugs in 2016 to facilities across the state (Gallizzi 2016). This program not only allows individuals in need to affordable healthcare, but it benefits the health of the greater community and the environment by reducing water pollution. If fewer antibiotics are being flushed down the toilet and instead are disposed of in an environmentally conscious way we will likely see less instances of antibiotic resistance among bacteria in the wastewater. However, no data have been collected to substantiate these claims.

We propose our project in order to explore if this program effectively is improving the health of Wyoming's environment. We will assay for the presence of antimicrobial resistant bacteria in counties that differentially utilize the WMDP. With data to back up the claims that Wyomingites are benefitting from less medication pollution in the wastewater, these programs may acquire resources allowing them to grow and help more people in the state. Contrarily, if we find data supporting hypotheses that the program is not effective, helpful evidence will exist to suggest that certain parts of the program be amended in order for it to become more successful.

Introduction:

Relevant Literature:

Overuse of antibiotics has led to a significant rise in antibiotic resistance, and it has been found that human waste contributes to this issue. Irrigation ditches, stream segments, overland flow paths

downstream of wastewater treatment plants have been shown to be important in the dissemination of antibiotic resistance (Pruden et al. 2012). Antibiotic resistance has also been observed downstream of landfills (Graham et al. 2011). Although the results suggest that human activity is a primary cause in this resistance, the exact parameters are not well understood, and the variables are not always well defined, especially in Wyoming, where to our knowledge there is no previous research of antibiotic resistance.

In order to test the presence of antibiotic resistance, several procedural steps can be taken. First, the wastewater can be plated onto an agar plate containing a specific antibiotic. Growth on the plate suggests a resistant colony (Paavilainen 2000). In order to assess the degree of resistance in a bacterial colony, a Kirby Bauer assay can be done, which allows multiple antibiotics to be tested on a single bacteria sample and examined for resistance (Hudzicki 2016). The sampled water can also be tested for ARGs or antibiotic resistance genes (Graham et al. 2011). The presence of these genes indicates that the bacteria within the sample have some form of antibiotic resistance. However, there are hundreds of genes that contribute to different types of resistance. One of these genes that contributes to antibiotic resistance is *ermB* (Lee et al. 2017). The presence of *ermB* in a sample indicates the presence of bacteria that are resistance to macrolides, a family of antibiotics that includes azithromycin, the most commonly prescribed antibiotic (Walker 2015). As previously noted, these techniques can be combined to illustrate a "whole picture" of antibiotic resistance. This can be examined and compared between counties that donate or do not donate to the WMDP to give a better understanding of the effects this program has on resistance in wastewater around Wyoming.

Preliminary Data:

The WMDP's Natasha Gallizzi gave preliminary data for this experiment. The data include total pounds donated per county for the years 2015 and 2016, as well as number of antimicrobials dispensed compared to the number of total medications dispensed for January through June 2015, and January through December 2016. Below are tables presenting the data.

Name of County	Pounds donated in 2015	Pounds donated in 2016
Albany	89.6	283.8

Carbon	0	0
Laramie	4381.9	3463.1
Niobrara	0	0
Sheridan	683.8	395.4
Sublette	0	0

Table 1. The pounds donated per county in the years 2015 and 2016. Information for every county in Wyoming was supplied by the WMDP, included in the table above is only the counties relevant to this research.

Date	Units of Antimicrobials Distributed	Total Units Distributed
Jan-June 2015	6,416	328,372
Jan-Dec 2016	1,252	433,950

Table 2. The number of antimicrobial units distributed as well as total units distributed for the dates given. For 2015 the data was for medication sent to the Downtown Clinic in Laramie, Wyoming as well as the mailed prescriptions. For 2016 the data were only for mailed prescriptions.

Conceptual Model

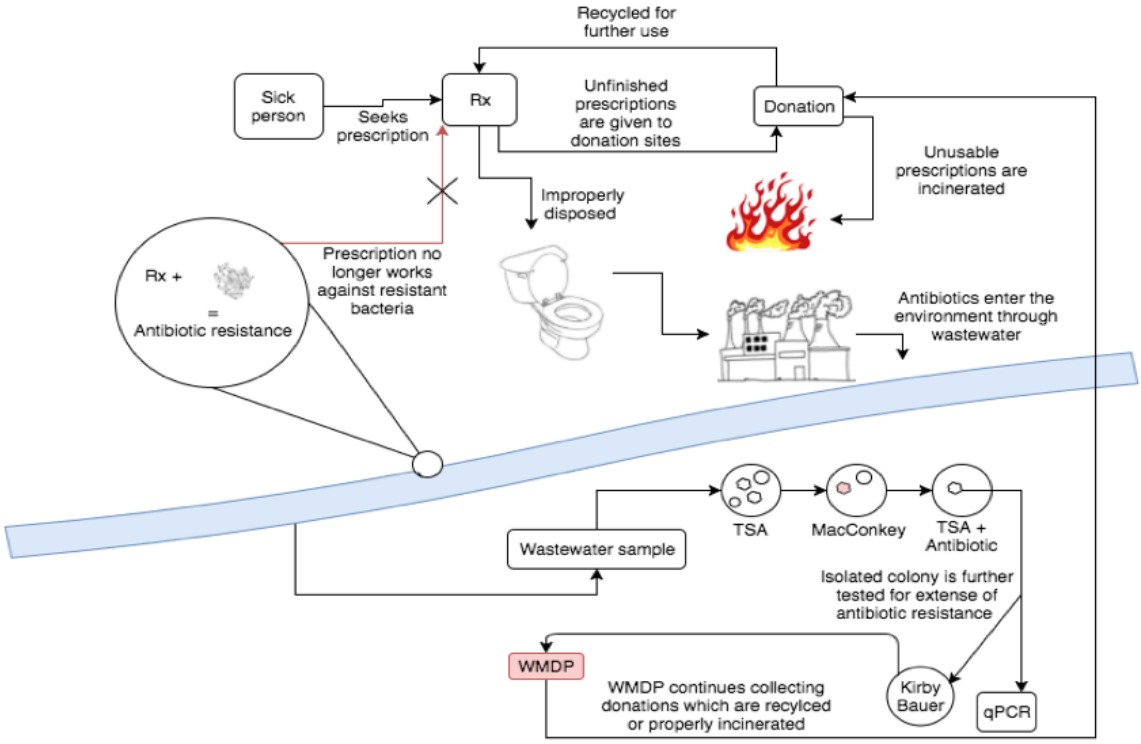

Figure 1: A general outline of the work to be done as well as the basics behind the antibiotic resistance that will be studied, as a result of improper disposal of antibiotic waste.

Justification of approach:

Several methods will be used in this experiment, including replica plating, a Kirby Bauer assay and qPCR. Sampling will be done at the influent sites at the treatment plants, and will be collected downstream from human activity, which has been shown to almost guarantee the presence of ARGs, or

antibiotic resistance (Berglund et al. 2014). All methods are established, and thoroughly cited in the literature.

For replica plating, a procedure similar to the method used by Paavilainen et al. (2000) will be used. Their research used agar plates with bacteria from skin samples that had been incubated until growth was observed. Plates with 100-1000 colonies were then replicated using a stamp covered in sterile velvet and plated on following plates (Paavilainen et al. 2000). These plates were then incubated and compared to the original plate for growth. For the Kirby Bauer assay, the procedure we will follow in this research is utilized and described by Hudzicki (2009). This procedure states that Mueller Hinton Agar is the best agar to use, due to its consistency in producing the reproducible results, it supports satisfactory growth for most bacteria, and there is significance literature to support susceptibility tests performed on this media (Hudzicki 2009). The procedure involved the sterile inoculation of a Mueller Hinton Agar plate with a single isolated bacterial colony, spread over the plate to produce a lawn of growth. Antibiotic disks were then placed on the agar, and after incubation the zones of inhibition were measured. Using a susceptibility table given in the research, the zones of inhibition were analyzed and the bacteria were determined to be resistant, intermediate or susceptible to each tested antibiotic (Hudzicki 2009). Our antibiotic choices for Kirby Bauer susceptibility testing are based on commonly prescribed antibiotics: Amoxicillin, Amoxicillin with clavulanic acid, Azithromycin, Cephalexin, and Ciprofloxacin (Walker 2015).

The use of qPCR was supported in several papers and is the standard procedure used for gene quantification on the DNA extracted from water samples (Berglund et al. 2015). We have selected to use qPCR primers specific for *ermB.* According to the Centers for Disease Control, Azithromycin (a macrolide) is the most commonly prescribed antibiotic which is why the *ermB* gene was selected.

Objectives:

1. To elucidate the positive effect of the Wyoming Medication Donation Program on antibiotic resistance in counties that use or do not use the recycling program.

2. To further our understanding of the implications of improper medication disposal on antibiotic resistance in wastewater.

3. To provide research findings and create informational materials that may be used to lead to more informed policy decisions about medication donation and disposal.

4. To lead to better awareness of the effectiveness of the WMDP.

Hypotheses:

1. Counties in Wyoming that do not donate antibiotics to the recycling program will have a higher rate of antibiotic resistant bacteria in wastewater.

2. Expression levels of *ermB* produced by qPCR from isolates sourced from counties that do not participate in the WMDP will be lower than expression levels of *ermB* sourced from counties that do participate in the program.

3. There will be a more extensive antibiotic resistance profile, as determined by Kirby Bauer susceptibility testing, for isolates in counties that do not participate in the Wyoming Medication Donation Program compared to counties in Wyoming that do participate.

Specific Aims:

1. We will culture influent water samples from treatment plants within Laramie, Albany, Carbon, Sheridan and Niobrara counties

2. We will utilize a replica plating technique on selective media that will distinguish ABR isolates from non-resistant isolates the water samples.

3. We will perform Kirby-Bauer susceptibility tests with the five most commonly prescribed antibiotics: Amoxicillin, Amoxicillin with Clavulanic acid, Azithromycin, Cephalexin, and Ciprofloxacin on resistant isolates.

4. Running qPCR on resistant isolates to test for the presence of *ermB*, a macrolide resistance gene.

Research Plan:

Overview

To test our hypothesis, we will collect wastewater samples of influent and effluent water from Carbon, Niobrara, Sublette, Sheridan, Laramie, Albany and Campbell counties. Laramie County,

Sheridan County, Campbell County and Albany County have had significant participation in the medication donation program while Carbon County, Sublette County, and Niobrara County have had no participation in the medication donation program. With the wastewater samples, a serial dilution will be performed to create a culture that can provide a countable number of colonies when plated. From the dilutions, we will first isolate colonies on TSA plates. Then, a replica plating technique will be used to inoculate MacConkey/MUG Agar, another TSA plate, and a separate TSA plate containing ampicillin. MacConkey/MUG agar will be used to isolate coliforms (suspected *Escherichia coli*), and the TSA with antibiotics will be used to identify resistant isolates. Once resistant coliforms are found, we will perform Kirby Bauer tests on these colonies to determine their susceptibility to five different antibiotics: Amoxicillin, Amoxicillin with Clavulanic Acid, Azithromycin, Cephalexin, and Ciprofloxacin. Alongside Kirby Bauer testing, qPCR will be performed to directly quantify the presence of *ermB* a macrolide antibiotic resistance gene.

	Hypothesis 1	Hypothesis 2	Hypothesis 3
qPCR	XXX		XXX
Kirby Bauer	XXX	XXX	
Replica plating	XXX		

Table 3: The overarching procedures used for this experiment and how they will tie in to the general overview of our research, related through the hypotheses.

Timeline:
The timeline for our project is outlined in the table below.

Projected Timeline	WEEK 0	WEEK 1	WEEK 2	WEEK 3	WEEK 4	WEEK 5	WEEK 6
Prepare media	XXX	XXX					
Growth		XXX	XXX				
Isolate colonies (replica plating)			XXX	XXX			
Kirby Bauer				XXX			
RNA prep				XXX			
qPCR				XXX	XXX		
Data					XXX	XXX	

Analysis							
Poster Creation						XXX	XXX

Table 4: The above timeline outlines the basic procedures over a 6 week time period. Week zero will be used to prep media as well as order primers for the qPCR, which will be ran in weeks three and four. In week 1 bacterial growth will begin on TSA plates, which will continue into week two. At this time point, the previous growth will be replica plated onto MacConkey and ampicillin agar to isolate fecal coliforms that are antibiotic resistant. Week three includes a Kirby Bauer assay of the isolated colonies from week two. Also during this week, preparation will be made for the qPCR, which will be run on weeks three and four. This prep includes scheduling the qPCR machine, as well as all RNA prep. Finally, starting in week four and ending in week six, the collected data will be analyzed and a poster will be created to present the experiment as a whole.

Methods

Twenty four hour composite samples of influent and effluent wastewater samples will be obtained by mail from six Wyoming counties: Niobrara, Sheridan, Campbell, Carbon, Laramie, and Albany. These water samples will be serially diluted so that the final count of colonies on the plate will be between 30 and 300. The remainder of the samples will be stored at -80ºC. The diluted samples will be spread on agar within 72 hours and incubated on TSA, Replica plating will be used on TSA, MacConkey/MUG, a control TSA plate and TSA plus ampicillin. This replica plating technique is outlined in Figure 2.

Once isolates are obtained, isolates will be frozen at -80ºC in 40% glycerol. Then a standard Kirby Bauer test will be completed (Hudzicki 2009). Isolates will be plated on Mueller Hinton agar and will be tested for resistance against 5 different antibiotics: Amoxicillin, Amoxicillin with Clavulanic Acid, Azithromycin, Cephalexin, and Ciprofloxacin. Zones of inhibition will be measured and recorded for each isolate and antibiotic. These zones of inhibition will then be compared to a standard table of sensitivity (Hardy Diagnostics 2015).

We will also be using the LightCycler® for qPCR to quantify the number of *erm*B macrolide resistance genes in each water sample. This instrument is housed in Dr. Naomi Ward's lab and we will be following standard protocol for the extraction of RNA and the qPCR procedure. We will extract RNA from bacterial samples using the Trizol RT extraction system (Invitrogen, Carlsbad, CA) following the manufacturer's instruction. The concentration and purity of RNA will be determined by

NanoDrop1000® spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE, USA). Complementary DNA (cDNA) will be synthesized from each RNA sample using a Reverse Transcription Kit and stored at -20ºC for later use in qPCR. We will use a primer corresponding to an ARG of interest, *ermB,* which shows resistance to macrolides such as azithromycin(Berglund 2015) and a gene that we know should be stably expressed in all bacteria, 16sRNA, to act as a control. Real-time quantitative PCR (qRT-PCR) will be performed with LightCycler® 96 in LightCycler® 8-Tube Strips with reaction volumes of 20 µl according to the procedure outlined in the 2005 study done by Bustin et al.

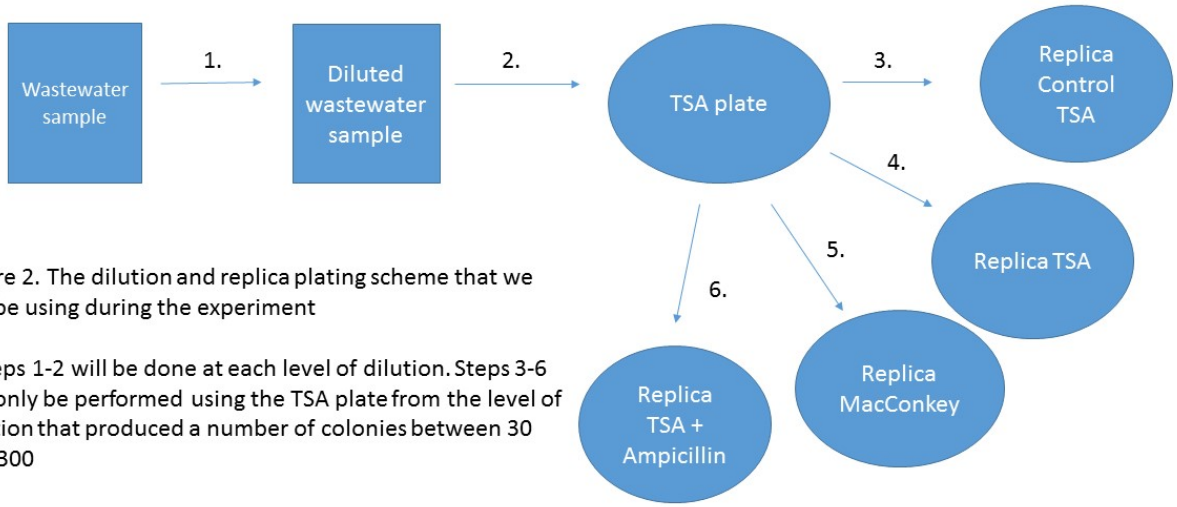

Figure 2. The dilution and replica plating scheme that we will be using during the experiment

* Steps 1-2 will be done at each level of dilution. Steps 3-6 will only be performed using the TSA plate from the level of dilution that produced a number of colonies between 30 and 300

*Any isolate from steps 3-6 that is both a coliform on the MacConkey Agar and is grown on a TSA plus ampicillin agar will be subjected to a Kirby-Bauer Susceptibility Test

Data Collection

For the replica plating procedure, data will be collected by counting the number of colonies that grow on the TSA plate, the MacConkey plate and the ampicillin plate. Growth of these colonies as well as an indication of lactose fermentation due to color change on the MacConkey plate will inform us that the isolated colony is a fecal coliform and is resistant to ampicillin. The Kirby Bauer assay will compare the diameters of zones of clearance for each antibiotic and each bacterial species. A sensitivity table will be used to determine how zone diameter relates to sensitivity (Hardy Diagnostics 2015). These tables

gives zones of inhibition measurements in millimeters that classify the measured zone for each bacteria as resistant, sensitive or intermediate to the antibiotic corresponding to the zone of inhibition.

We will collect our data for qPCR from the LightCycler, which will provide cycle quantification (Cq) values. Cq values will tell us the stability of the gene *ermB* in the sample, which we will compare for each of the colonies. The lower the Cq value, the higher *ermB* expression in our sample. Raw data throughout this experiment will include the number of countable colonies on the ampicillin plates, the data (Cq values) given by the LightCycler, as well as the measurements of the zones of inhibition in the Kirby Bauer Assay.

Day-to-day tasks, experiments, notations, and alterations in procedure will be stored in an official lab notebook, until the data can be analyzed and will be stored on the cloud (e.g. Google docs). All unexpected data and deviations from expected results will be written in the same lab manual and stored on the cloud until research can be done on how to properly analyze it.

The data collected in both procedures will be analyzed for statistical significance, through a Dunn's test and a one-way ANOVA test. An ANOVA one-way test will be used to see if there are significant differences between the collected means, i.e. means of differences in numbers of colonies on different plates and differences in ARG concentrations at different sites. The Dunn's test will then be used to detect which groups have means that are statistically different from one another. It assumes that the null hypothesis states that there are no differences between groups, meaning that there is no difference in number of resistant colonies at one county compared to the others.

Expected Results

Kirby Bauer assaying more sensitive isolates from utilize the WMDP. Cq values different between bacteria counties that utilize the

Counties **without** the WMDP:	Counties **without** the WMDP:
Low Cq values + More colonies on ampicillin plate + Significant resistance observed on the Kirby Bauer plate = High amount of antibiotic resistance	High Cq values + Less colonies on ampicillin plate + No significant resistance observed on the Kirby Bauer plate = Low amount of antibiotic resistance
What we expect	**What we DO NOT expect**

are expected to show the counties that of ARG will be isolated from WMDP than those

counties that do not. Specifically, Cq values in samples from Niobrara, Sublette, and Carbon counties will have lower Cq values than samples from Laramie, Sheridan and Campbell counties. We can interpret from this that the bacteria from counties that do not participate in the WMDP will exhibit more antibiotic resistance than the counties that do. There will be more copies of the RNA transcript of ARGs, therefore these bacterial strains are exhibiting more antibiotic resistance in counties that don't use the WMDP as compared to counties that participate. Unexpected results may include higher Cq values in Laramie, Sheridan and Campbell county samples or similar Cq values between treatment groups. We would conclude from this that antibiotic resistance is not more prevalent in counties that do not participate in the WMDP. If this is the case, we will look at other factors that may be influencing our results. These factors could include agricultural runoff from prophylactic antibiotic use or the ratio of prescriptions per person in each county. The unexpected data from our project could still increase the awareness among Wyoming healthcare providers about specific antibiotic resistance prevalence in their counties.

Support

This research will be funded by the University of Wyoming for the Fall 2017 Microbiology Capstone Course (MICR 4321) at a max budget of $1500 dollars. Our project supervisors are Rachel Watson, Dr. Gerard Andrews, and Dr. John Willford through the Microbiology Department.

References/Annotated References:

Berglund, B., Fick, J. and Lindgren, P.-E. Urban wastewater effluent increases antibiotic resistance gene concentrations in a receiving northern European river. Environ Toxicol Chem. 34(2015)192–196. doi:10.1002/etc.2784.

This article found through the UWYO Library database details the effect that antibiotics can have when combined with wastewater treatment. It is useful for qPCR techniques and information about antibiotics that could be tested. This source gave several ARGs that could be studied including *ermB*. This source is cited four times in this proposal, three times in justification of methods and once in materials and methods.

Centers for Disease Control and Prevention. Outpatient antibiotic prescriptions — United States, 2014. http://www.cdc.gov/getsmart/community/pdfs/annual-reportsummary_2014.pdf

This source tells us the national average of antibiotic prescriptions, helping us fill in gaps of preliminary data.

Centers for Disease Control and Prevention. Antibiotic/Antimicrobial Resistance. Web. (2017). https://www.cdc.gov/drugresistance/index.html

This government website provides information and facts about antibiotic resistance around the nation. It is referenced several times in the proposal.

"Conventional Activated Sludge Plant", CRS Water, (2017) http://watertreatment.net.au/services/sewage-\treatment/conventional-activated-sludge-plant/.

This source was accessed through the search engine Google. It explained how a conventional activated sludge treatment plant works, and was used to understand if there was a need to change sample collection procedures between water from this type of treatment plant and water from a natural lagoon system. This website is cited once in the material and methods section.

"Distribution of antibiotic resistance genes (ARGs) in anaerobic digestion and land application of swine wastewater" Environmental Pollution, 213 (2016), 751-759. https://ac-els-cdn-com.libproxy.uwyo.edu/S0269749116302196/1-s2.0-S0269749116302196-main.pdf?_tid=33f513f4-a0cc-11e7-84cf-00000aacb35e&acdnat=1506218636_4e8d8a73b1bd45bc03e27e2929ec9935.

This source was accessed through the University of Wyoming's Web of Knowledge Database. This source gave ARGs for tetracyclines, sulfonamide, and macrolide and although not all of these are studied in this research, this journal article gave several ARGs that we looked in to studying before our procedure was finalized. This paper is not cited in this proposal because it served as background knowledge.

"Dunn's test: Definition", Statistics How To, (2017) http://www.statisticshowto.com/dunns-test/.

This source was accessed through the search engine Google. It provided an overview of the Dunn's Test, as well as the formula to preform the test on our own data when we have reached the point where our data can be analyzed for statistical significance.

Gallizzi, Natasha, Wyoming Medication Donation Program, "Pounds of Donations Listed by County Years 2015 & 2016", (2015 & 2016).

Gallizzi, Natasha, Wyoming Medication Donation Program, "Total unit dispensed", (2015 &2016).Natasha Gallizzi of the WMDP gave both of the above sources through email exchange.
She provided a table that gave pounds donated in 2015 and 2016 per county as well as total units distributed compared to antimicrobial units distributed. These sources were essential in setting up our experimental procedure, as they allowed us to designate three counties who had not donated in 2015 and 2016 as the controls for the experiment and three counties that had as our experimental group. The information in these sources in presented in tables 1 and 2 in the preliminary data section.

Graham, David W., Olivares-Rieumont, Susana, Knapp, Charles W., Lima, Lazaro. Werner, David and Bowen, Emma, "Antibiotic Resistance Gene Abundances Associated with Waste Discharges to the Almendares River near Havana, Cuba", Environmental Science & Technology, 45, 2 (2011) 418-424, http://pubs.acs.org/doi/pdf/10.1021/es102473z.
This source was accessed through the University of Wyoming's Web of Knowledge Database. This journal gave good background information on antibiotic resistance in wastewater, and was very helpful in the relevant literature section of this proposal where it was cited twice.

Hardy Diagnostics. Hardydisk Antimicrobial Sensitivity Test (AST). 2015
https://catalog.hardydiagnostics.com/cp_prod/Content/hugo/HardyDiskASTProceduresandChart.pdf
This source was accessed via Google, and shows us the table of diameters that show the spectrum of resistance to our target antibiotics.

Hudzicki, Jan. "Kirby-Bauer Diffusion Susceptibilty Test Protocol", American Society for Microbiology, (2016) 1-23.http://www.asmscience.org/docserver/fulltext/education/protocol/protocol.3189.pdf?expires=1506224678&id=id&accname=guest&checksum=81F27B2658189A0ECEE340829AA2BA6D.
This source was accessed through the search engine Google, and gave Kirby Bauer procedure used in this research. It also provided the sensitivity tables that will be used to examine zones of inhibition, along with the tables given by Watson (2015). This source was cited once in the relevant literature section, three times in the

justification of methods, once in materials and methods, and once in data collection and analysis, for a total of 6 citations.

J. Hrenovic, I. Goic-Barisic, S. Kazazic, A. Kovacic, M. Ganjto, M. Tonkic. Carbapenem-resistant isolates of *Acinetobacter baumannii* in a municipal wastewater treatment plant, Croatia, 2014. Euro Surveill. 2016;21(15):pii=30195. DOI: http://dx.doi.org/10.2807/1560-7917.ES.2016.21.15.30195

This source had method sections similar in places to what we aim to do.

Jeff Donn, et al., Health Facilities Flush Estimated 250M Pounds of Drugs a Year USATODAY (Sept. 14, 2008, 12:00 PM), http://www.usatoday.com/news/health/2008-09-14-drugs-flush- water _N.html

This source estimates how many pounds of drugs are flushed down the toilet annually.

Lee, Jangwoo. Shin, Seung G., Jang, Hyun M., Kim, Young B., Lee, Joonyeob, Kim, Young M, "Characterization of antibiotic resistance genes in representative organic solid wastes: Food waste-recycling wastewater, manure, and sewage sludge", Science of the Total Environment, 579 (2017), 1692-1698.https://ac-els-cdn-com.libproxy.uwyo.edu/S0048969716326535/1-s2.0-S0048969716326535-main.pdf?_tid=b9bc0c68-a0cc-11e7-b5f6-00000aab0f6b&acdnat=1506218861_67ac4fbab1d1b269a86156b60949d3ed .

This source was accessed through the University of Wyoming's Web of Knowledge Database. It gave the ARG for tetracyclines, macrolides, quinolones, sulfonamides, beta-lactams, ciprofloxacin, azithromycin. Although this research does not evaluate all of these ARGs, this gave background knowledge on procedure, and it gave the ARG *ermB*, which is studied in this research. This paper is cited once in this proposal in the relevant literature section.

Manaia, Ceclia M., "Antibiotic Resistance In Wastewater: Origins, Fate and Risks", Paavilainen, T., Österblad, M., Leistevui, T., Huovinen P., Kotilainen, P., "Screening for Antimicrobial Resistnace in Normal Bacterial Flora of the Skin Using Replica Plating Method", European Journal of Clinical Microbiology & Infectious Diseases, 19(2000), 956-959.https://link-springer-com.libproxy.uwyo.edu/content/pdf/10.1007%2Fs100960000406.pdf.

This source was accessed through the University of Wyoming's Web of Knowledge Database. Although published some time ago, it gave very helpful information in designing the replica plating part of our procedure. It is cited 3 times in this proposal, once in relevant literature and twice in justification of methods.

Norman Hembach, Ferdinand Schmid, Johannes Alexander, Christian Hiller, EikeT.Rogall, Thomas Schwartz.

Occurrence of the mcr-1 Colistin Resistance Gene and other Clinically Relevant Antibiotic Resistance Genes in Microbial Populations at Different Municipal Wastewater Treatment Plants in Germany. Frontiers in Microbiology.Volume 8, Article 1282. 11 July 2017 doi: 10.3389/fmicb.2017.01282

This source is very recent and shows us what other researchers have done to locate resistance genes.

"One-way ANOVA in SPSS Statistics", Laerd Statistics, 1-2 https://statistics.laerd.com/spss-tutorials/one-way-anova-using-spss-statistics.php.

This source was accessed through the search engine Google. It provided an overview of a One-way ANOVA test, which will allow us to determine if there is statistically significant differences between the means in our collected data, which will allow us to preform a Dunn's Test.

Paavilainen, T., Österblad, M., Leistevuo, T., Huovinen, P., Kotilainen, P. Screening for Antimicrobial Resistance in Normal Bacterial Flora of the Skin Using the Replica Plating Method. European Journal of Clinical Microbiology and Infectious Diseases. 19(2012)12: 956–959

This source is cited three times in the proposal. It details the use of replica plating specifically for finding ABR bacteria. It is relevant to our methods as we will be using replica plating to isolate resistant colonies.

"Pipeline", National Small Flows Clearinghouse, 8, 2(1997)http://www.nesc.wvu.edu/pdf/WW/publications/pipline/PL_SP97.pdf.

This source was a pdf accessed through the search engine Google. It explained how a lagoon system can provide water treatment, and was used to understand if there was a need to change sample collection procedures between water from this type of treatment and a conventional activated sludge treatment plant. This pdf is cited once in the material and methods section. Although the source is old, it still gave very relevant and current information for how several of the counties in this study treat their water.

Prävention und Gesundheitsförderung, 3(2014), 180-184 .https://link-springer-com.libproxy.uwyo.edu/content/pdf/10.1007%2Fs11553-014-0452-3.pdf.

This source was accessed through the University of Wyoming's Web of Knowledge Database. This journal helped establish which ARGs are commonly found in wastewater antibiotic resistance studies. It provided background information, but was not cited in this proposal.

Pruden, Amy, Arabi, Mazdak, Stortboom, Heather N., "Correlation Between Upstream Human Activities and Riverine Antibiotic Resistance Genes", Environmental Science & Technology, 46(2012) 11541-11549, http://pubs.acs.org/doi/pdf/10.1021/es302657r.

This source was accessed through the University of Wyoming's Web of Knowledge Database. This journal addressed a hole in the literature – that it is difficult to establish all the controls/it's hard to say exactly what is causing antibiotic resistance. This information was very helpful in the relevant literature section where it is cited twice.

S A Bustin, V Benes, T Nolan, and M W Pfaffl. Quantitative real-time RT-PCR – a perspective. J Mol Endocrinol 34 (3) 597-601, 2005. doi: 10.1677/jme.1.01755

This source is cited in the methods section. It details the protocol for qrt-PCR. It was accessed through the University of Wyoming's Web of Knowledge Database.

Sanders ER. Aseptic Laboratory Techniques: Plating Methods. Journal of Visualized Experiments :JoVE.;(63):3064. doi:10.3791/3064. 2012

This is the source for the replicate plating method protocol. It was found on the University of Wyoming databases. It was used as background information.

Sven Jechalke, Simone Dealtry, Kornelia Smalla, Holger Heuer. Quantification of IncP-1 Plasmid. Prevalence in Environmental Samples. Applied and Environmental Microbiology. p. 1410–1413 Volume 79 Number 4. February 2013.

This source detailed the use of qPCR, which is helpful to our work. It was accessed through the University of Wyoming's Web of Knowledge Database.

Walker, Tracey, "5 most over-prescribed antibiotics", Drug Topics: Voice of the Pharmacist, (2015).http://drugtopics.modernmedicine.com/drug-topics/news/5-most-over-prescribed-antibiotics?page=full.

This source was accessed through the search engine Google, and was an article written to explain the highest prescribed antibiotics. This source gave good background knowledge on which types of antibiotic resistance this research should look for, based on over-prescription. It is cited once in the relevant literature section.

Wyoming Medication Donation Program. *Pounds of Donations Listed by County Years 2015 &2016*. (2017).

This resource was produced by the Wyoming Medication Donation Program and provided by Natasha Gallizzi; it contains current information about the areas of Wyoming that are most active in the donation program. It is important that this information is assessed as water samples are collected from various areas of the state.

Wyoming Department of Health. Wyoming Medication Donation Program. Web. (2017). https://health.wyo.gov/healthcarefin/medicationdonation/.

As the official website of the donation program, this website contains valuable information about the program guidelines including what can be donated and where it can be donated. It also provides other resources concerning donation programs.