BACTERIOPHAGE ISOLATION FROM WASTEWATER FOR BACTERIA PATHOGENIC TO HUMANS

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TYLER COREY BIRK

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by

TYLER COREY BIRK

APPROVED:

Dr. Crosby W. Jones, Chair
Professor of Biology

Dr. Greg Krukonis
Assistant Professor of Biology

April 23, 2018
Date Successfully Defended and
Approved by Advisory Committee

APPROVED:

Dr. Shirley M. Eoff
Director, Honors Program

May 10, 2018

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ABSTRACT

Pathogenic bacteria have become increasingly resistant to antibiotics and alternative treatments for infections are being developed. One such treatment, phage therapy, involves using bacteriophages to destroy the bacteria; however, they must first be isolated. Raw sewage is a commonly evaluated source due to the incredibly high quantity and diversity of bacteria found within it. This research aims to assess the quantity of phages against chosen pathogens in the sewage from the San Angelo Water Reclamation Facility. *Escherichia coli*, *Salmonella typhimurium, Serratia marcescens, Staphylococcus aureus, Staphylococcus epidermidis*, and *Streptococcus mutans* were evaluated because of the important roles they play in healthcare and the food industry. Quantities were assessed by forming lawns of bacteria and filtered sewage and counting the number of clearings, called plaques, that form. This method showed that phage could be isolated for four of the bacteria with two being found in similar quantities.

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INTRODUCTION

Antibacterial resistance is currently the greatest problem in infectious diseases that the world is facing per the World Health Organization's report in 2015 (1). This is largely due to the increase of "superbugs" or strains of bacteria that carry multiple antibiotic resistant genes. The most recognizable to the public is likely Methicillin-resistant *Staphylococcus aureus* (MRSA) due to its heavy media coverage. These strains are much more difficult to treat as the drugs historically used are no longer effective in eliminating them. Since these resistant strains continue to evolve at an increasing rate, the need for novel drugs to be developed by pharmaceutical companies has reached new levels (2). Unfortunately, despite the rising demand, pharmaceutical companies are hesitant to push large amounts of capital into the research and development of antibiotics as they are no longer as profitable. The heightened aversion to venturing too deeply into the antibiotic market stems from a multitude of factors including the surge of regulations, testing requirements, and the difficulty of creating a new and effective antibiotic, all of which have lengthened the development process and made the endeavor much more expensive (2). Justifying these costs is difficult for companies when their drug, if they succeeded in creating one that meets all standards and trials, inevitably falls victim to the same plight as the other antibiotics on the market and is rendered useless because the target strain develops a resistance to it. For these reasons and more, researchers have looked to different treatment methods to fight pathogenic bacteria.

One possible solution to this ever-growing problem could be found in bacteriophages which were originally discovered independently by Frederick Twort and Felix d'Herelle

(3,4). A bacteriophage is a virus that hijacks a bacterium's molecular machinery and forces it to serve as a factory producing mass quantities of the virus. As the phage finishes replicating, enzymes are released that break down the cell wall leading to lysis thus spilling the viral progeny into the environment. Not only does this kill an individual bacterium immediately, it also increases the likelihood of infecting other bacteria due to this increase in the number of phages in the environment. Unlike lytic viruses, temperate viruses can go through a lysogenic cycle in which the viral DNA will insert itself in the host's DNA and will be passed on to the daughter during cell division, until the conditions are ideal, at which point the viral DNA will excise itself and enter the lytic cycle. During either life cycle, the virus could potentially transfer DNA from one bacterial host to another in a process called transduction. Although it is not a novel technique, phage therapy could be a promising complementary or alternative strategy for combating antibiotic resistant pathogens. Phage therapy involves using lytic phages to infect and ultimately kill the infectious agent.

Experiments using phage therapy have been performed for nearly a century beginning with Felix d'Herelle who was a French-Canadian microbiologist. d'Herelle used the newly discovered phages to treat patients that were suffering from bacillary dysentery, or now commonly called shigellosis, which is known to be caused by a genus of bacteria called *Shigella* (4). Although d'Herelle and others created viable treatments, the life cycle of the phages was not fully understood. Early experiments were consequently abandoned by much of the world, except Russia, Poland, and the country Georgia. This was especially so when the first antibiotic was discovered and widely used during WWII (5). However, interest in the possibilities offered by phage therapy has resurged in recent years as concern for superbugs

increases. The increased worldwide interest has led to countries with a long history of using phage therapies, such as Georgia, to share their findings. This information, along with the advancements in virology as a whole, has stimulated researchers to re-evaluate the various advantages and disadvantages of using phage therapy over chemical antibiotics. In this regard, phage therapy has some major advantages.

The first advantage is that, unlike some antibiotics, a bacterium is unable to regain viability if it is successfully infected by an obligately lytic phage (6). This decreases the chance that the bacterium will incur or pass on any form of phage-resistant traits thus increasing the potential time that the phage can be used as treatment. Another advantage is that, if a phage with a limited specificity is chosen, the normal flora of the patient would not be disturbed (6). This is not true for many broad range antibiotics which can lead to a secondary infection in the GI tract by an antibiotic resistant bacterium like *Clostridium difficile*. A final advantage is its usefulness in penetrating the tough biofilm that is produced by some bacteria (7). A biofilm is a community of microbes that has embedded itself in a polysaccharide matrix bound to an inanimate surface. Once in a biofilm, the community is, as a whole, much more resistant to attacks by the immune system, phagocytosis, and antibiotics. Not only can a biofilm be tough to eradicate under normal circumstances but, if it forms on an inorganic implant in the body, it can go completely undetected by the immune system until it progresses too far to be treated by conventional means. In this situation, the implant is usually removed so that the biofilm does not slough off some of its layers and spread to other surfaces throughout the body. Phage therapy has the ability to break through the layers of the biofilm, in part due to the fast proliferation of phages and the close proximity of the bacteria

(7). However, it has been observed that this can lead to the development of phage resistance in the community at a higher rate. To combat this, it has been suggested that a combination of a phage cocktail, antibiotics, and antiseptics be used (8).

Despite these advantages, there are some concerns that must be considered before implementing phage therapy as a standard treatment in hospitals. Before a phage is used it is important to ensure that it is obligately lytic because if it can exhibit a lysogenic phase the chance of promoting the development of phage-resistance increases. The phages also must have a high virulence to ensure the death of the bacterium and a low rate of transduction to limit the dissemination of traits that could decrease the bacterium's sensitivity to phage therapy. Apart from the phage's behavior and virulence, its morphological characteristics should be observed, and its full genetic code should be sequenced to check for any red flags (6). While phages have demonstrated immense potential as a treatment for human infections, they may also possess widespread benefits for the environment and food industry.

The coral reefs around the world have been greatly reduced by destructive fishing, pollution, and rising greenhouses gases. This habitat is home to a large amount of biodiversity with many species being unique to the areas. Along with this reduction comes the weakening of the population thus opening them up for large-scale disease outbreaks. One such disease is caused by *Vibrio coralliilyticus* which typically infects hard coral but can also spread to soft coral, oyster larvae, and bivalve larvae (9). Treating this disease can be difficult because strains have already begun to show multiple antibiotic resistance with some strains showing resistance to commonly used drugs like ampicillin, amoxicillin, erythromycin, and sulphamethoxasole (10). When added at an early stage phage therapy has

been shown to help the coral defend against the pathogen and reduce the chance of spreading the disease to others (9). As this area of research continues, more uses for these phages in the environment could be discovered.

There are numerous ways in which phages can be used in the food industry for human, animal, and plant protection without weakening the antibiotics currently being used. In 2011 the global fruit and vegetable industry grew in value to a staggering $1.5 trillion despite the pressure caused by plant disease and adverse weather in some areas (11). Although the industry has seen growth, it is still hampered by the cost of the war against phytopathogens. When only considering the top eight US cash crops, the cost of treating pests and infections pre-harvest was estimated to be $300 billion (12). Historically the main treatments for these diseases have included chemical antibiotics, copper-based bactericides, and pesticides, but increased resistance and their environmental impact have led to countries banning some of the practices (11). Along with crop production, the spread of foodborne diseases through consumption is another strain on the US economy with these illnesses costing the US an estimated $152 billion dollars per year (13). Because these industries are so economically important, the search for alternative treatments for crop diseases and ways to kill bacteria on products has already led to multiple FDA and USDA approved phage preparations hitting the market for commercial use.

ListShield™ and PhageGuard Listex™ are two examples of phage preparations that target the pathogen *Listeria monocytogenes* which can be found in raw or packaged food products such as fresh catfish, ice cream, soft cheeses, and some fruit and vegetables (14,15). This is an important pathogen because *L. monocytogenes* infects about 1,600 people each

year with about 260 deaths (16). Listeriosis is usually caused by consuming contaminated food. The symptoms include: fever, diarrhea, confusion, and convulsions in most adults but can lead to miscarriage, or premature delivery in pregnant women (16). Listeriosis is typically treated with ampicillin or other B-lactams. However, if a patient is allergic to these, co-trimoxazole (a combination of trimethoprim and sulfamethoxazole) is the second-line therapy (17). Of the 202 strains of *Listeria monocytogenes* isolated and tested from food and the environment collected in France between 1996 to 2006, only 4 strains showed resistance. One of the strains was resistant to trimethoprim (17). If co-trimoxazole is used irresponsibly the gene that codes for its resistance could become more widespread. Using PhageGuard Listex™ on at-risk products could lengthen the lifespan of co-trimoxazole by limiting infections and thus lower the chance that incidental resistance will develop.

Bacteria pathogenic to plants and other crops could also be potentially treated with phage therapy. Phytopathogens can have a devastating impact on both local food stores and the global economy. However, due to the immense variation that is found in the bacterial strains that can infect one plant, it is difficult to isolate the appropriate phages necessary to effectively protect the plants (18). To fully treat the diseases a combination of phages is typically required. The other issues impeding the use of phages on a large scale for crop treatment is their low viability in the phyllosphere and their ability to access the bacteria. The phages have been shown to be susceptible to many environmental hazards including high temperature, pH changes, and rain (18). Furthermore, the most deleterious environmental hazard has been shown to be ultraviolet radiation originating from sunlight which can be disastrous for phage treatment. There have been multiple attempts to treat different strains of

Xanthomonas which can infect citrus, walnuts, tomatoes, and peach trees (11). From these experiments it has been found that suspending the phages in skim milk along with proper timing of their application can improve persistence (18).

While phage cocktails have been approved in the Western world for eradicating bacteria in food products, it has not yet been deemed safe enough for the treatment of diseases in humans. Understandably, this is not a simple process and it will take some time to conquer the hurdles that lie ahead before the FDA or other government entities approves it. To be approved, the FDA must be confident that the known and potential risks of a drug are outweighed by the benefits it provides to a patient (19). To make this decision the FDA takes a few things into consideration. First, they will use the currently available treatment options as a point of comparison for the potential new drug's risks. Next, the group that is proposing the use of the drug will need to submit at least two clinic trials that are well-designed and show that the treatment is effective and is not going to cause more harm than good to the patients. Finally, appropriate drug labels will need to be created that clearly outline how the drug will impact the consumer. Typically, the review process takes approximately 10 months after all pertinent information is provided (19).

While this may seem straightforward, these types of trials usually involve very stable chemical drug products and the regulations surrounding drug approval reflect this standard (20). This poses an issue for phage therapy because the phage cocktails that are used do undergo some changes to better adapt to the host bacteria that the treatment is targeting. It has been suggested that a special amendment for phage therapy should be made that also implements a strict monitoring system that will assess the development of resistance to

phages (20). Currently the only place that patients seeking phage therapy can go to receive treatment is the Eliava Phage Therapy Center in Tbilisi, Georgia (21). This facility advertises that they can successfully treat a variety of ailments such as skin, bone, eye, and urinary tract infections along with gastrointestinal diseases like irritable bowel disorder and bacterial gastroenteritis. Excluding travel costs, they state that, if the patient stays the recommended, 7-14 days, that the treatment will cost a total of €3900 or about 4,700 USD as of May 2018 (21). Despite already being regularly used in Georgia there have been very few published clinical studies that are well-conducted, randomized, double blind, and placebo controlled (20, 22). This issue is of the utmost importance because drug-approving bodies must be certain of a treatment's impact on the human body and without sufficient data on how phage cocktails work *in vivo* they will not be approved any time soon in the US (22).

In order to use bacteriophages, they must first be located. Many sources are used today including sewage, soil, and bacterial colonies. Raw sewage is a commonly evaluated source due to the incredibly high quantity and diversity of bacteria found within it that could be used to find a host for the bacteriophages (6). Cai et al. have shown that bacteria living in the sewage found at the beginning of wastewater treatment plants will reflect the human microbiome. This study also showed that the human gut microbiome was the greatest influence on the bacterial profile of this influent sewage. This study was unable to determine the bacteria to the species level. However, it showed that *Proteobacteria*, which includes *Escherichia* and *Salmonella*, made up 34% of the bacteria found (23). Therefore, using sewage as a source for the phages is a viable alternative to attempting to sample a significant portion of the San Angelo population as the bacteria in the wastewater represent those of the

entire population. It is important that sewage facilities around the world are examined because, much like soil, a virus found in one region could have key characteristics that make it useful for therapy that may not be found in a phage similar to one found elsewhere due to their high diversity (24). The San Angelo Water Reclamation Facility was chosen for this research because its potential as a source for bacteriophages had not been previously evaluated.

Escherichia coli, an intestinal colonizer, was used as the baseline for the project because data collected previously indicated phages could be reliably found in high numbers in this water source. The other bacteria selected for this study are the intestinal colonizers *Salmonella typhimurium* and *Serratia marcescens*, skin colonizers *Staphylococcus aureus* and *Staphylococcus epidermidis*, and an oral colonizer *Streptococcus mutans*. These bacteria were chosen because they each play an important role in healthcare and in agricultural systems around the world.

Escherichia coli is a gram-negative, facultative anaerobic rod and is a member of the Enterobacteriaceae family. Many *E. coli* strains are part of the normal flora of the gastrointestinal tract in humans and are usually nonpathogenic. However, some strains can cause infections accompanied by symptoms ranging from asymptomatic to life threatening with the site of infection often being strain specific. A well-documented and dangerous strain is the Shiga toxin producing *Escherichia coli* O157: H7 (25). This strain is a foodborne pathogen that causes dysentery, or bloody diarrhea, and is often associated with consuming undercooked beef, cheeses, yogurt, or raw unwashed vegetables. This strain is infamous for causing outbreaks on both small and large scales and there are, on average, 73,000 cases per

year in the United States (26). The treatment of these infections is typically handled by broad spectrum antibiotics that wreak havoc on the normal flora of the GI tract. For these reasons, *E. coli* is an important candidate for phage therapy.

Salmonella typhimurium is a gram-negative, facultative anaerobic rod that does not form capsules or spores. Per year, there are approximately one million cases of foodborne illnesses in the United States caused by *Salmonella* strains including *S. typhimurium*, *S. enteritidis*, and *S. typhi* (27). The most common symptoms of salmonellosis include diarrhea, fever, and abdominal cramps. The infection typically lasts about a week without treatment, but death can occur if the patient is elderly, young, immunocompromised or the diarrhea is severe enough (27). Infections usually occur when food, contaminated with the pathogen, is undercooked, and then ingested or handled raw. There are two prominent methods being tested to reduce *Salmonella* on foods such as poultry, eggs, pork, and produce. One method involves directly spraying phage preparations on the food item or fully submerging the item into the solution. This method has been shown to significantly reduce phage concentration of the three major *Salmonella* strains on multiple food matrices (28). The other major method being tested treats the animal instead of the product. The phage preparations were orally administered every two or three days to chickens directly infected with *S. typhimurium*. This experiment resulted in a significant reduction of salmonella in the chicken cecum if doses were given frequently (29). One issue they encountered was, after causing a reduction in bacteria, the number of bacteria began to rise once again. It was hypothesized that there were not enough phages circulating to encounter the remaining bacteria to completely eliminate the infection after already killing most of the population. In this case, it may be beneficial to

use a combination of phages and antibiotics to fully wipe out the bacteria. Both of these techniques will need many more trials to improve their effectiveness and reliability before they are brought to the market. However, if either of these methods is put to use, the number of infections and deaths caused by salmonellosis could be reduced.

Serratia marcescens is a gram negative, saprophytic rod, of the Enterobacteriaceae family. It is known for the bright red pigment, prodigiosin, it produces which has led to its use as a biological marker for studies examining the spread of bacteria. The infections are usually nosocomial (hospital acquired) and can cause urinary tract infections, endocarditis, septicemia and more depending on the site of infection (30, 31). *S. marcescens* is highly drug resistant with some strains being resistant to both extended-spectrum beta-lactamase and metallo-beta-lactamase drugs. These multi-drug resistant strains have been responsible for many hospital outbreaks when they contaminate soaps, handwashing stations, IV lines and more (31). Bacteriophages specific for this organism have been isolated from wastewater and soil and some of them, such as KSP90 and KSP100, have shown characteristics that would make them useful in phage therapy (32).

Staphylococcus aureus is a staphyloxanthin producing, gram-positive coccus that forms clusters. It is part of the normal flora of humans and is found in the anterior nares of about 30% of people which is only one of the many places it can be found (33). The pathogenic strains can be extremely dangerous, especially in a hospital setting where immunocompromised patients can easily be infected by consuming contaminated foods or through poor aseptic technique. The severity of the infection is dependent on the location of the infection, but it is known to cause sepsis, pneumonia, endocarditis, osteomyelitis, and

more (33). The danger posed by this bacterium today stems from the difficulty in its eradication due, in part, to its resistance to a wide range of antibiotics. This is further complicated because many people have developed allergies to the antibiotics. There are Methicillin-resistant *Staphylococcus aureus*, Vancomycin-resistant *Staphylococcus aureus,* and other resistant strains found in hospitals and contaminated foods (33). Dairy products are one of the food types that can carry these pathogenic bacteria and they are not always fully eliminated by the pasteurization process. To combat this, it has been suggested that phage cocktails be applied to the product after the pasteurization step because the treatment hampers the effectiveness of the phages (34).

Staphylococcus epidermidis is another gram-positive coccus that forms clusters and it is the most commonly isolated bacteria from human skin (35). *Staph. epidermidis* is an opportunistic pathogen that is primarily responsible for infections following the placement of an internal medical device. The bacteria are introduced to the medical device by either the patient's own skin or from the skin of one of the healthcare providers and can lead to infections of the bloodstream. Because *Staph. epidermidis* can form biofilms, it can be very difficult to treat such infections with standard chemical antibiotics (35). Phages that produce proteins that degrade the extracellular material of *Staph. epidermidis* biofilms have been discovered (36). These phages could yield promising results if combined with other antimicrobials. One study recommended coating medical implants or catheters with phage cocktails prior to their insertion (37).

Streptococcus mutans is a gram-positive, biofilm-forming coccus found in chains inside the mouth of humans. This bacterium is one of the primary bacteria in the onset of

dental caries which is one of the most common diseases in the world (34). These bacteria thrive in the mouth by breaking down sugars, such as sucrose, for use as a food source and as a building block for forming biofilms on the enamel of teeth. Once they form, these plaques produce high levels of acidic byproducts which wear down the enamel and produce cavities (38). Currently, these plaques can only be treated by scraping them off if it is early enough or drilling out the infected areas of the tooth and filling the hole if it has progressed too far. However, phages have been found in human saliva that, while they have a narrow host range, can reduce the growth and biofilm production of *Strep. mutans* (39).

All of the bacteria selected for this study have been important in the development and understanding of the mechanics of phage therapy. However, to compete against these devastating pathogens, the ideal phages need to be found which, in turn, means that their presence should be recorded in as many locations as possible. Studying phage presence is imperative in understanding their interactions with their hosts in their environment, observing any shifts in the viral population, and for identifying areas that could be considered as potential sources for novel phages. This research aims to assess the quantity of phages against the chosen pathogens in San Angelo's wastewater and whether related pathogens produce similar amounts. This will be answered by forming lawns with a mixture of bacteria and filtered sewage and counting the number of clearings, called plaques, that form after 24 hours of incubation. The plaques that form indicate the presence of a bacteriophage lineage that has developed and destroyed the bacteria in an area.

It was hypothesized that the common enteric bacteria, *E. coli* and *S. typhimurium*, would produce similar numbers of plaques because of their frequent interaction with humans

and in turn the wastewater system. However, *S. marcescens* is not as common an intestinal pathogen outside of hospital settings so it was predicted that it would produce very few if not the fewest plaques. Next, it was predicted that similar numbers of bacteriophages would be found for the two staphylococci, *Staph. aureus* and *Staph. epidermidis*, because they are both commonly found on or in humans. Finally, it was thought that *Strep. mutans* would be readily introduced into the wastewater system through oral care and would thus make wastewater a valid source for finding bacteriophages against it.

MATERIALS AND METHODS

Table number 1 displays each of the bacterial species used in this research along with their American Type Culture Collection (ATCC) type number and the site that they were originally isolated from.

TABLE 1. Strains used in the present study

Species	ATCC Number	Isolation
Escherichia coli	23848	N/A
Salmonella typhimurium	13311	Food poisoning; feces, human, 1911
Serratia marcescens	13880	Pond water
Staphylococcus aureus	13565	Ham involved in food poisoning
Staphylococcus epidermidis	49461	Clinical isolate
Streptococcus mutans	25175	Carious dentine

At the start of the project a pure culture of each of the six bacteria was made on Tryptic Soy Agar (TSA) (Acumedia Neogen Corp., Lansing, MI). Each of the bacteria came as a freeze-dried specimen and was revived using the guide produced by ATCC. The cultures were then re-streaked every two weeks to maintain the colonies and stored in the refrigerator after incubation.

Establishing a Baseline:

Using the *E. coli*, a trial run of the experiment was done to gather preliminary results as a point of comparison for further tests. To do this, a flame-sterilized loop was touched to the pure culture of *E. coli* and then mixed to suspend the bacteria in 5.0 mL of Tryptic Soy Broth (TSB) (Acumedia Neogen Corp., Lansing, MI). The culture was then allowed to incubate for 24 hours at 35°C. The opacity of this overnight culture was used as a reference point for the required opacity for the other bacteria. Frozen wastewater was thawed at room temperature. The wastewater was poured into a sterile Corning® 250 mL vacuum filtration/storage bottle system with a 0.22 µm cellular acetate membrane and filtered with a vacuum pump until only solid particulates remained in the upper chamber. Using a 10.0 mL pipette, 5 mL of the filtered sewage was dispensed into the tube of *E. coli* and left undisturbed for 15 minutes. Then, a 1.0 mL pipette was used to add various aliquots of the mixture (0.1 mL, 0.2, mL, 0.3 mL, 0.4 mL, 0.5 mL, 0.6 mL, 0.7 mL, 0.8 mL, 0.9 mL, 1.0 mL, 1.5 mL and 2.0 mL) to different vials of soft phage overlay agar (Nutrient broth 8g, NaCl 5g, Dextrose 1g, Bacteriological agar 8g, per 1000mL) that was kept liquefied at 45°C until it was ready to be used. The vial was then tapped three times to mix the contents and poured onto a plate of phage bottom agar (Nutrient broth 8g, NaCl 5g, Dextrose 1g, Bacteriological agar 15g, per 1000mL). The plate was then swiveled, until the entire plate was coated in liquid, and set aside until the top agar had solidified. These plates were incubated for 24 hours at 35°C. After incubation, the plates were evaluated and, because the higher volumes created too many plaques to count accurately, it was determined that, for future runs, 0.1 mL, 0.3 mL, 0.6 mL, and 0.9 mL would be used.

Bacteriophage Isolation and Enumeration:

Using the parameters determined in the previous section, phage isolation was done for each of the bacteria. Only one bacterium was tested at a time to ensure that each bacterium-phage mixture was given the same time for incubation. *Streptococcus mutans* did not produce consistent lawns on the phage bottom agar. Therefore, Brain Heart Infusion agar (BHI) (Acumedia Neogen Corp., Lansing, MI) was tested and produced much more reliable lawns when the bacterium was also grown in Brain Heart Infusion broth (Acumedia Neogen Corp., Lansing, MI). To quantify the plaques, each clearing was marked with a permanent marker and then counted and crossed out with a black pen. By counting in this manner, all the plaques were examined through both direct and indirect lighting before they were counted to ensure accuracy.

Bacteriophage Enrichment:

Because *Streptococcus mutans* and *Staphylococcus epidermidis* did not produce any plaques through the standard isolation method the wastewater was analyzed with a different method. The purpose of a phage enrichment experiment is to evaluate whether there are any trace levels of a bacteriophage in the source. This method, provided by the Center for Phage Technology at Texas A&M University, does not give insight into how prevalent the phages are, just if there are any at all that can infect a certain bacterium (40). A culture was made for both *Strep. mutans* and *Staph. epidermidis* and allowed to incubate overnight at 35°C. This was done by inoculating a 250 mL Erlenmeyer flask containing 100 mL of media with the selected bacterium. TSB was used for *Staph. epidermidis* and BHI broth was used for *Strep.*

mutans as these broths yielded high densities for their respective bacterium. After 24 hours of incubation, 10 mL of wastewater that had been thawed and filtered in the same manner as described earlier, was combined with 40 mL of sterile media and the 100 mL of overnight culture in a 250 mL culture flask. This mixture was once again incubated for 24 hours at 35°C to allow any bacteriophages against the bacteria in the solution enough time to infect and proliferate to an observable quantity.

The mixture was then completely filtered through a 0.22 um cellulose-acetate vacuum filter as described earlier. To 4.0 mL of TSB or BHI broth, inoculated and incubated with *Staph. epidermidis* and *Strep. mutans* respectively, 5.0 mL of the filtrate was added and left undisturbed at room temperature for 15 minutes. Then as before, aliquots were mixed with the soft phage overlay and poured onto the phage bottom agar. Aliquots of 0.1 mL, 0.3 mL, 0.6 mL, 0.9 mL, 1.5 mL, and 2.0 mL of mixture was used in this phase of the study. The *Strep. mutans* was poured onto plates of both phage bottom agar and BHI agar to account for the inconsistency of lawn formation on the phage bottom agar. Once the top agar gelled, the plates were placed in a 35°C incubator for 24 hours and afterward the plates were examined for the presence of plaques. This process was also repeated using fresh wastewater to ensure that freezing the water to preserve it did not damage the bacteriophages or reduce their viability.

Data Analysis:

Initially, the experiment was repeated ten times for each of the bacteria; however, there was poor lawn formation on some of the plates so true plaque amounts could not be

counted. This issue is reflected in the data as six runs were counted for *S. typhimurium* and nine for *E. coli*. The data were analyzed using the statistics software R. Because collected data were considered count data, they were put through a Poisson Regression analysis which compared the counts of each of the bacteria that produced plaques against those of the baseline *E. coli* to determine if they were statistically similar. However, because the data showed over dispersion a Quasi-Poisson Regression was used instead because it corrects for the issue. Next, the same process was done with *Staph. aureus* as the comparison point to test whether *S. marcescens* produced similar plaque counts to it at 0.9 mL. The results of these tests were used to draw conclusions as to the number of phages present in the wastewater in relation to one another.

RESULTS

From the experiment, it was found that phages against *Escherichia coli*, *Salmonella typhimurium*, and *Serratia marcescens* could be isolated at all four volumes of mixture. This was not the case for *Staphylococcus aureus* which was only isolated at 0.9 mL of mixture consistently and twice at 0.6 mL with one of them being the fresh sewage. Furthermore, the final two bacteria, *Staphylococcus epidermidis* and *Streptococcus mutans*, had no phage isolates even when higher aliquots were used. To further confirm the lack of bacteriophages against these species, the phage enrichment experiment outlined earlier was done on them. Although higher levels of mixture were used, and the culture was given a longer incubation time with a high concentration of sewage filtrate, no plaques were visible on the lawns that formed. For *Strep. mutans*, a more evenly dispersed lawn was produced in the plates that used BHI agar in lieu of the standard phage bottom agar that was used for the other bacteria. Because these two bacteria did not produce any plaques, their data did not fit a Poisson distribution and were not used in the analysis as their numerous zeros would skew the analysis. The distribution of counts for plates that successfully produced plaques followed a Poisson distribution which, in turn, means that the mean and the variance of the model was equal, and a Poisson Regression could be used for analysis.

S. typhimurium and *E. coli* plates produced similar plaque counts at all four quantities of mixture which can be inferred by the overlap in boxes and whiskers that was observed (FIG 1).

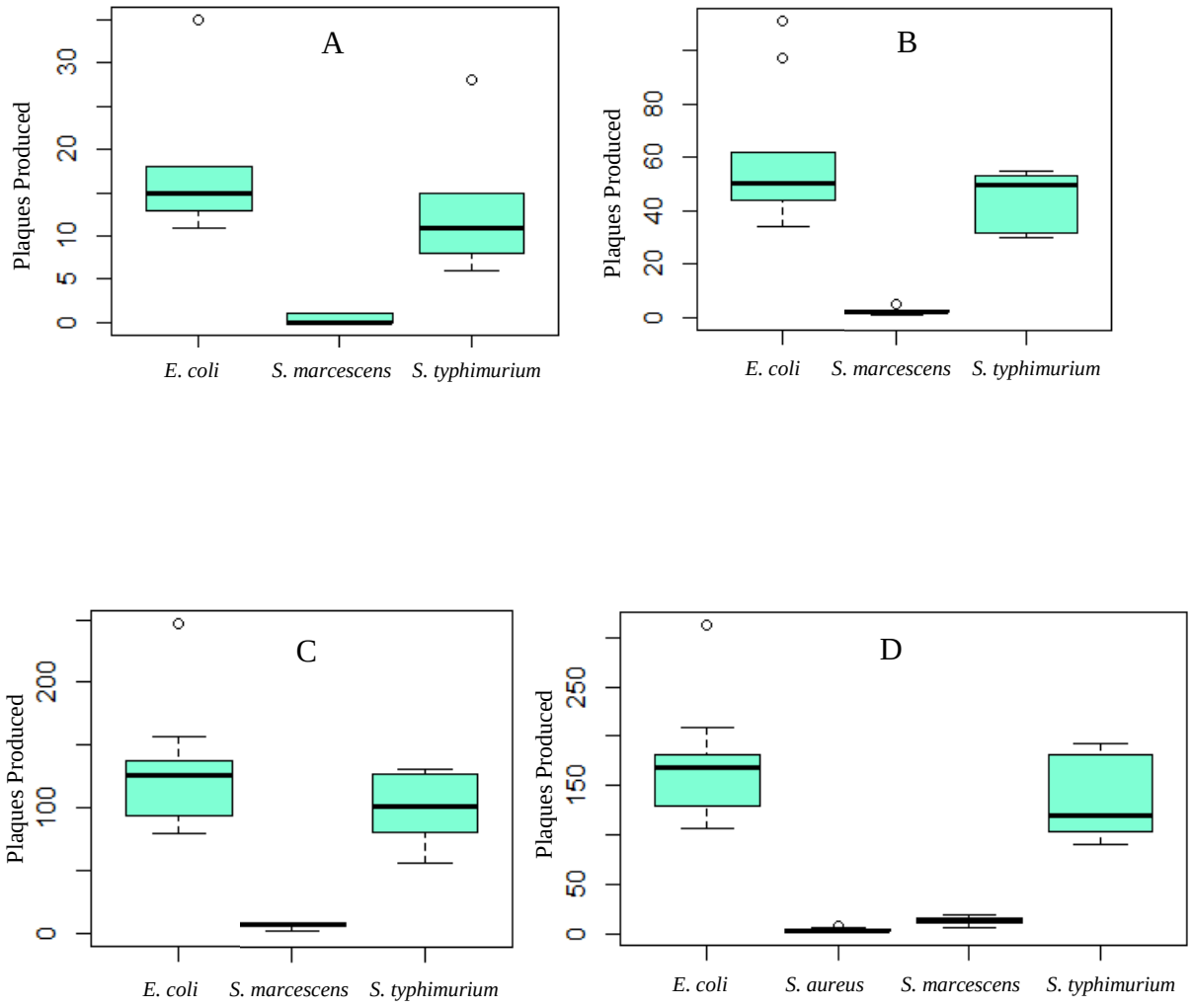

FIG 1 The number of plaques produced by *Escherichia coli, Staphylococcus aureus, Serratia marcescens, and Salmonella typhimurium* when 0.1 mL (A), 0.3 ml (B), 0.6 mL (C), and 0.9 mL (C) of sewage filtrate-pure culture mixture was added to the soft phage overlay.

Furthermore, this inference was able to be confirmed by putting *S. typhimurium* plate counts in a contrast matrix that modelled them against *E. coli's* and it was determined that the counts were all significantly similar (Quasi-Poisson regression, at 0.1 mL p-value = 0.3, at 0.3 mL p-value = 0.1, at 0.6 mL p-value = 0.50, at 0.9 mL p-value = 0.09 mL). While the

S. marcescens plates did produce plaques, they produced considerably fewer than those plated with *E. coli* (Quasi-Poisson regression, at all amounts, p-value < 0.05). Furthermore, at 0.9 mL *Staph. aureus* plates also produced plaques which, much like *S. marcescens*, contained fewer than those of *E. coli* (Quasi-Poisson regression, p-value < 0.05).

Because the *S. marcescens* and *Staph. aureus* plates produced so few plaques it was important to determine whether their counts were similar or different. Although the *Staph. aureus* count that was made when fresh wastewater was used is an outlier and is contained within the whisker of *S. marcescens* (FIG 2), their counts are not significantly similar (Quasi-Poisson regression, p-value = 0.03).

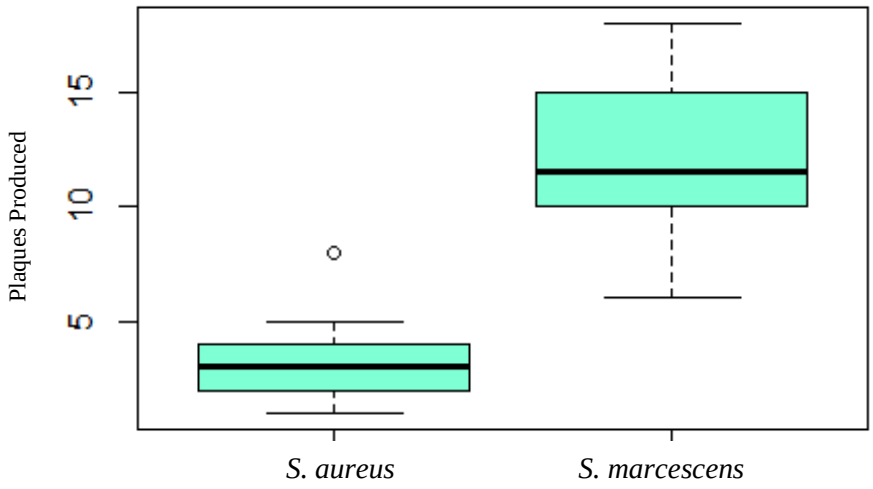

FIG 2 The number of plaques produced by only *Staphylococcus aureus* and *Serratia* when 0.9 mL of sewage filtrate-pure culture mixture was added to the soft phage overlay.

DISCUSSION

The original hypothesis was that the two bacteria tested in this study that can cause the greatest number of gastrointestinal infections, *E. coli* and *S. typhimurium*, would produce the highest number of plaques, followed by the staphylococci, *Staph. aureus* and *Staph. epidermidis*. The oral species, *Streptococcus mutans* was predicted to yield a minimal number and lastly *Serratia marcescens* was expected to produce the least. As per the results of the experiment it can be seen that this order was not correct. While it was true that *E. coli* and *S. typhimurium* yielded the most numerous plaques, the other bacteria did not yield the expected trend. Notably, little to no plaques were expected for *S. marcescens* but it yielded the third highest number of plaques. This was followed by *Staph. aureus* which was predicted to produce a similar number of plaques as *Staph. epidermidis*. However, *Staph. epidermidis* and *Strep. mutans* did not produce any plaques. Hence, the San Angelo Water Reclamation Facility appears to be a potential source for bacteriophages against *E. coli*, *S. typhimurium*, *S. marcescens* and *Staph. aureus* but not *Staph. epidermidis* and *Strep. mutans*. For bacteriophages to be found, it is necessary for the host bacteria to be present in the sample, in this case San Angelo sewage. The results seem to indicate that these species of bacteria are not equally likely to be found in sewage. It is important to search for explanations for these results to help shape future experiments.

As stated previously, *E. coli* and *S. typhimurium* plates displayed the highest number of plaques which indicates that there were more phages against them than any of the other bacteria. *E. coli* is part of the normal microflora of the human gut while *S. typhimurium* is not, which may be why the former seemed to have a slightly higher plaque amount on many

of the plates. It is expected that fecal waste from healthy humans would contain many more *E. coli* cells than *S. typhimurium* cells simply because the latter organism is less numerous in the guts of healthy people. However, it is also true that many animals, such as reptiles and amphibians carry *Salmonella* as normal microflora and therefore these organisms would be expected to be released into sewage from these sources (41,42). Despite this slight difference, according to the analysis, they did, in fact, produce similar plaque counts which suggests a similar number of phages in the wastewater. However, this may not indicate a direct correlation between the number of plaques and the levels of bacteria found in the sewage. This assumption can be drawn from studies that estimate the number of colony-forming units (cfu) per 100 mL to be ~10^6 for *E. coli* and ~10^2-10^4 for *S. typhimurium* (43,44). If these numbers are correct, then one would expect the numbers of bacteriophages specific for *E. coli* to be higher than the numbers specific for *S. typhimurium.* These findings are not consistent with and are not supported by previous research by Mandilara et al. which found a correlation between phage and host numbers in sewage (43). Their study also described a significant difference in the level of somatic coliphages, and *F*-specific coliphages in the raw sewage. Therefore, because in their study *E. coli* was used as the host for somatic coliphages and *S. typhimurium* was the host for *F*-specific coliphages, it is expected that they would get different numbers from those shown in the San Angelo wastewater. This difference in results could be due to several reasons. First, the number of bacteria in the local water source was not evaluated and because it is expected that fluctuations in the population of both species occurs, it is possible that the quantities of the two species were similar at the time of sampling (44). Secondly, our test may not be sensitive enough to discern a difference

between the two because of the imprecise method of creating the host culture broths. Visual assessment was used to determine the number of bacteria in the broth based on the turbidity of the broth and the exact cfu of the overnight cultures was not known. Therefore, there may have been more host cells of the *S. typhimurium* than the *E. coli*, thus allowing for more phage interactions with the host bacteria.

S. marcescens is known to be able to survive relatively harsh conditions, even when outside of a biofilm, and is found in a wide variety of environments including freshwater, soil, sewage, and other non-potable water. Multiple phages have been previously isolated from wastewater and environmental water that show lytic activity to clinical strains of *S. marcescens* (32, 45). This supports our findings that the phages against this bacterium can be readily isolated from the wastewater in San Angelo; however, it does not appear that the relative number of these phages have been previously analyzed. Because our strain was isolated from a freshwater source, we first speculated that the phages that we detected in the water might have been introduced from the environment and not via human waste. If this was true, it was believed that it might have been beneficial to redo this portion of the experiment using a known clinical strain of the bacteria to ensure that the phages isolated have some sort of direct clinical relevance. However, a previous study showed that there did not seem to be a correlation between the source of the bacterial strain and its ability to be lysed by the bacteriophages for *S. marcescens* (32). In other words, the bacteriophages appeared to be specific for receptors common across all strains of S. *marcescens*.

The other species of tested bacteria to display evidence of bacteriophages was *Staphylococcus aureus*. Many strains of these bacteria, including MRSA, have previously

been shown to be present in wastewater systems albeit at low volumes (46). The lower quantity of phages isolated makes sense if there is an actual correlation between phages and the quantity of bacteria in the sewage as it has been shown that less staphylococci can be found in the raw sewage of a treatment plant as compared to the *Enterobacteriaceae* which include the genera *Salmonella*, *Escherichia* and *Serratia* (23). This could explain why there were no plaques until 0.9 mL of the phage mixture was used. The presence of lytic phages against pathogenic strains of *Staph. aureus* has been documented in farm wastewater; however, the relative numbers of these phages was not analyzed in previous studies (47). By reporting the number of phages isolated along with the concentration of our mixture our data might be useful to researchers who want to isolate phages against this bacterium. The phages isolated might have clinical use in controlling infections since the strain used in this experiment was isolated from a food source that had been implicated in causing staphylococcal food poisoning. With increasing numbers of reports describing the presence of MRSA in wastewater facilities, an additional analysis of the San Angelo Water Reclamation Facility using a strain of *Staph. aureus* that is resistant to Methicillin could prove useful in the fight against this difficult to treat pathogen.

One of the tested species that did not produce any phages was *Staph. epidermidis*. While *Staph. epidermidis* can be an extremely successful nosocomial infectious agent, it is usually only found on healthy human skin and mucosal linings throughout the body. As with *Staph. aureus*, this bacterium likely makes up only a small portion of the bacterial profile in sewage because of its sensitivity to the conditions that are inherent in this water source. However, bacteriophages have reportedly been isolated from other sources. Such sources

include the anterior nares and through mitomycin C induction of prophages (48,49). These have yielded only temperate phages which are not preferred for therapy. There has apparently only been one study that has successfully isolated *Staph. epidermidis* phages from wastewater. Melo et. al. isolated a broad-range strictly lytic phage called SEP-1 (50). Despite using a similar enrichment method that was used to isolate SEP-1, no phages were found in the present study. One difference between the enrichment methods used in our study and the study in the earlier publication was that their sewage-bacteria mixture was agitated at a low rpm during the 24-hour incubation period. This agitation could have allowed for more host-phage interaction by limiting the number of bacteria that settled to the bottom of the broth and subsequently became unreachable to the phages. If the phages had been able to interact more freely with the bacteria, they may have been more readily capable of infection and thereby produce a detectable number of plaques. This could not be done in the present study because our lab did not have access to an incubation shaker so there was no way to continuously agitate the culture. In a future study it could be beneficial to try an enrichment experiment with *Staph. epidermidis* again and use the agitation method.

The other species tested in this study that did not produce any plaques, even after phage enrichment, was *Strep. mutans*. Very few phages have been isolated that can infect this bacterium and none of these have been from sewage (51). It seems that the present study is the first to evaluate the presence of phages for this bacterium in sewage as no other studies have been identified in the literature. Like those of *Staph. epidermidis*, most previously reported phages were isolated via mitomycin C induction of prophages which means they have very limited therapeutic use (51). The other phage that has previously been reported for

this bacterium originated from human saliva (39). As evidence of how rare this phage is, of the 81 saliva samples that were tested throughout the experiment, only one produced a bacteriophage (ϕAPM01) and in two other studies that tested saliva none were found. ϕAPM01 was shown to be serotype specific which means it has a very narrow host range and therefore may not be very useful in fighting dental caries. However, it did exhibit the ability to reduce the number of specific strains of *Strep. mutans* and their biofilms which, if combined with other phages or other antimicrobial agents, could be used to treat dental caries in their early stages.

Because the phages are in such low quantity in the oral cavity, it makes sense that very few of them would be released into the wastewater. While there are many oral streptococci species found in raw sewage, only a small portion of these are likely to be *Strep. mutans* when their abundance in the human microbiome is taken into account (23). In a study aiming to identify the healthy human bacterial profile, the average relative abundance of streptococci species was established using 127 samples of saliva. These data illustrated that the most prevalent oral streptococci were *Strep. parasanguis*, *Strep. salivarius*, and *Strep. infantis* with *Strep. mutans* not making the top eleven (52). All of this information supports this study's findings that there are no phages that can interact with these bacteria in the San Angelo wastewater system and future studies should take this information into consideration when attempting to isolate novel phages against it. However, as with *Staph. epidermidis*, the phage enrichment may need to be reevaluated using the methodology that agitates the mixture during the incubation period.

There are multiple future directions that should be considered after analyzing the results of this project, the first being increasing the number of species of bacteria that are included in the search for phages. While important, the bacteria originally chosen for this project did not nearly encompass all of those that could be potentially treated with phage therapy. One example would be *Pseudomonas aeruginosa.* This is a gram-negative bacterium that is multi-drug resistant and is often an opportunistic pathogen most notably in burns and in cystic fibrosis patients. Since it can produce biofilms and it has acquired antimicrobial resistance, it is a great candidate for phage therapy. Already there have been isolates of phages against this bacterium isolated from wastewater, and one has demonstrated the ability to significantly reduce the biofilms produced by clinical isolates of this bacterium when combined with chlorine (53). There are many other bacteria that could theoretically be eliminated with bacteriophages, in combination with antimicrobials, and the San Angelo wastewater could potentially contain some that have yet to be discovered.

Another step forward with this project would be to isolate specific phages from the plaques produced in this study in order to determine their characteristics and to get an idea of their host range. Before a phage can be considered for use as a treatment, its interactions must be known. The first characteristic that should be identified is the type of life cycle it undergoes. The isolated phage must undergo a lytic lifecycle to be considered useful because this lessens the chance of transduction and the development of phage resistant bacteria. Next, the host range should be determined to know whether it will begin to impact the patient's normal flora which nullifies one of the major advantages of using phages. Also, looking at these viruses under a transmission electron microscope would be advantageous in

understanding the phage. Knowing its morphological characteristics would help with determining its taxonomy, especially if combined with a genetic analysis.

Finally, more sources in San Angelo should be analyzed to look for the presence of other phages that could not be found in the raw sewage. Evaluating other phases in the water reclamation system could reveal different phages as there is a difference in the bacterial profile between raw sewage, activated sludge, and the effluent sewage. Furthermore, other locations in San Angelo, such as the Concho River, may reveal phages at different quantities and possibly with different characteristics.

In conclusion, although more work is needed to fully understand the full capabilities of these phages, they could be the answer to the increasing antimicrobial resistance. Even if they are not the final solution they could be a temporary stopgap to infection until another treatment is developed or new antibiotics are created. This call for phages as an alternative therapy cannot be answered without first knowing where they can be isolated. This study helps shed light on this issue by displaying the presence of bacteriophages against *E. coli, S. typhimurium, S. marcescens* and *Staph. aureus* in the wastewater of the San Angelo Water Reclamation Faculty. Equally important is knowing where not to look and the results of this study showed that this location was not a viable source for either *Staph. epidermidis* or *Strep. mutans*. Future studies can use this information to help determine where to search for novel phages.

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BIOGRAPHY

Tyler Corey Birk was born in Altus, Oklahoma but lived in Abilene, Texas for much of his life. He graduated from Wylie High School and chose to attend Angelo State University to major in biology in the hopes of going to dental school. While at ASU, Tyler presented research at both national and regional science and honors conventions. He also had the privilege to serve as the president of the Honors Student Association for two years.

After obtaining his Bachelor of Science in biology from ASU Tyler will attend dental school at the University of Texas Health Science Center in San Antonio. He will then either pursue a Postdoc in either Pediatric Dentistry or Advanced Education in General Dentistry. Tyler hopes to one day open his own dental practice.

Tyler can be reached at tylercbirk@gmail.com