VIRAL WARRIORS: SCREENING WATER SAMPLES FOR BACTERIOPHAGES AGAINST MULTIPLE DRUG RESISTANT BACTERIA

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VIRAL WARRIORS: SCREENING WATER SAMPLES FOR BACTERIOPHAGES AGAINST MULTIPLE DRUG RESISTANT BACTERIA

by

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ABSTRACT

Although mutation occurs randomly in nature and is passed randomly between bacterial species, the misuse and overuse of antibiotics in modern medicine has selected for antibiotic resistant organisms, resulting in an epidemic of antibiotic resistant infections. Used extensively in former Soviet Union countries with success, Western researchers have begun considering phage therapy for treatments, however it must be subjected to rigorous clinical trials before it can be approved by the FDA as a treatment method in North America (Gill *et al.* 2010; Abhilash *et al.* 2009).

In this study, phage screening was performed on eight MDR bacterial strains provided from LifeLabs and Royal Inland Hospital in Kamloops, B.C.: *E. coli* 15-102, 15-124, and 14-318; *Micrococcus luteus*; Methicillin Resistant Staphylococcus Aureus (MRSA) 1 and 2; *Serratia marcescens*; and Mycobacterium *smegmatis*. One non-resistant *E. coli* strain known to be killed by phages found in Kamloops sewage was used as a positive control. Seven water samples and one non-water sample were used in this experiment as a source of phages. Water samples were obtained from the Kamloops Sewage Treatment Plant, the Domtar pulp mill run-off, the Pacific Ocean, Bisaro Anima Cave, and alkaline ponds around Kamloops. The non-water sample was created from mixing dirt from Abbotsford, B.C. with sterile water. An additional enriched water sample was made through the incubation of broth culture, nutrient broth, and sewage water overnight at 37°C in an attempt to select for more strain-specific phages (Prescott *et al.* 2005). In addition, sterile water was used in the protocol as a negative control.

The successfulness of each phage screening trial was measured through the formation of plaques, which developed after plating the Multiple Drug Resistant (MDR) bacteria, molten agar, and phages for confluent growth on nutrient agar (Prescott *et al.* 2005). Of all the bacteria and environmental water samples, plaques only developed for the *E. coli* 14-318 strain using sewage water from the Kamloops Sewage Treatment Center. Using phage screening against these MDR bacteria allowed us to see that MDR pathogens present in our community are treatable with a potentially more beneficial and successful method to antibiotics.

Thesis Supervisor: Dr. Naowarat Cheeptham

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INTRODUCTION

BACKGROUND AND RATIONALE

Importance of Phage Therapy

Due to decades of extensive misuse and overuse of antibiotics in modern medicine, antibiotics that were once effective against pathogenic bacteria are now no longer sufficient because of rapid evolution and mutation of resistant bacterial species. Although novel antibiotics targeting MDR bacteria can be developed, pathogens ultimately end up becoming resistant to such drugs (Carlton 1999). To break this vicious cycle, phage therapy, which has been used extensively in former Soviet Union countries with success, is being reconsidered as a treatment method by Western researchers, who have exclusively relied on antibiotics to date. However, acceptance of this method has been difficult to obtain, and phage therapy must be subjected to rigorous clinical trials before it can be approved by the FDA as a treatment method (Gill *et al.* 2010; Abhilash *et al.* 2009).

Phage Therapy History

Phages were first discovered by British microbiologist Felix Twort in 1915, and later by French-Canadian microbiologist Felix d'Hérelle in 1917. Although Twort did not pursue his discovery, d'Hérelle investigated the nature and mechanism of phages as a therapeutic agent, and established phage therapy centers in the U.S., France, and Soviet Georgia (Carlton 1999). D'Hérelle's first use of phage screening was on French troops with severe hemorrhagic dysentery in July 1915, where he made bacterium-free filtrates of patients fecal samples and incubated them with isolated *Shigella* strains from the patients. He observed the appearance of clear plaques on agar plates, which he proposed were caused by a virus capable of parasitizing bacteria. These phages were later used in phage therapy trials in 1919 on hospital patients with severe dysentery, and after a single dose, the patients fully recovered (Sulakvelidze *et al.* 2001). The use of bacteriophages as therapeutic agents was later used extensively during World War Two, particularly by Soviet doctors to treat wound infections of troops on the battlefield. However the discovery of penicillin in 1928 caused a sharp decline in phage research in the West, which chose to prioritize treatment

of bacterial infections with antibiotics, leaving only the former Soviet Union countries still developing and utilizing phage therapy (Abedon *et al.* 2011).

Classification of Phages

Phages are classed into 13 different families according to their morphology, presence or absence of an envelope or lipid, and type of nucleic acid. Approximately 96% of phages are composed of an icosahedral head and tail and have double stranded DNA as their genome and are termed "tailed phages." Tailed phages are further classified by their morphological features into three families: *Myoviridae*, which possess a contractile tail and contain 93 species, including phages KVP20, KVP40, KVP241, and T-even; *Siphoviridae*, which possess a long non-contractile tail and contain 313 species, including phages Φ MR11 and λ ; and *Podoviridae*, which possess an extremely short tail and contain 50 species, including T7 phages. The other 4% of phages are classified as cubic, filamentous, or pleomorphic and contain double stranded or single stranded DNA or RNA (Matsuzaki *et al.* 2005).

Bacteriophage Life Cycle

Phages are viruses that infect and lyse specific bacteria through interacting with bacterial membrane receptors, disrupting bacterial metabolism, and eventually causing the cell to lyse after replicating their DNA inside of the bacterial host cell. (Gill *et al.* 2010; Abhilash *et al.* 2009). Phages can be divided into two groups according to their life cycle: lytic phages, which insert their DNA into bacteria and self-proliferate, leading to bacterial lysis, and lysogenic phages, which have an additional lysogenic cycle where their DNA is incorporated into the host genome and replicated as part of the host genome without lysing the cell. However, under environmental stress, such as changes in temperature, pH, and nutrients, the lytic cycle can be triggered, lysing the cell. As some lysogenic phages have toxic genes in their genome which can become incorporated into the bacterial genome, lytic phages are the most suitable therapeutic candidates. Alternatively, pseudolysogeny can occur, in which the phage genome enters the cell but cannot enter the lytic or lysogenic cycle. This typically occurs in nutrient-deprived conditions, when bacterial hosts cannot support DNA replication, and the phage genome will persist as a preprophage until nutrition is restored, at which point it can enter the lytic or lysogenic cycle (Figure 1; Feiner *et al.* 2015:used with permission).



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Figure 1. The lytic, lysogenic, and pseudolysogenic cycles. a. The lytic bacteriophage cycle, in which the phage replicates using host machinery and lyses the cell; b. The lysogenic cycle, where the phage genome is incorporated into the host chromosome as a prophage and persists in a dormant state until environmental stressors trigger the lytic cycle to commence; c. Pseudolysogeny, in which phage genome fails to replicate or establish itself as a prophage, occurring typically in nutrient-deprived conditions (Feiner *et al.* 2015, used with permission).

Mechanism of Bacteriolysis by Phages

The first step of bacteriolysis is phage infection, through which phages adsorb to a receptor on the bacterial surface, which is typically a protein or sugar. Phages generally only adsorb to specific bacterial strains or species, not across multiple species or genera, which makes bacteriophage therapy as a targeted therapeutic treatment so beneficial. After phage adsorption, phage DNA is injected into host cytoplasm and is either integrated into the host chromosome or replicated by host machinery and packaged into capsids, which are created during the late stage of phage infection. Tails are then attached to the DNA-filled head. The new phages then lyse the cell through the protein interactions of lysin, which degrades peptidoglycan, and holin, which form holes in the cell membrane, exposing the peptidoglycan layers to lysin. The released phages infect other bacterial cells following this, leading to lysis of the entire bacterial population. (Figure 3; (Matsuzaki *et ali.* 2005: used with permission)



Figure 2. Phage induced bacteriolysis: (1) Phage adsorption and DNA injection; (2) Phage DNA replication; (3) head and tail production; (4) holin and lysin synthesis; (5) DNA packaging; (6) complete phage assembly; (7) lysis of cell wall and release of phages; (8) circularization of phage DNA; (9) integration of phage DNA into host genome via lytic cycle (Matsuzaki *et al.* 2005, used with permission).

Bacteriophage Screening and Therapy Research

Pseudomonas aeruginosa

Vinodkumar *et al.* targeted 28 multidrug-resistant *Pseudomonas aeruginosa* strains in 1,647 septicemic mice using bacteriophages over a period of five years. Phages were isolated from raw sewage at a municipal sewage treatment plant after incubating sewage, nutrient broth, and *P. aeruginosa* at 58°C for 30 minutes in a water bath, following which a few drops of chloroform were added, the mixture was centrifuged, and the supernatant was filtered through a 0.22 μ l filter. The effectiveness of bacteriophage activity on *P. aeruginosa* was confirmed *in vitro* through placing phage isolate in wells on a *P. aeruginosa* lawn for 24 hours at 37°C, using sterile distilled water as a control. The phage strain used was effective against 74% of the *P. aeruginosa* strains. The 28 multidrug-resistant strains were resistant to almost all types of antibiotics, including β lactamases, and were used to introduce a fatal infection into the mice. A single injection of the phage strain administrated 45 minutes after the *P. aeruginosa* injection was sufficient to rescue 100% of the animals, and an injection when the mice were moribund was successful in rescuing approximately 50% of the mice (Vinodkumar *et al.* 2008).

A clinical trial by Wright *et al.* 2009 also tested phages against multiple-drug resistant *P. aeruginosa* in 24 patients with chronic otitis. Researchers found that the treatment patients showed significantly lower pathogenic *P. aeruginosa* levels 42 days after treatment compared to the placebo group and that no treatment-related adverse effect was reported. These results are indicative of phage therapy success in human clinical trials (Wright *et al.* 2009).

Enterococcus faecium

Biswas *et al.* 2002 used phage therapy on Vancomycin-resistant *Enterococcus faecium* infections in mice. Colonization of the gastrointestinal tract by Vancomycin-resistant *E. faecium* has become endemic in many hospitals and can lead to endocarditis, so a reliable treatment method is essential. Phages were isolated from municipal sewage through centrifugation and removal of the supernatant, following which the supernatant was added to precipitate in 10% polyethylene glycol, dissolved in SM buffer, and extracted with chloroform. This processed sewage was mixed with *E. faecium* and incubated for 20 minutes at 37°C, before being mixed with top agar and poured on agar plates and incubated overnight. Phages present in plaques were isolated and used in mice given a potentially fatal *E. faecium* injection. Phage injection 45 minutes after pathogen injection resulted in the rescue of 100% of the animals, and phage injection into moribund animals resulted in approximately 50% of the mice being rescued (Biswas *et al.* 2002).

Escherichia coli

Chibani-Chennoufi *et al.* collected diarrhea-associated *Escherichia coli* samples from pediatric diarrhea patients and environmental water samples, and isolated phages from fecal samples through centrifugation and filtration of the supernatant through a 0.45 µl filter. Phage plaque assays were successfully performed, and purified phages were given to mice with regular and ampicillin-resistant *E. coli* infections through drinking water. The results showed that some of the intestinal *E. coli* strains were lysed successfully, however, other strains were not. In particular, *E. coli* present in gut flora were only minimally affected by oral phage application, indicating that the phages were specific enough to only work against specific *E. coli* strains (Chibani-Chennoufi *et al.* 2004).

Benefits of Bacteriophage Therapy

As phages are composed mostly of nucleic acids and proteins, they are much less toxic than antibiotics, and phage therapy is seen as a superior method to antibiotics as it is much more specific and should be less likely to cause side effects or negative harm to the beneficial normal flora of the host (Abedon *et al.* 2011; Loc-Carillo *et al.* 2011; Matsuzaki *et al.* 2005). In addition, phages are reportedly very successful against bacteria that construct biofilms composed of a polysaccharide matrix that antibiotics cannot penetrate (Abhilash *et al.* 2009).

Bacteriophages are also notably more successful at completely killing the target bacteria species in comparison to antibiotics, and are also capable of increasing in number in an area specifically where the bacteria species is located, therefore phages themselves contribute to establishing the phage dose during phage therapy. Because of this, only a single dose of phages is generally required for treatment (Loc-Carillo *et al.* 2011).

Phages are also successful against extremely resistant bacteria strains. In addition to resistance to regular antibiotics, bacterial strains have been found to contain enzymes such as carbapenemases

and Extended-Spectrum Beta-Lactamases (ESBL), which further add to the broad resistance of the strains to treatment. Carbapenemases are β -lactamases which are capable of hydrolyzing carbapenems, cephalosporins, monobactams, and penicillins (Queenan *et al.* 2007). As multiple drug resistance (MDR) to antibiotics is easily passed through a plasmid during bacterial conjugation, carbapenems have been used to counteract MDR resistance, however this has led bacterial strains to develop resistance to carbapenems as well (Currie 2012). ESBLs are also β -lactamases found only amongst Gram negative bacteria, and mediate resistance to extended-spectrum cephalosporins and monobactams, but do not affect cephamycins or carbapenems (CDC 2010). Infections from bacteria which possess these enzymes renders antibiotic treatment highly unsuccessful, illustrating the importance of the development of an alternative treatment method, such as phage therapy.

In addition, phages are easily applied in the form of topical treatments and injections, and are versatile with regards to formulation development, as they can be used in combination with antibiotics or with other phage strains to increase the success of infection treatment (Loc-Carillo *et al.* 2011).

Drawbacks of Bacteriophage Therapy

The ideal phage is obligately lytic, stable in storage conditions and temperatures, subject to appropriate safety studies, specific to the target bacteria, and ideally, fully sequenced to ensure that it carries no toxin genes, which are difficult conditions to meet (Loc-Carillo *et al.* 2011). Even if a specific phage that is effective is found, it must be able to enter the lytic cycle to be used in treatment, as lysogenic phages will lay dormant until environmental stress is experienced by the host cell. Phages must also be able to be stored for long periods of time after they are isolated without breaking down, typically at -20°C (Prescott *et al.* 2005). Another issue is getting approval to put phage therapy on clinical trials so that it can be accepted as a treatment method in the west. Former Soviet countries do not face this problem, but for phage therapy to be put into use and approved by the FDA, rigorous clinical trials must be undergone. In addition, changing the western mindset to accept using viruses to treat infections presents another challenge. Phages only attacking a narrow host range is another problem, as isolating phages specific-enough for certain bacteria may take a lot of time and energy. Because of this, multiple phages may need to be

combined in a dosage in a so-called "phage cocktail" in order to most effectively target the bacteria (Loc-Carillo *et al.* 2011). Finally, phages have been known to contain toxin genes and regulate virulence in bacteria, so sequencing phages before they are put to therapeutic use is essential to ensuring the treatment will be beneficial (Wagner *et al.* 2002).

Bacteriophage Therapy in Combination with Other Treatment Methods

Although phages are used exclusively as a treatment method in Russia, countries like Poland use phages in combination with other treatment methods, in order to maximize treatment potential (Carlton *et al.* 1999). Although resistance to common antibiotics has become an issue, researchers are still finding novel antibiotics to which bacteria do not have resistance genes, which can potentially be used in combination with phages to ensure that all pathogenic bacteria are killed (Huff *et al.* 2004). Another novel treatment method exclusive to antibiotics and phage therapy is using clay to kill bacteria, as exhibited by the Kisameet Clay in the Heiltsuk First Nation territory in British Columbia, which has been shown to have antibacterial activity against MDR *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species (Behroozian *et al.* 2016). Phages could potentially be used in combination with this clay to give the most effective treatment possible for bacterial infections, much like the combination of antibiotics and phages.

SOURCES OF BACTERIOPHAGES

Water Samples Used

Using water samples from the Kamloops Sewage Treatment Plant, Domtar, the Pacific Ocean, Bisaro Anima Cave, alkaline ponds, and the non-water dirt sample in this project as a source of phages could have allowed for the discovery of novel phages which could have been extremely successful in lab. For instance, sewage contains many potentially pathogenic bacteria, such as *Escherichia coli* and *Enterobacter aerogenes*, which suggests that phages capable of successfully attacking these potentially dangerous and rapidly-evolving bacteria may also be present in the sewage sample from the Kamloops Sewage Treatment Center (Beaudoin *et al.* 2007). Likewise, cave water and Domtar runoff present extreme habitats, seeing as bacteria are forced to grow in energetically unfavourable and nutrient-limited conditions, and may host many novel and diverse bacteria species and their respective phages, which may be effective against other MDR bacteria (Barton *et al.* 2007). In addition, alkali pond water is extremely acidic and selects for resistant and adaptive bacteria such as *E. coli*. We can speculate that if *E. coli* is present excessively in alkali pond water, phages associated for *E. coli* are likely present as well (Parhad *et al.* 1974). Seawater has also been found to contain over 150 different isolates of bacteriophages and phages are shown to exceed bacterial concentration in seawater by a factor of 10, therefore seawater should have been a rich source of phages (Børsheim 1993). Soil was also speculated to contain bacteriophages effective against MDR bacteria, as soil contains many bacteria that can become pathogenic to humans, and an average of $1.5 \times 10^8 \text{ g}^{-1}$ phages, which is equivalent to 4% of the total population of bacteria (Ashelford *et al.* 2003).

The creation of an enrichment water sample was also attempted through the incubation of broth culture, nutrient broth, and filtered sewage water overnight at 37°C. Through exposing the bacteriophages present in the water sample to only one bacterial strain, it was projected that more successful phage screening trials would occur (Biswas *et al.* 2004; Chibani-Chennoufi *et al.* 2004; Prescott *et al.* 2005; Wright *et al.* 2009; Vinodkumar *et al.* 2008).

The successfulness of each environmental water sample was measured through the formation of plaques, which develop after plating the MDR bacteria, molten agar, and environmental water for confluent growth (Prescott *et al.* 2005). The development of plaques on the *E. coli* strains would further signal that bacteria possessing carbapenemases and ESBLs and are essentially untreatable can be treated successfully using phages. All plaques were sent to sequence the bacteriophages present to determine if novel phages had been found.

OBJECTIVE

The main objective of this project was to screen bacteriophages (bacteria-infecting viruses) from extreme habitats that specifically target the multiple drug resistant (MDR) bacteria. In particular, this experiment focused on the isolation of phages from local water samples, namely sewage water samples from the Kamloops Sewage Treatment Plant, and their effectiveness against MDR *E. coli* obtained from LifeLabs. We aimed to develop insight into the correct process, optimal concentrations, and proper conditions needed for phage isolation, and determine whether

successful bacteria-specific phages were capable of being screened, grown, and used against MDR bacteria.

The null hypothesis was that the MDR bacteria would be unaffected by bacteriophage screening and live regardless of the inoculation with phages.

MATERIALS AND METHODS

WATER SAMPLE COLLECTION

Water samples were obtained aseptically in sterile bottles from the Kamloops Sewage Treatment Plant, Domtar (three samples taken at 1.4 days, 2.3 days, and 3.3 days into the purification process, with the sample taken at 3.3 days being the final treated effluent), the Pacific Ocean (Bamfield, B.C.), Bisaro Anima Cave, and alkaline ponds. A water sample was also created from mixing dirt from Abbotsford, B.C. with sterile water. All samples were stored at 4°C until use.

WATER SAMPLE FILTRATION FOR BACTERIOPHAGE ISOLATION

Water samples were either syringe-filtered or pressure-filtered through a 0.22 μ m filter to remove bacterial particles, ensuring only phages were present. Samples containing a lot of particulate and debris were first filtered with a 0.45 μ m filter, and then a 0.22 μ m filter. In one trial, an enrichment water sample was created through the combination of nutrient broth, *E. coli* 14-318 bacterial culture, and sewage water incubated overnight at 37°C to promote the growth of strain-specific phages, before being filtered through a 0.22 μ m filter. The soil sample from Abbotsford was mixed with sterile water and let stand for 24 hours. It was then filtered using a 0.22 μ m filter.

CONTROLS

Positive and negative controls were used in the experiment. For the negative control, sterile water was plated in combination with the MDR bacteria, expecting that the bacteria would exhibit confluent growth and no plaque development would occur. The positive control involved plating the environmental water samples with a non-MDR strain of *E. coli* that was previously shown to be killed by phages present in the Kamloops Sewage Treatment Centre water sample, expecting that plaques would develop in this situation.

SEQUENCING

A plate containing plaques from sewage water which was successful against *E. coli* 14-318 was sent to GENEWIZ, Inc, in Seattle, USA, for sequencing. Following unsuccessful sequencing, new plates were sent to the University of Texas for sequencing. Both times, the standard laboratory procedure was repeated to ensure plaque formation, following which the fresh plate was wrapped in parafilm and shipped via express post on ice.

SAFETY PRECAUTIONS

The experiments were undergone in the laboratory in a biosafety cabinet so as to not contaminate other areas of the lab with the water samples and the MDR bacteria. All contaminated equipment was labelled and kept together and all samples were labelled and stored in an isolated area in the walk-in fridge. Proper biosafety training was also undergone in accordance with the requirements for Laboratory safety 2.

PROCEDURE



Figure 3. Overview of experimental procedure outlining the isolation and purification of bacteriophages from water samples, growth of bacteria in nutrient broth, and combination of phage water, bacteria, and molten agar on nutrient agar plate.

MDR Bacteria Preparation

Bacteria strains were confirmed to be multiple-drug resistant through a streak plate test on nutrient agar plates infused with tetracycline. Once resistance was confirmed, bacterial species were inoculated in nutrient broth overnight, shaking at 37°C, and then placed in the fridge to preserve cell density.

Isolation and purification of phage strains

Water samples were stored in the fridge at 4° C until ready for use. Samples were first filtered through a 0.45 μ m filter to remove bacteria and excess debris if needed, followed by a 0.22 μ m

filter, to leave only bacteriophages present in the water samples. The environmental water was stored at 4°C in fridge until needed.

In vitro confirmation of bacteriophage activity

In a test tube, 0.5 mL environmental water and 0.5 mL broth culture bacteria were mixed with 2.5 mL molten nutrient agar warmed to approximately 55°C, which was hot enough to pour easily but not hot enough to kill the bacteria, and then poured evenly onto nutrient agar plate. This step was also repeated with dilutions of 0.9 mL environmental water and 0.1 mL bacteria, but no significant differences in plaque formation were seen between the two dilutions. Petri plate lids were immediately placed onto plates to avoid contamination, and the plates were inverted and incubated at 37°C for 24 hours.

After incubation, the plates were checked for the presence of plaques. If plaques were found, the procedure was repeated again to ensure legitimate plaque formation, and the plates were wrapped in parafilm and sent via express post to be sequenced.

RESULTS

The combination of top molten agar, broth culture, and filtered environmental water samples was first attempted using the water samples from the Kamloops Sewage Treatment Center, the Pacific Ocean, and Domtar, however no plaques formed. The same protocol was repeated using strain-specific environmental water samples through the incubation of broth culture, nutrient broth, and environmental water overnight before filtering for isolated phages, however no plaque development was observed. Furthermore, plaque formation was not seen using water samples from Bisaro Anima Cave, alkali ponds, or the soil sample (Table1).

A more crude water sample was obtained from the Kamloops Sewage Treatment Centre, from which phages capable of successfully killing the MDR bacteria were only found. The only bacterial strain that developed plaques from this water sample was the *E. coli* 14-318 strain at both a 5:5 dilution and a 9:1 dilution of sewage water to broth culture (Table 2). A sewage water enrichment sample was prepared through the inoculation of broth culture in sewage water and nutrient broth overnight to induce strain-specific phages and was successful against the *E. coli* 14-318 strain. For the 5:5 broth dilution, 31 plaques were found, ranging in sizes from 1 mm to 5 mm and for the 5:5

sewage dilution 22 plaques were observed, sized 2 mm to 7mm. For the 9:1 broth dilution, 52 plaques were found ranging in size from 0.5 mm to 11 mm, and for the 9:1 sewage dilution, 39 plaques were noted from 1mm to 7 mm (Table 3). Overall, no significant difference was noted in the successfulness of phages between those present in the enrichment water and those in the sewage water, indicating that the enrichment water did not contain more strain-specific phages, and that the phages were equally as successful at lysing bacterial cells.

Table 1. The resistance, possession of carbapenemase and ESBL, and plaque presence observed of the MDR bacteria used in this project.

Strain	Resistance	Carbapenamase	ESBL	Plaque
				Presence
E. coli 15-102	Ampicillin ¹	+	+	-
E. coli 15-124	Ampicillin ²	+	+	-
E. coli 14-318	Ampicillin ³	+	+	+
Micrococcus luteus	TET (tetracycline-30 µg)	U^5	U	-
MRSA	TET (tetracycline-30 µg)	U	U	-
MRSA		U	U	-
Serratia	Tetracycline ⁴	U	U	-
Mybacterium	SXT (sulfamethoxozole-23.75	U	U	-
smegmatis	μ g and trimethoprim-1.25 μ g)			
Control : E. coli	None	U	U	+

¹Ampicillin, Amoxicillin/Clavulanic Acid, Piperacillin/Tazobactam, Cefalotin, Cefazolin, Cefoxitin, Cefixime, Ceftazidime, Ceftriaxone, Ertapenem, Meropenem, Amikacin, Gentamicin, Tobramycin, Ciprofloxacin, Tetracyclin, Nitrofurantoin, Trimethoprim/Sulfamethoxazole,

²Ampicillin, Amoxicillin/Clavulanic Acid, Piperacillin/Tazobactam, Cefalotin, Cefazolin, Cefoxitin, Cefixime, Ceftazidime, Ceftriaxone, Ertapenem, Meropenem, Ciprofloxacin, Tetracyclin, Trimethoprim/Sulfamethoxazole,

³Ampicillin, Amoxicillin/Clavulanic Acid, Piperacillin/Tazobactam, Cefalotin, Cefazolin, Cefoxitin, Cefixime, Ceftazidime, Ceftriaxone, Ertapenem, Meropenem, Amikacin, Gentamicin, Tobramycin, Ciprofloxacin, Tetracyclin, Nitrofurantoin, Trimethoprim/Sulfamethoxazole,

⁴Tetracycline, Gentamicin, Nitrofurantoin, Ceftriaxone, Tobramycin, Amikacin, Cefixime, Meropenem, Ertapenem

⁵Unknown

Water Sample Location	Plaque Presence	
Alkali Ponds	No	
Bisaro Anima Cave	No	
Domtar (three different samples)	No	
Kamloops Sewage Treatment Plant	Yes ¹	
Sea Water (Bamfield, B.C.)	No	
Soil Sample (Abbotsford, B.C.)	No	

Table 2. The water sample location and presence of plaques when water samples had been plated

 with MDR bacteria and molten agar.

¹ Worked against *E. coli* 14-318 and control *E. coli*

Table 3. The different water sample (enriched or regular sewage) to bacterial broth dilutions and the number and size of plaques present.

Water Sample Dilution	Number of Plaques	Plaque Size
500 μL Enrichment : 500 μL Bacteria	31	1 mm - 5 mm
500 μL Regular : 500 μL Bacteria	22	2 mm - 7 mm
900 µL Enrichment : 100 µL Bacteria	52	0.5 mm - 11 mm
900 μL Regular : 100 μL Bacteria	39	1 mm - 7 mm

Antibiotic Types	Resistance
Beta Lactams	ESBL, including carbapenemase
Aminoglycosides	Gentamicin, Tobramycin, Netilmicin
Quinolones	All
Tetracyclines	All
Furanes	All
Trimethoprim/Sulfonamides	All

Table 4. Additional resistance of *E. coli* 14-318 to beta lactams, aminoglycosides, quinolones, tetracyclines, furanes, and trimethoprim/sulfonamides.



Figure 4. Plaque presence after plating bacterial strain *E. coli* 14-318 with molten agar and sewage water and sewage broth samples in different concentrations: 1. 500 μ L sewage water to 500 μ L bacterial culture; 2. 500 μ L enrichment sewage to 500 μ L bacterial culture; 3. 900 μ L sewage water to 100 μ L bacterial culture; and 4. 900 μ L enrichment sewage to 100 μ L bacterial culture.

DISCUSSION

The absence of plaques found from plating a combination of top agar, broth culture, and filtered environmental water samples prompted the need for the growth and isolation of more strain-specific phages. This led to the creation of an enrichment broth through the inoculation of water samples, broth culture, and nutrient broth overnight before filtering the water for isolated phages.

The continued absence of phages inferred that either phages were not present in the water samples, or that phages specific enough to kill the MDR and control bacteria were not present. The latter point is supported, as the phages present in sewage water were successful against both the *E. coli* 14-318 strain and the control *E. coli* strain, however not against the *E. coli* 15-102 and 15-124 strains, indicating the specificity required for phages to attack a cell, even amongst bacteria of the same species (Chibani-Chennoufi *et al.* 2004). In addition, as sewage contains human excrement, which is rich in *E. coli* and other human pathogens, it was expected that phages capable of killing human pathogens would be present in the samples. However, the other water samples used came from environments that are not necessarily rich in human pathogens, which could account for the lack of phage specificity and plaque development.

A more crude sewage water samples was then put through the protocol, both as a standard filtered water sample and also as an enriched water sample, which was prepared through the incubation of broth culture, nutrient broth, and sewage water overnight. Sewage from sewage treatment centers is a rich source of phages and is used extensively in protocols in previous research as it contains a wide variety of human gut bacteria, potentially containing phages which specifically target human pathogens like the MDR bacteria used in the study. Due to the wide variety of bacteria present in sewage, many different bacteriophages could be present in one sample, which increases the chances of more MDR bacteria being targeted by bacteriophages. (Biswas *et al.* 2002; Vinodkumar *et al.* 2008; Wright *et al.* 2009). Phages have also been isolated from patient feces in the previous research, however only using feces from one subject likely narrows the amount of phages isolated (Chibani-Chennoufi *et al.* 2004).

Plaque presence was observed in both the *E. coli* 14-318 strain and the control *E. coli* strain, and there was not a noticeable difference in the number of plaques between the regular sewage sample and the enriched sample, indicating that the enriched sample did not improve specificity. These results were not expected, as enriched environmental water samples are used in most studies for phage screening, and were proposed to be more specific and successful against pathogens. Plaque size, however, differed considerably on the plates, ranging from 0.5 mm to 11 mm, potentially indicating that different phages were present (Biswas *et al.* 2004; Chibani-Chennoufi *et al.* 2004; Prescott *et al.* 2005; Wright *et al.* 2009; Vinodkumar *et al.* 2008).

The last water samples tested were from Bisaro Anima Cave and alkaline ponds, however no plaques developed after plating. Soil was also used as a phage source after being mixed with sterile water and filtered, but no plaques developed after plating. Along with the first water samples tested, it was inferred that phages did not exist in the water samples or that phage screening was not successful against the specific MDR bacteria. Compared to sewage water samples, which contain human excrement containing gut microbiota and potentially pathogens, extreme water and soil samples such as the Bisaro Amina Cave, alkali ponds, and soil samples used in this project are not necessarily exposed to the same species and strains of bacteria that associate with humans. Although the presence of *E. coli* in alkali ponds and soil samples has specifically been supported in previous studies, perhaps the phages present in these samples were not specific to the MDR E. coli used in this project (Børsheim 1993; Parhad et al. 1974). This postulation is supported as phages effective against one MDR E. coli strain were not effective against all of the MDR E. coli strains used, further illustrating the importance of specificity in phage screening. Indeed, the need for species- and strain-specific phages hinders the development of phage therapy as a therapeutic treatment method as such phages can be difficult to isolate and discover, rendering some bacterial infections untreatable. Although antibiotics negatively affect good gut microbiota, they are designed to kill all bacteria without needing specific parameters or specificity to do so, making antibiotics a broader and more effective treatment method theoretically. However, in the case of MDR bacteria, where antibiotics are useless, the time and energy spent finding phages specific to these bacteria is extremely important and critical to the treatment of these bacteria (Loc-Carillo et al. 2011).

Plates with *E. coli* 14-318 plaques were sent to be sequenced at GENEWIZ, Inc. Although a phage primer was used, phage genomes do not have the same conserved regions that bacterial genomes have, therefore the primer did not bind well to the *E. coli* 14-318 genome and the sequencing results were inconclusive (Hattful 2008). The plaques are now being sequenced at the University of Texas.

The results of this experiment were significant, as phages capable of killing MDR *E. coli* through phage screening were found to be present in local sewage and successful in multiple trials. Furthermore, the *E. coli* 14-318 strain possesses beta lactamases and is resistant to aminoglycosides, quinolones, tetracyclines, furanes, trimethoprims, and sulfonamides, which make antibiotic treatment nearly impossible. Therefore, the discovery of an alternative method to kill this bacteria strain is significant as no other common treatment method has been successful. In addition, these results contribute positively to phage therapy research and clinical trials in North America, and continued results of these trials will hopefully result in the FDA approving phage therapy as an alternative treatment method.

For future expansion of this research, more environmental water samples should be collected, including sewage and fecal samples from other cities and countries, considering feces is rich in phages specific to human pathogens and diverse sample sites could provide a wider range of phages. Phages could also be isolated from compost, landfills, and soil samples which are exposed to human wastes. In addition, more *E. coli* strains should be collected and screened to observe which strains the sewage phages are effective against, in order to further investigate the specificity of bacteriophages amongst a species.

Although there are downsides to phage therapy, such as the requirement for phages to be obligately lytic, stable in storage, safe in experimental studies, matched to specific bacteria, and fully sequenced to ensure they won't pass on toxicity, phage therapy is an effective therapeutic treatment method to treating bacterial infections, and could be used against bacteria which are resistant to antibiotics. This method has been used extensively in the former Soviet Union countries with success and is currently being researched extensively in the United States before it can be placed in clinical trials to be approved by the FDA as a treatment method. Along with phages being more

specific to target bacterial species and not having a negative effect on beneficial host microbiota, only one dose is needed for treatment as phages regulate their numbers based on the amount of target bacteria present and phages can be combined easily with other treatment methods, such as antibiotics and clay, to ensure the most effective and efficient treatment of bacterial infections. Although time, money, and energy need to be put into phage research and clinical trials, approval of this treatment method may be crucial for western medicine to counter the epidemic of antibiotic resistance. The benefits of phage therapy clearly outweigh the disadvantages.

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