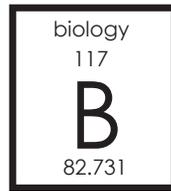


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CHARACTERIZING THE RESPONSE OF MULTIDRUG-RESISTANT *KLEBSIELLA* *PNEUMONIAE* SPECIES TO THE APPLICATION OF A PHAGE COCKTAIL

A RESEARCH PROPOSAL

by Steven Liu

Project Summary

The application of bacteriophages to treat bacterial infections is known as phage therapy, which takes advantage of bacteriophage's natural ability to infect and lyse bacterial hosts. Phages have been shaped by billions of years of evolution to be highly specialized deliverers of bactericidal agents to the cytoplasm of their target bacteria. Ever since discovery of bacteriophages in 1915, phage therapy was recognized as a potentially powerful tool for eliminating bacterial infections. The effectiveness of phage therapy can be increased by creating a mixture of multiple phages to target a wider variety of bacterial strains. Furthermore, phage therapy has many advantages over antibiotics. For example, phages replicate *in vivo*, so a smaller effective dose can be administered. Phages will also stop reproducing once the target bacteria is destroyed. Much of the evidence strongly shows that, appropriately administered, phage therapy is effective for treatment of bacterial infectious diseases, especially those caused by multidrug-resistant bacteria.

The worldwide spread of *Klebsiella pneumoniae* spp., which is resistance to a variety of antibiotics, threatens to revert modern medicine to a pre-antibiotic era. Infections caused by drug-resistant *K. pneumoniae* spp. are life threatening because of our limited ability to treat the associated disease symptoms. As a result, infections due to multidrug resistant *K. pneumoniae* spp. have an extremely high mortality rate. The design of a phage cocktail with potential therapeutic application against multidrug-resistant *K. pneumoniae* spp. may enable us to respond to a rapidly emerging generation of multidrug-resistant bacterial species.

Introduction

Basics of phage biology

Phages are naturally occurring viruses that specifically infect and lyse bacteria. The majority of reported phages are tailed phages, composed of an icosahedral head and tail, and all of them have double-stranded DNA as the genome (Matsuzaki, 2005). Phages can be further classified into two groups according to their life cycle. The lytic phage self-proliferates by intracellular development, maturation, and finally release of virions from the host cell resulting in the death of the bacteria. But the lysogenic phage integrates its phage genome into the bacterial genome, thereby allowing the phage genome to multiply synchronously with the host bacteria without destroying it. The generic life cycle of a phage begins with phage adsorption to a receptor on the bacterial surface, and then inserts phage DNA into the bacterial cytoplasm. The DNA is replicated, synthesized, assembled, and released from the bacteria (due to the lytic action of the proteins holin and endolysin), encoded by the phage genome, as shown in Figure 1. (Young, 1992). Distinct phage isolates achieve antibacterial activity by differing in their mechanism of entry or strategy of replication.

Phage therapy & its advantages

Phage therapy can be used for its lytic ability to control bacterial infection. There are multiple advantages of phage therapy over conventional chemical antibiotics. For example, there is no need to establish a dosage for phages because they are capable of replicating specifically where their hosts are located (Carlton, 1999). This eliminates the need to determine an effective dose of the drug which may be potentially dangerous if an excessive dose is administered. Only a single dose is required to achieve active therapy, resulting in greater bacterial clearance (Abedon & Abedon, 2010). In addition, phage therapy is minimally disruptive towards resident gut flora, unlike many chemical antibiotics, due to their host specificity. Phage host specificity also reduces the potential for inducing resistance by limiting the number of bacterial types, with which selection for specific phage-resistance mechanisms can occur (Hyman & Abedon, 2010; Skurnik et al., 2007; Gupta & Yogendra, 2011). From an economic perspective, the cost of phage production is relatively low compared with chemical antibiotics. Therapeutic phage products would therefore be more readily available to the general public, in contrast to several of the more expensive antibiotics available today (Skurnik et al., 2007; Kutter et al., 2010; Kramberger et al., 2010). The effectiveness of phage therapy can be increased by expanding the host-range of phages, because success depends largely on whether the phage can attach to the bacterial surface receptor. The host range may be limited to just a subset of strains within a single species, or it may include a broad range, spanning multiple bacterial genera (Hyman & Abedon, 2010).

Expanding host-range with phage cocktails

Phage infection can only occur after phage attachment to a bacterial cell receptor. Bacterial cell receptors may include components of the O-antigen, outer core oligosaccharide of the lipopolysaccharide, and a diversity of outer membrane proteins (Hashemolhosseini et al., 1994). Consequently, phage cocktails are prepared to broaden host range because the recognition of bacterial receptors is not universal (Chan et al., 2013). Phage cocktails are an amalgam of phages, each possessing different host ranges. Collectively,

they expand the host-range to confer a broader spectrum of activity against infectious bacteria (Fig. 2). Additionally, phage cocktails prevent the rapid emergence of phage-resistant strains. Bacteria may evolve resistance to a specific phage, but it would be unrealistic for the bacteria to simultaneously develop multiple resistance mechanisms to a collective group of phages (Goodridge, 2010; Tanji et al., 2005).

Recent advances of phage therapy

Currently, there are no commercially available therapeutic phages. The bulk of phage therapy research is concentrated in Eastern Europe and Soviet Union. The US and Europe lost much of their interest in phage therapy after the discovery of antibiotics, but Soviet research continued (Kutatekadze & Revaz, 2010). However, due to multidrug resistance, interest in phage therapy is increasing in the US and Europe leading to several clinical trials as shown in Table 1. A Phase II clinical trial for phage therapy was recently completed by Wright et al., who reported long-term positive results for ear infections, burn wounds, and leg ulcers caused by *Pseudomonas aeruginosa* infections (Merril et al., 2003; Harper & Enright, 2011). This demonstrates the effectiveness of phage therapeutics in *P. aeruginosa*, a bacterial species that has evolved multidrug resistance.

While there are no current therapeutic applications of phages, the United States Food and Drug Administration (FDA) has approved spraying meat with phages to eliminate *Listeria monocytogenes*, a highly virulent food-borne pathogen that was the primary cause of listeriosis, in August 2006 (Ramaswamy, 2007). ListShield is a phage cocktail containing six listeria-specific phages designed to attack a very broad spectrum of pathogenic *L. monocytogenes* strains. The phage preparation is sprayed on food surfaces, and later consumed without any significant side effects. Strict phage-host specificity targets only pathogenic *L. monocytogenes* strains. At the very least, this should demonstrate that direct human use of phages is likely to be very safe (Mai et al., 2010).

Current therapeutic treatment options

Excessive use of antibiotics has reinforced selective pressure for antibiotic resistance, accelerating the emergence of these “superbugs” (Drawz & Bonomo, 2010). As a result, our antibiotic armamentarium is exhausted, severely restricting our ability to respond to new multidrug-resistant threats. The antibiotic pipelines are nearly dry, failing to deliver novel antibiotics to match today’s multidrug resistance crisis. Only two new classes of antibiotics have been developed in the past 3 decades (Theuretzbacher, 2012; Gupta & Nayak, 2014). Treatment options for multidrug-resistant infections are extremely limited. While most isolates remain susceptible to colistin and tigecycline, their therapeutic viability is questionable. Recurring infection after treatment with tigecycline increased the MIC (minimum inhibitory concentration) significantly (Daly et al., 2007); colistin usage should be limited due to its neurotoxicity and nephrotoxicity (Zavascki et al., 2007).

Targeting *Klebsiella pneumoniae* carbapenemase (KPC)-producing *K. pneumoniae* with a phage cocktail

Klebsiella pneumoniae carbapenemase (KPC)-producing *K. pneumoniae* are becoming a growing menace due to their resistance to β -lactam antibiotics and nearly all commercially available antibiotics; this includes carbapenems, which are considered a drug of last resort against highly resistant microorganisms. The enzyme *K. pneumoniae* carbapenemase was first discovered in *K. pneumoniae* but has since been

found in other enterobacteriaceae. More than two million people are affected by antibiotic-resistant infections with at least 23,000 deaths. They are a leading cause of nosocomial infections. The first KPC-producing *K. pneumoniae* outbreaks were reported in hospitals in New York City before spreading globally to foreign healthcare environments (Bratu et al., 2005; Leavitt et al., 2007). KPC-producing *K. pneumoniae* are responsible for an estimated 9,000 drug-resistant infections per year and 520 attributed deaths (Bell et al., 2009). The attributable mortality rate due to KPC-producing *K. pneumoniae* bacteremia infections is 50%, the highest reported so far for any microorganism causing bacteremia, requiring more intensive patient care and exhausting the current antibiotic arsenal (Borer et al., 2009; Patel et al., 2008).

Hypothesis

A phage preparation consisting of multiple phages specific to *K. pneumoniae* will successfully infect and lyse bacterial host cells of various KPC-producing *K. pneumoniae* strains, due to the preparation's broad spectrum of activity.

Primary Objectives

- Screen fecal material obtained from the San Luis Obispo Wastewater Treatment facility as a source of *K. pneumoniae*-specific phage and inoculate with several representative strains of *K. pneumoniae* to perform a plaque assay.
- Isolate several phages based on their ability to produce lawns on the KPC-producing *K. pneumoniae* host bacterium and identify and classify the phages.
- Propagate phages until the desired yield of amplification is attained, and then purify and precipitate phages for use in in vitro assessments of its effectiveness.
- Test effectiveness by adding the phage cocktail to all KPC-producing *K. pneumoniae* strains grown in culture, and assessing the phage cocktail's ability to lyse the KPC-producing *K. pneumoniae* cultures.

Methods

Bacterial strain. Due to the moderate threat posed by the resistant *K. pneumoniae* strains, all microbial cultures will be handled according to the appropriate safety procedures and precautions corresponding to Biosafety Level 2 (Chosewood et al., 2009). Several representative strains (BAA-1705, BAA-1900, BAA-2078, BA-2342, BAA-2343 and BAA-2344) of *K. pneumoniae* that produce the enzyme KPC will be ordered from the American Type Culture Collection (ATCC) for phage isolation, amplification, and evaluation. All strains are resistant to a broad spectrum of antibiotics, including carbapenems, making them ideal models for the assessment of a phage cocktail. The strains will be grown at 37°C in the supplied ATCC® Medium. This media contains nutrient agar (beef extract, peptone, and agar). The strains will be stocked in nutrient broth containing 40% glycerol and stored at -80°C. Cultures will be stored at 4°C and transferred periodically.

Bacteriophage isolation and enrichment. Fecal material will be screened as a source for *K. pneumoniae* strain-specific phages. Fecal samples will be obtained from the San Luis Obispo Wastewater Treatment facility over a four month period. Portions of the samples will be added to volumes of early log-phase *K. pneumoniae* (grown at 37°C) containing 10 mM CaCl₂. These cultures will be incubated overnight at 37°C with shaking (200 rpm). Samples will be withdrawn and centrifuged in a microcentrifuge for 5 min. The supernatant will be removed and passed through a sterilized filter before being subjected to a plaque assay using *K. pneumoniae* as the host. Plaques appearing on lawns on the host bacteria will be further processed to achieve desired phage stock of phage isolate candidates (Bourdin et al., 2013).

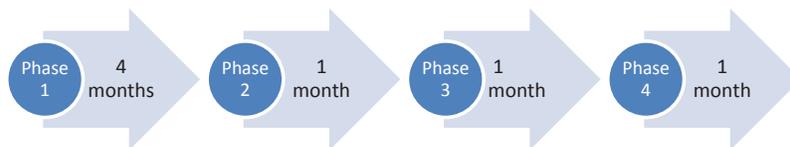
Phage morphology characterization. Identification and classification of phage candidates will be determined by observing the morphology of the phages by transmission electron microscopy. Phage particles will be sedimented at 25,000 g for 60 min. using a centrifuge, and then the phage particles will be washed twice in ammonium acetate (pH 7.2) and deposited on copper grids provided with carbon-coated Formvar films. They will be stained with 2% potassium phosphotungstate (pH 7.2) and examined in a Philips EM 300 transmission electron microscope (Bourdin et al., 2013).

Phage amplification and purification. Phage candidates will be amplified in a 2-L Erlenmeyer flask filled with 4 L of LB medium (grown at 37°C and pH maintained at 7.0 with NaOH 10%). The medium will be inoculated with 3% of an overnight *K. pneumoniae* culture. At OD₆₀₀ of 0.5, the bacteria will be infected with the phage lysates at a multiplicity of infection of 1. The flask will be placed in an incubator and shaken for 7 hours (400 rpm). Phages from the combined phage lysates will be separated from organic debris by low speed centrifugation, and then by additional ultracentrifugation to pellet the phages. The pellet will be re-suspended in phage buffer (150 mM NaCl and 10 mM Mg SO₄ in sterile water at pH 7.2) (Bourdin et al., 2013).

Phage cocktail testing. A 50 µl of *K. pneumoniae* ATCC BAA-1705, BAA-1900, BAA-2078, BA-2342, BAA-2343 and BAA-2344 overnight culture will be inoculated into 5 ml of LB broth and incubated with shaking at 37°C. The change of culture turbidity of OD₆₆₀ will be automatically recorded by photo recorder without fractionating the sample from the culture. When the culture reaches an OD₆₆₀ of 0.1, the phage cocktail will be added at a multiplicity of infection of 10. The subsequent OD₆₆₀ of the culture will be monitored. All assays will be performed in triplicate to confirm reproducibility and assess the effectiveness of the phage cocktail (Tanji et al., 2005). The effectiveness of the phage cocktail will be determined by measuring the optical density of the culture before and after treatment with the phage cocktail.

Statistical analysis. The OD₆₆₀ value of the bacterial culture before application of the phage cocktail will be recorded for each strain. After the addition of the phage cocktail, the subsequent OD₆₆₀ value of the bacterial culture will be recorded. The mean OD₆₆₀ values of the triplicate assay of all bacterial strains will be used for data analysis. An independent two-sample t-test will be performed to determine whether there is significant bacterial reduction after the application of a phage therapy. The data set is assumed to be equal in sample size and variance because it is the same population of bacteria being tested. Calculations will be performed in R, a free software programming for statistical computing. A significant difference will suggest that application of a phage cocktail is effective in bacterial reduction, and the opposite is true if there is no significant difference.

Timeline



Phase 1	A major portion of the research will be focused on screening fecal samples and selecting phages from an environmental source for use in a phage cocktail. Once appropriate phage candidates have been selected based on their ability to lyse KPC-producing <i>K. pneumoniae</i> strains, they will be enriched, amplified and purified.
Phase 2	Candidate phages will be identified and classified based on their morphology using transmission electron microscopy. This is to ensure they are suitable for use and do not produce any toxins.
Phase 3	Candidate phages will be amplified and purified to produce the final phage cocktail product.
Phase 4	The effectiveness of the phage cocktail will be tested by administering the phage cocktail to all cultures of KPC-producing <i>K. pneumoniae</i> strains, and assessing the optical density of the broth culture as a proxy of bacterial lysis.

Budget

Salary and Wages	\$2800
Equipment	\$0
Materials and Supplies	\$1,770
Travel Costs	\$0
Total Direct Costs	\$4,570

The undergraduate student researcher will be paid \$10 per hour and will spend 10 hours a week for 7 months on this research project. The student will therefore be paid a total of \$2800. KPC-producing *K. pneumoniae* strains ordered from ATCC will cost \$295 each, thus six strains of the bacteria will cost \$1,770 in total. All other equipment such as a spectrophotometer, ultracentrifuge, and a transmission electron microscope will be available for use due to collaboration with the Biology and Biomedical Engineering Department at California Polytechnic State University, San Luis Obispo. There will be no travel costs associated with this research.

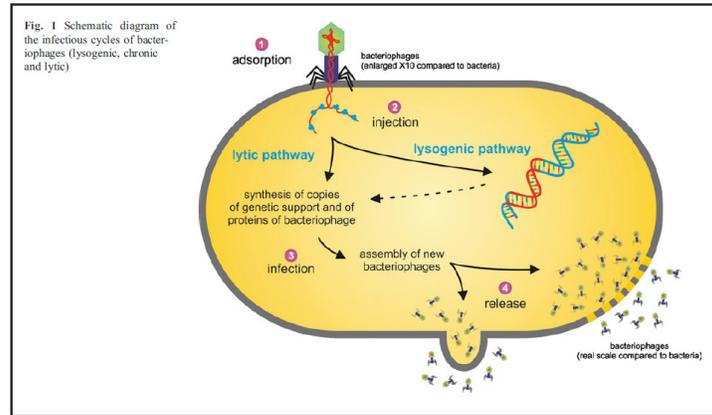


Figure 1. This shows the infectious life cycle of bacteriophages. It begins with adsorption, injection of genetic material, infection, and then the release of newly assembled bacteriophages.

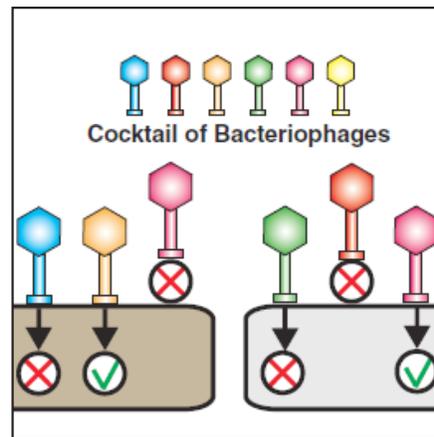


Figure 2. This illustrates the benefit of using a cocktail of bacteriophages by broadening the host-range to confer greater infectivity against multiple bacterial strains.

Target	Entity	Characteristics	Dosing	Phages ¹	Reduction ²	Comments	Ref.
<i>E. coli</i>	Cattle	Colonization	Oral	m × 4	–	Marginally positive reductions	[97]
<i>P. aeruginosa</i>	Human	Otitis	Topical	u × 6	10 ¹	Phase VII clinical trial; indication of efficacy with no adverse effects	[44]
<i>E. coli</i>	Food	–	–	m × 3	–	Reductions up to 100%	[79]
<i>Salmonella enteritidis</i> ser. Enteritidis	Poultry	Colonization	Spray	u × 3	10 ^{1.7}	Phage treatment prior to bacterial inoculation	[98]
<i>S. enteritidis</i> ser. Enteritidis	Poultry	Colonization	Oral	u × 4 or u × 45	–	Substantial early reductions in colonization (45–100%) but less later (15%)	[99]
<i>P. aeruginosa</i>	Murine	Burn wound	im., ip. or sc.	u × 3	–	12% mortality with phage treatment (ip.) and 94% without	[43]

Entries are listed in decreasing date and then author alphabetized order.
¹Lettering indicates different phage types – Myoviridae (contractile tails), Podoviridae (short tails), Siphoviridae (long noncontractile tails) and uncertain – with one letter for each phage used in a cocktail. Standard practice is to propagate cocktails as individual phage stocks that are then mixed together.
²Indicated is maximum average reduction seen.
 Enterococcus faecalis³: Vancomycin-resistant Enterococcus faecalis; im.: Intramuscular; in.: Intranasal; ip.: Intraperitoneal; m: Myoviridae; NA: Not applicable; p: Podoviridae; s: Siphoviridae; sc.: Subcutaneous; ser.: Serovar; u: Uncertain.

Table 1. This table highlights several recent investigations of bacterial reduction using phage cocktails.

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ABOUT THE AUTHOR

STEVEN LIU is a senior biological sciences student and microbiology minor. After graduation, he wishes to attend graduate school and then apply to an epidemiology or public health PhD program. His ideal career would be to join the Epidemic Intelligence Service with the Center for Disease Control and Prevention (CDC). Steven finds it amazing that the life sciences hold so much potential to benefit the health of humans. “Even after so many groundbreaking medical breakthroughs and advances, there still remains so much potential in the life sciences to improve the overall health of communities worldwide,” Steven said. He finds the piece he submitted to *Symposium* on antibiotic resistance is a serious topic that has yet to be fully understood. Steven believes that the impact of antibiotic resistance is important because many current medical processes and procedures require antibiotics. “I hope my piece will shed more light on antibiotic resistance and the severity of the situation,” he said. The labs and professors, such as Dr. Winstead and Dr. Black, in the College of Science and Math have inspired him to delve into the microbial world and the explore the huge impact that such microorganisms may have on our lifestyle.