Title of Document: THERAPEUTIC USE OF BACTERIOPHAGE AND ANTIBIOTIC FORMULATIONS FOR THE TREATMENT OF ANTIBIOTIC RESISTANT ACINETOBACTER BAUMANNII

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Abstract

Widespread use of antibiotics has enriched global bacteria populations for strains possessing antibiotic resistance (AR) genes. Proliferation of AR genes and mechanisms have resulted in numerous multidrug resistant (MDR) infections for which there are no effective treatments. *Acinetobacter baumannii* is a major cause of hospital acquired (nosocomial) infections and is associated with outbreaks of MDR infections. Virulent bacteriophages (phages) present a way to remedy bacterial infections, while also having built-in mechanisms to circumvent resistance. This proposed study aims to develop a phage therapeutic targeting antibiotic resistant *A. baumannii.* The phages chosen for the final formulation exhibited high bactericidal activity and were able to infect several strains of *A. baumannii* from a provided library. Additionally, the phage-antibiotic synergy (PAS) effect was investigated in formulations with sub-lethal doses of ampicillin and chloramphenicol. The effectiveness of the phage therapeutic at different multiplicity of infections (MOI) and antibiotic concentrations were assessed relative to standard

antibiotic doses. Well-plate studies suggest that higher MOI and antibiotic concentrations resulted in the greatest initial bactericidal effects, longest time to develop resistance, and lowest overall bacteria concentration. In future formulation studies, we would like to expand and optimize the current phage-antibiotic formulation and explore cocktail effects, whereby the formulation consists of a mixture of different phages that increases selective pressure.

Therapeutic Use of Bacteriophage and Antibiotic Formulation for the Treatment of Antibiotic Resistant *Acinetobacter baumannii*

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List of Abbreviations

Amp: ampicillin

APIC: Association of Professionals in Infection Control and Epidemiology

AR: antibiotic resistance

BSA: bovine serum albumin

Cam: chloramphenicol

CFU: colony forming units

ESKAPE: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter spp.*

ESP: electropositive silica gel particles

ICU: intensive care unit

LB: Luria-Bertani

MCB: master cell bank

MDR: multidrug resistant

MIC: minimum inhibitory concentration

Min: minocycline

MOI: multiplicity of infection in PFU/CFU

MPB: master phage bank

NDA: new drug application

OD: optical density

OD600: optical density at 600 nm

PBP: penicillin-binding protein

PBS: phosphate buffered saline

PFU: plaque forming units

PMB: polymyxin B

RPM: revolutions per minute

VNP: viral nanoparticles

WCB: working cell bank

WPB: working phage bank

Introduction

The discovery of antibiotics in the early 1900's ushered in a golden age of modern medicine. Sulfonamides and penicillin were the first effective antibiotics and were hailed as a panacea which would end the struggle against disease (1, 2). However, this was a fleeting reprieve as β-lactamases, bacterial enzymes which enable resistance to β-lactam antibiotics such as penicillin, were discovered (3–5). Numerous other classes of antibiotics were developed and were followed by the emergence of resistance mechanisms. Initially, this was somewhat inconsequential as there were multiple antibiotic options available. However, major issues have arisen with multidrug resistant (MDR) bacteria, against which standard antibiotics have either reduced effects or none at all (6). Currently, the only remaining treatment options for MDR infections are antibiotics of last resort, which have severe side effects and may cause long-term damage even if the bacterial infection is eliminated (7). Experts fear that it is only a matter of time until even these antibiotics become obsolete due to the emergence of resistant bacteria. Therefore, it is important that new treatment avenues be explored.

Utilizing bacteriophages to treat antibiotic resistant (AR) infections shows great promise, despite having been under-researched as a therapeutic for nearly a century in the US. However, they have seen use in Eastern Europe as a therapeutic agent, even before the advent of antibiotics. Phages are a specific subset of viruses which infect bacteria and archaea and replicate through a lytic and/or lysogenic cycle. Virulent phages are capable of replicating only through a lytic cycle whereas temperate phages are capable of replicating through both a lytic and a lysogenic cycle. Virulent phages are preferred for

therapeutic use as cell death and phage amplification occurs faster compared to temperate phages where lysogenic cycles may delay both. Phages infect bacteria in a highly specific manner through interactions between specialized phage proteins and cognate receptors on the bacterial surface. Once bound to the bacterium, the phage initiates an infection which eventually generates more phages and lyses the host bacterium. This results in a chain reaction, from which the new phages infect more bacteria until there are no susceptible and permissive bacteria remaining. Administering phages to eliminate pathogenic bacteria could be an effective treatment for bacterial infections and could be especially useful in cases where multidrug resistance is present (8). The purpose of this study was to generate phages capable of treating infections associated with clinically relevant AR diseases.

In this study, *Acinetobacter baumannii* was used to test phage treatments. *A. baumannii* is an aerobic, Gram-negative coccobacillus. It is a major cause of nosocomial and battlefield infections, is frequently associated with outbreaks of MDR infections, and has been declared one of the most serious ESKAPE organisms; a class of bacterial pathogens that are commonly known for their antimicrobial resistance. Antibiotic resistance in *A. baumannii* is mediated through a myriad of mechanisms contributing to the immense difficulty of treating the infections it causes (9). Phage variants active against *A. baumannii* will be derived and tested both individually and in combination in order to characterize their bactericidal activity *in vitro*. Additional experimentation will be performed to quantify the efficacy of phage cocktails at treating potentially lethal bacterial infections in combination with antibiotics.

Literature Review

Antibiotic Resistance

1.1 Overview

With the introduction of penicillin in the 1940's, the era of antibiotics began. New antibiotics were produced and prescribed in droves, even in situations where such treatment was unnecessary or inappropriate. However, the misuse of antibiotics is now recognized as the driving force behind increasing bacterial resistance to treatment. Antibiotics, when not administered appropriately, provide selective pressure to a population of bacteria, thus prompting the evolution and enrichment of AR populations (11). Countries with high rates of antibiotic use tend to have a greater issue with widespread infection from AR bacteria. For instance, Greece has three times the antibiotic consumption of the Netherlands and 51% of *S. aureus* bacterial infections in Greece are antibiotic resistant, compared to 1.6% of similar infections in the Netherlands (5). In the United States, where up to 50% of antibiotics prescribed are not needed or are not optimally effective as used, more than 2.8 million people every year fall ill due to resistant bacterial strains, resulting in more than 35,000 deaths every year (11, 12).

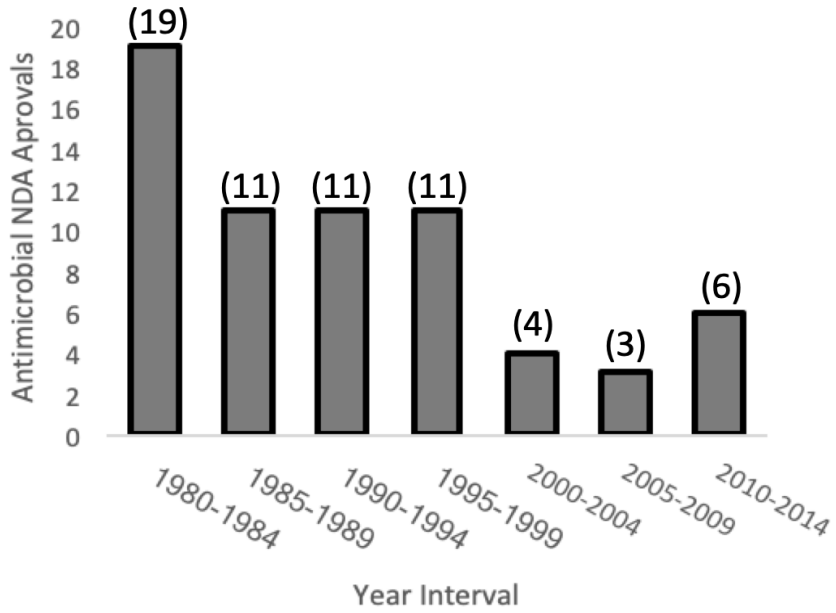

Figure 1: Trend of FDA antimicrobial NDA approval within 4-year intervals

Because of the lack of satisfactory treatment options, individuals who acquire infections of AR bacteria have been found to have significantly higher mortality rates than those infected by a more sensitive strain; 32% compared to 17% in one 30-day study of third-generation cephalosporin-resistant *E. coli* infected patients. After adjusting for confounding factors, patients with a cephalosporin-resistant *E. coli* bloodstream infection were estimated to have a 90% greater mortality risk than patients with a cephalosporin-susceptible strain of *E. coli* (14). Even when the resistant infection is successfully treated, survivors have longer hospital stays and recuperation periods, during which they are more likely to acquire a long-term disability as a result of their illness or the treatment administered. This results in both a reduced quality of life for the patient and increased strain on hospitals and healthcare systems. In the United States, the added burden on the healthcare system due to antibiotic resistant infections is estimated to be as high as $20 billion in direct costs, and more than $35 billion in lost productivity (5). In addition to an exacerbated financial burden, there is an immense time challenge in developing novel

and potent antibiotics. As the breadth of resistance expands, it takes longer to discover and approve a compound capable of circumventing this resistance. This trend is illustrated in **Figure 1** where the number of Food and Drug Administration (FDA) approved antimicrobial new drug applications (NDA) have been slowly decreasing in recent year (14).

The Centers for Disease Control and Prevention (CDC) recommends four strategies to combat the threat of antibiotic resistance: 1) The prevention of bacterial infections; 2) The development of a system for tracking data on resistant bacteria; 3) The improvement of policies concerning antibiotic stewardship, particularly the regulation of unnecessary antibiotic prescriptions; 4) The identification of novel drugs to combat bacterial infection, ideally in a continuous fashion in order to effectively combat evolving bacterial resistance (11, 15). Research regarding alternative solutions to combat antibiotic resistant bacterial infections is expanding and represents a crucial component of humanity's fight against disease.

1.2 Relevance to *Acinetobacter baumannii*

One common cause of antibiotic resistant bacterial infections is *Acinetobacter baumannii. A. baumannii* is one of the most successful pathogens in modern healthcare primarily due to its unparalleled ability to acquire antimicrobial resistance determinants (16). Instead of employing one or two resistance mechanisms like most bacteria, *A. baumannii* strains have been found to naturally possess β-lactamases, aminoglycoside-modifying enzymes, MDR efflux pumps, permeability defects, and modifications of common antibiotic target sites. In all, there are nearly 200 published resistance determinants found in *A. baumannii* strains (17). Due to the plethora of resistance

mechanisms associated with many *A. baumannii* infections, the only remaining treatment options for MDR infections rely on combinations of multiple antibiotics which almost always include colistin, an antibiotic of last resort (17).

The required use of colistin to treat MDR *A. baumannii* infections is not ideal. Colistin was originally removed from the pharmaceutical market in the 1970's due to its dose dependent neurotoxic and nephrotoxic side effects (18). While the neurotoxic effects of colistin are nearly always reversible, nephrotoxicity represents the greatest problem associated with its therapeutic usage. A study of 288 hospital patients given 317 colistin treatment regimens found that 95% of adverse reactions occurred in patients receiving no more than the recommended dose of the antibiotic (19). Neurotoxic effects were observed in 7.3% of patients, while 20.2% of patients experienced nephrotoxicity, with 1.9% experiencing acute tubular necrosis (19). These adverse reactions contributed to the deaths of 4.5% of patients treated with colistin (19). Additionally, *A. baumannii* is a frequent cause of urinary tract infections.

The emergence of colistin resistance, despite its judicious usage in the treatment of infections and adherence to the Association of Professionals in Infection Control and Epidemiology (APIC) 2010 guide to the elimination of MDR *A. baumannii* transmission in healthcare settings, illustrates just how dire the *A. baumannii* MDR situation is (20). Therefore, it is of paramount importance to pursue alternative methods of treating MDR *A. baumannii* infections, especially as the pipeline of antimicrobials that act through novel mechanisms remains barren (21).

Antibiotics are chemically defined antimicrobial agents used to treat bacterial infections. They are typically grouped into classifications based on their mechanism of

action or chemical structure. One common identifier for antibiotics is bactericidal versus bacteriostatic activity. Bactericidal antibiotics are those that directly elicit cellular death and lysis. This is effective at treating infections, however prolonged cellular lysis will often initiate an immune response due to an endotoxin surge after the release of cellular wall fragments and intracellular proteins. Potentially fatal inflammatory responses can result. Bacteriostatic antibiotics are those that inhibit growth of cells, resulting in delayed eradication of the bacteria. These antibiotics are often further grouped by their specific mechanism of action and cellular targets. The three broad mechanisms involve disrupting the bacterial cell envelope, inhibiting production of new proteins, and inhibiting DNA replication (22).

For this study, antibiotics are required for three reasons: as a tool for eradicating cultures if necessary, as a control to compare effectiveness of phage therapy, and to measure synergistic effects with phage. Four antibiotics representing diverse mechanisms of action were chosen. Studies have shown that use of multiple and diverse antibiotics offers several advantages. These include a wider antimicrobial spectrum, possible synergistic effects, and decreased chance of resistance emerging In this section, the classification and mode of action for each antibiotic studied will be discussed. Shown in ***Figure 2*** is the structure of the four antibiotics being studied.

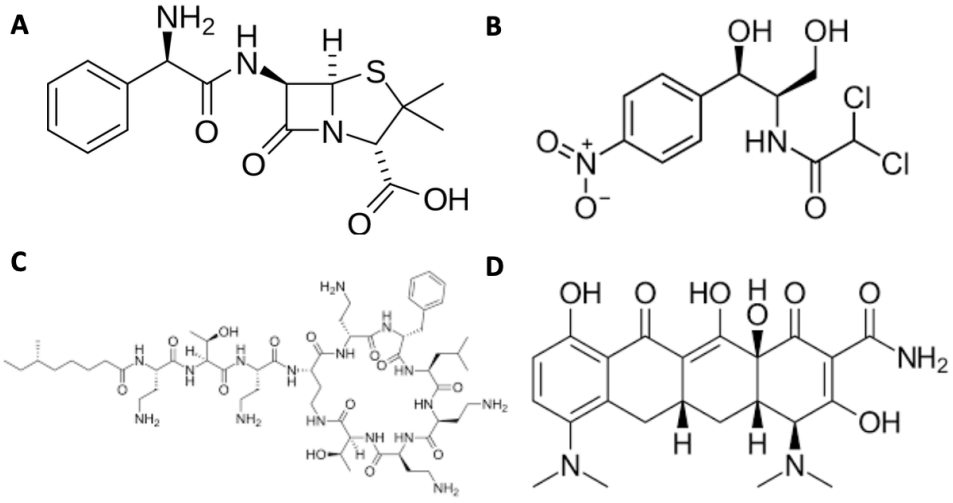

Figure 2: Structure of utilized antibiotics (A) ampicillin, (B) chloramphenicol, (C) polymyxin B, and (D) minocycline

The first antibiotic studied was ampicillin. This is classified as an aminopenicillin under the beta-lactams family. This bactericidal agent operates in two steps. In the first step, ampicillin binds to a surface receptor called membrane-bound penicillin-binding protein (PBP). These proteins regulate bacterial peptidoglycan cell wall synthesis (23). Peptidoglycan is an essential protein in maintaining cell wall integrity. Once bound, this inhibits the synthesis of peptidoglycan, ultimately resulting in cellular death and lysis. It is one of the most common antibiotics and is comparatively less toxic.

The second antibiotic utilized was chloramphenicol. This is a bacteriostatic antibiotic that inhibits protein synthesis. This is achieved by binding to the 50S ribosomal subunit. This inhibits peptidyl transferase, an enzyme that catalyzes amino acid elongation during protein synthesis. Without protein synthesis, growth is halted and ultimately the cells will die (24).

Polymyxin B is another bactericidal cell wall inhibitor that is only useful for treating gram-negative bacteria. The mechanism of action requires the α,γ-diaminobutyric

acid moiety of the polymyxin B bind to a negatively charged phosphate of a lipopolysaccharide molecule on the cell wall. This displaces divalent cations, destabilizing the lipopolysaccharide and increasing the permeability of the cell wall. This ultimately results in cytoplasmic leakage and cellular lysis (25). It is considerably toxic provided it can accumulate in renal cells and bind to surface proteins. This results in nephrotoxicity and renal dysfunction (26).

Minocycline was the final antibiotic investigated. This bacteriostatic antibiotic is grouped in the tetracycline family, which inhibits protein synthesis. It has multiple mechanisms of action, making it a very potent drug. The most prominent mechanism involves binding to the 30S ribosomal subunit and preventing aminoacyl-tRNA from binding. In doing so, protein synthesis is inhibited, and growth cannot continue. It has also recently been used for its anti-inflammatory effects (27).

Bacteriophages

2.1 Background

Bacteriophages are one of the potential alternative methods of treating antibiotic resistant bacteria. They are a type of virus that specifically infect only bacteria and are vastly abundant in nature, with an estimated 10^{32} total virus particles distributed throughout the planet (4). During lytic development, certain phages replicate by inserting their genetic material into a bacterium, then hijack the bacterial machinery to express their proteins and replicate their genetic material. They are then assembled inside of the bacterium before eventually causing the cell to lyse (28). Due to their abundance and

bactericidal activity, phage therapy is a promising yet insufficiently researched tool for solving the antibiotic resistance crisis.

Phages were first discovered in 1896 by Ernest Hanbury Hankin, a British bacteriologist working in India. He demonstrated that an unidentified substance in the waters of the Ganga and Yamuna rivers had the ability to kill cultures of cholera-inducing bacteria. He found that this "substance" was able to pass through filters with microscopic pores, and he published his work in the *Annals of the Pasteur Institute* (4). Reports of similar findings were abundant in the following years when Frederick Twort, also a British bacteriologist, proposed that the "substance" was a virus (29). In 1910, French-Canadian microbiologist Félix d'Herelle was studying patients with bacillary dysentery, and isolated what he called an "anti-Shiga microbe" by filtering stools from shigellosis patients. He found that the microbe was able to induce lysis in cultures of the bacilli, and named the microbe a "bacteriophage" (4).

D'Herelle continued his work, publishing numerous nonrandomized clinical trials. His work eventually caught on, and scientists around the world began researching phages (4). However, due to insufficient understanding of phage biology and inconsistent results in clinical trials, research into phage therapy was delayed in the United States. Once antibiotics were introduced, phage therapy research practically came to a halt in the Western world (30).

Recent research has expanded the knowledge of phage mechanisms and functionality considerably. Most phages consist of a capsid head, containing the phage DNA, as well as tail fibers. Phages either undergo the lytic or the lysogenic life cycle, or sometimes both. In both cycles, a phage infects a bacterium by binding to receptors on

the surface of the bacterium, and ejecting its DNA into the cytoplasm of the bacterium. In the lytic cycle, the phage is copied, and the phage genes are expressed to produce proteins needed for phage assembly. Progeny virions are then assembled inside of the bacterium until the bacterium lyses. In the lysogenic cycle, the phage DNA is integrated into the bacterial chromosome through recombination, creating a prophage. In this case, the cell survives. Latent prophage is stored in the bacterial chromosome until it induces and thus resumes the lytic cycle (28).

Today, with antibiotic resistance becoming more prevalent, there is a pressing need for an alternative treatment for bacterial infections. The spotlight in the United States is beginning to return to phage therapy research. However, there are many barriers to phage therapy becoming useful for treatment in humans in the United States mainly due to a lack of an appropriate regulatory framework that can address the specifics of viral therapeutics (29).

2.2 Research Applications

Despite the potential hurdles for making phage therapies into viable treatments, they have great potential for research and development. This is partially due to the fact that viruses have evolved to act as a packaging entity in which their proteins self-assemble to form a capsid that transports and protects the nucleic acids essential to replication. To take advantage of this functionality, researchers have genetically or chemically modified phages in order to alter their surfaces or contents to create viral nanoparticles (VNP) with specific properties useful for expanding applications in biotechnology and various industries. Research with phages has directly contributed to

developments in the fields of agriculture, food safety, diagnostic testing, pharmaceutical modeling, and other health-related fields.

VNPs have profound implications in the biotechnology sector, given that phages are incapable of integrating and replicating in mammalian cells and are biodegradable due to their protein-based structure, thereby minimizing their persistence *in vivo* (30). One highly investigated application is that of the phage display, in which unique polypeptides are bound to the protein coat of the phage through chemical conjugation and complexation or genetic modification. These proteins can be used in pharmaceutical modeling to investigate receptor-ligand interactions between the desired antigen and antigen variants, captured in phage display libraries. In the same sense, protective antigens can be forcibly expressed on phages in vaccines in order to activate the innate immune system by uptake from antigen-presenting cells, thereby initiating a cytokine profile against the epitope of interest (9). Beyond ligands and antigens, phages can also be manipulated to express antibodies or enzymes to enhance immunotherapy treatments. One such treatment utilizes a genetically modified filamentous phage that displays a cocaine-sequestering antibody, which inhibits its activity and prevents it from influencing the central nervous system thereby treating addiction (31).

Another potential application of VNPs is in diagnostic testing in a process referred to as phage typing. A process that began in the early 1960s, phages have been used in conjunction with fluorescently labeled antibodies to identify unknown microbial strains. The unique specificity between a phage and its target permits this generalization (32). The procedure has been refined through analysis of plaques resulting from phage-

induced lysis and delivery of reporter genes that results in expression of measurable proteins, such as fluorescent compounds (9).

Phages are a novel alternative to other food sterilizing chemicals because of their specificity to bacteria. The recent emergence of phage applications in the food safety industry has produced a variety of products that combat common pathogens found on raw meat. One such application is the distribution of lytic phages on chicken breasts via a disinfectant spray that directly targets and eliminates *Salmonella* (33). Bactericidal sprays such as these can eliminate the potentially pathogenic bacteria without introducing a new organism that can be harmful to the consumer.

Phages have also been used to combat plant infections that have plagued the agriculture industry. One study displayed the effectiveness of a phage product that reduces the impact of bacterial wilt (a bacterial infection that kills crops) and paves the way for a more sustainable approach to agriculture (34). These are just a few examples that display the impact phages have had in the food safety and agricultural industries. Much research is to be done in these respective fields with phages, but given the current literature it is clear that the potential applications of phages are wide-reaching and practical.

2.3 Therapeutic Applications

Though not as thoroughly investigated, phages also have many potential therapeutic applications. Given the increasingly threatening rise of antibiotic resistant bacteria, phages can offer a viable alternative to traditional antibiotics as a sort of "biological drug."

2.3.1 Advantages Over Antibiotics

The evolution of increasingly antibiotic resistant strains of bacteria far outpaces the rate of research and development of novel antibiotics (5). Phages have an advantage over antibiotics in this arena because they are one of the most abundant organisms on earth (35). For virtually every bacteria on earth, there is a phage to which it is susceptible. Additionally, coevolution between phages and the bacteria they target prevents the emergence of long term resistance. In one study, researchers illustrated the adaptability of *V. cholerae* phages to counteract the resistance their bacterial target developed toward them. While the various strains of *V. cholerae* used in the study employed a CRISPR/Cas system (an enzymatic system which allows for the insertion and/or deletion of DNA sequences) to inhibit the assembly of certain nucleic acids necessary for lytic infection, the phage also used a CRISPR/Cas system to work around the phage inhibitory chromosomal island allowing it to infect the bacterial target (36).

Another advantage that phages have over antibiotics is significantly lower toxicity. In a 2017 study by Debarbieux et al, it was found that the lysis of *E. coli* cells by β-Lactams released more endotoxins than lysis by phages. Endotoxins, such as lipopolysaccharides, can be found in the outer membrane of gram-negative bacteria. They cause an immune response in the tissue affected by the infection and are responsible for the physiological symptoms of infection and septic shock. Additionally, in this study, it was shown that the phages were much faster at lysing the *E. coli* than the antibiotics *in vitro* (37).

In addition to lower toxicity, phages are also far less disruptive to normal fauna than antibiotics (38). Due to their lack of target specificity, antibiotics kill “good”

bacteria as well as the bacteria causing the infection. In fact, development of broad-spectrum antibiotics was favored until the drawbacks of such treatments became more apparent (39). Unlike antibiotics, phages kill only their narrow host range and are therefore much more effective at killing the bacteria that should be removed and avoiding the bacteria that is useful and necessary in the body.

Phages are also capable of auto-dosing. Phages replicate at the site of infection within their target bacteria (40). This maintains and increases titer of phage at the infection site thus when the bacterial hosts are at high density dosing and re-dosing the infection with killing agents (41). When the infection has subsided and hosts are no longer available for the phage, the phage replication subsides as well.

2.3.2 Use Against Biofilms

Another benefit of bacteriophages is their ability to penetrate biofilms. whereas antibiotics cannot. Biofilms consist of bacteria that attach to a surface and create an extracellular matrix for support, essentially creating a three-dimensional bacterial colony. Studies show that phage treatments are able to penetrate and destroy biofilms more effectively than antibiotics due to the extracellular polymeric substance (EPS) depolymerases that they may produce (42). Biofilms are often resistant to antibiotics and other antimicrobial agents, and biofilm infections usually require treatment with powerful antibiotics for a prolonged period of time (5). Phages, however, are capable of spreading radially throughout the biofilm while undergoing the lytic cycle and in some cases can destroy it with one dose (43). As biofilms age, they become more resistant to antibiotics and stronger doses must be used. However, phages have been shown to be effective against biofilms of various stages of maturation (44). Antibiotics have also been found to

be less effective against biofilms containing multiple species of bacteria, which are more common in natural conditions than single-species biofilms while phage cocktails can effectively target multiple species of bacteria present in biofilms (45). Biofilms consist of bacteria that produce polymers to provide structure, giving them their three-dimensional shape. Besides targeting the bacteria present in biofilms, phages can depolymerize the biofilm support structure by producing depolymerases (44).

2.3.3 Phage and Bacteria Generation

In addition to being advantageous compared to antibiotics, phages are extremely abundant in nature. While it takes many years and millions of dollars to find antibiotics, finding phages that have not yet been collected and characterized is rather facile. Isolating phages from wastewater samples is a common component of experimental design in phage studies. In one study, researchers took waste effluent samples from a water treatment plant and were able to isolate twelve phages to which their studied strains of *L. monocytogenes* were sensitive (46).

A method of phage isolation using electropositive silica gel particles (ESPs) was proven effective in efficiently isolating phage strains in large water samples of varying quality (47). Since most phages have a negative electrostatic charge in water, the positive charge of the ESPs allowed them to capture the phages. The researchers tested this method to compare it to traditional methods using river water from several locations. They found that ESP had the ability to isolate far more phages than traditional methods used on the same water samples, especially in samples with a low concentration of phages.

2.3.4 Factors Affecting Efficacy

Despite their many advantages over antibiotics, phages have several factors affecting their efficacy as a therapeutic. Antibiotics have a broader range of bacterial targets than a single serotype of phage. Therefore, the species of bacteria causing the infection must be known in order to successfully treat it with a phage therapeutic, whereas antibiotics can be prescribed without the knowledge of which bacterial strain is causing the infection. This means more extensive test procedures must be used to identify which bacteria are present and which phage is needed to target it. Using a single phage serotype in a therapeutic will also likely not be effective for an infection by multiple strains or species of bacteria. Because of this, phage cocktails, or a combination of several types of phages, are more effective as a therapeutic for bacterial infections involving multiple bacterial species (47).

Additionally, multispecies biofilms as opposed to single species biofilms can affect the efficacy of single serotype phage treatments (45). A dual-species biofilm composed of *S. aureus* and a variety of co-species was inoculated with phage phiIPLA-RODI, a phage to which neither of the species in the biofilm were sensitive (45). The results indicated that the presence of the phage impacted the co-species with *S. aureus.* In one example, after inoculation, while the number of *S. aureus* decreased, the number of the co-species in that trial, *L. plantarum,* increased significantly (45). The complex relationship of multispecies biofilms in relation to phage therapies should be further studied.

Another factor affecting the efficacy of phage therapies is the method of delivery. Phages can be administered via injection, nasal sprays, food additives, spray-dry

powders, and tablets. *In vivo* factors that then become relevant are the pH of the digestive tract, penetration into the target areas of the body, and natural clearance by the patient's immune system. In most cases, adding certain salts, gelatin, and bovine serum albumin (BSA) to liquid formulations of phage treatments is what helps maintain phage viability (48). One paper reported that freeze drying phages maintains phage genetic and physical stability better than liquid formulations, but that the process of freeze drying leads to a rapid decline in viable phages (48). Materials such as egg whites, organ extracts, and yeast extracts have been shown to be good additives to protect the phages against this drop in viability. However, in practice these animal-sourced materials could create issues as allergens if used in human therapies (48). Despite these many complex factors to phage therapy, recent successes show the potential of this treatment method in mainstream medicine.

2.3.5 Recent Developments in Therapeutic Success

Though phage therapy is not yet a part of mainstream American medicine, phage cocktails procured from Eastern Europe have been used to treat wounds that do not respond to antibiotics at the Wound Care Center in Lubbock, Texas (49). Other studies, particularly in the US, have shown the effectiveness of phage therapy in vitro. One study showed the effectiveness of phage therapy against a *Clostridium difficile* infection in an *in vitro* colon model (50).

In 2009, the first placebo-controlled, double-blinded human clinical trial of a phage therapeutic was conducted at a university hospital in the United Kingdom. Patients with chronic otitis and ear infections due to antibiotic-resistant *Pseudomonas aeruginosa* were either treated with a placebo or with a phage cocktail consisting of 6 different

phages (51). *P. aeruginosa* counts were significantly lower in phage-treated patients 42 days post-treatment, whereas there was no significant change in *P. aeruginosa* counts for placebo-treated patients.

2.4 FDA Regulatory Framework

Despite progress in research for phages as a therapeutic, FDA regulations are still a work in progress for phages. Some applications, such as use in agriculture and livestock, where regulations are less stringent than the regulations for human treatment, have already been approved. In 2006, the Listex P100 phage was deemed "Generally Regarded as Safe" by the FDA, who also approved Intralytix's Listshield, which is a phage that kills the food-borne *Listeria monocytogenes* bacteria (52). These approvals are the first regulations of phages as food additives by the FDA, although there is also approval for the use of phages as pesticides on crops (53).

Phage therapies to be directly used on humans have not yet had specific guidelines set out for them, but it appears as though the FDA is proceeding with caution, requiring approval for a single phage strain (54). Since phage cocktails are the preferred method of administration, and have been proven to be more practical and effective, experts are hoping for FDA approval, however the FDA has a history of opposing drug cocktails unless all of the components are individually and collectively proven both safe and effective (54).

Scientists are arguing for the approval of phage therapies based on some of the fundamental characteristics of phages themselves. Phages are inherently non-harmful to humans because they are not human pathogens and cannot use human or other eukaryotic hosts to replicate. If they do exhibit any of the characteristics that could make them

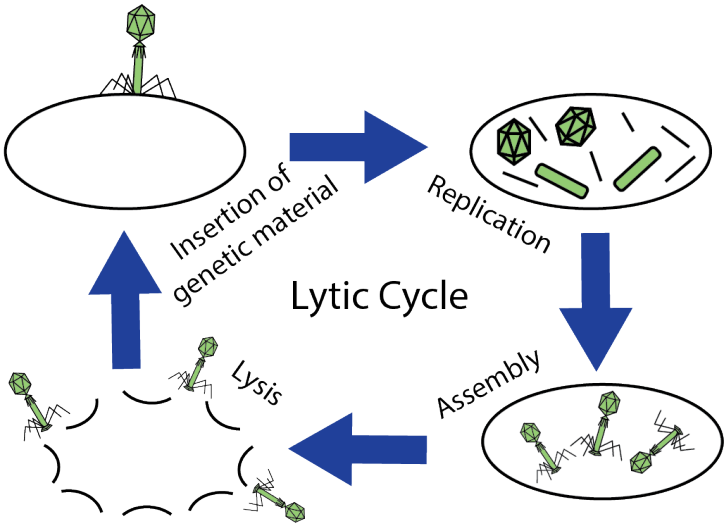

Figure 3: Phages that undergo the lytic cycle quickly lyse bacteria.

harmful to humans, bioinformatic methods could be used to determine what the harmful characteristics are, so that phages could be easily categorized and labeled as "safe" or "unsafe" (53).

In preparation for the coming FDA regulations, ways of getting phage-based therapeutics approved through unconventional or non-canonical channels are being explored. One such method is to modify the phages themselves. In the event that the FDA does not approve phage cocktails, it is possible to engineer phages to infect more than one strain of bacteria. A phage enters a bacterium via an enzyme on its tail which creates a hole in the capsid coat of the bacterium, as shown in step one of the Lytic cycle depicted below in ***Figure 3***. This then means that creating phages with more than one type of enzyme would allow them to infect multiple types of bacteria (54).

This idea leads to other possibilities for modifying the phages, such as adding even more enzymes, and suggests a potential for scientists to create effective phage therapeutics without the FDA's approval of phage cocktails.

2.5 Combination Treatment with Antibiotics and Bacteriophages

Combination therapies have previously been shown to be an effective treatment method against non-bacterial illnesses by targeting both specific and general mechanisms of a disease (55). In the case of bacteriophages and antibiotics, a combination treatment takes advantage of the phage mechanisms described above to increase a bacteria's susceptibility to the antibiotics, making overall treatment more effective. Selective pressure by phages will frequently reintroduce antibiotic susceptibility into formerly resistant bacterial strains (56). Indeed, numerous studies have found increased killing efficacy through synergistic antibiotic-bacteriophage treatments in both planktonic as well as biofilm forming bacteria (57, 58). This phenomenon of bacteriophages re-sensitizing resistant bacteria to antibiotics is potentially illustrated in a 2019 study of phage-antibiotic synergy and biofilm forming *Staphylococcus aureus* and *Pseudomonas aeruginosa*; greater killing was observed in experiments where phage was administered before antibiotics rather than simultaneous administration (59).

Not only do antibiotics become more effective when used in combination with phage, but phage also deliver greater killing efficacy when used in combination with antibiotics. A 2018 study examining the underlying mechanisms of phage-antibiotic synergy (PAS) observed synergy in both Gram-positive and Gram-negative bacteria when cells were stressed with β-lactam antibiotics and phage. Stress from the antibiotics caused swelling in the bacterial cells resulting in greater phage production after delayed lysis (60).

Acinetobacter baumannii

4.1 Selection

A. baumannii was chosen from a selection of 15 bacterial species identified by the Centers for Disease Control and Prevention as the top antimicrobial resistance threats (11). The other bacteria considered were *Clostridium difficile, Klebsiella pneumoniae, Escherichia coli, Enterobacter cloacae, Enterobacter asburiae, Neisseria gonorrhoeae, Campylobacter jejuni, Enterococcus faecium, Pseudomonas aeruginosa, Salmonella enterica, Shigella dysenteriae, Staphylococcus aureus, Streptococcus pneumoniae,* and *Mycobacterium tuberculosis.* Our selection criteria had four main components; 1) extent and severity of antibiotic resistant disease caused by the pathogen, 2) accessibility of relevant bacterial strains and corresponding phages, 3) breadth of current phage therapy research targeting the pathogen, and 4) likelihood of being able to isolate novel virulent phages active against the clinically relevant bacterial strains considered in 2.

Following criteria 1 and 2 narrowed the list to *C. difficile, E. cloacae, A. baumannii, S. dysenteriae* and *M. tuberculosis*. *C. difficile* was ruled out because of difficult culturing conditions, a lack of good sources for finding novel phages, and the prevailing notion that all phages found to be active against the bacterium are likely to be necessarily lysogenic. *S. dysenteriae* was ruled out because there are already non-antibiotic based treatments in development and because the majority of its phages possess genes coding for endotoxins. *M. tuberculosis* was ruled out because of a low abundance of environmental phages and due to difficulties in administering phage therapeutics as noted by other researchers. The final choice of *A. baumannii* over *E. cloacae* was based

on the greater disease incidence and severity of *A. baumannii* and its broader antibiotic resistance profile.

4.2 Clinical Significance

With growing antibiotic resistance, the threat associated with infections increases profoundly in the hospital setting. As one of the most common and dangerous gram-negative pathogens, *A. baumannii* is mainly associated with pneumonia and bloodstream infections though it has also been implicated in urinary tract infections and post-surgery complications in immunocompromised and long-term hospital patients. *A. baumannii* is the causative agent behind up to 10% of intensive care unit (ICU) acquired pneumonias and a substantial portion of seasonal pneumonia in tropical regions (61, 62). Being implicated in 1.3% of all bloodstream infections, *A. baumannii* is the 10th most common microbial cause of such ailments and is associated with crude mortality rates up to 43.4%, the third highest for all causative agents (63).

A. baumannii has developed nearly every mechanism for resistance including modification of enzymes and target-site, production of β-lactamases, permeability defects, and efflux pumps. By 2007, in certain clinical settings, 70% of *A. baumannii* isolates were found to be MDR and were the direct cause of 2-10% of nosocomial infections (64). The diversity of resistance pathways has resulted in resistance to nearly every family of antibiotics and has contributed to its evasiveness in treatment (17). Conventional antimicrobial treatments are reaching a standstill as the population is evolving faster than the drugs used to target it. New treatments in radioimmunotherapy, bactericidal gene therapy, and nanoparticles have potential, but are proving expensive

and need much more time to develop. But with renewed interest in phages, a new treatment modality is possible (65).

4.3 Current Phage Research

In order to improve the ability to use phages as a treatment, they should be characterized to improve understanding and utilization. Characterization of phages targeting *A. baumannii* began in 2010 when with ϕAB2 and nine other phages with different host ranges were isolated from catheter washings, raw sewage, and wastewater (66). Additional phages have been found in local bodies of water as well as other environments in a clinical setting (67–71). With a limited pool of well-characterized phages, the therapeutic possibilities are still being investigated. *In vitro* studies and *in vivo* studies with mice have begun in recent years and have seen positive results. Phage therapy has even helped a patient suffering from *A. baumannii* complicated necrotizing pancreatitis in a clinical study, returning him to a stable health condition (72). More research is required to improve the efficiency and viability of using phage cocktails through discovery of new virulent phages, analysis of synergistic effects, and optimal culture conditions.

Materials and Methods

Bacteria and bacteriophage strain acquisition and growth conditions

Initial studies conducted used AC54 *Acinetobacter baumannii* strain (AB-Felix) and the BS46 bacteriophage purchased from the Félix d'Hérelle Reference Center for Bacterial Viruses at Laval University, Canada. To aid in developing a phage library, 12 additional strains of phages and bacterial hosts were provided through a limited purpose cooperative research and development agreement (LP-CRADA) for material transfer with the Naval Medical Research Center at Frederick, Maryland. All *A. baumannii* bacteria and phage strains were propagated at 37 °C in lysogeny broth (LB) or on LB agar plates unless otherwise indicated. The phage strains were presumed to all be virulent. A non-pathogenic *E. coli* strain (*E. coli*-pGlo) was provided by the UMD Bioprocess Scale-up Facility and a virulent mutant lambda phage (λ-mut) was provided by Dr. Vincent Lee of UMD's department of cell biology and molecular genetics. This *E. coli*-phage pair was propagated the same as the *A. baumannii* bacteria and phages.

Propagation and master cell bank maintenance of bacterial hosts

For host propagation, a single colony of *A. baumannii* was lifted from an LB agar plate or a sample of thawed cells, either straight from the vendor or from a previously generated master cell bank (MCB). These were placed in a 15 mL culture of LB broth. This culture was expanded overnight at 37 °C with shaking. 100 µL of this culture was used to inoculate 100 mL of fresh LB broth in a shake flask and expanded until the optical density (OD) at 600 nm (OD600) was observed to be roughly 1.0. Optical density at 600 nm is the industry standard for enumerating bacteria count as it measures the light

scatter from the cells. Scatter due to cell death is negligible as they undergo autolysis, which removes cellular debris. This was measured using a laboratory scale spectrophotometer in a disposable cuvette. The instrument has a range of linearity between 0.00 and 0.600. For samples with an OD greater than 0.600, they were diluted to the range of linearity. To create the MCB, the culture was then centrifuged at 5000 revolutions per minute (rpm) for 10 minutes to form a bacterial pellet which was then resuspended in 1 mL of LB broth. Approximately 0.6 mL of this sample was mixed with 0.6 mL of glycerol to get a glycerol concentration of 50% w/v. This was stored in a cryovial at -80 °C. To create a working cell bank (WCB), 100 µL of the culture was streaked on an agar plate that was wrapped in parafilm and allowed to grow overnight at 37 °C. Once the plate was confluent, the plate was stored at 4 °C and remained viable for up to two weeks.

Development of standard/growth curve for bacterial host enumeration/growth kinetics

100 µL of an overnight culture from a viable WCB was used to inoculate 100 mL of fresh LB broth in a shake flask. Every hour for 12 hours, 4 mL were aliquoted off and the OD at 600 nm was measured. The sample was then serially diluted up to 10^{-9} fold in LB broth and 100 µL was spread in triplicate on agar plates and incubated overnight at 37 °C. The serial dilution that yielded between 10-100 colonies was counted and used to correlate the OD at the time point and the number of colony forming units (CFU).

Minimum inhibitory concentration (MIC) determination

Concentrated antibiotic solutions of ampicillin (Amp), chloramphenicol (Cam), polymyxin B (PMB), and minocylin (Min) were prepared in 1.00 X phosphate-buffered saline (PBS). This buffer solution consisted of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4. 2-fold serial dilutions in PBS were then prepared from these concentrated stocks. Bacteria in mid-log phase were added to 1.6 mL of fresh LB in a 24-well plate to an OD600 between 0.1 and 0.2 (final volume 1.8 mL) and 200 µL of the antibiotic dilutions were added to give a final volume of 2.0 mL per well. Mid-log phase for this study was defined as 2-3 hours after exponential growth started. The plates were incubated in a plate reader with shaking for 48 hours and OD600 was monitored over time. The MIC was determined as the lowest concentration tested at which there was a significant delay in or decreased rate of growth as compared to a control well prepared with PBS with no antibiotic.

Propagation and master phage bank maintenance of bacteriophage

100 µL of an overnight bacteria culture from a viable WCB was used to inoculate 100 mL of fresh LB broth in a shake flask. Once the culture reached the mid-log phase, 100 µL of phage stock was added to the culture. The culture shaken at 37 °C for approximately 6 hours. A 4 mL aliquot was drawn off every hour for an OD600 measurement to ensure a drop viable cell density corresponding to cell death and phage propagation. If this drop was observed, the remaining culture was spun down at 5000 rpm for 10 minutes to remove cellular debris. The supernatant was then filtered through a 0.22 µm pore-size millipore filter to remove any remaining debris and was stored in a 4 °C refrigerator. This constituted the master phage bank (MPB).

Plaque assay for phage enumeration

A 15 mL overnight culture of the host bacterium was prepared in LB broth. 200 µL of this culture was spread on an LB plate. 10-fold serial dilutions of the phage sample were prepared by dilution with PBS and 15 µL of each dilution was added to the spread plate of bacteria in a single drop. The plate was incubated overnight at 37 °C and the number of plaques in the first dilution which produce enumerable plaques instead of a single contiguous zone of clearance (typically 10-20 plaques). This was used to calculate the number of plaque forming units (PFU) per mL for the phage sample. Four typical plaque assays are illustrated in **Figure 4.** In this figure, Plate A illustrates clustered plaques that are overlapping infectious phage particles too close together to provide accurate enumeration. Plate B illustrates confluent bacteria with no infectious phage particles. Plate C illustrates the resurgence of individual bacteria colonies that developed resistance to the initial phage. Plate D illustrates isolated plaques with sufficient resolution such that phage titer can be enumerated.

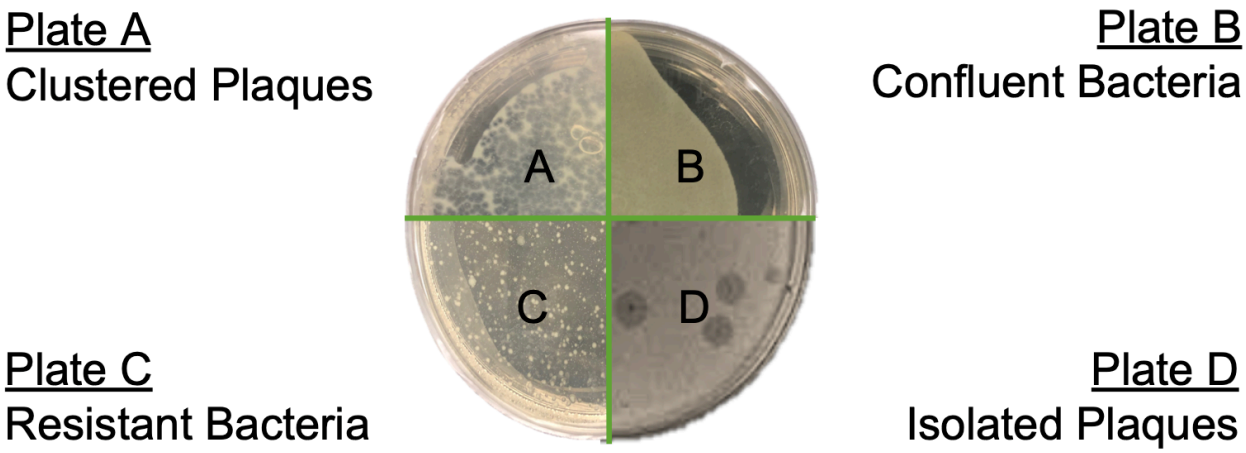

Figure 4: Representation of four typical plaque assay results where (A) has numerous overlapping plaques, (B) no plaques, (C) development of resistant bacteria, and (D) has isolated plaques

Development of one-step killing curve for phage growth kinetics

2 mL of an overnight culture was used to inoculate 100 mL of fresh LB broth in a shake flask. Once the culture reached the mid-log phase, 100 µL of phage was added to the culture. Every 30 minutes for 5 hours, 1.5 mL of the culture was transferred into a cuvette and the OD600 was measured. The sample was then transferred to a microcentrifuge tube and spun down at 5000 rpm for 5 minutes to remove cellular debris. The phage concentration in the supernatant at each timepoint was then determined by plaque assay.

Results

Time-Delayed Phage Treatment of *E. coli*

While in the process of acquiring *A. baumannii* bacteria and phage strains, some proof of concept studies were carried out using *E. coli*-pGlo and its paired phage, λ-mut. The *E. coli*-pGlo was grown in shake flasks at 37 °C and was infected with a serial dilution of phage at early log-phase (**Figure 5**). The time before lysis of the culture was observed increased as the phage dilution increased and all dilutions were able to cause a similar amount of lysis. The correspondence between the observed decrease in OD600 of the culture and the change in phage titer was investigated further. *E. coli* in the early-log phase were inoculated with a phage at an MOI of 0.5. The samples of the culture were collected each timepoint were then taken, the cells pelleted, and the supernatant titered for phage (**Figure 6**). A spike in phage titer coincided with the leveling off of the OD600 curve and at the next time point, the OD600 started to decrease. This corresponds well with the predicted observations for culture lysis that is driven by bactericidal action of a lytic phage.

A. baumannii Phage Typing

The most efficient bacterial host for testing is marked by rapid growth kinetics, indicating viable and robust cells, and the ability to be infected by multiple strains of phage. The first stage to formulate a phage therapeutic was to identify the bacterial host that best displayed these traits. To achieve this, each of the 12 bacteria strains provided by the Naval Medical Research Center were spread onto LB plates and 10 μL of undiluted phage stocks of each of the 12 corresponding phage strains was applied to each

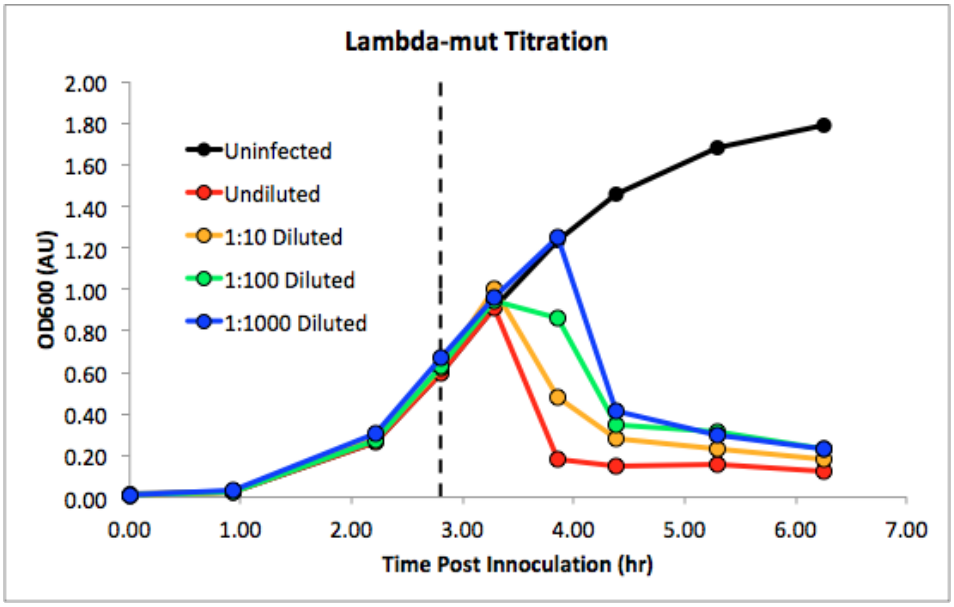

Figure 5: Phage titration curves at different dilutions of phage tested in shake flasks. Increaseing the phage dilution delayed the inset of cell killing but did not change the final extent of culture lysis.

plate in a single drop. The plates were incubated overnight and the appearance of zones of clearance were used to identify cross susceptibility (**Figure 7.A**). For the remainder of this discussion, bacteria strains will be named AB# and the phage strains will be named ϕ#. The number indicates the bacterial host and phage pair. For example, in **Figure 7.A**, ϕ4 and ϕ8 were able to infect and kill AB4. A typical plaque assay is shown in **Figure 7.B** where a quantifiable amount of plaques were observed at the 10^{-8} dilution. Using this method, the titer for ϕ4 was determined to be roughly $10^{11.5}$ PFU/mL.

All phage strains were able to infect their specific bacterial host, except for strain 1, potentially because the phage had poor bactericidal efficacy or was weakened in transit. Additionally, all phage strains other than ϕ1, ϕ3, and ϕ4 produced a distinct halo surrounding the plaque, likely indicating diffusion of a polysaccharide depolymerase . The cross infectivity and titer of each phage strain is shown in **Figure 8.A** and **Figure**

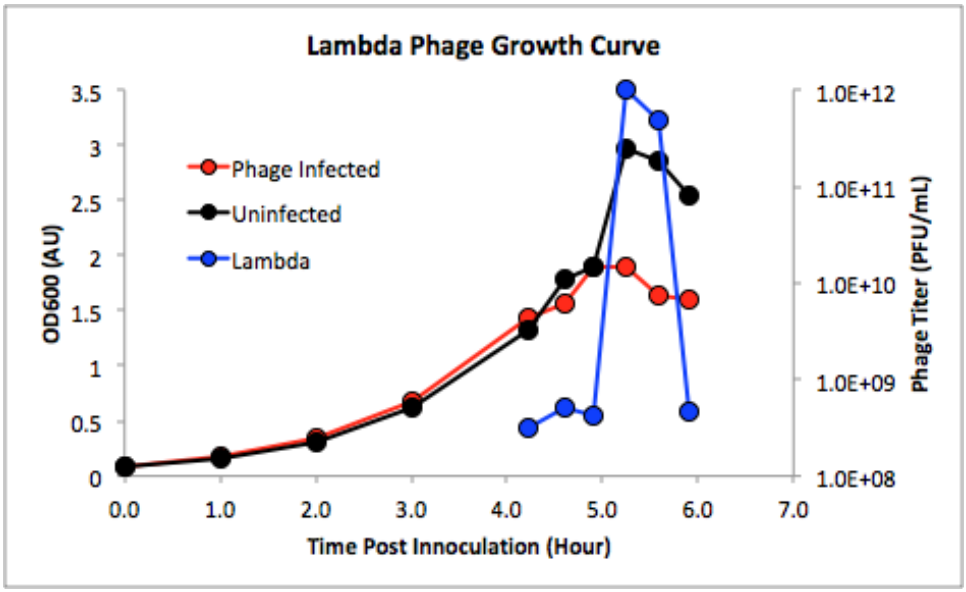

Figure 6: Single step phage killing curve in shake flasks. The sudden rise in phage titer corresponds to leveling off of the growth curve for the infected bacteria while the uninfected control continues growing.

8.B. It can be shown that bacteria AB4, AB5, AB7, AB9, and, AB11 are susceptible to more than one phage strain, with AB7 and AB11 vulnerable to three.

To further narrow down the bacteria selection, a growth curve was generated for each strain of bacteria that was susceptible to more than one phage or for phage strains that were able to infect two or more bacteria. These results are illustrated in **Figure 8.D** where log-phase was determined to be roughly between 1 and 4 hours post inoculation. Strains AB4, AB5, AB7,8, AB10, and AB11 sustained growth for 10 hours, after which there was a steady decline. AB10 had an extended stationary phase up until 20 hours post inoculation. AB4 had a rapid death phase beyond 9 hours. Additionally, AB9, AB10, and AB11 all had a doubling time of roughly 20.5 hours, the fastest out of the 7 strains. Of these, AB9 was chosen for further characterization.

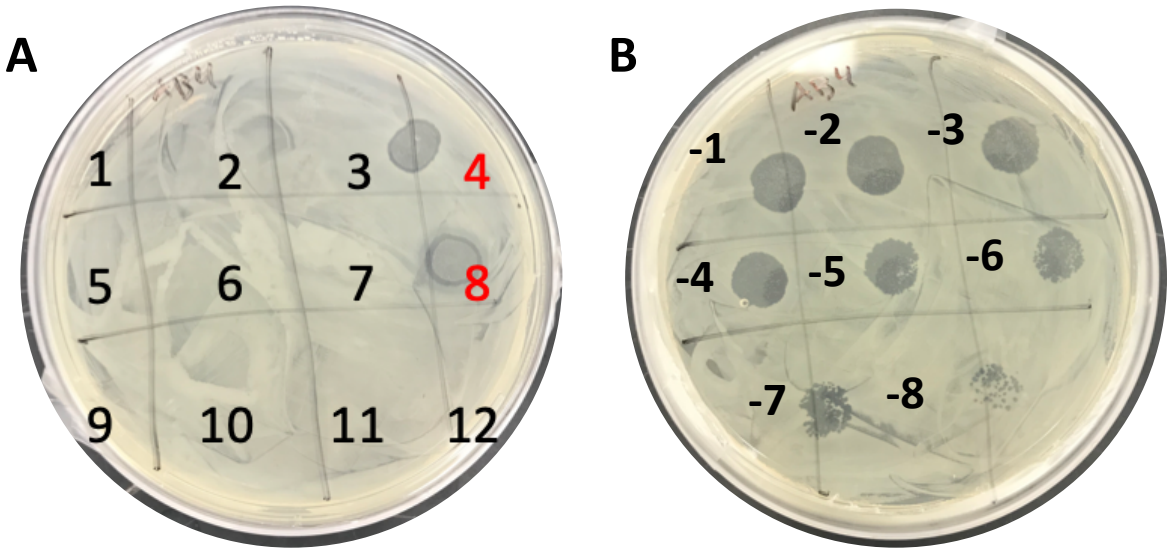

Figure 7: LB Agar plates for (A) determining cross infectivity of AB4 and (B) determining ϕ4 titer. The different phage strains are indicated numerically in A and the log10 of the dilution used for titering ϕ4 corresponding to each drop is indicated in B.

Phage Titration

The next stage of the study was to assess the effect of multiplicity of infection (MOI), in terms of PFU of phage per bacterium, on initial growth suppression and time until resistance emerged. An MOI ranging between 0.0001 and 10 was tested in a 24 well-plate using AB9 and ϕ9 and AB-Felix and ϕ-Felix. Unless otherwise noted, all of the following experiments use these same bacteria-phage pairings and the phage strain is indicated simply as "Phage." The OD600 readings for each well were monitored over 48 hours and are shown in **Figure 9**. There are three key trends observed in this dataset. First, as MOI increases, the initial bactericidal effects increase as well. This is consistently illustrated in the 0 to 3 hour post-inoculation range where the MOI 10 sample immediately dropped to an OD600 around 0.1. Samples with a lower MOI achieved the same minimum OD600, but required more time. All samples less than MOI 10 first exhibited limited growth, indicating that not every cell was infected at the initial

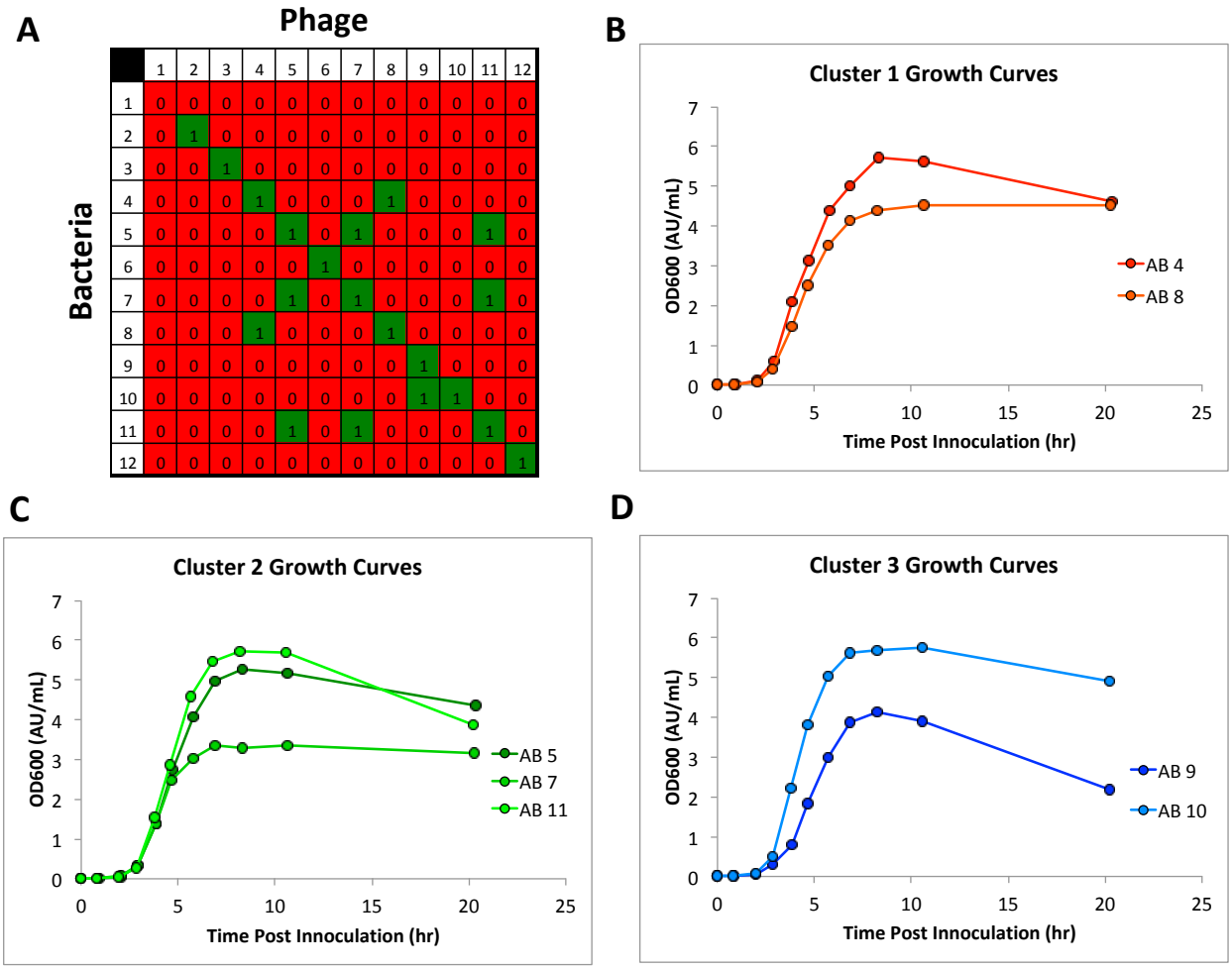

Figure 8: Summary of naval medical research center phages and bacterial hosts (A). 0 indicates an incompatible bacteria and phage pair and a 1 indicates a compatible pair. Three clusters of bacteria-phage cross reactivity were identified. The growth of the host bacteria in each cluster was characterized in shake flasks (B, C, D).

timepoint. However, even for MOI 0.0001, the bactericidal effects of the phage dominated the natural growth of the bacteria within 3 hours. The next parameter of interest is the time to develop resistance, represented by significant growth following prolonged cell death. For nearly all MOI, the time to develop resistance was roughly the same at 7 hours post-inoculation. Finally, the doubling times of the bacteria in each phage-treated well were longer than the uninfected control and the initial doubling time from that same well for the lower MOI wells where some initial growth was observed. This is consistent with a decreased fitness of the resistant bacteria indicating a trade off

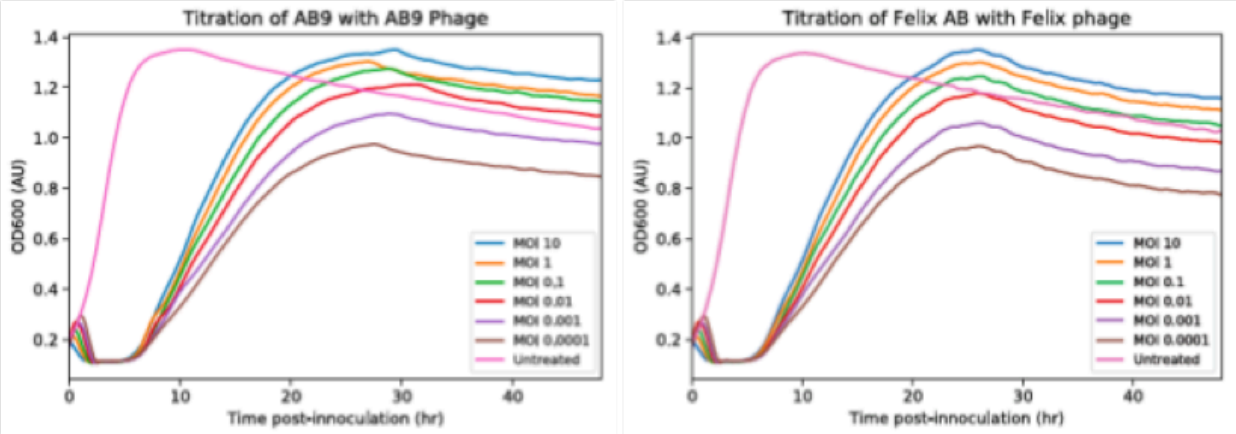

Figure 9: Phage titration curve depicting the bactericidal kinetics at different MOIs tested in 24-well plates. Higher MOIs resulted in more rapid killing while lower MOIs were associated with a slower growth rate once resistance emerged.

with phage susceptibility. However, decreased nutrient availability in the growth medium due to initial growth of the bacteria and production of phage would also likely result in a longer doubling time and decreased maximum OD600 as was observed. This depletion of nutrients as well as their initial abundance is not reflective of the local environment of a bacterial infection and accordingly no follow up work was done looking at changes in growth rate at these later timepoints.

Antibiotic Titration:

The susceptibility of AB9 and the AB-Felix to Amp, Cam, PMB, and Min was determined in an automated plate reader using 24-well plates. The growth curves of the bacteria when Amp and Cam were introduced to the AB9 strain showed a characteristic sigmoidal shape followed by gradual decrease in OD600 (**Figure 10**). A Savitzky-Golay filter with a window size of 135 minutes and a polynomial order of 3 was used to smooth the data in order to eliminate noise related to growth in the microplate reader. For Amp, the lowest two concentrations were ineffective at slowing or halting bacterial growth for

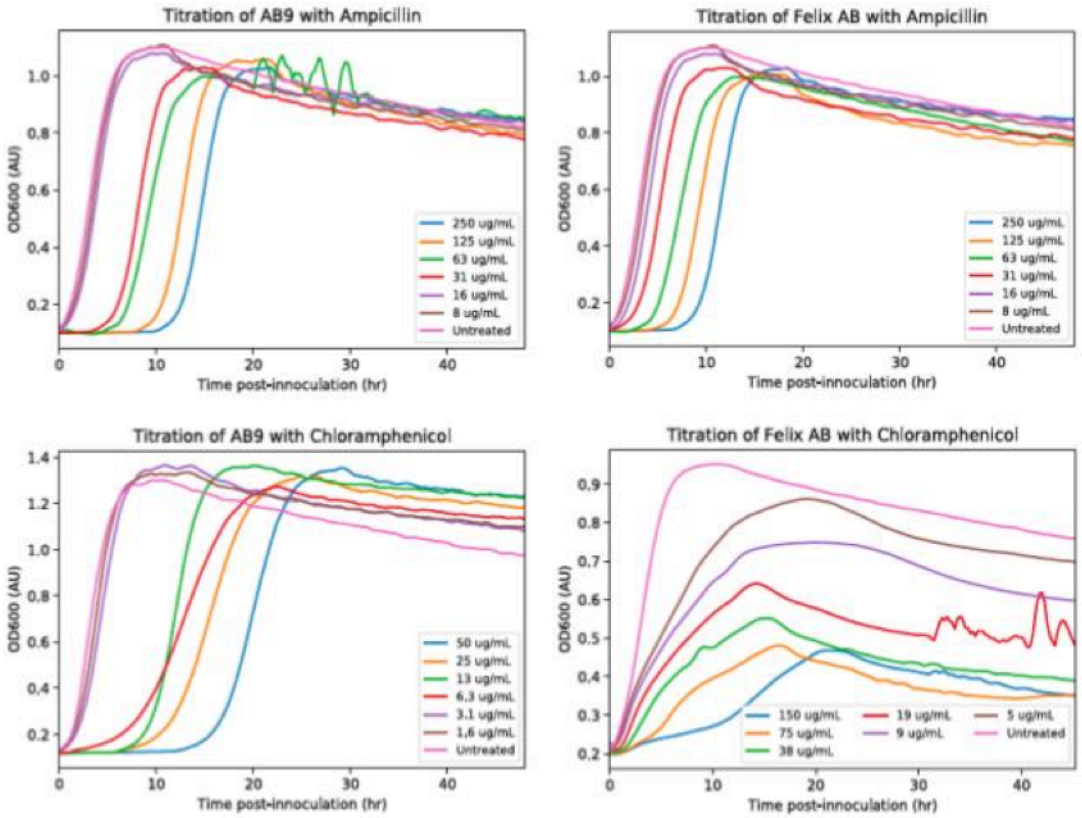

Figure 10: Titration of ampicillin and chloramphenicol. Increasing Amp concentrations were associated with a longer time before resistance merged for both bacterial strains. Increasing Cam concentrations were still associated with an increased time before resistance emerged for the AB9 bacteria, but for AB-Felix there was a lower rate of growth observed for lower concentrations of Cam while at higher concentrations initial growth was suppressed.

both bacteria; while for Cam, there was a differential effect based on the AB strain- the two lowest concentrations had no effect on the growth of AB9, but were somewhat effective in slowing, though not halting, the growth of Felix. For both antibiotics and bacterial strains, the degree to which growth was affected increased with increasing antibiotic concentrations. The MIC for Amp of AB9 was determined to be 31 µg/mL and 6.3 µg/mL for Cam. While the growth of AB9 does appear to be significantly delayed at concentrations of 6.3 µg/mL Cam, growth is first completely arrested at 13 µg/mL. The susceptibility of AB-Felix to Amp was similar to AB9 with an MIC of 31 µg/mL.

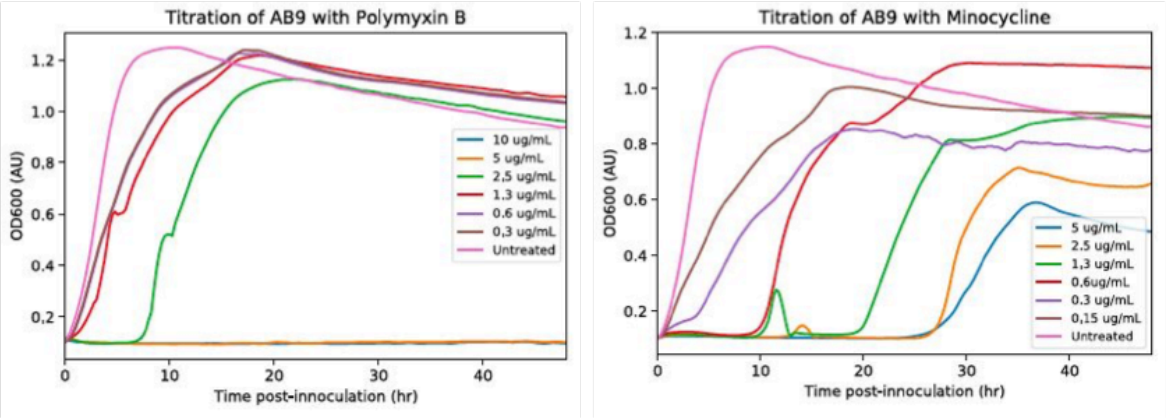

Figure 11: Titration of polymyxin B and minocycline AB9. The MIC for PMB is 2.5 µg/mL, and no resistance in the bacteria evolves above this concentration. The MIC for Min is between 0.3 µg/mL and 0.6 µg/mL, and the growth curves for AB9 to which Min has been added are complex in shape.

However, growth of AB-Felix in the presence of Cam was more complex and did not follow the characteristic delayed sigmoidal growth curve (**Figure 10**). Initial growth was similar to the untreated control at 9 µg/mL but after 2 hours there was a significant decrease in growth rate and a reduced maximum OD600. This effect increased as the concentration of Cam was increased up to 75 µg/mL after which increasing antibiotic concentration did not cause further decrease in initial growth rate or maximum OD600. As such, two MICs were established; 9 µg/mL for growth rate reduction and 75 µg/mL for growth delay.

Additional testing was attempted using the more clinically relevant PMB and Min (**Figure 11**). The MIC for PMB for AB9 was determined to be 2.5 µg/mL; above this concentration, no resistance emerged, either in liquid culture or on plates. For the same strain, the MIC for Min was 0.6 µg/mL for complete arrest of growth and 0.15 µg/mL for growth rate reduction. Interestingly, at higher concentrations of Min (1.3 to 2.5 µg/mL); a bactericidal effect is observed. Increasing OD600 is followed by a sharp decrease and a

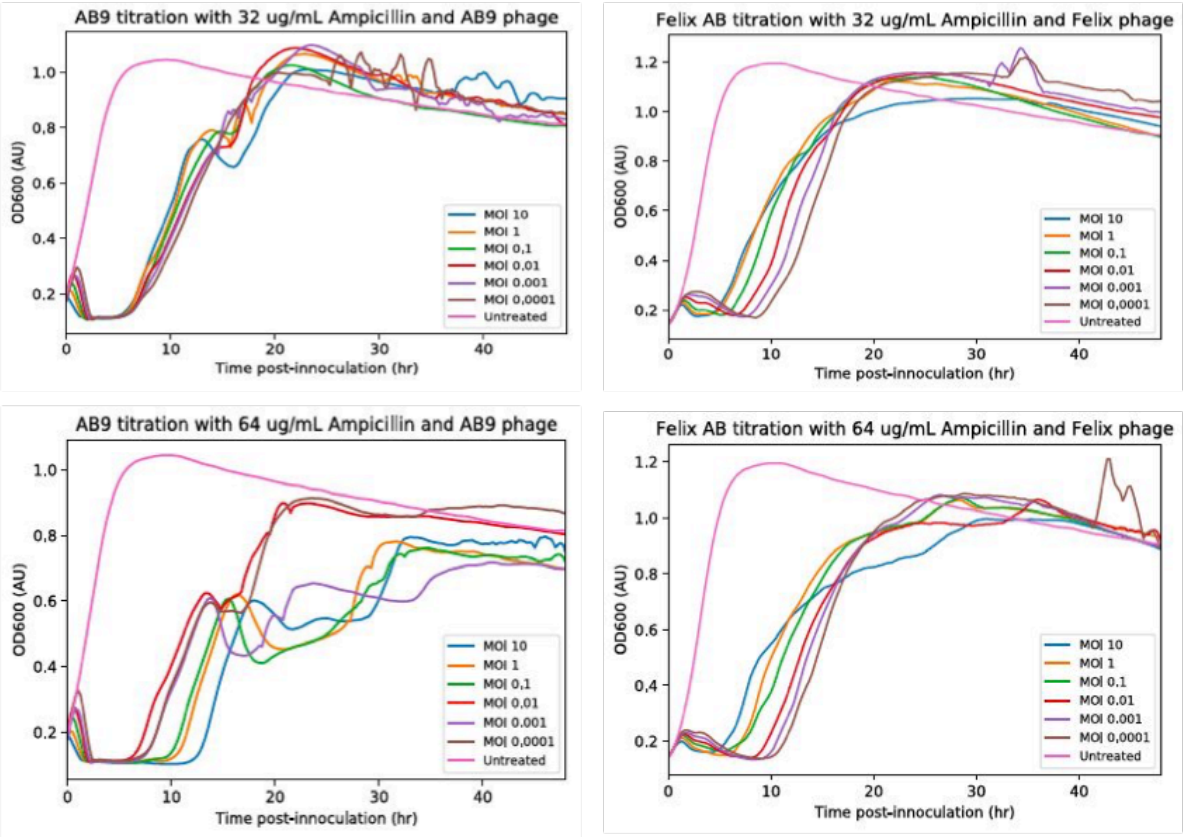

Figure 12: Titration of phage in the presence of two concentrations of Amp. At the higher Amp concentration, increasing MOI was associated with a longer time before resistance emerged for the AB9 bacteria while at the lower Amp concentration there was little difference. For AB-Felix, a higher MOI was associated with more rapid lysis at both Amp concentrations while lower MOIs resulted in prolonged gradual lysis of the culture.

plateau, indicating bacterial death followed by a lack of further growth until a second generation of resistance is observed. Interestingly, the sudden drop in OD600 is not observed at 0.6 µg/mL but there is a plateau followed by continued growth. This is consistent with a brief period during which the rate of bacteria death equals the rate of bacteria growth until only resistant bacteria remain. This would also explain the unusual growth characteristics observed for the AB-Felix bacteria treated with higher concentrations of Cam. The AB-Felix strain was significantly more susceptible to both PMB and Min with MICs of 0.1 and 0.05 µg/mL, respectively (data not shown). At these

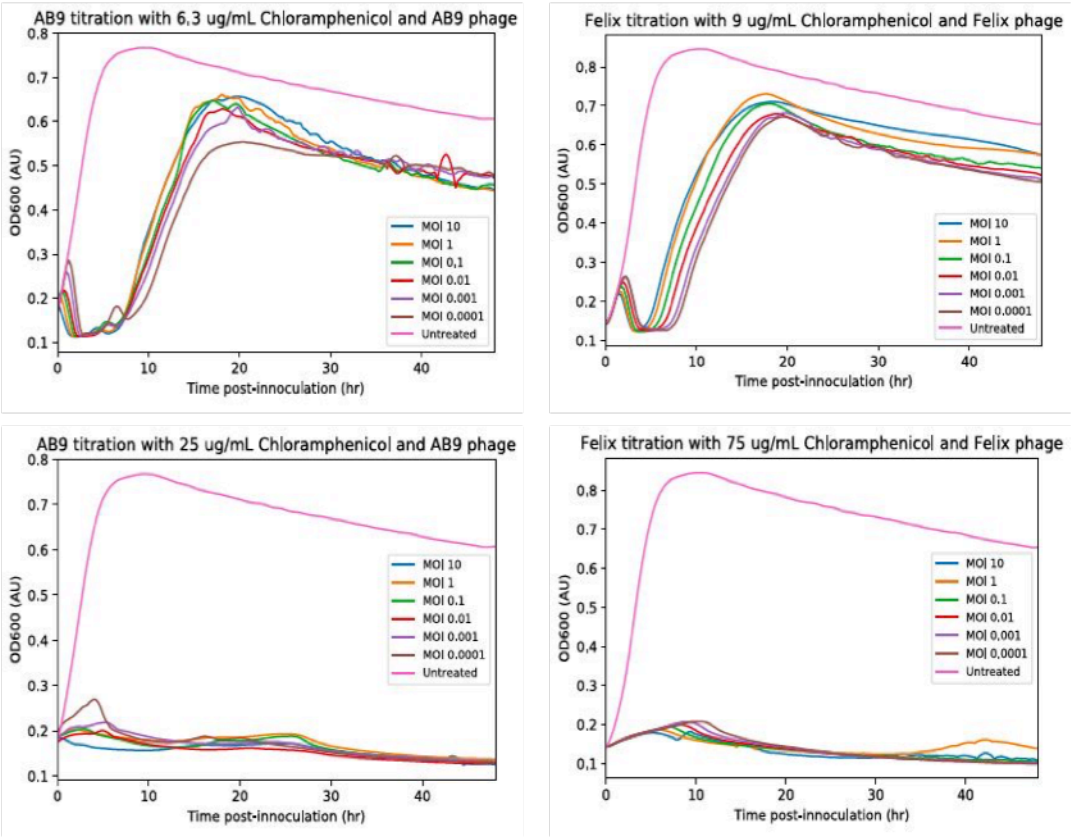

Figure 13: Titration of the phage in the presence of two concentrations of Cam. For both bacteria at the higher Cam concentration a higher MOI is associated with more rapid lysis of the culture and significant resistance was not observed at any MOI. At the lower Cam concentration higher MOIs were associated with more rapid killing but lower MOIs were associated with a longer time before resistance emerged.

concentrations there was no growth observed over the entire 48 hour observation period and at a 2-fold lower concentration the growth was indistinguishable from the untreated control. For these reasons, combination testing was only carried out with phage and Amp and Cam as PMB and Min had too many confounding factors to properly take into account.

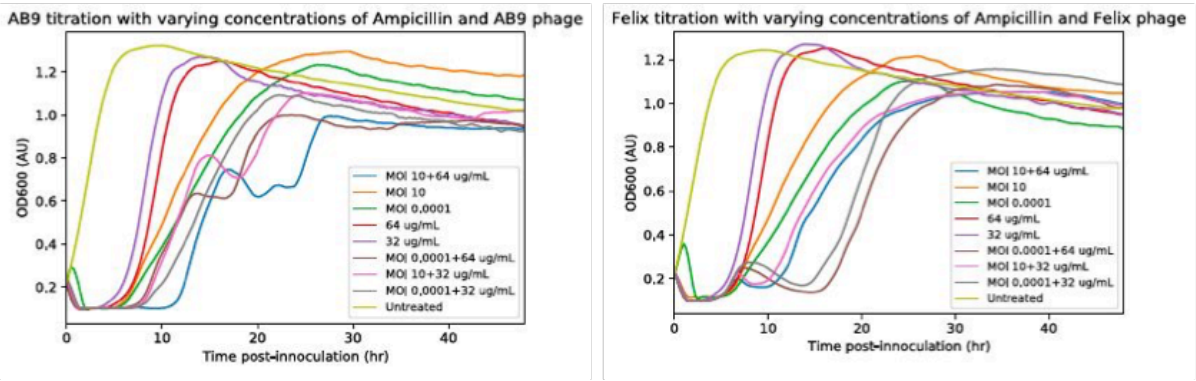

Figure 14: Combination treatment of bacteria with phage and ampicillin, varying the concentration concentrations of both bactericidal agents. In general, combining both phage and antibiotic at any concentrations outperformed either treatment given alone. For AB9, the higher MOI and higher Amp concentration were associated with the longest time before resistance emerged. For AB-Felix, using the lower MOI and the highest concentration of Amp resulted in the longest time before resistance emerged.

Combination Treatment of *A. baumannii* with Amp or Cam and Phage 9 at fixed antibiotic concentrations

The two strains of *A. baumannii* were tested with their paired phage and two concentrations of antibiotic. For Amp, the MIC and a 2-fold higher concentration were used (**Figure 12**) and for Cam the MIC for delayed growth and the MIC for reduced growth rate were used (**Figure 13**). For AB9, the initial killing of the bacteria occurs a single time point earlier (15 minutes) for the samples treated with the higher concentration of Amp. Initial resistance in the 32 μg/mL treated samples occurs at nearly the same time. However, the samples treated with a higher MOI of phage display a second drop in OD600 before recovering and attaining a similar maximum OD600. When the higher concentration of Amp is used, this effect is present at all MOIs tested and the second drop in OD600 is maintained for a significantly extended period of time at the higher MOIs tested. Additionally, the time before resistance first emerges is significantly increased in these samples when a higher MOI is used. While intriguing, the

second drop in OD600 is less relevant in the context of a bacterial infection. The observed delay before resistance first emerges in the samples treated with the higher concentration of Amp is much more significant and is indicative of a synergistic effect between the phage and antibiotic. For AB-Felix, increasing the MOI resulted in more rapid initial killing but resistance emerged significantly quicker than when a lower MOI was used. Interestingly, while increasing the concentration of Amp caused it to take longer overall for resistance to emerge, it did not cause any significant change in how much longer it took for resistance to emerge between any two different MOIs used for the same concentration of Amp. This has implications for designing a phage therapeutic where the choice of a high or low dosage of phage may depend on the antibiotic used in combination and also which phage or combination of phages is being used.

For both AB9 and AB-Felix, treatment with the higher, delayed growth MIC of Cam in combination with phage resulted in bacteria death without the emergence of resistance over the entire 48 hour time course. In both cases, a higher MOI of phage was associated with a more rapid lysis of the culture. At the MIC for reduced growth rate, both bacteria strains were killed more rapidly when a higher MOI of phage was used and the time before resistance first emerged increased as the MOI of phage was lowered. The AB9 bacterial also displayed a second rapid drop in OD600 shortly after resistance first emerged that is reminiscent of what was observed at the higher concentrations of Min tested. This second period of lysis occurred more quickly when a higher MOI was used. This phenomenon is very intriguing in the sense that it resembles what would be expected for cells infected by a lysogenic phage when a stressor triggers the lytic cycle. All of the phages used are reported to be virulent but this does not preclude the possibility of a

prophage being present in the AB9 genome. This opens the possibility of identifying additional temperate phages capable of infecting these bacteria which may be made virulent through genetic manipulation.

Same Experiment Comparisons of Different *A. baumannii* Treatments

For better context, multiple different antibiotic and phage combinations were tested again in parallel in a single 24-well plate experiment (**Figure 14**). Both Amp concentrations previously tested in combination with different MOIs of phage were included. To best illustrate differences caused by varying MOIs, MOIs of 10 and 0.0001 were used. For both AB9 and AB-Felix, the combination of an MOI of 0.0001 and the lower Amp concentration (32 μg/mL) outperformed either individual treatment. Additionally, for AB9, the treatment that gave the longest lasting reduction in OD600 before resistance was observed was an MOI of 10 and an Amp concentration of 64 μg/mL while for AB-Felix the treatment with an MOI of 0.0001 and 64 μg/mL of Amp caused the longest lasting reduction in OD600, not counting the slight rise that occurs starting at around 6 hours post treatment. This is consistent with the prediction that using multiple treatments in combination will be more effective than a single treatment and with the previously observed trends for bactericidal effects of various MOIs of phage when tested with Amp.

Discussion

Combination of Ampicillin and Phage

The combination treatment of both ampicillin and phage demonstrated a greater bactericidal efficacy than either treatment alone. In general, the initial lysis of the culture is driven by which agent acts more rapidly. The bactericidal action of beta-lactam antibiotics is through interference with cell wall synthesis leading to eventual rupture of the cell while phage-mediated lysis does not necessarily require host cell growth. This would imply that initial lysis would be phage driven. However, at an MOI of 10 it is entirely feasible for many more than 10 phages to absorb to a single bacterial cell resulting in a population of uninfected cells that continue dividing while the phages replicate. This same issue is not faced by antibiotics at the concentrations used and likely results in their action being the dominant player for the initial lysis observed. This is consistent with our observations in **Figure 14** where the initial drop in OD600 was very similar across all treatments with the same Amp concentration. The added benefit of the phages in these scenarios would be in scavenging all of the Amp resistant bacteria remaining in the culture. This is consistent with our results demonstrating a prolonged time before resistance emerges when both phages and Amp are used as compared to either used individually.

Combination of Chloramphenicol and Phage

The combination of both chloramphenicol and phage demonstrated a greater bactericidal efficacy than either treatment alone. As opposed to Amp and phage treatment where both agents exhibit bactericidal activity, Cam is primarily bacteriostatic through

translational inhibition. This produces a conundrum where if the dose of Cam used is too great then it will prevent translation of phage proteins just as it does bacterial proteins. This will result in a delay in lysis of the bacteria by phage but also prevent significant bacterial growth as any Cam resistant bacteria will be scavenged by the phage. This is consistent with our observations where treatment of bacteria with Cam at the growth delaying MIC prevented significant initial growth and resulted in a prolonged, slow period of lysis. At the growth rate lowering MIC, there was a slight delay before notable lysis occurred but it still proceeded fairly rapidly. The fact that the emergence of resistance was not observed at the higher Cam concentration is likely due to an inherent delayed bactericidal effect of bacteriostatic antibiotics where cellular damage accumulates overtime without the production of fresh proteins eventually resulting in cell death.

Polymyxin B and Minocycline

Testing of phages in combination with either PMB or Min was unproductive. In both cases, using the MIC of either antibiotic in combination with even an MOI of 0.0001 of phage resulted in complete cell death or the arrest of growth with no resistance being observed within 48 hours. This is not entirely surprising as these antibiotics were chosen for testing due to their extremely powerful antimicrobial activity and successful use as a monotherapy in the clinical setting. Additionally, attempts to test with phages on PMB or Min resistant bacteria were not made because antibiotic resistant bacteria taken from liquid cultures or antibiotic plates were not viable when re-streaked or reinoculated preventing any further study. The initial emergence of Min resistant bacteria followed by a rapid die off at higher concentration of antibiotic was briefly investigated. As this

mirrors what could be observed with the induction of the lytic cycle of an unknown prophage, aliquots of the filtered media from those wells were applied in a single drop to a spread plate of the bacteria. No plaques were observed following overnight incubation which indicates either the absence of phage or an extreme preference for the lysogenic cycle. These possibilities were not investigated further.

Future Directions

Over the course of this study, the scope and goal of the research shifted. Due to difficulty with co-evolving phage and bacteria, the focus shifted to studying the synergistic effects of phage-antibiotic treatment. However, time became a limiting factor for following every potential direction of this new focus. This leaves several interesting avenues of research open for study in the future.

Firstly, the results of microplate studies should be corroborated by shake flask experiments. While our results are compelling, finding these synergistic effects mirrored in the results of a larger scale experiment would provide further, more robust support for the increased efficacy of phage-antibiotic treatment. Ultimately, the most compelling results would come from studying the synergistic effects of phages and antibiotics *in vivo*.

Secondly, in addition to varying the concentrations of antibiotic and phage, taking a look at the timing of each treatment would be useful. Previous research with other bacterial species has established that, while antibiotic and phage being administered simultaneously has a strong killing effect, administering phage several hours before administering antibiotic can have a stronger effect (73). Our research was primarily concerned with different phage titers and antibiotic concentrations. Reexamining the synergy of the phages and antibiotics used in this study with a staggered administration methodology could potentially elucidate further the interaction between these two treatments. This is highlighted in our results with Cam where the initial bacteriostatic effects prevented rapid action by the phage. Administering the phages quickly once

resistance to Cam starts to emerge would allow them to function unhindered by the antibiotic.

Finally, the design of the formulation should be expanded. The initial study design involved investigating the efficacy of a cocktail of multiple phages compared to that of a single phage strain. The inclusion of multiple phage strains would suppress growth for a longer duration as it presents additional requirements for developing resistance. To investigate the effect of a phage cocktail, a series of 24-well plate studies would be conducted where different mixtures of phages would be placed in each well. A test of ANOVA would be used to determine if concentration and phage identity had an effect on time to develop resistance in order to select the most potent cocktail.

Additionally, while not a goal of this study, the results with AB9 and Min open up the possibility of a new project looking for prophages in a number of pathogenic bacteria. The wildtype *E. coli* λ phage is a temperate phage but there are a number of known mutations that prevent it from undergoing the lysogenic cycle and instead becoming a virulent phage. One of the main issues with the use of temperate phages for antimicrobial therapy is the potential for them to facilitate the transfer or acquisition of pathogenicity associated genes through the lysogenic cycle. Introduction of mutations into these temperate phages to prevent the lysogenic cycle would ameliorate this concern and could significantly broaden the number of therapeutically useful phages. This is especially true for pathogens such as *C. difficile* for which only lysogenic phages have been described.

Conclusion

The potential for phages to combat bacterial infections may have been indirectly recognized in ancient times, but it is becoming increasingly relevant to the practice of modern medicine. As bacteria grow increasingly resistant to antibiotic treatment, phages can only become more important in disease prevention. Fortunately, phages have a myriad of potential applications, many of which can be explored in future research.

The necessity to develop unique methodologies for the delivery of phage cocktails also poses many possible research questions. Phages are incredibly widespread and diverse, meaning that no single methodology will prove ideal for all treatments. In a similar vein, boundless potential for research on new phages remains. Given that phages are the most prevalent replicating entities on Earth, novel phage research and new applications for known phages remain very promising avenues for further consideration.

Phages have an array of useful applications beyond replacing antibiotics, such as in combating contaminations which may not be treatable through antibiotics alone. In recent studies, phages have been shown to be effective in the treatment of bacterial biofilms, which antibiotics are not able to destructively target. Thus, the possible applications and avenues for future research with phages can take on many different routes beyond therapeutic purposes, such as in the food industry, sanitation and filtration systems, and hospital safety measures.

Antibiotics may be losing their former potency, but that does not make them completely irrelevant. An interesting avenue for further testing could be to test the efficacy of a synergistic treatment that includes both phages and antibiotics. Past tests have supported the idea that combinations may be more effective than the exclusive use of either

treatment (59). The use of antibiotics and phages in combinations provides an interesting avenue of study, and further testing is undoubtedly necessary.

With multiple research fronts, a host of useful applications, and largely unexplored solutions by mainstream pharmaceuticals, phages represent a research goal for scientists and are at the forefront of the post-antibiotic era.

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