AMERICAN UNIVERSITY OF BEIRUT

ESTABLISHMENT OF A BACTERIOPHAGES PLATFORM: ALTERNATIVE THERAPEUTIC OPTION

by
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ABSTRACT
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Background: Pathogenic *Escherichia coli*, common water contaminant, cause a large number of morbidity and mortality worldwide. Unfortunately, unrestricted use of antibiotics led to the appearance and spread of antibiotic resistant isolates. Regardless of the numerous efforts to overcome antibiotic resistance, this threat to public health is but only increasing. This stresses the need for an alternative therapeutic modality for antibiotics such as bacteriophages.

Bacteriophages are bacteria-killing viruses; lytic bacteriophages are one type of phage that drives the direct targeted killing of bacteria. Unlike lysogenic phages that possess an integrase gene, lytic phages do not. The integrase gene gives phages the ability to incorporate their genetic material into the host genome, thus allowing them to remain dormant in the host. Bacteriophage therapy relies on the use of lytic phages as it aims to treat infections as quickly and effectively as possible. Therefore, we decided to isolate phages against *E. coli* ATCC 25922 from Ain El-Mreiseh and Ramlet El-Bayda sewage samples.

Methods: In the current study, two sewage samples were collected from Ain El-Mreiseh and Ramlet El-Bayda sewage sources. Isolation of phage was done by Double Agar Layer Assay method using *E. coli* ATCC 25922 as host strain. Moreover, phages were characterized on the basis of host range, latent period, burst size, bacteriolytic activity, and biofilm inhibition and elimination capability.

Results: Seven distinct phages were isolated against *E. coli* ATCC 25922 from Ain El-Mreiseh and Ramlet El-Bayda sewage samples. Mean phage titer of above seven phage isolates ranged between 10^{10} and 10^{15} Plaque Forming Units/ml. AM 02 recorded the widest host range followed by AM 01 while AM 03, AM 04, RB 01, RB 02, and RB 03 displayed the lowest host range. RB 03 was found to have the shortest latent period and the smallest burst size. In addition, all 7 phages showed considerable bacterial killing effect, and a potential role in preventing *E. coli* ATCC 25922 biofilm formation; and the phages AM 03, AM 04, RB 01, RB 02, and RB 03 were able to significantly eliminate mature biofilm.

Conclusion: We successfully isolated and characterized seven bacteriophages. Several phages are potential candidates to be included in our phage library giving there promising bacterial killing effect, and ability in biofilm prevention and elimination.

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ABBREVIATIONS

ABR	Anti-bacterial resistance
AM	Ain El-Mreiseh
AR	Antibiotic Resistance
CRISPR-Cas	Clustered regularly interspaced short palindromic repeats-Cas
DLA	Double Agar Overlay Assay
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
E. coli ATCC 25922	*Escherichia coli* ATCC 25922
Lysin	Endolysin
MBEC	Minimum biofilm eradication concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
OMV	Outer Membrane Vesicles
P. aeruginosa	*Pseudomonas aeruginosa*
PAM	Protospacer-adjacent motif
PAS	Phage-Antibiotic Synergy
Phage	Bacteriophage
RB	Ramlet El-Bayda
RBPs	Receptor-Binding Proteins
RNA	Ribonucleic acid
WHO	World Health Organization

CHAPTER I

INTRODUCTION

A. Antimicrobial Resistance

Antimicrobial resistance (AMR) is the phenomenon through which microorganisms like bacteria, viruses, parasites, and fungi acquire new characteristics that allow these pathogens to overcome the action of commercially available drugs. AMR thus equips pathogens with the ability to become resistant against their elimination, making infections harder to treat. This manuscript will solely focus on resistance acquired by bacteria, also referred to as antibacterial resistance (ABR) (Prestinaci, Pezzotti, & Pantosti, 2015).

In the early 1940s (Peacock & Paterson, 2015), antibiotics that provided treatment against bacterial infections were discovered and introduced to the public. Shortly after, Alexander Fleming – the microbiologist who discovered penicillin – warned the public in an interview with *The New York Times* about penicillin misuse that could possibly lead to resistant bacteria. 20 years after penicillin discovery, penicillin-resistant *Staphylococcus aureus* strains emerged, thus confirming Fleming's fears. This paved the way to antibacterial resistance, a current worldwide public health problem (Figure 1). Various antibiotics were later released to the market to combat bacterial strains that may either harbor or lack resistance genes (Browne et al., 2020). Although antibiotics were initially highly effective in clearing various bacterial infections, some of them were rendered ineffective due to the spread of AMR. This necessitated the production of new antibiotics, to target AMR pathogens. However, the discovery of new drugs is laborious and may take up to years, which made it difficult to keep up with the emergence and spread of AMR, especially with bacteria's ability to quickly develop resistance against any antibiotic newly released into the market, or antibiotic still undergoing clinical trials (Organization, 2020) (Browne et al., 2020).

In 2019, the World Health Organization (WHO) declared AMR as a silent pandemic killing more than 70,000 people across the globe every year. This number is expected to increase tremendously to reach about 10 million deaths by 2050 (de Kraker, Stewardson, & Harbarth, 2016).

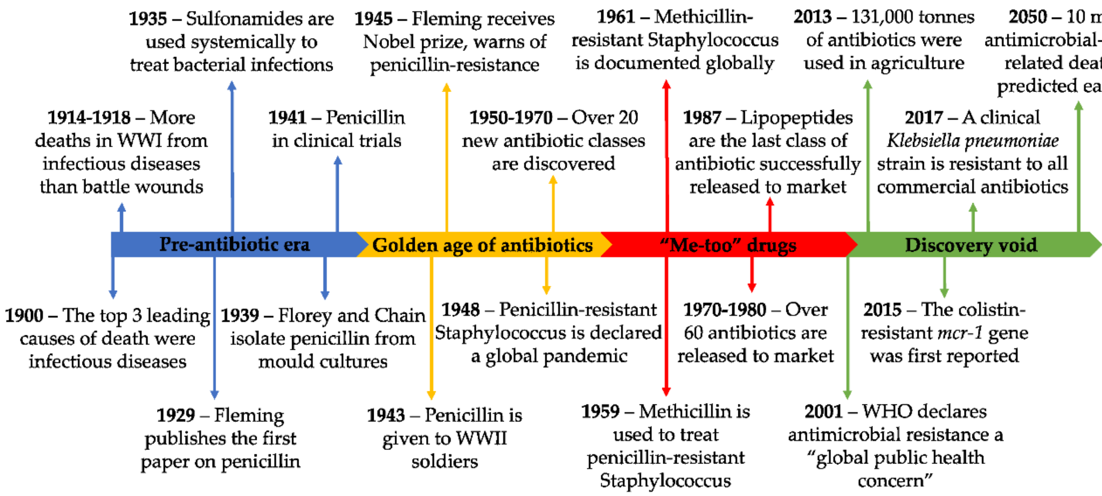

Figure 1: A schematic representation of the ABR timeline taken from (Browne et al., 2020).

1. Factors contributing to the origins of the ABR crisis

One of the main origins of ABR within a bacterium is the bacterial community. Microbes in general, and particularly bacteria, share extremely small genetic material, with minimal capacity of resistance genes carriage. Thus, bacterial evolution and adaptation do not solely rely on the bacterium itself, but on the cooperation of bacterial cells in one community, thus allowing the transfer of genes between these cells that may be assisted by the rapid bacterial generation time. The above two factors ensure the delivery of newly acquired resistance genes throughout the generations (Michael, Dominey-Howes, & Labbate, 2014).

One of the main drivers of acquired antibiotic resistance is the genetic exchange among bacteria. The three major known modes of genetic exchange are bacterial conjugation, transduction, and transformation (Sun, 2018). During bacterial conjugation the *tra* genes found along the conjugative plasmids (donor plasmid), encode for sex pilus formation between the donor and the recipient cell, to ensure a safe transfer of DNA plasmid copies (Virolle, Goldlust, Djermoun, Bigot, & Lesterlin, 2020). Bacterial transformation on the other hand doesn't rely on cell-cell contact or on the presence of viable donor cells but counts instead on the bacterial uptake of the residual persistent DNA in the bacterial surrounding. The bacterial cell will either degrade the transferred DNA via bacterial nucleases, or will integrate it within the bacterial chromosome (Johnston, Martin, Fichant, Polard, & Claverys, 2014). The third known mode of genetic exchange between bacteria is transduction, in which genetic materials are transmitted from one bacterial species to the other through the aid of a mediator virus. A certain type of virus known as bacteriophages with a bacterial infecting tendency, which will incorporate certain bacterial host genes within its viral genome, will deliver these genes to the newly infected bacterial host.

Other bacteria may acquire their resistance through mutations within their genome. Similarly, resistance acquired through mutations may limit the effect of antibiotics, if not abolish it. These mutations may allow a bacterium to eliminate a certain antibiotic upon its entry into the pathogen by 1) using specific enzymes, 2) outflowing the antibiotic using efflux pumps, 3) modifying the antibiotic target and thus altering its effect. In contrast, mutations may altogether prevent the entry of antibiotics by changing the entryways into the bacterium. If an antibiotic is manufactured to target a specific protein or a particular step within the bacterial life cycle, a bacterium can develop a new replicative life cycle that ensures its survival against the antibiotic target (Figure 2) (Kapoor, Saigal, & Elongavan, 2017).

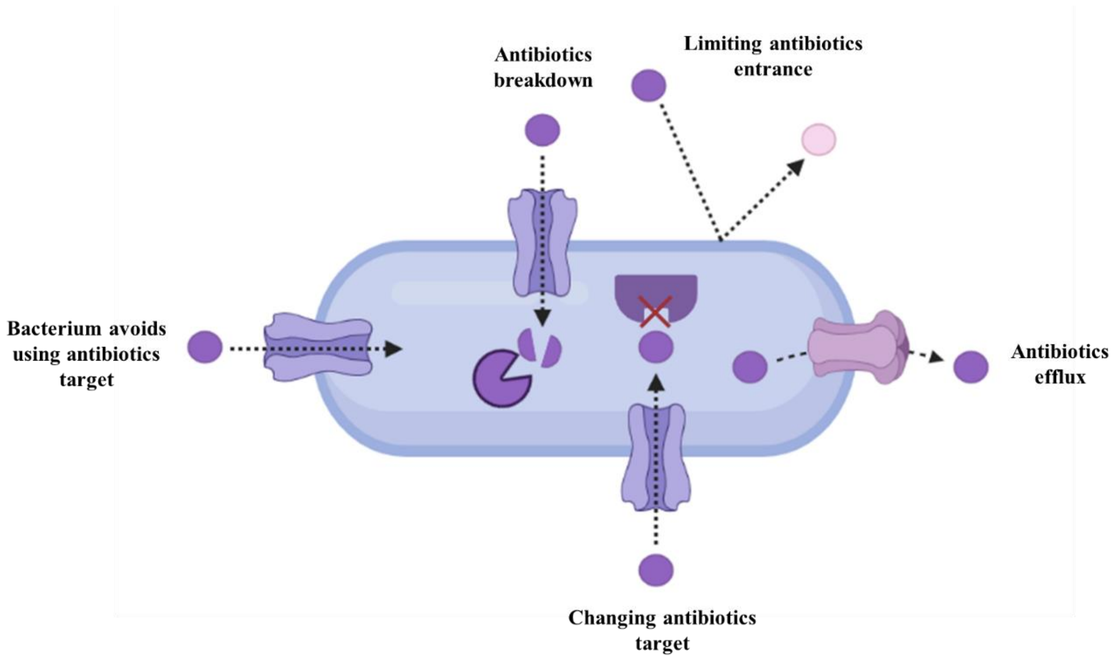

Figure 2: Schematic representation of the defense strategies used by the bacterium to resist the effect of antibiotics.

While some bacterial strains acquire their resistance, others demonstrate an intrinsic natural resistance against antibiotics, which is naturally shared among a certain bacterial species, even without any previous antibiotic exposure. One of the most common natural resistance mechanism is the presence of efflux pumps that may develop into multi-efflux pumps (Reygaert, 2018).

Humans have played a major role in the development and spread of AR. It has been estimated that farm animals consume through their water and food, volumes of antibiotics exceeding the total volume consumed by humans within the whole globe (Wegener32, 2012). The widespread adoption of antibiotic use in food animals, paved the way for the appearance of the first AR coliform bacteria in turkeys administered Streptomycin in their food in 1951 (Starr & Reynolds, 1951). Since then, resistance to antibiotics, such as tetracyclines, sulfonamides, β-lactams and penicillin, has increasingly been observed (Ma, Xu, Tang, Li, & Zhang, 2021). Studies have indicated that the long-term use of antibiotic led to the development and spread of resistant strains between animals, and between animals and humans. Furthermore, AR resistant bacterial transmission to humans is no longer limited to raw food but transgressed to include processed food at all stages (Chang, Wang, Regev-Yochay, Lipsitch, & Hanage, 2015).

In addition, hospitals play a central role in AR spread between patients, as it has been estimated that 5–15% of all hospitalized patients acquire so-called Healthcare Associated Infections, ranging between multidrug-resistant and pan-drug resistant (D'Accolti, Soffritti, Mazzacane, & Caselli, 2019).

The current drastic situation drove scientists towards searching for alternatives to antibiotics, particularly natural alternatives, and in our case, we have decided to tackle bacteriophages as natural alternatives to antibiotics.

B. Definition and Discovery of Bacteriophages

Bacteriophages or "bacteria eaters" in Greek, are filterable viruses that infect or kill bacterial cells without causing any harm to host cells (Kasman & Porter, 2020). Bacteriophage, commonly referred to as "phages, are the most abundant and diverse biological entities on the planet, with more than 10^{31} phages worldwide, each differing in their morphology, shape, and size (Dion, Oechslin, & Moineau, 2020). Bacteriophages coexist along with their bacterial host, where they dwell inside to manufacture new viral particles. Bacteriophage capability of lysing bacterial host within broth or agar cultures, shed the lights on their role in eradicating and eliminating pathogens in humans and animals (E. C. Keen, 2012).

When tackling bacteriophage discovery, scientists often rely on Frederick Twort and Felix d'Herelle's bacteriophage observation from the early 90's. However, it has been reported that the first ever bacteriophage observation took place way before, particularly in 1896. Ernest Hankin, an English bacteriologist, reported the first bacteriophage observation while observing a limitation to the *Vibrio cholera* growth within the collected water sample from Ganges and Jumna rivers in India (Abedon, Thomas-Abedon, Thomas, & Mazure, 2011). The same incident was then reported by a Russian bacteriologist, Gamaleya, while working on *Bacillus subtilis* (Sulakvelidze, Alavidze, & Morris, 2001).

Phages were never explored until the early twentieth century, by Frederick Twort, English physician, and microbiologist. A limitation in the *Micrococcus* growth, the glassy-transparent zone, was observed in one of the agar plates growing *Micrococcus* colonies, like that observed by Hankin and Gamaleya. The obtained glassy-transparent area, which was thought to be a bacterial contaminate, was further explored (Taylor, 2014). Samples were isolated from the glassy-transparent area and were administered to a new generation of *Micrococcus*, yielding the same glassy appearance. Samples were also obtained from the new plate with growth limitations and were administered to a new generation of *Micrococcus*. The same results were again observed, demonstrating that the agent was transmissible. Twort then tried to streak the sample obtained on an agar plate to check for any *Micrococcus* growth, but none was observed, indicating a lethality against *Micrococcus* colonies. The obtained sample was then subjected to a Chamberland candle filtration (fine porcelain filter) to trap any bacterial contaminants, before administering it to a new generation of *Micrococcus*, which yielded a consistent bacterial lethality and glassy-transparent appearance (Duckworth, 1977).

Twort suggested three hypotheses to explain these observations. The first suggested hypothesis was that the cultured generation of *Micrococcus* was secreting enzymes that were small enough in size to pass through filters. He hypothesized that secreted enzymes were causing *Micrococcus* lysis, thus stimulating further enzyme production. This transmission was thought to "almost be considered as an acute infectious disease of *Micrococci*". The second hypothesis pointed toward a change in the *Micrococcus*' life cycle, while the third hypothesis depicted the glassy-transparent sample as a microscopic virus. However, neither of his hypotheses were further investigated due to the World War I eruption. Still, he published a paper in the *Lancet* describing a "glassy and transparent" transformation on agar plates where bacteria would not grow even when sub-cultured, after which he joined the Royal Army Medical Corps in 1915 (E. Keen, 2015).

Two years later, Felix d'Herelle, a Canadian French microbiologist, validated Twort's third hypothesis while examining French soldiers having *severe hemorrhagic dysentery* on the borders of Paris. Herelle started his work by collecting fecal samples from the infected soldiers, to prepare a bacterium-free sample, which would later be mixed with *Shigella* strains isolated from the patients. The mixture obtained was either introduced into animals or streaked on an agar medium to record the bacterial growth. The same glassy-transparent observation was recorded by d'Herelle while observing his plates. The obtained findings were then published by d'Herelle in the *Comptes rendus de l'Academie des sciences*, where he described these glassy-transparent zones as "obligate intracellular parasite" of a bacterium. Thus, validating Twort's third hypothesis (Chanishvili, 2012; Summers, 2017). D'Herelle conducted an efficacy examination to detect the effectiveness of the isolated and propagated phages in the elimination of bacteria. In other words, he tested the possibility of phages to be used as treatment against bacteria. Bacteriophage examination indicated that not all applied phages were equally effective when used as treatments and that each required a specific means of preparation and application (Monk, Rees, Barrow, Hagens, & Harper, 2010).

His work on phage therapy has known success and he thus expanded his work on phage therapy by targeting patients infected with cholera and other enteric diseases. Lieutenant-Colonel Morison relied on d'Herelle's work to combat and save the lives of millions of patients infected with cholera and dysentery in India (Monk et al., 2010). However, Alexander Fleming's penicillin discovery and the use of penicillin as the main treatment option for bacterial infections in World War II, overshadowed phage discovery and therapy. Their application was thus narrowed down to include only Poland and the Ex-Soviet

Union (Moelling, Broecker, & Willy, 2018), before regaining popularity in 1977 when the whole genome of the first phage was sequenced (Gaynes, 2017; Zrelovs, Dislers, & Kazaks, 2021).

Notwithstanding its long history, phages still lag behind in the race of antimicrobials. Nevertheless, phages have succeeded in eliminating resistant bacterial infections, after antibiotic treatment failed. For example, a patient suffering from recurrent subphrenic abscess caused by multi-drug resistant *E. coli* was successfully treated with a 33 day course of bacteriophage (Kwarciński et al., 1994). In addition, phages have been used as a final resort to treat newborns suffering from cerebrospinal meningitidis caused by *Klebsiella pneumonia*, after antibiotic treatment failed. Oral administration of phages over a period of 5 weeks eliminated the infection completely (Strój, Weber-Dabrowska, Partyka, Mulczyk, & Wójcik, 1999). Moreover, clinical improvements have been recorded in patients suffering from infectious allergoses after phage administration. Patients were divided into three groups, the first group received only phages, second received antibiotics, while the third received a combination of both. Clinical improvement was observed among 86 % of the phage treated cases, 48 % of the antibiotic treated cases, 83 % of the phage-antibiotic treatment combination (Sakandelidze, 1991).

C. Bacteriophage Composition and Structure

Phages are simple viruses and like other animal viruses, they vary in morphology, shape, and size. Their genetic material may exist in the form of single stranded, or double stranded, Deoxyribonucleic acid (DNA) or Ribonucleic acid (RNA), It may be encapsulated by a protein capsid-head (Figure 3.A), consisting of several copies of one or more different proteins. Through electron microscopy and genome sequencing, scientists noticed that the phage head can appear in two different forms, either as an icosahedral head (20 sides) or as a filamentous head. Most icosahedral-based phages share a tail that assists the phage in the bacterial attachment step, which will be discussed at a later stage in this manuscript. In contrast, a minority of icosahedral-head phages, along with all filamentous-head phages, lack a phage tail and appear solely as icosahedral and filamentous heads, respectively (Figure 3.B) (Tavares, 2018).

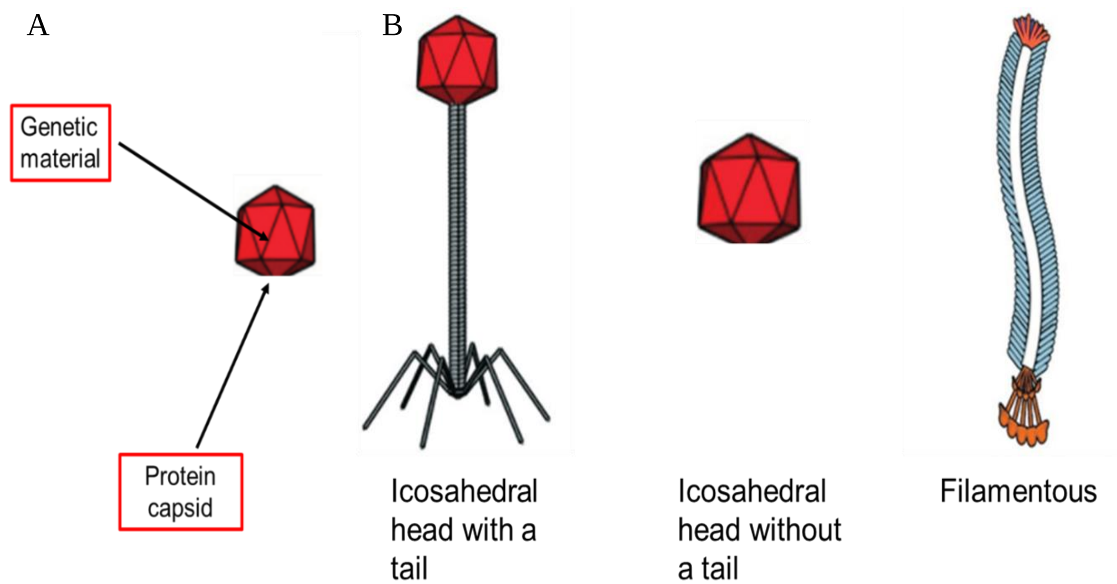

Figure 3: Schematic representation of the phage structure. A) Schematic representation of the phage head. B) Schematic representation of all three different phage structures.

D. Bacteriophages Life Cycles

Bacteriophages have four main life cycles: lytic, lysogenic, pseudo-lysogenic, and chronic life cycles. However, it is worth mentioning that a new phage life cycle was discovered, which is the Cryptic life cycle (Figure 4) (Olszak, Latka, Roszniowski, Valvano, & Drulis-Kawa, 2017) (X. Wang & Wood, 2016).

1. Lytic life cycle

The cycle starts with the adsorption stage in which the phage attaches through its Receptor-Binding Proteins (RBP) to a specific receptor on the bacterial cell surface such as LPS, OmpA and OmpC, Vi-antigen, flagella, and pili. Phages evolved to produce several enzymes such as polysaccharide depolymerases, which aid in the recognition and degradation of capsular polysaccharides and extracellular polysaccharides that might mask bacterial receptors. The phage then proceeds to inject its genetic material into the cytoplasm of the bacterial cell. Once inside the cytoplasm, the phage genetic material hijacks the cell's replication and translation mechanisms and makes use of the host cell's metabolic machinery to make large amounts of viral components. The latter also referred to as virions, keep replicating and maturing until reaching about 200 virions per one infected bacterial cell. Once enough virions have matured and accumulated, a specialized phage-encoded enzyme, particularly endolysin, also referred to as “lysin”, and the protein “holin”, will degrade the bacterial cell wall, allowing the release of virions to the surrounding environment and subsequently lysis of the infected bacterium (Figure 4) (Stone, Campbell, Grant, & McAuliffe, 2019).

2. Lysogenic life cycle (temperate phages)

Similarly, lysogenic phages start by attaching to their host bacterial cell and then penetrating its cell membrane to inject their genetic material. However, their genetic material does not hijack the bacterial replication machinery as in the lytic life cycle, but instead integrates into the host chromosome with the aid of phage-encoded integrases; the phage genetic material incorporated into the bacterial genome is referred to as a prophage. Bacteria harboring the prophage, also known as “lysogen bacteria”, remain viable and constantly propagate the prophage within every bacterial division, thus enabling the prophage-encoded gene for transcription (Howard-Varona, Hargreaves, Abedon, & Sullivan, 2017)

However, the exposure of the lysogeny bacterium to any stressing conditions such as UV light, low nutrient conditions, or chemicals like mitomycin C, will trigger the “lysogenic conversion” phenomenon. This transitions the lysogenic life cycle into the lytic cycle, causing bacterial death and yielding phage virion release to the environment (Figure 4) (Lin, Koskella, & Lin, 2017).

3. Pseudo-lysogenic life cycle

Phages may pass through a transitional stage known as the pseudo-lysogenic life cycle, owing to stressful living conditions (starvation). In this cycle, the phage prophage does not cause bacterial lysis as per the lytic phage, and does not integrate and divide along the bacterial chromosomes, but is instead preserved in the form of linear or circular plasmids (Fortier & Sekulovic, 2013). Restoring the ideal growth conditions (nutritional supplies) for bacteriophages allows them to overcome the unstable development stage, also known as "stalled development", and thus commit to either the lytic or lysogenic life cycles (Figure 4) (Clokie, Millard, Letarov, & Heaphy, 2011; Łoś & Węgrzyn, 2012).

4. Chronic life cycle

Chronic phages, unlike the previously mentioned phages, fall under two main categories. They can display a chronic-lysogenic life cycle, characterized by the integration of phage genetic material within the bacterial host chromosome, without releasing any virions to the surrounding environment and without causing any harm to the harboring bacterium (Hobbs & Abedon, 2016). In contrast, they may follow a pure chronic life cycle, during which continuous production and release of new active virion particles take place, without causing any harm or death to the housing bacteria (Figure 4) (Howard-Varona et al., 2017).

5. Cryptic life cycle

Nevertheless, the pseudo-lysogenic and chronic are not the only subcategories of the lytic and lysogenic life cycles, as a newly classified subcategory was announced, which is the cryptic life cycle. Cryptic phages are the outcome of lysogenic mutagenesis in which the phage prophage remains trapped within the bacterial chromosome. While normally, exposing bacteria to any stressing conditions causes prophage detachment from the bacterial genome, unlike lysogenic prophages, cryptic prophages do not transition to the lytic cycle and exit the bacterial host cell in an inactive form without causing any harm to it (X. Wang & Wood, 2016). These prophages can harbor various genes that can influence bacterial metabolism, division, and susceptibility to antibiotics and phage therapy (Ragunathan, Vanderpool, & O'Toole, 2019).

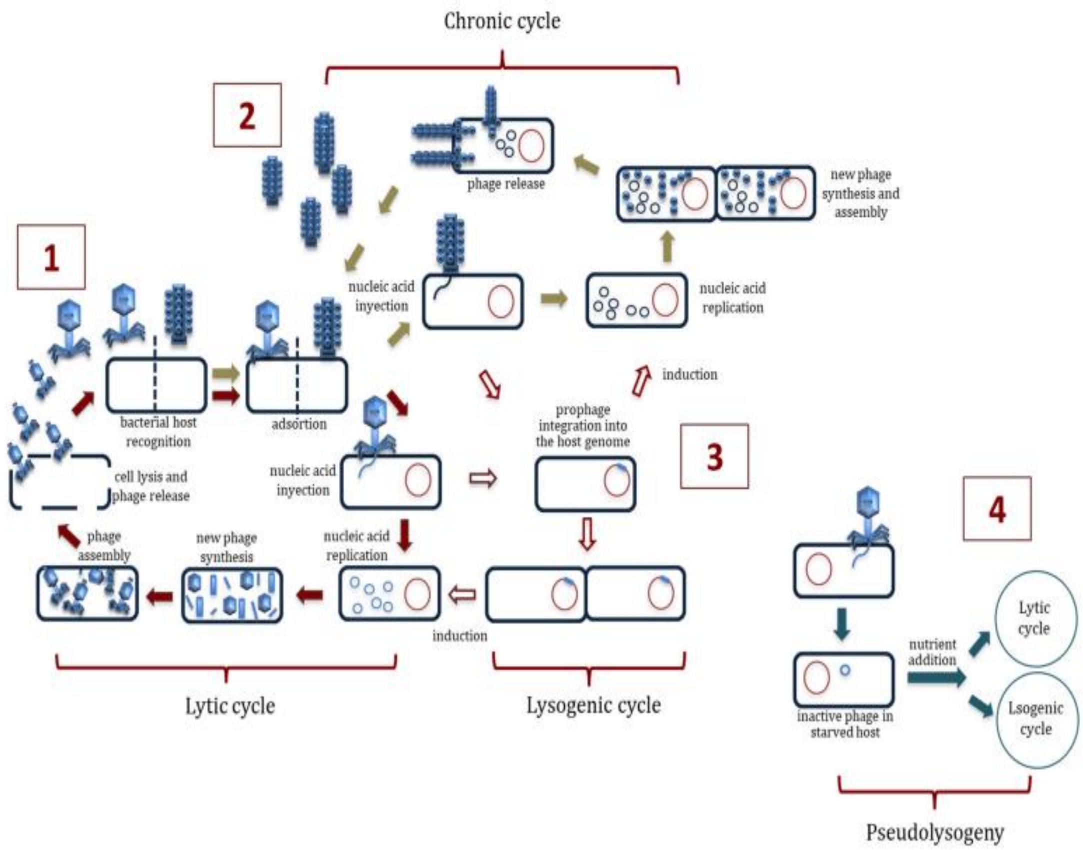

Figure 4: A schematic representation of the bacteriophage life cycles. 1) Lytic life cycle, 2) Chronic life cycle, 3) Lysogenic life cycle, and 4) Pseudo-lysogenic life cycles, taken and modified from (Sieiro et al., 2020).

E. Phage therapy

Phage therapy is the administration of phages to treat and eliminate a certain bacterial infection (Brives & Pourraz, 2020). Phage delivery approaches fall into 4 main categories which are: conventional phage therapy, phage-derived lytic enzyme, combinatorial phage therapy, and bioengineered phages (Lin et al., 2017).

1. Conventional phage therapy

Conventional phage therapy is defined as the administration of lytic phages solely to eliminate a certain bacterial infection. Phages administered in a conventional phage therapy approach can either be monophages, single phage administration, or polyphages, multiple phages administration. Monophages are applied for screening, controlling, and examining the efficacy of developed phage therapy against animal models suffering from multidrug resistant bacterial infections. However, monophages are constantly subjected to environmental stressing conditions, and most importantly bacterial resistance (Gordillo Altamirano & Barr, 2019).

Minimal to insufficient successful applications of monophage therapy have been reported. To overcome the monophage therapy limiting factors, polyphage therapy, or what is known as cocktail phage therapy, was established. Phage cocktails were designed to target a single bacterial strain, multiple strains belonging to the same species, or even multiple strains belonging to different species (Gordillo Altamirano & Barr, 2019).

One of the most promising applications of cocktails phages was the administration of a combination of 5 different phages to treat rabbits suffering from an enteric infection. A decrease in the *Vibreo cholera* bacterial load was recorded in mice orally treated with the above cocktail of phages (B. K. Chan, Abedon, & Loc-Carrillo, 2013), (Jaiswal, Koley, Ghosh, Palit, & Sarkar, 2013). Despite the promising findings and outcomes, polyphage preparation is also subjected to various challenges that include: 1) a complex and long preparation process, and 2) triggering an immune response against the delivered phage cocktail (Gordillo Altamirano & Barr, 2019). Owing to the previously mentioned limits, scientists followed a new phage therapy approach that included the use of both monophages and polyphages, followed by sequential delivery of phages, rather than a simultaneous one. This innovative approach was referred to as "sequential phage therapy". This therapy approach facilitated the activity of the applied phages and allowed phages to overcome any arising bacterial resistance. In addition to that, the sequential technique limited the bacterial propagation and granted the immune system the time needed to naturally eliminate the targeted bacteria (Gordillo Altamirano & Barr, 2019; Hall, De Vos, Friman, Pirnay, & Buckling, 2012).

2. Phage-derived lytic enzymes

As mentioned earlier, lytic phages rely on two enzymes encoded by the phage genome to lyse the bacterial cell wall during the lytic life cycle. These enzymes are the "lysin" and "holin" enzymes (Oliveira, São-José, & Azeredo, 2018). Once the phage hijacks the cell's replication and translation mechanisms, phage genes will be expressed, and these two enzymes will aid in the hydrolysis of the cell wall and progeny release. During viral assembly, the phage-encoded holin, a transmembrane protein, accumulates in the bacterial cell membrane, and controls the phage latent period, which is defined by the timing of phage-induced host cell lysis. In addition, holin proteins will trigger at the end of the lytic cycle, the opening on the cytoplasmic side of the cell membrane. The opening created by the holin protein, allows the lysin protein, a peptidoglycan hydrolase, to flow and hydrolyze the bacterial cell wall, thus releasing the assembled progenies. Most researchers focused their work on lysin alone, as lysin can hydrolyze the bacterial cell wall independently from holin, while holin cannot perform this function (Lin et al., 2017).

An exogenous administration of phage lysin against MDR bacterial respiratory pathogens, aided in the hydrolysis of various bacterial peptidoglycan bonds, without triggering an adverse immune response, and without causing any phage resistance (Vázquez, García, & García, 2018). In addition, lysin proved to play a major role in combating bacteremia in mice, viable bacteria in the blood, caused by multidrug-resistant *Acinetobacter baumannii, Streptococcus pneumoniae*, and others (Lood et al., 2015; Witzenrath et al., 2009).

3. Combinatorial phage therapy

Antimicrobial resistance was the main reason that drove scientists towards phage therapy as an alternative to antibiotics. However, despite all advantages of phage therapy over antibiotic use, bacteria ended up developing phage resistance, thus worsening the bacterial resistance threat worldwide. The plan to overcome this ongoing and growing crisis involved merging both antibiotics and phages and applying them in a synergistic fashion or what is known as Phage-Antibiotic Synergy (PAS) (Comeau, Tétart, Trojet, Prère, & Krisch, 2007; Tagliaferri, Jansen, & Horz, 2019).

PAS was primarily investigated by *Comeau AM et al* in 2007, where phage lytic activity against *E. coli* was further enhanced with the administration of sublethal concentrations of β-Lactam and quinolone antibiotics. The above phage-antibiotic combination yielded an increase in the phage-bacterial adsorption rate, accompanied by a shorter phage latent period (Comeau et al., 2007). Furthermore, a study conducted by *Ryan EM et al* in 2012 confirmed the major role of PAS in biofilm control and eradication. Where the minimum biofilm eradication concentration (MBEC) of cefotaxime against *E. coli* ATCC 11303, the lowest concentration of cefotaxime required to eradicate *E. coli* ATCC 11303, decreased from 256 to 128 and 32 µg/mL upon cefotaxime combination with medium and high titers of T4 phage, respectively. In addition, T4-cefotaxime combination, displayed a decrease in T4 latent period from 24 min to 18 min, in addition to an increase in T4 phage titer, indicating a better phage replication and adsorption rate (Ryan, Alkawareek, Donnelly, & Gilmore, 2012).

However, restoring bacterial sensitivity to a specific antibiotic remains the main goal of any phage-antibiotic combination (B. K. Chan et al., 2018). *Chan BK et al* in 2016, conducted a study to evaluate the role of PAS in resolving aortic prosthetic graft infection by *P. aeruginosa*. The phage targeted within the study, OMKO1, is a lytic phage that utilizes the outer membrane porin M (OprM) of the multidrug efflux systems MexAB and MexXY as a receptor-binding site. The administration of OMKO1 on its own triggered phage resistance, dedicated to changes in the efflux pump mechanism. The above mutation restored the bacterial sensitivity to Ceftazidime, Ciprofloxacin, Tetracycline, and Erythromycin (B. K. Chan et al., 2016).

Moreover, the combinatorial phage therapy was developed to include the administration of phage encoded enzymes, particularly lysins along with antibiotics. Wang et al in 2015 proved that the administration of PlyCD-lysin (PlyCD1-174), yielded greater lytic activity and a broader lytic spectrum against *C. difficile* strains while remaining inactive against eukaryotic cells. Furthermore, vancomycin-PlyCD1-174 application proved to be more bactericidal against *C. difficile,* than vancomycin alone (Q. Wang, Euler, Delaune, & Fischetti, 2015).

4. Bioengineered phages

Lysin is species specific as it targets certain peptidoglycan domains of specific bacterial species cell wall. The peptidoglycan targets of these lysins are well-conserved and play a major role in the cell wall composition. Nevertheless, the general structure of phage lysin differs depending on the bacterial host nature, whether gram negative or gram positive. Lysins from bacteriophages that infect Gram-positive bacteria consist mainly of two main domains, N-terminal catalytic domain, and a C-terminal cell wall binding domain responsible for the high specificity of lysins. Gram-negative lysins, on the other hand, lack a cell wall binding domain, and consist instead of an individual catalytic domain with terminal-charged residues for cell wall binding (Ghose & Euler, 2020). Bioengineered phages have played a major role in lysin domain fusion procedure where two-gram positive lysin phages with similar host range specificity, are fused to provide a novel lysin with greater lytic activity. Yang et al in 2014 constructed a chimeric lysin named ClyH, by fusing the catalytic domain of Ply187 lysin with the cell wall binding domain of phiNM3 lysin, both sharing the same lytic activity against certain methicillin-resistant *Staphylococcus aureus* clinical isolates. ClyH proved to have higher efficacy than the parental lysin, and its administration in MRSA infected mice yielded a decrease in the MIC when compared to the administration of each lysin on its own. In addition, single intraperitoneal administration of ClyH saved the MRSA infected mice from death (Yang et al., 2014).

CHAPTER II

OBJECTIVES AND AIMS

The World Health Organization (WHO) has long recognized the need for an improved and coordinated global effort to contain AMR. In 2017, the WHO provided for the first time a list of 12 antibiotic-resistant "priority pathogens" bacterial families, that posed the greatest threat to human health. These families were later categorized into 3 categories, critical, high, and medium priority, based on their impact on health and the urgent need for new antibiotic development. The critical category comprises *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella spp.*, *Escherichia coli (E. coli)*, *Serratia spp.*, *and Proteus spp*, posing severe and deadly infections such as bloodstream infections and pneumonia (Tacconelli, Magrini, Kahlmeter, & Singh, 2017).

Many studies have highlighted the major role of sewage as a major reservoir for resistance bacteria, particularly the ones belonging to the critical category. Nonetheless, both treated and untreated sewage sources play a major role in the spread of undegradable Antimicrobial Resistant Genes (ARG) among the residing bacterial community, thus facilitating bacterial resistance (Fouz et al., 2020; Rizzo, Fiorentino, & Anselmo, 2013). Unfortunately, inadequate wastewater treatment and disposal, particularly in low- and middle-income countries have eased the spread of AMR in aquatic environments (Larsson, 2014; Mendelson et al., 2016). In addition to being categorized under the critical antibiotic resistant category, a recent study has displayed the critical role of waterborne *E. coli* as a major reservoir for AMR worldwide (Haberecht et al., 2019).

E. coli is a gram-negative bacterium that belongs to the *Enterobacteriaceae* family and is the most prevalent commensal inhabitant of the gastrointestinal tracts of humans and warm-blooded animals, while rarely causing disease to their host. It is however considered one of the most common human and animal opportunistic pathogens, owing to its ability to survive both aerobic and anaerobic conditions along with its wide availability (Yoon, Jeong, Kwon, & Kim, 2009).

Lebanon on the other hand, share along with the Eastern Mediterranean countries a prevalence of resistance higher than several European countries (Salem, Dahdouh, & Daoud, 2013). A study conducted by *Dagher LA et al* in 2021 to assess the water quality in 14 different rivers water, detected the presence of fecal coliforms (facultatively anaerobic, rod-shaped, gram-negative, non-sporulating bacterium, that residues in the intestines of warm-blooded animals) and *E. coli* in 96.29% and 95.5% of the samples, respectively. Out of the isolated samples, 45.8% were classified as MDR (Dagher, Hassan, Kharroubi, Jaafar, & Kassem, 2021). In addition, a study conducted on 5 major rivers in Lebanon, showed major dissemination of MDR *E. coli* and *Klebsiella pneumoniae* isolates, among the collected samples (Moussa, Abboud, & Tokajian, 2021).

Given the above, this study aims to isolate and characterize bacteriophages with lytic activity against multi-drug resistant *Escherichia coli* from Ain El-Mreiseh and Ramlet El-Bayda sewage water. The obtained phages will represent part of the phage library, which will cover ultimately several bacterial species.

CHAPTER III

MATERIALS AND METHODS

A. Bacterial Strains and Growth Conditions:

Bacterial strains used in this study are listed in **Table 1**. *E. coli* strains were stored at -20°C in 60% glycerol and cultivated at 37°C, with shaking at 160 rpm, in liquid Luria–Bertani broth (LB) or plated on solid LB medium with 1.5% agar. The plates with a solid medium were incubated at 37°C for 24 h. The phage isolation process was performed at 37°C using *E. coli* ATCC 25922 as a screening isolate, under aerobic conditions without shaking. The biofilm assay was performed using the *E. coli* 49-448 clinical isolate, which was grown at 37°C without shaking in a 96 well plate.

Table 1: Bacterial strains used in the study.

Bacterial strain	**Source or other characteristics**
***E. coli* LABORATORY STRAINS**	
ATCC® 25922™	Serotype 06, Biotype 01
***E. coli* CLINICAL STRAINS**	
7- 1146	Urine
8-1150	Wound
17-1164	Urine
18-1166	Urine

22- 1176	Urine
39-242	Urine
49-448	Urine
64- 201680081	Blood
65- 202350075	DTA
66- 202340112	Sputum
67- 202350068	Sputum
71-203070013	Urine

B. Isolation of Bacteriophage from Ain El-Mreiseh and Ramlet El-Bayda Urban Sewage:

Sewage samples were collected in a 50 ml flacon tube from Ain El-Mreiseh and Ramlet El-Bayda untreated sewage sources in Beirut. The collected falcon tubes were placed on the bench for 24 hours at room temperature to sediment all the debris. The next day, 20 ml of each of the sewage sample was transferred into its corresponding Erlenmeyer flask, followed by the administration of 2.5 ml 0.5 McFarland *E. coli* ATCC 25922, and 2.5 ml LB broth into each flask. Both flasks were then cultivated overnight at 37^{o}C with shaking at 160 rpm. The obtained phage lysate was centrifuged at 5000 rpm for 15 min at room temperature, and then filtered through 0.4- & 0.22-micron filters to remove any residual bacterial cells. To observe plaques formed by millions of phages against *E. coli* ATCC 25922 colonies, the Double Agar Overlay Assay (DLA) was performed. Briefly, standard Petri dishes were filled with 25 ml of 0.8-1 % LB agar, yielding the bottom LB agar. Then, a 10 µL diluted phage sample was mixed with 1 ml of *E. coli* ATCC 25922 cells (mid-log phase)in a 15 ml falcon tube, and the tube was left to incubate at room temperature for 10 min. Approximately 3 mL of the top agar (LB with 0.6% agar) at 50°C was then added on top of the phage-bacteria mixture, and the mixture was poured on top of the bottom LB agar. The plates were left to air dry and were then incubated at 37°C overnight to visualize any phage plaques. The obtained plaques were then placed in a 1.5 ml Eppendorf tube containing 500 μL SM buffer (100 mM NaCl, 10mM $MgSO_4$, and 50mM Tris-HCl, PH 7.5). The tubes were then centrifuged at 10,000 × *g* for 2 min, to collect the supernatant. The tubes were then left at 4°C for 24 h to allow phage to diffuse into the buffer at 4^{o}C.

C. Purification of the Isolated Phages:

The aim of the following experiment was to determine the Plaque Forming Unit per ml (PFU/ml) for all seven phages. One hundred μL of the phage mixture stored in SM buffer were transferred into a 15 ml flacon tube, followed by the addition of 10 ml LB broth and 100 μL of 0.5 MacFarland *E. coli* ATCC 25922. The flacon tube was cultivated overnight at 37^{o}C with shaking at 160 rpm. The obtained phage lysate was centrifuged at 5000 rpm for 15 min at room temperature, and then filtered through a 0.4- & 0.22-micron filters to remove any residual bacterial cells. Then the DLA technique was conducted, and the phages were again isolated, centrifuged, and stored in 500 μL SM buffer at 4^{o}C. The same procedure was repeated at least 3 times or until reaching homogeneous plaques morphology, to determine each phage's PFU/ml.

PFU/ml= (number of plaques per plate)/(dilution factor)(lysate volume)

D. Bacteriophage Turbidity Testing

Bacteriophage turbidity assay was applied as a primary screening method to identify the phage host range. The turbidity testing assays will allow us to screen our phages against previously sequenced bacterial *E. coli* clinical isolates. Three 15 ml falcon tubes were assigned for each phage turbidity assay, a testing tube having 990 µL LB broth, 10 µL phage lysate, and 3ml 0.5 MacFarland of the *E. coli* clinical isolate we aim to test, a positive control tube having LB broth and 3ml 0.5 MacFarland of the *E. coli* clinical isolate, and a negative control having LB broth only. Then all three tubes' optical densities were measured, before placing them in the incubator at 37^{o}C with shaking. The optical density was again measured after 4 hours of incubation, then the tubes were returned to the incubator. The optical density was again measured after 24 hours of incubation. The optical density of the testing tube was compared at each time point to that of the positive control to record any decrease in the optical density, which consequently indicates activity of the phage against the tested *E. coli* clinical isolate. The following technique was conducted against all bacterial isolates mentioned in Table 1.

E. Bacteriophage Spot Lysis Assay

The first host range screening method was the turbidity testing, in which phages were placed in a 3 ml 0.5 MacFarland of the *E. coli* clinical strains, the tubes were then incubated at 37^{o}C with shaking overnight. The results obtained during the turbidity testing were confirmed using the spot lysis assay on a double agar layer plate. Briefly, standard Petri dishes were filled with 25 ml of LB agar. Then, 3 ml of LB medium, supplemented with 0.6

% agar were mixed with 990 µL LB broth and 3 ml of 0.5 McFarland *E. coli* ATCC 25922 cells and were then poured on top of the LB bottom agar. Ten-fold dilutions of phage lysate were prepared in LB broth and spotted onto the surface of the double agar layer plates with a tested host. Plates were incubated overnight at 37°C without shaking. The plates were then examined the next day for any plaque formation. The results observed will be differentiated into 2 different categories based on the observation of plaques : (+) for plaque observation, and (-) for no plaques. The following technique was conducted against all bacterial isolates that provided a clarity among the turbidity testing tube or recorded a decrease in the optical density when compared to the positive control.

F. Bacteriolytic Activity Determination

The bacteriolytic activity of all seven phages was evaluated in a 96 well-plate against *E. coli* ATCC 25922, *E. coli* 39-242, *E. coli* 49-448, *E. coli* 64-201680081, and *E. coli* 71-203070013 at OD 600 nm for 13 hours. The absorbance of the wells harboring phage-bacteria mixture at MOI 10, 1, 0.1, and 0.01 was compared to that of the positive control containing 0.5 McFarland host bacterial cells and LB broth, and to that of the negative control wells containing LB broth only. Five wells were designated for each MOI per run.

G. One-Step Growth Curve

The following experiment was conducted to detect the number of phage particles released from the infected *E. coli* ATCC 25922 cells in one hour while plating the bacterial-viral mixture every 5 minutes. Two 15 ml falcon were labeled for each time point as follows:

10^{-2} and 10^{-4}, respectively. 990 µL LB broth was added to each 15 ml falcon tube. The phages lysate was then diluted to reach an MOI of 1. Then, 0.1 ml of the obtained diluted phages were added on top of the adsorption tube containing 9.9 ml LB broth. At t=0 minutes, 0.01 ml of the adsorption tube mixture was transferred into the 10^{-2} tube, the tube was properly vortexed before transferring 0.01 ml of its mixture into the 10^{-4} tube. 3 ml 0.6 % agar was added on top of both tubes, before pouring the obtained mixture into its corresponding LB agar plate. The adsorption tube was left to incubate for 5 minutes at room temperature. At t=0 minutes, 0.01 ml of the adsorption tube mixture was transferred into the 10^{-2} tube, the tube was properly vortexed before transferring 0.01 ml of its mixture into the 10^{-4} tube. 3 ml 0.6 % agar was added on top of both tubes, before pouring the obtained mixture into its corresponding LB agar plate. The same procedure was repeated till reaching t=60 minutes. All plates were left to air dry at room temperature before placing them in the incubator at 37°C for 24 hours. The plates were then removed from the incubator, and the number of plaques observed per each plate was recorded.

H. Effect of Phages on Bacterial Biofilm Formation-Inhibition of Biofilm Formation (IF) Treatment Assay

The following experiment was executed in a 96-well plate against *E. coli* ATCC 25922 bacterial strain. The chosen bacterium was streaked on an LB agar plate, and the plate was left to incubate at 37°C overnight. Then, a single bacterial colony was inoculated using a sterilized loop into a 3 ml LB broth in a 5 ml polystyrene tube. The obtained tube was then incubated at 37°C with shaking at 160 rpm overnight. 500 µL of the overnight bacterial culture were transferred into a 5 ml polystyrene tube having 2 ml LB broth while adjusting the LB-bacterial mixture to a 0.5 McFarland optical density. Hundred-fold dilution of the resulting bacterial solution was prepared by adding 100 μL of the bacterial solution to a 9900 μL LB broth. 150 μL of the diluted bacterial solution were added to all wells but the negative control while adding 200 μL of the LB broth to the negative control wells.

Within this experiment, two antifungals were used as controls, the anidulafungin and micafungin. Anidulafungin and micafungin are semisynthetic echinocandins that inhibit 1,3-β-d-glucan synthase, an enzyme involved in Aspergillus and Candida species cell walls synthesis. Nevertheless, Rogiers O et al in 2018, proved that anidulafungin reduced the abundancy of S. aureus poly-β-(1,6)-N-acetylglucosamine, a staphylococcal biofilm matrix polymer, inside serum-coated triple-lumen catheters placed in 24-well plates (Rogiers et al., 2018). In addition, micafungin demonstrated a major role in eliminating *Pseudomonas aeruginosa* biofilms in vitro and in vivo, as *Pseudomonas aeruginosa* has a 1,3-β-d-glucan in its cell wall (Kissoyan, Bazzi, Hadi, & Matar, 2016).

A phage isolated against *Salmonella infantis* (Sal INF 122131 M) was applied as negative control, which was proven to lack any activity against *E. coli* ATCC 25922 using the spot testing assay.

Fifty μL of AM 01, AM 02, AM 03, AM 04, RB 01, RB 02, RB 03, anidulafungin, micafungin, DMSO (diluted in LB broth), 0.9 % NaCl, and Sal INF 122131 M were added to their designated wells, while 50 μL of diluted *E. coli* ATCC 25922 was added to all positive control wells. The plate was then incubated at 28°C for 24 hours without shaking. The planktonic cells were then removed, and the plate was washed three times with 200 μL 1X PBS. The plate was left to air dry at room temperature for 30 minutes. All wells were later stained with 100 μL of 0.1 % crystal violet to measure the optical density (OD) of the crystal violet-stained biofilm matrix, and the plate was left to incubate at room temperature for 20 minutes. Crystal violet was then removed, and all wells were properly washed three times with 1X PBS to eliminate excess crystal violet stain. The plate was left to air dry for 30 minutes. The biofilm matrix was then detached from the wells with the administration of 95 % ethanol, the plate was left again to incubate at room temperature for 10 minutes. Then, the content of the 96-well round bottom plate was transferred into a 96-well flat bottom plate to check the plate's optical density at 595 nm.

I. Phages Biofilm Clearance Ability-Post Biofilm Formation (PF) Treatment Assay

The same treatments applied for the IF assay was applied to the PF assay. 200 μL of the diluted bacteria were added into all wells except for the negative control wells having 200 μL LB broth only. The plate was then incubated at 28°C for 24 hours without shaking. Planktonic cells were removed the next day, followed by three times washes with 1X PBS. One hundred fifty μL LB broth were added to all wells, followed by the addition of 50 μL of AM 01, AM 02, AM 03, AM 04, RB 01, RB 02, RB 03, anidulafungin, micafungin, DMSO (diluted in LB broth), 0.9 % NaCl, and Sal INF 122131 M to their designated wells, while 50 μL of diluted *E. coli* ATCC 25922 and 50 μL LB broth were added to positive and negative control wells, respectively. The plate was then incubated at 28°C for 5 hours without shaking. The planktonic cells were then removed, and the plate was washed three times with 200 μL 1X PBS. The plate was left to air dry at room temperature for 30 minutes. All wells were later stained with 100 μL 0.1 % crystal violet to measure the optical density (OD) of the crystal violet-stained biofilm matrix, and the plate was left to incubate at room temperature for 20 minutes. Crystal violet was then removed, and all wells were properly washed three times with 1X PBS to eliminate excess crystal violet stain. The plate was left to air dry for 30 minutes. The biofilm matrix was then detached from the wells with the administration of 95 % ethanol, the plate was left again to incubate at room temperature for 10 minutes. Then, the content of the 96-well round bottom plate was transferred into a 96-well flat bottom plate to check the plate's optical density at 595 nm.

CHAPTER IV

RESULTS

A. Isolation of Seven phages from Ain El-Mreiseh and Ramlet El-Bayda:

The DLA performed using Ain EL-Mreiseh enriched sewage sample yielded various plaque morphologies (Figure 5). All the obtained plaques were isolated; however, only four plaques were further examined throughout our study. The examined plaques are: (A) Circular clear zone with a small size (Escherichia phage AM 01), (B) Circular clear zone with a large size (Escherichia phage AM 02), (C) Circular halo zone with a small size (Escherichia phage AM 03), and (D) Circular halo zone with a large size (Escherichia phage AM 04) (Figure 5).

Similarly, different plaque morphologies were observed after performing the DLA using Ramlet El-Bayda enriched sewage sample (Figure 6). All the obtained plaques were isolated; however, only three plaques were further examined throughout our study. The examined plaques are: (A) Circular clear zone with a large size (Escherichia phage RB 01), (B) Circular clear zone with a medium size, and (C) Circular clear zone with a small size (Figure 6).

All the isolated plaques were placed in a 1.5 ml Eppendorf tube containing 500 μL SM buffer (100 mM NaCl, 10mM $MgSO_4$, and 50mM Tris-HCl, PH 7.5), the tube was then centrifuged, and the supernatant was stored at 4^{o}C.

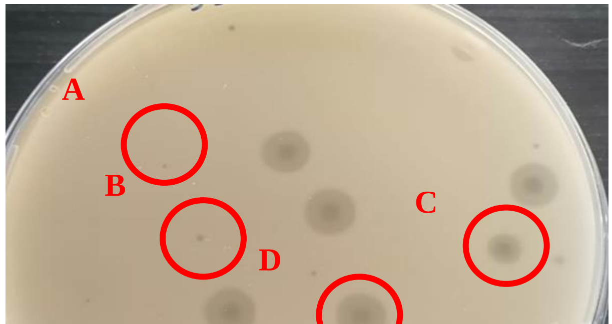

Figure 5: A) Escherichia phage AM 01, B) Escherichia phage AM 02, C) Escherichia phage AM 03, and D) Escherichia phage AM 04 among Ain El-Mreiseh DLA plate against E. coli ATCC 25922.

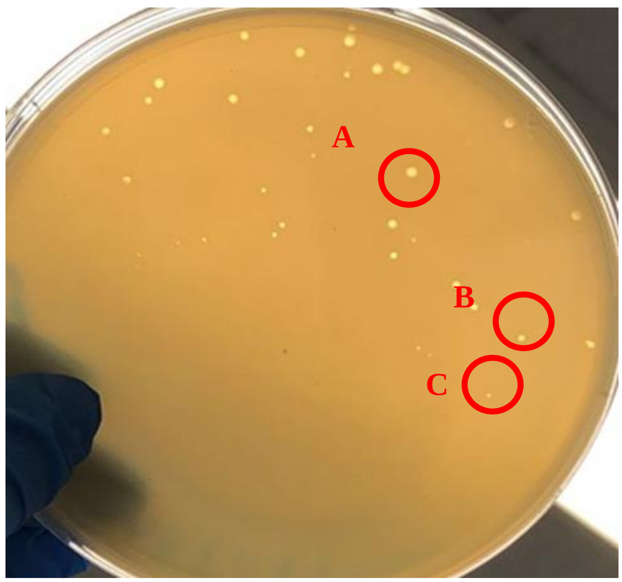

Figure 6: A) Escherichia phage RB 01, B) Escherichia phage RB 02, and Escherichia phage RB 03 among Ramlet El-Bayda DLA plate against E. coli ATCC 25922.

B. Phages Purification Assay

The DLA experiments performed in triplicates for each phage yielded the PFU/ml recorded in Table 2.

Table 2: A table representing the PFU/ml of Ain El-Mreiseh and Ramlet El-Bayda phages.

Official Name	Number of Plaques	Dilution Factor	Plaque Forming Unit/ml
Escherichia phage AM 01 (or AM 01)	4.5	$1/10^{10}$	$4.5x10^{12}$
Escherichia phage AM 02 (or AM 02)	5	$1/10^{8}$	$5x10^{10}$
Escherichia phage AM 03 (or AM 03)	1	$1/10^{8}$	$1x10^{10}$
Escherichia phage AM 04 (or AM 04)	9.66	$1/10^{9}$	$9.66x10^{11}$
Escherichia phage RB 01 (or RB 01)	5.7	$1/10^{12}$	$5.7x10^{14}$
Escherichia phage RB 02 (or RB 02)	10	$1/10^{12}$	$1x10^{15}$
Escherichia phage RB 03 (or RB 03)	4	$1/10^{9}$	$4x10^{11}$

C. Turbidity and Spot Lysis Assay

Turbidity test confirmed phage activity against *E. coli* ATCC 25922, *E. coli* 39-242, *E. coli* 49-448, *E. coli* 64-201680081 and *E. coli* 71-203070013 previously sequenced bacterial strains (Table 3). Activity is represented as a decrease in the turbidity of the testing tube OD at 4 and 24 hours when compared to the bacterial culture (PC) un-inoculated with phage suspension as a control (Table 4). As expected, all phage isolates yielded a clear suspension and a decrease in their testing tube OD against *E. coli* ATCC 25922 when compared to *E. coli* ATCC 25922 PC's OD. On the other hand, five phages (AM 01, AM 02, RB 01, RB 02, and RB 03) testing tubes appeared cleared and displayed a decrease in their OD against *E. coli* 49-448 (Figure 7), while only four phages (AM 01, AM 02, AM 03, and AM 04) yielded an activity against *E. coli* 39-242. Nonetheless, two phages (AM 01 and AM 02) showed activity against E. coli 71-203070013, whereas only one phage (AM 02) showed activity against E. coli 64-201680081. It was noticed that none of the phages provided any activity against *E. coli* 7- 1146, *E. coli* 8-1150, *E. coli* 17-1164, *E. coli* 18-1166, *E. coli* 22-1176, E. *coli* 65- 202350075, *E. coli* 66- 202340112, and *E. coli* 67- 202350068.

Table 3: Sequence type and other data for selected MDR and virulent Escherichia coli strains.

E. coli strains	Sequence type	Plasmids	Host type	Disease
E. coli 39-242	127	Col156	Human/Animals	Sepsis/UTI
E. coli 49-448	453	Col(BS512)/ IncFII	Animals	UTI
E. coli 64-201680081	131	Col(BS512)/ IncFIA/ IncFIB(AP001918)	Human/Animals	UTI
E. coli 71-203070031	69	Col156/ Col440I/ IncFIA/ INCFIB(AP001918) IncQ1	Human/Animals	Sepsis/UTI

Table 4: Testing tube OD in comparison to *E. coli* strains PC OD at 4 and 24 hours

***E. coli* strains**	**Clarity and decrease in the OD at all measuring timepoints**	**Turbidity and increase in the OD at all measuring timepoints**
ATCC 25922	AM 01, AM 02, AM 03, AM 04, AM 05, AM 06, & AM 07	None
7- 1146	None	AM 01, AM 02, AM 03, AM 04, AM 05, AM 06, & AM 07

8-1150	None	AM 01, AM 02, AM 03, AM 04, AM 05, AM 06, & AM 07
17-1164	None	AM 01, AM 02, AM 03, AM 04, AM 05, AM 06, & AM 07
18-1166	None	AM 01, AM 02, AM 03, AM 04, AM 05, AM 06, & AM 07
22-1176	None	AM 01, AM 02, AM 03, AM 04, AM 05, AM 06, & AM 07
39-242	AM 01, AM 02, AM 03, & AM 04	RB 01, RB 02, & RB 03
49-448	AM 01, AM 02, RB 01, RB 02, & RB 03	AM 03 & AM 04
64- 201680081	AM 02	AM 01, AM 03, AM 04, RB 01, RB 02, & RB 03
65- 202350075	None	AM 01, AM 02, AM 03, AM 04, AM 05, AM 06, & AM 07
66- 202340112	None	AM 01, AM 02, AM 03, AM 04, AM 05, AM 06, & AM 07
67- 202350068	None	AM 01, AM 02, AM 03, AM 04, AM 05, AM 06, & AM 07
71- 203070013	AM 01 & AM 02	Am 03, AM 04, RB 01, RB 02, & RB 03

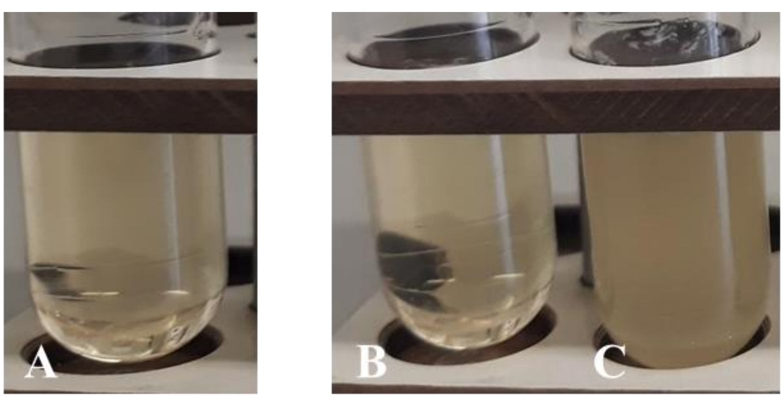

Figure 7: Turbidity testing results of RB 01 against *E. coli* 49-448. A) RB 01 testing tube against *E. coli* 49-448, B) Negative control tube, and C) Positive control tube.

A spot lysis assay was performed to determine the bacterial host range for all seven phages and to confirm the results obtained during the turbidity testing. The results indicated that the phages were capable of infecting *E. coli* ATCC 25922, *E. coli* 39-242, *E. coli* 49-448, *E. coli* 64-201680081 and *E. coli* 71-203070013 strains and yielding a clear plaque (Tables 5 and 6). All phage isolates produced an activity against *E. coli* ATCC 25922. Five phages (AM 01, AM 02, RB 01, RB 02, and RB 03) infected *E. coli* 49-448, while only four phages (AM 01, AM 02, AM 03, and AM 04) infected *E. coli* 39-242. Two phages (AM 01 and AM 02) were able to infect *E. coli* 71-203070013. Whereas only one phage (AM 02) was able to infect *E. coli* 64-201680081. None of the phages could infect *E. coli* 7- 1146, *E. coli* 8-1150, *E. coli* 17-1164, *E. coli* 18-1166, *E. coli* 22-1176, E. *coli* 65- 202350075, *E. coli* 66-202340112, and *E. coli* 67- 202350068 (Figure 8).

Table 5: Spot lysis assay results of AM phages against *E. coli* strains.

***E. coli* strains**	**AM 01**	**AM 02**	**AM 03**	**AM 04**
ATCC 25922	+	+	+	+
7- 1146	-	-	-	-
8-1150	-	-	-	-
17-1164	-	-	-	-
18-1166	-	-	-	-
22-1176	-	-	-	-
39-242	+	+	+	+
49-448	+	+	-	-

64-201680081	-	+	-	-
65-202350075	-	-	-	-
66-202340112	-	-	-	-
67-202350068	-	-	-	-
71-203070013	+	+	-	-

Table 6: Spot lysis assay results of RB phages against E. coli strains.

***E. coli* strains**	**RB 01**	**RB 02**	**RB 03**
ATCC 25922	+	+	+
7- 1146	-	-	-
8-1150	-	-	-
17-1164	-	-	-
18-1166	-	-	-
22-1176	-	-	-
39-242	-	-	-
49-448	+	+	+
64- 201680081	-	-	-
65- 202350075	-	-	-

66- 202340112	-	-	-
67- 202350068	-	-	-
71- 203070013	-	-	-

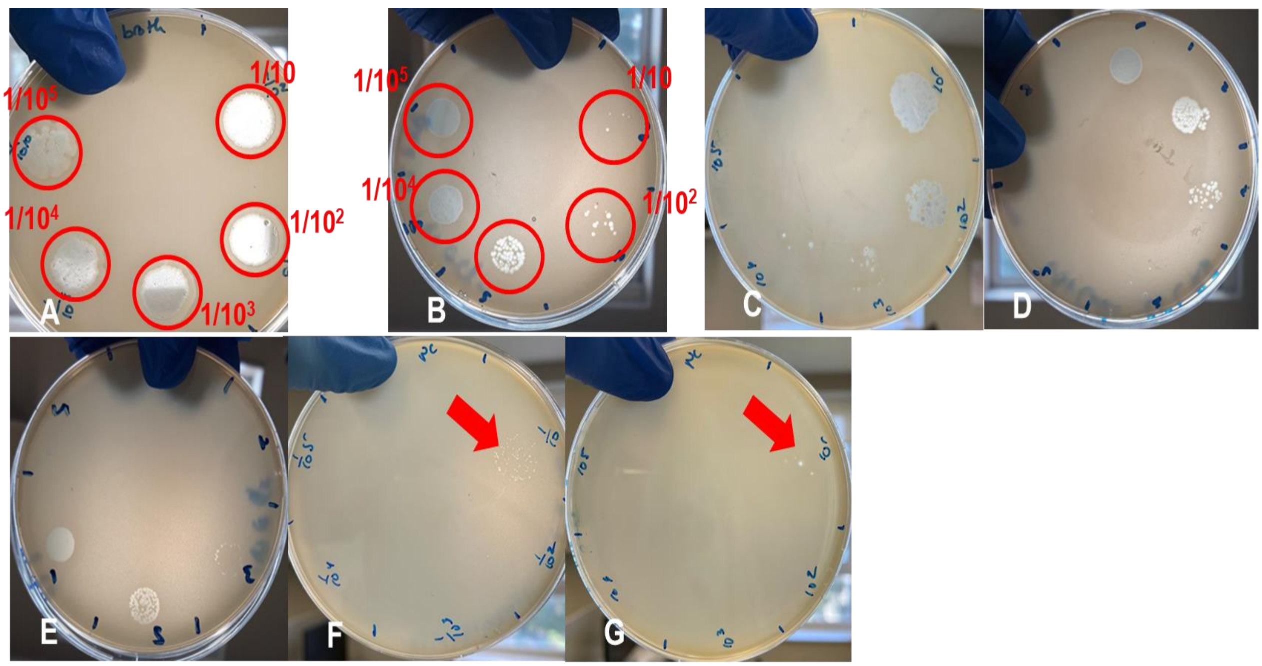

Figure 8: Spot lysis assay results of A) AM 04 against *E. coli* ATCC 25922, B) RB 01 against *E. coli* ATCC 25922, C) AM 01 against *E. coli* 49-448, D) RB 03 against *E. coli* 49-448, E) AM 03 against *E. coli* 39-242, F) AM 04 against *E. coli* 39-242, and G) AM 02 against *E. coli* 71-203070013.

D. Bacteriolytic Activity Determination

The bacteriolytic activity of all seven phages was determined against *E. coli* ATCC 25922 at MOI 10, 1, 0.1, and 0.01. As shown in Figure 9 , the absorbance of *E. coli* ATCC 25922 cultured without phages increased continuously within 24 hours. In figure 9, AM 01 absorbance increased gradually to reach a peak between 1.5-2 hours at MOI 10-0.01, then decreased remarkably to reach a minimum between 3.5-5 hours at different MOIs. The absorbance of the cultures with AM 01 remained stable at its lowest level till the end of the experiment. Similar graphs were observed for AM 02, AM 04, and RB 02, but at different time points. AM 03 and unlike all the other phages displayed a bacteriostatic activity, where a stable absorbance to the minimum was maintained at all MOIs until 5 hours when the absorbance started to remarkably increase again. Nonetheless, RB 01 absorbance increased to reach a peak between 1-1.5 hours, then decreased to reach a minimum between 2-3 hours. The absorbance started to increase remarkably after 6 hours at all MOIs but MOI 10 where a stable absorbance was maintained at the lowest level till the end of the experiment. RB 03 displayed an increase followed by a decrease and a stable absorbance to the minimum profile, before observing a bacterial regrowth after 10.5 hours.

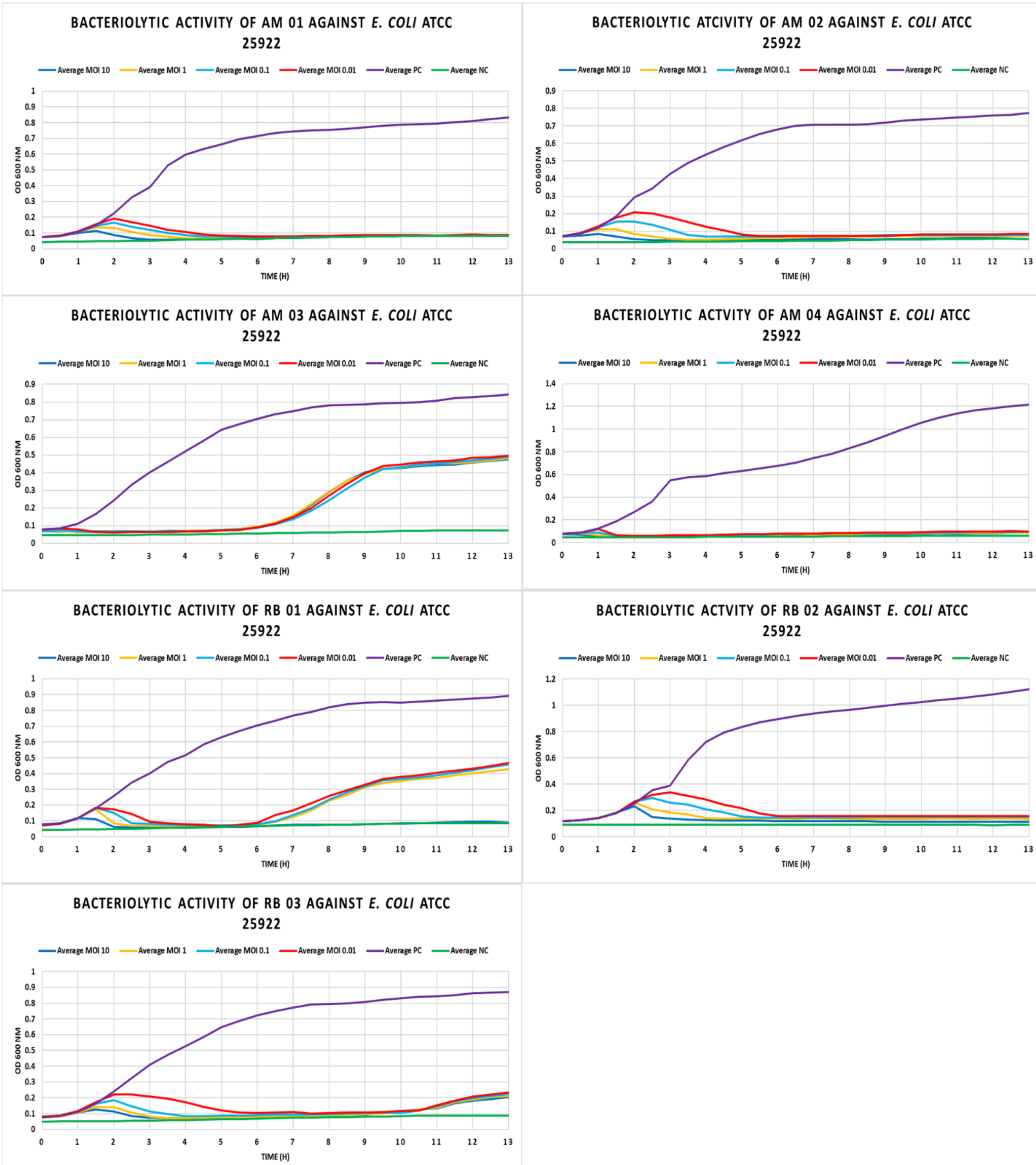

Figure 9: Bacteriolytic activity of all 7 phages against E. coli ATCC 25922.

E. One-Step Growth Curve

Single step growth experiment was performed to determine the latent time and phage burst size. As shown in Figure 10, the latent period for AM 04, RB 01 and RB 03 was about 35 minutes for AM 04 and RB 01, and 20 minutes for RB 03, and the PFU was 494, 142, and 30 per infected host cell, respectively.

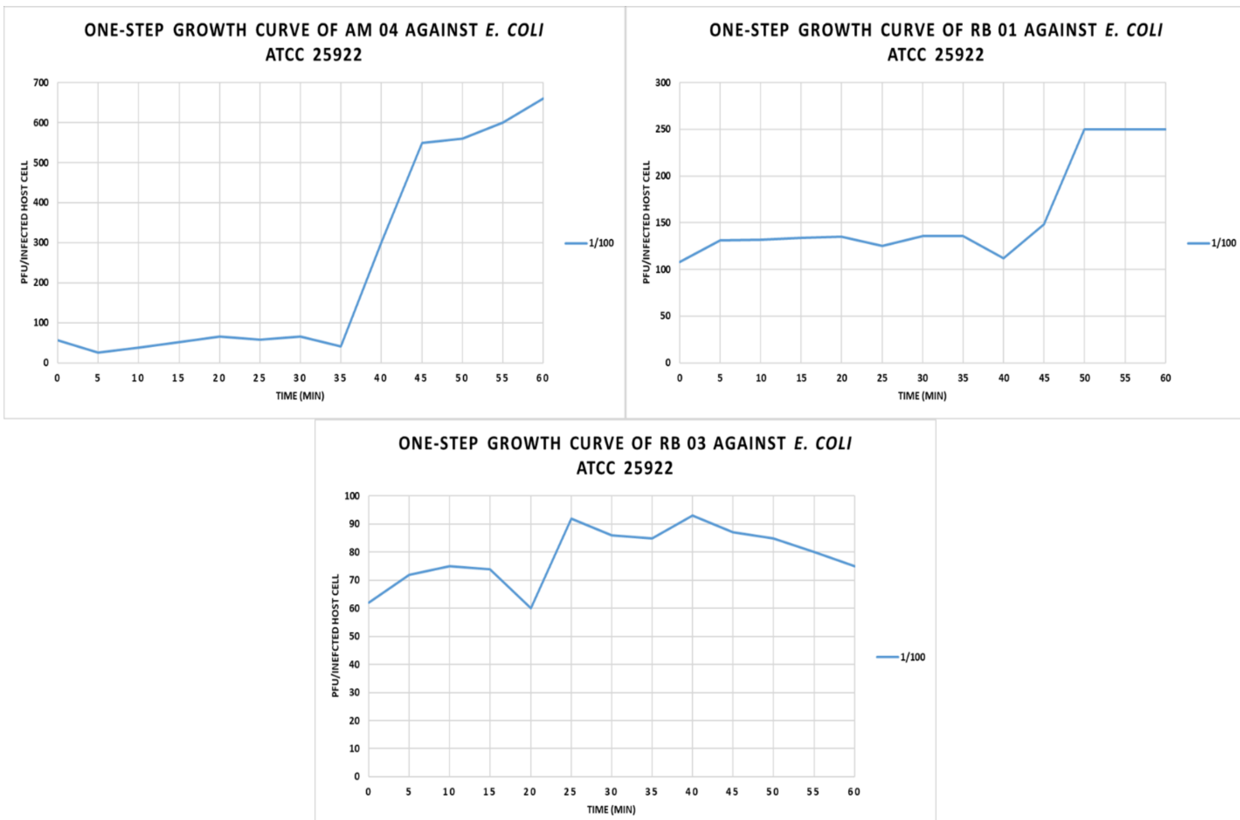

Figure 10: One-step growth curve of AM 04, RB 01, and RB 03 against E. coli ATCC 25922.

F. Effect of Phages on Bacterial Biofilm Formation-Inhibition of Biofilm Formation (IF) Treatment Assay

The capability of all seven phages (MOI 10) along with the anti-fungal treatments in preventing biofilm formation of *E. coli* ATCC 25922 was detected 24 hours after coculture using the crystal violet staining technique. As shown in figure 11, compared to *E. coli* ATCC 25922 cultured alone, the wells with bacteria cocultured with the 7 phages showed complete inhibition of biofilm formation. Very few biofilms could be detected when the *E. coli* ATCC 25922 was incubated with both anidulafungin and micafungin.

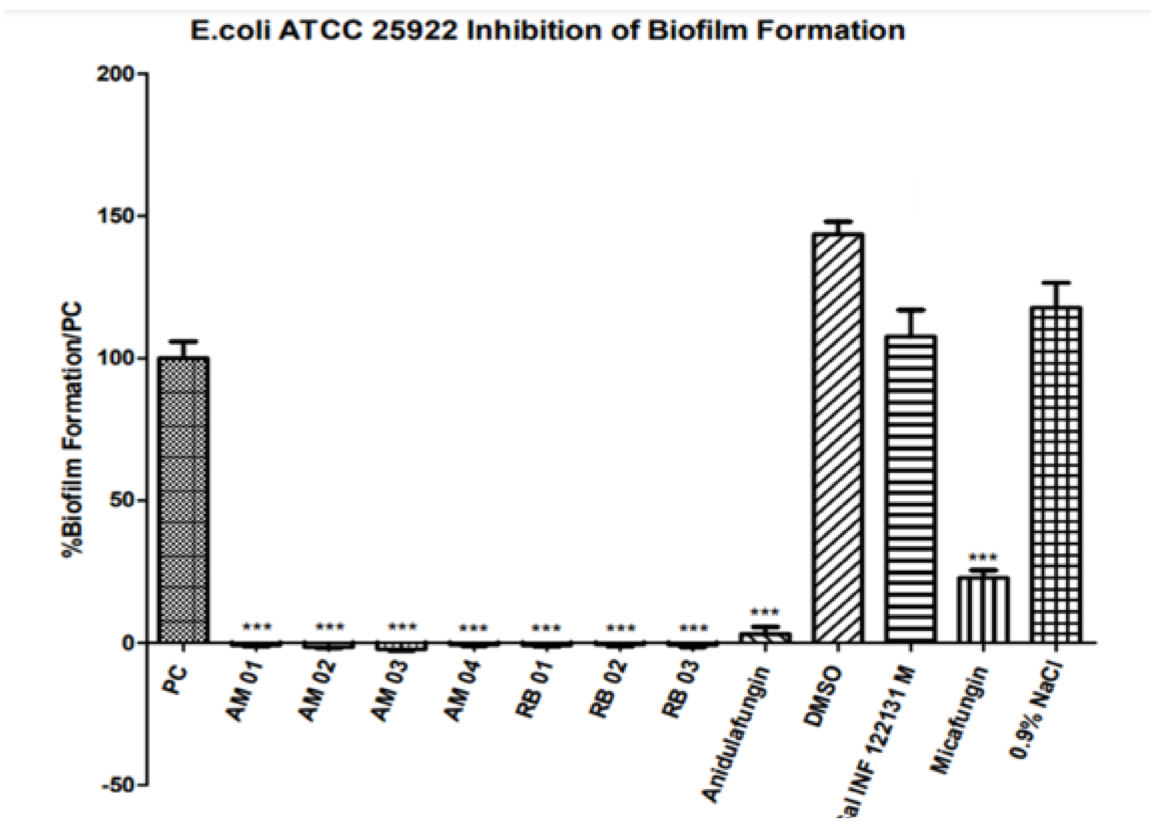

Figure 11: Inhibition of biofilm formation ability of the phages, micafungin, and anidulafungin. We used manutanlyte to calculate significance in comparison to the positive control, and we considered $p<0.05$ (***) an indication of significance.

G. Phages Biofilm Clearance Ability-Post Biofilm Formation (PF) Treatment Assay

The capability of all seven phages along with the anti-fungal treatments in clearing the mature biofilm was determined in figure 12. Treatment with AM 03, AM 04, RB 01, RB 02, and RB 03 caused a significant decrease in biofilm biomass of *E. coli* ATCC 25922 when compared to the untreated PC wells. However, AM 01, AM 02, anidulafungin and Micafungin showed no decrease in biofilm clearance efficiency after treatment.

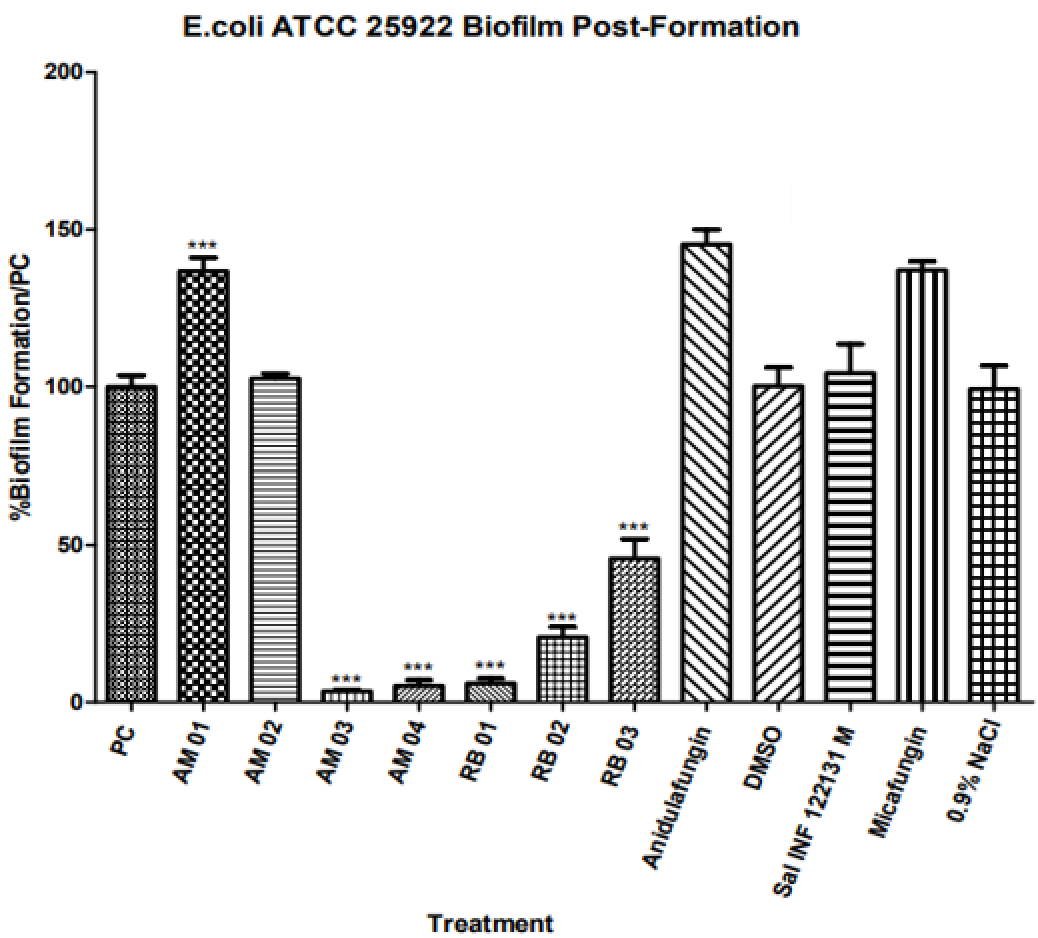

Figure 12: Biofilm clearance ability of the phages, micafungin, and anidulafungin. We used manutanlyte to calculate significance in comparison to the positive control, and we considered $p<0.05$ (***) an indication of significance.

CHAPTER V

DISCUSSION

AR remains one of the major threats to human health around the world with no promising new antibiotics to combat MDR pathogens, particularly *E. coli*, which stresses the need for an urgent antibiotic alternative. As part of our long-term objective of the establishment of a bacteriophage library, in this study, we isolated seven phages against an ATCC strain of *E. coli* and tested their activity against *E. coli* strains isolated from the urine of UTI patients, and blood. Untreated sewage samples were used in this study as an abundant and broadly diverse source for isolation of novel phages against *E. coli* (bacteriophages can be found wherever their host cells reside), which were subsequently characterized.

We counted on the DLA to count the number of newly synthesized phages appearing in the form of plaques on the surface of the top agar. The DLA conducted using Ramlet El-AByda and Ain El-Mreiseh filtered sewage samples against MDR *E. coli* ATCC 25922 provided various plaque shapes and sizes; however, we decided to further investigate 3 phages from Ramlet El-Bayda (RB 01, RB 02, and RB 03), and 4 from Ain El-Mreiseh (AM 01, AM 02, AM 03, and AM 04). AM 01, AM 02, RB 01, RB 02, and RB 03 exhibited clear and discrete plaques of different sizes, while AM 03 and AM 04 exhibited large turbid halo shaped plaques (Figure 5 and 6). Traditionally, clear plaque appearance has been linked to lytic life cycles while turbid-halo appearance may imply a temperate phage (Lwoff, 1953). The above assumption suggests that AM 01, AM 02, RB 01, RB 02, and RB 03 are more likely to be lytic rather than temperate phages, unlike that of AM 03 and AM 04.

To further investigate whether AM 03 and AM 04 are lysogenic phages, and to check if they are originally harbored within the *E. coli* ATCC 25922 we ran its genome sequences along the “Phaster” software to check for any prophage genome along the host genome (Figure 13). The Phaster software indicated the 3 intact prophages, 3 questionable prophages, and 4 incomplete prophages along the *E. coli* ATCC 25922 genome. Therefore, AM 03 and AM 04 might be representing the prophages harbored within the host genome, which shifted to the lytic cycle upon bacterial stress, causing bacterial lysis. In our study we subjected all 7 phages to 3 consecutive cycles of purification to obtain a uniform plaque morphology along each plate, to determine the PFU for each phage. The obtained PFU/ml for all 7 phages ranged between 1×10^{10} to 1×10^{15} PFU/ml. Phages exhibiting both high titers and lytic profiles are ideal candidates for biocontrol application (Snyder, Perry, & Yousef, 2016). After determining the PFU for each phage one-step growth curve assay was conducted, and surprisingly large halo shaped plaques were observed along the plates of RB 01 and RB 03 (Figure 14), which were proven to provide a uniformed morphology before conducting the one-step growth curve experiment. The obtained halo shaped plaques resembled that of AM 03 and AM 04, which supported our assumption that AM 03 and AM 04 phages might represent the prophages harbored within the bacterial genome that shifted to the lytic cycle upon host stress.

Region	Region Length	Completeness	Score	# Total Proteins	Region Position	Most Common Phage	GC %	Details
1	12.5Kb	incomplete	40	15	106745-119266	PHAGE_Phage_Gifsy_1_NC_010392(2)	49.16%	Show
2	5.1Kb	questionable	70	6	1685829-1690985	PHAGE_Stx2_c_Stx2a_F451_NC_049924(3)	54.94%	Show
3	11.4Kb	questionable	80	11	1725965-1737438	PHAGE_Stx2_c_1717_NC_011357(3)	47.45%	Show
4	6.3Kb	incomplete	60	7	1755449-1761764	PHAGE_Stx2_c_Stx2a_F451_NC_049924(3)	44.74%	Show
5	34.8Kb	questionable	70	15	2492733-2527618	PHAGE_Sodali_phiSG1_NC_007902(1)	51.78%	Show
6	18.7Kb	questionable	80	30	2914661-2933389	PHAGE_Pectob_CBB_NC_041878(2)	50.37%	Show
7	13.6Kb	incomplete	30	14	3476764-3490377	PHAGE_Salmon_118970_sal3_NC_031940(2)	46.03%	Show
8	37.9Kb	intact	150	42	3490197-3528128	PHAGE_Entero_mEp460_NC_019716(23)	50.09%	Show
9	49.3Kb	intact	150	67	3891555-3940922	PHAGE_Entero_lambda_NC_001416(23)	50.29%	Show

Figure 13: Prophages along *E. coli* ATCC genome.

The ideal phage for phage therapy is a phage that targets a single bacterial species while targeting many if not all strains belonging to the targeted species (Hyman, 2019). We conducted the host range determination assay to qualify potential phages for cocktail administration (Benjamin K. Chan & Abedon, 2012). None of the phage's recorded an activity within the turbidity testing assay against *E. coli* 7- 1146, *E. coli* 8-1150, *E. coli* 17-1164, *E. coli* 18-1166, *E. coli* 22-1176, E. *coli* 65- 202350075, *E. coli* 66- 202340112, and *E. coli* 67- 202350068, while all phage provided a strong active against *Escherichia coli* ATCC 25922 (Table 4). Moreover, AM 02 displayed the broad host range as it targeted 4 clinical strains, unlike AM 03, AM 04, RB 01, RB 02, and RB 03 unlike AM 03, which are more host specific. The confirmatory spot testing technique yielded the same results (Tables 5 and 6).

In order to further investigate the lytic ability of our phages we performed the bacteriolytic activity. The bacteriolytic activity of AM 01, AM 02, AM 04, RB 01, RB 02 and RB 03 phages against *E. coli* ATCC 25922 at different MOIs exhibited a remarkable potential role of phages in controlling the *E. coli* infections, due to bacterial number rapid decrease to the minimum shortly after coculturing. AM 04 recorded a complete bacterial elimination, which indicates that AM 04 is highly likely lytic and not lysogenic, unlike AM 03 that marked a stable absorbance to the minimum level till 5.5 hours followed by an increase in the absorbance, indicating a lysogenic activity against *E. coli* ATCC 25922. However, it should be noted that the number of bacterial cells began to rise within all treated wells of AM 03 after 5.5 hours of coculturing. RB 01 displayed an MOI dependent profile (Bacterial elimination at MOI 10). High concentration of phages is essential to avoid phage resistance and properly eliminate the infection (Wright, Hawkins, Anggård, & Harper, 2009). Bacterial regrowth was detected along AM 03, RB 01, and RB 03 profiles. Nevertheless, the bacterial regrowth scenario can't be avoided whether phages were applied in vitro or in vivo due to bacteria-virus co-evolution, which facilitated the evolution of several bacterial innate and adaptive immune systems targeting all stages of the phage life cycle, thus rendering its parasitic effect. An example of the adaptation system is the bacterial ability to eliminate or reducing the interaction between the RBP and its bacterial receptors through receptor alteration as in the case *ompU Vibrio cholerae* receptor modification which facilitates the bacterial resistance to vibriophage ICP2 (Seed et al., 2014). Another main phage-specific defenses developed by bacterial cells is the Restriction-modification system which consist of two compartments, the restriction endonucleases and methyltransferase which will target the bacteriophage genetic material located in the bacterial cytoplasm. The bacterial restriction endonuclease will target specific short DNA motifs located along both bacterial and phage DNA; however, the bacteria will modify its genome using the methyltransferase enzyme, therefore escaping the restriction enzyme activity (Rusinov, Ershova, Karyagina, Spirin, & Alexeevski, 2018). For that sake, further studies must be conducted to evaluate first the effect of our 7 phages in bacterial elimination in vivo.

Short latent period and large burst size are essential features for highly effective phages serving as therapeutic agents (Bull & Gill, 2014). Phages with a short latent period and large burst size are more effective in inactivating bacteria (Duc, Son, Honjoh, & Miyamoto, 2018). Out of the tested phages RB 03 displayed the shortest latent period and 30 PFU/ml. Large-halo shaped plaques were observed along the plates of RB 01 and RB 03 (Figure 14), which were proven to provide a uniformed morphology before conducting the one-step growth curve experiment. The obtained halo shaped plaques resembled that of AM 03 , which supported our assumption that AM 03 phages might represent the prophage harbored within the bacterial genome that shifted to the lytic cycle upon host stress.

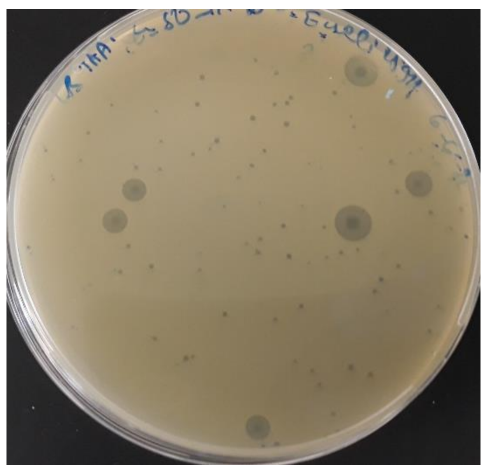

Figure 14: Halo shaped phages observed along RB 01 one-step growth curve plate against *E. coli* ATCC 25922.

One of the most common broad bacterial defense mechanisms is the formation of a biofilm matrix, which can trap the phage upon entry, therefore reducing the phage RBP interaction with their corresponding bacterial receptor (Flemming & Wingender, 2010).

Nonetheless, various studies have been reported targeting phages with parasitic activity against *E. coli* species, with these phages displaying biofilm elimination capacities (Montso, Mlambo, & Ateba, 2021; Pei & Lamas-Samanamud, 2014; Sanchez et al., 2022). In this study, we examined the ability of our 7 phages in inhibiting the formation of biofilm by *E. coli* ATCC 25922, which has been used as a positive control when testing *E. coli* strains biofilm formation capacities (Whelan, O'Grady, Corcoran, Finn, & Lucey, 2020). All 7 phages showed an excellent effect in preventing the formation of biofilm at 24 hours after coculture with an MOI 10, where almost no bacterial growth was detected after 24 hours of incubation. In addition, both micafungin and anidulafungin which were proven to inhibit the formation of biofilm in *Pseudomonas aeruginosa* and *Staphylococcus aureus* respectively exhibited a significant effect in preventing the formation of biofilm in the 24 hours incubated *E. coli* ATCC 25922 (Kissoyan et al., 2016; Rogiers et al., 2018). Nevertheless, we moved on to further investigate the biofilm elimination capacity of our phages, by testing mature *E. coli* ATCC 25922 biofilm elimination capacity.

The phages AM 02, AM 03, RB 01, RB 02, and RB 03 reduced significantly mature biofilm (biofilm post-formation) after treatment for 24 h. Although AM 01 and AM 02 showed significant inhibition of biofilm formation, they lacked any mature biofilm removal capability. Interestingly, phages AM 01 and AM 02 behaved similarly in terms of bacteriolytic activities and biofilm inhibiting effect but quite differently in the disaggregation of a mature biofilm. This might be due to the ability AM 03, AM 04, RB 01, RB 02, and RB 03 to produce polysaccharide depolymerases that will degrade the biofilm matrix. For instance, K5-2 and K5-4, Two T7-like Bacteriophages, were proven to encode for two capsule depolymerases against *Klebsiella pneumoniae* species, which allowed the phages to come in contact with the targeted bacteria (Hsieh, Lin, Lin, Chen, & Wang, 2017). Although various studies categorized halo shaped phages as temperate phages, other studies have shown that halo shaped phages can play an important role in exopolysaccharide depolymerization and biofilm degradation (Oliveira et al., 2018), and this might be the case of AM 03. However, we need to sequence our phages to determine whether the above hypotheses are valid or not.

In conclusion, we isolated seven phages against *E. coli* ATCC 25922 from Ain El-Mreiseh and Ramlet El-Bayda untreated sewage sources. All phages but AM 03 exhibited a lytic activity against their host strain. AM 02 displayed a wide host range unlike AM 03, AM 04, RB 01, RB 02 and RB 03 which are more specific towards their host strain. More importantly, several phages exhibited varying abilities in inhibiting and eliminating biofilm. Based on the results obtained and experiments conducted, RB 03 is considered a good candidate for *E. coli* ATCC 25922 infection elimination. RB 03 displayed an inhibition of the bacterial growth at phage doses (not MOI dependent), shortest latent period, and inhibition of biofilm formation and post formation capabilities. Although RB 03 shared a relatively narrow host range, yet the following situation can be adjusted by broadening the spectrum of screened *E. coli* clinical isolates. Another good candidate for *E. coli* ATCC 259522 infection elimination is AM 02. AM 02 displayed the broadest host range, a complete inhibition of bacterial growth within the bacteriolytic activity determination assay, in addition to its biofilm formation inhibition capability.

Compared to the conventional antibiotic therapy, phage therapy displays some disadvantages owing some gaps in knowledge and regulation of phage administration. One of the main issues is the route of administration. For example, phage administered intravenously are subjected to rapid elimination by our immune cells. In addition, delivery of aerosols phages can be impaired by mucus and biofilm residing in the lungs, leading to phage loss (Romero-Calle, Guimarães Benevides, Góes-Neto, & Billington, 2019). Aside of phage route of administration, we lack a common valid protocol for the frequency and duration of phage treatment (Sabouri Ghannad & Mohammadi, 2012). Moreover, phage is subjected to various biosafety limitations, owing the ability of the phages to harbor toxins, antibiotic resistance genes, virulence genes (Romero-Calle et al., 2019).

Future studies would focus on extended characterization of the isolated phages, including Transmission Electron Microscopy imaging to detect the phages morphology, genome sequencing of the isolated phages, check the phages PH and thermal stability, and finally check the phages efficiency in vivo and in vitro.

BIBLIOGRAPHY

Abedon, S. T., Thomas-Abedon, C., Thomas, A., & Mazure, H. (2011). Bacteriophage prehistory: is or is not Hankin, 1896, a phage reference? *Bacteriophage, 1*(3), 174-178.

Brives, C., & Pourraz, J. (2020). Phage therapy as a potential solution in the fight against AMR: obstacles and possible futures. *Palgrave Communications, 6*(1), 1-11.

Browne, K., Chakraborty, S., Chen, R., Willcox, M. D., Black, D. S., Walsh, W. R., & Kumar, N. (2020). A New Era of Antibiotics: The Clinical Potential of Antimicrobial Peptides. *International Journal of Molecular Sciences, 21*(19), 7047. Retrieved from https://www.mdpi.com/1422-0067/21/19/7047

Bull, J. J., & Gill, J. J. (2014). The habits of highly effective phages: population dynamics as a framework for identifying therapeutic phages. *Front Microbiol, 5*, 618. doi:10.3389/fmicb.2014.00618

Chan, B. K., & Abedon, S. T. (2012). Chapter 1 - Phage Therapy Pharmacology: Phage Cocktails. In A. I. Laskin, S. Sariaslani, & G. M. Gadd (Eds.), *Advances in Applied Microbiology* (Vol. 78, pp. 1-23): Academic Press.

Chan, B. K., Abedon, S. T., & Loc-Carrillo, C. (2013). Phage cocktails and the future of phage therapy. *Future Microbiol, 8*(6), 769-783. doi:10.2217/fmb.13.47

Chan, B. K., Sistrom, M., Wertz, J. E., Kortright, K. E., Narayan, D., & Turner, P. E. (2016). Phage selection restores antibiotic sensitivity in MDR Pseudomonas aeruginosa. *Sci Rep, 6*, 26717. doi:10.1038/srep26717

Chan, B. K., Turner, P. E., Kim, S., Mojibian, H. R., Elefteriades, J. A., & Narayan, D. (2018). Phage treatment of an aortic graft infected with Pseudomonas aeruginosa. *Evol Med Public Health, 2018*(1), 60-66. doi:10.1093/emph/eoy005

Chang, Q., Wang, W., Regev-Yochay, G., Lipsitch, M., & Hanage, W. P. (2015). Antibiotics in agriculture and the risk to human health: how worried should we be? *Evolutionary applications, 8*(3), 240-247.

Chanishvili, N. (2012). Chapter 1 - Phage Therapy—History from Twort and d'Herelle Through Soviet Experience to Current Approaches. In M. Łobocka & W. Szybalski (Eds.), *Advances in Virus Research* (Vol. 83, pp. 3-40): Academic Press.

Clokie, M. R., Millard, A. D., Letarov, A. V., & Heaphy, S. (2011). Phages in nature. *Bacteriophage, 1*(1), 31-45. doi:10.4161/bact.1.1.14942

Comeau, A. M., Tétart, F., Trojet, S. N., Prère, M. F., & Krisch, H. M. (2007). Phage-Antibiotic Synergy (PAS): beta-lactam and quinolone antibiotics stimulate virulent phage growth. *PLoS One, 2*(8), e799. doi:10.1371/journal.pone.0000799

D'Accolti, M., Soffritti, I., Mazzacane, S., & Caselli, E. (2019). Fighting AMR in the Healthcare Environment: Microbiome-Based Sanitation Approaches and Monitoring Tools. *Int J Mol Sci, 20*(7). doi:10.3390/ijms20071535

Dagher, L. A., Hassan, J., Kharroubi, S., Jaafar, H., & Kassem, II. (2021). Nationwide Assessment of Water Quality in Rivers across Lebanon by Quantifying Fecal Indicators Densities and Profiling Antibiotic Resistance of Escherichia coli. *Antibiotics (Basel), 10*(7). doi:10.3390/antibiotics10070883

de Kraker, M. E., Stewardson, A. J., & Harbarth, S. (2016). Will 10 Million People Die a Year due to Antimicrobial Resistance by 2050? *PLoS Med, 13*(11), e1002184. doi:10.1371/journal.pmed.1002184

Dion, M. B., Oechslin, F., & Moineau, S. (2020). Phage diversity, genomics and phylogeny. *Nature Reviews Microbiology, 18*(3), 125-138. doi:10.1038/s41579-019-0311-5

Duc, H. M., Son, H. M., Honjoh, K.-i., & Miyamoto, T. (2018). Isolation and application of bacteriophages to reduce Salmonella contamination in raw chicken meat. *LWT, 91*, 353-360. doi:https://doi.org/10.1016/j.lwt.2018.01.072

Duckworth, D. (1977). Who Discovered Bacteriophage? *Bacteriological reviews, 40*, 793-802. doi:10.1128/MMBR.40.4.793-802.1976

Flemming, H. C., & Wingender, J. (2010). The biofilm matrix. *Nat Rev Microbiol, 8*(9), 623-633. doi:10.1038/nrmicro2415

Fortier, L. C., & Sekulovic, O. (2013). Importance of prophages to evolution and virulence of bacterial pathogens. *Virulence, 4*(5), 354-365. doi:10.4161/viru.24498

Fouz, N., Pangesti, K. N., Yasir, M., Al-Malki, A. L., Azhar, E. I., Hill-Cawthorne, G. A., & Abd El Ghany, M. (2020). The contribution of wastewater to the transmission of antimicrobial resistance in the environment: implications of mass gathering settings. *Tropical medicine and infectious disease*, *5*(1), 33.

Gaynes, R. (2017). The Discovery of Penicillin—New Insights After More Than 75 Years of Clinical Use. *Emerging Infectious Diseases, 23*(5), 849-853. doi:10.3201/eid2305.161556

Ghose, C., & Euler, C. W. (2020). Gram-Negative Bacterial Lysins. *Antibiotics (Basel), 9*(2). doi:10.3390/antibiotics9020074

Gordillo Altamirano, F. L., & Barr, J. J. (2019). Phage Therapy in the Postantibiotic Era. *Clin Microbiol Rev, 32*(2). doi:10.1128/cmr.00066-18

Haberecht, H. B., Nealon, N. J., Gilliland, J. R., Holder, A. V., Runyan, C., Oppel, R. C., . . . Ryan, E. P. (2019). Antimicrobial-Resistant Escherichia coli from Environmental Waters in Northern Colorado. *J Environ Public Health, 2019*, 3862949. doi:10.1155/2019/3862949

Hall, A. R., De Vos, D., Friman, V. P., Pirnay, J. P., & Buckling, A. (2012). Effects of sequential and simultaneous applications of bacteriophages on populations of Pseudomonas aeruginosa in vitro and in wax moth larvae. *Appl Environ Microbiol, 78*(16), 5646-5652. doi:10.1128/aem.00757-12

Hobbs, Z., & Abedon, S. T. (2016). Diversity of phage infection types and associated terminology: the problem with 'Lytic or lysogenic'. *FEMS Microbiol Lett, 363*(7). doi:10.1093/femsle/fnw047

Howard-Varona, C., Hargreaves, K. R., Abedon, S. T., & Sullivan, M. B. (2017). Lysogeny in nature: mechanisms, impact and ecology of temperate phages. *Isme j, 11*(7), 1511-1520. doi:10.1038/ismej.2017.16

Hsieh, P.-F., Lin, H.-H., Lin, T.-L., Chen, Y.-Y., & Wang, J.-T. (2017). Two T7-like Bacteriophages, K5-2 and K5-4, Each Encodes Two Capsule Depolymerases: Isolation and Functional Characterization. *Scientific Reports, 7*(1), 4624. doi:10.1038/s41598-017-04644-2

Hyman, P. (2019). Phages for Phage Therapy: Isolation, Characterization, and Host Range Breadth. *Pharmaceuticals (Basel), 12*(1). doi:10.3390/ph12010035

Jaiswal, A., Koley, H., Ghosh, A., Palit, A., & Sarkar, B. (2013). Efficacy of cocktail phage therapy in treating Vibrio cholerae infection in rabbit model. *Microbes Infect, 15*(2), 152-156. doi:10.1016/j.micinf.2012.11.002

Johnston, C., Martin, B., Fichant, G., Polard, P., & Claverys, J.-P. (2014). Bacterial transformation: distribution, shared mechanisms and divergent control. *Nature Reviews Microbiology, 12*(3), 181-196. doi:10.1038/nrmicro3199

Kapoor, G., Saigal, S., & Elongavan, A. (2017). Action and resistance mechanisms of antibiotics: A guide for clinicians. *Journal of anaesthesiology, clinical pharmacology, 33*(3), 300-305. doi:10.4103/joacp.JOACP_349_15

Kasman, L. M., & Porter, L. D. (2020). Bacteriophages. *StatPearls [Internet]*.

Keen, E. (2015). A century of phage research: Bacteriophages and the shaping of modern biology. *BioEssays, 37*. doi:10.1002/bies.201400152

Keen, E. C. (2012). Phage therapy: concept to cure. *Front Microbiol, 3*, 238. doi:10.3389/fmicb.2012.00238

Kissoyan, K. A., Bazzi, W., Hadi, U., & Matar, G. M. (2016). The inhibition of Pseudomonas aeruginosa biofilm formation by micafungin and the enhancement of antimicrobial agent effectiveness in BALB/c mice. *Biofouling, 32*(7), 779-786. doi:10.1080/08927014.2016.1199021

Kwarciński, W., Lazarkiewicz, B., Weber-Dabrowska, B., Rudnicki, J., Kamiński, K., & Sciebura, M. (1994). [Bacteriophage therapy in the treatment of recurrent subphrenic and subhepatic abscess with jejunal fistula after stomach resection]. *Pol Tyg Lek, 49*(23-24), 535.

Larsson, D. G. (2014). Antibiotics in the environment. *Ups J Med Sci, 119*(2), 108-112. doi:10.3109/03009734.2014.896438

Lin, D. M., Koskella, B., & Lin, H. C. (2017). Phage therapy: An alternative to antibiotics in the age of multi-drug resistance. *World J Gastrointest Pharmacol Ther, 8*(3), 162-173. doi:10.4292/wjgpt.v8.i3.162

Lood, R., Winer, B. Y., Pelzek, A. J., Diez-Martinez, R., Thandar, M., Euler, C. W., . . . Fischetti, V. A. (2015). Novel phage lysin capable of killing the multidrug-resistant gram-negative bacterium Acinetobacter baumannii in a mouse bacteremia model. *Antimicrobial agents and chemotherapy, 59*(4), 1983-1991.

Łoś, M., & Węgrzyn, G. (2012). Pseudolysogeny. *Adv Virus Res, 82*, 339-349. doi:10.1016/b978-0-12-394621-8.00019-4

Lwoff, A. (1953). Lysogeny. *Bacteriol Rev, 17*(4), 269-337. doi:10.1128/br.17.4.269-337.1953

Ma, F., Xu, S., Tang, Z., Li, Z., & Zhang, L. (2021). Use of antimicrobials in food animals and impact of transmission of antimicrobial resistance on humans. *Biosafety and Health, 3*(1), 32-38. doi:https://doi.org/10.1016/j.bsheal.2020.09.004

Mendelson, M., Røttingen, J. A., Gopinathan, U., Hamer, D. H., Wertheim, H., Basnyat, B., . . . Balasegaram, M. (2016). Maximising access to achieve appropriate human antimicrobial use in low-income and middle-income countries. *Lancet, 387*(10014), 188-198. doi:10.1016/s0140-6736(15)00547-4

Michael, C. A., Dominey-Howes, D., & Labbate, M. (2014). The antimicrobial resistance crisis: causes, consequences, and management. *Front Public Health, 2*, 145. doi:10.3389/fpubh.2014.00145

Moelling, K., Broecker, F., & Willy, C. (2018). A wake-up call: we need phage therapy now. *Viruses, 10*(12), 688.

Monk, A. B., Rees, C. D., Barrow, P., Hagens, S., & Harper, D. R. (2010). Bacteriophage applications: where are we now? *Lett Appl Microbiol, 51*(4), 363-369. doi:10.1111/j.1472-765X.2010.02916.x

Montso, P. K., Mlambo, V., & Ateba, C. N. (2021). Efficacy of novel phages for control of multi-drug resistant Escherichia coli O177 on artificially contaminated beef and their potential to disrupt biofilm formation. *Food Microbiology, 94*, 103647. doi:https://doi.org/10.1016/j.fm.2020.103647

Moussa, J., Abboud, E., & Tokajian, S. (2021). The dissemination of antimicrobial resistance determinants in surface water sources in Lebanon. *FEMS Microbiol Ecol, 97*(9). doi:10.1093/femsec/fiab113

Oliveira, H., São-José, C., & Azeredo, J. (2018). Phage-Derived Peptidoglycan Degrading Enzymes: Challenges and Future Prospects for In Vivo Therapy. *Viruses, 10*(6). doi:10.3390/v10060292

Olszak, T., Latka, A., Roszniowski, B., Valvano, M. A., & Drulis-Kawa, Z. (2017). Phage life cycles behind bacterial biodiversity. *Current medicinal chemistry, 24*(36), 3987-4001.

Organization, W. H. (2020). Lack of new antibiotics threatens global efforts to contain drug-resistant infections. *New release Geneva*.

Peacock, S. J., & Paterson, G. K. (2015). Mechanisms of methicillin resistance in Staphylococcus aureus. *Annual review of biochemistry, 84*, 577-601.

Pei, R., & Lamas-Samanamud, G. R. (2014). Inhibition of biofilm formation by T7 bacteriophages producing quorum-quenching enzymes. *Appl Environ Microbiol, 80*(17), 5340-5348. doi:10.1128/aem.01434-14

Prestinaci, F., Pezzotti, P., & Pantosti, A. (2015). Antimicrobial resistance: a global multifaceted phenomenon. *Pathog Glob Health, 109*(7), 309-318. doi:10.1179/2047773215y.0000000030

Ragunathan, P. T., Vanderpool, C. K., & O'Toole, G. (2019). Cryptic-Prophage-Encoded Small Protein DicB Protects <i>Escherichia coli</i> from Phage Infection by

Inhibiting Inner Membrane Receptor Proteins. *Journal of Bacteriology, 201*(23), e00475-00419. doi:doi:10.1128/JB.00475-19

Reygaert, W. C. (2018). An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS microbiology, 4*(3), 482-501. doi:10.3934/microbiol.2018.3.482

Rizzo, L., Fiorentino, A., & Anselmo, A. (2013). Advanced treatment of urban wastewater by UV radiation: Effect on antibiotics and antibiotic-resistant E. coli strains. *Chemosphere, 92*(2), 171-176. doi:10.1016/j.chemosphere.2013.03.021

Rogiers, O., Holtappels, M., Siala, W., Lamkanfi, M., Van Bambeke, F., Lagrou, K., . . . Kucharíková, S. (2018). Anidulafungin increases the antibacterial activity of tigecycline in polymicrobial Candida albicans/Staphylococcus aureus biofilms on intraperitoneally implanted foreign bodies. *J Antimicrob Chemother, 73*(10), 2806-2814. doi:10.1093/jac/dky246

Romero-Calle, D., Guimarães Benevides, R., Góes-Neto, A., & Billington, C. (2019). Bacteriophages as Alternatives to Antibiotics in Clinical Care. *Antibiotics (Basel), 8*(3). doi:10.3390/antibiotics8030138

Rusinov, I. S., Ershova, A. S., Karyagina, A. S., Spirin, S. A., & Alexeevski, A. V. (2018). Avoidance of recognition sites of restriction-modification systems is a widespread but not universal anti-restriction strategy of prokaryotic viruses. *BMC Genomics, 19*(1), 885. doi:10.1186/s12864-018-5324-3

Ryan, E. M., Alkawareek, M. Y., Donnelly, R. F., & Gilmore, B. F. (2012). Synergistic phage-antibiotic combinations for the control of Escherichia coli biofilms in vitro. *FEMS Immunol Med Microbiol, 65*(2), 395-398. doi:10.1111/j.1574-695X.2012.00977.x

Sabouri Ghannad, M., & Mohammadi, A. (2012). Bacteriophage: time to re-evaluate the potential of phage therapy as a promising agent to control multidrug-resistant bacteria. *Iran J Basic Med Sci, 15*(2), 693-701.

Sakandelidze, V. M. (1991). [The combined use of specific phages and antibiotics in different infectious allergoses]. *Vrach Delo*(3), 60-63.

Salem, S., Dahdouh, E., & Daoud, Z. (2013). Resistance of gram-negative bacilli in Lebanon. *International Scholarly Research Notices, 2013*.

Sanchez, B. C., Heckmann, E. R., Green, S. I., Clark, J. R., Kaplan, H. B., Ramig, R. F., . . . Maresso, A. W. (2022). Development of Phage Cocktails to Treat E. coli Catheter-Associated Urinary Tract Infection and Associated Biofilms. *Frontiers in Microbiology, 13*. doi:10.3389/fmicb.2022.796132

Seed, K. D., Yen, M., Shapiro, B. J., Hilaire, I. J., Charles, R. C., Teng, J. E., . . . Camilli, A. (2014). Evolutionary consequences of intra-patient phage predation on microbial populations. *Elife, 3*, e03497. doi:10.7554/eLife.03497

Sieiro, C., Areal-Hermida, L., Pichardo-Gallardo, Á., Almuiña-González, R., De Miguel, T., Sánchez, S., . . . Villa, T. G. (2020). A hundred years of bacteriophages: Can phages replace antibiotics in agriculture and aquaculture? *Antibiotics, 9*(8), 493.

Snyder, A. B., Perry, J. J., & Yousef, A. E. (2016). Developing and optimizing bacteriophage treatment to control enterohemorrhagic Escherichia coli on fresh produce. *International journal of food microbiology, 236*, 90-97.

Starr, M. P., & Reynolds, D. M. (1951). Streptomycin resistance of coliform bacteria from turkeys fed streptomycin. *American Journal of Public Health and the Nations Health, 41*(11_Pt_1), 1375-1380.

Stone, E., Campbell, K., Grant, I., & McAuliffe, O. (2019). Understanding and Exploiting Phage-Host Interactions. *Viruses, 11*(6). doi:10.3390/v11060567

Strój, L., Weber-Dabrowska, B., Partyka, K., Mulczyk, M., & Wójcik, M. (1999). [Successful treatment with bacteriophage in purulent cerebrospinal meningitis in a newborn]. *Neurol Neurochir Pol, 33*(3), 693-698.

Sulakvelidze, A., Alavidze, Z., & Morris, J. G., Jr. (2001). Bacteriophage therapy. *Antimicrobial agents and chemotherapy, 45*(3), 649-659. doi:10.1128/AAC.45.3.649-659.2001

Summers, W. C. (2017). Félix Hubert d'Herelle (1873-1949): History of a scientific mind. *Bacteriophage, 6*(4), e1270090-e1270090. doi:10.1080/21597081.2016.1270090

Sun, D. (2018). Pull in and Push Out: Mechanisms of Horizontal Gene Transfer in Bacteria. *Frontiers in Microbiology, 9*. doi:10.3389/fmicb.2018.02154

Tacconelli, E., Magrini, N., Kahlmeter, G., & Singh, N. (2017). Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. *World Health Organization, 27*, 318-327.

Tagliaferri, T. L., Jansen, M., & Horz, H. P. (2019). Fighting Pathogenic Bacteria on Two Fronts: Phages and Antibiotics as Combined Strategy. *Front Cell Infect Microbiol, 9*, 22. doi:10.3389/fcimb.2019.00022

Tavares, P. (2018). The Bacteriophage Head-to-Tail Interface. *Subcell Biochem, 88*, 305-328. doi:10.1007/978-981-10-8456-0_14

Taylor, M. W. (2014). The discovery of bacteriophage and the d’herelle controversy. In *Viruses and man: a history of interactions* (pp. 53-61): Springer.

Vázquez, R., García, E., & García, P. (2018). Phage Lysins for Fighting Bacterial Respiratory Infections: A New Generation of Antimicrobials. *Frontiers in Immunology, 9*. doi:10.3389/fimmu.2018.02252

Virolle, C., Goldlust, K., Djermoun, S., Bigot, S., & Lesterlin, C. (2020). Plasmid Transfer by Conjugation in Gram-Negative Bacteria: From the Cellular to the Community Level. *Genes, 11*(11), 1239. Retrieved from https://www.mdpi.com/2073-4425/11/11/1239

Wang, Q., Euler, C. W., Delaune, A., & Fischetti, V. A. (2015). Using a novel lysin to help control Clostridium difficile infections. *Antimicrobial agents and chemotherapy, 59*(12), 7447-7457.

Wang, X., & Wood, T. K. (2016). Cryptic prophages as targets for drug development. *Drug Resist Updat, 27*, 30-38. doi:10.1016/j.drup.2016.06.001

Wegener32, H. C. (2012). *Antibiotic resistance—linking human and animal health*. Paper presented at the Improving food safety through a one health approach: workshop summary.

Whelan, S., O'Grady, M. C., Corcoran, D., Finn, K., & Lucey, B. (2020). Uropathogenic Escherichia coli Biofilm-Forming Capabilities are not Predictable from Clinical Details or from Colonial Morphology. *Diseases, 8*(2), 11. Retrieved from https://www.mdpi.com/2079-9721/8/2/11

Witzenrath, M., Schmeck, B., Doehn, J. M., Tschernig, T., Zahlten, J., Loeffler, J. M., . . . Schütte, H. (2009). Systemic use of the endolysin Cpl-1 rescues mice with fatal pneumococcal pneumonia. *Critical care medicine, 37*(2), 642-649.

Wright, A., Hawkins, C. H., Anggård, E. E., & Harper, D. R. (2009). A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant Pseudomonas aeruginosa; a preliminary report of efficacy. *Clin Otolaryngol, 34*(4), 349-357. doi:10.1111/j.1749-4486.2009.01973.x

Yang, H., Zhang, Y., Yu, J., Huang, Y., Zhang, X. E., & Wei, H. (2014). Novel chimeric lysin with high-level antimicrobial activity against methicillin-resistant Staphylococcus aureus in vitro and in vivo. *Antimicrobial agents and chemotherapy, 58*(1), 536-542. doi:10.1128/aac.01793-13

Yoon, S. H., Jeong, H., Kwon, S.-K., & Kim, J. F. (2009). Genomics, biological features, and biotechnological applications of Escherichia coli B:"Is B for better?!". *Systems biology and biotechnology of Escherichia coli*, 1-17.

Zrelovs, N., Dislers, A., & Kazaks, A. (2021). Motley Crew: Overview of the Currently Available Phage Diversity. *Frontiers in Microbiology, 11*. doi:10.3389/fmicb.2020.579452