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Methods for Isolating Bacteriophage of Bacterial Pathogens and Possible Bacteriophage Isolation for *Micrococcus luteus*

By

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To the Graduate Faculty:

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Thesis Abstract-Idaho State University (2018)

In recent years there has been a great increase in the cases of multidrug resistant bacterial infections. These infections are becoming more dangerous as fewer known antibiotics are able to be used to treat them. One of these worrisome bacteria is *Acinetobacter baumannii*. *A. baumannii* has become a major contributor to nosocomial infections in recent years. With its ability to attach to sterile medical surfaces, produce a biofilm, and be resistant to disinfectants it is growing increasingly difficult to treat. *Micrococcus luteus*, while not typically thought of as a pathogen, is also capable of producing biofilm and is showing signs of developing antibiotic resistance as well. Because of this it is important to investigate new ways of treating antibiotic resistant bacteria. One way to do so is with bacteriophage therapy that will to target specific pathogens and clear the infection. Bacteriophage are able to diffuse through biofilms and less prone to have bacteria become resistant to them. This research looks at methods used to isolate novel bacteriophage for *A. baumannii* and *M. luteus* from dairy cattle feces.

Keywords: Antibiotic resistance, *Acinetobacter baumannii*, *Micrococcus luteus*, Bacteriophage therapy

I. *Acinetobacter baumannii*

History

Acinetobacter baumannii is an aerobic, non-fermentative, gram-negative coccobacillus bacterium considered to be a ubiquitous organism. It is often found in soils and water all over the world (16). The *Acinetobacter* genus was first classified in 1986 using DNA-DNA hybridization. Since then, more than 12 separate species of *Acinetobacter* have been identified. Currently, 56 separate species of the *Acinetobacter* genus have been identified according to the International Journal of Systematic and Evolutionary Microbiology (3). *A. baumannii* specifically was identified in 1998. It was among the top three pathogens to cause nosocomial infections in Taiwan from 2003-2009 and its incidence has only continued to increase worldwide since (8). Due to the emergence of it as a pathogen within military medical facilities in the Middle East it has earned the nickname "Iraqibacter". *A. baumannii* is now one of the most common causes of wound infections seen in soldiers coming back from that area of the world (11)

A. baumannii can survive in various environments making it an increasingly important organism in the health care field. It has been reported to thrive in hospital conditions, being resistant to disinfectants as well as antibiotics. In addition to *A. baumannii*'s ability to resist antibiotics, disinfectants, and detergents it is also extremely resistant to prolonged periods of desiccation (7-9,16,34). Currently new drugs, such as tigecycline, and old ones, like colistin, are still affective when used with other antibiotics. Although resistance to these cocktails has begun to be reported in clinical strains (7).

Disease

Acinetobacter baumannii has become a pathogen of note within the last few years, primarily for its ability to develop resistance to currently used antibiotics (8,9,16,19,20,23,26,34,35,41). There are three classes of drug resistance that this bacteria falls into, multidrug resistant (MDR), extensively drug resistant (XDR), and pan drug resistant (PDR) (12). Multidrug resistant *A. baumannii* (MDRAB) refers to strains that are resistant to at least three of the five main types of antibiotics: cephalosporins, carbapenems, β-lactamase inhibitors, fluoroquinolones, and aminoglycosides. Resistant to aminopenicillins have begun to emerge (8,9,16,19,20,23,26,34,35,41).

A. baumannii can be considered an opportunistic pathogen and is a contributor of hospital acquired (nosocomial) infections. In recent years it has become a major cause of nosocomial infections and has been classified as one of the most important hospital acquired pathogens. World Health Organization has listed *A. baumannii* as 'critical' meaning that urgent attention is needed for new antibiotics and treatment (26).

A. baumannii infection is an issue primarily for those with weakened immune systems, such as those in the intensive care unit (ICU) or the burn units. *A. baumannii* has been known to cause pneumonia, urinary tract infections (UTI), infections in the blood stream, post-surgical complications, and wound infections (8,9,16,19,20,23,26,34,35,41). Because of the tendency of *A. baumannii* to be drug resistant, the mortality rate from hospital acquired infections has been estimated to be 20-60% (41). This indicates a higher incidence of morbidity, mortality, and can increase the cost of trying to treat it (39).

The role of *A. baumannii* in war wounds has greatly increased, with many soldiers returning from Iraq and Afghanistan with infections of this bacterium. The CDC performed a survey in 2008 that indicated that *A. baumannii* was the number one cause of opportunistic infections within the ICUs of Taiwan. The history of the bacterium indicates that most of the drug resistances it has developed has occurred in the 40 years since the 1970s, when it was susceptible to most antibiotics (23).

Another antibiotic used to treat MDRAB, when other antibiotics no longer work, has been carbapenem, however resistance to this drug is also on the rise. One of ways in which *A. baumannii* shows resistance to carbapenems is through the gene bla_{NDM}, which encodes for a carbapenemase. This gene has been shown to be responsible for spread of carbapenemase amongst various gram-negative bacteria and *A. baumannii* could be one of the reservoirs for the spread of this gene. This gene has now been seen in other species of *Acinetobacter* as well, such as *A. lwoffii* and *A. schindleri* which had not previously been identified as clinically significant isolates. With the introduction of the bla_{NDM} gene these species are now being looked into as having a greater clinical significance (26).

A. baumannii also shows increased expression of efflux pumps belonging to the resistance-nodulation-division (RND) that can eliminate various molecules from the cell, including many different forms of antibiotics. Three of these pumps have been currently identified in *A. baumannii*; AdeABC, AdeFGH, and AdeIJK. Combined these three pumps efflux almost all classes of antibiotics used to treat infection with *A. baumannii* (29).

One of the major contributors to the virulence of *A. baumannii* is its ability to produce biofilms. Biofilms are made from extracellular polysaccharides and are known to protect the bacteria that produce them; by preventing desiccation, nutrient depletion, and making it difficult

for antibiotics to reach the bacterial cells. Some of the genes that are involved in biofilm formation are biofilm-associated protein (Bap), outermembrane protein A (OmpA), and the pilus usher-chaperone assembly system. These genes have recently been shown to be upregulated because of various chemical quorum signals and electrical signals sent between cells (39). Often the upregulation of these genes is triggered by environmental stressors; desiccation, nutrient deprivation, and antibiotics.

Biofilm

One of the survival methods of *A. baumannii* is to attach to both biological and inanimate objects. This adherence increases the probability of survival and allows for biofilm formation (9,41). A biofilm is an adaptive mechanism produced by bacterial cells that allow them to withstand harsh conditions. Biofilm formation offers further protection from the environment and from antibiotics and disinfectants than antibiotic resistance genes alone (9,39,41). They provide an intrinsic resistance antibiotics, environmental stressors, and products of our own immune system (13). These films make the infections difficult to treat and have led to a necessity of alternative treatments. These treatments may involve the use of Bacteriophage, which are small enough to pass through the biofilm and infect the bacterial cells (9,13,39,41).

Biofilms are a major contributor to infection of biomaterials, which aid in the survival of bacteria in chronic infections and may confer added resistance to antibiotics. They are complex structures surrounding bacteria of the same, or mixed, species. Biofilms are no longer defined as single species cultures, but rather as multicellular populations of cells. However, in the case of human infections most of biofilms are composed of a single bacterial species (32).

Biofilms are primarily composed of two components, exopolysaccharide (EPS) and glycocalyx. These two components offer the protection that bacteria find in biofilms. They allow the bacteria to survive environmental stressors and antibiotic exposures, and may even play a part in reducing the stress of nutrient depletion. These two compounds aren't the only components found within the biofilm however, there are also various internal structures and channels. These structures and channels are thought to allow bacterial cells to send signals to each other about the conditions of the environment that they are in and may be involved in adherence to surfaces (18).

One of the main genes responsible for biofilm formation is the *bap* gene. In *A. baumannii,* and other biofilm producing bacteria, biofilm-associated protein (Bap) promotes the initial attachment for cell-to-cell interactions. It can enhance adhesion to epithelial cells as well as to inorganic surfaces, such as hospital equipment (39). Bap is expressed at the cell surface and is known to have a roll in attachment of the cells to surfaces and may play a part in invasion of host tissue. However, the Bap gene has not been shown to be directly linked to genes for EPS secretion, so its role is likely limited to adhesion and attachment during biofilm formation (17). BAP has also been noted to self-assemble in strains of *S. aureus*, making functional amyloid aggregates that the cells attach with and the biofilm matrix is built from. It is the insoluble nature of the N-terminal region that allows for this aggregation and is one area of the protein found to be conserved over many strains. This conserved region may then offer a targeted area for treatment (39).

Other factors come to play in the ability of *A. baumannii* to form a protective biofilm. One of the efflux pumps in *A. baumannii,* AdeABC, is positively controlled by a Two-Component System (TCS) AdeRS. The AdeABC genes are usually involved in antibiotic

resistance and they have been seen to be upregulated in strong biofilm forming strains of *A. baumannii* as well. The AdeRS gene is encoded directly upstream of *ade*ABC and mutations in *adeRS* have caused an increased expression of the AdeABC pump. The increase in the AdeABC pump results in decreased susceptibility to many antibiotics and an increase in biofilm production. What environmental factors trigger the activation of the AdeRS system is still not yet understood. However, targeting this gene may offer a way of reducing biofilm formation, antibiotic resistance, and virulence in *A. baumannii* strains (26).

One of the difficulties with treating infections by organisms that produce biofilms is that there are no antibiotics specific to biofilms currently available. There are some drugs used to suppress biofilm formation currently in various research phases, but none have made it to clinical trials yet. One of the reasons for this is the poor amino acid sequence similarity of Bap between strains of bacteria, only the structure of this protein remains similar to one another. This is problematic when the antibiotics are attempting to target the ability of the bacteria to adhere to surfaces and to each other (39).

Treatments

Due to the rapid increase in MDR *A. baumannii* and other infections, antimicrobial peptides (AMPs), have been targeted as a novel treatment method but currently there are few that have made it to the clinical trials stage of development. None of these have shown much of an effect on biofilm-associated infections, making them less reliable to use in *A. baumannii* infections (1).

AMPs still offer an avenue of possible treatment due to their ability to show broad spectrum action against Gram-negative bacteria, Gram-positive bacteria, viruses, fungi, and

parasites; they cannot currently be used on their own due to being unable to diffuse through biofilms. This has led to research studying whether AMPs coupled with known antibiotics may have an inhibitory effect on biofilm producing bacteria. The combination of AMPs and antibiotics may make bacteria that were once antibiotic resistant susceptible again. The use of AMPs in combination with imipenem or ciprofloxacin have been shown to increase the effectiveness of the two antibiotics. Indicating that the use of AMPs in conjunction with antibiotics may be one method to counter MDRAB (1).

Inhibiting the production of biofilms by *A. baumannii* would result in some sensitivity to antibiotics returning as the antibiotics would be able to make it to the cells in the absence of the biofilm. Studies have reported that the use of polymyxins have been able to inhibit biofilm formation. These antibiotics are currently used as a last resort antibiotic for many MDR- gram negative infections (35).

In order for these antibiotics to be effective, the minimum inhibitory concentrations (MIC) of these drugs needs to be maintained. Sub-MIC levels have been shown to increase biofilm formation and expression of the Ade family of efflux pumps. The resulting increase in efflux pumps gives higher antibiotic resistance to the surviving cells. As a result, it is exceedingly important to constantly monitor the level of polymyxins in a patient's blood, to make sure increased antibiotic resistance was not being facilitated (35). Looking into ways that can reduce biofilm formation or compounds that can diffuse through biofilms may be the next treatment methodology to consider.

One drug of choice used to treat MDR *A. baumannii* (MDRAB) in the past has been colistin. It acts on gram negative bacteria by disrupting the outer membrane. However there has been an alarming increase in the number of infections showing building resistance to this

antibiotic, with two main mechanisms of resistance. One of the means by which *A. baumannii* has developed resistance to colistin is in the production of biofilm. The cationic antimicrobial peptide of colistin has difficulty in making it through the lipopolysaccharide component of the biofilm. This defense mechanism has resulted in recent outbreaks of these drug resistant bacteria in hospitals around the globe, predominately in Asia and the Middle East (12).

Strains of *A. baumannii* that had a complete loss of lipopolysaccharide (LPS) or merely a modification of the lipid A moiety of LPS returned susceptibility to vancomycin, azithromycin, and rifampin. This is likely the case due to the lack of LPS causing the permeability of the outer membrane to change and allow for the increased susceptibility. However, for mutations that only modified the LPS the resistance to those antibiotics was maintained (12).

The colistin resistant strains of *A. baumannii* were also found to have much lower growth rates than non-resistant strains which may affect the over all fitness of the strain. The ability to form biofilm and consequently resist many antibiotics and disinfectants remains unaffected by the change in the LPS, although given the decreased growth rate the biofilm may be slower to form and thus reduce the virulence of the strains (12).

Most antibiotics are not designed to reduce biofilm formation and thus biofilms offer protection to the organisms by keeping the antibiotics away from the cells. N-acetycysteine (NAC) has been shown to control the growth of biofilm on several clinical bacteria; such as *P. aeruginosa*, *S. pneumoniae*, *S. epidermidis*, E. *coli*, and *E. faecalis*. Tigecycline (TGC) is a broad-spectrum antibiotic used to treat various drug resistant bacteria. It has also been shown to have an inhibitory affect on biofilm formation by *A. baumannii*. Used together the NAC could suppress the biofilm formation and the TGC could attack the bacteria itself, leading to a treatment of MDR organisms that is more effective than the use of either antibiotic on their own.

Currently, NAC has already been shown to reduce the biofilm formation in *A. baumannii* and thus, when combined with TGC, represents a possible therapy for *A. baumannii* infection (13).

Currently the best way to control the growth of *A. baumannii* is to use pasteurization, ultraviolet light, chemical sanitizers, ozone, and photocatalysis. Unfortunately, many of these methods are harmful to humans or surface materials, making them less than ideal to treat the bacterium. Some standard methods to protect from infection, such as ethanol or alcohol rubs, enhance the virulence of multidrug-resistant *A. baumannii*, making the control of MDRAB more difficult. The lack of methods to treat this bacterium highlights the need for alternative strategies to prevent its spread (8).

II. Micrococcus luteus

History

Micrococcus luteus is gram-positive cocci that group in tetrads and belong in the family *Micrococcaceae*. Among *Micrococcus* species *M. luteus* is the most common found on the skin of humans (31,36). It is normal flora for the skin and mucous membranes, along with other members of *Micrococcaceae* and opportunistic pathogens, such as *Staphylococcus* species (15,36,38). Until 1975 there were 8 subgroups of micrococci, but the utilization of DNA sequencing has rearranged the classification of *Micrococcus* species reducing the number of subgroups to 6. The other two were moved to the genus *Staphylococcus*, showing the relatively close relationship of those two genera. *M. luteus* forms yellow colonies on blood agar, which resemble *Staphylococcus* specie. These colonies are catalase positive and coagulase negative. *M.*

luteus is bacitracin susceptible which is one of the features that distinguishes it from *Staphylococcus* species (36).

M. luteus is usually considered to be a non-pathogenic but in immunocompromised patients it has been shown to be an opportunistic pathogen. In this grouping of patients, *M. luteus* has been known to be the causative agent of intracranial abscesses, pneumonia, septic arthritis, and meningitis (2,15,33,36). By 1985 there had only been two cases of meningitis caused by *M. luteus* reported, the numbers have since increased, but it is still considered to be a rare cause of disease. Fosse *et. al.* reported a third from a 57-year-old woman, after the addition of a ventriculo-peritoneal shunt to deal with a meningeal hemorrhage, and a basilar bifurcation aneurysm (15). As of 1997 *M. luteus* has also been associated with infections of indwelling intravenous lines, continuous ambulatory peritoneal dialysis fluids, ventricular shunts, and prosthetic valves. Some of these infections can be quite serious, such as septic arthritis, meningitis, and endocarditis (33). Infecting similar areas as *Staphylococcus epidermidis*, another opportunistic pathogen that is closely related to the *Micrococcus* genus (36).

Disease

Given that *M. luteus* has the designation of normal flora, it may often be labeled as a contaminate in blood specimens when it should be flagged as the possible cause of the infection. This designation often delays the appropriate treatment of the bacteria and allows for biofilm formation, making it harder to treat (33). Since 1999, *Micrococcus* species have become increasingly important due to a rise in nosocomial infections, or infections of prosthetic material, leading to life threatening infections. This has been especially severe in immunocompromised patients. In 1999 the first cases of a chronic cutaneous infection by *M. luteus* was reported, in which the patient did not have a prosthetic device and did not have systemic disease, although

they were immunocompromised (38). This represented a new area in of infection by *M. luteus* as an opportunistic pathogen.

Infections by *M. luteus* typically occur in locations of the body that are remote from host defense. This can lead to serious life-threatening infections by an organism that very rarely causes infection. One of the leading sources of entry of *M. luteus* is plastic surfaces with intravenous cannulae. The biofilm produced by *M. luteus* allows the organism to attach to the plastic and when mixing with host fluids it is distributed into the host's body. The fact that *M. luteus* is normal flora and so rarely causes infection makes the diagnosis of this bacterium more difficult and that can lead to life threatening situations (38).

A survival mechanism for *M. luteus* is its ability to maintain viability in low nutrients environments. Research has indicated that even after 3 months of nutrient deprivation, nearly 50% of *M. luteus* cells in culture can be resuscitated back into growing normally by the addition of nutrient rich media. Stability of DNA maintenance and membrane proteins help to ensure that basic function of the cells are maintained during periods of starvation and dormancy. This slowing of metabolic processes into dormancy can lead to an indirect method of antibiotic resistance. When conditions aren't favorable to cell growth the cells can go into dormancy and wait out the bad conditions for up to 6 months or more, making treatment more difficult (30).

The ability to undergo dormancy has been shown to prevent bacteria from being culturable in chronic infections, allowing them to hide from conventional diagnosis. It is seen in some more notable pathogens, for example *Mycobacterium tuberculosis*, and is an avenue that the World Health Organization (WHO) is considering as a target for treatment. This could also be used for other bacteria that evade antibiotics by dormancy, such as *M. luteus*. 18 proteins have been identified as being upregulated in a state of dormancy for *M. luteus*. Several proteins appear

to be tied to anaplerotic metabolism, theoretically to deal with the lack of nutrients available (27).

In combination with biofilm production this state of dormancy may help protect the bacteria even from bacteriophage. The reduced metabolism means that it is harder for the bacteriophage to hijack the cell to produce the proteins it need given that most cellular functions are slowed or turned off (18). In these conditions lysogenic phage may be more likely to decrease the population of cells left in the system (32).

M. luteus has been shown to have resistances to penicillin, ampicillin, and erythromycin. Some strains have been found to be oxacillin resistant as well and are showing similar resistance patterns to multidrug resistant *Staphylococcus aureus* (MRSA). This is worrisome in that it could indicate that *M. luteus* may be on its way to being as difficult to treat as a MRSA infection (36).

Biofilm

Because of its ubiquitous nature *M. luteus* can be found outside of the medical field but still cause problems that are similar to those seen in the medical industry. Specifically, one of these problems is biofilm formation. This process forms extra-cellular polysaccharides (EPS) that has the potential to disrupt medical filtration membranes; it is also one of the ways in which organisms can colonize indwelling catheters and other medical devices. Due to its ability to form biofilms on the membranes of water treatment plants the EPS produced by *M. luteus* has been investigated. Some membranes, or medical surfaces, have a greater likelihood of EPS binding and increasing biofilm density than others. Feng *et al*. noted that hydrophilic polyvinylidenefluoride (PVDF) membranes had EPS adhere to them more easily than

hydrophobic polypropylene (PP) membranes. However, reduced flow due to EPS blockage was still seen in both (14).

Biofilms are composed of more than just EPS they are also highly heterogeneous and have channels and structures within them to allow for nutrient spread and to resist environmental stresses. Some of these channels and structures allow bacteria to signal to one another about the environment around them. The structure of the polysaccharides also influences the properties of the molecules that hold the biofilm together, specifically glycocalyx, which provides protection from antimicrobial agents as well as biological stresses (18).

Biofilms are largely responsible for the ability of bacteria to cause endocarditis, UTI, chronic otitis media, chronic bacterial prostatitis, and respiratory tract infections. Many of which *M. luteus* has recently become an opportunistic pathogen of. The biofilm formation can complicate the healing process even if it doesn't cause a dangerous infection, which can lead to other serious healthcare issues (32).

Treatments

M. luteus, while typically considered normal flora, has been implicated in some nosocomial infections of immunocompromised patients. Antibiotic resistance is one of the major issues dealt with in both nosocomial and community acquired infections, becoming an ever more serious issue. *M. luteus* has been shown to have moderate levels of multidrug resistance, and research done by Bonjar in 2004 indicates that some herbal remedies used in Asia do have antibiotic properties against *M. luteus*, leading to possible future methods of treatment. These treatments still need to be investigated in a clinical setting however, and may be some years away from approval (2).

The antibiotic of choice for treating *M. luteus* is rifampicin as it has been shown to be most active against *M. luteus*. Rifampicin has been shown to be quite affective when used singularly. While *M. luteus* has begun showing resistance to teicoplanin, another former drug of choice, when given in the absence of rifampicin (36). The lack of research studies done on treatments for *M. luteus* infection, coupled with the appearance of antibiotic resistance suggests that new treatments need to be investigated. Preferably before resistances out pace known methods of treatment.

III. Bacteriophage

What are they

A bacteriophage is a virus that utilizes bacterial cells as their host. Bacteriophages are one of the most abundant organisms on earth. Because of this and their specificity to their hosts they have been used in drug discovery and human health practices (32,40). They have been classified into 13 families according to their morphologies, types of nucleic acid, and whether they have an envelope. Many bacteriophages have icosahedral head, tails which act as the attachment sight, and double stranded DNA genomes. There are three main groups of tailed phages; *Myoviridae*, *Siphoviridae*, and *Podoviridae* that they are separated into these groups by their morphology. *Myoviridae* have contractile tails, *Siphoviridae* have long non-contractile tails, and *Podoviridae* have extremely short tails (28).

Bacteriophage were discovered in 1896 by Ernest Hankin and were first described for their antibiotic properties, but they weren't recognized as viruses until 1916 by Felix d'Hérelle. d'Hérelle initially isolated bacteriophage whose hosts were cholera, bubonic plague, and anthrax

and was the first to suggest that bacteriophage might be used as a treatment for infections. These discoveries occurred 20 years prior to the application of penicillin as an antibiotic (28,32). However, the usage of penicillin combined with theoretical concerns about foreign DNA led to most western countries abandoning the development of bacteriophage as a form of therapy. The research into bacteriophage as a viable therapy continued in Eastern Europe and the Soviet Union (28).

Each phage consists of nucleic acid, either DNA or RNA, and proteins that make up the outer structures of the phage. Many of these proteins exhibit some form of enzymatic activity. The variety of types and shapes of bacteriophage is wide; there are filamentous, icosahedral phage, phage with tails, phage without tails, and some that have a lipoprotein envelope around the main protein head. Like all other viruses, bacteriophages require host cells in order to replicate themselves and do so in one of two different cycles; the virulent lytic cycle or the more dormant lysogenic cycle (32).

Bacteriophages are viruses that specifically target bacterial species. They can be found almost anywhere that bacteria can be found and are exceedingly common, with concentrations of $3.16x10^4$ phage per ml having been detected in sewage samples (18). If the bacteriophage is an environment that bacteria can survive in then bacteriophage will also be found there. The viral receptors are designed to attach to specific antigens on the surface of bacterial cells, making them very selective. Bacteriophage are natural parasites of bacteria and are generally extremely host-specific. This specificity makes them ideal for possible treatments of bacteria on surfaces and possibly active infections as they would not harm the human host (8,18,20,41). The use of bacteriophage is already gaining attention in controlling specific bacterial foodborne pathogens (8).

Most of the time bacteriophage can be physically found near their bacterial hosts. Thus, areas where certain pathogens can be found are the most likely places to find bacteriophage specific to the pathogen. In order to infect their hosts bacteriophage, recognize various outer cell wall components. They can recognize flagella, lipopolysaccharides, and various outer membrane proteins (21).

There has been evidence that some bacteriophage contain genes for the production of polysaccharide depolymerase enzymes that can be imbedded in the outer protein shell of the phage particles. These enzymes allow phage to break down the polysaccharides in biofilm so that they can travel through the matrix to reach and infect bacterial cells. During this process, these new holes in the biofilm could allow for the passage of other molecules into the biofilm, such as antibiotics. The depolymerases have been seen in both lytic and lysogenic phages (18).

A number of factors have an effect on the ability of phage to grow or what form they may take, such as ions, pH, temperature, and the environment they are in can affect their absorption rate and infective ability. Additions of calcium and magnesium to the environment of the phage can increase the ability of phage to attach to the host cell (24).

Lysogenic vs Lytic

One of the issues with propagating lytic phage using bacteria is that they may be contaminated by temperate phages, also known as prophages. Under certain conditions prophages, which exist within the bacterial genome, may pop out at irregular times forming plaques similarly to lytic phage. Prophages are replicable objects within a bacterial genome that contain an independent set of genes; they are also known as lysogenic phage (6).

Bacteriophage that go through the lytic cycle invade specific bacteria and lyse the cells after causing significant metabolic disruptions. This cycle of replication reduces the bacterial population by removing cells from the environment (10). During the lytic cycle phage attach to bacterial surfaces and inject their DNA, or RNA, into the host cell (32). The first step in the lytic cycle is attachment. During this step the phage will attach to a specific protein or sugar on the surface of the bacterial cell. Usually these attachment zones are specific to a bacterial species or even select strains of a bacterial species. Phage that can attach across species or even across genera are very rare (10,28). It is during the attachment step where lytic phage get their host specificity, binding to unique structures on the surface of specific bacterial hosts (32).

Once the phage is attached it will inject its genetic material into the bacteria (10,28). The nucleotide sequences then hijack the mechanisms of the host cells to replicate their genetic code and transcribe and translate their unique proteins. The capsid is constructed from some of these phage proteins and the replicates of the phage genome are inserted into the capsid. For phage that contain a tail, it is the last part added to the capsid/head and then the bacterial cell is lysed by endolysin and holin and the phage particles are released back into the environment to infect other bacterial host cells. Endolysin breaks down the peptidoglycan and holin causes holes to form in the cellular membrane (28,32).

Only lytic bacteriophage are capable of being used for bacteriophage therapy, as since they directly lead to the lysis of bacterial cells. Lysogenic phage can remain dormant for too long and come out irregularly and thus cannot be adequately controlled for use as a therapeutic agent (32).

Lysogenic phage on the other hand integrate their genetic information into the hosts, either in the form of a plasmid or sometimes into the host chromosome itself. At this point they

are known as prophage within the bacterium. Phage will remain inside the bacterium until something triggers a shift to the lytic cycle. It could take many generations of the bacterium before the conditions for the shift are reached, allowing for another method of replication of the phage. Each subsequent generation containing the prophage can individually be triggered into the lytic cycle and thus more cells can release more bacteriophage (28,32).

Once the phage genome is integrated into the bacterial genome it can be multiplied many times without destroying the bacterial cell. The absorption of the phage genome also gives the bacterial cells some resistance to the infection of other phages that are related to the lysogenic phage. Lysogenic phages can also carry toxic genes in their genome, adding to the virulence of the bacterium they reside in (28). This chance of carrying a toxic gene is another reason why lysogenic phage are not good avenues to look at for bacteriophage therapy.

Bacteriophage therapy

Bacteriophage therapy represents one of the new methods for control of bacterial infections. Their potential use is a real possibility, due to their ability to multiply exponentially and their mode of action, which is completely different than antibiotics. For these reasons bacteriophage can be used against multidrug resistant strains of bacteria (20,28,41). Bacteriophages are viruses that specifically target and lyse bacterial cells. Bacteriophage even have the ability to move through the protective biofilm bacteria may produce. This means that they can function in conditions where antibiotics are unable to reach. Because of the selective nature of bacteriophages, they are more likely to be safe for use on plants and animals and are not infective to anything other than their specific bacterial host (28,41).

Bacteriophage can be isolated from nearly everywhere sea water, sewage water, sludge ponds, feces, food, etc. (24). They typically utilize the bacteria that are already present in the sample as their hosts. Their host specificity has given rise to their evolution alongside the specific bacteria they infect (16,32).

Bacteriophage have a different mode of action than antibiotics making them ideal to use individually or combined with antibiotics, increasing the effectiveness of the antibiotics when faced with multi-drug resistant bacteria. The ability to develop resistance to phage attack is significantly much slower than the development of drug resistance, making phage a more viable method of treatment. Phage also can rapidly respond to resistances that bacteria develop to evade the infection by phage, in an evolutionary arms race. Subsequently the cost to develop a phage cocktail is surprisingly cheaper than the development of new antibiotics (28,32).

Given that these bacteriophages only target bacterial cells and can be so specific as to only infect certain strains of bacteria, they are very unlikely to cause a problem within humans if used for treatment (21,37). Phage have been approved to treat infections in both in plants and animals, and studies have been done on the effectiveness of treating dysentery, skin infections, pulmonary infections, meningitis, and infected wounds in humans. In 2002, a study investigated the efficacy of phage therapy in treating infections of vancomycin resistant *Enterococcus faecium* in mice and noted that the pathogen was resolved in nearly all the infected mice (32).

Bacteriophage isolation offers a possible alternative for biological control of pathogenic bacteria (21,37). The use of phage as a treatment for infection is not new. In 1919 bacteriophages were used as therapeutic agents to treat bacterial dysentery and continued to be used up until 1940. With the arrival of antibiotics, however, the use decreased as it was easier to obtain new

and effective antibiotics. The rise in multidrug resistant bacterial pathogens has led to a resurgence in the idea of using bacteriophage as a method of treatment (20).

Bacteriophages have already been used to treat foods contaminated with *Campylobacter*, *Enterobacter*, *E. coli* O157, *Listeria*, *Salmonella*, and *Staphylococcus*; in addition to the non-food contaminate *Pseudomonas aeruginosa*. These methods have been shown to reduce the bacterial contaminates by 1-5 log (8). In 2006 the FDA approved methods of bacteriophage treatment for control of *Listeria* contamination in food preservation. A cocktail of six listeriaphage in a mixture was sprayed on the surface of ready-to-eat meat and poultry products to reduce *Listeria* contamination in those foods. In 2007 the European Food Safety Authority also approved the use of bacteriophage as a biological control agent against *Listeria* (21,23,37). With the approval of the use of bacteriophage in food presentation a door has been opened to consider bacteriophage treatment as a candidate for biocontrol agent in with human applications (37).

The aforementioned biocontrol methods have been primarily used on liquid foods and looking at their effectiveness on solid surfaces has not yet been significantly studied. For fighting infection by *A. baumannii*, the bacteriophage must be effective on hard surfaces, such as beds and equipment used in the ICU (8). It is possible that adding bacteriophage to a hand rub or cream may afford a new method for preventing contamination of medical personal (23).

The most effective bacteriophages for use as therapy are those that have high efficacy of adsorption, short latent periods, and large burst (plaque) sizes. This insures that the largest number of bacteria are infected and lysed by the viruses. Bacteriophages may offer better treatment for organisms that produce biofilm by triggering the destruction of the film. This has been seen as biofilm reduction facilitated by polysaccharide-degrading enzymes which some

bacteriophage possess. The biofilm reduction can sometimes be the identified as the presence of a halo surrounding the phage plaques on media plates (9).

Bacteriophage cocktails are typically used to broaden the host range specificity of the treatment. This allows for a broader coverage of various receptors within a genus, or even within a species when bacteriophage are specific down to strain. Understanding what receptors specific phage use can be useful in designing phage cocktails for better treatment capabilities. These cocktails are also a means of getting around the adaptive immunes system of bacterial cells. Some CRISPR sequences deal with some phage but with the use of a cocktail another phage in the mix can target the bacterial cells instead. The use of cocktails with antibiotics may also increase the effectiveness of both (21). To combat the host-specific nature of bacteriophage, treatments may be designed with use of cocktails of phage known to be infectious to a species of bacteria. These may be used in combination with antibiotics to increase the effectiveness (34).

The in vitro activity of bacteriophage cocktails has been observed to have an efficacy rage of 80-100% in mice, using various bacterial species. Treating the same infections with ciprofloxacin only had a range of 50-80%. When antibiotics and phage cocktails were used together there was a 100% clearance of the bacterial infection. For these studies the best way to administer the phage was through intraperitoneal injection (32).

The combination of antibiotics and bacteriophage has been shown to increase the effectiveness of both. Specifically, antibiotics and a cocktail of phage show the greatest effect on bacterial populations. The use of both has been shown to decrease the development of resistances to antibiotics and increase the susceptibility to bacteriophage infection. The order in which the cocktails are given also appears to influence the inhibitory qualities of the mixtures. If the antibiotic is given prior to the phage cocktail then the phage cocktail is better able to reduce the

cell count of the bacteria. Whereas the addition of the antibiotic at the same time as the phage has shown to reduce the viability of the phage and thus the bacterial cell count was not lowered as expected. This may also be the case when anti-biofilm bacteriophage are used within the phage cocktail, antibiotic resistant bacteria won't be released from the compromised biofilm if bacteriophage have infected the cells (10). Because of the valuable use of phage cocktails, it is important to continue isolations of novel bacteriophage (34).

Interestingly, it has been shown that bacteria that gain bacteriophage resistance lose their virulence. These less virulent bacteria can then be utilized in vaccine development, giving bacteriophage therapy a different angle to study if the target pathogen becomes resistant to the phage. These bacterial strains are weakened overall and less likely to survive environmental stressors than wild type bacteria. Unlike previous studies, Capparelli *et al*. showed that bacteriophage therapy could be administered 2 weeks after bacterial infection are still effective in infecting and lysing bacterial cells (5).

Some of the difficulties involved with using bacteriophage for treatment is the ability to large-scale produce stable bacteriophage products. The conditions have to be standardized for the phage to remain viable. pH, temperature, bacterial strain, and media composition are the most important factors to maintaining the stability of the particles. These conditions can vary between particular bacteriophage. It is also critical to do titers on each culture grown as the smallest variation in these variables can lead to extreme changes in the amount of viable phage that can be obtained (40).

An additional problem using bacteriophage as a method of treatment is that bacteria have an adaptive immune system to protect themselves from viruses. This system known as clustered regularly interspaced short palindromic repeats (CRISPR) are found in about half of the known

bacterial species. These sequences are often of similar size and nucleotide sequence as phage, plasmids, and other foreign DNA. These sequences are transcribed into RNA that interacts with CRISPR associated proteins (Cas) or with RNase III associated with Cas proteins to recognized and chop up foreign nucleotide sequences. CRISPR allows bacteria to resist being hijacked by foreign genes, preventing the bacteria from making copies of the foreign message and ultimately lysed by phage. This immune response makes the continued research into novel bacteriophage very important. The CRISPR system may not recognize sequences of completely novel phage, offering a way around the defense system (4).

Yet another difficulty with using bacteriophage as a treatment is in the reduction of biofilm. Some bacteriophage have the ability to break down biofilms with the enzyme polysaccharide depolymerase, allowing the phage to reach the cells within. This procedure is limited by the ability to break down the entire biofilm. The depolymerase found in bacteriophage are very specific to certain polysaccharides, if the EPS surrounding the cells is changed slightly then the depolymerase would no longer be effective in breaking down the biofilm. This gives bacterial cells a method of resistance to these bacteriophages. Using bacteriophage alone in these instances may not be enough to completely remove the bacterial cells (18).

The actions of bacteriophage within a biofilm depend on a variety of factors, including the structure of the biofilm, the size of the phage, the slow bacterial metabolism, and the burst size of the phage as it lyses the bacterial cell. The burst size is measured by the amount of new phage particles that are released once the cell is lysed. As mentioned previously some phage contain enzymes that can break down the matrix of biofilms. These enzymes offer yet another avenue of possible treatment for biofilm forming infections. If biofilm can be reduced around the infection, then the bacterial cells can be targeted by both phage and antibiotics (32).

Bacteriophage have already been used in biological control of biofilms in the medical field. In 2006 bacteriophage were introduced to the hydrogel coating of a catheter and it was discovered that there was a much lower amount of biofilm that formed in the catheter than in the untreated catheters. In another experiment done in 2010, phage was passed through a catheter that had already been coated with a biofilm and noted that the biofilm was destroyed by the phage cocktail that was added. The biggest complication in the utilization of phage to disrupt biofilms is the same as using phage to directly treat infections is the stability of the phage. Keeping the phage in a solution where they remain viable for a period is a challenge as the conditions may vary depending on the type of phage present (32).

The utilization of genetically modified, nonreplicating, bacteriophage is also being considered as a possible method of treatment. The ability of nonreplicating M13 phage to block the growth of *Helicobacter pylori* was explored. In mice, the administration of this genetically modified phage showed that while the virus didn't multiply in *H. pylori* the growth of the bacterium was greatly reduced (28).

The use of phage therapy does bring other concerns, such as the release of endotoxins (lipopolysaccharide) by gram negative bacteria upon lysis. It may be possible to genetically modify to produce restriction endonuclease that would digest the bacterial genome and thus reduce the lipopolysaccharides produced and minimize endotoxin release (28).

There are other concerns that exist when it comes to bacteriophage therapy. One of these is the potential detrimental effect of introducing foreign genetic material into the patients' system. The concern is that it may introduce undesirable traits and genes into the human system. One way around this is to consider the enzymes encoded for in phage genomes and using them as a treatment instead of the phage themselves (28,32). Specifically, enzymes that degrade

bacterial cell walls, lytic enzymes that break down peptidoglycans which would be very effective on Gram positive bacteria; such as *Micrococcus luteus, Streptococcus pyogens, Bacillus anthracis*, and possibly MRSA. These enzymes are known as phage endolysin, or lysin (28).

The use of phage lysin on its own may have a greater activity on bacterial cells than antibiotics that inhibit peptidoglycan production. The antibiotics only lyse the cells when they are undergoing active division whereas phage lysin can lyse the bacterial cells at any point in their replicative cycle. Thus lysin enzymes can have a lytic effect on bacteria that are resistant to peptidoglycan inhibitor antibiotics. The phage lysin is as specific to bacterial species as the phage that produce them, with the exception of an enterococcal phage, and thus can be used to target specific bacterial infections and leave normal flora alone (28). All of these options show that research into the discovery of novel bacteriophage is important for various new treatment methods for antibiotic resistant bacteria.

CHAPTER 2

ISOLATION OF POSSIBLE BACTERIOPHAGE FOR *MICROCOCCUS LUTEUS* AND METHODS FOR ISOLATING NOVEL BACTERIOPHAGE OF BACTERIAL PATHOGENS

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ABSTRACT: The rising numbers of antibiotic resistance in bacteria has led to a crisis in treatment options. *Acinetobacter baumannii* is an example of bacteria that have developed a dangerous level of multidrug resistance. Not only does it have genes allowing for the resistance to antibiotics, but it also produces a biofilm that protects it. In recent years, *A. baumannii* has become a major contributor to nosocomial infections making it critical to develop new treatment methods. *Micrococcus luteus*, while typically not thought of as a pathogen, it is also developing a resistance to antibiotics. *M. luteus* is capable of forming a biofilm on its own making it worrisome as it has increasingly been noted as an opportunistic pathogen.

One potential new treatment of antibiotic resistance is the development of bacteriophage therapy, using bacterial viruses to target the infection and treat it. This study looks at methods for isolating novel bacteriophage from dairy cattle feces, specifically for the biofilm producers *A. baumannii* and *M. luteus*.

KEYWORDS: Antibiotic resistance, *Acinetobacter baumannii*, *Micrococcus luteus*, Bacteriophage therapy

INTRODUCTION

One of the rising concerns within the medical health profession is the distinct increase in cases of multidrug-resistant bacteria. Fewer antibiotic treatments are maintaining their effectiveness against both gram-positive and gram-negative bacteria. By definition, multidrug-resistant organisms (MDROs) show resistance to more than one antibiotic, leading to difficulties in finding treatment that is effective. Two gram-negative bacteria that are of huge concern are *Klebsiella pneumoniae* and *Acinetobacter baumannii*, in which some strains are showing

resistance to all available antibiotic agents (1). This study focused on *A. baumannii* and *M. luteus* as a gram-negative and a gram-positive bacterium showing antibiotic resistance and biofilm formation.

A. baumannii is a gram negative, aerobic, non-fermentative, coccobacillus bacterium that can be found nearly everywhere, it is considered ubiquitous, and can survive very harsh environments, including surfaces in healthcare facilities (2-5). This is problematic because of the growing antibiotic resistance co-occurring with this organism (3,6-9). *A. baumannii* has been categorized as an opportunistic pathogen but in the past few years it has caused many infections in both immunocompromised and healthy individuals (6,7,10). It is responsible for hospital-acquired (nosocomial) pneumonia, urinary tract infection (UTI), bloodstream infections, wound infections (11-14). *A. baumannii* is the fifth most common cause of nosocomial infections worldwide, partly due to the rapid development of antibiotic resistance (13). This has led to it classification as a 'critical' organism by the WHO (7).

Some of the antibiotic resistance in bacteria have come from genes encoded for within the bacterial genome, but some have also come from the biofilm the bacteria can form (6,11,14,15). A biofilm is defined as a population of either prokaryotic or eukaryotic cells. They used to be thought of as being composed of a single species, as is the case in most clinical situations, but they may be composed of numerous species (6,16,17). These biofilms likely are one of the major factors determining whether antibacterial treatments fail, it is hard for antibiotics to get through the thick polysaccharide and make it to the bacterial cells (8,15-18). The ability to form a biofilm greatly contributes to the virulence of the bacteria that produce them (6,13). Biofilms likely play a role in the ability of *A. baumannii* to survive long periods of

time in environments that lack moisture, or even in the presence of disinfectants. This allows the organism to cling to and survive on otherwise sterile surfaces, as in clinical environments (6).

Biofilms are formed primarily by the secretion of exopolysaccharide (19). Within these polysaccharide polymers lay channels and streamers which are thought to allow communicate about the environment around them. The thick polysaccharide doesn't just protect against outer environmental stressors, but may also provide a reservoir for nutrients (16,17,19). They have been associated with infections causing endocarditis, UTI, chronic otitis media, chronic bacterial prostatitis, and respiratory tract infections seen with cystic fibrosis patients. Bacteria that can form biofilm can attach to and colonize a wider variety of surfaces than bacteria that are unable to form biofilm (6). In some instances, the organisms capable of forming biofilm that may cause infections from medical equipment may be normal flora of the skin, oral cavity, urinary tract, reproductive system, and gastrointestinal tract (6,16). This is the case in the instances of infection by *Micrococcus luteus*.

Micrococcus luteus has been designated normal flora of the oral cavity. It is a gram positive that group in tetrads (20,21). Like *A. baumannii*, *M. luteus* is ubiquitous in the environment, however, it is rarely the source of disease. Because of the classification as normal flora it is often considered a contaminate when seen in cultured specimens. This can delay treatment in instances where *M. luteus* is the opportunistic pathogen and the source of the infection. As a pathogen *M. luteus* has typically been seen in cases of immunocompromised individuals, however it is increasingly being seen in relation to biomedical procedures (22). It has been associated with septic arthritis, meningitis, endocarditis, intracranial abscesses, and pneumonia (21,23). Typically, these infections have been traced back to medical equipment such as indwelling intravenous lines, continuous ambulatory peritoneal dialysis fluids, ventricular

shunts, and prosthetic valves (21,22). These are similar areas that infections by coagulase negative *Staphylococcus* are seen, which *M. luteus* is a close relative of (21).

M. luteus has several ways of avoiding antibiotics even though there have rarely been associated with antibiotic resistance. One way, which is like *A. baumannii*, is its ability to form a biofilm thus protecting it from environmental stressors. *M. luteus* biofilm gives it similar protections as other biofilm forming organisms; desiccation resistance, antibiotic resistance, and ways to avoid nutrient deprivation (17). A second, and more unique method, is dormancy. The cells of *M. luteus* can decrease their metabolism in order to survive conditions that would be otherwise fatal to the cells (24). These cells can survive three to six months in this dormant state and still return to an active metabolic state and begin to divide to increase the population (25). This dormant behavior prevents most antibiotics from acting on the organism (26). Without actively dividing cells antibiotics that target peptidoglycan production are ineffective, and without normal metabolic processes occurring antibiotics that target metabolism also fail to work (24-26).

With these means of avoiding antibiotics via biofilms, genetics, or dormancy different treatment options need to be considered. *A. baumannii* gains antibiotic resistance very quickly, through genetic adaptation or biofilm formations, and has become a major cause of nosocomial infections (3,6-9,11,13-15). *M. luteus*, while typically considered normal flora, has started to be seen more frequently as an opportunistic pathogen. Delay in diagnosing *M. luteus* has made it more difficult to treat, likely do to dormant cells or biofilm formation (17,20-22,24,26). These antibiotic resistance, or avoidance, mechanisms need alternate treatment options to control the infections.

Recently there has been interest in looking at bacteriophage as a new method of treatment for these harder to treat infections (6,11,16,27-31). Bacteriophage were discovered in 1896 by and were first described for their antibiotic properties. In 1916 Felix d'Hérelle recognized them as viruses. Initially phage whose hosts were cholera, bubonic plague, and anthrax were isolated and it was at that time that bacteriophage might be used as a treatment for infections. While these discoveries occurred 20 years prior to the application of penicillin as an antibiotic, the usage of penicillin combined with theoretical concerns about foreign DNA led to most western countries abandoning the development of bacteriophage as a form of therapy. The research into bacteriophage as a viable therapy continued in Eastern Europe and the Soviet Union (16,31).

Bacteriophage present a new form of treatment because their ability to multiply exponentially, their mode of action, the ability to move through protective biofilms, and their selective nature (6,11,16,27-31). Phage are viruses that specifically target and lyse bacterial cells. They can be isolated from nearly everywhere sea water, sewage water, sludge ponds, feces, food, etc. and they typically utilize the bacteria that are already present in the sample as their hosts (28).

Because phage only target specific bacterial species, or even specific strains, they are more likely to be safe for use on plants and animals (29). Their ability to filter through biofilms implies that they can function in conditions where antibiotics are unable to reach (6,11,27). Phage inject their genetic material into bacterial cells and hijack the cells own mechanics to replicate the phage particles, which is completely different than the mode of action of antibiotics (16,31). This means that they can be used against multidrug resistant strains of bacteria (6,11,27,32).

This study looks at three slightly varying methods of isolating lytic bacteriophage for *A. baumannii* ATCC 19606 and *M. luteus*. These bacteria were chosen because of their ubiquitous nature and their ability to form biofilms. The same methods were used on both types of bacteria to see if similar methodologies can be used to isolate bacteriophage for both gram-negative and gram-positive bacteria. Dairy cattle feces was utilized as the source to look for novel bacteriophage, due to the ubiquitous nature of these bacteria. Dairy cattle feces has not readily been studied for bacteriophage isolation, yet the close interaction of these animals with human pathogens has. The feces may be a good source for possible bacteriophage for *A. baumannii*, *M. luteus*, and other pathogenic bacteria such as *E. coli* and *S. enterica*.

METHODS AND MATERIALS

Method 1: One-week incubation

The first method that was utilized to isolate bacteriophage involved two separate week-long incubations. 5 grams of dairy cattle feces was added to 50 mL of Luria-Bertani (LB) broth. The broth contained 10mM of CaCl2, which has been known to facilitate phage attachment (28). 1 mL of a turbid *A. baumannii* or *M. luteus* culture was added to the feces and LB broth. The *A. baumannii* culture was grown overnight but no longer than 24 hours as biofilm growth in the culture can prevent even dispersal of cells in the culture. *M. luteus* was grown for 24-36 hours as it had a slower growth rate than *A. baumannii* and thus took longer to get to a turbid culture. These cultures were then grown for a week to allow for phage to interact with the bacterial cells and replicate within them. The *A. baumannii* and feces culture was incubated at 37˚C for the week, whereas the *M. luteus* and feces culture was incubated at room temperature (RT) for the week, due to their varying growth conditions.

After the week incubation the cultures were centrifuged at 10,000 rpm for 5 minutes. The supernatants were then filtered through a .45 µm filter and again through a .22 µm filter to ensure that none of the bacteria from the culture would be present in the phage enrichment solution. 1 mL of the enrichment was added to fresh 50mL of LB,CaCl2 along with 1 mL of fresh pathogen culture (grown the same as previous). These cultures incubated for another week in the same conditions as they were in the first round of incubation.

Once the incubation was complete the cultures were once again centrifuged at 10,000 rpm for 5 minutes, and then the supernatant was filtered in the same manner as the previous incubation. The second-round enrichment was then combined with fresh pathogen and plated in an overlay method. This involves mixing 300 µL of enrichment with 300 µL of pathogen in 2.5 mL of soft agar (.5% agar instead of 2%). This solution was then poured on LB, 10mMCaCl2 thick poured agar plates. The liquid was gently swirled until the entire surface of the plate was covered. The plates were left undisturbed on a flat surface for an hour to allow the soft agar to solidify before they were moved for incubation. The plates containing the *A. baumannii* plus enrichment were incubated overnight at 37˚C while the *M. luteus* plus enrichment plates were incubated for 36 hours at RT.

Once the bacterial lawns had grown the plates were examined for clearings in the lawn. When symmetric clearings are formed by virus particles they are referred to as plaques. If plaques are present they can be utilized for further isolation of the phage, by two different methods to be discussed later.

Controls were done using 300 µL of sterile water with 300 µL of pathogen in 2.5 mL of soft agar. No plaques should be seen on the control plates, only smooth bacterial lawns.

Method 2: Overnight incubation

The second method used to isolate bacteriophage was done very similarly to the first. The main difference being that instead of each incubation being done for a week, they were only allowed to incubate for 12-48 hours depending on the pathogen. The same measurements of 50mL LB with 10mM CaCl2, 5 g of feces, and 1 mL of fresh pathogen were used in the initial incubation. 50 mL fresh broth with 1 mL enrichment and 1 mL pathogen were used in the second incubation. The solutions were filtered the same between each step and the final enrichment was plated in the soft agar overly method with 300 µL of enrichment with 300 µL of pathogen and 2.5 mL of soft agar. The plates were incubated the same as in the first method, and each plate was again examined for plaque formation. Controls were also done in the same manner as in the previous method.

Method 3: Overnight incubation without pathogen

The third method utilized for phage isolation changed a little bit more from the first two. Due to the large quantity of left over enrichment the measurements were decreased for the incubating samples. The measurements instead were 5 mL of broth inoculated with .5 g of feces that were left to incubate overnight. There was no addition of pathogen in the initial incubation. The following day the enrichments were centrifuged and filtered as before then used in the second incubation. 5 mL of broth were combined with 100 µL of turbid pathogen culture and 100 µL of enrichment. The cultures were incubated in the same fashion as the first two methods before being centrifuged, filtered, and plated.

Control plates were done in the same fashion as the previous two methods.

Phage Isolation

When plaques are seen on the plates they need to be further isolated to insure that a single phage is present in an isolated culture. Because the initial enrichment came from a mixed culture it is possible that there may be more than one phage present on the plates. This is done in one of two ways; the first is to core a selection of isolated plaques. Typically, this procedure is done with at least 5 separate plaques. The extraction was done by extracting a 1 mm diameter section of plate that contains a single plaque, using a pasture pipette for ease of transfer. This agar piece was placed in 1 mL fresh LB, 10mM CaCl2 and placed in a 4˚C refrigerator overnight to allow the phage to elute out of the agar and into the broth without allowing for bacterial growth. The next day the solutions were plated using the soft agar overlay method. After an overnight incubation the plates were examined for plaques.

The second method of phage isolation was done when the plaques are too close together to be extracted individually using the pipette method. Instead an overlay of 10 mL of LB, 10mM CaCl2 broth was added to the top of the soft agar plates and they were left on a flat surface overnight. The overnight incubation at RT acts in the same manner as the 4˚C incubation, it allowed for the phage to elute out of the agar and into the broth. The broth was then centrifuged and filtered given that bacterial cells can grow at RT. The sample was then diluted from 10^1 to 10^{10} and plated in the soft agar overlay method and incubated overnight. The plates were then examined for plaque formation.

RESULTS

Control plates:

Growth on the control plates was typically a uniform bacterial lawn showing few if any deformations of the agar (see Figure 1). There was an issue with some of the *A. baumannii* control plates in that they showed a swirl pattern characteristic of biofilm formation. Within the swirls clearings could be seen that were similar to plaque formations, but this did not occur on every control plate (see Figure 2). *M. luteus* control plates all showed the same uniform bacterial lawn growth across the soft agar surface.

One-week incubation:

The plates made from the enrichments that were left to incubate for a weeks' time showed no visible plaques. Each plate, for both *A. baumannii* and *M. luteus*, showed uniform bacterial lawns that looked no different than the control plates.

Overnight incubation:

The plates made from the enrichments that were left to incubate overnight showed plaque like clearings on the *A. baumannii* plates (see Figure 3). No plaque like clearings were seen on the *M. luteus* plates, and a weak lawn was noted after 18 hours. Plates were looked at again at 36 hours. They showed a much thicker and uniform lawn but no plaque-like clearings were noted.

Overnight incubation without pathogen:

The plates made from the enrichment that lacked pathogen in the first round showed plaque-like clearing zones for both *A. baumannii* and *M. luteus*. The *M. luteus* plates were left to

grow for 36 hours to show adequate thickness of the lawn. The plates showed 5-10 plaque-like clearings per plate (see Figure 4), plaques were cored from one plate and a broth overlay was performed on another.

Plaque Isolation:

Because of the issue seen with the *A. baumannii* control plates neither coring or broth overlay were completed with these samples. The cored samples for *M. luteus* showed few possible plaque-like clearings, but the broth overlay dilutions showed a great reduction in bacterial growth (see Figure 5,6). Bacterial cell density was taken from the same turbid culture in the same measurements across all 10 dilution plates. The 10^{10} dilution showed not only a reduction in bacterial growth but plaque-like clearings as well (see Figure 7).

DISCUSSION

Three slightly varying methods were used to try and isolate novel bacteriophage from dairy cattle feces, with *A. baumannii* or *M. luteus* as their hosts. The first method of enriching for a week with pathogen and feces, then again for a week with the enrichment and fresh pathogen, showed no isolation of phage. The lack of any visible plaques on the plates could be from the conditions in the media after a weeks' worth of growth. Bacteriophage are relatively delicate; they have a small range of pH and temperature that they remain viable in. Once the bacteria have reached stationary phase and start lysing or dying off the cellular components they release into the culture may reduce the viability of the phage in the solution.

The extended exposure to bacterial cells also increases the likelihood that the cells that remain in the culture may have developed a resistance to the phage present. Bacteria have innate

defense against the attachment or injection of the phage genome and after a period of time many of the cells in the solution may be showing those resistance. One of the most well-known defense mechanisms in bacteria is the CRISPR system and it has been a target of study in recent years (33). The resistant cells would then become the more prevelant cells in the culture and reduce the likelihood of recovering phage.

The results of the one-week incubation lead to the changes to overnight incubation method. The bacterial cultures were still in the log phase in an overnight culture and thus may have actively dividing phage in the culture as well. The addition of the pathogen in the initial incubation would allow for any phage that utilize the pathogen as a host to replicate in the initial enrichment. This was possibly seen with *A. baumannii*, since plaque-like clearings were seen on the sample plates. However, it can't be said for certain that this was the cause of phage for *A. baumannii*, given the plaque-like clearings that were noted on some of the control plates as well.

There is the possibility that the clearings seen on the control plate, and thus on the experimental plates, may have been caused by lysogenic phage (prophage) being triggered to enter the lytic cycle of their division. Something within the environmental conditions of the incubating plates may have initiated the lysing of cells by phage that otherwise remain dormant within the bacterial genome. For this reason, most studies considering bacteriophage therapy look specifically for lytic phage, phage that enter a cell and ultimately lyse it. Lysogenic phage enter the cell but then integrate into the genome of the cell to be replicated with the bacterial genome. They may remain dormant within the bacterial strain for many generations before something triggers their transition to the lytic cycle of phage replication (16,31).

The clearings could also be caused by the presence of biofilm formation in the starter culture. The polysaccharide polymers may cause the swirl patterns seen on the control plate and may block bacterial growth from some sections of the plate. One way to combat the possibility of biofilm formation in the starter culture causing the issues seen in the control plates would be to do a growth curve assay on the *A. baumannii* strain used. This would ensure that the starter culture was in the log phase of growth and that biofilm formation was at a minimum. This was the suspected cause of the issues with phage isolation for *A. baumannii* in this study as the appearance of the swirl patterns and clearings were only seen periodically on the control plates.

Another way to verify that what was seen on the experimental plates may have been phage is for a spot test to be performed. A spot test is done by creating a bacterial lawn on a plate using soft agar containing the pathogen that has been allowed to solidify. The phage enrichment samples are then spotted onto the soft agar surface in increasing dilutions. Once dry the plates are incubated and then examined for clearings that reduce with each dilution (34).

These swirl patterns were not seen with *M. luteus*, but neither were any plaque-like clearings. Further adjustments to the methods were then done to facilitate a larger number of possible phage in the enrichment. Instead of adding pathogen to the first incubation the feces was incubated overnight on its own. This increased the likelihood of the phage present in the sample would be amplified before pathogen was added to it. The second incubation included the pathogen to further amplify any phage that were present that could use it as a host. It was with this method that plaque-like clearings were seen with *M. luteus*.

Single phage cores were taken and re-plated from these initial samples and the initial results were inconclusive, showing plaque-like clearings but not in the increased number that

was expected or showing no plaques at all. It is possible that the diffused phage number was too high in these samples, as they were not diluted out and re-plated. Diluting the cored samples and plating may offer more conclusive results.

Broth overlays were also done on the initial samples and the serial dilutions of the broth overlay showed a reduction in growth of the bacterial lawn. The 10^{10} dilution also showed an abundant number of small plaque-like clearing zones. While these results do not conclusively indicate that phage has been isolated for *M. luteus* they do make a strong suggestion that this is the case. Further dilution of the broth overlay would need to be done to get better spacing between plaque-like clearings so single phage coring could be done to ensure isolation.

This study did not conclusively show the isolation of bacteriophage from both *A. baumannii* and *M. luteus*, but it did suggest that novel phage may be present in the samples. Future research needs to be done to verify that what was seen is in fact bacteriophage acting on the bacteria present, but it is reasonable to conclude that the methods used within this study can be used to isolate bacteriophage. This approach was used to look for novel phage from dairy cattle feces, an area that has previously not been looked at for novel phage, to identify phage that may be candidates for use in bacteriophage therapy. The discovery of novel bacteriophage is important given the sharp increase in MDRO being seen in both nosocomial and community acquired infections. New therapies offer a different way to combat severe infections that may otherwise kill patients when conventional antibiotics don't work. Bacteriophage also can move through biofilms increasing their activity at clearing infection when compared to antibiotics alone.

APPENDIX A

Figures

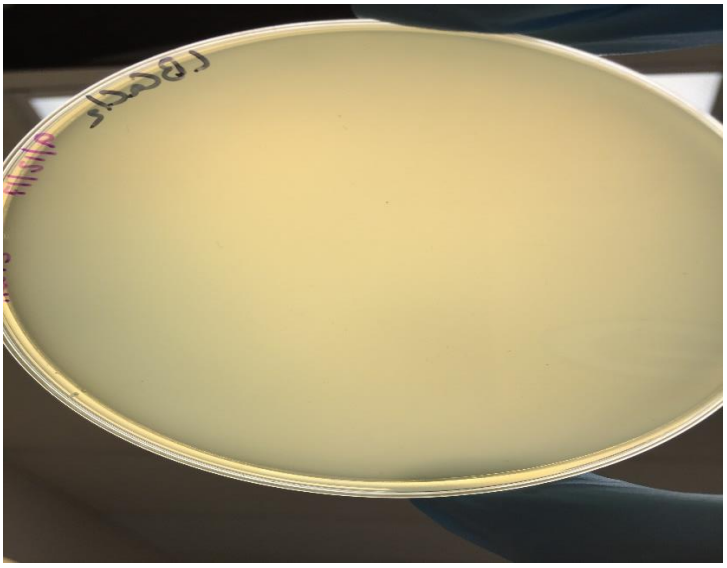

Figure 1: Photograph of a control plate for *M. luteus* after 36 hours incubation at RT, showing a uniform bacterial lawn

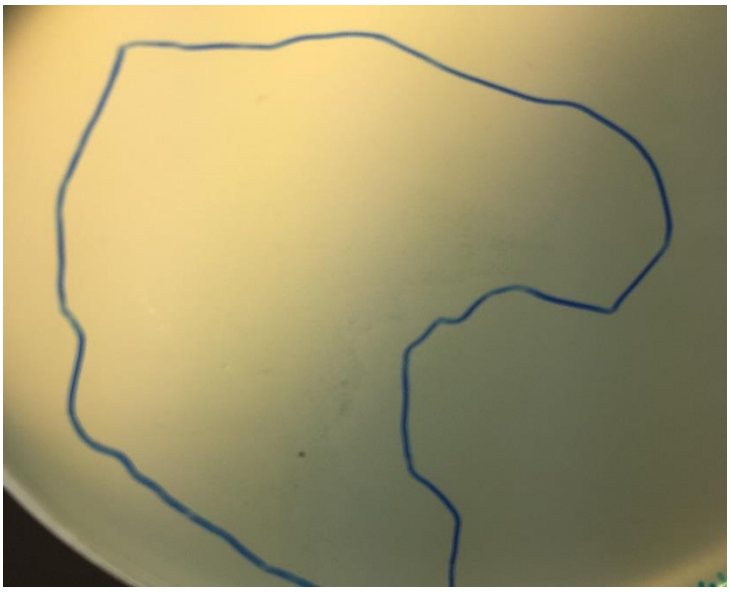

Figure 2: Picture of a control plate for *A. baumannii* after 18-hour incubation at 37˚C, showing the swirl pattern containing plaque-like clearings.

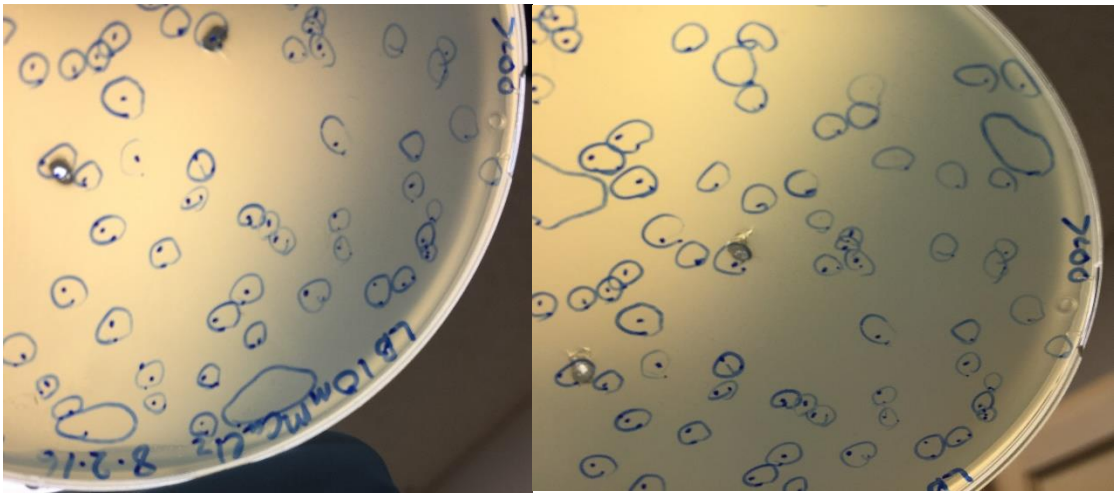

Figure 3: Photographs of soft agar overlay plates for *A. baumannii* after 18-hour incubation at 37˚C, showing scattered clearings consistent with possible plaque formation. The large holes in the agar are from coring isolated plaques for further testing.

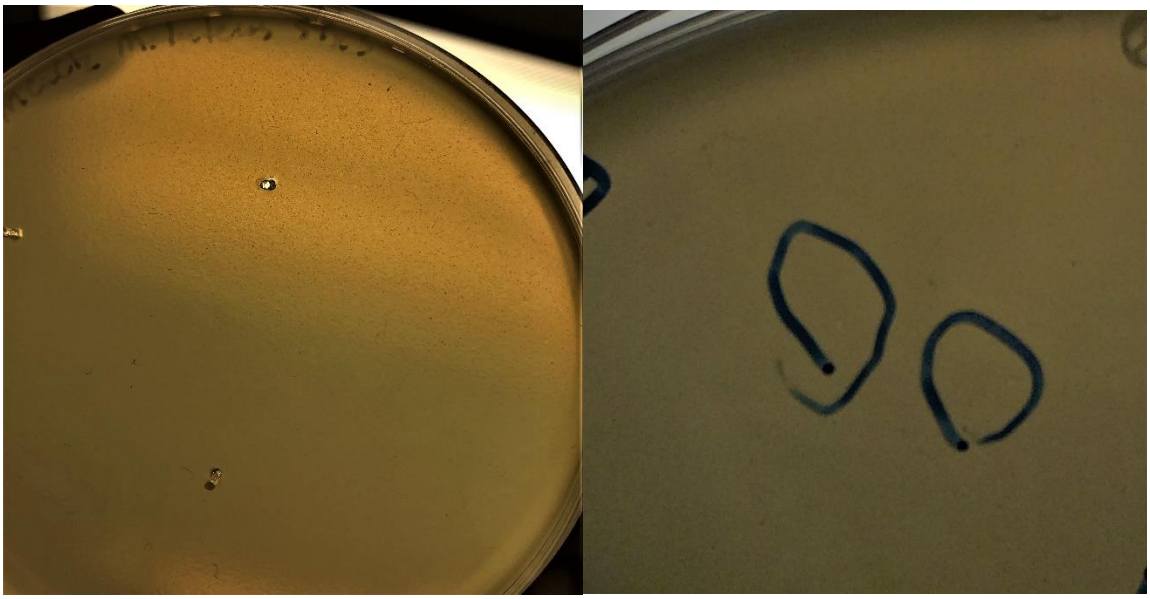

Figure 4: Photographs of soft agar overlay plates for *M. luteus* after 36-hour incubation at RT, showing cored holes with a few plaque-like clearings (left) and circled plaque like clearings in the agar (right).

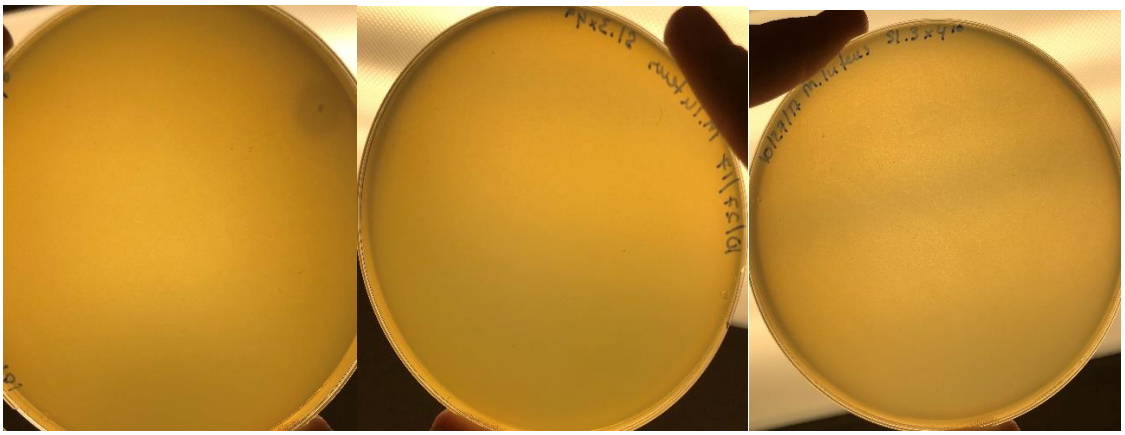

Figure 5: Photographs of *M. luteus* broth overlay plating after 36 hours of incubation at RT, showing decreasing bacterial growth with increasing dilution of broth overlay stock solution. A thick lawn for the 10^8 dilution (left), thinner lawn for the 10^9 dilution (middle), thin lawn with plaque-like clearings for the 10^{10} dilution (right).

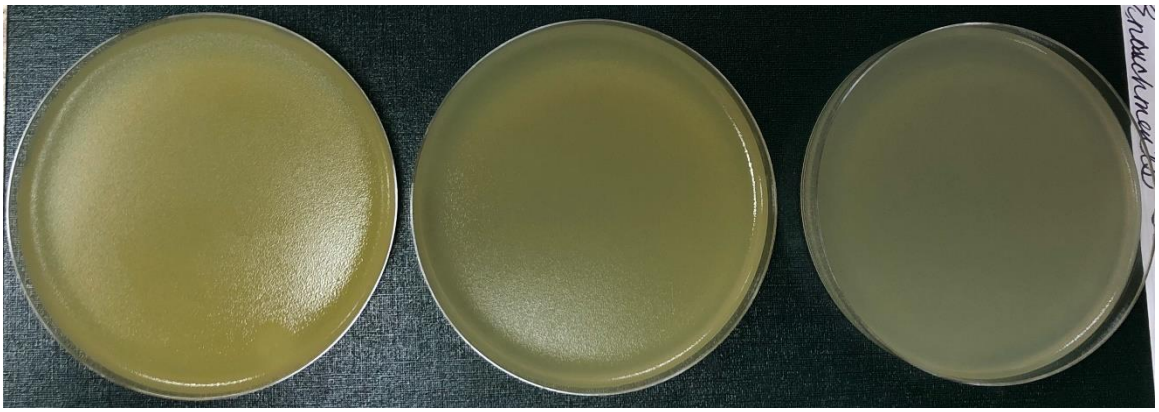

Figure 6: Photograph of *M. luteus* broth overlay plating after 36 hours of incubation at RT, showing decreasing bacterial growth from dilution 10^8 (left) to dilution 10^{10} (right).

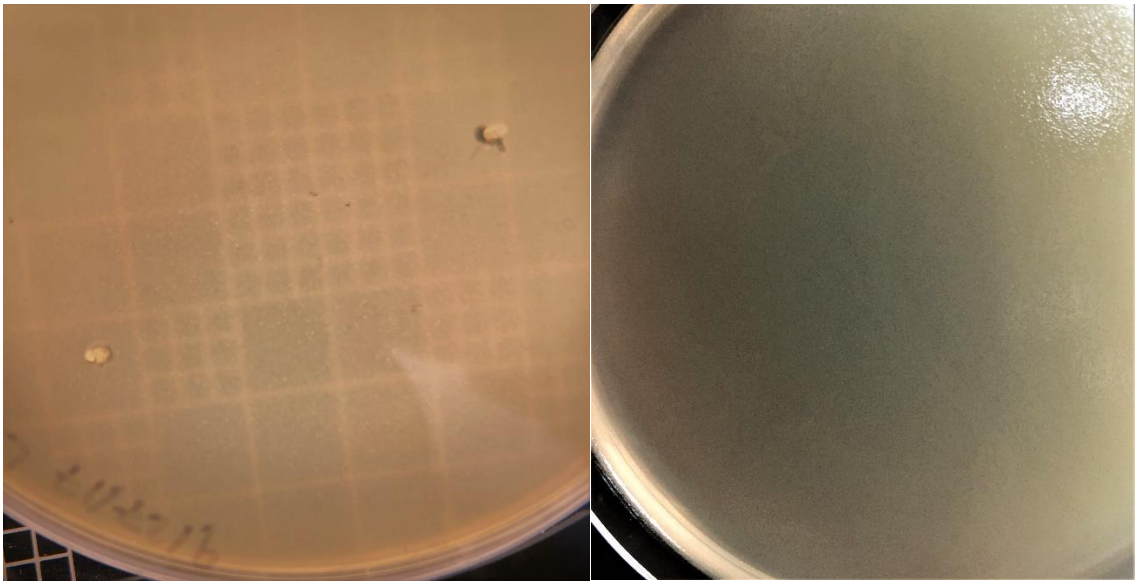

Figure 7: Photograph of *M. luteus* broth overlay plating after 36 hours of incubation at RT, showing phage-like plaques on a light box for the dilution 10^{10}(left), and a view using a dark background (right).

References:

1. **Magiorakos, A.P., Srinivasan, A., Carey, R.B., Carmeli, Y., Falagas, M.E., Giske, C.G., Harbarth, S., Hindler, J.F., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D.L., Rice, L.B., Stelling, J., Struelens, M.J., Vatopoulos, A., Weber, J.T., Monnet, D.L**. (2012) Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology and Infection* **18**:268-281

2. **Peng, F., Mi, Z., Huang, Y., Yuan, X., Niu, W., Wang, Y., Hua, Y., Fan, H., Bai, C., Tong, Y.** (2014). Characterization, sequencing and comparative genomic analysis of vB_AbaM-IME-AB2, a novel lytic bacteriophage that infects multidrug-resistant *Acinetobacter baumannii* clinical isolates. *BioMed Central Microbiology* **181**(14):1-14

3. **Jin, J., Li, ZJ., Wang, SW., Wang, SM., Huang, DH., Li, YH., Ma, YY., Wang, J., Liu, F., Chen, XD., Li, GX., Wang, XT., Wang, ZQ., Zhao, GQ.** (2012). Isolation and characterization of ZZ1, a novel lytic phage that infects *Acinetobacter baumannii* clinical isolates. *BioMed Central Microbiology* **156**(12):1-8

4. **Chang, KC., Lin, NT., Hu, A., Lin, YS., Chen, LK., Lai, MJ.** (2011) Genomic analysis of bacteriophage ϕAB1, a ϕKMV-like virus infecting multidrug-resistant *Acinetobacter baumannii*. *Genomics* **97**:249-255

5. **Chen, LK., Liu, YL., Hu, A., Chang, KC., Lin, NT., Lai, MJ., Tseng, CC.** (2013) Potential of bacteriophage ϕAB2 as an environmental biocontrol agent for the control of multidrug-resistant *Acinetobacter baumannii*. *BioMed Central Microbiology* **154**(13):1-10

6. **Yele, AB., Thawal, ND., Sahu, PK., Chopade, BA.** (2012) Novel lytic bacteriophage AB7-IBB1 of *Acinetobacter baumannii*: isolation, characterization and its effect on biofilm. *Archives Virology*. **157**:1441-1450

7. **Malaka De Silva, P., Kumar, A.** (2017) Effect of Sodium Chloride on Surface-Associated Motility of *Acinetobacter baumannii* and the Role of AdeRS Two-Component System. *Journal of Membrane Biology* **251**:5-13

8. **Bardbari, A.M., Arabestani, M.R., Karami, M., Keramat, F., Aghazadeh, H., Alikhani, M.Y., Bagheri, K.P.** (2018). Highly synergistic activity of melittin with imipenem and colistin in biofilm inhibition against multidrug-resistant strong biofilm producer strains of *Acinetobacter baumannii*. *European Journal of Clinical Microbiology & Infectious Diseases* **37**:443-454

9. **Bouvet, P.J.M., JeanJean, S., Vieu, J.F., Dijkshoorn, L.** (1990) Species, Biotype, and Bacteriophage Type Determinations Compared with Cell Envelope Protein Profiles for Typing Acinetobacter Strains. Journal of Clinical Microbiology **28**(2):170-176

10. **Farmer, NG., Wood, TL., Chamakura, KR., Everett, GFK.** (2013). Complete Genome of *Acinetobacter baumannii* N4-Like Podophage Presley. *Genome Announcements* **1**(6):1-2

11. **Dubrovin, EV., Popova, AV., Kraevskiy, SV., Ignatov, SG., Ignatyuk, TE., Yaminsky, IV., Volozhantsev, NV.** (2012). Atomic Force Microscopy Analysis of the *Acinetobacter baumannii* Bacteriophage AP22 Lytic Cycle. *PLOS one* **7**(10):1-9

12. **Sato, Y., Unno, Y., Ubagai, T., Ono, Y.** (2018). Sub-minimum inhibitory concentrations of colistin and polymyxin B promote *Acinetobacter baumannii* biofilm formation. PLOS one. **March 19, 2018**: 1-18

13. **Tiwari, V., Patel, V., Tiwari, M.** (2018) *In-silico* screening and experimental validation reveal L-Adrenaline as anti-biofilm molecule against biofilm-associated protein (Bap) producing *Acinetobacter baumannii*. *International Journal of Biological Macromolecules* **107**:1242-1252

14. **Jeon, J., Kim, J., Yong, D., Lee, K., Chong, Y.** (2012) Complete Genome Sequence of the Podoviral Bacteriophage YMC/09/02/B1251 ABA BP, Which Causes Lysis of an OXA-23-Producing Carbapenem-Resistant Acienetobacter baumannii Isolate from a Septic Patient. Journal of Virology **86**(22):12437-12438

15. **Feng, J., Liu, B., Xu, J., Wang, Q., Huang, L., Ou, W., Gu, J., Wu, J., Li, S., Zhuo, C., Zhou, Y.** (2018) *In vitro* effects of N-acetylcysteine alone and combined with tigecycline on planktonic cells and biofilms of *Acinetobacter baumannii*. *Journal of Thoracic Disease* **10**(1):212-218

16. **Parasion, S., Kwiatek, M., Gryko, R., Mizak, L., Malm, A.** (2014) Bacteriophages as an Alternative Strategy for Fighting Biofilm Development. *Polish Journal of Microbiology* **63**(2):137-145

17. **Hughes, K.A., Sutherland, I.W., Jones, M.V.** (1998) Biofilm susceptibility to bacteriophage attack: the role of phage-borne polysaccharide depolymerase. *Microbiology* **144**:3039-3047

18. **Farshadzadeh, Z., Taheri, B., Rahimi, S., Shoja, S., Pourhajibagher, M., Haghighi, M.A., Bahador, A.** (2018) Growth Rate and Biofilm Formation Ability of Clinical and Laboratory-Evolved Colistin-Resistant Strains of *Acinetobacter baumannii*. *Frontiers in Microbiology* **9**:1-11 doi: 10.3389/fmicb.2018.00153

19. **Feng, L., Li, X., Song, P., Du, G., Chen, J.** (2014) Physicochemical properties and membraned biofouling of extra-cellular polysaccharide produced by a *Micrococcus luteus* strain. *World Journal of Microbiology and Biotechnology* **30**:2025-2031

20. **Mukamolova, G.V., Yanopolskaya, N.D., Votyakova, T.V., Popov, V.I., Kaprelyants, A.S., Kell, D.B.** (1995) Biochemical changes accompanying the long-term starvation of Micrococcus luteus cells in spent growth medium. Archives in Microbiology **163**:373-379

21. **Seifert, H., Kaltheuner, M., Perdreau-Remington, F.** (1995) *Micrococcus luteus* Endocarditis: Case Report and Review of the Literature. *Zentralblatt für Bakteriologie* **282**:431-435

22. **Peces, R., Gago, E., Tejada, F., Laures, A.S., Alvarez-Grande, J.** (1997) Relapsing bacteraemia due to *Micrococcus luteus* in a haemodialysis patient with a Perm-Cath catheter. *Nephrology Dialysis Transplantation* **12**:2428-2429

23. **Fosse, T., Peloux, Y., Granthil, C., Toga, B., Bertrando, J., Sethian, M.** (1985) Meningitis Due to *Micrococcus luteus*. *Infection* **13**:280-281

24. **Kaprelyants, A.S., Kell, D.B.** (1993) Dormancy in Stationary-Phase Cultures of *Micrococcus luteus*: Flow Cytrometric Analysis of Starvation and Resuscitation. Applied and Environmental Microbiology **59**(10):3187-3196

25. **Mukamolova, G.V., Yanopolskaya, N.D., Votyakova, T.V., Popov, V.I., Kaprelyants, A.S., Kell, D.B.** (1995) Biochemical changes accompanying the long-term starvation of *Micrococcus luteus* cells in spent growth medium. *Archives of Microbiology* **163**:373-379

26. **Mali, S., Mitchell, M., Havis, S., Bodurin, A., Rangel, J., Olson, G., Widger, W.R., Bark, S.J.** (2017) A Proteomic Signature of Dormancy in the Actinobacterium *Micrococcus luteus*. *Journal of Bacteriology* **199**(14):1-15

27. **Kang, H.W., Kim, J.W., Jung, T.S., Woo, G.J.** (2013) wksl3, a New Biocontrol Agent for *Salmonella enterica* Serovars Enteritidis and Typhimurium in Foods: Characterization, Application, Sequence Analysis, and Oral Acute Toxicity Study. *Applied and Environmental Microbiology* **79**(6):1956-1968

28. **Li, L., Zhang, Z.** (2014) Isolation and characterization of a virulent bacteriophage *SPW* specific for *Staphylococcus aureus* isolated from bovine mastitis of lactating dairy cattle. *Molecular Biology Reports* **41**:5829-5838

29. **Shin, H., Lee, J.H., Kim, H., Choi, Y., Heu, S., Ryu, S.** (2012) Receptor Diversity and Host Interaction of Bacteriophages Infecting *Salmonella enterica* Serovar Typhimurium. *PLOS ONE* **7**(8):1-11

30. **Warner, C.M., Barker, N., Lee, S.W., Perkins, E.J.** (2014) M13 bacteriophage production for large-scale applications. *Bioprocess Biosystems Engineering* **37**(10):2067-2072

31. **Matsuzaki, S., Rashel, M., Uchiyama, J., Sakurai, S., Ujihara, T., Kuroda, M., Ikeuchi, M., Tani, T., Fujieda, M., Wakiguchi, H., Imai, S.** (2005) Bacteriophage therapy: a revitalized therapy against bacterial infectious diseases. *Journal of Infection and Chemotherapy* **11**:211-219

32. **Jin, J., Li, ZJ., Wang, SW., Wang, SM., Huang, DH., Li, YH., Ma, YY., Wang, J., Liu, F., Chen, XD., Li, GX., Wang, XT., Wang, ZQ., Zhao, GQ.** (2012). Isolation and characterization of ZZ1, a novel lytic phage that infects *Acinetobacter baumannii* clinical isolates. *BioMed Central Microbiology* **156**(12):1-8

33. **Cady, K.C., Bondy-Denomy, J., Heussler, G.E., Davidson, A.R., O'Toole, G.A.** (2012) The CRISPER/Cas Adaptive Immune System of *Pseudomonas aeruginosa* Mediates Resistance to Naturally Occurring and Engineered Phages. *Journal of Bacteriology* **194**(21):5728-5738

34. **Ghajavand, H., Esfahani, B.N., Havaei, A., Fazeli, H., Jafari, R., Moghim, S.** (2017) Isolation of bacteriophages against multidrug resistant Acinetobacter baumannii. Research in Pharmaceutical Sciences **12**(5):373-380

CHAPTER 3

Future Directions

The overarching goal of this study was to look into methods for the isolation of novel bacteriophage from dairy cattle feces. It specifically was looking to isolate phage from *A. baumannii* and *M. luteus* because of the antibiotic resistance and biofilm formation seen in them. However, antibiotic resistance is not a clear problem for *M. luteus* at this time the bacterium has been able to cause chronic infection due to biofilm formation and dormancy. Dairy cattle feces offered a novel source for phage isolation given that it had not been studied in detail and the ubiquitous nature of *A. baumannii* and *M. luteus* made it possible that novel bacteriophage using them as hosts may be present within the sample.

Three slightly varying methods of bacteriophage isolation were used in the study. They varied on time of incubation, addition of pathogen in the first incubation, and overall size of the enrichment samples. Each enrichment was plated with pathogen doing the soft agar overlay and incubated for 18-36 hours at the optimal growth temperature for the pathogen present. The method involving overnight incubation minus pathogen showed plaque-like clearings in the agar for both *A. baumannii* and *M. luteus*. *A. baumannii* had an issue of similar, albeit *varying* sized, plaque-like clearings as the test plates making it hard to conclusively say that the clearings were caused by bacteriophage.

The possible bacteriophage seen on the *M. luteus* plates were further isolated and the broth overlay phage extraction showed reduction in bacterial growth in dilutions 10^8, 10^9, and 10^{10} with a high number of plaque-like clearings seen on the final plate. This reduction of growth with the appearance of plaque-like clearings suggest the presence of bacteriophage, but given the

plaques were too close together to individually isolate it is not conclusive of bacteriophage presence.

Further dilutions of the broth overlay for *M. luteus* needs to be done in order to reduce the number of plaques seen on the plates. From there the plaques can be cored and plated again. This will confirm the presence of phage and following rounds of coring will ensure that the phage have been isolated, in the event there is more than one type of phage present in the enrichment. These isolated phages can then be characterized by their plaque size, shape of the bacteriophage, and what kind of nucleotides the genome is made from. The optimal conditions for phage viability can also be explored using spot testing to look at differing temperatures and pHs.

Beyond these standard characteristics growth reduction in liquid media can be done to look at the ability of the phage to infect planktonic cells. Using aged cultures that have already developed a biofilm, the phage can be studied to see if it has the capability to reduce biofilm as well as being lytic against *M. luteus*.

In order to see if the plaque-like clearings on the *A. baumannii* plates the issue of plaque-like clearings being seen on the control plates needs to be dealt with. Doing growth curves on *A. baumannii* would mean that the starter cultures for the experiment will be in the log phase of growth and be less likely to have biofilm already in the culture tube, since different strains may have slightly different growth rates. Spot testing can also be done to verify that what has already been enriched is in fact bacteriophage since dilutions should show a decrease in plaque-like clearings from a complete clearing of the spot area to a few plaques seen in the spot area. This reduction would indicate that bacteriophage are present in the enrichment sample.

One limit to this study is the viability of bacteriophage themselves. Slight variations in pH and temperature in the enrichment media can be enough to prevent the attachment of phage that are present in the media. This can make things difficult for the initial isolation and further confirmation of novel bacteriophage. Further work would need to be done to optimize the conditions for retention of phage. This is difficult considering that varying phage have different optimum pH and temperature for attachment.

Another limit is that bacteria have innate defenses against bacteriophage infection. If bacterial cells are left in the culture with phage for an extended period it is possible that they will develop a resistance to the phage attachment or the injection of the phage genome. It will be important to make sure bacterial cells are removed from phage stocks as to not introduce resistant bacteria into the experiments as they could reduce the appearance of plaques on the plates.

Although this study had limitations it has shown the possibility of obtaining novel bacteriophage that may one day be used as bacteriophage therapy to treat infections by multidrug resistant bacteria. Given the ever-increasing incidents of antibiotic resistance seen in worldwide infections, new forms of treatment need to be developed. Using this methodology may offer a way to isolate novel bacteriophage to be used in the treatment of other pathogens that are showing antibiotic resistance.

References:

1. Bardbari, A.M., Arabestani, M.R., Karami, M., Keramat, F., Aghazadeh, H., Alikhani, M.Y., Bagheri, K.P. (2018). Highly synergistic activity of melittin with imipenem and colistin in biofilm inhibition against multidrug-resistant strong biofilm producer strains of *Acinetobacter baumannii*. *European Journal of Clinical Microbiology & Infectious Diseases* **37**:443-454

2. Bonjar, G.H.S. (2004) Evaluation of Antibacterial Properties of Iranian Medicinal-Plants against *Micrococcus luteus*, *Serratia marcescens*, *Klebsiella pneumoniae* and *Bordetella bronchoseptica*. *Asian Journal of Plant Sciences* **3**(1):82-86

3. Bouvet, P.J.M., JeanJean, S., Vieu, J.F., Dijkshoorn, L. (1990) Species, Biotype, and Bacteriophage Type Determinations Compared with Cell Envelope Protein Profiles for Typing *Acinetobacter* Strains. *Journal of Clinical Microbiology* **28**(2):170-176

4. Cady, K.C., Bondy-Denomy, J., Heussler, G.E., Davidson, A.R., O'Toole, G.A. (2012) The CRISPER/Cas Adaptive Immune System of *Pseudomonas aeruginosa* Mediates Resistance to Naturally Occurring and Engineered Phages. *Journal of Bacteriology* **194**(21):5728-5738

5. Capparelli, R., Nocerino, N., Iannaccone, M., Ercolini, D., Parlato, M., Chiara, M., Iannelli, D. (2010) Bacteriophage Therapy of *Salmonella enterica*: A Fresh Appraisal of Bacteriophage Therapy. *Journal of Infectious Disease* **201**:52-61

6. Ceglarek, I., Piotrowicz, A., Lecion, D., Miernikiewicz, P., Owczarek, B., Hodyra, K., Harhala, M., Górski, A., Dąbrowska, K. (2013) A novel approach for separating bacteriophages from other bacteriophages using affinity chromatography and phage display. *Scientific Reports* **3220**(3):1-6

7. Chang, KC., Lin, NT., Hu, A., Lin, YS., Chen, LK., Lai, MJ. (2011) Genomic analysis of bacteriophage ϕAB1, a ϕKMV-like virus infecting multidrug-resistant *Acinetobacter baumannii*. *Genomics* **97**:249-255

8. Chen, LK., Liu, YL., Hu, A., Chang, KC., Lin, NT., Lai, MJ., Tseng, CC. (2013) Potential of bacteriophage ϕAB2 as an environmental biocontrol agent for the control of multidrug-resistant *Acinetobacter baumannii*. *BioMed Central Microbiology* **154**(13):1-10

9. Dubrovin, EV., Popova, AV., Kraevskiy, SV., Ignatov, SG., Ignatyuk, TE., Yaminsky, IV., Volozhantsev, NV., (2012). Atomic Force Microscopy Analysis of the *Acinetobacter baumannii* Bacteriophage AP22 Lytic Cycle. *PLOS one* **7**(10):1-9

10. Escobar-Páramo, P., Gougat-Barbera, C., Hochberg, M.E. (2012) Evolutionary dynamics of separate and combined exposure of *Pseudomonas fluorescens* SBW25 to antibiotics and bacteriophage. *Evolutionary Applications* **5**:583-592

11. Farmer, NG., Wood, TL., Chamakura, KR., Everett, GFK. (2013). Complete Genome of *Acinetobacter baumannii* N4-Like Podophage Presley. *Genome Announcements* **1**(6):1-2

12. Farshadzadeh, Z., Taheri, B., Rahimi, S., Shoja, S., Pourhajibagher, M., Haghighi, M.A., Bahador, A. (2018) Growth Rate and Biofilm Formation Ability of Clinical and Laboratory-Evolved Colistin-Resistant Strains of *Acinetobacter baumannii*. *Frontiers in Microbiology* **9**:1-11 doi: 10.3389/fmicb.2018.00153

13. Feng, J., Liu, B., Xu, J., Wang, Q., Huang, L., Ou, W., Gu, J., Wu, J., Li, S., Zhuo, C., Zhou, Y. (2018) In vitro effects of N-acetylcysteine alone and combined with tigecycline on planktonic cells and biofilms of *Acinetobacter baumannii*. *Journal of Thoracic Disease* **10**(1):212-218

14. Feng, L., Li, X., Song, P., Du, G., Chen, J. (2014) Physicochemical properties and membraned biofouling of extra-cellular polysaccharide produced by a *Micrococcus luteus* strain. *World Journal of Microbiology and Biotechnology* **30**:2025-2031

15. Fosse, T., Peloux, Y., Granthil, C., Toga, B., Bertrando, J., Sethian, M. (1985) Meningitis Due to *Micrococcus luteus*. *Infection* **13**:280-281

16. Ghajavand, H., Esfahani, B.N., Havaei, A., Fazeli, H., Jafari, R., Moghim, S. (2017) Isolation of bacteriophages against multidrug resistant *Acinetobacter baumannii*. *Research in Pharmaceutical Sciences* **12**(5):373-380

17. Gregorio, E.D., Del Franco, M., Martinucci, M., Roscetto, E., Zarrilli, R., Di Nocera, P.P. (2015) Biofilm-associated proteins: news from *Acinetobacter*. *BMC Genomics* **933**(16):1-14

18. Hughes, K.A., Sutherland, I.W., Jones, M.V. (1998) Biofilm susceptibility to bacteriophage attack: the role of phage-borne polysaccharide depolymerase. *Microbiology* **144**:3039-3047

19. Jeon, J., Kim, J., Yong, D., Lee, K., Chong, Y. (2012) Complete Genome Sequence of the Podoviral Bacteriophage YMC/09/02/B1251 ABA BP, Which Causes Lysis of an OXA-23-Producing Carbapenem-Resistant *Acinetobacter baumannii* Isolate from a Septic Patient. *Journal of Virology* **86**(22):12437-12438

20. Jin, J., Li, ZJ., Wang, SW., Wang, SM., Huang, DH., Li, YH., Ma, YY., Wang, J., Liu, F., Chen, XD., Li, GX., Wang, XT., Wang, ZQ., Zhao, GQ. (2012). Isolation and characterization of ZZ1, a novel lytic phage that infects *Acinetobacter baumannii* clinical isolates. *BioMed Central Microbiology* **156**(12):1-8

21. Kang, H.W., Kim, J.W., Jung, T.S., Woo, G.J. (2013) wksl3, a New Biocontrol Agent for *Salmonella enterica* Serovars Enteritidis and Typhimurium in Foods: Characterization, Application, Sequence Analysis, and Oral Acute Toxicity Study. *Applied and Environmental Microbiology* **79**(6):1956-1968

22. Kaprelyants, A.S., Kell, D.B. (1993) Dormancy in Stationary-Phase Cultures of *Micrococcus luteus*: Flow Cytrometric Analysis of Starvation and Resuscitation. *Applied and Environmental Microbiology* **59**(10):3187-3196

23. Lee, CN., Tseng, TT., Lin, JW., Fu, YC., Weng, SF., Tseng, YH. (2011). Lytic Myophage Abp53 Encodes Several Proteins Similar to Those Encoded by Host *Acinetobacter baumannii* and Phage phiKO2. *Applied and Environmental Microbiology* **77**(19):6755-6762

24. Li, L., Zhang, Z. (2014) Isolation and characterization of a virulent bacteriophage SPW specific for *Staphylococcus aureus* isolated from bovine mastitis of lactating dairy cattle. *Molecular Biology Reports* **41**:5829-5838

25. Magiorakos, A.P., Srinivasan, A., Carey, R.B., Carmeli, Y., Falagas, M.E., Giske, C.G., Harbarth, S., Hindler, J.F., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D.L., Rice, L.B., Stelling, J., Struelens, M.J., Vatopoulos, A., Weber, J.T., Monnet, D.L. (2012) Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology and Infection* **18**:268-281

26. Malaka De Silva, P., Kumar, A. (2017) Effect of Sodium Chloride on Surface-Associated Motility of *Acinetobacter baumannii* and the Role of AdeRS Two-Component System. *Journal of Membrane Biology* **251**:5-13

27. Mali, S., Mitchell, M., Havis, S., Bodurin, A., Rangel, J., Olson, G., Widger, W.R., Bark, S.J. (2017) A Proteomic Signature of Dormancy in the Actinobacterium *Micrococcus luteus*. *Journal of Bacteriology* **199**(14):1-15

28. Matsuzaki, S., Rashel, M., Uchiyama, J., Sakurai, S., Ujihara, T., Kuroda, M., Ikeuchi, M., Tani, T., Fujieda, M., Wakiguchi, H., Imai, S. (2005) Bacteriophage therapy: a revitalized therapy against bacterial infectious diseases. *Journal of Infection and Chemotherapy* **11**:211-219

29. Montaña, S., Palombarani, S., Carulla, M., Kunst, A., Rodriguez, C.H., Nastro, N., Vay, C., Ramirez, M.S., Almuzara, M. (2017) First case of bacteraemia due to *Acinetobacter schidleri* harboring blaNDM-1 in an immunocompromised patient. *New Microbe and New Infection* **21**:28-30

30. Mukamolova, G.V., Turapov, O.A., Kazarian, K., Telkov, M., Kaprelyants, A.S., Kell, D.B., Young, M. (2002) The rpf gene of *Micrococcus luteus* encodes an essential secreted growth factor. *Molecular Microbiology* **46**(3):611-621

31. Mukamolova, G.V., Yanopolskaya, N.D., Votyakova, T.V., Popov, V.I., Kaprelyants, A.S., Kell, D.B. (1995) Biochemical changes accompanying the long-term starvation of *Micrococcus luteus* cells in spent growth medium. **Archives of Microbiology 163**:373-379

32. Parasion, S., Kwiatek, M., Gryko, R., Mizak, L., Malm, A. (2014) Bacteriophages as an Alternative Strategy for Fighting Biofilm Development. *Polish Journal of Microbiology* **63**(2):137-145

33. Peces, R., Gago, E., Tejada, F., Laures, A.S., Alvarez-Grande, J. (1997) Relapsing bacteraemia due to *Micrococcus luteus* in a haemodialysis patient with a Perm-Cath catheter. *Nephrology Dialysis Transplantation* **12**:2428-2429

34. Peng, F., Mi, Z., Huang, Y., Yuan, X., Niu, W., Wang, Y., Hua, Y., Fan, H., Bai, C., Tong, Y. (2014). Characterization, sequencing and comparative genomic analysis of vB_AbaM-IME-AB2, a novel lytic bacteriophage that infects multidrug-resistant *Acinetobacter baumannii* clinical isolates. *BioMed Central Microbiology* **181**(14):1-14

35. Sato, Y., Unno, Y., Ubagai, T., Ono, Y. (2018). Sub-minimum inhibitory concentrations of colistin and polymyxin B promote *Acinetobacter baumannii* biofilm formation. *PLOS one*. **March 19, 2018**: 1-18

36. Seifert, H., Kaltheuner, M., Perdreau-Remington, F. (1995) *Micrococcus luteus* Endocarditis: Case Report and Review of the Literature. *Zentralblatt für Bakteriologie* **282**:431-435

37. Shin, H., Lee, J.H., Kim, H., Choi, Y., Heu, S., Ryu, S. (2012) Receptor Diversity and Host Interaction of Bacteriophages Infecting *Salmonella enterica* Serovar Typhimurium. *PLOS ONE* **7**(8):1-11

38. Smith, K.J., Neafie, R., Yeager, J., Skelton, H.G. (1999) *Micrococcus* folliculitis in HIV-1 disease. *British Journal of Dermatology* **141**:558-561

39. Tiwari, V., Patel, V., Tiwari, M. (2018) In-silico screening and experimental validation reveal L-Adrenaline as anti-biofilm molecule against biofilm-associated protein (Bap) producing *Acinetobacter baumannii*. *International Journal of Biological Macromolecules* **107**:1242-1252

40. Warner, C.M., Barker, N., Lee, S.W., Perkins, E.J. (2014) M13 bacteriophage production for large-scale applications. *Bioprocess Biosystems Engineering* **37**(10):2067-2072

41. Yele, AB., Thawal, ND., Sahu, PK., Chopade, BA. (2012) Novel lytic bacteriophage AB7-IBB1 of *Acinetobacter baumannii*: isolation, characterization and its effect on biofilm. *Archives Virology*. **157**:1441-1450