Phage Therapy: A Solution to Antibiotic Resistance?

A project submitted

by

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Certification Statement

This is to certify that the project titled “Phage therapy in combating multi-drug resistant bacteria” submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy from the Department of Pharmacy, BRAC University constitutes my own work under the supervision of Professor Eva Rahman Kabir, Ph.D, Chairperson, Department of Pharmacy, BRAC University and M. Zulfiquer Hossain, Ph.D, Associate Professor, Department of Pharmacy, BRAC University. Throughout the project I have given appropriate credit where I have used the language, ideas or writings of another.

Signed

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Counter signed by the supervisor

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Dedicated to my sister

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I would like the express how grateful I am to the Almighty for keeping me in good health and allowing me to purse my ambitions through the knowledge I have acquired in life.

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Abstract

Antibiotic resistance has increased immensely within the last few decades rendering many widely used medicines obsolete. This has led to interest in alternative methods of treatment for infectious diseases. Bacteriophage therapy can provide a well needed solution to this problem. Phages are viruses which infect bacteria and therefore express bacteriocidal properties. They are highly selective of the bacteria they infect and as a result do not harm host cells. Since the discovery of phages in 1915 there has been an increase in the amount of research carried out to develop a therapeutic use. Phage life cycles are better understood now and their ability to adapt to changes in their host bacterial characteristics, reduces chances of phage resistance. Phage therapy is being developed by various companies for the past few decades, with most products pending clinical trials or market approval. The manufacture of phages include steps beginning from genomic identification of phages, isolation and purification and finally to formulation development. Manufacture of phages is different from manufacture of other therapeutic products as it is a living entity and needs very precise conditions for production. Presently there are treatment options which have shown effectivity when administered as a solid dosage form, topically and nasally. Due to the nature of phage therapy it has the potential to cure diseases in a wide range of patients suffering from various ailments which prevents use of antibiotics to cure diseases and may be the solution to the present global crisis of antibiotic resistance.

Contents

List of acronyms

ICTV: International Committee on Taxonomy of Viruses

DNA: Deoxyribonucleic acid

WHO: World Health Organization

ROS: Reactive oxygen species

TLRs: Toll-like receptors

KCs: Kupffer cells

TNF: Tumor necrosis factor

IL: Interleukin

RNA: Ribonucleic acid

IBD: Inflammatory bowel syndrome

GST: Glutathione S-transferase

LPS: Lipopolysaccharides

CDC: Centre for Disease Control and Prevention

AIDS: Acquired immunodeficiency syndrome

NGC: Next-generation sequencing

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1. Introduction

Growing resistance of an increasing number of bacterial species to antibiotics have forced scientists to look into new methods of treating bacterial diseases (Merril et al., 1996). Bacteriophages were discovered, at different occasions, by two different scientists: Frederick Twort, a British pathologist, in 1915 and again by Félix d'Hérelle, a Canadian microbiologist, in 1917. After this discovery the interest in the field of phages have grown. Due to the bacteriocidal properties of phages it has become more evident that it will be useful in therapy to cure diseases.

1.1 Bacteriophage

The term *bacteriophage* was given by D'Hérelle which means "bacteria eater" do define the agent's bacteriocidal properties. Most phages have a size ranging from 24-200nm. Phages are simple organisms which contain genetic material surrounded by a protein capsid. The genetic material is composed of nucleic acids. The nucleic acid can be present as single or double stranded DNA or RNA. Phages have three basic structural forms, an icosahedral (20-sided) head with a tail, an icosahedral head without a tail or a filamentous form (Britannica, 2017). Bacteriophages are viruses which infect bacteria, each phage is suited to infecting only a certain type of bacteria, making them highly selective. Bacteriophages have been used to successfully treat infections associated with bacteria a decade before the discovery of penicillin, however these successes were limited after their initial hype due to incomplete understanding of phage biology leading to clinical failures (Hanlon, 2007). The main reasons for discarding phages as an effective treatment option are due to the inability to recognize the narrow host range of phages, failure to efficiently purify phages to remove impurities and underestimation of the ability to remove phage particles from the body by the reticuloendothelial (Appendix A1) system (Merril et al., 1996).

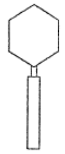
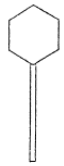
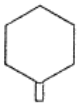
The main problem of antibiotic resistance is associated with changes in several genetic and fundamental structures of the bacteria in order to survive. Phages have the ability to modify themselves whenever the bacteria do so which reduces the chances of phage resistance, unlike antibodies which are not self-modifying entities. This leads to lower chances of human hosts being susceptible to infection in the context of phage therapy. The purpose of this paper is to

understand how phage therapy is used and its success so far in treating bacterial diseases. The findings will then be used to evaluate the application of this therapy practically.

1.2 Taxonomy of Bacteriophages

Taxonomy (Appendix A2) of viruses give information regarding bacteriophage families and species. Table 1.1 shows how bacteriophages can be classified into 10 different types depending on some of their properties. The table provides information on the order, family, number of species, properties and generalized shape of different bacteriophages (ICTV, 2016).

Table 1.1: Taxonomy of bacteriophages.

Order	**Family**	**Properties**	**Number of species in the family**	**Generalized shape**
Caudovirales	Myoviridae	Contractile tail	276	
Caudovirales	Siphoviridae	Noncontractile long tail	553	
Caudovirales	Podoviridae	Short tail	132	
Unassigned	Microviridae	ssDNA (C), 27nm, 12 knob like capsomes	21	
Unassigned	Corticoviridae	dsDNA (C), complex capsid, lipids, 63nm	1	

Unassigned	Tectiviridae	dsDNA (L), inner lipid vesicle, pseudo-tail, 60nm	5	
Unassigned	Leviviridae	ssRNA (L), 23nm, like poliovirus	4	
Unassigned	Cystoviridae	dsRNA (L), segmented, lipidic envelope, 70-80nm	1	
Unassigned	Inoviridae	ssDNA (C), filaments or rods, 85-1950 x 7nm	31	
Bunyavirales	Plasmaviridae	dsDNA (C), lipidic envelope, no capsid, 80nm	1	

1.3 Antibiotic resistance

Antibiotics are the most commonly prescribed drugs in countries, both developed and developing, around the world (Gebeyehu, Bantie, & Azage, 2015). The discovery of antibiotics has allowed a cure for many untreatable and life threatening diseases. However with every significant infectious disease caused by bacteria that emerges, there are strains which are resistant to all types of antibiotics (Cirz et al., 2005). Evidence suggests that when faced with agents (antibiotics) that cause DNA damage the bacteria respond by induction of proteins that promote mutations in their genome, resulting in formation of genes resistant to antibiotics (Cirz et al., 2005).

Resistance is observed when there is a modification of bacterial genes leading to significant changes in protein production and therefore behavior of bacteria towards antibiotics. Lateral gene transfer, also known as horizontal gene transfer, is a clinically significant route through which resistant genes are acquired by bacteria (Cirz et al., 2005). Lateral gene transfer is the transmission of genes between individual cells generating not only new gene assortments but also help move genes throughout populations and from species to species.

Antibiotic resistance genes are spread due to reasons which include overuse and misuse of antibiotics in medical, veterinary and agriculture settings (Tao et al., 2016). According to a WHO report, 80% of all antibiotics are used in the community of which around 20-50% are used inappropriately (WHO, 2007). Misuse and overuse refers to use of antibiotics when it is not necessary. A common example of this practice in a healthcare setting is justifying the use of antibiotics only through observed symptoms of a patient. Symptoms of an illness do not provide sufficient information about the type of bacteria which has caused the infection and it is necessary to perform a bacterial culture test to successfully identify the causative pathogen. Self-diagnosis of an illness is also a contributing factor of antibiotic overuse. Antibiotic resistance is widespread in the last two decades due to the inadequate intake of the prescribed antibiotic dose. After a patient receives an antibiotic prescription they usually take the medication for the first few days of the illness and stop as soon as they see the symptoms of their illness diminishing instead of finishing the whole prescribed dose. This practice allows the bacteria which are still present inside the body to replicate and acquire immunity to the low concentrations of antibiotics. Moreover an extension of this practice is observed when patients store a part of their medication course in reserve for a future occasion either for themselves or others (Gebeyehu et al., 2015).

1.4 Examples of antibiotic resistance

Some of the most common and extensive antibiotic resistance cases include, but are not limited to:

- methicillin resistance in *S. aureus*
- vancomycin resistance in enterococci
- extended-spectrum beta-lactamase production by gram-negative bacilli and

- penicillin resistance in *Streptococcus pneumoniae* (Lindgren, Karlsson, & Hughes, 2003).

Ciprofloxacin, a member of the quinolone family, functions by interfering with rejoining of DNA ends in bacteria causing premature DNA hydrolysis by the bacteria, disrupting normal cellular function. Resistance is observed in *Escherichia coli* which possess mutation in genes that encode the topoisomerases (Appendix A3) allowing rejoining of the DNA strands or genes that affect either cell permeability or drug export. (Cirz et al., 2005).

B. pseudomallei is found to be resistant to many first and second generation antibiotics such as:

- cephalosporins
- penicillins
- macrolides
- colistin
- rifamycins and
- aminoglycosides (Guang-Han et al., 2016).

A viable and necessary candidate for phage therapy is *P. aeruginosa* due of its high levels of antibiotic resistance (Hall, Vos, Friman, Pirnay, & Buckling, 2012). *P. aeruginosa* colonizes and infects human hosts due to its ability to easily adapt to its environment causing serious infections the most common of which is pneumonia.

P. aeruginosa currently shows resistance to several antibiotics that include:

- penicillin G
- aminopenicillin
- penicillin G combined with beta-lactamase inhibitors
- aminopenicillin combined with beta-lactamase inhibitors
- first and second generation cephalosporins
- piperacillin
- tazobactam
- cefepime
- ceftazidime
- aminoglycosides

- quinolones
- carbapenems
- colistin and
- fosfomycin (Yayan, Ghebremedhin, & Rasche, 2015).

1.5 Body's immune response

The immune system of the body is very particular about the substances allowed to circulate freely though the body. Any foreign substance triggers an almost immediate response to neutralize or remove the 'threat'. Viruses are potent activators of the signal pathways leading to increased cytokine or reactive oxygen species (ROS) production. The effects are due to the presence of viral proteins on the surface of the virus as well as endotoxins. For practical implementation of bacteriophages in treatment this must be assessed to evaluate the fate of the phage once inside the body.

Viral proteins and exdotixins are antigens which trigger an immune response. The response is initiated due to the presence of Toll-like receptors (TLRs) expressed in sentient cells such as macrophages and dendritic cells that make up the first line of defense for the human body. Pro-inflammatory signals are activated when TLRs bind to antigens which in turn leads to Kupffer cells (KCs) in the liver being activated. KCs produce various cytokines, essentially tumor necrosis factor (TNF)-α, interluekin-1β (IL-1β), interluekin-6 (IL-6) and interluekin-8 (IL-8) (Friedman, 2000). Cytokines are important in cell signaling and have an impact on the behavior of the cells they come into contact with. Therefore TLRs play an important role in recognizing invading pathogens through recognition of pathogen specific molecular patterns (Kawai & Akira, 2010). In theory, administration of a large quantity of phages could lead to stimulation of the body's immune system and cause anaphylaxis (Appendix A4) so its concentrations need to be carefully evaluated and studied before administration. However till date no reports of anaphylaxis have been documented and so the risk can be considered minimal (Doss, Culbertson, Hahn, Camacho, & Barekzi, 2017).

2. Overview of bacteriophage therapy

Bacteriophage, or in short phage, therapy utilizes the principle that bacteria are susceptible to infection themselves by viruses. Viruses that infects bacteria are known as bacteriophages. Viruses are not considered to be alive universally as they require a live host in order to replicate by utilizing the host's molecular composition. The presence of a host cell is vital for viral life cycle stages (Gross, 2006). As with any infection, phages attack the host bacterial cells and interfere with bacterial metabolism leading to death of the bacteria by cell lysis (Guang-Han et al., 2016).

2.1 Highlights in the development of phage therapy in the 20th century

Since the discovery of phages, more and more research has been done to characterize and understand how phages function. The dates in Table 2.1 marks some of the key aspects in the history of phage therapy in the 20th century. The events which have taken place during this time have allowed phage therapy to evolve and reach its present state.

Table 2.1: Highlights in the development of phage therapy in the 20th century (R.Merril, Scholl, & Adhya, 2003)

Dates	Events
1915	Discovery of bacteriophage.
1925	Sinclair Lewis' novel "Arrowsmith" published. A novel about a doctor who discovers a phage that destroys bacteria.
1926	d'Herrelle uses phage therapy to treat avian typhosis in chicken, Shigella dysentery in rabbits and bacillary dysentery in humans.
1930s	• United States pharmaceutical companies market phage products: Eli Lilly market Staphylojel, E. R. Squibb & Sons phage for Staphylococcus and Abbott Laboratories combined phage for Staphylococcus and colon bacillus. • d'Herrele establishes Tbilisi Phage Institute.
1932	Morison reports successful phage therapy for cholera epidemic.
1933	Study on problems of commercial phage products.
1934	Council on Pharmacy and Chemistry of the American Medical Association

	concluded that phage therapy had value in treatment of diseases.
1937	Asheshov conducted experiments to determine if phages could affect experimental infections.
1940s	Antibiotics overshadow phage therapy.
1943	Dubos rescues mice infected intracerebrally with *Shigella dysenteriae.*
1963	Stent presented theoretical reasons for failure of phage therapy in Molecular Biology of Bacterial Viruses.
1969	Inchley showed that the reticulo–endothelial system (mainly liver) removes intravenously injected phage.
1971	Ochs and colleagues begin long series of studies using phage to probe human immune system
1973	Geier shows oral phage ineffective for systemic distribution
1980s	Smith and Huggins perform several phage therapy experiments, including one that shows phage can be more effective than antibiotics.
1981	Institute of Immunology and Experimental Therapy in Wrocow began treated humans with phage therapy. Over the next 20 years, over 1,300 patients were treated.
1990s	• Biotech industry begins exploring phage therapy in Western countries. • Pharmacokinetics of phage therapy studied.
1996	• Lederberg comments encourage phage therapy re-examination. • Merril and colleagues develop therapeutically more effective long-circulating phage.

2.2 Highlights in the development of phage therapy in the 21st century

As more information is learned by studying phages, the interest in phage therapy is growing. The dates in Table 2.2 marks some important events which have transpired during the 21st century and they show that progress is being made in this field of healthcare.

Table 2.2: Highlights in the development of phage therapy in the 21st century (Salmond & Fineran, 2015)

Dates	Events
2002	The diversity of phages becomes apparent (ecology and viromics)
2003	First synthetic genome synthesized (ΦX174)
2007	Adaptive phage immunity of CRISPR–Cas demonstrated
2012	Cas9 RNA-guided nuclease used for genome editing
2013	Phase I/II clinical trial initiated for PhagoBurn (phage therapy)

2.3 Phage life cycle

Phages display steps in their life cycles which are common to all viruses: adsorption, separation of nucleic acids from protein coat, expression and replication of the nucleic acids, virion (Appendix A5) assembly and finally release and transmission (Weinbauer, 2004). Figure 2.1 gives a visual representation of the phage life cycle.

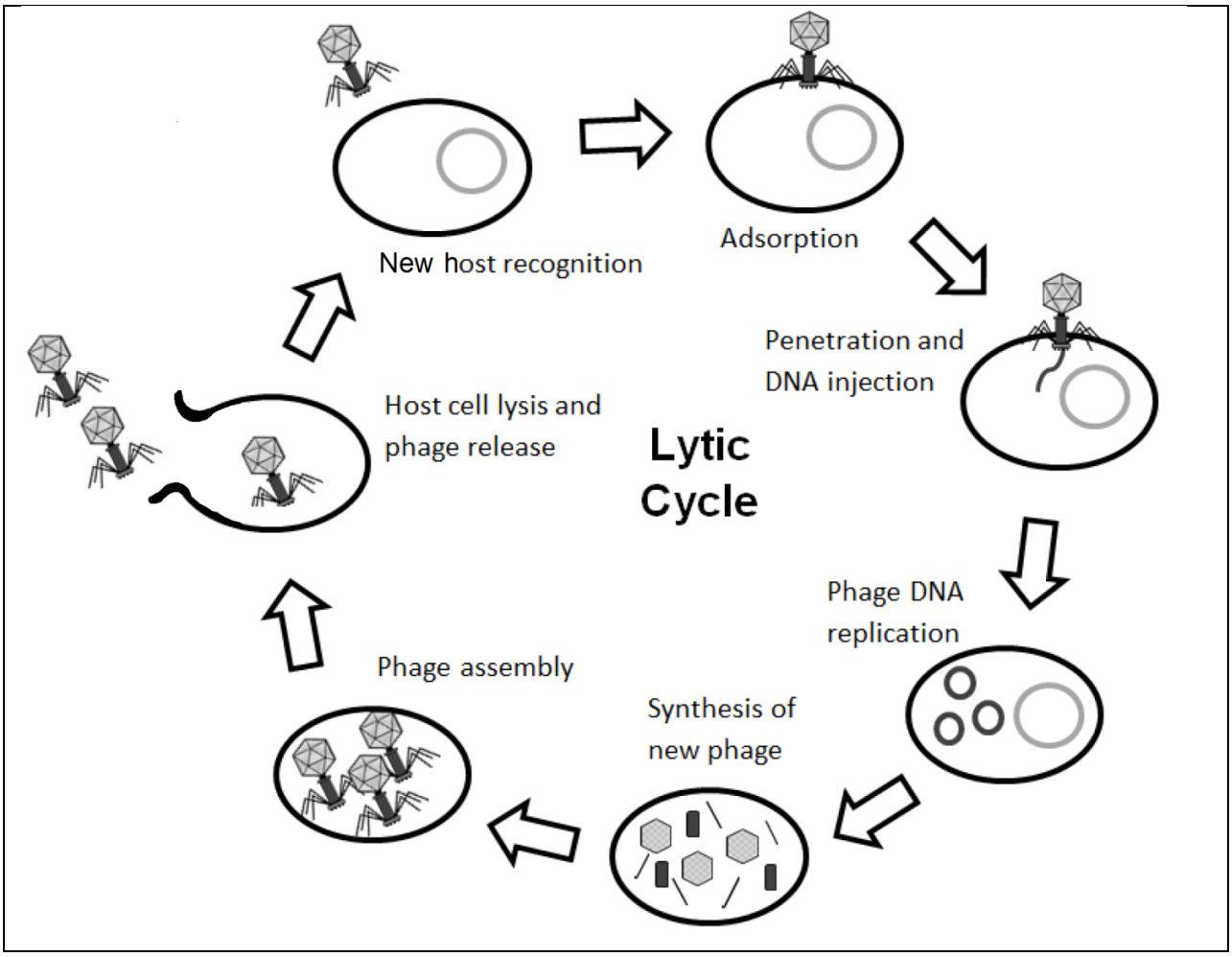


Figure 2.1: Phage life cycle

Host cell recognition is the initial step in phage infection. Adsorption refers to the adhesion to the surface of an object, in this case attachment of the phage to the bacteria. Adsorption is a reversible step as there may be a possibility that the phage will not infect the bacteria. This is the step where the specificity of the phage is expressed as it will only bind to its designated bacterial host. After the bacterial host is identified to be ideal, by its surface structures, the phage will form an irreversible bond with the bacterial host. The viral genome composed of nucleic acids becomes separated from the protein coat followed by the formation of a hole in the bacterial cell surface, the hole is made with the help of phage enzymes. The viral gene is then inserted into the host via the hole, this step is referred to as insertion. The outer casing of the phage, known as the capsid, remains on the surface of the bacteria (Weinbauer, 2004).

Following viral gene insertion, the phage gene is translated by the bacterial ribosomes to form phage proteins. The formation of phage proteins and their assembly to make new phages represent the reproductive stage of the phage life cycle. The phages rapidly reproduce at this stage by utilizing host resources and processes to produce virions or new phages. These are essentially viral particles that include a viral genome enclosed by a capsid protein casing (Gross, 2006). The transcription process for the formation of virions utilizes the host bacterial RNA polymerase enzyme. The viral genome is divided into transcription units which correspond to the early, middle and late genes based on their timing of expression during the phage life cycle (Swapna, Kumari, & Nagaraja, 2015).

The phages have genetic material which encode for viral proteins such as endolysin and holin which lyse bacteria host cells from within. Holin is a small protein which functions by accumulating inside the host cell and allows the endolysin to carry out its function. The endolysin degrades the peptidoglycan, a structural component of the bacterial wall, reducing the structural integrity of the bacterial cell and therefore allows the release of the phage virions (Doss et al., 2017). Virions are released when burst size is reached, which is the term used to describe the number of particles released in one infection cycle (Paepe & Taddei, 2006). The release is consistent with bacterial cell rupture or lysis (Gross, 2006). Released viroids can perform the next stage in the life cycle by infecting other surrounding bacterial cells leading to widespread bacterial lysis eventually eliminating the bacterial pathogen from the system of the human host.

The multiplication rate of phages can be assessed by the help of two parameters: the burst size and the latency period (the time between infection and lysis of the host) (Paepe & Taddei, 2006). Viruses also possess certain life history traits: multiplication rate in a host, survival outside the host, and mode of transmission (Gross, 2006). Therefore when studying the viral life cycle, the aforementioned traits need to be studied. Determining the host range of the phage is important when selecting a viable phage for therapy (Guang-Han et al., 2016). A large host range would signify that a specific phage can act upon a number of different bacteria, allowing the approach to not become limited to one specific bacteria.

Some of the most prominent characteristics of the phages which make them a suitable choice over antibiotics include: their high specificity, ability to replicate at the site of infection, ability

to reach areas in the body with poor blood circulation, reliability to not cause any microbial imbalance and absence of any serious side effects (Guang-Han et al., 2016). All this is achieved without any disruption of the human body's natural microflora (Appendix A6) (Semler, Goudie, Finlay, & Dennisa, 2014) reducing or eliminating any chances of dysbiosis. Phage therapy is extremely specific and so phages do not attack eukaryotic calls, making it ideal for use in humans (Doss et al., 2017).

2.4 Existence of phages within the human body

Bacteriophages can be found in every place that hosts bacteria. Phages are dependent on bacteria as they serve as being bacterial parasites. It is estimated that for every bacterial cell there are about 10 phages, making phages the most abundant living entity on the planet (Santos, Carvalho, Azeredo, & Ferreira, 2014). Infection, for the purpose of phage therapy, does not only depends on the immediate bacterial host of infection, but also on the human host. In humans the microbiome is biogeographically structured across the four major habitats: theoral cavity, the gastrointestinal tract, the vagina, and the skin. Each body site is distinctive and therefore the interactions between the phages and the bacteria are also unique.

It has recently been found that based on metagenomic analyses (Appendix A7) and epifluorescence microscopy (Appendix A8), microecology of bacteria is affected by phages. In fact, a large proportion of the population of microbiome in various habitats inside the body are virus-like particles. Occasionally there is an imbalance in the natural numbers of the microbiome, defined as dysbiosis (Appendix A9), leading to various diseases. One such disease associated with dysbiosis is periodontal disease. Phage therapy can provide a way of treating such a condition (Wahida, Ritter, & Horz, 2016).

Other disorders linked with unbalanced microbiome populations include, gastrointestinal diseases, obesity, non-alcoholic fatty liver disease, cancer, brain development, and psychiatric disorders. Emerging evidence suggests that phages stably attach to mucosal walls and provide protection from bacterial attack. One prominent example in which phage activity and shifts in bacterial populations have been described to cause significant changes is inflammatory bowel disease (IBD) (Wahida et al., 2016). IBD is a term mainly used to describe two conditions that involve inflammation of the gut (gastrointestinal tract) and the colon (large intestine).The main

symptoms of IBD include; pain, swelling or cramping in the abdomen, recurring or bloody diarrhea, weight loss and extreme tiredness.

Phages are salient in controlling bacterial communities and also direct the complex relationship between humans and their bacterial companions. Metagenome research will allow a better understanding of how phages influence the changes in bacterial genomes making them either more or less virulent. Even though this therapy has been seen to be useful in some European countries for decades, there is widespread skepticism regarding phage therapy because bacteriophages are generally associated with diseases. Concerns about safety and efficacy must be thoroughly addressed by rigorous scientific and clinical studies.

2.5 Phage adaptability

Phages have been shown to adapt to the changes of bacteria, where populations of resistant bacteria can potentially be neutralized by phages. This is a perfect example of coevolution showing how high infectivity of phages corresponds to greater resistance in bacteria and vice versa (Hall et al., 2012). In order to survive the continuous onslaught of phages, bacteria tend to develop survival characteristics which would enable them to fend off the attack. This behavior is present in all types of organisms, that when threatened, organisms tend to build better defense mechanisms to ensure their survival. These adaptive or evolutionary changes in the bacteria therefore tend to promote counteractive changes in the infecting phages. This leads to coevolution of the organisms, where both organisms evolve that is dependent solely upon each other. The easiest and most obvious way a bacteria can defend itself is by inhibiting the phage from attaching to its surface. Changes in bacterial cell structure could inhibit the ability of the phage to bind to the bacteria. The phage therefore needs to modify its attachment structures in order to bind to the bacterial host. The changes to both of these organisms would be initiated from a change in their genetic sequences which code for the cell structure in the case of the bacteria and the attachment structure in case of the phage.

Another possible way is the production of certain enzymes by the bacteria which would degrade phage genetic materials so they are not translated. Phages could respond to this with protecting elements which would prevent degradation by the bacteria. The ability of the phage to respond to

the changes of the bacteria allows a wider stability in treatment options. In most cases pathogenic bacteria can adapt significantly after the initial onset of a disease. In case of phage therapy, the phages do not need to be changed every time the bacterial structure is modified. The time taken to diagnose any changes in bacterial structures or mechanisms would be too large since by that time more of the bacteria could change in the infected host. Phage therapy provides a live approach in the treatment of bacterial diseases as it can modify itself to meet any changes in the target bacteria.

2.6 Emergence of resistant variants: a challenge in phage therapy

Phage resistance in bacteria have been seen to arise. Physical properties which can induce the onset of this resistance include environmental conditions and phage concentrations. There are a few ways this resistance can be brought about. In a study, mutations which appear to be due to heritable or transmissible genes in the bacteria has been confirmed by the continual growth of the bacteria even when phage concentrations are high. Resistant bacteria have not been provided with any other special conditions compared to the phage susceptible strains. Although the number of resistant bacteria had been negligible at the beginning of the experiment, it was found to become more prominent as the susceptible strains slowly died out (Cairns, Timms, Jansen, Connerton, & Payne, 2009). Like any other group of interrelating organisms, bacteria and phages demonstrate changes in their population dynamics dependent upon each other (Santos et al., 2014). There is an evolutionary pressure on hosts by infectious parasites to adapt in order to survive infection, expressed either genetically, phenotypically (Appendix A10) or both (Wielgoss, Bergmiller, Bischofberger, & Hall, 2015).

Lotka-Volterra equations can be used to explain the population relationship between a bacteria and a bacteriophage (Santos et al., 2014). The model starts with a population of bacteria (prey) and phage (predator). Initially a food source is provided to the bacteria to allow growth and increase in population. The food source of the phage therefore depends entirely on the population of the bacteria and it is accurately assumed that the phages do not stop infecting the bacteria. Subsequently increases in phage populations are observed as replication occurs extensively. Increase in phage population leads to reduction in bacterial populations. In case of this particular relationship, phage populations do not die out as a result of decline in bacterial populations.

Existing bacterial presence is usually associated with resistance which is most probably acquired by the ability of the bacteria to adapt to phage infection in order to survive.

A concern with phage therapy was addressed which provided information that phages could assist in transferring of DNA from one bacterium to another. The result of this form of transfer could lead to the transfer of several unwanted genes which may cause increased pathogenicity, virulence and resistance. Therefore phages which do not possess the ability to package additional host DNA or require host DNA to synthesize its own should be used, the success of the method has been shown to work very well in phage therapy (Wittebole, Roock, & Opal, 2014). To combat the emergence of resistant bacteria due to phages it is necessary to select phages which do not exhibit a lysogenic state in their replication process and only replicate using a lytic state. The lytic state corresponds to the lysis of the bacterial cell only whereas the lysogenic state allows viral genome integration into the bacterial cell to be expressed in certain distinct events many bacterial generations later. The lysogenic state is observed as an extended part of the lytic state of a bacteriophage as shown in Figure 2.2. The lysogenic cycle starts after the insertion of viral genome into the host bacteria. The inserted genetic sequence is incorporated into the host genome and upon host replication is copied into the genome of the offspring as the parent genetic sequence. The genome is copied numerous time from generation to generation without any changes to the host cell functions, only when there are certain environmental triggers the phage genome is expressed to carry out the rest of the life cycle of the phage.

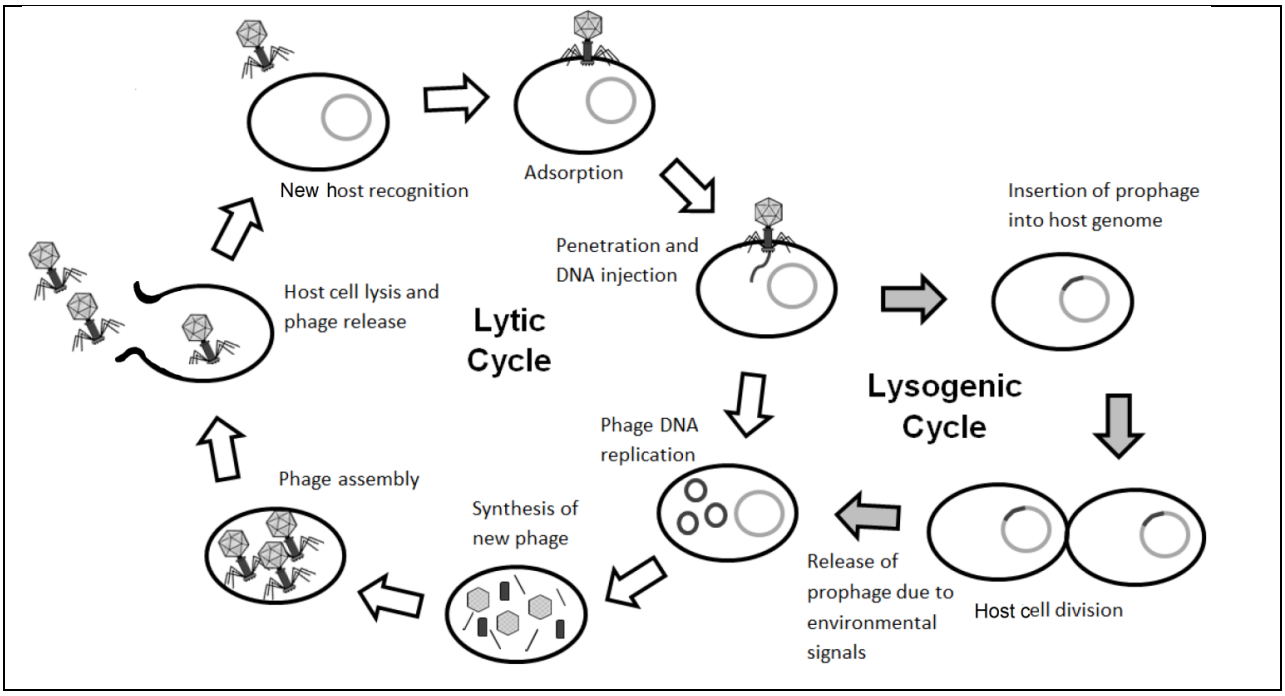


Figure 2.2: The lysogenic cycle

3. Bacteriophage selection, isolation, purification and manufacture

Phage isolation needs to be performed before it can be used in therapy. Unlike bacteria, which can be grown in cultures in the presence of suitable media, phages are cultured in the presence of a living host. Therefore when dealing with bacteriophages it is necessary to first culture bacteria which will serve as the host to the phages. Bacterial cultures are usually composed of broth or agar mediums (Jensen et al., 2015). A viral plaque is a visible structure formed due the infection of cell cultures by viruses. In case of the virus being a bacteriophage the plaque will be formed on bacterial culture plates. The plaques are zones which appear to be clear of bacteria as the bacterial cells have been killed by the phages. Populations of viruses can be quantified by the help of plaques using colony counters and in order to give more precise numbers staining techniques can be used. Usually the appearance of the plaques can give information regarding the degree of infection. Highly virulent or lytic strains give clear plaques whereas turbid plaques are observed when only a fraction of the hosts have been killed which the living hosts being fully or partially resistant.

The need for purification is for the removal of impurities. For this therapy the impurities include bacterial proteins, DNA, lipopolysaccharides, peptidoglycans, etc. all of which are present in bacterial cells. Prophages are another very common and unwelcome impurity in bacteriophage cultures (Ceglarek et al., 2013). Prophages are viral genes which are present inside bacteria which have been infected, possessing a unique property to replicate and produce phages when activated. Prophages display individual specificity, immunogenicity as well as other biological properties and are composed of many macromolecules (Ceglarek et al., 2013). Purification of phages are performed by separation of lysates from the freshly obtained plaque. Incubation of the plaque containing the phages is performed to allow complete lysis of bacterial cells followed by removal of lysates with the help of a filter usually having a pore size of 0.45 μm (Kot, Kilstrup, Vogensen, & Hammer, 2016).

Purification is usually performed utilizing size-exclusion chromatography or chromatofocusing (Appendix A11) processes. To obtain the best results with greatest success and promise the approach of monolithic anion-exchange chromatography is used. Bacteriophage surfaces are contoured with affinity tags (Appendix A12) such as glutathione S-transferase (GST) and His-tag allowing binding to affinity resins (Ceglarek et al., 2013). In order to state with confidence the

identity of the phage it is necessary to perform a Phage Genomic DNA sequencing procedure. The DNA can be extracted using a Qiagen Lambda KIt using phenol/chloroform then precipitated with ethanol (Hsu, Lin, Pan, Hsieh, & Wang, 2013).

3.1 Selection of suitable phages for therapy

Not all phages will be ideal for phage therapy. At present there are two reasons which weigh heavily on the selection of phages for therapy: the large natural variation of phages and target bacteria, and the regulatory framework for clinical application of phages (Mirzaei & Nilsson, 2015). Development of new phage therapy products do not cost any more than the amount needed for new antibiotic development, which has an estimated cost of about US$ 10–50 million (Brussow, 2012).

There are certain criteria which have to be met in order for phages to be viable for use in therapy. Initially before administration, like any other form of medication, phages should exhibit good pharmacokinetics (Appendix A13) and pharmacodynamics (Appendix A14). Pharmacokinetics refers to the movement of drugs within the body while pharmacodynamics refers to the effects of a drug and its mechanisms of action. In phage therapy the phage is the drug and so its properties inside the patient needs to be ideal. Good pharmacokinetics is the ability of the phage to reach and attack the bacteriophage at the site of infection. Good primary pharmacodynamics would represent the extent of the phage's antibacterial virulence, and good secondary pharmacodynamics would be the low potential for phages to do harm to the patient's body. An ideal phage to be used in therapy should be easily absorbed into and distributed throughout the body. It must reach the target site in a relatively small time interval and be able to infect bacterial cells. Replication of the phages inside bacterial cells should also be rapid in order to increase its number in a given time. After elimination of pathogenic bacteria from the body, the phage need to be either metabolized from the body, excreted or both without any changes in body physiology. In a study it was estimated that less than 1% of orally administrated phages for therapy have been recovered from the stool of patients (Sarker et al., 2012).

3.2 Endotoxin removal

Initiation of immune response is not the only concern associated with phage therapy. Another important assessment is necessary which will demonstrate the safety of the therapy and that is

endotoxin removal. Endotoxins are not constituents of phages themselves but are present in phage isolates. After the phage purification process, there are remnants of the bacterial host culture still present. These serve as impurities in the phage isolate and contain endotoxins mostly originating from the cells of gram-negative bacteria, specifically they are lipopolysaccharides found on the surface of gram-negative bacteria (Raetz, 1990).

Various techniques are utilized for endotoxin removal. Ultra filtration is a common technique for purification of biological macromolecules. Affinity techniques can also be used to effectively remove lipopolysaccharides (LPS) using immobilized ligands such as polymyxin B, L-histidine, poly-L-lysine, poly(ethyleneimine), PEGylated polypeptides and L-serine immobilized on polyvinylidene fiber. Ionic and ion-dipole interactions with a high negative charge can also be exploited for purification purposes. Organic solvents such as 1-butanol and 1-octanol can be used to remove endotoxin efficiently from aqueous bacteriophage lysates while still being able to retain the infectivity of the bacteriophages. This method is efficient, scalable and cost-effective and therefore a significant method of endotoxin removal (Szermer-Olearnik & Boratyński, 2015). Exdotoxins can also be removed using a commercially available technique that utilizes the principle of affinity chromatography. EndoTrapH Blue contains a high endotoxin affinity ligand with affinity matrix derived from bacteriophages and is proteinaceous. The affinity ligands are immobilized in agarose beads and so do not leak into the extract prominently. (Merabishvili et al., 2009) Any form of phage related study needs highly purified particles. Endotoxins possess immunogenicity which allows for cell-mediated immune response generation. In another study, seven different endotoxin removal strategies which were composed of Endotrap HD column purification and/or CsCl density centrifugation in combination with Endotrap purification, followed by organic solvent (1-octanol), detergent (Triton X-100), enzymatic inactivation of the endotoxin using alkaline phosphatase and CIM monolytic anion exchange chromatography were compared. Caesium Chloride density purification, one of such strategies, was found to provide the highest amount of endotoxin removal at 99%. Other methods did not give the same promising results and additional tests lead to loss of the valuable bacteriophage titer (Belleghema, Merabishvilib, Vergauwenc, Lavigned, & Vaneechouttea, 2017).

Vaccine purification can be used as a reference to phage purification. This is due to the similarity between vaccines and phages. Phages and vaccines are both of viral origin making their isolation, culture and purification methods very similar, if not identical. During vaccine purification, liquid chromatography in a three step batch operation has been relied upon to provide a way to remove undesired impurities. This method leads to the binding of the desired molecule to the stationary phase while the impurities flow out through the column along with the mobile phase. The desired molecule which remains bound to the stationary phase is then recovered by the help of different fluids containing suitable conductivity, pH, polarity and chaotropacity (Appendix A15) (Iyer et al., 2012). Chaotropacity refers to the entropic disordering of lipid bilayers and other biomacromolecules which is caused by substances dissolved in water. Liquid chromatography therefore utilizes a large amounts of buffers and is a time consuming process overall. Despite these drawbacks the desired substance can be successfully separated in situations where there is a large amount of impurities present, the process is economical as a smaller amount of stationary phase can be used to trap the molecule of interest instead of the impurities being trapped to a large amount of stationary phase (Iyer et al., 2012). Liquid chromatography can therefore be used in phage therapy to remove residual substances which originate from the bacteria culture medium in which the phages have been grown. In the case of there being lower to moderate amounts of impurities present, another process known as flow-through chromatography is used which binds the impurities to the stationary phase allowing the desired substance to flow through with the mobile phase. The flow-through method is the most suitable for endotoxin removal (Woo et al., 2011).

The generation of endotoxins is most prominent after successful phage therapy. Successful phage therapy refers to the ability of the phage to cause lysis in the bacterial cell leading to bacterial death. Therefore after cell lysis the contents of the bacterial cells are expelled into the surroundings leading to severe inflammatory responses (Nobrega, Costa, Kluskens, & Azeredo, 2015). Among the bacterial components released during cell lysis, endotoxins are the most harmful to the body. If the amount of endotoxins in the body increase the patient may go into septic shock. Septic shock is a condition where the body experiences drastic changes in a short amount of time as a result of infection. It is very dangerous as it can cause severe problems such as sudden blood pressure drops and affected normal functioning of body organs, leading to organ failure. Endotoxins are usually neutralized and removed by the liver and the kidney respectively.

Patients with reduced hepatic and renal function have a higher chance of suffering from septic shock but in most cases it is triggered by a sudden increase in endotoxin levels in the blood before it can be neutralized or removed by the body. As in the case of antibiotic treatment of gram-negative bacteria, patients treated with phage therapy need to be monitored for symptoms of septic shock. Patients receiving phage therapy could take oral administrations of activated charcoal and maintain a healthy diet intake to reduce the incidences of septic shock. Activated charcoal is beneficial in toxin removal as it contains various micropores (Appendix A16) which have large surface areas and can adsorb small toxic particles.

3.3 Manufacture of Phages for therapy

Manufacture of phage therapy products are similar to that of any other biological products. The first step is genomic identification followed by phage matching (isolation and modification), formulation development and finally treatment. After the initial genomic screening of the phages to be used to confirm the identity of the phage, manufacturing steps can be taken.

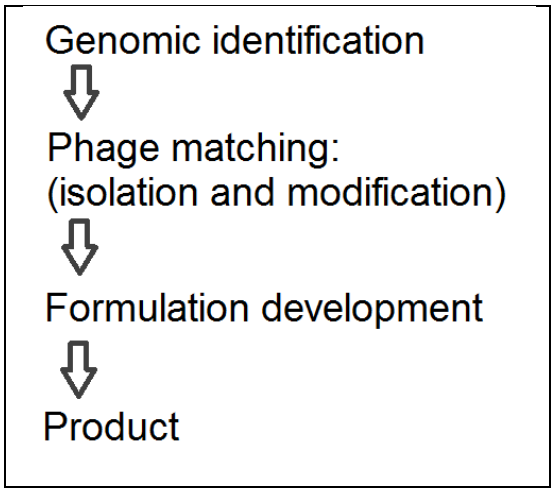


Figure 3.1: Steps in the manufacture of phages for therapy

3.3.1 Genomic identification or sequencing

In order to sequence genomes we must use whole genome sequencing. Whole-genome sequencing is the most comprehensive method for analyzing the genome. Genomic information has been instrumental

in identifying inherited disorders, characterizing the mutations that drive cancer progression and tracking disease outbreaks. Genome sequencing provides information about the identity of an organism, its virulent properties, the genes responsible for the temperate lifestyle of the organism and its genetic stability. This rapidly reduces sequencing costs and the ability to produce large volumes of data making whole-genome sequencing a powerful tool for genomics research. While whole-genome sequencing is commonly associated with sequencing human genomes, it can be modified to meet any organism specific requirements which include all species, such as agriculturally important livestock, plants or disease-related microbes. There are several key whole genome sequencing methods. The type used in case of bacteriophages is small genome sequencing. Small genome sequencing involves sequencing the entire genome of a bacterium, virus or other microbe and then comparing the sequence to a known reference. The process is used for organisms having small genome sequences, usually less than or equal to 5 Mb. Sequencing gives a detailed description of the organism and its characteristics. Small genome sequencing utilizes next-generation sequencing (NGS), allowing DNA libraries for small genomes to be prepared in 2 days without utilizing labor-intensive cloning steps (Illumina, 2017). NGS is a DNA sequencing technology that can be used to sequence entire genomes or constrained to specific areas of interest, including all 22,000 coding genes or small numbers of individual genes. The main reason for using NGC is due to its ability to replace characterization of pathological organisms by morphology, staining and metabolic criteria by genomic definition (Behjati & Tarpey, 2013). The aforementioned traits need to be deduced by utilization of a lot of time and resources while not always providing fruitful results making NGS a very popular method of organism characterization.

3.3.2 Phage matching

Phage matching is essentially isolation of the phages and their modification in order to make them express the necessary characteristics needed in therapy. Phages are grown on bacterial cultures so their grown rates depend on their ability to infect the bacteria and reproduce. The phages are isolated and impurities are removed. Impurities include host cell proteins, host cell DNA, residual reagents, endotoxins, pyrogenic endotoxins, hemolysins, etc. Impurities can be removed using centrifugation and cellulose acetate filters (Brown, Petrovski, Dyson, Seviour, & Tucci, 2016). After purification of the phages they can either be directly incorporated into therapeutic use by dosage formulation or the phages can undergo modifications. Modifications are performed when there are some characteristics of the phages which need to be enhanced or

eliminated. When desired properties are detected after genomic screening, the properties are amplified by genetic modifications. As technologies become more advanced it is possible now to study the mechanisms utilized by phages in providing antibacterial properties. Phage genomes can now be altered to meet the demands associated with therapy. Such demands include use of lytic phages, reduced antigens on the phage surface, insertion of affinity tags on phage surfaces, increased ability to bind with bacterial cells and wider host range. Changes of certain gene sequences in the phage could lead to the phage having only a lytic state and not a lysogenic state. Reducing the number or composition of antigens on the surface of the phages would lead to lower chances of immune response generation by the patient's body towards the phage. Improvements in the structural components which are used in binding to the bacteria would allow stronger and faster attachment to the bacterial host leading to increased rate of bacterial death. A wider host range can be achieved by making changes to the genes expressing bacterial specificity (Nobrega et al., 2015). Therefore allowing minor changes in a single phage to give relief from a number of pathogenic bacteria instead of having to isolate, culture and purify phages from the beginning in order to treat every bacterial pathogen singularly is both cost-effective and time efficient.

3.3.3 Formulation development

During formulation development of phages it is important to assess various parameters to understand which reagents, excipients (Appendix A17) and manufacturing procedures need to be used. Phage formulation is different from that of chemical medicines. The temperature and pH are some of the most important aspects in the manufacturing process which needs to be optimal to prevent any damage to the phages. The formulation depends on the dosage form to be prepared, however a specific set of steps need to be followed in all cases before formulation development as shown in Figure 4. Firstly the vector of the therapy must be designed. This includes the type of phage to be used, productivity levels of the phage, cell cycle and single or cocktail phage use. Manufacturing can then proceed. After which it is necessary to determine therapy delivery and its potency. The route of administration, dosage and frequency as well as purity of the product are included in therapy delivery and potency. During the manufacturing of the phage product the ability of the process to adapt to existing production equipment and techniques are assessed (Biologics, 2017). The scale of manufacture will determine the capacity

of the machinery needed. During manufacture sufficient equipment needs to be present to make sure that the product can be analyzed as required to ensure that quality of the product is maintained uniformly.

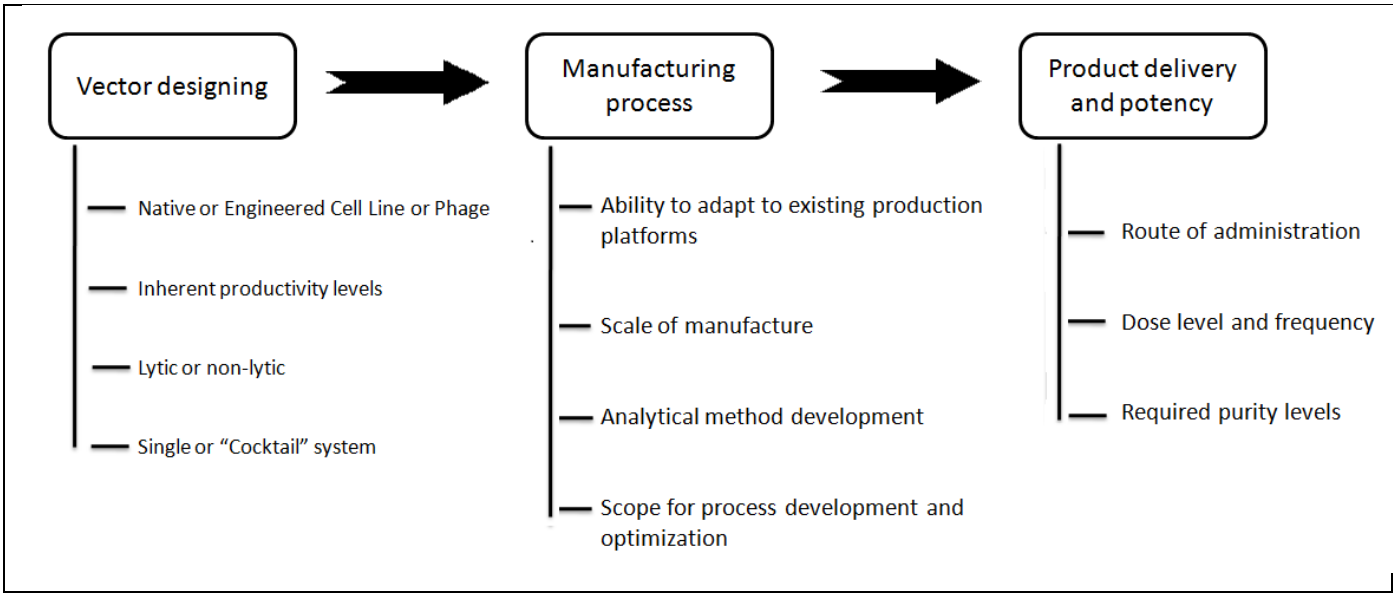


Figure 3.2: Phage formulation development

4. Treatment

Phage therapy can be effectively used against a wide range of infections involving but are not limited to burns, guts and respiratory infections as well as systemic infections. The route of administration of phage therapy and the formulation used is dependent on the site and nature of the infection. Even through products used for phage therapy are sensitive to handle, several companies are investing their efforts towards research and development of phage therapy (Appendix A18).

4.1 Solid dosage administration

Administration of bacteriophages is similar to that of other medications. The route of administration depends on the site of the infection. For systemic use the most acceptable and easy to use method is oral administration. Phages have been formulated for administration in solid dosage. When solid dosage forms of suppositories and troches were used against *R. equi*, bacterial cell lysis was observed. When stored at 4 °C and protected from light, the phages remained stable for 60 days in the suppository formulation and 90 days in the troches (Browna et al., 2016). When given orally phages have a lower effectiveness due to the conditions inside the stomach and intestines. The presence of the low pH of the stomach coupled with the enzymes of the intestines decrease the activity of phages. The problem can be overcome by encapsulation that can be used to protect the phages during oral administration. Alginate/$CaCO_3$ microcapsules having a size ranging from 124–149μm has been used successfully to facilitate oral phage delivery. The method allowed gastrointestinal protection of the phages and also provided a means for sustained release of phages as opposed to an immediate release accompanied with rapid removal. Furthermore the phages showed excellent stability when stored at 4 °C for 6 months and have been observed to remain stable at room temperatures for at least two weeks (Colom et al., 2017). Another method used in encapsulation is the utilization of liposomes (Appendix A19) to enclose the phages (Colom et al., 2015). Both these encapsulation methods have shown therapeutic effects even after administration was stopped, showing that they possess an enhanced ability to provide therapy over time. In case of rectal administration of solid doses the activity problems associated with stomach and intestinal conditions can be bypassed, making this route of administration more viable for use in phage therapy.

4.2 Topical administration

Various experiments have been carried out showing the success of bacteriophage therapy in topical administration. Phage therapy has provided positive results in the treatment of infected venous ulcers (Markoishvili, Tsitlanadze, Katsarava, Morris, & Sulakvelidze, 2002). Venous ulcers are wounds which are thought to be a result of improper functioning of venous valves. They form around the lower parts of the legs and the ankles, when the valves that prevent the backflow of blood do not function properly, leading to increased blood pressure in veins. The ulcers are usually open in nature and therefore are prone to bacterial infections. Systemic antibiotic therapy has provided little relief as the medication does not have direct access to the wound as blood does not get pumped efficiently to the legs. A proven solution to this problem is a wound dressing preparation known as PhagoBioDerm which consists of an antibiotic and lytic bacteriophages impregnated in a biodegradable polymer (Markoishvili et al., 2002). This principle can be used to treat other types of topical infections where the permeability of systemic medication is low. Large open wounds can also be healed faster and with lower incidences of life threatening infection occurrences.

Other topical formulations have been tested out using phages to treat other local infections caused by bacteria. It was found that when cetomacrogol cream aqueous, aqueous cream and cetrimide cream aqueous preparations were formulated using phage and used against *P. Acnes*, they were capable of releasing the phage which caused the lysis of the bacteria. This activity was seen for at least 90 days when the formulations were stored at 4 °C in a light-protected container (Browna et al., 2016). The information gives evidence of the stability of the formulation. However it is seen that phages show better lytic activity when used in a preparation using hydrophilic bases as opposed to hydrophobic bases. A reason for this difference could be the presence of sodium lauryl sulphate in greater concentrations in the emulsifying ointment as opposed to the aqueous cream. Use of sodium lauryl sulphate has shown virucidal or virus killing activity as it possesses the ability to denature proteins, making its use an undesirable trait. Not only is the composition a problem in the formulation, the high viscosity of the ointment, compared to the cream, also presents a barrier to phage release. The use of certain components have shown a phage release inhibition. Zinc oxide has appeared to inhibit phage release by binding to the phage protein and starch has reduced phage release due to its thickness. However,

zinc oxide in the preparation appears to show the more phage release inhibitory action (Browna et al., 2016).

4.3 Nasal administration

The nose allows a direct opening between the inside of the body and the environment, thereby causing it to be the most common pathway through which infections are contracted. Even though there are various barriers which trap most particles and microorganisms, some still find their way to the lungs and lead to various respiratory diseases. Emergence of multidrug-resistant strains of *Yersinia pestis,* a zoonotic bacteria, may result in epidemics of untreatable plague and so have created the demand for phage therapy to provide a solution. Between 2010-2015 there have been 3248 cases of plague reported worldwide including 584 deaths (WHO, 2017). Plague is not a new emerging disease. It has been around for centuries. During the fourteenth century, plague was responsible for the deaths of an estimated 50 million people in Europe and was widely known as the "Black Death". There are two main clinical forms of plague, bubonic and pneumonic with bubonic being the most common form. It is a very serious disease with about 30-60% case-fatality ratio (Appendix A20) in case of the bubonic kind and 30-100% case-fatality ratio for the pneumonic kind, if left untreated. Infected individuals initially develop a "flu-like" symptom after an incubation period of 1-7 days. Typical symptoms are the sudden onset of fever, chills, head and body-aches and weakness, vomiting and nausea. Bubonic plague is characterized by painful swollen lymph nodes or 'buboes'. The bacteria that causes plague is usually found in small animals and their fleas. Humans become contaminated by bites from infected fleas or direct contact with infected materials or through inhalation (WHO, 2017). In a study, purified phage designated as φA1122 was administered in human monocytes and hepatocytes as well as infected mice to determine its activity against *Yersinia pestis*. Results showed that there was a lack of cytotoxicity (Appendix A21) in the human host cells and the therapy showed promising results in treating the infection (Filippov et al., 2012).

B. cenocepacia is a highly antibiotic resistant bacteria known to cause respiratory infections. Experiments conducted on infected mice using aerosol phage therapy was found to have a significant decrease in bacterial loads in the isolated lung tissues of the mice, within 2 days (Semler et al., 2014) demonstrating the effectiveness of phage therapy.

5. Recommendations

Bacteriophages can be considered an emerging domain in the treatment of bacterial infections. Though not widely approved, they have been found to be effective in various critical conditions to combat bacterial infections.

5.1 Immunocompromised human host

An immunocompromised patient will not have the same immune response initiating ability as a regular patient. This list of patients are usually composed of those on chemotherapy (cancer), immunosuppressive therapy (post transplantation patients, rheumatologic disorders) or suffering from acquired immunodeficiency diseases (AIDS, post-splenectomy) (Bajaj & Tombach, 2016). These patients have a weakened immune system and their bodies usually have a hard time dealing with the increasing amounts of medicines they need to take. Patients suffering from genetic diseases such as cystic fibrosis (Appendix A22) have been reported to acquire respiratory infections caused by *Staphylococcus aureus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and *Burkholderia cepacia* leading to septicemia, respiratory failure, and reduced life expectancy *(Semler et al., 2014).*

The mechanism of phage therapy relies mainly on any one or both of the following principles. Firstly by directly killing the bacterial host during the lytic phase by the help of virions and secondly by initiation of an immune response with the help of particles generated by the phages or phage constituents themselves which are mainly made up of parts of the bacterial cells (Borysowski & Go´rski, 2007). Patients suffering from reduced immune activity will be most affected when the immune system needs to play a vital part in removal of bacterial infections associated with phage therapy. Immunosuppressed patients do not have enough white blood cells to recognize the progress of the bacteriophages in eradicating the bacterial pathogen. The bacteria will remain active although the phages have presented them as an antigen to the human body's immune system, possibly resulting in phage resistance.

5.2 Patients suffering from hepatic and renal dysfunction

In patients suffering from hepatic (liver) and renal (kidney) dysfunction, disease treatment with antibiotics can be challenging. Different antibiotics are metabolized and eliminated in different

ways. Antibiotics can be metabolized solely by the liver, eliminated directly by the kidney or experience excretion by a combination of both. The pharmacokinetic properties of antibiotics usually depend upon the family it belongs to.

Antibiotics belong to any of the following families:

- sulfonamides,
- beta-lactams,
- glycopeptides,
- polymixins,
- tetracyclines,
- aminoglycosides,
- macrilides,
- oxazolidinones,
- lincosamides,
- amphenicons,
- streptogramins,
- antimycobacterials,
- quinolones and
- the others are miscellaneous (Rang, Ritter, Flower, & Henderson, 2016).

The antibiotics can present serious side effects including hepatitis, acute renal failure, hypersensitivity reactions, nephrotoxicity and gastrointestinal disturbances, to name a few (Rang et al., 2016). Patients suffer from renal or hepatic failure for various reasons.

Renal failure (acute or chronic) is caused by:

- Blood or fluid loss
- Blood pressure medications
- Heart attack
- Heart disease
- Infection
- Liver failure

- Long term intake of aspirin, ibuprofen (Advil, Motrin IB, others), naproxen (Aleve, others) or related drugs
- Severe allergic reaction (anaphylaxis)
- Severe burns
- Severe dehydration

The most common causes of chronic liver failure (where the liver fails over months to years) include:

- Hepatitis B
- Hepatitis C
- Long-term alcohol consumption
- Cirrhosis
- Hemochromatosis (an inherited disorder that causes the body to absorb and store too much iron)
- Malnutrition

The causes of acute liver failure, when the liver fails rapidly, however, are often different. These include:

- Acetaminophen (Tylenol) overdose
- Viruses including hepatitis A, B, and C (especially in children)
- Reactions to certain prescription and herbal medications
- Ingestion of poisonous wild mushrooms

For patients suffering from renal or hepatic inefficiency, use of antibiotics becomes life threatening in some cases due to the heightened side effects experienced by them, compared to a healthy patient suffering from bacterial disease.

Studies on animals have shown that phage therapy is suitable for use as its excretion from the body does not solely depend upon kidney and liver function. Phages used in therapy have been observed to be removed by hepatic Kupffer cells. The main pathway of phage elimination however is caused by the systemic inflammatory response of the body. Removal of phages before they can express their therapeutic potential is undesirable and is usually caused by

specific immunogenic characteristics of the phage, duration of phage exposure, dose of the phage, route of administration and excipients used in therapy formulation. High levels of anti-phage antibodies have the ability to effectively neutralize phages but this undesirable effect can be counteracted by changing the timing, frequency and dose of phages (Hodyra-Stefaniak et al., 2015). This therefore allows phage therapy to be used as an alternative to antibiotic therapy in patients suffering from hepatic and renal inefficiency after clinical trials are successfully carried out.

5.3 Nosocomial infections

According to the Centre for Disease Control and Prevention (CDC), some of the most common nosocomial infections are urinary tract infections, respiratory pneumonia, surgical site wound infections, bacteremia, gastrointestinal and skin infections. A bacterial strain named *Klebsiella pneumoniae* are opportunistic pathogens acquired by human hosts associated to hospitalization. Patients who have compromised immune systems along with diabetes mellitus or chronic pulmonary obstruction are the primary sufferers of an attack by the pathogen (Podschun & Ullmann, 1998). *Klebsiella*, a Gram-negative enteric bacterium has been found to cause pyrogenic liver abscess (Appendix A23) in recent years. It has been observed that the capsule of the bacterium is an important virulence vector of the strain (Hsu et al., 2013). This pathogenic strain has shown resistance to a wide range of antibiotics making it very difficult to cure. Current evidence implicate that the main reason for resistance is due to plasmid transmission within the bacterial strains (Nathisuwan, Burgess, & II, 2001).

Klebsiella pneumoniae are most frequently resistant to:

- aminoglycosides,
- fluoroquinolones,
- tetracyclines,
- chloramphenicol
- trimethoprim or sulfamethoxazole (Hudson, Bent, Meagher, & Williams, 2014).

With the increase in antibiotic resistance it has become important to find alternatives which can effectively cure this disease and therefore reduce mortality rates. Thus phage therapy can be used as an effective therapeutic agent.

5.4 Use of a combination of bacteriophages

Bacteria have the potential to become resistant to the phages in order to survive. They can modify any of the sites where phages bind and prevent attachment. A way to overcome this problem is to use a combination of bacteriophages which will all be attacking the bacteria together, reducing the chances of resistance. If a small number of the phages in the cocktail prove to be ineffective as a result of resistance, other phages will be able to infect the bacteria in their absence. It has been shown that, by treating bacteria with a combination of phages the results show more promise. Experimentally the mutation frequency of bacteria were reduced when a phage cocktail was used. The lytic disintegration, breakdown and rate of the bacterial cells was increased compared to monophage (Appendix A24) treatment. The same experiment when reproduced using a murine (Appendix A25) bacteremia models, showed similar results and administration into the host and furthermore did not produce any adverse effects 30 days after injection (Gu et al., 2012).

T4 phages which are virulent phages, propagating by serial lytic infection, did not produce a lysogenic state which refers to a viral replication step that incorporates the viral gene into the host genome or forms a circular replicon (Appendix A26) inside the host cytoplasm (Denou et al., 2009). The process destroys the host bacterial DNA making it unlikely that unwanted genes will be transferred. (Denou et al., 2009) Safety of a given phage combination cannot be achieved by genome analysis by itself. In vivo tests must be carried out in both animals and human beings. When adults had an oral administration of nine phages similar to T4, no adverse effects were observed. The stool sample contained only 1% of the total oral phage concentration (Sarker et al., 2012).

Referring to the use of a combination of phages alone is not enough, whether the administration of the combination be simultaneous or sequential, leading to pronounce differences. Addition of phages simultaneously tends to yield results most successful for use in short term strategies while sequential addition provides equally and potentially better results over long term strategies (Hall et al., 2012). Phage Therapy is a reality in Russia where a number of companies, the most prominent being Microgen, sell a large list of different phage cocktails for diverse bacterial infections and medical indications (Brussow, 2012). Using a combination of phages provide another very important solution to a different problem of practicality of this therapy. Since phage

therapy is very specific, the etiology of the host organism needs to be determined in order to choose the corresponding effective phage. Using 'cocktails' of phages allows there to be a instantly available treatment option without waiting helplessly as the bacterial ethiology (Appendix A27) is being determined (Wittebole et al., 2014).

6. Concluding remarks

Phage therapy represents a very practical solution for tackling antibiotic resistance depending on the willingness and interest in pursuing this field of therapy. Its advantages have so far outweighed the challenges it possesses in combating multi-drug resistance of pathological bacteria. Phages have been extensively studies for the past few decades and this has allowed a wider and better understanding of the characteristics and mechanisms utilized by bacteriophages. Although there has been concern about the challenges that phage therapy possess in theory, they have not been practically proven. One of the most attractive aspects of this therapy is the inability of phages to attack body cells of the human patient undergoing therapy as well as the reduced number of side effects usually associated with antibiotic therapy. Use of natural phage combinations are a very good starting point of this therapy as genetic modification of phages is also possible to yield better success in treatments as well as reduce the costs of production. Purification methods of phage therapy have been developed making its use more widespread and eliminating human host specificity allowing uniform results. Isolation of phage from cultures is relatively easier compared to antibiotic isolation. Genomic sequencing of the phages allows complete identification of phage types and can be used to tailor specific medication. Any minor changes to phages can also be made as a result when the genome is known allowing ideal properties to be incorporated. Investment in this field of study is very low mainly due to the overdependence upon traditional chemical medicines but may be overcome in the near future as people become more aware of the benefits of phage therapy. The most important aspect of this therapy is its ever present potential for adaptability whether by natural or artificial methods to combat bacterial infections. The phage adapts to the bacteria and so does not need to be discarded every time a pathological strain is brought to light. Administration of the phages are not limited to single dosage form types but can be manufactured as needed depending on the type of infection and its site of action. As the number of effective antibiotics reduce, the pressure mounts on healthcare professionals to find an alternative before it is too late. This scarcity has given rise to the funding of research groups and companies to begin production of phage products which can be seen in their pipeline. Currently bacteriophages are in various phases of clinical trials, which when completed and approved, may be next in line to cure bacterial infection. Of any other alterative which is proposed none have the historical evidence to support

its claims like phage therapy does so we believe that if anything can relieve us of the complications as a result of antibiotic resistance, it will be phage therapy.

7. References:

Bajaj, S. K., & Tombach, B. (2016). Respiratory infections in immunocompromised patients: Lung findings using chest computed tomography. *Elsevier*. doi: 10.1016/j.jrid.2016.11.001

Behjati, S., & Tarpey, P. S. (2013). What is next generation sequencing? *Research in Practice*. doi: 10.1136/archdischild-2013-304340

Belleghema, J. D. V., Merabishvilib, M., Vergauwenc, B., Lavigned, R., & Vaneechouttea, M. (2017). A comparative study of different strategies for removal of endotoxins from bacteriophage preparations. *Elsevier, 132*, 153–159.

Biologics, C. (2017). Production of Phages for Clinical Trials. Retrieved 15 May, 2017, from http://www.cobrabio.com/Services/Viruses/Case-Studies/Production-of-Phages-for-Clinical-Trials

Borysowski, J., & Go´rski, A. (2007). Is phage therapy acceptable in the immunocompromised host? *Elsevier*.

Britannica, E. (2017). Bacteriophage. Retrieved May 15, 2017, 2017, from https://www.britannica.com/science/bacteriophage

Brown, T. L., Petrovski, S., Dyson, Z. A., Seviour, R., & Tucci, J. (2016). The Formulation of Bacteriophage in a Semi Solid Preparation for Control of Propionibacterium acnes Growth. *PLOS ONE*. doi: 10.1371/journal.pone.0151184

Browna, T. L., Thomasa, T., Odgersa, J., Petrovskib, S., Sparka, M. J., & Tucci, J. (2016). Bacteriophage formulated into a range of semisolid and solid dosage forms maintain lytic capacity against isolated cutaneous and opportunistic oral bacteria. *Journal of Pharmacy and Pharmacology*. doi: 10.1111/jphp.12673

Brussow, H. (2012). What is needed for phage therapy to become a reality in Western medicine? *Elsevier Inc.*

Cairns, B. J., Timms, A. R., Jansen, V. A. A., Connerton, I. F., & Payne, R. J. H. (2009). Quantitative Models of In Vitro Bacteriophage–Host Dynamics and Their Application to Phage Therapy. *PLOS Pathogens, 5*(1).

Ceglarek, I., Piotrowicz, A., Lecion, D., Miernikiewicz, P., Owczarek, B., Hodyra, K., . . . Dabrowska, K. (2013). A novel approach for separating bacteriophages from other bacteriophages using affinity chromatography and phage display. *SCIENTIFIC REPORTS*. doi: 10.1038/srep03220

Cirz, R. T., Chin, J. K., Andes, D. R., Crecy-Lagard, V. d., Craig, W. A., & Romesberg, F. E. (2005). Inhibition of Mutation and Combating the Evolution of Antibiotic Resistance. *PLoS Biology, 3*(6).

Colom, J., Cano-Sarabia, M., Otero, J., Aríñez-Soriano, J., Cortés, P., Maspoch, D., & Llagostera, M. (2017). Microencapsulation with alginate/CaCO3: A strategy for improved phage therapy. *SCIENTIFIC REPORTS*. doi: 10.1038/srep41441

Colom, J., Cano-Sarabia, M., Otero, J., Cortés, P., Maspoch, D., & Llagostera, M. (2015). Liposome-Encapsulated Bacteriophages for Enhanced Oral Phage Therapy against Salmonella spp. *Applied and Environmental Microbiology, 81*(14). doi: 10.1128/AEM.00812-15

Denou, E., Bruttin, A., Barretto, C., Ngom-Bru, C., Brüssow, H., & Zuber, S. (2009). T4 phages against Escherichia coli diarrhea: Potential and problems. *Elsevier Inc.*

Doss, J., Culbertson, K., Hahn, D., Camacho, J., & Barekzi, N. (2017). A Review of Phage Therapy against Bacterial Pathogens of Aquatic and Terrestrial Organisms. *MDPI*. doi: 10.3390/v9030050

Filippov, A. A., Sergueev, K. V., He, Y., Huang, X. Z., Gnade, B. T., Mueller, A. J., . . . Nikolich, M. P. (2012). Bacteriophage therapy of experimental bubonic plague in mice. *Adv Exp Med Biol, 954*, 337-348. doi: 10.1007/978-1-4614-3561-7_41

Friedman, S. L. (2000). Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. *J Biol Chem, 275*(4), 2247-2250.

Gebeyehu, E., Bantie, L., & Azage, M. (2015). Inappropriate Use of Antibiotics and Its Associated Factors among Urban and Rural Communities of Bahir Dar City Administration, Northwest Ethiopia. *PLOS ONE*. doi: 10.1371/journal.pone.0138179

Gross, L. (2006). Success Comes at a Cost, Even for Phages. *PLoS Biology, 4*(7). doi: 10.1371/journal.pbio.0040227

Gu, J., Liu, X., Li, Y., Han, W., Lei, L., Yang, Y., . . . Feng, X. (2012). A Method for Generation Phage Cocktail with Great Therapeutic Potential. *PLOS ONE, Volume 7*(Issue 3).

Guang-Han, O., Leang-Chung, C., Vellasamy, K. M., Mariappan, V., Li-Yen, C., & Vadivelu, J. (2016). Experimental Phage Therapy for Burkholderia pseudomallei Infection. *PLOS ONE*. doi: 10.1371/journal.pone.0158213

Hall, A. R., Vos, D. D., Friman, V.-P., Pirnay, J.-P., & Buckling, A. (2012). Effects of Sequential and Simultaneous Applications of Bacteriophages on Populations of Pseudomonas aeruginosa In Vitro and in Wax Moth Larvae. *Applied and Environmental Microbiology, 78*(16), 5646–5652.

Hanlon, G. W. (2007). Bacteriophages: an appraisal of their role in the treatment of bacterial infections. *Int J Antimicrob Agents, 30*(2), 118-128. doi: 10.1016/j.ijantimicag.2007.04.006

Hodyra-Stefaniak, K., Miernikiewicz, P., Drapała, J., Drab, M., Jończyk-Matysiak, E., Lecion, D., . . . Dąbrowska, K. (2015). Mammalian Host-Versus-Phage immune response determines phage fate in vivo. *Nature Scientific Reports*. doi: 10.1038/srep14802

Hsu, C.-R., Lin, T.-L., Pan, Y.-J., Hsieh, P.-F., & Wang, J.-T. (2013). Isolation of a Bacteriophage Specific for a New Capsular Type of Klebsiellapneumoniae and Characterization of Its Polysaccharide Depolymerase. *PLOS ONE, 8*(8).

Hudson, C. M., Bent, Z. W., Meagher, R. J., & Williams, K. P. (2014). Resistance Determinants and Mobile Genetic Elements of an NDM-1-Encoding Klebsiella pneumoniae Strain. *PLOS ONE, 9*(6).

ICTV. (2016). Taxonomy. Retrieved 15 May, 2017, from https://talk.ictvonline.org/taxonomy/

Illumina. (2017). Whole-genome sequencing. Retrieved 11 May, 2017, from https://www.illumina.com/techniques/sequencing/dna-sequencing/whole-genome-sequencing/small-genomes.html

Iyer, G., Ramaswamy, S., Cheng, K.-S., Sisowath, N., Mehta, U., Leahy, A., . . . Chung, F. (2012). Flow-Through Purification of Viruses- A Novel Approach to Vaccine Purification. *Elsevier*.

Jensen, K. C., Hair, B. B., Wienclaw, T. M., Murdock, M. H., Hatch, J. B., Trent, A. T., . . . Berges, B. K. (2015). Isolation and Host Range of Bacteriophage with Lytic Activity against Methicillin Resistant Staphylococcus aureus and Potential Use as a Fomite Decontaminant. *PLOS ONE*. doi: 10.1371/journal.pone.0131714

Kawai, T., & Akira, S. (2010). The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol, 11*(5), 373-384. doi: 10.1038/ni.1863

Kot, W., Kilstrup, M., Vogensen, F. K., & Hammer, K. (2016). Clear Plaque Mutants of Lactococcal Phage TP901-1. *PLOS ONE*. doi: 10.1371/journal.pone.0155233

Lindgren, P. K., Karlsson, A. s., & Hughes, D. (2003). Mutation Rate and Evolution of Fluoroquinolone Resistance in Escherichia coli Isolates from Patients with Urinary Tract Infections. *American Society for Microbiology, 47*, 3222–3232. doi: 10.1128/AAC.47.10.3222–3232.2003

Markoishvili, K., Tsitlanadze, G., Katsarava, R., Morris, J. G., Jr., & Sulakvelidze, A. (2002). A novel sustained-release matrix based on biodegradable poly(ester amide)s and impregnated with bacteriophages and an antibiotic shows promise in management of infected venous stasis ulcers and other poorly healing wounds. *Int J Dermatol, 41*(7), 453-458.

Merabishvili, M., Pirnay, J.-P., Verbeken, G., Chanishvili, N., Tediashvili, M., Lashkhi, N., . . . Vaneechoutte, M. (2009). Quality-Controlled Small-Scale Production of a WellDefined Bacteriophage Cocktail for Use in Human Clinical Trials. *PLOS ONE, 4*(3).

Merril, C. R., Biswas, B., Carlton, R., Jensen, N. C., Creed, G. J., Zullo, S., & Adhya, S. (1996). Long-circulating bacteriophage as antibacterial agents. *Proc. Natl. Acad. Sci. USA, 93*.

Mirzaei, M. K., & Nilsson, A. S. (2015). Isolation of Phages for Phage Therapy: A Comparison of Spot Tests and Efficiency of Plating Analyses for Determination of Host Range and Efficacy. *PLOS ONE*. doi: 10.1371/journal.pone.0118557

Nathisuwan, S., Burgess, D. S., & II, J. S. L. (2001). Extended-Spectrum b-Lactamases: Epidemiology, Detection, and Treatment. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy, 21*(8), 920-928.

Nobrega, F. L., Costa, A. R., Kluskens, L. D., & Azeredo, J. (2015). Revisiting phage therapy: new applications for old resources. *Elsevier*. doi: 10.1016/j.tim.2015.01.006

Paepe, M. D., & Taddei, F. (2006). Viruses' Life History: Towards a Mechanistic Basis of a Trade-Off between Survival and Reproduction among Phages. *PLoS Biology, 4*(7).

Podschun, R., & Ullmann, U. (1998). Klebsiella spp. as Nosocomial Pathogens: Epidemiology, Taxonomy, Typing Methods, and Pathogenicity Factors. *Clinical Microbiology Reviews*, 589–603.

R.Merril, C., Scholl, D., & Adhya, S. L. (2003). The prospect for bacteriophage therapy in Western medicine. *Nature Reviews Drug Discovery, 2*. doi: 10.1038/nrd1111

Raetz, C. R. H. (1990). Biochemistry of Endotoxins. *Annual Reviews Inc.*

Rang, H. P., Ritter, J. M., Flower, R. J., & Henderson, G. (2016). *Rand and Dale's Pharmacology 8th edition*. Churchill, Livingstone: Elsevier.

Salmond, G. P. C., & Fineran, P. C. (2015). A century of the phage: past, present and future. *Nature Reviews Microbiology*. doi: 10.1038/nrmicro3564

Santos, S. l. B., Carvalho, C., Azeredo, J., & Ferreira, E. n. C. (2014). Population Dynamics of a Salmonella Lytic Phage and Its Host: Implications of the Host Bacterial Growth Rate in Modelling. *PLOS ONE, 9*(7).

Sarker, S. A., McCallin, S., Barretto, C., Berger, B., Pittet, A.-C. c., Sultana, S., . . . Brussow, H. (2012). Oral T4-like phage cocktail application to healthy adult volunteers from Bangladesh. *Elsevier Inc.*

Semler, D. D., Goudie, A. D., Finlay, W. H., & Dennisa, J. J. (2014). Aerosol Phage Therapy Efficacy in Burkholderia cepacia Complex Respiratory Infections. *Antimicrobial Agents and Chemotherapy, 58*(7), 4005–4013. doi: 10.1128/AAC.02388-13

Swapna, G., Kumari, V., & Nagaraja, V. (2015). Different Modes of Transactivation of Bacteriophage Mu Late Promoters by Transcription Factor C. *PLOS ONE*. doi: 10.1371/journal.pone.0129504

Szermer-Olearnik, B., & Boratyński, J. (2015). Removal of Endotoxins from Bacteriophage Preparations by Extraction with Organic Solvents. *PLOS ONE*. doi: 10.1371/journal.pone.0122672

Tao, W., Zhang, X.-X., Zhao, F., Huang, K., Ma, H., Wang, Z., . . . Ren, H. (2016). High Levels of Antibiotic Resistance Genes and Their Correlations with Bacterial Community and Mobile Genetic Elements in Pharmaceutical Wastewater Treatment Bioreactors. *PLOS ONE*. doi: 10.1371/journal.pone.0156854

Wahida, A., Ritter, K., & Horz, H.-P. (2016). The Janus-Face of Bacteriophages across Human Body Habitats. *PLOS Pathogens*. doi: 10.1371/journal.ppat.1005634

Weinbauer, M. G. (2004). Ecology of prokaryotic viruses. *Elsevier*.

WHO. (2007). A safer future: global public health security in the 21st century. Retrieved April 07, 2017, 2017, from http://www.who.int/whr/2007/en/

WHO. (2017, April 2017). Plague. Retrieved May 04, 2017, 2017, from http://www.who.int/mediacentre/factsheets/fs267/en/

Wielgoss, S., Bergmiller, T., Bischofberger, A. M., & Hall, A. R. (2015). Adaptation to Parasites and Costs of Parasite Resistance in Mutator and Nonmutator Bacteria. *Oxford Journals*. doi: 10.1093/molbev/msv270

Wittebole, X., Roock, S. D., & Opal, S. M. (2014). A historical overview of bacteriophage therapy as an alternative to antibiotics for the treatment of bacterial pathogens. *Landes Bioscience*. doi: 10.4161/viru.25991

Woo, M., Khan, N. Z., Royce, J., Mehta, U., Gagnon, B., Ramaswamy, S., . . . Cheng, K. S. (2011). A novel primary amine-based anion exchange membrane adsorber. *J Chromatogr A, 1218*(32), 5386-5392. doi: 10.1016/j.chroma.2011.03.068

Yayan, J., Ghebremedhin, B., & Rasche, K. (2015). Antibiotic Resistance of Pseudomonas aeruginosa in Pneumonia at a Single University Hospital Center in Germany over a 10-Year Period. *PLOS ONE*. doi: 10.1371/journal.pone.0139836

Appendix:

A1: Reticuloendothelial system: This is a part of the immune system that consists of the phagocytic cells and is located in the reticular connective tissue. The cells are primarily monocytes and macrophages which protect the body by eliminating foreign objects.

A2: Taxonomy: The theory and practice of grouping individuals into species, arranging species into larger groups, and giving those groups names, thus producing a classification

A3: Topoisomerase: Topoisomerases are enzymes that participate in the over-winding or under-winding of DNA. The winding problem of DNA arises due to the intertwined nature of its double-helical structure. During DNA replication and transcription, DNA becomes over-wound ahead of a replication fork. If left unabated, this torsion would eventually stop the ability of DNA or RNA polymerases involved in these processes to continue down the DNA strand.

A4: Anaphylaxis: Anaphylaxis is a severe, whole-body allergic reaction to a chemical that has become an allergen. An allergen is a substance that can cause an allergic reaction.

A5: Virion: Virion is the complete, infective form of a virus outside a host cell, with a core of RNA or DNA and a capsid.

A6: Microflora: The community of microorganisms, including bacteria, fungi and algae, that live in a particular habitat or in or on another living organism.

A7: Metagenomic analysis: Analysis of genomes contained with an environmental sample

A8: Epifluoresence microscope: A fluorescence microscope is a conventional compound microscope that has been equipped with a high-intensity light source (usually a mercury arc lamp) that emits light in a broad spectrum from visible through ultraviolet.

A9: Dysbiosis: Dysbiosis (also called dysbacteriosis) is a term for a microbial imbalance or maladaptation on or inside the body, such as an impaired microbiota.

A10: Phenotype: The set of observable characteristics of an individual resulting from the interaction of its genotype with the environment.

A11: Chromatofocusing: Chromatofocusing is a protein-separation technique that allows resolution of single proteins and other ampholytes from a complex mixture according to differences in their isoelectric point. Chromatofocusing utilizes ion exchange resins and is typically performed on fast protein liquid chromatography (FPLC) or similar equipment capable of producing continuous buffer gradients though this is not a requirement.

A12: Affinity tags: Affinity tags are highly efficient tools for purifying proteins from crude extracts.

A13: Pharmacokinetics: The branch of pharmacology concerned with the movement of drugs within the body.

A14: Pharmacodynamics: The branch of pharmacology concerned with the effects of drugs and the mechanism of their action.

A15: Chaotropacity: Chaotropicity describes the entropic disordering of lipid bilayers and other biomacromolecules which is caused by substances dissolved in water.

A16: Micropores: A very narrow pore, especially in a material.

A17: Excipients: An excipient is a substance formulated alongside the active ingredient of a medication, included for the purpose of long-term stabilization, bulking up solid formulations that contain potent active ingredients (thus often referred to as "bulking agents", "fillers", or "diluents"), or to confer a therapeutic enhancement on the active ingredient in the final dosage form, such as facilitating drug absorption, reducing viscosity or enhancing solubility. Excipients can also be useful in the manufacturing process, to aid in the handling of the active substance concerned such as by facilitating powder flowability or non-stick properties, in addition to aiding *in vitro* stability such as prevention of denaturation or aggregation over the expected shelf life. The selection of appropriate excipients also depends upon the route of administration and the dosage form, as well as the active ingredient and other factors.

A18: The commercial use of phages can be differentiated into different types of work being carried out by the following organizations, such as:

1. Emphasizing pre-clinical phage therapy research and development (R&D):

AmpliPhi Biosciences

Enbiotix

Fixed Phage

InnoPhage

Intralytix

Pherecydes Pharma

Sarum Biosciences

Synthetic Genomics

Technophage

2. Emphasizing the development of products that do not employ replication-competent phages:

   AvidBiotics

   Eligo Bioscience

   Enbiotix

   Phico

3. Involved primarily in phage product distribution, some R&D may be involved as well:

   Biochimpharm

   Imbio

   Microgen

4. Facilitate patient phage therapy treatment:

   Center for Phage Therapy

   Eliava Phage Therapy Center

   Globalyz Biotech

   Novomed

   Phage Therapy Center

   Phage International

5. Emphasizing phage-mediated biocontrol (that is not strictly "therapy"):

   APS Biocontrol

   Epibiome

   InnoPhage

Intralytix

Micreos Food Safety

Omnilytics

Phage Biotech

Phagelux

Technophage

6. Market products as phage lysates rather than in terms of the phages themselves:
   Delmont
7. Involved in the use or development of enzybiotics:
   GangaGen
   Lysando GmbH
   Micreos Food Safety
   New Horizons Diagnostics?
8. Emphasize phage-based bacterial detection technologies:
   Sample6
9. Address issues of phage-associated industrial contamination:
   Phage Consultants
10. Emphasize phages in other biotechnology products:
    Versatile BioSciences

A19: Liposomes: A minute spherical sac of phospholipid molecules enclosing a water droplet, especially as formed artificially to carry drugs or other substances into the tissues.

A20: Case-fatality ratio: is the proportion of deaths within a designated population of "cases" (people with a medical condition), over the course of the disease.

A21: Cytotoxicity: Cytotoxicity is the quality of being toxic to cells.

A22: Cystic fibrosis: Cystic fibrosis is a progressive, genetic disease that causes persistent lung infections and limits the ability to breathe over time.

A23: Pyrogenic liver abscess: A pyogenic liver abscess (PLA) is a pocket of pus that forms in the liver in response to an infection or trauma.

A24: Monophage: A single phage.

A25: Murine: Relating to or affecting mice or related rodents.

A26: Replicon: A replicon is a DNA molecule or RNA molecule or a region of DNA or RNA, that replicates from a single origin of replication.

A27: Etiology: The cause, set of causes or manner of causation of a disease or condition.

CRISPR-Cas: CRISPR stands for Clustered regularly interspaced short palindromic repeats. They are segments of prokaryotic DNA containing short, repetitive base sequences.