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**EXPLORING THE USE OF
BACTERIOPHAGES AS AN
ALTERNATIVE TREATMENT
OF EQUINE DISEASES**

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ABSTRACT

Oona Kaartinen: Exploring the Use of Bacteriophages as an Alternative Treatment of Equine Diseases

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Antibiotic resistance is a growing global health threat, necessitating the development of alternative treatments. Bacteriophages, viruses that specifically infect and lyse bacteria, serve as a potential solution for combating infections caused by resistant bacterial strains. Phage therapy has been used since the early 1900s, particularly in the pre-antibiotic era, but its application and research declined due to the rapid rise and high effectiveness of antibiotic-based treatments. However, the increased prevalence of antibiotic resistance has renewed the interest in phages as therapeutic tools.

The aim of this thesis is to explore the structure and function of bacteriophages, their historical use, and potential applications in veterinary medicine, particularly in the treatment of equine diseases. Phage therapy's differences from, and key advantages over, antibiotics are also examined. Additionally, the role of genetic engineering in enhancing the safety and efficacy of phage therapy is assessed. The main challenges surrounding phage therapy, including regulatory aspects, resistance and immune responses, are also discussed.

Replacing antibiotics partially or completely with bacteriophages promotes sustainable development in several ways. Phage therapy can reduce the need for antibiotics, decreasing their release into waterways and the environment. This, in turn, could slow the spread of antibiotic resistance, ultimately benefiting public health. In addition, maintaining microbial balance in horses contributes to their overall well-being and supports the sustainability of broader animal and microbial populations. In veterinary medicine, phage therapy represents a promising area of research that is potentially more accessible compared to strictly regulated human medicine.

Based on this literature review, phage therapy alone is not yet effective enough to fully replace antibiotics. However, a combined approach using both antibiotics and bacteriophages could be an effective treatment strategy. In some cases, phages could serve as a complete substitute, particularly for preventive measures. If proven to be successful in veterinary field, the acceptance of phage therapy in human medicine may become more feasible.

Although phage therapy has potential, it comes with a few notable challenges. These include bacterial resistance to phages and possible immune system reactions. Furthermore, while genetic engineering and advancements in molecular biology offer ways to overcome some previous limitations in phage therapy, they also raise new technical and ethical concerns. Further research, international collaboration, and updated regulatory frameworks are essential to integrate phage therapy into mainstream medicine and veterinary practice.

Keywords: bacteriophages, phage therapy, antibiotic resistance, AMR, bacterial infections, genetic engineering, equine diseases

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TIIVISTELMÄ

Oona Kaartinen: Bakteriofagien käyttö hevossairauksien vaihtoehtoisena hoitomuotona
Opinnäytetyö
Tampereen yliopisto
Tekniikan ja luonnontieteiden kandidaattiohjelma
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Antibioottiresistenssi on maailmanlaajuinen terveysuhka, joka vaatii vaihtoehtoisten hoitomenetelmien kehittämistä. Bakteriofaagit, bakteereja infektoivat ja tuhoavat virukset, tarjoavat lupaan ratkaisun resistenttien bakteerikantojen aiheuttamien infektioiden torjuntaan. Vaikka faagiterapiaa on käytetty 1900-luvun alusta lähtien, sen käyttö väheni antibioottien yleistyessä. Antibioottiresistenssin lisääntyminen on kuitenkin herättänyt uudelleen kiinnostuksen faagipohjaisia hoitoja kohtaan.

Tässä kandidaatintyössä tarkastellaan bakteriofagien rakennetta ja toimintaa, niiden käyttöä historiassa sekä mahdollisia sovelluksia eläinlääketieteessä, erityisesti hevostautien hoidossa. Lisäksi tarkastellaan geenitekniikan roolia faagiterapian turvallisuuden ja tehokkuuden parantamiseksi. Myös faagiterapiaan liittyviä keskeisiä haasteita, kuten resistenssin kehittymistä ja immuunivasteita, käsitellään.

Antibioottien osittainen tai täydellinen korvaaminen bakteriofaageilla tukee kestävästä kehitystä monin tavoin. Faagiterapia voi vähentää antibioottien tarvetta ja näin ollen vapautumista resistenssiin ja ympäristöön. Tämä puolestaan voisi hidastaa antibioottiresistenssin leviämistä, millä on positiivinen vaikutus myös kansanterveyteen. Lisäksi hevosten mikrobitasapainon ylläpitäminen edistää hevosten hyvinvointia ja laajemman eläin- ja mikrobipopulaation kestävyyttä. Eläinlääketieteessä faagiterapia on lupaava tutkimusalue, sillä sen käyttöönotto on sääntelyn kannalta joustavampaa kuin ihmislääketieteessä.

Tämän kirjallisuuskatsauksen perusteella faagiterapia ei ainakaan vielä voi korvata antibiootteja täysin. Yhdistetty lähestymistapa, jossa käytetään sekä antibiootteja että bakteriofageja, voisi kuitenkin olla tehokas hoitomuoto. Joissakin tapauksissa faagit voivat toimia täydellisenä korvikkeena, erityisesti ennaltaehkäisevissä hoidoissa. Jos faagiterapia osoittautuu menestyksekkääksi eläinlääketieteessä, sen hyväksyminen ihmislääketieteessä voisi olla helpompaa.

Tutkimusten perusteella faagiterapialla on paljon potentiaalia, mutta siihen liittyy myös haasteita, kuten faagiresistenssi ja immuunireaktiot. Vaikka geenitekniikalla ja molekyylibiologian menetelmillä on mahdollisuus voittaa faagiterapian aiemmat ongelmakohdat, ne herättävät samalla uusia teknisiä ja eettisiä huolenaiheita. Faagiterapian laajempi käyttöönotto edellyttää lisätutkimusta, kansainvälistä yhteistyötä ja ajantasaista sääntelyä niin eläin- kuin ihmislääketieteessä.

Avainsanat: bakteriofagit, faagiterapia, antibioottiresistenssi, mikrobilääkeresistenssi, bakteeri-infektiot, geenitekniikka, hevossairaudet

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ABBREVIATIONS

AMR	Antimicrobial resistance
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
crRNA	CRISPR RNA
dsDNA	Double-stranded DNA
GA	Gibson Assembly
GMP	Good Manufacturing Practice
MDR	Multidrug-resistant
PAM	Protospacer Adjacent Motif
phage	Bacteriophage
RCR	Rolling-circle replication
sgRNA	Single guide RNA
TAR	Transformation-associated recombination
tracrRNA	Trans-activating crRNA

1. INTRODUCTION

Antimicrobial resistance (AMR) is a growing global concern. In 2023, the World Health Organization (WHO) described AMR as one of the greatest threats to human health worldwide, at all income levels. It directly affects public health, but also has serious effects on animals and plants, leading to reduced productivity and threatened food security. (World Health Organization, 2023) According to an article published in *The Lancet*, 4.95 million deaths were linked to bacterial AMR in 2019, out of which 1.27 million were directly attributable. (Murray et al., 2022) It is estimated that antibiotic resistance could lead to approximately 300 million premature deaths by 2050, and result in economic losses of up to 100 trillion U.S. dollars (Munita & Arias, 2016).

The increased use of antibiotics has been shown to be strongly associated with the development of AMR. A Finnish study focusing on macrolide-resistant *Streptococcus pyogenes* demonstrated the direct connection between resistant strains and the use of erythromycin, an antibiotic belonging to the class of macrolides (Bergman et al., 2004). Globally, many antibiotics that have been effective in the past are losing their efficacy due to adaptive mechanisms of pathogens. This trend has been accelerating due to overprescribing of antibiotics and misusing them in viral infections, where they are clearly ineffective (Llor & Bjerrum, 2014). Furthermore, the spread of AMR is not limited to clinical settings, as antibiotics are often used in agriculture at even lower thresholds.

Antibiotics are widely used in veterinary medicine and agriculture to prevent and treat infections. In the U.S., around 8 billion animals are treated by 10 different antibiotics each year or at some point during their lifetime. Most of the antimicrobial classes used in agriculture overlap with those used in human medicine. (Jassim & Limoges, 2014) It is estimated that around 75% of the antibiotics used are not fully metabolized, but instead are excreted in waste, eventually contaminating the environment. This is referred to as antibiotic pollution, which further promotes the spread of AMR (Chee-Sanford et al., 2009).

The rise of antimicrobial resistance has driven researchers to look for alternative methods to combat bacterial infections. One promising approach is the use of bacteriophages, or phages for short. These are viruses that selectively infect and kill bacteria. Unlike antibiotics, phages are highly specific to their bacterial hosts, which minimizes the risks

to beneficial microflora. However, these natural bacteria eaters function differently depending on their morphology and life cycle, which makes it crucial to conduct extensive research before their widespread applications. Improper understanding and use may lead to unintended ecological consequences, such as immunological responses or phage resistance.

Phage therapy in veterinary medicine presents an intriguing area of research. As phages selectively target bacteria without disrupting the beneficial microbiota, they could serve as a viable alternative to antibiotics. Compared to human medicine, ethical considerations and regulatory framework surrounding phage therapy in animals may pose fewer challenges, as the threshold for adopting new treatments in veterinary medicine is often lower.

Equines are valuable animals, playing a major role in the livestock industry. The American Horse Council (AHC) stated that the equine industry made a contribution of 177 billion U.S. dollars to the economy in 2023 (American Horse Council, 2023). Unfortunately, horses are highly susceptible to various bacterial infections. The increasing prevalence of antimicrobial resistance has made the treatment of these diseases even more challenging (Kabir et al., 2024). Given the economic and health-related impact of bacterial infections in horses, it is essential to explore alternative treatments.

The aim of this study is to explore the use of bacteriophages as an alternative treatment to antibiotics, specifically in equine diseases. The goal is not only to evaluate the effects of phage therapy on horses and the environment but also to compare its efficacy to traditional antibiotic treatments. How have phages already been used and how could they be used in the future? Could they serve as a full replacement for antibiotics? What kinds of challenges are related to phage therapy, and are there any ways to solve them with modern technologies? How can genetic engineering enhance phage-based treatments, and what kinds of technologies could be used? The paper begins with an overview of bacteriophages, including their discovery, morphology, function and life cycles in Chapter 2. Chapter 3 provides a detailed explanation of phage therapy and compares it to antibiotic treatments. Applications and possibilities of phage therapy in the treatment of equine diseases are explored in Chapter 4, followed by a discussion on genetic engineering for safer and more efficient phage therapy in Chapter 5. Challenges associated with the implementation of phages in veterinary medicine are addressed in Chapter 6. Finally, key findings are summarized in Chapter 7.

2. BACTERIOPHAGES

Bacteriophages are viruses that specifically infect and kill bacteria (Chanishvili, 2016). In the same way as other viruses, they require a living host to propagate. With an estimated global population of 10^{31} particles, phages are the most abundant biological systems on Earth (Comeau et al., 2008). Despite their potential to effectively kill the host, they have been shown to play a crucial role in microbial communities by driving the evolution and genetic diversity of bacteria (Lehti et al., 2017). Owing to their unique characteristics, bacteriophages have emerged as an increasingly intriguing field of study in recent years, especially for medical applications. This chapter explores the discovery, structure and function of bacteriophages, as well as their two main life cycles.

2.1 Discovery

In 1915, an English physician Frederick Twort, attempted to propagate the vaccinia virus on agar plates. Instead of vaccinia, he observed that only contaminating bacteria were growing - or so he thought at first. After some time, Twort realized that instead of living cells, these were zones of dead bacteria. Unable to fully explain the cause and verify his findings, Twort documented his observations. (Twort, 1915)

Two years after Twort's discovery, a French-Canadian microbiologist Felix d'Herelle became convinced that he had found a virus capable of infecting and lysing bacteria. He described these viruses as bacteriophages. Later d'Herelle attempted to use phages as therapeutic agents against bacterial infections. Despite the limited understanding of phage biology at the time, he was able to conduct some successful treatments. (Keen, 2015)

The discoveries of Twort and d'Herelle were followed by expanded research of bacteriophages and their possible applications in medical field. However, after Alexander Fleming discovered antibiotics in 1928 (Fleming, 2001), their superior efficacy and reliability in the treatment of bacterial infections led to the decline of phage therapy. Limited knowledge of phage biology, combined with the widespread adoption of antibiotics, resulted in the abandonment of phage therapy in Western medicine (Hanlon, 2007). The role of antibiotics, along with their advantages and limitations compared to phages, are explored in more detail in Chapter 3.

2.2 Morphology and function

All bacteriophages share common viral properties, meaning they rely on the host cell's machinery for replication and have relatively small genomes. Phages exhibit high host specificity, with different phage types targeting specific bacterial species, or in some cases even strains. It is believed that at least one type of bacteriophage exists for every bacterial species. Phage genomes can range from 3.4 kb up to 500 kb and may consist of either RNA or DNA. (Keen, 2015)

The structure of a phage is typically symmetric and consists of a protein capsid protecting the viral genome and ensuring its stability until infection. The capsid is often icosahedral in shape, providing an optimal balance between efficacy and stability. This structure minimizes the genetic information required to encode the capsid proteins while maximizing the volume for storing the viral genome. (Tavares, 2018) Despite the wide variety of bacteriophage morphologies, the most common ones carry double-stranded DNA (dsDNA) within the capsid, which is attached to a tail (Figure 1) (Strathdee et al., 2023).

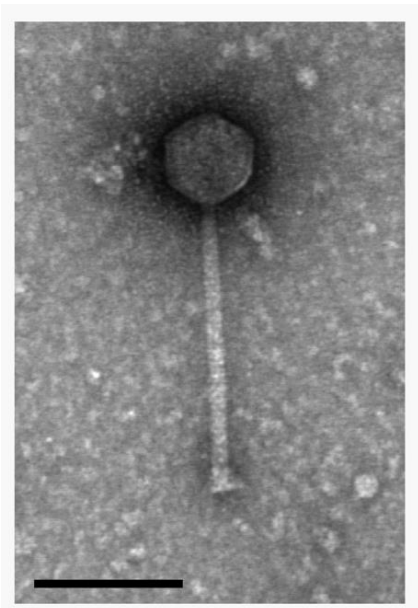


Figure 1. Bacteriophage structure, adapted from (Strathdee et al., 2023).

Phage tail is a special structure used for both host cell recognition and genome injection. The viral genome is injected through the tail tube, and tail fibers are responsible for host cell recognition. (Maghsoodi et al., 2019) Tailed dsDNA phages, classified under *Caudoviricetes*, account for approximately 96% of all known phages and are further categorized based on their tail structures. The tail fibers at the end of the tail play a crucial role in host specificity by binding to specific receptors on the bacterial cell surface. These can

vary depending on phage, but can be, for instance, lipopolysaccharides, teichoic acids or flagella. (Taslem Mourosi et al., 2022) This specificity allows phages to selectively target and eliminate pathogens without disrupting the beneficial microbiota, making them valuable in medical applications. However, due to the fast replication and mutation rates of bacteria, changes in the bacterial cell receptors are possible, which can ultimately result in unsuccessful viral infection.

Viruses are classified based on the nucleic acid that forms their genome. DNA viruses contain either single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA), whereas RNA viruses have genomes composed of either single-stranded RNA (ssRNA) or double-stranded RNA (dsRNA). In each case, the genome can be linear or circular. (Speir & Johnson, 2012) As previously mentioned, dsDNA is the most common genome type among phages, and thus this study focuses exclusively on these types of viruses. The genetic code of viruses contains instructions for proteins essential to their life cycle. These include structural proteins that form the viral capsid, as well as enzymes and proteins necessary for replication, immune evasion, and host cell interaction. Additionally, viruses have regulatory elements to control gene expression. (Rampersad & Tennant, 2018)

DNA replication and transcription systems in viruses are diverse and complex, but they follow similar patterns. The production of new copies of phage DNA is essential for assembling new viruses, and replication typically relies entirely on the host cell's machinery. However, some larger dsDNA viruses can encode their own DNA replication enzymes. In addition to DNA replication, mRNA must be generated to produce new phage particles. Both mRNA synthesis and DNA replication occur in three phases: initiation, elongation and termination. (Rampersad & Tennant, 2018)

In transcription initiation, RNA polymerase recognizes the promoter sequences of the viral genome. Transcription factors facilitate the binding of the polymerase to the promoter. Once the initiation is completed, RNA polymerase unwinds the DNA and progresses along the template strand, synthesizing mRNA. Termination signals within the viral genome instruct the polymerase to release the newly synthesized mRNA, which is then translated into functional viral proteins on the host cell's ribosomes. (Louten, 2016)

The mechanism of viral DNA replication depends on the structure of the genome, as circular and linear DNA replicate differently. However, it adheres to common principles, beginning at the origin of replication (Ori) within the viral genome. Initiator proteins bind to these sequences, unwind the DNA and recruit the replication machinery. During elongation, DNA polymerases synthesize new DNA strands, using the separated original

strands as templates to create complementary strands. Replication is completed as termination sites are reached, and ligase enzymes seal the nicks in the DNA to ensure strand continuity. Once replication is complete, newly synthesized viral genomes are packaged into new phage particles during assembly. (Strauss & Strauss, 2008)

Phages with a circular genome typically use rolling-circle replication (RCR), which involves continuous and unidirectional DNA synthesis. In this case, leading and lagging strands are synthesised separately, and initiation takes place at two different origins. RCR is also used by plasmids, but the mechanism differs. In the case of phages, termination of the leading strand replication immediately initiates another round of replication, which enables the rapid propagation of viruses. On the contrary, in healthy bacterial cells the plasmid copy number is tightly regulated. Therefore, the replication initiator is inactivated after only one round of replication. (Wawrzyniak et al., 2017) RCR is illustrated in Figure 2.

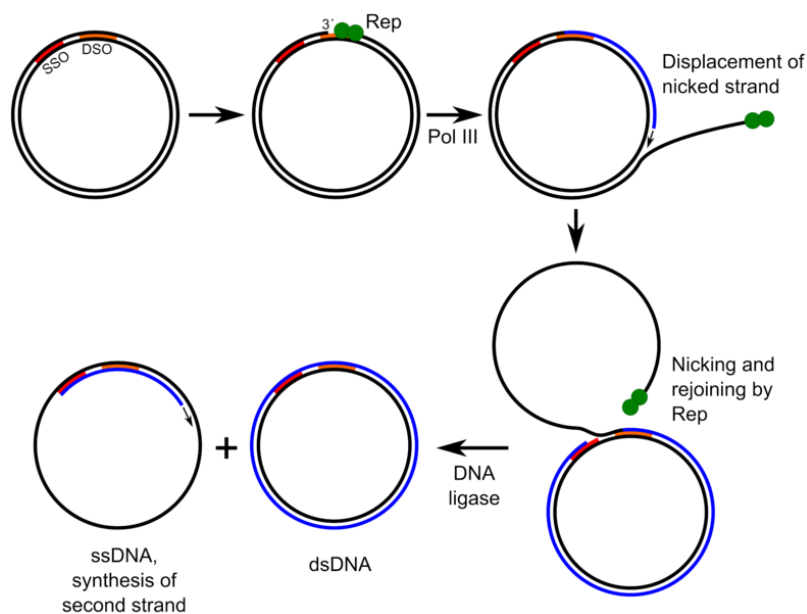


Figure 2. "Rolling-circle replication", CC BY-SA 4.0 (Vornholt, 2015).

Phage infection is a several step process, involving attachment, genome injection, phage DNA replication and transcription, viral particle synthesis, phage assembly and finally release. Tailed phages recognize their host by the receptors on the cell surface, which triggers the release of the genetic material from the phage head into the cytoplasm. It is crucial that the delivery of the viral genome into the host cell occurs without compromising cell viability, thus allowing successful propagation to take place. After genome injection, DNA replication and transcription proceed as described in the previous chapter,

which is followed by phage assembly. Once constructed, progeny phages are released from the bacterial cell to continue their infection cycle.

Attachment and genome injection are mediated by the phage tail. Although the precise mechanisms are not yet fully understood, several models have been proposed. For example, the infection mechanism of phage T4, which targets *Escherichia coli*, has been studied. After binding to the bacterial cell membrane, the phage T4 tail undergoes a conformational change, transitioning from a high-energy state to a low-energy state. This transformation provides the force needed for the needle-like tip of the tail tube to pierce through the outer membrane and periplasmic space. (Maghsoodi et al., 2019) The tip of the tail exhibits lysozyme-like activity, degrading the peptidoglycan layer beneath the periplasmic space. Finally, contact between the tail tube and the cytoplasmic membrane initiates the release of the phage DNA into the host cell. (Rossmann et al., 2004)

Once inside the host cell, phage particles are synthesized using the bacterial replication machinery. During this process, the host cell's DNA is fragmented into smaller pieces, preventing normal bacterial function and ensuring efficient viral particle construction. As described in the previous chapter, the viral genome must be both replicated and transcribed into mRNA to produce the necessary proteins for the assembly. Some phages rely on multiple copies of a single capsid protein, while others encode two or more distinct capsid proteins. To form an icosahedral shell, at least 60 copies of a capsid protein are required, along with other structural proteins that contribute to proper geometry and stability. (Aksyuk & Rossmann, 2011)

The empty protein shell formed before DNA packaging is known as a prohead. Once the prohead is assembled, packaging of the phage DNA begins. This process is facilitated by a terminase or packaging enzyme complex and is powered by ATP hydrolysis (Fujisawa & Morita, 1997). During this stage, some bacterial DNA fragments may also be mistakenly packaged into phage particles, a phenomenon further discussed in Chapter 2.4. In addition to DNA packaging, tails are assembled separately and later attached to the mature phage head, completing virion formation (Aksyuk & Rossmann, 2011).

The final step in the infection cycle is the release of fully assembled phages from the bacterial cell. Double-stranded DNA phages typically achieve this through the coordinated action of holins and endolysins, leading to host cell lysis at the end of the replication cycle (Young, 2002). Holins accumulate in the cytoplasmic membrane and, at a specific time, induce the formation of pores that disrupt the membrane integrity. These pores allow endolysins to reach the cell wall, where they start to degrade the peptidoglycan

layer. (Wu et al., 2021) This ultimately leads to cell rupture and the release of phage progeny, allowing them to infect new host cells.

2.3 Life cycles

Bacteriophages have been identified to proliferate through four different life cycles, of which the lytic and lysogenic cycles are the most extensively studied. Virulent phages follow the lytic mode, whereas temperate phages can undergo the lysogenic cycle. The key difference between the two cycles is the lysogenic phages' ability to integrate and remain dormant in the host genome until triggered to propagate. (Makky et al., 2021) Identifying the life cycle of a phage is crucial, as only lytic phages can be used for therapeutic applications. However, temperate phages could be genetically modified to become obligately lytic, thereby expanding their potential use in phage therapy.

In the lytic cycle, the host cell is immediately transformed into a phage factory to synthesize multiple copies of bacteriophages. This process involves early gene expression, genome replication and late lytic gene expression. Ultimately, the phage particles are assembled and the host cell lyses, releasing the mature virions as described in Chapter 2.2. (Strathdee et al., 2023)

In contrast, during the lysogenic cycle, temperate phages integrate their genetic material into the host cell genome, forming a prophage. Instead of instant expression, the so-called prophage remains dormant in the genome until triggered to enter the lytic cycle. Hence, the viral genes replicate passively as the bacterial cells divide. Lytic genes are blocked by lysis repressors until a chemical or physical stress triggers the lysogenicity to switch into lytic mode. (Makky et al., 2021) The lysogenic cycle is represented in Figure 3.

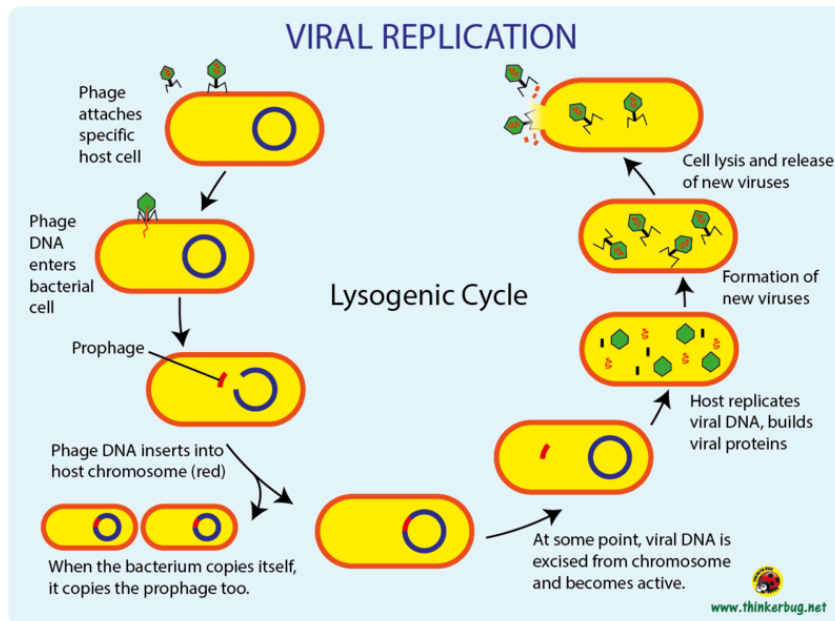


Figure 3. "Viral Replication", CC BY-SA 4.0 (Thinkerbug, 2021).

2.4 Transduction

When bacteriophages propagate, fragments of bacterial DNA may accidentally be incorporated into newly assembled phage particles. Following the release, when the progeny phages infect new bacterial cells, they inject not only viral genetic material but also pieces of bacterial genome. This process is known as transduction, a key mechanism of horizontal gene transfer driving the natural evolution and genetic diversity of bacteria. Transduction is further categorized into three main types: generalized, specialized and lateral transduction. (Chiang et al., 2019)

Out of the three transduction types, generalized and specialized transduction are the most studied. In generalized transduction, any part of the bacterial genome may be transferred to another bacterium. This occurs when a bacteriophage mistakenly packages random bacterial DNA during the phage assembly stage of the lytic cycle. However, the occurrence of this error is rare, with a probability of around 0.1%. (Makky et al., 2021) In contrast, specialized transduction includes the transfer of specific bacterial genes. During the lysogenic cycle, genes located next to the integration site of the prophage may be included in the excised DNA. As a result, both viral and bacterial genetic material can be packaged into phage capsids, and thus the formed viral and bacterial DNA hybrids are transferred to new bacterial cells. (Chiang et al., 2019) Generalized and specialized transduction are illustrated in Figure 4.

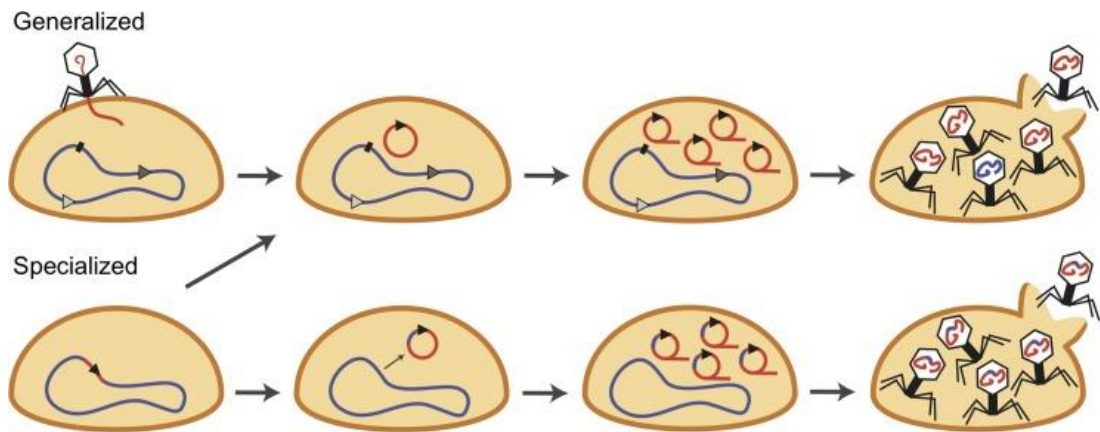


Figure 4. Generalized and specialized transduction, adapted from (Chiang et al., 2019).

The third, less common and more recently discovered type of transduction occurs due to an abnormal life cycle of a phage. In this case, the prophage begins replication before excising from the bacterial genome, which leads to the packaging of long bacterial DNA fragments into phage particles. (Chee et al., 2023) Lateral transduction has been observed in phages infecting *Staphylococcus aureus*, contributing to the development of AMR and the spread of virulence factors (Fillol-Salom et al., 2021).

To ensure stable transduction, the injected genetic material must be integrated into the bacterium's genome through homologous recombination. This process involves the exchange of nucleotide sequences between two matching DNA molecules and thus requires sequence similarity from the incoming and the host cell DNA. If this process fails, it is unlikely that the foreign genetic material will remain inside the cell during the divisions.

3. PHAGE THERAPY AS AN ALTERNATIVE TO ANTIBIOTICS

In 1928, the medical field took a massive step forward when Alexander Fleming discovered penicillin, the first natural antibiotic against multiple bacterial strains (Fleming, 2001). Just over a decade after, it was already approved for clinical use and produced on a large scale. This was followed by the discovery of several more classes of antibiotics that also started to enter the market. (Hutchings et al., 2019) These substances eliminate bacteria through different mechanisms, such as disrupting bacterial cell wall formation or inhibiting protein synthesis. Antibiotics quickly became widely used, but not long after Fleming's discovery, the first case of a resistant strain was already detected (Uddin et al., 2021).

Antibiotic resistance poses a serious threat to both humans and animals. Bacterial strains that are resistant to all known antibiotics are emerging, making it essential to explore alternative treatments capable of combating these superbugs. One such treatment, already used before the antibiotic era, has recently regained scientific interest. Phage therapy was explored and even implemented before the discovery of antibiotics, and scientists are now reevaluating its potential. With significant advancements in synthetic biology and genetic engineering, the use of bacteriophages as therapeutic tools is becoming a promising alternative in both human and veterinary medicine.

3.1 Antibiotic resistance and the return of phage therapy

Antibiotic resistance arises from the improper and excessive use of antibiotics (Chin et al., 2023). The rapid adaptation and evolution of bacteria enable gene mutations that favour their survival to spread, and in antibiotic-rich environments, these mutations are related to resistance. Horizontal gene transfer is responsible for transmitting genetic material through three key mechanisms: conjugation, transformation and transduction. In conjugation, two bacterial cells exchange genetic material via a pilus. In contrast, transformation occurs when bacteria take up genetic material from dead bacteria in the environment. Transduction, discussed in more detail in the previous chapter, involves a bacteriophage transferring genetic material from one bacterial cell to another.

Antibiotics are widely used in medicine but even more extensively in animal husbandry. Often, they are not only applied for bacterial infections, but rather as a precautionary measure. A significant portion of the antibiotics used is not fully absorbed and eventually

ends up in the environment, leading to antibiotic pollution. This exposure applies selective pressure on microbial populations: bacteria having the ideal mutations to resist the destroying mechanisms of the antibiotics survive and proliferate. As these bacteria replicate, they give rise to superbug strains. These bacteria can spread antibiotic resistance genes to other bacteria, including human pathogens, posing a serious threat to public health. (Chin et al., 2023)

Equines are highly susceptible to multiple bacterial infections. They spend a significant amount of time outdoors, so the risk of exposure to antibiotic resistant bacteria is higher than that for humans. In recent years, studies have shown a rising prevalence of multi-drug-resistant (MDR) bacteria among horses. MDR pathogens are resistant to more than three different antibiotic classes. (Kabir et al., 2024) When resistant bacterial strains spread among equines, they can also be transmitted to humans who work or spend time around them, further increasing the threat to public health.

The use of bacteriophages for the treatment of bacterial infections is called phage therapy. Due to the increase of antibiotic resistance over the past decades, there has been a renewed interest in this approach. Multiple case studies and clinical trials have demonstrated phage therapy's potential, especially in treating MDR bacterial infections. While phage therapy is still not widely available or adopted in mainstream medicine, continuous research and regulatory progress are facilitating its clinical applications.

The realization of phages' potential more than 100 years ago by Felix D'Herelle was followed by extensive research and experimentation worldwide. In 1925, D'Herelle and Alexander Yersin treated patients suffering from cholera, a bacterial infection in the small intestine, and out of a group of 16, all recovered. The mortality rate of a placebo group was 60%, whereas that of the phage-treated group was only 8%. (Marongiu et al., 2022) This success opened the doors for the therapeutic application of phages.

In one Indian region heavily affected by cholera outbreaks, phage therapy was administered as both a preventive and therapeutic measure for the population. As a result, no epidemics occurred during the treatment period, whereas in the neighbouring region over 1,500 people died from the disease in 1933 (Summers, 1993). Interest in phage therapy grew in the U.S. as well, where physicians conducted studies on its effectiveness. For instance, in one trial phage therapy resulted in over 90% success rate in the treatment of 57 patients with staphylococcal skin infections (Crutchfield, 1930).

However, due to the superior efficacy of antibiotics discovered in 1928, and at the time, a limited knowledge of phage physiology and safety, phage therapy was largely abandoned in most countries. Despite this, it remained in use in Georgia, Poland and Russia,

where research and clinical applications continued. However, regulatory frameworks for phage therapy differ between these countries, affecting its availability. (Yang et al., 2023)

In the former Soviet Union, where antibiotics were both scarce and often less effective, phage therapy was used to combat various bacterial infections. Clinical trials were conducted during the Winter War against Finland in 1939-1940, where phage therapy showed its efficacy against gas gangrene (Marongiu et al., 2022). Many other trials were also carried out, although with varying success rates.

Phages have been explored in veterinary medicine and studied in animal models since their discovery. Especially in the early stages of phage therapy, experiments to assess its efficacy were conducted in animals. However, the majority of the reports focus primarily on farm animals, as well as rats and mice. (Bianchessi et al., 2024) A few studies have also focused on equines - these are further explored in Chapter 4.

Over the past hundred years, numerous successful cases and trials have demonstrated the potential of bacteriophages in therapeutic use, even without a full understanding of phage biology or possibilities of genetic engineering. With today's advanced scientific knowledge and tools, solutions to previously unsuccessful phage treatments could be found, making the method more effective and reliable than before.

3.2 Phage therapy

At present, phage therapy lacks market approval in Western medicine. However, in some cases where antibiotic treatment has completely failed, emergency approvals for the use of phage therapy have been admitted. Such cases have been reported in France, Belgium, Poland, Australia and the U.S. (Patey et al., 2018) Since these are only exceptions, no officially registered phage therapy products are available, making it difficult to access ready-to-use phages. In special cases where phage therapy is considered, products can be sought from Russian or Georgian suppliers (Patey et al., 2018).

Bacteriophages are typically used either as a cocktail of multiple phage types or as a treatment with one specific species isolated from the environment of the target pathogen. Lytic phages are generally preferred for phage therapy owing to their immediate bactericidal action. (Kortright et al., 2019) Additionally, phage therapy has been studied in combination with antibiotics. Phages and antibiotics may work independently, or in synergy, resulting in more effective outcome of the treatment. Synergy between antibiotics and phage therapy has been observed, though most studies have been conducted in animal models rather than clinical trials. This synergy may occur through different mech-

anisms. For instance, a lytic phage that associates with a mechanism of antibiotic resistance in the target bacteria, may help to reduce the resistance. (Gu Liu et al., 2020) This helps to fight against bacteria that already have resistance to the antibiotic.

The delivery mechanism of phages depends on the type of infection. For wounds, phages can be applied topically as a cream formulation, which requires only minimal modification. In contrast, oral administration poses more challenges. Gastric acidity may inactivate phages, which must be taken into consideration. To overcome this issue, techniques such as alkanisation or encapsulation in acid-resistant capsules or pills, can be used. (Patey et al., 2018)

The use of bacteriophages in medicine requires high levels of purification, precise formulation and accurate dosing. Even with these measures, ensuring consistent quality remains more difficult for biological drugs compared to chemical alternatives. In vitro studies often fail to accurately predict the behaviour of phages in complex biological systems. Moreover, just as bacteria may develop a resistance to antibiotics, they can also evolve resistance to phages. Addressing these challenges is crucial before phage therapy can be widely implemented as a routine treatment. More clinical trials and investments are needed to ensure the efficacy and safety of phages as therapeutic tools.

3.3 Phage preparation

The success of phage therapy relies on the quality of the phage preparation. The stability, sterility and cytotoxicity of phages must be characterized, and impurities like endotoxins should be avoided. However, since these are biological drugs, the stability cannot be fully guaranteed. Usually phages are used in the form of a water suspension, where they are partially stable with the protein structure (Hibstu et al., 2022).

Phage particles are produced with well-known bacterial strains in precise and optimized conditions. Virus particles propagate inside their bacterial hosts, after which the cells lyse, and progeny phages are released into the environment. (Bretaudeau et al., 2020) Since phages are produced in bacterial cell cultures, the generated solutions include endotoxins, nucleic acids, peptidoglycan and other bacterial impurities. The removal of these particles is critical to ensure the safety of the phage products.

The purification of phage solutions can be carried out using different methods, including filtering, centrifugation and chromatography, which are widely used. This step is crucial to minimize the risks of proinflammatory responses. In the worst cases solutions containing bacterial endotoxins can result in intravascular coagulation, organ failures or even fatality. One possibility for purification is to use polyethylene glycol mediated precipitation

with cesium chloride gradient ultracentrifugation. Although the method is effective, it is time-consuming and may cause phages to lose their efficacy. Other possible methods are Tangential Flow Filtration and resin and membrane-based chromatography. (Roshankhah et al., 2023) All methods have advantages, but they often come with scalability issues and chemical additives can cause problems. Membrane chromatography could possibly be the best option for large-scale phage purification, since it is quick and doesn't include harmful additives (Roshankhah et al., 2023).

Before the therapeutic use of phages, they must be well characterized. This includes the determination of phage's host range, its ability to kill bacteria efficiently, as well as genome sequencing to detect possible antibiotic resistance and virulence genes. With new and developed methods, deeper analysis of phage particles and their functions is possible.

The characterization of phages provides essential information about their safety and potential. Viruses that have the possibility to infect eukaryotic cells should not be used in veterinary or human medicine. In addition, temperate phages or phages carrying toxin genes should not be considered. Morphological characterization can be done using Transmission Electron Microscopy or Atomic Force Microscopy, and Whole Genome Sequencing or Polymerase Chain Reaction could be utilized in genetic characterization. To determine the host range and phage adsorption, a spot test and adsorption assay can be used. Mass Spectrometry and X-ray Crystallography provide tools for more detailed functional and structural analysis. (Necel et al., 2020)

The stability of phage particles is a key factor in their therapeutic use. Proper formulation and storage under optimal pH and temperature conditions are crucial, although these factors vary depending on the delivery route. Organic solvents such as ethanol and isopropanol play an important role in liquid formulations and phage encapsulation. However, especially at high concentrations, these solvents can inactivate and damage phages. (Cao et al., 2023) Understanding phage morphology is essential when designing an optimal formulation, as structural features, like long protein tails or lipid components, can significantly influence the stability of the phage in different solvents.

Proper phage purification, characterization, formulation and storage are crucial to ensure successful treatment. These factors ensure the safety, stability and efficacy of phage therapy. Modern technologies make all this easier than ever before.

3.4 Phage libraries

Phage libraries are collections of properly characterized bacteriophages for different purposes, including molecular biology, biotechnology, drug discovery and therapeutic use. These libraries can consist of natural phages isolated from the environment, or genetically engineered phages tailored for specific needs. For safe and effective phage therapy, well-characterized and studied phages are required. (Gibson et al., 2019)

Precisely constructed, characterized and properly maintained phage libraries provide several advantages for clinical applications. Extensive phage analyses offer better understanding about their properties, while rapid screening enables the identification of the most effective phages for each infection. In the so called “sur-measure” approach, custom phage preparations are developed to match patient-specific needs (Pirnay et al., 2011). Customized treatments are possible by selecting or engineering phages to match infection-specific bacterial strains. Phage libraries also enable the development of phage cocktails targeting a broad range of bacterial strains and thus reduce the risk of phage resistance (Gibson et al., 2019).

Well-designed phage libraries provide valuable tools for improving the efficacy, safety and reliability of phage therapy. By maintaining diverse collections of phages, researchers and clinicians can find solutions for new bacterial infections or those that no longer respond to antibiotics. Continuous research and the integration of bioinformatics will most likely improve the development of phage libraries in the future.

3.5 Phage therapy compared to antibiotics

Phage therapy differs from traditional antibiotic treatments in several ways. Regarding their function, phages typically disrupt multiple processes within bacterial cells, ultimately leading to their lysis. On the contrary, antibiotics are designed to interfere with a specific process of the cell. In terms of resistance, whether it is against antibiotics or phages, it works by blocking the treatment’s mode of action.

The high specificity of phages ensures the elimination of the targeted pathogen, leaving the healthy microbiota intact. In the case of antibiotics, they often have a broad-spectrum effect, which can have negative impacts on the patient’s own microflora. A disrupted microbiome often leads to various other issues related to optimal immune system function. Allergic reactions, nausea, vomiting, gastrointestinal effects, neurological effects and fungal infections have been associated with antibiotic use (Mohsen et al., 2020). The severity and occurrence of these side effects depend on the antibiotic class used.

Several differences exist in the practical use of these treatments. Antibiotics have a short time between the diagnosis and treatment, as well as constant dosing. Phage therapy, however, requires a long time between diagnosis and treatment, which can pose challenges. Antibiotics are widely accepted in Western medicine, which is currently not the case with phage therapy. Diffusion through cell membranes makes it possible to treat intracellular bacteria with antibiotics. In contrast, phages are often unable to penetrate the intracellular matrix of eukaryotic cells. A key advantage of phage treatment over antibiotics is their self-amplifying action, meaning that they propagate only when the target bacteria is present. Consequently, a single dose may be sufficient for treatment. (Kortright et al., 2019) Phages also do not persist in nature after the treatment in the same way as antibiotics, leading to fewer ecological consequences.

There are a few major problems that are often discussed in the case of phage therapy. First, there is a significant variation among phages, with around 90% able to infect and kill the bacteria in a desirable manner (Nilsson, 2014). Additionally, as previously mentioned, bacteria can develop resistance to phages, which can complicate the treatment. Immunological responses in patients can occur, but a greater risk may be the rapid release of bacterial toxins upon cell lysis (Nilsson, 2014). More about the challenges around phage therapy are explored in Chapter 6.

Combining phage therapy with antibiotics may help to address some of the challenges. Studies have shown synergy between these two treatments, leading to improved phage adsorption and shortened latent periods. This combination is particularly beneficial for bacterial biofilms, which are challenging to treat and often resistant to antibiotics. (X. Li et al., 2021)

Challenges and advantages of phage therapy and antibiotics are summarized in Table 1. Many of the challenges surrounding phage therapy could possibly be solved with modern technologies discussed in Chapter 5. More about possible solutions is explored in Chapter 6.

Table 1. Challenges and advantages of antibiotics and bacteriophages

	Bacteriophages	Antibiotics
Advantages	<ul style="list-style-type: none"> - Self-amplifying action - Activity against antibiotic resistant bacteria - Preserves healthy microbiota - Doesn't persist in nature - Could be genetically modified 	<ul style="list-style-type: none"> - Widely accepted and available - Diffusion through cell membranes - Ease of use - Mass production
Challenges	<ul style="list-style-type: none"> - Not allowed as a routine treatment - Phage resistance - Immunological responses - Unable to penetrate the intracellular matrix of eukaryotic cells - Complex production - Stability and storage limitations 	<ul style="list-style-type: none"> - Antibiotic resistance and the rise of "superbugs" - Microbiome imbalances - Accumulation in the environment - Different types of side effects: allergic reactions, gastrointestinal effects, nausea, vomiting, neurological effects, fungal infections - No self-replication - Long-term health effects

4. PHAGE THERAPY FOR EQUINE DISEASES

Bacterial infections are particularly common in equines, spreading easily both within the stable and between different stables through human contact. Effective control of these diseases is crucial owing to the economic and social significance of horses. Since the discovery of antibiotics, they have been the primary treatment for bacterial infections; however, AMR is an increasing concern in equine populations. Additionally, antibiotics are associated with several adverse effects, such as disruptions of the gut microbiota, allergic reactions and loss of appetite (Kabir et al., 2024). These factors highlight the importance of exploring alternative treatments, including phage therapy, for bacterial infections.

4.1 Staphylococcal Superficial Pyoderma

Staphylococcal skin infections appear quite frequently among horses. This is mainly due to their thick fur and exposure to wet environments, which make it difficult to manage these conditions. The treatment typically involves long-term antibiotic use, which can disrupt the microbiota and lead to various other issues. Moreover, *Staphylococcus aureus*, in particular, is one of the most resistant skin-infection causing pathogens (Foster, 2017). Consequently, on top of the microbiome disruption, antibiotic treatment may be completely ineffective in the case of resistant strains. At worst, these two combined may weaken the immune system and result in even worse infection.

There are relatively few studies on the use of bacteriophages in the veterinary field, but some of them have shown promising results. One successful clinical trial, reported in 2010, explored the use of phage therapy against *Pseudomonas aeruginosa* in dogs with otitis. In this study, a phage cocktail consisting of six strains was prepared and used, leading to a reduction in bacterial cell counts in all the dogs treated. (Hawkins et al., 2010) This success inspired researchers to further explore the use of phages in veterinary medicine.

A more recent trial investigated the efficacy of phage therapy for treating Staphylococcal Superficial Pyoderma in equines. The therapy focused on the treatment against *S. aureus*, which can be found on healthy equine skin, but poses a risk if it invades to underlying epithelial tissues. A cocktail of two lytic phages belonging to the *Podoviridae* family were used for the treatment. These phages had 100% in vitro inhibition coverage against eight *S. aureus* strains that were screened for the preparation. Bacterial strains were

characterized using matrix-assisted laser desorption ionization time-of-flight mass spectrometry, and they were used for the propagation of phages. (Marshall & Marsella, 2023)

The trial involved 20 horses suffering from skin infections caused by *S. aureus*. These horses received treatment with phages and a placebo at different infection sites, administered once daily for four weeks. However, unlike the successful phage therapy against *P. aeruginosa* in dogs, this study did not result in a significant reduction in bacterial cell counts. (Marshall & Marsella, 2023)

Several factors may explain why phage therapy was not successful in this case. Although the therapy may have been effective against *S. aureus*, it is possible that other cocci strains continued to proliferate. Cell counts were recorded without identifying specific bacterial strains, so other strains may have colonized the infection sites while *S. aureus* was destroyed. The study also failed to control the underlying cause of the pyoderma, which could have contributed to the lack of improvement. Furthermore, equine skin infections may be difficult to treat with phages, as they get easily exposed to harsh conditions, such as UV rays, wind and rain. These factors may inactivate phages and cause issues related to their stability.

While phage therapy holds potential as an alternative treatment for equine pyoderma, further research is necessary to refine its application. To improve treatment efficacy, phage cocktails should target multiple bacterial strains, not just one, and the underlying cause of the infection must be more clearly understood. In addition, more detailed analysis of bacterial strains before and after the treatment would provide better information about the success of the treatment.

4.2 Bacterial Keratitis

The large eye size of horses makes them susceptible to various corneal infections, including bacterial keratitis, which is usually caused by *Staphylococcus*, *Streptococcus* and *Pseudomonas* species. Among these, *Pseudomonas aeruginosa* is known for its rapid infection, which can lead to blindness if not treated effectively. (Furusawa et al., 2016) This pathogen can be transmitted through a contaminated environment or horse-to-horse transmission, and is able to exist as biofilms, making it a serious concern (Kabir et al., 2024). Furthermore, *P. aeruginosa* has developed resistance to multiple antibiotics, making treatment even more difficult (Pang et al., 2019).

Although no studies of phage therapy for bacterial keratitis directly in horses have been conducted, it has been studied in mouse models with positive results. In a study published in 2016 in Japan, broad host-range phages from the *Myoviridae* and *Podoviridae*

families were isolated from sewage water and used to treat a mouse model simulating equine bacterial keratitis. The mice were infected with *P. aeruginosa* strains that cause eye infections in both dogs and horses. The phage therapy was highly effective, killing the bacteria when administered within three hours of infection. The phages demonstrated an adsorption rate of more than 80% within 30 seconds of being applied to the host cells. (Furusawa et al., 2016)

This study is particularly relevant for the equine veterinary field, as it suggests that phage therapy could potentially replace antibiotics currently used in racehorse eye drops. Traditionally, horses' eyes are washed with antibiotics after a race to prevent infections, but phage therapy could be a more sustainable and targeted alternative. The high adsorption rate demonstrated in the study is crucial, since the eye drops are often rapidly washed out in tears. If horses' eyes are washed immediately after a race, phage therapy could work to prevent infection. However, after three hours, phage therapy becomes less effective, as the bacteria may have already invaded deeper layers of the corneal stroma, where the phages cannot reach. The study demonstrated that 6-12 hours after bacterial infection, phages did not prevent bacterial growth (Furusawa et al., 2016). Therefore, the best outcome would most likely be achieved by administering phage therapy as a preventive measure before the race to avoid the infection in the first place.

In contrast to the study focusing on Staphylococcal skin infections, in this case, the underlying cause of the disease was clearer. In addition, more detailed analysis of phage morphology and function was conducted with electron microscopy and adsorption rate analysis. The treatment was well designed based on both the phages' and the target bacteria's functional features. Based on the investigation of the most effective phages, a cocktail of the two best against *P. aeruginosa* was selected. (Furusawa et al., 2016) All these factors resulted in positive and useful outcomes, considering further development and implementation of phage therapy.

4.3 Future prospects and importance

Equines play a significant role both economically and socially. The industry directly supports commercial sports, wholesale trade, hospitality, real estate, agriculture and livestock. In addition to these, horses are involved in law enforcement, military operations, public transport, tourism and safari expeditions. (American Horse Council (AHC), 2023) Unfortunately, the presence of infectious diseases significantly limits the potential of the equine industry.

Antibiotics have long been essential tools for preventing and treating bacterial infections in horses. However, AMR in equines has become a serious threat over the past few decades, with spreading pathogens that are increasingly difficult to treat. Beyond the economic losses to the equine industry, resistant pathogens present in the environment can also transfer their resistance genes through horizontal gene transfer, posing a major threat to human health. Some pathogens, such as *Rhodococcus equi*, can even directly infect humans (Weinstock & Brown, 2002).

The excessive, and in the equine industry often preventive, use of antibiotics exerts selective pressure on bacteria, driving the emergence of AMR strains. In addition, antibiotics negatively affect the complex equine digestive system and microbiota, leading to various issues in their immunity and overall well-being. Normal gut flora is essential for preventing pathogen colonization and supporting efficient digestion but also has an influence on the gut-brain axis and behaviour. Various cases of severe side effects caused by antibiotics have been reported in recent years. (Kabir et al., 2024) New methods for both preventing and treating bacterial infections in horses are needed. As discussed in the previous chapters, the industry has started to explore the use of bacteriophages as a potential treatment.

At present, phage therapy has not developed sufficiently to entirely replace antibiotics. Despite that, the combined use of antibiotics and bacteriophages could be a viable approach. In preventive applications, phages could possibly substitute antibiotics completely. This would eliminate the need for prophylactic antibiotic use.

Based on the few studies available, using a cocktail of two or more broad-range phages is more effective than using only one. Often bacterial infections are caused by multiple species, which is why a wide host range is important. Combining multiple phages with a wide host range eventually results in an even more effective outcome. The use of phage cocktails has also been shown to reduce the formation of phage-resistant bacteria. (Furusawa et al., 2016)

Phage therapy for horses could have multiple positive impacts on the equine industry, the environment and human health. Reduced antibiotic use would directly decrease the development of resistant bacterial strains and their associated negative effects. This, in turn, would lessen the burden on both veterinary and human healthcare sectors while supporting the success of the equine industry. If phage therapy proves to be effective in equines, it could potentially be adapted for use in human medicine as well.

It is crucial to develop innovative solutions before antimicrobial resistance becomes entirely uncontrollable, although the situation is already alarming. Continued research and

investments in alternative treatments, such as phage therapy, are crucial steps towards mitigating the growing threat of AMR.

5. GENETIC ENGINEERING OF PHAGES

Genetic engineering of bacteriophages refers to the modification of their genomes to enhance phages' properties. With various tools and advanced methods of molecular biology, it is possible to increase their therapeutic potential and provide more targeted treatments than before. The desired modifications, complexity of the genome as well as the final use determine which method would be the best in each case. In recent years, advanced sequencing techniques combined with new genome editing methods have revolutionized bacteriophage engineering, enabling the generation of phages with unique features.

5.1 Traditional methods

Traditional methods include techniques using homologous recombination, which refers to the exchange of similar nucleotide sequences between two DNA molecules. This can be achieved in different ways, including the phage crosses method and homologous recombination between a plasmid and the wild-type phage genome. In the phage crosses method, two parental phages are used to generate mutant progeny phages, whereas in the plasmid-based method only one phage and bacterial cell carrying a plasmid are required. (Chen et al., 2019)

In the traditional phage crosses method, host cells are infected with two different phages carrying at least two selective markers. This allows for homologous recombination between parental phage genomes, after which the resulting progeny are screened for the desired phenotypes. If successful recombinants are obtained, they are purified for further analysis. (Chen et al., 2019) However, this method only allows for the combination and exchange of existing phenotypes rather than the introduction of specific genetic modifications, which limits its use. The phage crosses method is illustrated in Figure 5.

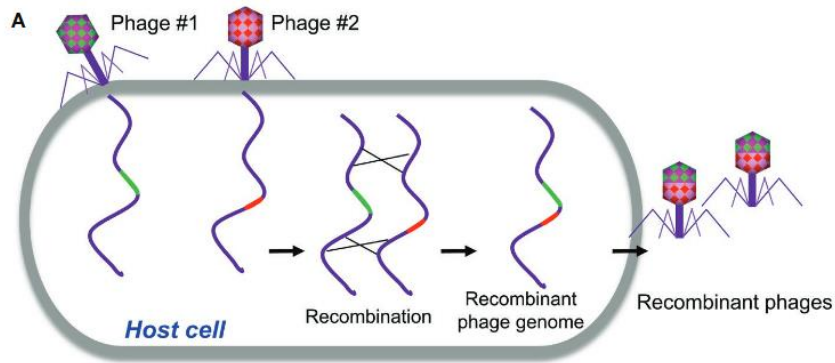


Figure 5. Phage crosses method, adapted from (Chen et al., 2019).

Homologous recombination between a phage genome and a plasmid enables the generation of recombinant phages with gene insertions, deletions and replacements (Chen et al., 2019). A plasmid carrying the desired mutation is constructed and can be transformed into bacterial cells using electroporation (Oda et al., 2004). Bacteria are then infected with the target phage that is being modified. If recombination takes place successfully between the plasmid and the phage DNA, the resulting progeny phages contain the intended modifications. (Chen et al., 2019)

Traditional homologous-recombination-based methods are time-consuming and often have low efficiency, making it challenging to create phages with desired traits. These methods rely on the natural recombination machinery of the host cell and may lead to unpredictable outcomes. Extensive screening is required to isolate successfully modified phages. In contrast, new approaches such as CRISPR-Cas-based editing and synthetic genome assembly, provide more precise and efficient tools for faster large-scale phage engineering.

5.2 Modern methods

Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-based gene editing techniques allow scientists to selectively modify genomes in a cost-effective and precise way. CRISPR-Cas9, in particular, is a technology that has gained significant attention in recent years. In addition to editing genomes of living organisms, this method can also be used to modify bacteriophages.

The CRISPR-Cas systems occur naturally in some bacteria and archaea, enabling them to defend against phages and other external genetic elements. The mechanism works by integrating short viral sequences into the cell's CRISPR locus, allowing the organism

to recognize and clear future infections. The CRISPR array consists of repeated sequences separated by spacers, and can be found in both chromosomal and plasmid DNA. (Rath et al., 2015)

The spacers function as a molecular memory, allowing prokaryotes to recognize matching foreign genomes. By acquiring new spacers, new foreign DNA can be recognized. Following spacer acquisition, CRISPR RNA (crRNA) is expressed, and with the aid of RNA polymerase, pre-crRNA is transcribed into mature crRNA. Finally, crRNA matches with the target sequence, while guiding a Cas protein to cleave the invading genetic elements. Some CRISPR-Cas systems require a trans-activating crRNA (tracrRNA) to function properly. (Rath et al., 2015)

CRISPR-Cas-based genome editing relies on two key features of the prokaryotic adaptive immune system. Complementary base pairing between crRNA and the original spacer helps to locate the target sequence, while the Cas protein's nuclease activity facilitates the cleavage of the DNA (Zhang et al., 2022). The CRISPR-Cas complex binds to the target sequence with the aid of crRNA, which has a complementary sequence to the DNA. Cas proteins cleave the DNA by creating a double-strand break at the target site. CRISPR-Cas systems can be further classified into different types depending on the activity of the Cas proteins. (T. Li et al., 2023)

Among the various CRISPR-Cas systems, CRISPR-Cas9 is one of the most widely used in genome editing. In this technique, a synthetic RNA, called a single guide RNA (sgRNA), which is a simplified version of crRNA, is used to guide Cas9 to cleave the DNA at a specific site, upstream of the Protospacer Adjacent Motif (PAM) sequence. After the cleavage, the cell's own DNA repair machinery is used to introduce the desired mutations. (T. Li et al., 2023) For instance, the modifications can be cloned into a donor plasmid or a repair template, which is transformed into a bacterial host cell. The DNA break is repaired via recombination with the DNA that carries the desired modifications (Zhang et al., 2022). The CRISPR-Cas9 method is represented in Figure 6.

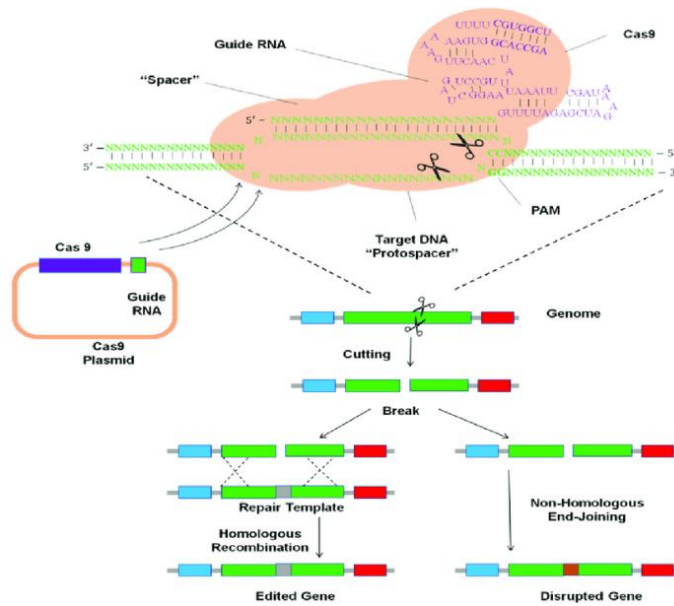


Figure 6. CRISPR-Cas9 method, adapted from (Upadhye J et al., 2023).

The natural CRISPR-Cas9 system has been extensively studied in order to modify it to meet scientific needs. Its efficiency and precision make it an extremely useful tool for genetic engineering in various scientific disciplines, including phage therapy. Future advancements in this technology will most likely enhance its accuracy and usability.

Synthetic biology includes the artificial design and synthesis of new biological particles and systems, including DNA fragments, that do not yet exist in nature. This approach can be used to create phages with interesting features for therapeutic use. New strategies enable the construction of genomes with better properties, such as more efficient bacterial cell detection and a wider host range.

Following the sequencing and design with computer software, various strategies can be utilised in the construction of new DNA fragments. For instance, Transformation-Associated Recombination (TAR) and Gibson Assembly (GA) have been studied and used in phage genome construction. With chemically synthesized oligonucleotides, it is possible to create completely novel bacteriophages (Sun et al., 2023). CRISPR-Cas systems can also be used as a tool in synthetic biology.

GA allows for the *in vitro* assembly of several DNA fragments to form a large DNA construct, while TAR uses yeast cells for the assembly. In GA, DNA fragments are joined together chemically in a single reaction, with the aid of DNA polymerase, endonuclease and DNA ligase. TAR, on the other hand, is a cell-based mechanism, relying on yeast's recombination machinery to assemble the fragments. (Sun et al., 2023)

With synthetic biology, it is possible to design and create new phages for therapeutic applications. With advanced DNA sequencing technologies, oligonucleotide synthesis

and DNA assembly methods, it is possible to build new genomes with novel characteristics. These techniques open possibilities for developing bacteriophages with improved specificity and efficiency in therapeutic use.

6. CHALLENGES

Although phage therapy presents a promising alternative for treating bacterial diseases, several regulatory and technical challenges must be addressed. One major hurdle is the variation in legislation between countries, which complicates access to suitable phages for medical and veterinary use. Additionally, the cost of phage therapy remains high compared to traditional antibiotics due to the lack of similar large-scale production.

From a technical perspective, challenges regarding the treatment include issues related to the formulation, delivery and dosage. Successful phage therapy should be effective while minimizing negative side effects. Furthermore, phage resistant bacteria and biofilms are also significant concerns, requiring more research.

6.1 Regulatory aspects

The lack of regulatory approval of phage therapy remains one of the greatest limitations of the treatment in most countries. In the EU and the U.S., phage products fall under pharmaceutical legislation, and the efficacy, safety and quality must be guaranteed by a manufacturer following Good Manufacturing Practise (GMP) standards (Pires et al., 2020). However, even in the absence of efficacy data and general flexibility, in some countries phage therapy can be approved if antibiotic treatment has failed to bring the desired result (Strathdee et al., 2023). In addition, for veterinary use and animal feed, the legislation is less strict. Some phage products have already received “Generally Recognized as Safe” (GRAS) status, and can be used as a preventive measure in animal food (Pires et al., 2020).

In order to fall under the requirements of the legislation, phage products must go through strict purification and formulation processes. The absence of endo- and exotoxins, as well as other impurities, must be guaranteed to minimize immunological responses. Phages must also have a good self-life in the formulation. (Pires et al., 2020) In case they lose their activity, the treatment will most likely be unsuccessful. The formulation of a phage product must always be designed individually, since the properties of each phage type may significantly differ depending on the structural features of the strain.

The availability of phage products for medical and veterinary use is currently low, with the exception of Georgia, Russia and Poland (Yang et al., 2023). Updated policies are needed to provide phage therapy as a routine treatment. Fortunately, some countries are loosening the restrictions, and phage therapy can be provided especially in life-

threatening cases. Although the regulatory framework differs, these countries include the United Kingdom, France, Belgium, Australia, India, China and the U.S. (Yang et al., 2023).

6.2 Technical challenges

One of the main technical challenges in phage therapy is related to the development of phage resistant bacteria. Bacteria may develop resistance against phages as in the case of antibiotics. Different resistance strategies include, for instance, modification of bacterial cell surface receptors or blocking phage DNA transcription, replication or phage protein synthesis. In addition, phage DNA degradation by CRISPR-Cas results in unsuccessful phage infection.

Phage resistance is a frequently discussed topic in phage therapy, and several strategies have been developed to minimize it, or to combat already phage resistant bacteria. Genetic engineering opens new possibilities, for example, bacteria that have developed phage resistance through the modification of cell surface receptors, could be treated with a phage that has been modified to recognize the new receptors. To prevent the formation of phage resistant bacteria, it has been shown that phage cocktails work better than treatment with only one phage strain (Zalewska-Piątek, 2023).

The possibility of some bacteria to form biofilms can pose difficulties for phage therapy. Often, phages are not able to penetrate through a thick matrix formed by the bacteria, which makes the treatment ineffective. However, phages have developed strategies to overcome this issue. With depolymerases, enzymes that degrade bacterial polysaccharides, phages can get better access to the biofilm (Pires et al., 2020). In terms of phage therapy, natural phages not carrying this feature could potentially be used in combination with enzymes that target the specific biofilm.

On top of possible phage resistance and ineffectiveness against bacterial biofilms, the immunogenicity of phages may result in unsuccessful phage therapy. Phage-specific humoral memory, as well as the rapid lysis of bacterial cells, may both pose challenges. Especially in high doses of phage therapy, the release of large amounts of bacterial endotoxins can trigger inflammation (Krut & Bekeredjian-Ding, 2018). Consequently, it is critical to design the phage product and dosage carefully to avoid unintended consequences. In terms of the adaptive immune response, the recognition and destruction of phages as viral particles may cause problems for the treatment (Krut & Bekeredjian-Ding, 2018).

7. CONCLUSIONS

Increasing antibiotic resistance and the negative side effects of these drugs necessitate the exploration of other, more sustainable alternatives. Based on this literature review, bacteriophages represent an intriguing area of research, and phage therapy will most probably increase in the future as an alternative to antibiotics. However, since phages function as biological drugs, thorough research is required before their widespread application in mainstream medicine and the veterinary field.

Although phage therapy was previously displaced by antibiotics due to their superior efficacy, advancements in molecular biology and genetic engineering now provide new opportunities to overcome previous limitations. Clinical trials have proven the potential of phage therapy even before proper understanding of phage morphology and function, so with today's scientific expertise, it is valid to take another look at these viruses.

Antibiotic resistance is rising rapidly in equine populations due to the high frequency of bacterial infections and thus antibiotic use. A significant portion of these drugs ends up in the environment as antibiotic pollution, giving rise to MDR strains. These bacteria pose a serious risk to public health, as they can transfer antibiotic resistance genes to human pathogens. Given this, phage therapy could serve as an excellent alternative in the equine industry, decreasing the spread of resistant strains and health risks for both animals and humans. If proven successful in the veterinary field, phage therapy could be considered in human medicine more easily.

Answers to the research questions were mostly found, although phage therapy remains an area requiring more research, and many aspects are still not fully understood. History has shown the efficacy of phage therapy, and with today's scientific knowledge and advancements, it could be more effective than ever before. While phage therapy shows vast potential, it is not yet effective enough to completely replace antibiotics. However, a combined approach using both antibiotics and phages could effectively treat many infections, reducing antibiotic use and mitigating environmental pollution. In cases of preventive antibiotic use, phages could potentially serve as a full replacement.

Despite its promise, challenges such as phage resistance and immunological responses must be addressed. Genetic engineering, especially CRISPR-Cas9-based methods and synthetic biology, expand the possibilities of phage therapy, but at the same time, raise more ethical concerns. More research is needed, and international collaboration is crucial to establish phage libraries and share knowledge. Regulatory frameworks should be

updated to align with current scientific advancements and facilitate the development of phage-based treatments.

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