Phage Therapy: A Potential Novel Therapeutic Treatment of Methicillin-Resistant *Staphylococcus aureus*

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Abstract

The emergence of multidrug-resistant bacterial strains, especially in the clinical setting, has renewed interest in alternative treatment methods. The utilization of prokaryotic viruses in phage therapy has demonstrated potential as a novel treatment method against multidrug-resistant bacterial infections. As the post-antibiotic era quickly approaches, the development and standardization of phage therapy is critically relevant to public health. This review serves to highlight the development of phage therapy against methicillin-resistant *Staphylococcus aureus* (MRSA), an antibiotic-resistant bacterial strain responsible for severe clinical infections.

Keywords: Bacteriophage therapy, MRSA, antibiotic resistance, virulence factors

1.0 Introduction

1.1 Brief History of Phage Therapy

Phages were discovered independently in 1915 by the British microbiologist Felix Twort and in 1917 by French-Canadian microbiologist Felix d'Herelle. Responsible for the systematic investigation of the nature of bacteriophages, d'Herelle, in 1921, first utilized phages for the treatment of dysentery in Paris, France[1]. This treatment resulted in the rapid recovery of patients and brought relevance to phage therapy as a clinical treatment method. Continued study and clinical use led to d'Herelle becoming the leading expert on phage therapy in this period. Throughout the early

20th century, d'Herelle and other microbiologists isolated phages to treat pathogenic bacteria such as *Shigella dysenteriae, Salmonella typhi, Escherichia coli, Pasteurella multocida, Vibrio cholerae, Yersinia pestis, Pseudomonas aeruginosa, Neisseria meningitis,* and various strains of *Streptococcus*[2]. By 1931 d'Herelle had established phage therapy centers worldwide in the United States, France, and Soviet Georgia. While phage therapy showed promise, it was weighed down by a few major problems. These problems included host range, genetic variation, and the inability to consolidate the value of phage in preventing infectious disease. These problems eventually led to the fall of phage therapy. In this period, phage therapy was poorly incorporated into medicine, lacking theories that could be integrated with other notions of conventional medicine[3]. When antibiotics were discovered to be an efficacious treatment method against bacterial infections, phage therapy was abandoned.

At the turn of the 21st century, the field of medicine faced a new challenge. The mass application of antibiotics in the 20th century led to the prevalence of antibiotic-resistant bacterial strains[4-6]. The inability to treat these bacterial infections with standard antibiotics makes them a significant threat to public health. The continued prevalence of antibiotic-resistant bacterium has led to the need for new novel antimicrobial agents. This need has renewed interest in phage therapy as a potential novel treatment. When considering phage therapy in this modern era, there are three significant characteristics of phages that lead to their consideration as a potential treatment method: 1) Host specificity: Phage targets bacteria with high specificity. This characteristic ensures that phage treatment would only infect the target bacteria while natural microbiota is unaffected. 2) Genetic engineering: Genetic engineering was not an available option in the early stages of phage therapy. With current advances in science, we can now engineer phages to express traits of potential value. 3) Phages are ideal candidates for co-therapy with antibiotics: Co-therapy involves using both antibiotics and phage therapy to treat multidrug-resistant bacteria. The advancement of science since the discovery of phages in the early 20th century has led to a greater understanding of phages and an increased ability to utilize them for the benefit of public health[7-11].

The discovery of antibiotics revolutionized modern medicine, but the increased prevalence of antibiotic-resistant bacteria[4-6] has threatened their effectiveness. As we enter the 21st century, the prevalence of antimicrobial resistance (AMR) in bacteria has increased due to the massive and sometimes inappropriate use of antibiotics. Antibiotic-resistant bacterial infections account for over 2.8 million infections and 35,000 deaths annually in the

United States alone[5]. The continued occurrence and prevalence of antibiotic-resistant bacterial strains is considered a serious threat to global health and the economy. The Institutes of Medicine estimates that the annual cost of antibiotic-resistant bacterial infections in the United States is approximately 4 to 5 billion dollars[12,13]. Increased prevalence of antibiotic-resistant bacterial strains, as well as a decrease in antibiotic development, is a critical issue in the field of medicine[14,15]. These issues have exacerbated the need for new, novel treatment methods for antibiotic-resistant bacterial infections.

In recent research into antibiotic alternatives, bacteriophages and their components have gained relevance as potential novel treatment methods[7-11]. Phage therapy utilizes phage particles that specifically infect and lyse bacterial cells. A significant benefit of phage therapy is host specificity; phages only infect prokaryotic cells and cannot infect eukaryotic cells. However, new alternative treatment methods for bacterial infections are subject to technical and regulatory challenges. Challenges of alternative treatment methods such as phage therapy include activity spectrum, pharmacokinetics, immune response, manufacturing logistics, regulation, quality control, and market acceptance[16]. While these alternative treatments may not replace antibiotics completely, it has been suggested that use in unison with antibiotics could be a potentially viable method for treating multidrug-resistant bacterial strains[17-19]. This review will focus on developing phage therapy specifically against methicillin-resistant *Staphylococcus aureus* (MRSA), a severe threat to public health.

2.0 Phage Therapy

2.1 Methicillin-Resistant S. aureus

This review focuses on one of the most common and relevant multidrug-resistant bacterial strains, Methicillin-resistant *S. aureus* (MRSA). MRSA, commonly found in healthcare facilities, has been classified as a severe threat to public health[5,20]. The prevalence of MRSA infections in healthcare facilities poses a major threat to patients with compromised immune systems and is responsible for co-infections. *S. aureus* infections are characterized by red, swollen pustules on the skin's surface accompanied by a fever. Infections are commonly treated with antibiotics; however, the methicillin-resistant variant of *S. aureus* is resistant to standard clinical antibiotic treatment. Untreated MRSA infections can lead to pneumonia and, in severe cases, sepsis. Literature has determined that the current

mortality rate of MRSA infections is approximately 32 percent[21,22]. Considering this, the development of alternative treatment methods is essential to the preservation of public health. Figure 1 depicts the chronological map of *S. aureus* treatment. In 1940, the discovery of penicillin as a miracle drug offered unlimited hope to bacterial control; however, within the space of two years, *S. aureus* developed resistance to penicillin[7,23-25]. By 1960 over 80% of *S. aureus* strains had developed resistance to penicillin[23,25]. Methicillin was introduced in 1961 as an alternative treatment of *S. aureus*. Only a year later, *S. aureus* developed resistance to this antibiotic as well[25]. The first outbreak of MRSA was recorded in 1968, followed by the second and third outbreaks between 1970 and 1980[25]. By 1980 MRSA had spread worldwide. In 1990, vancomycin became the drug of choice against MRSA[25,26]. However, there was an observed rise in intermediate vancomycin resistance, leading to the occurrence of complete vancomycin resistance in 2002[25,26]. Since 2002, MRSA prevalence and a decrease in antibiotic development created a severe risk to public health. Several researchers have delved into antibiotics against MRSA; however, none have reached clinical applicability[27,28]. For instance, in 2019, Nicolas et al. showed that peptidomimetics-cyclic heptapseudopeptides were effective against MRSA in both mild and severe sepsis, and these antibiotics did not pose any health threat to humans in an *in vitro* study[27]. Further animal studies also confirmed that there was no toxicity recorded for mouse models and zebrafish embryos[27]. In another study in the same year, Geitani et al. reported that two novel peptides named "LL-37 and CAMA" were potent against clinical isolates of MRSA[28]. The progress of antibiotic development for MRSA has since declined due to the cost of production for these highly specialized semi-synthetic compounds. Due to this decline in antibiotic drug development paired with the increased cost of these drugs, bacteriophage therapy has once again become relevant in the field of therapeutics. In 2009, a group of researchers examined the safety of bacteriophage-based formulations for treating wounds caused by *S. aureus*[29]. In phase I clinical trial, they reported no safety concerns with the use of bacteriophage treatment; nonetheless, they encouraged a vigorous test for the efficacy of the phage preparations in a phase II trial[29]. In 2013, a bacteriophage lysin named "PlySs2", an aminopeptidase was reported to have bactericidal activity, exhibiting a MIC of 16 μg/ml for MRSA with a single dose of 2-mg of PlySs2 being potent enough to confer 92% protection against MRSA in mice[30]. This peptide showed notable broad lytic activity also against *S. pyogenes*, with high thermostability, hence presenting as a good candidate for MRSA therapeutic[30].

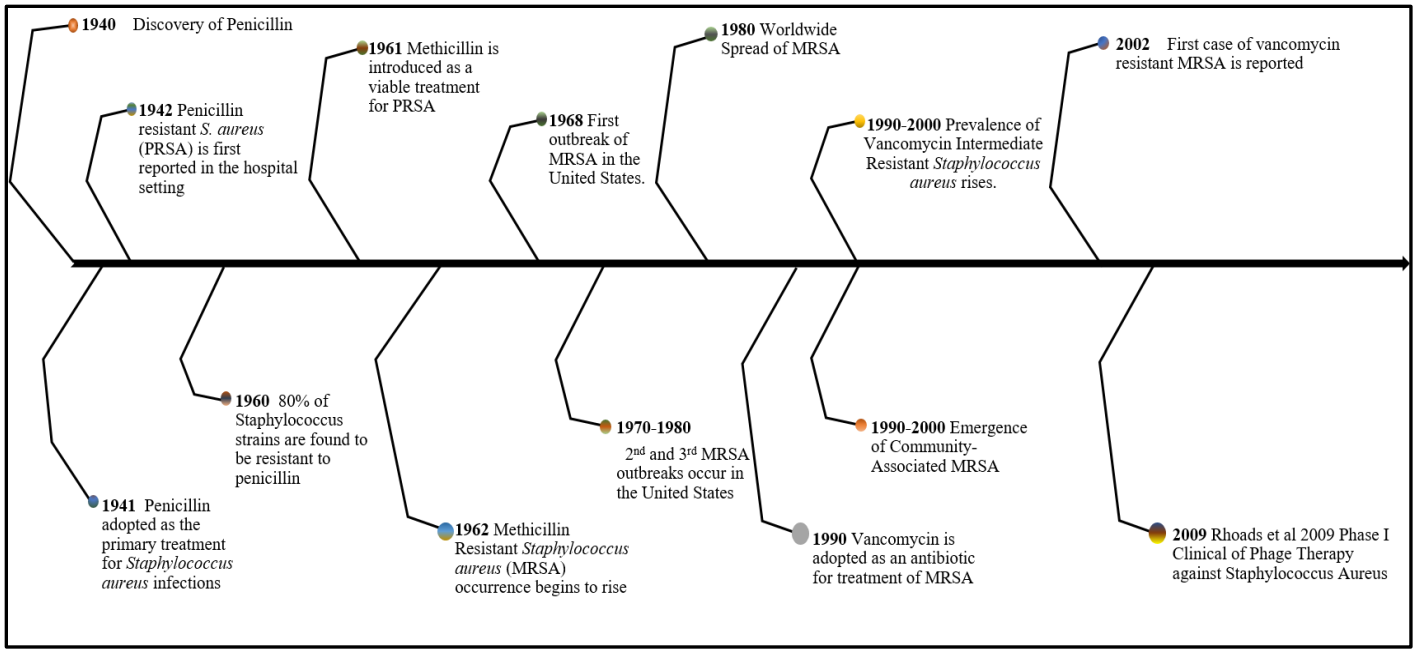

Figure 1. A chronological map of S. aureus treatment, evolution, and impact.

2.0 Virulence Factors Associated With MRSA

Antibiotic resistance is the primary clinical obstacle for the treatment of MRSA infections. Therefore, it is crucial to understand the virulence factors that facilitate this resistance. MRSA infections are resistant to beta-lactam antibiotics such as penicillin and semi-synthetic antibiotics such as methicillin, which were the standard treatment of *S. aureus* before MRSA[23]. To understand the virulence factors that allow for MRSA's antibiotic resistance, it is essential to also understand the evolution of *S. aureus* infections. As figure 1 outlines, *S. aureus* has gradually developed resistance to antibiotics, starting with penicillin in the form of penicillin-resistant *S. aureus* (PRSA), which was first reported in 1942[7,24]. The virulence factor present in PRSA was determined to be the gene *blaZ*[7,31]. This gene inhibits the binding of penicillin-binding proteins (PBPs) that function to disrupt peptidoglycan cross-linking during cell wall synthesis[23].

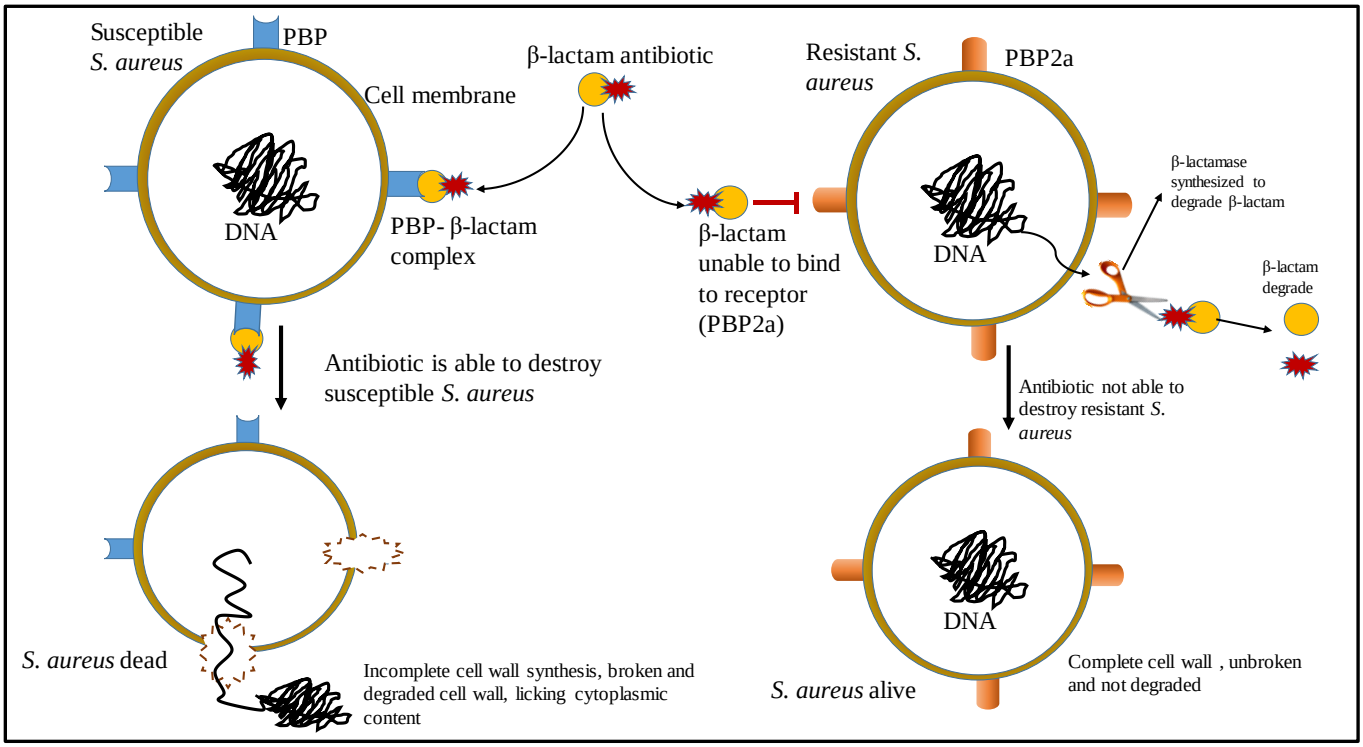

Figure 2. Diagrammatic illustration of the mechanism of inhibition of antibiotic resistance of MRSA

As shown in Fig.2, this inhibition is achieved through the production of beta-lactamase enzymes and structural alteration of the PBP receptor[23]. This virulence factor resulted in methicillin becoming the new standard antibiotic treatment. *S. aureus* and PRSA eventually developed new virulence factors for resistance of methicillin resulting in MRSA[7,24]. Methicillin resistance results from the development of a mobile genetic element called the staphylococcal cassette chromosome *mec* (SCC*mec*)[24]. The SCC*mec* genetic element contains the gene *mecA* that inhibits methicillin binding to the PBP 2a receptor[24,32]. Methicillin utilizes the PBP 2a receptor for the disruption of peptidoglycan cross-linking during cell wall synthesis. This structural change influenced by *mecA* results in methicillin resistance.

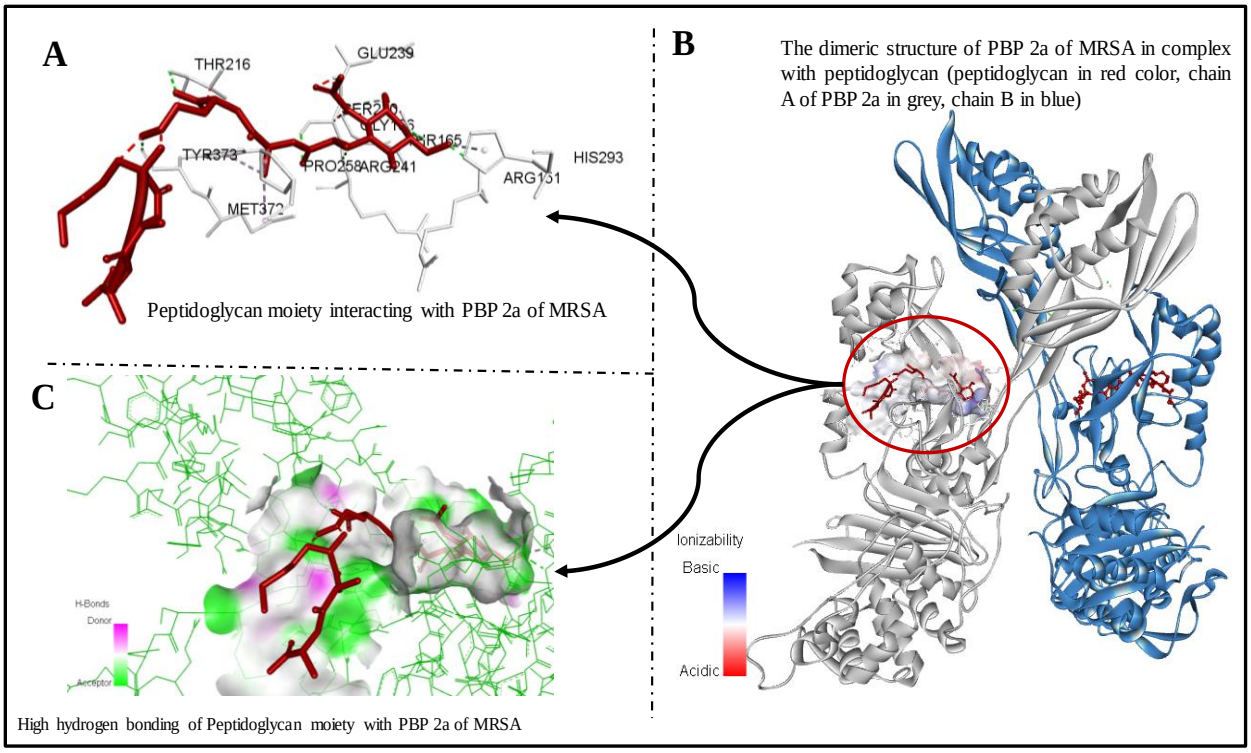

Figure 3. The interaction of the dimeric PBP 2a with peptidoglycan moiety of MRSA**.**

As shown, the dimeric PBP 2a binds to peptidoglycan moiety from MRSA. The PBP 2a structure (3ZG5) was extracted from the RCSB website (https://www.rcsb.org/structure/3ZG5), with the PBD ID, 3ZG5[35]. Molecule - ligand interactions were analyzed using Biovia Discovery Studio 2021 Client (BIOVIA Discovery Studio Visualizer-https://discover.3ds.com/discovery-studio-visualizer). As shown in figure 2A, the dimeric molecule binds to peptidoglycan via a minor cleave found in both monomers (chain A, and chain B). PBP 2a establishes hydrogen bonds with peptidoglycan moiety at the following amino acids in the binding site; ARG151 (bond distance, 2.33Å), THR165 (bond distance, 3.36Å), THR216 (bond distance, 2.75Å), SER240 (bond distance, 2.76Å), ARG241 (bond distance, 2.85Å), TYR373 (bond distance, 2.52Å), GLY166 (bond distance, 3.73Å), HIS293 (bond distance, 3.84Å). It also interacts with the peptidoglycan molecule via alkyl hydrophobic interactions in PRO258 (bond distance, 5.31Å) and MET372 (bond distance, 4.00Å).

Otero et al. demonstrated that there exists an allosteric control of *S. aureus* penicillin-binding protein 2a that allows for methicillin resistance. In β-lactam susceptible *S. aureus*, the transpeptidase activity of their PBPs is absent. Consequently, β-lactam permanently acrylates the active site serine[33]. However, MRSA PBP 2a is impervious to β-

lactam acylation; hence the dd-transpeptidation reaction is carried out, thus producing the cell wall of the bacteria. As shown in Figure 3, the PBP 2a enzyme, a dimeric molecule, is shown bound to a peptidoglycan moiety during the bacterial cell wall synthesis process.

The ability of MRSA to acquire mobile genetic elements carrying a variety of virulence factors has led to significant variation among MRSA strains[24]. Virulence factors that have been highlighted in literature include Panton-Valentine leucocidin[24,34] (PVL), PSM cytolysins, and toxic shock syndrome toxin-1[24,35]. These exotoxins are responsible for MRSA's increased virulence and exceptional ability to evade the immune system[24]. The evolution of *S. aureus* and its virulence factors has increased the threat of these infections to public health. A list of some of the virulence factors of MRSA is shown in Table 1.

Virulent Factor	**Function**	**Reference**
cap5	Capsular polysaccharide-antiphagocytic factor Clearance	[36]
pvl	Exotoxin	[33]
cap8	Capsular polysaccharide-antiphagocytic factor Clearance	[37]
ssp	*Staphylococcus* serine protease, proteolytic processing	[38]
sdrE	serine-aspartate repeat proteins, binds to extracellular matrix proteins, e.g. fibronectin, fibrinogen, collagen, and elastin	[39]
clfB	Adhesin, binds fibrinogen	[34]
fnbA	biofilm f production, Adhesin, binds fibrinogen	[40]
sdrD	serine-aspartate repeat proteins, binds to extracellular matrix proteins, e.g. fibronectin, fibrinogen, collagen, and elastin	[39]
tst	Exotoxin, toxic shock syndrome toxin-1	[34]

icaD	Polysaccharide intercellular adhesion, biofilm production	31,40,41
cna	biofilm production, Adhesin, binds collagen	40
ebpS, *fib*, *bap*, *icaA*	biofilm production	40
blaZ, *tetK*, *ermC*, *tetM*, *mecA*	Antimicrobial resistance	31

Table 1. A list of some virulence factors in methicillin-resistant *S. aureus*

4.0 S. aureus Biofilm as a Physical Shield Against Antibiotics

The formation of biofilms by Staphylococcus spp is a crucial adaptation for bacterial survival, thus protecting it from harsh environmental factors, antibiotics, and even the bacterial host immunity[42]. In *Staphylococcus epidermidis*, the discovery of poly-N-acetylglucosamine (PNAG) and polysaccharide intercellular adhesin (PIA) was the first factor shown to mediate biofilm formation[43,44]. The discovery of multiple biofilm formation factors in *S. aureus* such as the LPXTG-cell wall-anchored biofilm-associated protein (BAP)[45], fibronectin-binding protein (FnBP)[46], cell wall anchored clumping factor A (ClfA), cell wall-anchored clumping factor B (ClfB), S. *aureus* surface protein G (SasG), *S. aureus* surface protein C (SasC), *S. aureus* protein A (Spa)[44] as well as other genes such as *ebpS*, *fib*, and *icaA*[40] elucidated the mechanisms of action involved in antibiotic resistance employed by *S. aureus* via its biofilm formation. Some cytoplasmic proteins have also been implicated in biofilm phenotypes[47]. The effect of phages on the formation and maturation of biofilms has been studied; Gabisoniya et al. showed that pretreatment of *Pseudomonas aeruginosa* with vB-Pa 4 and vB-Pa 5 phages of *P. aeruginosa* prevented the formation of biofilms[48]. Similar prospects of phage applicability have been studied in *S. aureus.* Using a bioluminescent *S. aureus* and its phage as study subjects, Kelly et al., demonstrated complete elimination of biofilms of the bacteria in as short as 72 hours[49].

5.0 Lytic Phage for Phage Therapy

Phages suggested for phage therapy utilize a lytic mechanism for the infection of bacterial cells. The lytic lifestyle is comprised of five stages: attachment, penetration, biosynthesis, maturation, and lysis. In the attachment stage,

phages utilize their tailspike proteins to interact with specific bacterial surface receptors of the lipopolysaccharide membrane. This interaction has been observed at the molecular level in a variety of phage families[50-52]. As previously mentioned, phages are characterized by a narrow host range and may infect only one species or strain of bacteria within a species[53]. This specificity is unique and can be exploited for targeted treatment of bacterial infections in phage therapy and identification of bacterial pathogens in phage typing[54,55]. Following attachment to the host cell membrane, the phage utilizes its tail machinery to penetrate the cell membrane and inject its viral genome[56,57]. The biosynthesis step of this mechanism is carried out through the synthesis of virus-encoded endonucleases to degrade the bacterial chromosome. The virus then utilizes the functions of the host cell to replicate, transcribe, and translate viral components for the assembly of a progeny[58]. Assembly of the newly synthesized virions, termed maturation[59], is followed by the disruption of the host cell membrane by phage proteins holin or lysozyme. This disruption leads to the lysis of the host cell and the release of the progeny to infect other bacterial cells.

6.0 Phages Against MRSA

6.1 Identification of Phages Against MRSA

Phages are characterized by a narrow host range and may infect only one species or strain of bacteria within a species. The development of phage therapy for specific bacterial strains requires the identification, isolation, and characterization of phages that exhibit lytic lifestyles in the desired bacterial target. Lytic phages offer the greatest therapeutic potential due to their consistent, lethal effects on their host[60]. According to literature, all known phages associated with the *Staphylococci* family of bacteria belong to the order of Caudovirales and are primarily members of the families *Siphoviridae* and *Myoviridae*[61-64]. The use of phage typing for the identification of *S. aureus* infections in the clinical setting[65] served to develop a library of phages specific to this bacterial genus. This library is integral in the screening of phages for lytic activity against MRSA. As the prevalence of MRSA increases, the ability to identify *S. aureus* phages that carry out lytic lifestyles in MRSA is a vital step in the development of viable treatments. Isolation of phages from the order Caudovirales followed by characterization and *in vitro* testing is a viable method for identification of *S. aureus* phages with lytic lifestyles within MRSA[66-70]. Phages are known to be abundant in any ecosystem in which their bacterial host is present[68]. Literature has been able to utilize samples, primarily from

healthcare facility sewage, for the isolation of *S. aureus* phages[66-69]. Characterizations of these isolates through double-layer plaque assay (DLA) and electron-microscopy have resulted in the identification of *S. aureus* phages belonging to the *Siphoviridae* and *Myoviridae* families[66-68].

Phages of the order Caudovirales are classified structurally into three families of tailed bacterial viruses: *Myoviridae* (long contractile tails), *Siphoviridae* (long non-contractile tails), and *Podoviridae* (short non-contractile tails)[67]. One of the renowned prototypic phages from the *Podoviridae* is the *Salmonella* phage known as P22[71] and its phylogenic relative the ε34 phage, which also infects *Salmonella* spp[72]. All three families of Caudovirales feature non-enveloped protein shell heads containing a single linear dsDNA molecule. The dsDNA genomes of these phages encode from 27-600 genes clustered according to function arranged in large operons. Caudovirales are found in over 140 prokaryotic genera representing most branches of the bacterial phylogenetic tree. With a wide variety of host ranges, some members of this order can infect members of multiple genera of bacteria while others show high specificity[67].

6.2 Host Range

A significant obstacle in the development of phage therapy is the host range of phages. Infection specificity of phages can often lead to difficulties in the development of efficacious phage therapy methods. The host range of *S. aureus* phages against clinically isolated MRSA strains can be determined through *in vitro* assays. Against isolates of clinical and community-related MRSA infections, phage host ranges have shown wide variation as naturally expected. We contribute this wide variation to the high specificity between phages and their target host. Literature has been able to identify a variety of phages with host ranges suitable for phage therapy against MRSA[73-78].

Phages selected for the treatment of MRSA infections should exhibit a broad host range against clinically relevant strains. Literature has outlined several polyvalent phages that could be utilized for phage therapy[60]. A phage that has exhibited a broad host range against MRSA is the phage MR003[77]. This phage, a member of the Caudovirales family, has been observed to infect 97% of clinical and community MRSA strains[77]. This host range is significantly higher than other *S. aureus* phages that typically infect from 20% to 73% of MRSA strains[76,78]. The host specificity of phage MR003 is hypothesized to result from the genomic structure of the tailspike and baseplate structures of the

virus. Comparative genomic studies of MR003 to common *S. aureus* phage SA012 revealed that these two phages share homology in ORF117 and ORF119, responsible for receptor binding to host cells. Therefore, it was determined that differences in the tailspike and baseplate structures seem to be the key contributing factor to the broad host specificity in MR003[79]. Another relevant phage is phage 812. *In vitro* studies have shown this phages ability to kill 95% of 782 clinical *S. aureus* isolates[80]. Phage 812 is closely related to phage K, which demonstrates an extensive host range against MRSA. Phage K has also been shown to be effective against MRSA strains that are vancomycin-resistant and teicoplanin resistant[60]. *In vitro* study demonstrated that 39 out of 53 clinically isolated strains were sensitive to phage K and that insensitive strains could be treated with variants of phage K[81]. Genomic studies of phages that are potential candidates for phage therapy against MRSA infections could be helpful in identifying factors that influence host range[77-79].

A method utilized to increase the host range of phage treatments against MRSA is phage cocktails. Phage cocktails address the challenge of limited host ranges by incorporating multiple phages with varying host ranges in solution. This method has been shown to increase the infectivity of phages against MRSA[76]. An experimental phage cocktail of four *S. aureus* phages that infected 37.5%, 26.7%, 21.4%, and 19.6% of clinical MRSA isolates, respectively, resulted in a cocktail that could infect 66% of clinical MRSA isolates[76]. Phage cocktails allow for the lysing of MRSA bacterial strains without the host range limitations associated with individual phage treatments. While phage cocktails provide greater ease of use, a potential downfall of this method is the greater complexity in manufacturing and possible clinical outcomes[82]. While individual phage therapy only requires the isolation of one specific phage, phage cocktails require the isolation and purification of multiple phages, which in turn increases the complexity of manufacturing.

6.3 MRSA Phages As Therapeutic Agents

6.3.1 Biological Considerations

Phage therapy, first used almost a century ago, is driven by the continued occurrence and prevalence of antibiotic-resistant bacterial strains. While the discovery of antibiotics negated the need for new antimicrobial agents in the 20th century, antibiotic resistance in the 21st century has renewed the need for new antimicrobial agents. The

rise of phage therapy as a potential novel therapeutic method is facilitated by our improved understanding of phage biology, genetics, immunology, and pharmacology. Aspects of phage therapy that once hindered its efficacy have now been standardized to improve treatment success. Regulatory requirements of phage therapy call for strictly lytic phages confirmed antimicrobial activity against the target pathogen and the removal of contaminating bacterial debris and endotoxins[83]. Identifying the bacterial host cell receptor for any therapeutic phage is also vital in the long-term success of phage therapy. Identification of these receptors can provide insight into phage resistance, evolutionary trade-offs, and the use of co-therapies that are less likely to generate phage-resistant hosts.

Phages that feature lytic lifestyles are ideal for the success of phage therapy. The use of temperate or lysogenic phages is highly inadvisable in phage therapy as their ability to lysogenize cells is hindered by the rise of homoimmunity in a bacterial population and the possibility of lysogenic conversion[84,85]. Lysogenic conversion can lead to bacterial populations gaining new, often pathogenic genetic traits, such as phage-encoded toxins[84] or antimicrobial-resistant determinants[85]. Despite these drawbacks and potential hazards, temperate or lysogenic phages have shown potential to be utilized through genetic manipulation of their life cycle[60]. Research has demonstrated that two distinct mutations, *vir*, and clear plaque, can essentially change temperate phages into obligately lytic phages[60]. Both mutations affect the repressor protein of the phage, inhibiting its ability to become a prophage or carry out lysogenic conversion[60]. A *vir* mutant has already been successfully utilized in an animal study, showing promise for this method[60].

While lytic phages are considered the standard for phage therapy, there are still some concerns about their abilities. Scientific understanding of phages has been greatly advanced since their discovery a century ago. However, our knowledge of phages is still limited. The genomes of lytic phages can contain greater than 50% hypothetical genes with no known function[86], as well as encode auxiliary proteins that alter bacterial physiology in ways that are not fully known[86]. The number of genes and auxiliary proteins that we are currently unaware of makes abortive infections a major concern. Abortive infection is a method of bacterial defense in which the bacterial cell upon infection kills itself to ensure the replication of a phage is stopped. This mechanism could possibly lead to the bacterial host acting as a reservoir inside the human body for phage DNA with unknown functions. This concern is also shared with

mutant phages such as *vir* and clear plaque, especially considering that temperate phages typically carry a wide range of virulence factors[60]. Continued research of phage genetics is key in ensuring the safety of phage therapy.

7.0 Comparison of phages to Antibiotics

Phages and antibiotics both serve as antibacterial agents functioning to lyse or inhibit the persistence of bacterial infections. While both agents have a similar function, they feature several key differences that determine their appropriateness for situational usage.

The use of antibiotics has been observed to have adverse health effects in some situations[87]. Adverse health effect of antibiotics includes instances of anaphylaxis, nephrotoxicity, cardiotoxicity, hepatotoxicity, neurotoxicity, and several gastrointestinal and hematological complications[87]. The most common adverse effect of antibiotic treatment is an allergic reaction, which is prominent in children[87]. These allergic reactions are most commonly the product of high tissue concentrations[88-90]. The safety of phage therapy has not been as extensively studied, especially in western medicine. However, new studies have deemed phage therapy practices such as oral administration as safe[89-94]. In oral administration, the translocation of phage across the intestinal epithelium into the blood has been suggested as beneficial to the host[95]. The benefit of this translocation is the downregulation of immune response to indigenous gut microbiota antigens through the inhibition of interleukin-2, tumor necrosis factor, and interferon-gamma production[95]. This downregulation, in addition to phage host specificity, protects the natural gut microbiota. The protection of natural gut microbes is a typical criticism of antibiotics. The immunological response to phage therapy may be beneficial in healthy patients; however, literature disputes the safety of treatment in patients with compromised immune systems[96-98]. The immunological response is especially significant in the context of MRSA infections that are prominent in patients who are immunocompromised. Patient-to-patient variation in the study of phage therapy has been an area of concern. While transduction may be beneficial to natural gut microbes, there is concern that this characteristic could also be related to the disruption of normal intestinal barrier function. This disruption could potentially lead to disorders such as Crohn's disease, inflammatory bowel disease (IBS), and type 1 diabetes[99]. Literature has determined that there is variation in the inflammatory response to phage therapy based on the site of infection[100]. The study of phage therapy is relatively new, and there are many characteristics such as

immunological response and physiological response that require further study to comprehensively assess the safety of phage therapy.

Host specificity is a defining characteristic of phage therapy. The broad use of antibiotics has been documented for their adverse effects on the human gut microbiome that sometimes lead to diarrhea and *C. difficile* infection[101]. Other potential outcomes of antibiotic perturbations in the gut microbiome include asthma, obesity, and diabetes[102-104]. Phage therapy is highly specific to bacterial species and strain, resulting in less irritation of the natural gut microbes while still effectively reducing the presence of pathogens[105,106]. As discussed in the host range section of this review, the specificity of phages can sometimes lead to the inability to treat an infection colonized by multiple bacterial species. A common clinical example of this scenario is burned victims who typically suffer infections colonized by more than one singular bacterial strain[107]. The development of phage cocktails that are effective against a range of bacterium present in an infection can increase the host range of treatment, which in turn results in more effective treatment of the infection. It is important to note that the success of phage cocktail treatment is dependent on the ability to identify the pathogens present. While phage cocktails address complex infections and the limitations of host specificity, they result in major logistical challenges[82]. Phage cocktails present limitations in development, large-scale production, and distribution, a distinct advantage of broad-spectrum antibiotics.

An interesting characteristic of phage therapy is the relationship between geographic location and phages used for treatment. Studies have shown that phages show high specificity to bacterial targets from their indigenous region[94,108]. These studies utilized Russian *E. coli* phage cocktails for the treatment of microbiologically determined *E. coli* diarrhea in Bangladesh[94]. The treatment resulted in no improvement of clinical outcome. Results suggested that phage cocktails are better adapted to local bacteria populations[108], and that bacterial host range can be restricted both spatially and temporally[109]. A suggested solution to this challenge is the development of phage cocktails with regional specificity for the clinical setting[110]. In the context of MRSA infections, as well as other antibiotic-resistant bacterial strains, this means that the phages that can be used to target these bacteria are likely found in the same enviroment[111]. While this high specificity provides challenges in production that are not common with broad-spectrum antibiotics, it does have some benefits. Regions that have limited access to antibiotics would greatly benefit from the ability to

isolate phages that could be utilized for specific phage therapy of regionally prevalent pathogens. The utilization of phage therapy in these regions would also positively impact the economic burden that the cost of antibiotic treatment entails. Antibiotics have been a cornerstone of clinical treatment for over a century, but the increased prevalence of antibiotic-resistant bacterial strains has required the development of new novel treatments. The limited adverse effect, target specificity, and abundance of phages in the natural world make phage therapy a potentially viable treatment.

Antibiotic	**Protein**	**Antimicrobial Mechanism**	**Bacterial Resistance Mechanism**	**Resistance Gene**
Penicillin	Beta-lactamase	Binding to penicillin binding proteins (PBPs) disrupts peptidoglycan cross linking during cell wall synthesis resulting in lysis.	Production of Beta-lactamase enzymes and alteration of PBP[23]	blaZ[23,31]
Methicillin	Beta-lactamase 2a	Binding to PBP 2a disrupts peptidoglycan cross linking during cell wall synthesis resulting in lysis.	Structural change of PBP 2a[32]	mecA[23]
Vancomycin (glycopeptides)	D-ala: D-lac ligase D-ala: D-ser ligase	Interaction with uncross-linked peptidoglycan pentapeptides results	Alteration of structure of cell wall	vanA[23,26] vanB[23,26]

		in inhibition of the extracellular steps of peptidoglycan synthesis.	peptidoglycan precursors[26]	
Phage	**Protein**	**Antimicrobial Mechanism**	**Bacterial Resistance**	
S. aureus Phages Trsa205[76] Trsa207[76] Trsa220[76] Trsa222[76] SA003[77] MR003[77] LS2a[112] Regional Phages[94,108]	Tailspike Proteins	Phage tailspike proteins specifically target receptors on the lipopolysaccharide membrane to initiate penetration, replication, synthesis, assembly, and release, resulting in lysis of the bacterial host[50-52].	The evolutionary rate of bacteria to develop resistance to phage treatment is significantly slower than bacterial development of antibiotic resistance.[7]	

Table 2. Mechanisms of Therapeutics against S. aureus A brief comparative description of antibiotic and potential phage S. aureus treatment methods, mechanism, and resistance.

7.1 Clinical Challenges of Phage Therapy against MRSA

The lack of validated and adequately controlled clinical trials is a challenge to progressing phage therapy into standard clinical practice[113]. The pharmacological characteristics of phages hinder their standardization in clinical trials. A primary pharmacological concern is the self-replicating nature of phages; unlike conventional drug treatments, phage therapy requires awareness of various novel kinetic phenomena[114]. Determining dosage is

particularly challenging since phages can increase upon infection of the target bacteria exponentially. Experimental design of clinical trials utilizing phages requires standardization and guidance using tailored pharmacokinetic models for specific systems. The establishment of these models as standard practice would significantly advance the use of phage therapy in clinical trials[114].

Another challenge in the clinical use of phages for the treatment of bacterial infections is the delivery of phage virions to the location of the infection. Phages require direct contact with the target bacteria to carry out infection and lysis. The broad distribution of phage in the body cannot effectively treat the target infection. Literature has exhaustively examined methods of delivery in animal models, revealing that administration of phages into the intramuscular, subcutaneous, or intraperitoneal have shown significant influence on the success of phage therapy[112,115,116]. Intraperitoneal injection of phage MR11, an *S. aureus* phage, demonstrated the ability to eradicate MRSA infections in mice models[116]. Animal trials have demonstrated the abilities of phage therapy as a novel therapeutic against MRSA and worked towards standardization of dosages for adequate treatment. Dose-response studies in white rabbits have demonstrated the effectiveness of phage therapy against *S. aureus* via subcutaneous injection. This study concluded that high concentrations of the phage L2Sa, an *S. aureus* phage, were shown to prevent abscesses caused by infection[112].

While phage monotherapy has shown promise, combination therapy or phage cocktails also offer a broad range of activities against bacterial infections. Phage cocktails, as previously described in this review, consist of the combination of several phages with various host ranges. This combination addresses the limitations of monotherapies host range and reduces the potential development of phage resistance in bacteria. While phage cocktails feature a broader host range, it has been shown that they significantly increase the challenge of assessing inflammatory response, potential gene transfer, and the development of multi-phage resistance[117]. Further study and standardization of phage cocktail therapy are required to determine their effectiveness as well as efficacy fully.

7.2 Human Clinical Trials

Human clinical trials for phage therapy against MRSA are limited due to the challenges previously mentioned. Standardization of clinical trials requires preliminary studies to determine the adequate dosage, delivery, and host response. The use of animal models has mainly been beneficial to the progression of standardized phage therapy

methods[112,116,117]. The select phage therapy clinical trials that have been conducted show promise for using phages against MRSA infections.

One notable clinical trial of bacteriophage therapy, referenced throughout literature, addressed the safety of phage therapy through a phase I trial[29]. Rhoads et al in 2009 focused on the treatment of venous leg ulcers in humans. This trial treated ulcers with bacteriophages targeted against *Pseudomonas aeruginosa, S. aureus, and Escherichia coli*. Results of this phase I trial concluded that there were no adverse events attributed to the phage therapy and that between test and control groups, there was no significant difference ($p>0.05$) in the frequency of adverse events, rate of healing, or frequency of healing[29]. Phase I clinical trial was successful in demonstrating the safety of phage therapy in humans[29]. While Rhoads et al showed promise, phase II trials of phage therapy must be carried out to determine efficacy.

Recent interest in phage therapy has resulted in the increased involvement of pharmaceutical companies in phage research and clinical trials. Novolytics (UK) has recently announced that phage cocktail gels that target MRSA are in the developmental stage[118]. This phage cocktail would serve to treat nasal carriage of MRSA as well as skin infections and indwelling medical devices[118]. While phase II and phase III trials have not been announced for phage therapy treatment of MRSA infections, it can only be assumed that they are on the horizon. Continued research into phage pharmacokinetics, stability, delivery, partnered with the development of novel formulations and exhaustive clinical trials will eventually allow phage therapy to reach widespread clinical application. As shown in Table 3, *S. aureus* and its associated strains introduce challenges to standard antibiotic treatment methods. While antibiotic resistance is the result of bacterial evolution, antibiotic-resistant bacteria remain susceptible to phage infection, hence continued study of phage therapy could potentially replace or synergize with antibiotic treatment of MRSA.

Strain of *S. aureus*	**Antibiotic Treatment**	**Resistance Developed**	**Potential Phage Treatments**
S. aureus	Penicillin	Resistance results in PRSA[23]	SA003[77] Trsa205[76]

Penicillin Resistant *S. aureus*	Methicillin	Resistance results in MRSA[23]	Trsa207[76] Trsa220[76] Trsa222[76] Phage Cocktail (Trsa205, Trsa207, Trsa220, Trsa222)[76] MR003[77] L2Sa[112] Regionally Isolated Phage Cocktails[94,108] Regionally Isolated Phages[94,108]
Methicillin Resistant *S. aureus*	Vancomycin	Resistance results in VRSA[23,26,119]	
Vancomycin Resistant *S. aureus*	Quinupristin/Dalfopristin	Partial results in (mrMRSA)[23,120]	
Multi-drug Resistant *S. aureus*	Varies in accordance with resistance	Resistant to Standard Treatments[120]	

Table 3. Strains of S. aureus, the antibiotic they developed resistance against, and the potential phage treatments options.

Conclusion

The increased prevalence and occurrence of antibiotic-resistant bacteria is a major threat to public health, especially the notorious antibiotic-resistant *S. aureus*. While antibiotic dose-response has been standardized, consideration of MRSA phages varied replication factors is crucial for the determination of standard relative dosage for 'killing' titers. Additionally, MRSA phages multiplication is incumbent on host availability; for this reason, an initial "killing titer" might tremendously increase after phage administration through the phage's replicative process. An added dimension in phage biology is its ability to co-evolve with its host; this added advantage over antibiotics enhances the need to study MRSA phages as therapeutic tools against the bacteria. Hence, a clearer insight into MRSA

phage biology, pharmacokinetics, and pharmacodynamics will provide the requisite avenue for the broad application of phage therapy. It is undoubtedly that an alternative treatment method for these antibiotic-resistant bacteria such as MRSA is essential to counteract human infections[5] and the economic burden they present[12,13]. MRSA being one of the most prevalent antibiotic-resistant bacterial strains, is an immediate and severe threat to public health[5,20]. The utilization of lytic *S. aureus* phages for MRSA treatment shows potential as a treatment method. Literature has outlined the potential benefits of phage therapy against MRSA due to their host specificity, wide diversity, and success in animal and limited clinical trials. While phage therapy against MRSA requires further study, literature to this date suggests that phage therapy shows favorable potential as a novel treatment.

References

1. Dublanchet, A., & Fruciano, E. (2008). [A short history of phage therapy]. Med Mal Infect, 38(8), 415-420. https://doi.org/10.1016/j.medmal.2008.06.016 (Breve histoire de la phagotherapie.)
2. Abedon, S. T. (2015). Ecology of Anti-Biofilm Agents I: Antibiotics versus Bacteriophages. Pharmaceuticals (Basel), 8(3), 525-558. https://doi.org/10.3390/ph8030525
3. Fruciano, D. E., & Bourne, S. (2007). Phage as an antimicrobial agent: d'Herelle's heretical theories and their role in the decline of phage prophylaxis in the West. Can J Infect Dis Med Microbiol, 18(1), 19-26. https://doi.org/10.1155/2007/976850
4. WHO (2020). Antimicrobial Resistance. https://www.who.int/healthtopics/antimicrobial-resistance
5. CDC. (2019). Antibiotic Resistance threats in the United States. https://www.cdc.gov/DrugResistance/Biggest-Threats.html
6. DM, L. (2009). Has the era of untreatable infections arrived? Journal of Antimicrobial Chemotherapy (64), 29-36.
7. Lin, D. M., Koskella, B., & Lin, H. C. (2017). Phage therapy: An alternative to antibiotics in the age of multi-drug resistance. World J Gastrointest Pharmacol Ther, 8(3), 162-173.
8. D'Accolti, M., Soffritti, I., Mazzacane, S., & Caselli, E. (2021). Bacteriophages as a Potential 360-Degree Pathogen Control Strategy. Microorganisms, 9(2). https://doi.org/10.3390/microorganisms9020261
9. Lukacik, P., Barnard, T. J., & Buchanan, S. K. (2012). Using a bacteriocin structure to engineer a phage lysin that targets Yersinia pestis. Biochem Soc Trans, 40(6), 1503-1506. https://doi.org/10.1042/BST20120209
10. Lukacik, P., Barnard, T. J., Keller, P. W., Chaturvedi, K. S., Seddiki, N., Fairman, J. W., Noinaj, N., Kirby, T. L., Henderson, J. P., Steven, A. C., Hinnebusch, B. J., & Buchanan, S. K. (2012). Structural engineering of a phage lysin that targets gram-negative pathogens. Proc Natl Acad Sci U S A, 109(25), 9857-9862. https://doi.org/10.1073/pnas.1203472109
11. Ghosh, C., Sarkar, P., Issa, R., & Haldar, J. (2019). Alternatives to Conventional Antibiotics in the Era of Antimicrobial Resistance. Trends Microbiol, 27(4), 323-338. https://doi.org/10.1016/j.tim.2018.12.010
12. . Morehead MS, S. C. (2018). Emergence of global antibiotic resistance. Prime Care (45), 467-484.
13. O''Neill, J. (2016). Tackling drug-resistant infections globally: Final report and recommendations The Review on Antimicrobial Resistance, https://amrreview.org/Publications.html.
14. Ardal C, B. M., Laxminarayan R, McAdams D, Outterson K et al. (2019). Antibiotic Development- Economic, regulatory, and societal challenges. National Review Microbiology.
15. Sharland MGS, H. B., Moja L, Pulcini C, Zeng M et al. (2019). Eml expert committee and antibiotic Working Group. Essential Medicines list becomes a global antibiotic stewardship tool. Lancet Infectious Disease. (19), 1278-1280.

16. Reindel, R., & Fiore, C. R. (2017). Phage Therapy: Considerations and Challenges for Development. Clin Infect Dis, 64(11), 1589-1590. https://doi.org/10.1093/cid/cix188
17. Chan, B. K., Abedon, S. T., & Loc-Carrillo, C. (2013). Phage cocktails and the future of phage therapy. Future Microbiol, 8(6), 769-783. https://doi.org/10.2217/fmb.13.47
18. McCallin, S., Sarker, S. A., Sultana, S., Oechslin, F., & Brussow, H. (2018). Metagenome analysis of Russian and Georgian Pyophage cocktails and a placebo-controlled safety trial of single phage versus phage cocktail in healthy *Staphylococcus aureus* carriers. Environ Microbiol, 20(9), 3278-3293. https://doi.org/10.1111/1462-2920.14310
19. Chen, L., Yuan, S., Liu, Q., Mai, G., Yang, J., Deng, D., Zhang, B., Liu, C., & Ma, Y. (2018). *In Vitro* Design and Evaluation of Phage Cocktails Against Aeromonas salmonicida. Front Microbiol, 9, 1476. https://doi.org/10.3389/fmicb.2018.01476
20. CDC. (2019). Methicillin-resistant *Staphylococcus aureus* (MRSA). https://www.cdc.gov/mrsa/
21. Pastagia, M., Kleinman, L. C., Lacerda de la Cruz, E. G., & Jenkins, S. G. (2012). Predicting risk for death from MRSA bacteremia. Emerg Infect Dis, 18(7), 1072-1080. https://doi.org/10.3201/eid1807.101371
22. Kaye, K. S., Anderson, D. J., Choi, Y., Link, K., Thacker, P., & Sexton, D. J. (2008). The deadly toll of invasive methicillin-resistant *Staphylococcus aureus* infection in community hospitals. Clin Infect Dis, 46(10), 1568-1577. https://doi.org/10.1086/587673
23. Rasmussen, G., Monecke, S., Brus, O., Ehricht, R., & Soderquist, B. (2014). Long term molecular epidemiology of methicillin-susceptible *Staphylococcus aureus* bacteremia isolates in Sweden. PLoS One, 9(12), e114276.
24. Otto, M. (2012). MRSA virulence and spread. *Cell Microbiol*, *14*(10), 1513 1521. https://doi.org/10.1111/j.1462-5822.2012.01832.x
25. DeLeo, F. R., & Chambers, H. F. (2009). Reemergence of antibiotic-resistant *Staphylococcus aureus* in the genomics era. *J Clin Invest*, *119*(9), 2464-2474.
26. D'. Meziane-Cherif, F. A. S., Ahmed Haouz, Patrice Courvalin. (2012). Structural and Functional Characterization of VanG D-Ala:D-Ser Ligase Assocaited with Vancomycin Resistance in Enterococcus faecalis. Journal of Biological Chemistry (45), 287.
27. Nicolas, I., Bordeau, V., Bondon, A., Baudy-Floc'h, M., & Felden, B. (2019). Novel antibiotics effective against gram-positive and -negative multi-resistant bacteria with limited resistance. PLoS biology, 17(7), e3000337.
28. Geitani, R., Ayoub Moubareck, C., Touqui, L., & Karam Sarkis, D. (2019). Cationic antimicrobial peptides: alternatives and/or adjuvants to antibiotics active against methicillin-resistant *Staphylococcus aureus* and multidrug-resistant Pseudomonas aeruginosa. BMC microbiology, 19(1), 54.
29. Rhoads, D. D., Wolcott, R. D., Kuskowski, M. A., Wolcott, B. M., Ward, L. S., & Sulakvelidze, A. (2009). Bacteriophage therapy of venous leg ulcers in humans: results of a phase I safety trial. J Wound Care, 18(6), 237-238, 240-233. https://doi.org/10.12968/jowc.2009.18.6.42801
30. Gilmer, D. B., Schmitz, J. E., Euler, C. W., & Fischetti, V. A. (2013). Novel bacteriophage lysin with broad lytic activity protects against mixed infection by Streptococcus pyogenes and methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother*, *57*(6), 2743-2750. https://doi.org/10.1128/AAC.02526-12
31. Demir, C., Demirci, M., Yigin, A., Tokman, H. B., & Cetik Yildiz, S. (2020). Presence of biofilm and adhesin genes in *Staphylococcus aureus* strains taken from chronic wound infections and their genotypic and phenotypic antimicrobial sensitivity patterns. Photodiagnosis and photodynamic therapy, 29, 101584. https://doi.org/10.1016/j.pdpdt.2019.101584
32. Stapleton, P. D., Shah, S., Ehlert, K., Hara, Y., & Taylor, P. W. (2007). The beta-lactam-resistance modifier (-)-epicatechin gallate alters the architecture of the cell wall of *Staphylococcus aureus*. Microbiology (Reading), 153(Pt 7), 2093-2103. https://doi.org/10.1099/mic.0.2007/007807-0
33. Otero, L. H., Rojas-Altuve, A., Llarrull, L. I., Carrasco-López, C., Kumarasiri, M., Lastochkin, E., Fishovitz, J., Dawley, M., Hesek, D., Lee, M., Johnson, J. W., Fisher, J. F., Chang, M., Mobashery, S., & Hermoso, J. A. (2013). How allosteric control of *Staphylococcus aureus* penicillin binding protein 2a enables methicillin resistance and physiological function. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(42), 16808–16813.
34. Hoppe, P. A., Holzhauer, S., Lala, B., Bührer, C., Gratopp, A., Hanitsch, L. G., Humme, D., Kieslich, M., Kallinich, T., Lau, S., Leistner, R., Niebank, M., Pokrywka, A., Ringe, H., Schaper, A. S., Schröder, J. T.,

Schwarz, C., Staab, D., Stegemann, M. S., Thee, S., Krüger, R. (2019). Severe infections of Panton-Valentine leukocidin positive *Staphylococcus aureus* in children. Medicine, 98(38), e17185.

35. McDevitt, D., Nanavaty, T., House-Pompeo, K., Bell, E., Turner, N., McIntire, L., Foster, T., & Hook, M. (1997). Characterization of the interaction between the *Staphylococcus aureus* clumping factor (ClfA) and fibrinogen. Eur J Biochem, 247(1), 416-424. https://doi.org/10.1111/j.1432-1033.1997.00416.x
36. van Wamel, W., Xiong, Y. Q., Bayer, A. S., Yeaman, M. R., Nast, C. C., & Cheung, A. L. (2002). Regulation of *Staphylococcus aureus* type 5 capsular polysaccharides by agr and sarA *in vitro* and in an experimental endocarditis model. Microbial pathogenesis, 33(2), 73–79.
37. Sutter, D. E., Summers, A. M., Keys, C. E., Taylor, K. L., Frasch, C. E., Braun, L. E., Fattom, A. I., & Bash, M. C. (2011). Capsular serotype of *Staphylococcus aureus* in the era of community-acquired MRSA. FEMS immunology and medical microbiology, 63(1), 16–24. https://doi.org/10.1111/j.1574-695X.2011.00822.x
38. Rice, K., Peralta, R., Bast, D., de Azavedo, J., & McGavin, M. J. (2001). Description of staphylococcus serine protease (ssp) operon in *Staphylococcus aureus* and nonpolar inactivation of sspA-encoded serine protease. Infection and immunity, 69(1), 159–169. https://doi.org/10.1128/IAI.69.1.159-169.2001
39. Liu, H., Lv, J., Qi, X., Ding, Y., Li, D., Hu, L., Wang, L., & Yu, F. (2015). The carriage of the serine-aspartate repeats protein-encoding sdr genes among *Staphylococcus aureus* lineages. The Brazilian journal of infectious diseases: an official publication of the Brazilian Society of Infectious Diseases, 19(5), 498–502. https://doi.org/10.1016/j.bjid.2015.07.003
40. Serray, B., Oufrid, S., Hannaoui, I., Bourjilate, F., Soraa, N., Mliji, M., Sobh, M., Hammoumi, A., Timinouni, M., & El Azhari, M. (2016). Genes encoding adhesion factors and biofilm formation in methicillin-resistant *Staphylococcus aureus* in Morocco. Journal of infection in developing countries, 10(8), 863–869.
41. Zhao, H., Xu, S., Yang, H., He, C., Xu, X., Hu, F., Shu, W., Gong, F., Zhang, C., & Liu, Q. (2019). Molecular Typing and Variations in Amount of tst Gene Expression of TSST-1-Producing Clinical *Staphylococcus aureus* Isolates. Frontiers in microbiology, 10, 1388.
42. Rode, Tone Mari, Solveig Langsrud, Askild Holck, and Trond Møretrø. "Different patterns of biofilm formation in Staphylococcus aureus under food-related stress conditions." *International journal of food microbiology* 116, no. 3 (2007): 372-383.
43. O'Gara JP. ica and beyond: biofilm mechanisms and regulation in Staphylococcus epidermidis and Staphylococcus aureus. FEMS Microbiol Lett. 2007; 270(2):179–88. PMID: 17419768
44. Zapotoczna, M., O'Neill, E., & O'Gara, J. P. (2016). Untangling the diverse and redundant mechanisms of Staphylococcus aureus biofilm formation. *PLoS pathogens*, *12*(7), e1005671.
45. Cucarella C, Solano C, Valle J, Amorena B, Lasa I, Penades JR. Bap, a Staphylococcus aureus surface protein involved in biofilm formation. J Bacteriol. 2001; 183(9):2888–96. PMID: 11292810
46. O'Neill E, Pozzi C, Houston P, Humphreys H, Robinson DA, Loughman A, et al. A novel Staphylococcus aureus biofilm phenotype mediated by the fibronectin-binding proteins, FnBPA and FnBPB. J Bacteriol. 2008; 190(11):3835–50. doi: 10.1128/JB.00167-08 PMID: 18375547
47. Foulston L, Elsholz AKW, DeFrancesco AS, Losick R. The Extracellular Matrix of Staphylococcus aureus Biofilms Comprises Cytoplasmic Proteins That Associate with the Cell Surface in Response to Decreasing pH. Mbio. 2014; 5(5)
48. Gabisoniya, T. G., M. Zh Loladze, M. M. Nadiradze, N. K. Chakhunashvili, M. G. Alibegashvili, N. G. Tamarashvili, and V. A. Pushkina. "Effects of bacteriophages on biofilm formation by strains of Pseudomonas aeruginosa." *Applied biochemistry and microbiology* 52, no. 3 (2016): 293-297.
49. Kelly, D., O. McAuliffe, R. P. Ross, and A. Coffey. "Prevention of Staphylococcus aureus biofilm formation and reduction in established biofilm density using a combination of phage K and modified derivatives." *Letters in applied microbiology* 54, no. 4 (2012): 286-291.
50. Steinbacher, S., Miller, S., Baxa, U., Weintraub, A., & Seckler, R. (1997). Interaction of Salmonella phage P22 with its O-antigen receptor studied by X-ray crystallography. Biol Chem, 378(3-4), 337-343. https://doi.org/10.1515/bchm.1997.378.3-4.337
51. Nilsson, N., Malmborg, A. C., & Borrebaeck, C. A. (2000). The phage infection process: a functional role for the distal linker region of bacteriophage protein 3. J Virol, 74(9), 4229-4235. https://doi.org/10.1128/jvi.74.9.4229-4235.2000

52. Susskind, M. M., & Botstein, D. (1978). Molecular genetics of bacteriophage P22. Microbiol Rev,42(2), 413. https://www.ncbi.nlm.nih.gov/pubmed/353481
53. Ayariga Joseph, Venkatesan Karthikeya, Ward Robert, Wu Hongzhuan, Jackson Doba, Villafan Robert. 2018. Initiation of P22 Infection at the Phage Centennial. Frontiers in Science, Technology, Engineering and Mathematics: A Peer-Reviewed Journal. Vol.2.issue 2.
54. Doss, J., Culbertson, K., Hahn, D., Camacho, J., & Barekzi, N. (2017). A Review of Phage Therapy against Bacterial Pathogens of Aquatic and Terrestrial Organisms. Viruses, 9(3). https://doi.org/10.3390/v9030050
55. Mehndiratta, P. L., & Bhalla, P. (2012). Typing of Methicillin resistant *Staphylococcus aureus* : a technical review. Indian J Med Microbiol, 30(1), 16-23. https://doi.org/10.4103/0255-0857.93015
56. Andres, D., Hanke, C., Baxa, U., Seul, A., Barbirz, S., & Seckler, R. (2010). Tailspike interactions with lipopolysaccharide effect DNA ejection from phage P22 particles *in vitro*. J Biol Chem, 285(47), 36768-36775. https://doi.org/10.1074/jbc.M110.169003
57. Prokhorov, N. S., Riccio, C., Zdorovenko, E. L., Shneider, M. M., Browning, C., Knirel, Y. A., Leiman, P. G., & Letarov, A. V. (2017). Function of bacteriophage G7C esterase tailspike in host cell adsorption. Mol Microbiol, 105(3), 385-398. https://doi.org/10.1111/mmi.13710
58. Levinthal, C. (1956). The Mechanism of DNA Replication and Genetic Recombination in Phage. Proc Natl Acad Sci U S A, 42(7), 394-404. https://doi.org/10.1073/pnas.42.7.394
59. Levinthal, C., & Fisher, H. W. (1953). Maturation of phage and the evidence of phage precursors. Cold Spring Harb Symp Quant Biol, 18, 29-33. https://doi.org/10.1101/sqb.1953.018.01.008
60. Nicholas H. Mann,The potential of phages to prevent MRSA infections, Research in Microbiology, Volume 159, Issue 5,2008, Pages 400-405, ISSN 0923-2508, https://doi.org/10.1016/j.resmic.2008.04.003.
61. Deghorain, M., & Van Melderen, L. (2012). The Staphylococci phages family: an overview. Viruses, 4(12), 3316-3335. https://doi.org/10.3390/v4123316
62. Lindsay, J.A. Genomic variation, and evolution of *Staphylococcus aureus*. Int. J. Med. Microbiol. 2010, 300, 98–103.
63. Brussow, H.; Canchaya, C.; Hardt, W.D. Phages and the evolution of bacterial pathogens: From genomic rearrangements to lysogenic conversion. Microbiol.Mol. Biol. Rev. 2004, 68, 560–602.
64. Kwan, T.; Liu, J.; DuBow, M.; Gros, P.; Pelletier, J. The complete genomes and proteomes of 27 *Staphylococcus aureus* bacteriophages. Proc. Natl. Acad. Sci. USA 2005, 102, 5174-5179.
65. Wentworth, B.B. Bacteriophage Typing of the Staphylococci. Bacteriol. Rev. 1963, 27, 253–272.
66. Eman Rashad Ahmed Mahmoud, H. A. H. A., Amal Saeid Mohamad Abo-senna, Omnia Karem M. Riad & Maha Mohamad Abd Al – Rahman Abo- Shadi. (2021). Isolation and characterization of six gamma-irradiated bacteriophages specific for MRSA and VRSA isolated from skin infections. Journal of Radiation Research and Applied Sciences, 14:1, 34-43. https://doi.org/10.1080/16878507.2020.1795564
67. Andrew M.Q. King, M. J. A., Eric B. Carstens, Elliot J. Lefkowitz. (2012). Order - Caudovirales. Virus Taxonomy Elsevier, 39-45. https://doi.org/doi.org/10.1016/B978-0-12-384684-6.00001-X.
68. Kitamura, N., Sasabe, E., Matsuzaki, S., Daibata, M., & Yamamoto, T. (2020). Characterization of two newly isolated *Staphylococcus aureus* bacteriophages from Japan belonging to the genus Silviavirus. Arch Virol, 165(10), 2355-2359. https://doi.org/10.1007/s00705-020-04749-6
69. Nasser, A., Azizian, R., Tabasi, M., Khezerloo, J. K., Heravi, F. S., Kalani, M. T., Sadeghifard, N., Amini, R., Pakzad, I., Radmanesh, A., & Jalilian, F. A. (2019). Specification of Bacteriophage Isolated Against Clinical Methicillin-Resistant *Staphylococcus aureus*. Osong Public Health Res Perspect, 10(1), 20-24. https://doi.org/10.24171/j.phrp.2019.10.1.05
70. Golnar Rahimzadeh, P. G., and Mohammad Sadegh Rezai. (2016). Characterization of Methicillin-Resistant *Staphylococcus aureus* (MRSA) Phages from Sewage at a Tertiary Pediatric Hospital. Archives of Pediatric Infectious Diseases, 5(1). https://doi.org/10.5812/pedinfect.39615 (e39615)
71. Jeremie Williams, Karthikeya Venkatesan, Joseph Atia Ayariga, Doba Jackson, Hongzhuan Wu, Robert Villafane. 2018. A genetic analysis of an important hydrophobic interaction at the P22 tailspike protein N-terminal domain. Archives of Virology.

72. Ayariga Joseph, Venkatesan Karthikeya, Ward Robert, Wu Hongzhuan, Jackson Doba, Villafan Robert. 2018. Initiation of P22 Infection at the Phage Centennial. Frontiers in Science, Technology, Engineering and Mathematics: A Peer-Reviewed Journal. Vol.2.issue 2.
73. Mariem N. Mohammed-Ali, N. M. J. (2015). Isolation and Characterization of Bacteriophage against Methicillin Resistant *Staphylococcus aureus*. Journal of Medical Microbiology and Diagnosis 5(1), 213. https://doi.org/10.4172/2161-0703.1000213
74. O'Flaherty, S., Ross, R. P., Flynn, J., Meaney, W. J., Fitzgerald, G. F., & Coffey, A. (2005). Isolation and characterization of two anti-staphylococcal bacteriophages specific for pathogenic *Staphylococcus aureus* associated with bovine infections. Lett Appl Microbiol, 41(6), 482-486. https://doi.org/10.1111/j.1472-765X.2005.01781.x
75. Danovaro, R., Corinaldesi, C., Dell'anno, A., Fuhrman, J. A., Middelburg, J. J., Noble, R. T., & Suttle, C. A. (2011). Marine viruses and global climate change. FEMS Microbiol Rev, 35(6), 993-1034. https://doi.org/10.1111/j.1574-6976.2010.00258.x
76. Mujib A. Abdurahman, I. l. T., Inci Durukan, Mona Khorshidtalab, Ali O. Kilic. (2021). Four Temperate Bacteriophages from Methicillin-resistant *Staphylococcus aureus* Show Broad Bactericidal and Biofilm Removal Activity. Kafkas Universitesi Veteriner Fakultesi Dergisi, 27(1), 29-36. https://doi.org/10.9775/kvfd.2020.24680
77. Jeon, J., D'Souza, R., Hong, S. K., Lee, Y., Yong, D., Choi, J., Lee, K., & Chong, Y. (2014). Complete Genome Sequence of the Siphoviral Bacteriophage YMC/09/04/R1988 MRSA BP: A lytic phage from a methicillin-resistant *Staphylococcus aureus* isolate. FEMS Microbiol Lett, 359(2), 144-146. https://doi.org/10.1111/1574-6968.12580
78. Peng, C., Hanawa, T., Azam, A. H., LeBlanc, C., Ung, P., Matsuda, T., Onishi, H., Miyanaga, K., & Tanji, Y. (2019). Silviavirus phage MR003 displays a broad host range against methicillin-resistant *Staphylococcus aureus* of human origin. Appl Microbiol Biotechnol, 103(18), 7751-7765. https://doi.org/10.1007/s00253-019-10039-2
79. Dakheel, K. H., Rahim, R. A., Neela, V. K., Al-Obaidi, J. R., Hun, T. G., Isa, M. N. M., & Yusoff, K. (2019). Genomic analyses of two novel biofilm-degrading methicillin-resistant *Staphylococcus aureus* phages. BMC Microbiol, 19(1), 114. https://doi.org/10.1186/s12866-019-1484-9
80. Abatángelo V, Peressutti Bacci N, Boncompain CA, Amadio AA, Carrasco S, et al. (2017) Broad-range lytic bacteriophages that kill *Staphylococcus aureus* local field strains. PLOS ONE 12(7): e0181671.
81. Stephen C. Becker, Juli Foster-Frey, David M. Donovan, The phage K lytic enzyme LysK and lysostaphin act synergistically to kill MRSA, *FEMS Microbiology Letters*, Volume 287, Issue 2, October 2008, Pages 185–191.
82. Chan, B. K., Abedon, S. T., & Loc-Carrillo, C. (2013). Phage cocktails and the future of phage therapy. Future Microbiol, 8(6), 769-783. https://doi.org/10.2217/fmb.13.47
83. Young, R., & Gill, J. J. (2015). MICROBIOLOGY. Phage therapy redux--What is to be done? Science, 350(6265), 1163-1164. https://doi.org/10.1126/science.aad6791
84. Fortier, L. C., & Sekulovic, O. (2013). Importance of prophages to evolution and virulence of bacterial pathogens. Virulence, 4(5), 354-365. https://doi.org/10.4161/viru.24498
85. Haaber, J., Leisner, J. J., Cohn, M. T., Catalan-Moreno, A., Nielsen, J. B., Westh, H., Penades, J. R., & Ingmer, H. (2016). Bacterial viruses enable their host to acquire antibiotic resistance genes from neighbouring cells. Nat Commun, 7, 13333. https://doi.org/10.1038/ncomms13333
86. Philipson, C. W., Voegtly, L. J., Lueder, M. R., Long, K. A., Rice, G. K., Frey, K. G., Biswas, B., Cer, R. Z., Hamilton, T., & Bishop-Lilly, K. A. (2018). Characterizing Phage Genomes for Therapeutic Applications. Viruses, 10(4). https://doi.org/10.3390/v10040188
87. Granowitz, E. V., & Brown, R. B. (2008). Antibiotic adverse reactions and drug interactions. Crit Care Clin, 24(2), 421-442, xi. https://doi.org/10.1016/j.ccc.2007.12.011
88. Rouveix, B. (2003). Antibiotic safety assessment. Int J Antimicrob Agents, 21(3), 215-221. https://doi.org/10.1016/s0924-8579(02)00354-0
89. Shehab, N., Patel, P. R., Srinivasan, A., & Budnitz, D. S. (2008). Emergency department visits for antibiotic-associated adverse events. Clin Infect Dis, 47(6), 735-743. https://doi.org/10.1086/591126

90. Naqi SA, Sahin N, Wagner G, Williams J. Adverse effects of antibiotics on the development of gut-associated lymphoid tissues and the serum immunoglobulins in chickens. American Journal of Veterinary Research. 1984 Jul;45(7):1425-1429. PMID: 24049911.
91. Bruttin, A., & Brussow, H. (2005). Human volunteers receiving Escherichia coli phage T4 orally: a safety test of phage therapy. Antimicrob Agents Chemother, 49(7), 2874-2878. https://doi.org/10.1128/AAC.49.7.2874-2878.2005
92. Merabishvili, M., Pirnay, J. P., Verbeken, G., Chanishvili, N., Tediashvili, M., Lashkhi, N., Glonti, T., Krylov, V., Mast, J., Van Parys, L., Lavigne, R., Volckaert, G., Mattheus, W., Verween, G., De Corte, P., Rose, T., Jennes, S., Zizi, M., De Vos, D., & Vaneechoutte, M. (2009). Quality-controlled small-scale production of a well-defined bacteriophage cocktail for use in human clinical trials. PLoS One, 4(3), e4944. https://doi.org/10.1371/journal.pone.0004944
93. McCallin, S., Alam Sarker, S., Barretto, C., Sultana, S., Berger, B., Huq, S., Krause, L., Bibiloni, R., Schmitt, B., Reuteler, G., & Brussow, H. (2013). Safety analysis of a Russian phage cocktail: from metagenomic analysis to oral application in healthy human subjects. Virology, 443(2), 187-196. https://doi.org/10.1016/j.virol.2013.05.022
94. Sarker, S. A., Sultana, S., Reuteler, G., Moine, D., Descombes, P., Charton, F., Bourdin, G., McCallin, S., Ngom-Bru, C., Neville, T., Akter, M., Huq, S., Qadri, F., Talukdar, K., Kassam, M., Delley, M., Loiseau, C., Deng, Y., El Aidy, S., Berger, B., & Brussow, H. (2016). Oral Phage Therapy of Acute Bacterial Diarrhea with Two Coliphage Preparations: A Randomized Trial in Children From Bangladesh. EBioMedicine, 4, 124-137. https://doi.org/10.1016/j.ebiom.2015.12.023
95. Gorski, A., Wazna, E., Dabrowska, B. W., Dabrowska, K., Switala-Jelen, K., & Miedzybrodzki, R. (2006). Bacteriophage translocation. FEMS Immunol Med Microbiol, 46(3), 313-319. https://doi.org/10.1111/j.1574-695X.2006.00044.x
96. Hodyra-Stefaniak, K., Miernikiewicz, P., Drapala, J., Drab, M., Jonczyk-Matysiak, E., Lecion, D., Kazmierczak, Z., Beta, W., Majewska, J., Harhala, M., Bubak, B., Klopot, A., Gorski, A., & Dabrowska, K. (2015). Mammalian Host-Versus-Phage immune response determines phage fate *in vivo*. Sci Rep, 5, 14802. https://doi.org/10.1038/srep14802
97. Park, K., Cha, K. E., & Myung, H. (2014). Observation of inflammatory responses in mice orally fed with bacteriophage T7. J Appl Microbiol, 117(3), 627-633. https://doi.org/10.1111/jam.12565
98. Borysowski, J., & Gorski, A. (2008). Is phage therapy acceptable in the immunocompromised host? Int J Infect Dis, 12(5), 466-471. https://doi.org/10.1016/j.ijid.2008.01.006
99. Tetz, G., & Tetz, V. (2016). Bacteriophage infections of microbiota can lead to leaky gut in an experimental rodent model. Gut Pathog, 8, 33.
100. Pincus, N. B., Reckhow, J. D., Saleem, D., Jammeh, M. L., Datta, S. K., & Myles, I. A. (2015). Strain Specific Phage Treatment for *Staphylococcus aureus*. Infection Is Influenced by Host Immunity and Site of Infection. PLoS One, 10(4), e0124280. https://doi.org/10.1371/journal.pone.0124280
101. Rea, K., Dinan, T. G., & Cryan, J. F. (2016). The microbiome: A key regulator of stress and neuroinflammation. Neurobiol Stress, 4, 23-33.
102. Metsala, J., Lundqvist, A., Virta, L. J., Kaila, M., Gissler, M., & Virtanen, S. M. (2015). Prenatal and post-natal exposure to antibiotics and risk of asthma in childhood. Clin Exp Allergy, 45(1), 137-145.
103. Cox, L. M., & Blaser, M. J. (2015). Antibiotics in early life and obesity. Nat Rev Endocrinol, 11(3), 182-190. https://doi.org/10.1038/nrendo.2014.210
104. Mikkelsen, K. H., Allin, K. H., & Knop, F. K. (2016). Effect of antibiotics on gut microbiota, glucose metabolism and body weight regulation: a review of the literature. Diabetes Obes Metab, 18(5), 444-453. https://doi.org/10.1111/dom.12637
105. Mai, V., Ukhanova, M., Reinhard, M. K., Li, M., & Sulakvelidze, A. (2015). Bacteriophage administration significantly reduces Shigella colonization and shedding by Shigella-challenged mice without deleterious side effects and distortions in the gut microbiota. Bacteriophage, 5(4), e1088124. https://doi.org/10.1080/21597081.2015.1088124

106. Galtier, M., De Sordi, L., Maura, D., Arachchi, H., Volant, S., Dillies, M. A., & Debarbieux, L. (2016). Bacteriophages to reduce gut carriage of antibiotic resistant uropathogens with low impact on microbiota composition. Environ Microbiol, 18(7), 2237-2245. https://doi.org/10.1111/1462-2920.13284
107. Servick, K. (2016). DRUG DEVELOPMENT. Beleaguered phage therapy trial presses on. Science, 352(6293), 1506. https://doi.org/10.1126/science.352.6293.1506
108. Bourdin, G., Navarro, A., Sarker, S. A., Pittet, A. C., Qadri, F., Sultana, S., Cravioto, A., Talukder, K. A., Reuteler, G., & Brussow, H. (2014). Coverage of diarrhoea-associated Escherichia coli isolates from different origins with two types of phage cocktails. Microb Biotechnol, 7(2), 165-176. https://doi.org/10.1111/1751-7915.12113
109. Koskella, B. (2014). Bacteria-phage interactions across time and space: merging local adaptation and time-shift experiments to understand phage evolution. Am Nat, 184 Suppl 1, S9-21. https://doi.org/10.1086/676888
110. Niu, Y. D., Johnson, R. P., Xu, Y., McAllister, T. A., Sharma, R., Louie, M., & Stanford, K. (2009). Host range and lytic capability of four bacteriophages against bovine and clinical human isolates of Shiga toxin-producing Escherichia coli O157:H7. J Appl Microbiol, 107(2), 646-656. https://doi.org/10.1111/j.1365-2672.2009.04231.x
111. Latz, S., Wahida, A., Arif, A., Hafner, H., Hoss, M., Ritter, K., & Horz, H. P. (2016). Preliminary survey of local bacteriophages with lytic activity against multidrug resistant bacteria. J Basic Microbiol, 56(10), 1117-1123. https://doi.org/10.1002/jobm.201600108
112. Wills, Q. F., Kerrigan, C., & Soothill, J. S. (2005). Experimental bacteriophage protection against *Staphylococcus aureus* abscesses in a rabbit model. Antimicrob Agents Chemother, 49(3), 1220-1221. https://doi.org/10.1128/AAC.49.3.1220-1221.2005
113. Furfaro, L. L., Payne, M. S., & Chang, B. J. (2018). Bacteriophage Therapy: Clinical Trials and Regulatory Hurdles. Front Cell Infect Microbiol, 8, 376. https://doi.org/10.3389/fcimb.2018.00376
114. Payne, R. J., & Jansen, V. A. (2003). Pharmacokinetic principles of bacteriophage therapy. Clin Pharmacokinet, 42(4), 315-325. https://doi.org/10.2165/00003088-200342040-00002
115. Ryan, E. M., Gorman, S. P., Donnelly, R. F., & Gilmore, B. F. (2011). Recent advances in bacteriophage therapy: how delivery routes, formulation, concentration, and timing influence the success of phage therapy. J Pharm Pharmacol, 63(10), 1253-1264. https://doi.org/10.1111/j.2042-7158.2011.01324.x
116. Matsuzaki, S., Yasuda, M., Nishikawa, H., Kuroda, M., Ujihara, T., Shuin, T., Shen, Y., Jin, Z., Fujimoto, S., Nasimuzzaman, M. D., Wakiguchi, H., Sugihara, S., Sugiura, T., Koda, S., Muraoka, A., & Imai, S. (2003). Experimental protection of mice against lethal *Staphylococcus aureus* infection by novel bacteriophage phi MR11. J Infect Dis, 187(4), 613-624. https://doi.org/10.1086/374001
117. Parracho, H. M., Burrowes, B. H., Enright, M. C., McConville, M. L., & Harper, D. R. (2012). The role of regulated clinical trials in the development of bacteriophage therapeutics. J Mol Genet Med, 6, 279-286. https://doi.org/10.4172/1747-0862.1000050
118. Monk, A. B., Rees, C. D., Barrow, P., Hagens, S., & Harper, D. R. (2010). Bacteriophage applications: where are we now? Lett Appl Microbiol, 51(4), 363369. https://doi.org/10.1111/j.1472-765X.2010.02916.x
119. McGuinness, W. A., Malachowa, N., & DeLeo, F. R. (2017). Vancomycin Resistance in *Staphylococcus aureus*. *Yale J Biol Med*, *90*(2), 269-281. https://www.ncbi.nlm.nih.gov/pubmed/28656013
120. Merlino, J., Watson, J., Rose, B., Beard-Pegler, M., Gottlieb, T., Bradbury, R., & Harbour, C. (2002). Detection and expression of methicillin/oxacillin resistance in multidrug-resistant and non-multidrug-resistant *Staphylococcus aureus* in Central Sydney, Australia. *J Antimicrob Chemother*, *49*(5), 793-801. https://doi.org/10.1093/jac/dkf021