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Bacteriophage-Liposomes Complex, a Bi-therapy System to Target *Streptococcus pneumonia* and Biofilm: A Research Protocol

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Abstract
Introduction: *Streptococcus pneumoniae* is a gram-positive bacterium, which is the leading cause of death for young children, elderly population, and immunocompromised patients. Its ability to mutate and become resistant to some of the strongest antibiotics makes them difficult to treat and increases the risk of disease spread. Although the development of stronger antibiotics to treat such microbes may be an option, they potentially pose a dangerous threat to the body. As such, a viable treatment option to fight against antimicrobial resistance has yet been found.
Methods: The study focuses on utilizing a bi-therapy system to target *S. pneumoniae* in biofilm, which is the site of emerging antibiotic resistant mutants, by creating levofloxacin-liposomes carrying phages and testing them both *in vitro* and *in vivo*.
Anticipated results: Using bacteriophage therapy and applying bacteriophage-antibiotic synergy, it is hoped to augment the potency of the treatment while lowering its side-effects. The Cp-1 bacteriophage-liposomes complexes are expected to be specific to the *S. pneumoniae* to carry antibiotics to sites of infection.
Discussion: The therapy could ensure targeted bacterial lysis and site-directed delivery of low-dose drugs to decrease the toxicity effect of the antibiotics. Once the efficacy is established and is proven to be significant, its potency can be tested in BALB/cByJ mice models before bringing this therapy to animal trials then human clinical trials.
Conclusion: Bacteriophages are very attractive therapeutic agents that effectively target pathogenic bacteria, safe for the human body, and highly modifiable to combat newly emerging bacterial threats. In addition to its many benefits, the use of bacteriophages could significantly reduce healthcare costs. The potential use of bacteriophages-liposomes complexes could be translated to treat respiratory infections in humans after confirming its efficacy *in vitro* and *in vivo* studies.

Keywords: biofilm; *Streptococcus pneumoniae*; bacteriophage Cp-1; antibiotic-resistance; antibiotic liposome delivery; pneumococcal infection

Introduction

Streptococcus pneumoniae infection has been the leading cause of death for children under the age of five, and a contributing factor to the mortality rates of elderly and immunocompromised patients [1–3]. This gram-positive bacterium primarily spreads through airborne droplets resulting in millions of infections and over one million deaths per year [4]. Furthermore, some pneumococcal infection survivors can later develop long-term complications, including loss of hearing, neurological deficits, and neuropsychological impairments [5–7].

S. pneumoniae acts as an opportunistic pathogen and can infect the human nasopharynx before migrating to the lower airways and other lung tissues leading to respiratory infections [8]. Nasopharynx infection by *S. pneumoniae* is typically asymptomatic and its colonization of the host precedes disease [5,9]. Previous work demonstrated that pneumococcal biofilm formation begins during nasopharyngeal colonization [10]. These biofilms are structures composed of microbial cells, extracellular polymeric substances, and provide an environment for the genetic exchange of materials to increase antimicrobial resistance [11]. Their matrix, composed primarily of polysaccharides, is resistant to antibiotics and is associated with chronic infections [12]. *S. pneumoniae* strains that are effective colonizers of the nasopharynx are known to be better at forming biofilms and biofilm-specific antibacterial resistance on epithelial cells than other colonizer strains [13–15]. Collectively, these findings underlie how *S. pneumoniae* biofilm is formed and established before a patient is diagnosed for therapy.

S. pneumoniae infections are typically treated using beta-lactam and fluoroquinolone (e.g., levofloxacin) antibiotics [16]. They work by preventing DNA replication in bacteria and causing bacterial cell death [17]. While traditional antibiotic treatment might be bactericidal against

the planktonic form of *S. pneumoniae*, it becomes ineffective in the face of biofilm-specific antibacterial resistance of the *S. pneumoniae* biofilm [18–20]. Increased resistance, in this regard, is partially due to the reduced infiltration of antibiotics into the biofilm structure but is more likely attributed to adaptive phenotype changes of the biofilm bacteria [4,21,22]. Additionally, the biofilm environment provides a fertile ground for the emergence of antibiotic-resistant *S. pneumoniae* mutants; the mutation rate of cells in biofilms is higher than planktonic cells [22]. These mutations give rise to antibiotic-resistant strains due to modifications of the antibiotic-targeted proteins, antibiotic drugs, or antibiotic transport [23]. Resistant strains have been reported for both aforementioned family-drugs further reducing the ability of antibiotic-based therapy to eliminate *S. pneumoniae* infections. To that end, the breakdown of formed biofilm provides for an appealing therapeutic intervention against *S. pneumoniae* infection.

With the antibiotic resistance crisis and urgent search for alternative clinical treatments for bacterial infections, bacteriophages have re-emerged as a promising alternative to antibiotics for clinical use [24,25]. Bacteriophages are viruses that infect bacteria to hijack the cell's machinery and reproduce new phages. After new bacteriophages are assembled, they begin lysing the cell to burst out and infect nearby bacteria [26]. Bacteriophage's inability to interact directly with eukaryotic cells [27] suggests that they cannot infect human cells and are possibly safe for therapeutic use.

Bacteriophages targeting and eliminating *S. pneumoniae* have successfully been identified and isolated [25]. However, in the context of biofilm degradation, only bacteriophage-encoded enzymes have been tested against *S. pneumoniae* [28]. In particular, Cpl-1 and Cpl-7 lysozymes derived from Cp-1, a *S. pneumoniae*-specific lytic bacteriophage, were found to induce bacterial cell lysis and biofilm degradation [25,28,29] (Figure 1). Furthermore, the synergistic approach of phage therapy with antibiotics has displayed efficient elimination of several bacterial infections [30–32], though this is yet to be demonstrated for *S. pneumoniae*. infections.

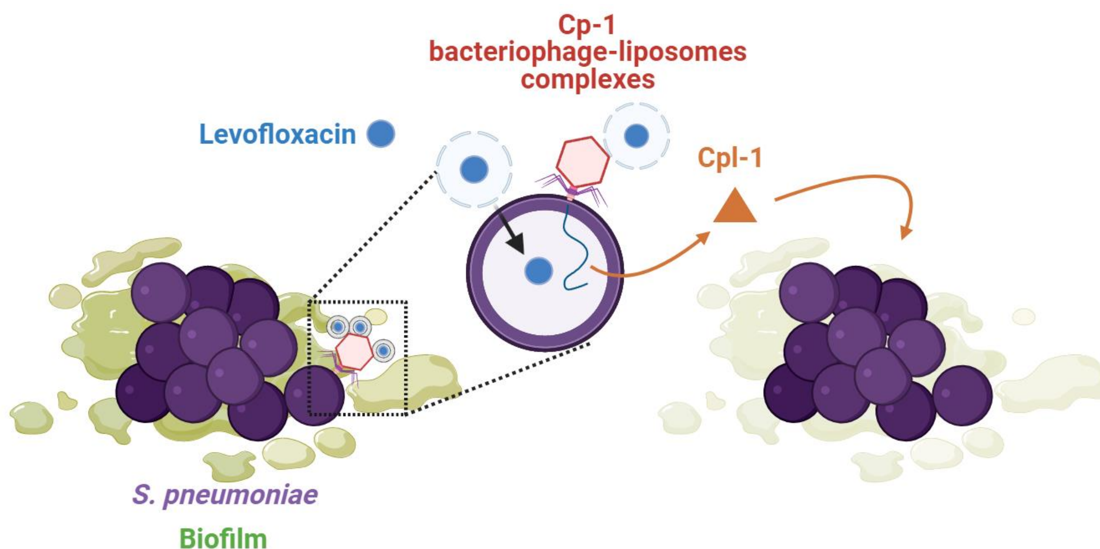

Figure 1. Outline of Cp-1 bacteriophage-liposome complexes' mechanism of action. The Cp-1 bacteriophage selectively targets *S. pneumoniae* and localizes the levofloxacin-containing liposomes to the sites of bacterial infection. Both levofloxacin and bacteriophage genome enter the bacterial cell, leading to its death. Additionally, the bacteriophage genome encodes for the Cpl-1 enzyme, which is capable of degrading the surrounding biofilm.

Lipid vesicles as drug carriers are currently one of the most promising delivery approaches for antibiotics [33,34]. Antibiotic-loaded liposomes have previously received US Food and Drug Administration (FDA) approval for clinical use due to their improved pharmacokinetics, biodistribution, decreased toxicity and lack of immune system activation [34–37]. Moreover, liposomes loaded with an active compound can be coupled with bacteriophages [38]. Notably, the composition of the liposome and its similarity to the bacterial membrane mediates their fusion. The process promotes accumulation of the antibiotic within the bacteria consequently bypassing several mechanisms of antibiotic resistance [39].

Therefore, we hypothesize that using Cp-1 as a biofilm-degrading bacteriophage to deliver antibiotic-loaded liposomes offers an effective and novel strategy in overcoming biofilm-based *S. pneumoniae* infections. Here, we describe the formation of Cp-1 bacteriophage-antibiotic-loaded liposomes complexes, dubbed Cp-1 LevoLipo, and the efficiency of this novel bi-therapy, both *in vitro* and *in vivo*, against *S. pneumoniae* infections. The use of this therapy would not only ensure biofilm breakdown but also

antibiotic localization and accumulation at the site of *S. pneumoniae* infection (Figure 1).

Methods

Cp-1 bacteriophages have been shown to infect *S. pneumoniae* [40] and disrupt the biofilm. Thus, using them to develop bacteriophage-liposomes complexes seems like a potential synergistic therapeutic avenue. Using the detergent depletion technique [41] combined with antibiotic addition, positively charged liposomes containing levofloxacin would be generated. They would then be conjugated with negatively charged Cp-1 bacteriophages [38] to obtain Cp-1 bacteriophage-liposomes complexes.

LevoLipo Complexes Formation

To create the liposomes, levofloxacin powder would be added to a mixture of 1,2-dioleoyl-3-trimethylammonium propane chloride (DOTAP) and 1,2-dioleoyl-sn-glycerophosphocholine (DOPC) lipids dissolved in chloroform (30:70 mass ratio of DOTAP/DOPC) at a ratio of 1:100 (levofloxacin to lipids). This mixture would then be dried with a nitrogen evaporator, which should leave a transparent film at the bottom of the tube. Once dried, a phosphate buffer (10mM NaH_2PO_4, pH 7.0, 100mM NaCl) containing cetyltrimethylammonium bromide (CTAB) would be added to the film obtained previously to a molar ratio of 3:1 (CTAB/lipids-levofloxacin molar ratio). Following CTAB addition, the samples would be sonicated and dialyzed in phosphate buffer, then eluted through Sephadex G-50 beads to get rid of excess CTAB [38] (Figure 2) and purify the liposomes carrying levofloxacin (LevoLipo).

Cp-1 Phage Coupling to LevoLipo Complexes

To allow the Cp-1 phage to carry the positively charged liposomes, a capsid protein will need to be modified to have a negatively charged region. The targeted capsid protein is gp9, which composes the majority of the phage proteins at around 90% of total proteins. Addition of 8 glutamic acids would create a negatively charged region at the C-terminus of gp9, as its first 48 amino acids have been shown to be cleaved off [40], using a PCR mutagenesis-based approach (Figure 2). To form the complex of phage-liposomes (Cp-1 LevoLipo), simply mix the negatively-charged mutated phages with the positively-charged purified liposomes [38] (Figure 2).

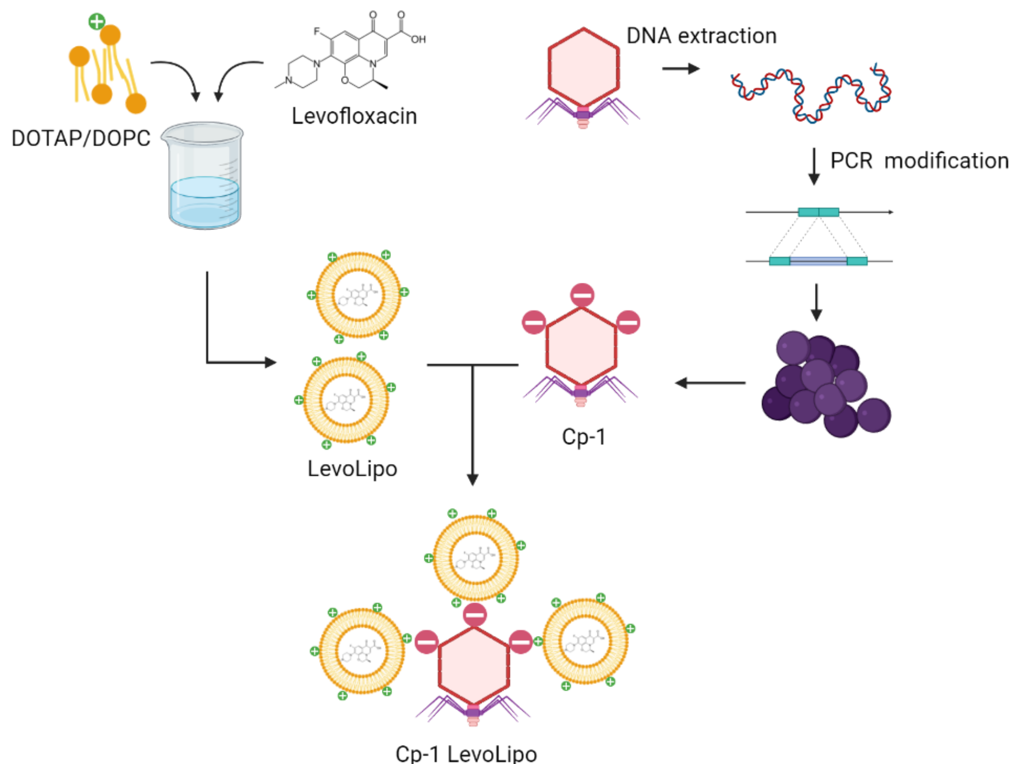

Figure 2. Formation of Cp-1 LevoLipo bi-therapy system. To generate the LevoLipo particles, DOTAP and DOPC lipids would be mixed with levofloxacin powder. After drying this mixture, the pellet would then be re-solubilized with a detergent (CTAB). To generate the negatively charged Cp-1 phages, a PCR based approach would be utilized to add eight glutamic acids to the C-terminal region of the gp9 protein using the purified Cp-1 DNA. Once the modifications are added to the gene, they would be stitched back in the bacteriophage DNA and transformed in *S. pneumoniae* to generate the new modified bacteriophage. These charged phage particles and LevoLipo particles would then be mixed together to generate the bi-therapy system.

To confirm that the complexes are formed, the samples would be fixed onto a copper grid using 1% glutaraldehyde for 5 mins at room temperature and stain using 2.5% phosphotungstic acid and 2.5% trehalose at pH 7.0. Then, the Cp-1 LevoLipo complexes would be imaged by transmitted electron microscopy [38].

Bactericidal Activity Testing of Cp-1 LevoLipo Complexes *In Vitro*

To test the bactericidal efficiency of each treatment *in vitro*, bacterial lawns of *S. pneumoniae* will be used for the antibiogram test [42]. The test consists of placing filter-paper disks imbued with different concentrations of levofloxacin (standards from company), the solution of levofloxacin-liposomes (LevoLipo), the solution of Cp-1 bacteriophages, or of the solution containing Cp-1 bacteriophages carrying levofloxacin-liposomes (Cp-1 LevoLipo) on blood agar plates of bacterial lawns (Figure 3). After incubation at 37℃ with 5% CO2 for 24hrs, the growth inhibition zones formed around the filter-paper disks would be measured. By comparing the Cp-1 LevoLipo inhibition zones to the levofloxacin standards, an optimal concentration of Cp-1 LevoLipo will be determined for follow-up *in vivo* experiments. The same experiment would also be repeated with *S. pneumoniae* in biofilm models using the same protocol. To favor biofilm formation of *S. pneumoniae*, the bacteria would be grown in Todd-Hewitt broth supplemented with 500µM of zinc [43] prior to transfer the newly made biofilm onto blood agar plate to create a bacterial lawn (Figure 3). Each *in vitro* experiment will be repeated at least 3 times (biological replicates). Once Cp-1 LevoLipo complexes are confirmed to be more effective at eliminating *S. pneumoniae in vitro* than the other treatments, an *in vivo* assay to assess infection treatment will be carried out.

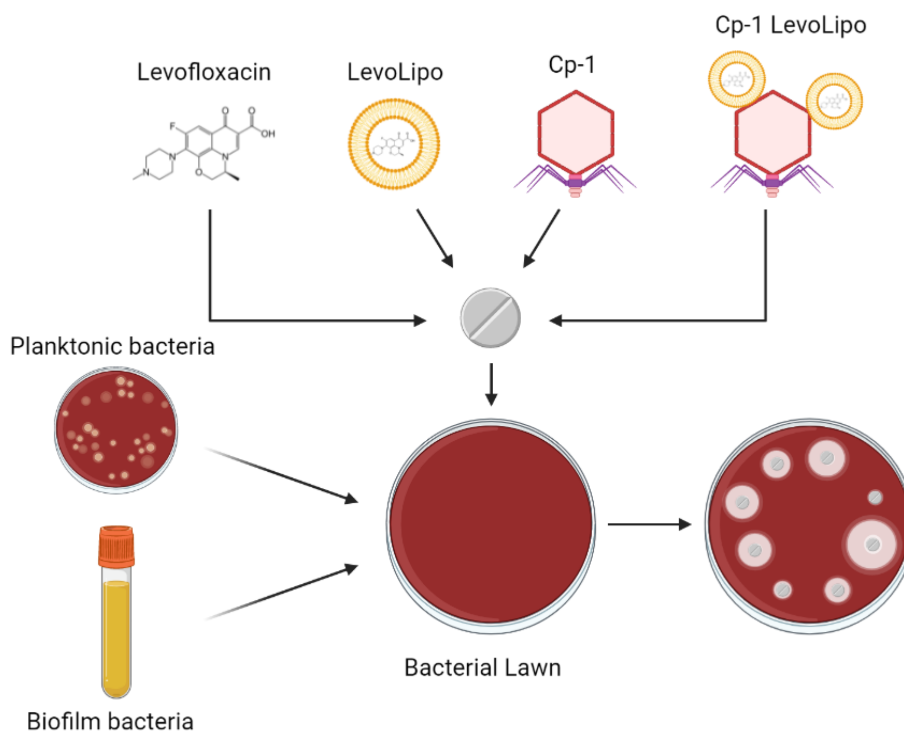

Figure 3. Assessment of Cp-1 LevoLipo bi-therapy system's ability to kill *S. pneumoniae* bacteria *in vitro*. Filter-paper disks loaded with either Levofloxacin, LevoLipo, Cp-1, or Cp-1 LevoLipo would be incubated on bacterial lawns of blood agar plates. The degree of growth inhibition surrounding each filter paper would be used to measure treatment effectiveness. Additionally, *in vitro* experiments would also be repeated to measure treatment effectiveness in the presence of biofilm. The biofilm formation would be stimulated by growing the bacteria in Todd-Hewit broth in presence of zinc.

In vivo Bactericidal Activity of Cp-1 LevoLipo Complexes

Next, a set of *in vivo* experiments would be carried out to assess the effectiveness of the bi-therapy system and to monitor potential side effects. A previous study has detailed the infection of the BALB/cByJ mice with *S. pneumoniae* and confirmed the formation of biofilm *in vivo* [10]. Using this same protocol, 6–12-week-old female mice will be infected via intranasal injection of $5x10^6$ colony forming unit (CFU) of *S. pneumoniae* in PBS buffer [43]. After 48 hours, mice were treated with 10.6 mg/kg [44] every 8hrs of levofloxacin [45], a solution of LevoLipo, a solution of Cp-1, or a solution of Cp-1 LevoLipo via subcutaneous

injection (Figure 4). To assess potential bacteriophage, levofloxacin, or bi-therapy system side effects, mice behavior, grooming, activity, and stool samples would be monitored [34–36] (Figure 4).

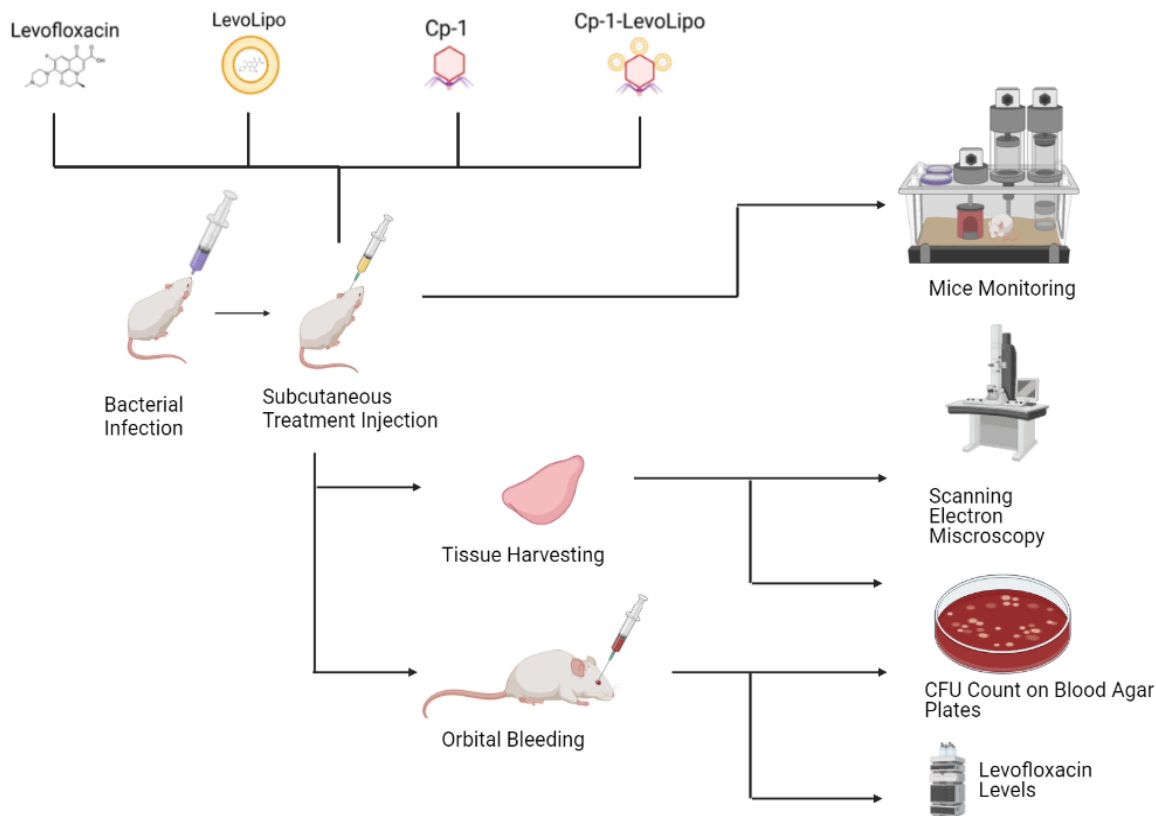

Figure 4. *In vivo* assessment of Cp-1 LevoLipo bi-therapy system's ability to kill *S. pneumoniae*. BALB/cByJ mice would be infected with bacteria by intranasal injection and subsequently treated with either Levofloxacin, LevoLipo, Cp-1, or Cp-1-LevoLipo via subcutaneous injection. Mice would then be monitored to assess for treatment side effects. Mice lungs and nasal septum would be harvested and either homogenized and plated on blood agar plates to measure bacterial CFUs or analyzed by SEM. Finally, mice blood would be collected and used to measure Cp-1 bacteriophage levels and measure levofloxacin blood levels.

To determine bacterial colonization, the mouse nasal septum and lungs were harvested and homogenized. Samples would be serially diluted and plated onto tryptic soy agar with 5% sheep blood, from which the colony forming unit (CFU) count of each sample would be determined [10]. Additionally, tissue samples harvested would be assessed by scanning electron microscopy (SEM) to evaluate biofilm persistence [10] (Figure 4). Next, *S. pneumoniae* CFU in the blood would be determined by collecting blood samples 48 hours post-bacterial inoculation via orbital bleeding [43] and plating serial dilutions onto blood agar plates as described above (Figure 4). These blood samples would also be used to determine levofloxacin blood levels via high performance liquid chromatography (HPLC) as described in [45,46] (Figure 4) and Cp-1 bacteriophage levels by co-incubating the bacteriophages with *S. pneumoniae* bacteria on blood agar plates in a technique called Double Agar Overlay [47,48].

Results

For the antibiogram assay, it is expected that the disks containing Cp-1 LevoLipo will have less bacterial growth around them, compared to the other treatments. Additionally, LevoLipo or levofloxacin alone should have similar sized growth inhibition zones, as the antibiotic delivery by the liposomes should not alter its efficiency. It is expected that only the Cp-1 phages and the Cp-1 LevoLipo complexes to have growth inhibition regions in the biofilm antibiogram assay, where Cp-1 LevoLipo complexes would have the larger inhibition regions. For the *in vivo* experiments, mice treated with the Cp-1 LevoLipo are expected to have minimal side effects, as monitored by levofloxacin blood levels and mice general health conditions and have the least *S. pneumoniae* present in tissues samples following CFU counting after plating and SEM imaging.

Discussion

Phage therapy is becoming a promising treatment alternative for bacterial infections [49]. Since its discovery, extensive research has been conducted to utilize bacteriophage as a potential therapy in the midst of widespread use of antibiotics and rising antimicrobial resistance (AMR) [50]. However, there must be some considerations regarding phage therapy experimentation since several reports of successful phage therapy were tested in humans [50] came with limitations. For instance, there were neutralizing antibodies that appeared in humans after administering phages [51]. These antibodies interfere with treatment but could be solved by administering higher titers of phages. Rapid clearance of phages from the bloodstream is another major limitation in humans. Some phage therapeutics that have failed in mice showed phages being cleared by the spleen, liver, and kidneys [52]. Therefore, there should be careful examination of phage strains that would stay in circulation longer and avoid being recognized by filtering organs when choosing the bacteriophage. In addition, optimizing phage preparation before an injection is of high importance to clear off any host bacteria or debris. Very pure lysates need to be prepared to prevent any endotoxins or live bacteria into the body.

Bacteriophages are effective agents to fight against bacteria and have proven to be a better alternative to antibiotics [53]. However, to improve the efficacy even further, a co-therapy treatment would be experimented to administer both bacteriophages and antibiotics. If the bacterium acquires resistance to phages, it is unlikely for that mutation also to cause resistance to antibiotics as well and vice versa. Thus, the probability of acquiring double mutations to resist both bacteriophages and antibiotics is unlikely within a single bacterium cell. It would likely take 10^{13} bacterial divisions for such a double mutation to occur [51,54]. This low probability suggests that co-administration of antibiotics and phage therapy could prevent resistance in *S. pneumoniae*.

The chosen bacteriophage is a lytic Cp-1 family of phages that targets *S. pneumoniae* [55]. Cp-1 bacteriophages can encode for Cpl-1 lysozyme which is a glycosidase that cleaves the glycosidic bonds in complex sugars [56–58], which allows them to be very efficient in disintegrating the *S. pneumoniae* biofilms [28,59]. Cp-1 bacteriophages would help eliminate biofilm-specific antibiotic resistance to allow for the effective deliverance of antibiotics as a co-therapy treatment option.

There are many advantages with the use of liposomes as carriers for the antibiotics in this theorized system. Liposomes provide stability and protection from an unwanted breakdown in the body [60]. This helps by increasing circulation time and prevents repeated administration or high dose concentrations [37]. They also provide targeted delivery and controlled release of drugs. A positively charged liposome would target a negatively charged bacterial cell wall through electrostatic interactions [60,61]. The physicochemical properties would help in the accumulation of drugs at infected sites and direct interaction with bacteria [39]. While these liposomes could deliver antibiotics to other bacteria in the region, the bulk of antibiotic delivery would occur at the *S. pneumoniae* infection due to bacteriophage-directed therapy. This should prevent side effects associated with wide-spread antimicrobial treatment such as diarrhea.

Although the phages and antibiotics could be administered separately, it was decided that they would be delivered together as part of the improved system. Liposomes that contain the antibiotics would fuse with bacteriophages in hopes of targeting and aggregating bacteriophages at the site of *S. pneumoniae*, allowing for the localization of antibiotics. Thus, the targeted drug delivery system via bacteriophage would improve the effectiveness of the delivery while lowering the toxicity of the antibiotics on normal healthy tissues [60]. The bi-therapy system would increase biofilm penetration, lower off-target toxicity, and improve the overall efficacy.

The presence of levofloxacin-resistant *S. pneumoniae* might render the antibiotic ineffective, and thus, the expectation for the bacteriophage is to eliminate these strains. Only a few strains inhabiting the respiratory tract are typically resistant to levofloxacin. Hence, the intention to choose levofloxacin as the antibiotic to study the efficiency of the bacteriophage-liposomes complexes was to prove that this model would overcome the challenge that levofloxacin would have if administered alone. Since levofloxacin is widely used as a treatment for respiratory infection, a larger group of patients who are undergoing that treatment could be impacted. If the bacteriophage-liposomal delivery of levofloxacin could eliminate levofloxacin-resistant *S. pneumoniae* by targeting the biofilm, a more effective therapy could be developed.

Conclusions

The rise of antibiotic resistance among *S. pneumoniae* has driven the research of a possible synergistic therapy. While there are no direct therapies against *S. pneumoniae* biofilm, bacteriophages are very attractive therapeutic agents that effectively target pathogenic bacteria, safe for the human body, and highly modifiable to combat newly emerging bacterial threats [62]. This approach would act as a stepping-stone in commencing Cp-1 LevoLipo clinical trials. While the project's primary value stems from increased efficacy and biofilm elimination, the bi-therapy also diminishes dosage-related toxicities of antibiotics through bacteriophage-directed localization. In addition to its many benefits, the use of bacteriophages could significantly reduce healthcare costs [63]. Furthermore, the potential use of bacteriophages-liposomes complexes could be translated to treat respiratory infections in humans after confirming its efficacy *in vitro* and *in vivo* studies. Overall, it reduces the burden of new antibiotic development in the

long-term as it allows us to reuse pre-existing antibiotics that were rendered ineffective and eliminates one of the drivers of antibiotic-resistance mutations: biofilm.

List of Abbreviations Used
AMR: antimicrobial resistance
CFU: colony forming unit
Cp-1 LevoLipo: Cp-1 bacteriophage carrying LevoLipo
CTAB: cetyltrimethylammonium bromide
DNA: deoxyribonucleic acid
DOPC: 1,2-dioleoyl-sn-glycerophosphocholine
DOTAP: 1,2-dioleoyl-3-trimethylammonium propane chloride
LevoLipo: liposome containing levofloxacin
PBS: phosphate-buffered saline
SEM: scanning electron microscopy
TEM: transmitted electron microscopy

Conflicts of Interest
The authors declare that they have no conflicts of interest.

Ethics Approval and/or Participant Consent
No ethics approval and/or participant consent was required as this is a proposed protocol. If the experiments were to ever be done, they would require such approval.

Authors' Contributions
All authors have contributed equally to this research.
EA: made contributions to the literature research, drafted the manuscript, and gave final approval of the version to be published.
SA: made contributions to the design of the study, drafted the manuscript, and gave final approval of the version to be published.
AGL: made contributions to the design of the study, drafted the manuscript, and gave final approval of the version to be published.
DJ: made contributions to the literature research, drafted the manuscript, and gave final approval of the version to be published.

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