An overview of phage therapy as an alternative option of MDR bacteria and possible future of COVID-19 treatment

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of

Master of Science in Biotechnology

Department of Mathematics and Natural Sciences

BRAC University, Bangladesh

September, 2022

Declaration

It is hereby declared that

1. The thesis submitted is my/our own original work while completing Maters of Science degree at BRAC University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
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Approval

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Ethics Statement

This material is an original work, which has not been previously published elsewhere. It is my own research and analysis in a truthful and complete manner. The paper properly credits all the sources used (correct citation).

Abstract

One of the greatest difficulties that humanity will confront in the near future is antibiotic resistance. (Bacterio) phage therapy is a valuable therapeutic alternative to antibiotics, and while the concept is almost as ancient as phage discovery, antibiotic discovery and development in the mid-twentieth century hampered its widespread adoption. The global problem of multidrug-resistant bacterial infections demand immediate action, including the invention of antibiotic-free medicines. Phages can down regulate excessive inflammatory reactions relevant in clinical course of COVID-19. This paper is focused on the existing regulatory framework to which such therapy should adhere and reviewed the current obstacles and shortcomings in scientific development for bacteriophage therapy and assume that the PT may participate a therapeutic role in terms of COVID-19 treatment.

Acknowledgement

First of all, I wish to declare my humble gratitude to Almighty Allah, who has bestowed the gift of outmost mercy, and given me the strength, patience and understanding required to complete this work amid this Covid- 19 situation.

I convey my special thanks to Professor A. F. M. Yusuf Haider, PhD, Chairman, Department of Mathematics and Natural Sciences, BRAC University, who always has tremendous support for every student. I express my gratitude to my supervisor, Iftekhar Bin Naser, PhD, Assistant Professor, Department of Mathematics and Natural Sciences, BRAC University, for his encouragement and continuous guidelines for this research. I would like to express my deepest respect to Mahboob Hossain, PhD, Professor, Department of Mathematics and Natural Sciences, BRAC University for always being a great inspirer.

I sincerely thank my beautiful family who trusted in me and is always there for me. This journey would be incomplete without some dear friends. Afroza Khanam Anika, Fariha Ferdousi and Tasfia Supti were my greatest supports while I did this work. I am grateful to all of these people.

Sincerely,

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List of Acronyms

AMR- Antimicrobial Resistance

MDR- Multidrug Resistant

qPCR- quantitative polymerase chain reaction

API's- Active Pharmaceutical Ingredients

GMP- Good Manufacturing Practice

ICU- Intensive Care Unit

MLST- Multilocus- sequence typing

ELISA- Enzyme Linked Immunosorbant Assay

ECMO- The Extracorporeal membrane oxygenation

CRAB- Carbapenem-resistant Acinetobacter baumannii

APT- Adaptive Phage Theraputics

PPE- Personal Protective Euipment

BSL- Biosafety Level

SIP- Shanghai Institute of Phage

SARS-CoV2- Severe acute respiratory syndrome coronavirus 2

Introduction

And

Literature Review

1. Introduction

1.1 Background:

More than 15 classes of antimicrobials have been discovered in the history of antimicrobial drug development, and they have become a cornerstone in the control and management of microbial infections, saving many lives. Antimicrobic therapy has proven to be one of the most effective treatments in clinical medicine. Antimicrobial resistance has emerged in the microbiota of many ecological compartments as a result of the widespread and often indiscriminate use of antimicrobials in human and veterinary medicine, as well as in agriculture. Multidrug resistance among bacterial pathogens is particularly concerning, as it threatens to significantly limit our ability to control infectious diseases. If no quick action is taken, the mortality toll from multidrug-resistant bacterial infections is expected to exceed 10 million by 2050. Despite the fact that phages have been used to treat a variety of diseases for over a century, their practical application in medicine is still confined to a few countries, as noted above. Because phages, unlike ordinary pharmacological products, are living creatures, one of the major considerations when considering their usage for therapy and prophylaxis is their safety. Following the encouraging outcomes of recent case studies using specially engineered phages, phage therapy has attracted renewed interest on a global scale. Since the COVID-19 pandemic epidemic, researchers have suggested the use of phages in many elements of pandemic containment, such as phage therapy for SBIs and phage display for antiviral antibody screening. The potential of phages in COVID-19 patients with SBIs, contrasts with the outpouring of research on anti-SARS-CoV-2 antibodies using phage display techniques, but is little understood. In this paper, we'll first go over the history and current condition of phage therapy, then talk about the biggest difficulties it has to overcome when treating Covid-19 infection (especially SBI's).

1.2 Key Words:

Phage therapy, MDR, Antimicrobials, COVID-19, SBI

1.3 Aim of the study:

The aim of this study is to find out the general scenario of phage therapy, along with the current outcome of phage therapy on Covid-19 treatment.

2. Literature review:

2.1 General Aspects of the Use of Phages as Antimicrobials

Our ability to control bacterial infectious diseases has been severely harmed by the advent of multidrug-resistant bacteria (MDR bacteria). According to a recent study, MDR infections now cause over one million fatalities each year, and the future looks significantly worse [1,2]. As a result, immediate preventative measures are required. Controlling the spread of antibiotic resistance, inventing new medicines, and discovering alternative therapeutics are among the techniques proposed. Bacteriophages (phages), bacterial viruses, were discovered in the second decade of the twentieth century and were quickly applied to the treatment of bacterial infectious disorders with promising results [3]. With the exception of the Soviet Union and a few Eastern European countries, phage therapy was abandoned after the discovery of antibiotics (due to hindered access to antibiotics there)[4]. Both advantages and disadvantages of phage therapy are discussed in following paragraph.

2.2 Advantages

(a) **Activity against antibiotic-resistant bacteria.** Bacteria, even MDR strains, can be infected and killed by phage [5]. This is the most evident benefit of using phage therapy to combat antibiotic resistance in today's world. Furthermore, the therapeutic phage composition may be chosen to impose an evolutionary trade-off in which bacterial resistance to phage evolves

at the expense of antibiotic susceptibility [6]. According to this logic, phage-antibiotic combination therapy has a significant potential to effectively combat antibiotic resistance by eradicating resistant bacteria while also inhibiting the spread of resistance genes [7,8]. The phage–antibiotic synergy, often known as the PAS effect, has been documented in a number of publications. As a result, combination therapy may be the safest and most beneficial option, as it reduces resistance and pathogenicity. [9–11].

(b) **Specificity.** Many phages have a high specificity for their host bacterial strains, making them a very selective therapeutic for preventing dysbiosis in the healthy microbiota. In contrast to antibiotics, phages only infect strains from the same genus or species, and frequently only one or a few strains within a species, protecting the normal microbiota and reducing side effects [12]. The bacterial receptor that the virions recognize through one or more receptor-binding proteins [13] is the source of phage specificity, which can also be influenced by post-entry anti-phage defensive mechanisms [14]. Phages can be polyvalent (i.e., have a wide host range) if they identify a receptor that is found in multiple bacteria, or they can be very specific if they bind receptors that are exclusive to a single bacterial variety. Another theory is that the phage uses a receptor that is only produced in specific circumstances, limiting its infectivity.

(c) **Multiplication at the site of the infection (auto-dosage).** Phages can multiply at the infection site. The phages will multiply and produce progeny once they reach the bacterium they are targeting. As a result, phage therapy might be considered an auto-dosage treatment if enough phage particles reach the infection site. Furthermore, phages would be removed in the absence of bacterial hosts if the infection is completely controlled. As a result, anytime an auto-dosed, "active" treatment is obtained, infection eradication can be elicited with only one injection [16].

(d) **Ubiquity and diversity**. Phages can be found in almost any habitat [17], and they play a critical role in ecosystems by regulating bacterial populations, including the human microbiota [18]. The key practical consequence of such ubiquity and diversity is the ease with which new phages can be discovered, in contrast to the existing slow rate of antibiotic development.

(e) **Evolvability.** As phages are evolving entities, directed evolution techniques can be used to optimize them. When compared to conventional treatments, which are chemical compounds that are stable, this offers up a lot more possibilities. Evolvability in phages can be used for a variety of purposes, including boosting lytic capability, improving paricle stability, expanding

the host range, and combating bacterial resistance. The Appelmans' procedure, for example, relies on spontaneous mutation and recombination among phages in a cocktail to create phage variants capable of infecting previously non-susceptible bacterial strains [19,20].

(f) **Safety.** The phageome is the collection of phages carried by humans.

Their biological activities, beyond bacterial population regulation, are still unknown. Their broad existence in the human body, on the other hand, appears to be a favorable safety signal. Furthermore, clinical research and phage-treated food consumption have shown that phages are harmless. The production of bacterial endotoxins after the lysis of the targeted bacterial cells could be an issue with phage therapy. It should be highlighted, however, that comparable discoveries have been found with conventional therapy with some antibiotics, and that the existing research does not support the notion that phage administration causes harmful inflammatory reactions. Phages may also infiltrate tissues that aren't directly targeted by the treatment, but these interactions don't appear to be harmful.

2.3 Weaknesses

(a) **Phage-resistance.** Bacteria can develop resistance to phage infection in the same way as antibiotic resistance does. The most typical option is to employ phage cocktails, rather than a single phage, and/or a "à la carte" selection of phages for each infecting isolate. This reduces the chances of the host developing resistance to all phages at the same time [26]. The "step-by-step" method, in which phages are separated against phage-resistant bacterial mutants in consecutive screening rounds to acquire new phages capable of infecting resistant variations, is an intriguing approach. This technique mimics the natural antagonistic co-evolution that occurs during treatment, resulting in a phage cocktail capable of infecting both the original bacterium and the anticipated resistant versions [27]. Furthermore, the formation of phage-resistant bacteria is not always a disadvantage, since it might result in a decline in the bacterial host's fitness or pathogenicity [28], or it can resensitize bacteria to antibiotics [9].

(b) **Specificity.** This feature has the potential to be a double-edged sword. Phage specificity necessitates thorough susceptibility testing of each bacterial pathogen before to therapy, which may be a problem for some acute infections that require immediate attention. Furthermore, to offer significant coverage of bacterial variety, this specificity may necessitate the establishment of vast phage libraries and/or substantial sampling and screening activities. This can be a difficult task, and it has raised significant regulatory concerns because, under the existing structure, each phage must be reviewed and approved individually. Furthermore, as a personalized treatment, the eventual necessity to design a new phage preparation for each bacterial infection limits economic profitability and can be considered as a severe disadvantage by pharmaceutical companies. Again, phage cocktails that target different receptors or different bacterial strains can be a potential solution.

(c) **In vivo phage activity.** There isn't always a link between a phage's in vitro and in vivo behavior, especially when it comes to propagation capabilities. This is related to the complexity of bodily fluids as well as ecological interactions in vivo [29,30]. Furthermore, phage propagation is dependent on the bacterial host's physiological condition, which may or may not be appropriate for infection in vivo (for example, whether the bacterium is embedded or not within a biofilm, the expression of receptor molecules, and so on) [31]. Furthermore, because phages are larger than antibiotics, they disperse less effectively. In vivo, where various physical barriers are faced, this constraint is exacerbated. As a result, infection is unlikely at low phage and bacterial densities, and the threshold densities required to achieve phage infection may sometimes necessitate the use of extremely high phage dosages [32,33].

(d) **Immune response.** As phages are composed of biomacromolecules, they have the potential to induce an immunological response when administered [34]. Immune reactions to phage components are generally not seen to be an issue for the person receiving treatment, while they do play a role in the phage therapy's success [35]. On the one hand, the immune response may cause phages to be removed from the system [36], but this effect can be mitigated by

changing dose, administration route, and other parameters. Synergy with the immunological antimicrobial response, on the other hand, appears to be important for treatment success [37], however some evidence suggests that phage therapy can be used successfully in immunocompromised patients as well [38].

2.4 Obtaining Therapeutic Phage Preparation:

The main steps for therapeutic phage suspensions suitable for use in clinical setting are summarized in figure 1.

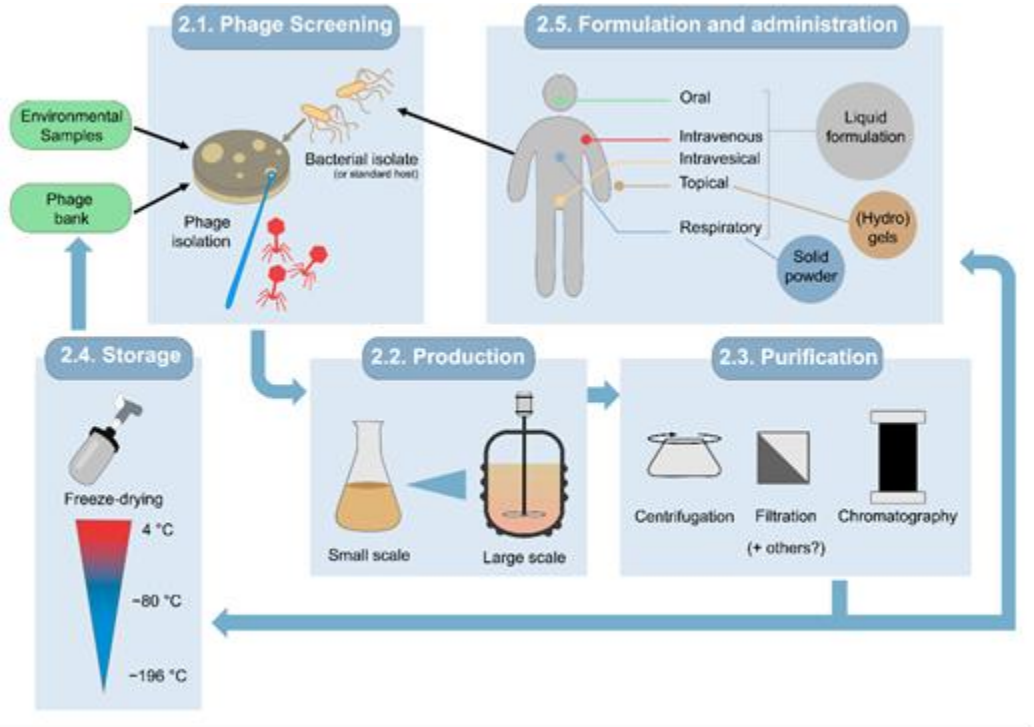


Figure 1. Steps in preparation of phage suspensions suitable for phage therapy, including screening, propagation, purification, storage, and formulation.

2.5 Selection of Screening Host Strains and Phages Intended for Therapy

The discovery and selection of phages and bacterial strains appropriate for phage production is the first step in the phage therapy process. Phages are the most abundant biological organisms on our planet. They can be found in any environment, even in harsh conditionsphage "hunting" sites will be determined by the bacteria to be eradicated [43], because phages infecting a specific host can usually be isolated from habitats where that bacterium lives. However, because the resident bacteria (including the pathogenic one) are likely lysogenic, tolerant, or resistant to the associated phages, it may be difficult to detect phages active against a bacterial isolate from samples from the same patient. As a result, alternate sampling locations are suggested. In nature, many harmful bacteria have free-living variations, making it possible to locate therapeutic phages in environmental samples. A large diversity of phages active against human infections can be found in human-made places or infrastructures, such as wastewater treatment facilities [44].

The phage screening parameters will be determined by the target bacterium once samples have been gathered. In general, phages can be obtained by propagating them on reference strains or clinical isolates [45]. The bacterium of interest may not be cultureable in some situations, so a surrogate host may be utilized instead (e.g., Mycobacterium smegmatis is strongly preferred for the isolation of mycobacteriophages over the pathogenic, slowly growing Mycobacterium tuberculosis [46]). Isolation with multiple bacterial strains may be preferred if a broad-range phage is desired (for example, one that targets several strains), though single strain enrichment does not always preclude finding broad-range phages [43].

2.6 Small- and Large-Scale Production Processes

The double-layer agar method is commonly used to produce phages, as it allows for the isolation of individual phages and then liquid culture of single plaques [49]. Centrifugation, filtering, and/or other purification procedures are used to generate a pure phage lysate (containing just one kind of lysis plaque) (see below). Using bioreactors of various sizes [50], which allow continuous and semi-continuous production, the process can be scaled up and improved to an

industrial level. The latter appears to be the most advantageous for large-scale uniform production since it prevents phage-bacteria co-evolution (albeit it may be operationally complex) [51].

2.7 Purification of Phage Solutions

The fundamental purpose of this procedure is to keep phage suspensions free of bacterial toxins, lipopolysaccharide, and other cellular debris [52]. Additional processes, such as dialysis, ultrafiltration, or treatment with organic solvents, may be required in addition to centrifugation and membrane filtration. The relevant tests should be done to ensure the absence of harmful components, and if necessary, additional purification procedures such as specialized filtration, affinity chromatography, tangential flow filtering, and/or CsCl gradient ultracentrifugation can be utilized. Anion exchange chromatography, as illustrated by Convective Interaction Media® (CIM) columns, could offer an alternative. These more specialized techniques frequently necessitate time-consuming phage tuning (or groups of related phages), but also allow the concentration of viral particles in lysates with low phage titers.

2.8 Storage

Phages can be stored at a variety of temperatures, including 4 degrees Celsius, 80 degrees Celsius, or liquid nitrogen (196 degrees Celsius), or freeze-dried [54,55]. Protecting lysates from evaporation or contamination is often enough to keep their titer from deteriorating over time. To prevent or prolong the loss of infectivity, additives can be applied. The most commonly utilized supplements are Mg^{2+} and Ca^{2+} ions (about 10 mM in the form of $CaCl_2$ or $MgSO_4$), which are given to the culture medium before infection to aid adsorption and are then present in the recovered lysate [54,56]. Cryopreservants such as disaccharides (lactose, sucrose, trehalose) or polyethyleneglycol, as well as gelatin or Ficoll, are other popular additives [57,58]. Adsorption of phages to cell debris can result in a significant reduction in titer, signaling that eliminating cellular impurities from a crude lysate is equally crucial for preservation. Furthermore, reducing

the number of passes is critical since many rounds of propagation paired with phages' high mutation frequencies can result in genetic variations between the parent isolate and its progeny [54].

2.9 Formulation and Administration

Because phages are essentially protein structures, they are vulnerable to proteases, some chemical compounds, extreme temperatures, pH, and ionic strength. As a result, it's critical to utilize the right formulation to ensure that the phage titer is constant during formulation and storage, as well as in the in vivo setting where they're used. Again, the best formulation conditions may change based on the unique phage, therefore in the case of phage cocktails, this merits careful consideration because each type of phage may require specifically customized conditions [59]. The route of distribution, which, in turn, is dependent on the infection site, is the most important variable of phage therapy formulations:

(a) Gastrointestinal infectious illnesses can be treated with oral medication. Oral phages given as water-based liquid suspensions without further protection have been found to survive stomach passage and be recovered in the feces in some cases [60–62]. Because phages are commonly synthesized in sterile buffers such as phosphate-buffered saline (PBS), the bacterial growth medium, ordinary saline, or water, the formulation effort for liquid phage suspensions is typically modest [61,63–65]. More complex formulations designed specifically for oral administration can help phages survive the harsh conditions of the gastrointestinal tract. Encapsulating phages, for example, shields them from the highly acidic stomach environment and digestive enzymes [66]. Furthermore, their release can be activated in a regulated manner, such as pH-dependent release, in which capsules are programmed to become permeable at particular pHs depending on the intended site of action, such as the stomach (pH 1–3), small intestine (pH 5.5–6.5), or colon (pH 6.5–7.2) [67]. Polysaccharides, natural or synthetic plastic polymers, liposomes, and micelles are among the natural and synthetic polymers that offer great flexibility for adapting phage encapsulation and subsequent release to varied medicinal applications [68,69].

(b) Skin infections, wounds, burns, ulcers, and osteoarticular infections are all treated with phages applied topically [70]. Topically applied phages in liquid, semi-solid, and liposome-encapsulated formulations, as well as phage-immobilized wound dressings, have been reported [63]. When employing liquid preparations, they can be dripped directly into the affected area or put in a gauze soaked in the solution. Gel or cream formulations, on the other hand, can be used to circumvent some of the constraints of liquid preparations, with hydrogels preferred over organic solvent-based gels. This is especially important in the treatment of burn wounds, because hydrogels maintain the wound moist while also promoting phage stability [63]. Commercial infection-control treatments can also be utilized as a base for topically delivering phages, but it's important to check whether the product's composition affects phage infectivity [71].

(c) Preparing phages as stable liquid formulations for intranasal instillation or nebulization, or as a solid powder in an inhalable form, is required for local phage treatment of respiratory illnesses [72]. Liquid suspensions are the most popular formulations for respiratory infections due to their relative ease of preparation. Nebulization of liquid phage suspensions has yielded inconsistent results, implying that temperature, relative humidity, nebulization-induced mechanical stress, system delivery efficiency, and the nature of the phage itself all play a role. In the case of dry powder inhalation, freeze-drying or spray-drying are two approaches for obtaining solid phage formulations. In general, both techniques subject phages to a variety of challenges that may affect their infectivity [73], however phage preservation can be improved by controlling critical parameters and adding appropriate excipients, such as polymers for encapsulation [74,75].

(d) In the treatment of systemic infections, intravenous injection is suggested [76]. Liquid phage suspensions are commonly utilized in this instance, which are typically produced in aqueous buffers adequate for inoculation [64,72].

(e) The use of intravenous instillation of liquid phage formulations to treat genitourinary tract infections has also been reported [77,78].

2.10 Quality Criteria for Therapeutic Phage Preparations

Phage preparations must meet certain quality criteria that ensure their safety for clinical usage, as well as extensive traceability documentation, as they are items intended for human medicine. The phage preparation supplier must set up the manufacturing and delivery parameters in line with the applicable regulations (see Section 4) and to achieve the approved quality attributes. These quality controls for phage-based products would typically include:

(a) **Phage identity**. Each phage's identity is determined by its unique genomic sequence [79,80]. Metagenomics has been proposed as a quality control tool for some vaccines [81], and has therefore been used to evaluate the composition of commercial phage products [82,83]. This method allows for the detection of biological impurities as well as the identification of the active substance. While random mutations are unavoidable during propagation, they should be minimized as much as possible through process design (e.g., minimizing subcultivation steps), and functional properties should be tested on a regular basis with validated quality controls, as even single-nucleotide polymorphisms can cause significant phenotypic changes. In other circumstances, however, a highly discriminating PCR-based genotyping approach may suffice [79]. On a case-by-case basis, the maximum allowable degree of genetic divergence between the master batch and the phage population in the therapeutic product, as well as the frequency of such quality checks, should be modified [79].

(b) **Phage Titer.** The double-layer agar method is used to determine the titer of each particular phage. Another option is to use lethality curves, which measure the optical density of phage-infected bacterial cultures to determine the kinetics of phage-induced lysis [84]. Other methods for quantifying phages, including as qPCR and ELISA, can be utilized, however they do not necessarily quantify infectious viral particles, whereas double-layer and lethality assays do [79].

(c) **General Purity.** High-performance liquid chromatography, along with mass spectrometry if necessary, is used to determine the purity and proper composition of biopharmaceuticals. These techniques can be used to recognize phage capsid proteins, poisons, and other bacterial proteins. Because the essential production with pathogenic bacterial hosts poses a danger, quality guidelines should specify maximum levels for contaminants such as

toxins or bacterial DNA, which must be checked using specific and appropriate molecular biology methods as described below.

(d) **Toxins.** Endotoxins have been quantified using a variety of in vitro methods, including gel-clot, turbidimetric, and chromogenic tests. The limulus amebocyte lysate assay is the most extensively used of the latter [85]. A reporter cell line can be employed when this experiment is not relevant, such as when the masking effect is present [86]. Various commercial assays, such as ELISA or assays based on reporter cell lines, can be used to detect other hazardous bacterial proteins.

(e) **Contaminating Nucleic Acids.** To assess the concentration of contaminated nucleic acids, quality controls may be required (i.e., non-phage nucleic acids). qPCR is used to assess for the existence and concentration of leftover nucleic acids.

(f) **Other Quality Controls.** Current sterility and general quality control rules for medicines should also apply to phage-based medications [87]. Total microbial load, pH, osmolarity, visual appearance, and/or maximum water content (in lyophilized preparations) are some of the criteria that should be examined [79].

2.11 Regulation for Phage Preparations

The lack of suitable regulation is perhaps the biggest roadblock to phage therapy's adoption in medicine. If phage preparations are classified as "classic" medicinal items, they must adhere to all applicable laws and regulations governing the manufacture and quality of such products. This essentially implies they must adhere to GMP (good manufacturing practices) guidelines, which has provided a significant challenge for the development of medical phage formulations in terms of increasing prices or complicating management for large-scale production [88].

Although therapeutic phages are considered pharmaceutical products, the phage's nature distinguishes them from standard antimicrobial therapy. Indeed, phage specificity, virus–host co-evolution, or a complex in vivo pharmaco-logical behavior have all had a negative impact on the results of several recent phage therapy clinical studies [33,89–91]. As a result, the clinical trial to market road appears to be a long way off. The measures by which we evaluate phage therapy

could possibly be specifically modified and may not be the same as those used to evaluate chemotherapeutics, according to a preliminary conclusion [92]. So far, customized formulations, or phage preparations particularly conceived and produced to combat the infection present in a single patient, have been the most successful strategy to using phage treatment in the clinic. In reality, in most countries, the compassionate use of phages for certain patients who have no other treatment choices is the present regulatory framework for phage therapy [4,88]. Some of the harsher standards can be avoided within this framework, but each instance must still be approved by the competent authorities.

In Belgium, a new technique has allowed for a more systematic and practical approach to individualized phage therapy, as well as increased manufacturing and handling flexibility (e.g., by not forcing strict GMP compliance). The key to this strategy is to think of phage products as magistral preparations, because they are less regulated in terms of production and marketing than pharmaceutical medicines [93,94]. Phages are recognized as active pharmaceutical ingredients (APIs) (substances employed in a finished pharmaceutical product to achieve a pharmacological action) that are subjected to an external quality assessment based on a specific monograph. A specialized phage bank, in which each conserved phage should be certified to be used as an API in such a way that the certification covers all important quality criteria, would be a viable infrastructure to operate under this regulatory framework. In Spain, magistral formulations are governed by European standards, the Royal Spanish Pharmacopoeia, and a consensus document that specifies the requirements for APIs to be used in magistral formulations, as well as the principles of proper elaboration and quality control. The term "magistral formulation" is defined in these documents as "the drug intended for an individualized patient to explicitly fulfill a detailed medical prescription of the active ingredients that it contains, prepared by a pharmacist or under his direction according to the rules of proper preparation, prepared by a pharmacist or under his direction" and quality control developed for this purpose, dispensed in a pharmacy or pharmaceutical service, and with enough user information" To employ phages in magistral formulations, official permission from the appropriate agency (in Spain, the AEMPS) is required, as well as a specific monograph issued by the supplier indicating the "laws of proper production and quality control." The typical monograph developed in Belgium can be found as Supplementary Material in reference [94], and it summarizes most of the concerns discussed in this review. While this radical structure may be realistic for the time being, many countries have

yet to adopt it. Although phages have been used as compassionate medicines in Spain, we are unaware of any examples of phage therapy being used under this regulatory framework. In any event, it's evident that phage therapy will likely fall outside of standard drug restrictions. Adapting or developing a specialized regulator for phage therapy has already been suggested [95,96]. The main goals of such a regulation should be to (a) develop adequate criteria for assessing the quality, efficacy, and safety of phage products; (b) simplify formal procedures for administering personalized phage therapy; and (c) distribute responsibility and compensation fairly among the involved actors.

Regardless of the regulatory status of phage therapy in most countries, including Spain, phage preparations for therapy are available commercially (e.g., through the Eliava Institute in Georgia, which retains and promotes the Eastern tradition in phage therapy) or by contacting academic or clinical institutes dedicated to phage therapy research and promotion.

2.12 Clinical Trials and Prospects for Phage Therapy

An adapted regulation in favour of personalized phage therapy (possibly following the Belgian example) can be a shortcut to get phage therapy to the clinic and even to the market in Spain, notwithstanding the potential for controversy [97]. However, as previously stated, once phage treatment becomes more widely used in clinical practice and/or the number of untreatable infections rises, individualized phage therapy may become impractical (unless health-care and research systems are adequately supported to fulfill the demand). A more traditional, market-based strategy would undoubtedly be preferred in this case [89]. The latter would still necessitate phage formulations going through the standard medication development pipeline, which includes demonstrating efficacy in randomized clinical trials. Indeed, the absence of meaningful data under existing clinical trial criteria is one of the greatest roadblocks to phage therapy's practical prospects. Despite the recent publication of a large number of generally successful case reports, the phage research community has yet to produce convincing randomized clinical trial data demonstrating phage therapy efficacy. When we look at the two most well-known recent phage therapy trials that failed (the PhagoBurn trial and the Nestlé Bangladesh diarrhea trial), the

evidence suggests a lack of understanding of complex phage behavior in vivo, as well as incorrect assumptions about pathogenic bacteria susceptibility [33,90]. Both of these factors make standardized, large-scale studies extremely difficult. The above-mentioned trials, for example, found a lower phage dosage at the infection site than expected, as well as resistance to the cocktails among the infecting bacteria. One of the study's authors later noted that the polymicrobial character of some infections makes them difficult to treat with phage therapy alone, and even more difficult to conduct a successful randomized controlled trial [89]. As a result, one feasible option for successful clinical trials is to focus solely on well-characterized diseases, with special attention paid to the appropriateness of the targeted pathogens [98]. In fact, the only phase 2 clinical trial with positive efficacy outcomes to date took this approach, choosing a single-pathogen illness (chronic otitis caused by antibiotic-resistant Pseudomonas aeruginosa) and assessing the identification and number of bacteria present in each patient [99]. To ensure the future success of clinical trials, other concerns such as phage production (according to GMP), storage, product shelf-life, dosage, and correct formulation/administration must be thoroughly reviewed and adjusted. Regardless of the potential roadblocks, a number of phage clinical trials are presently underway at various stages, with both predetermined phage cocktails and individualized interventions, ensuring phage therapy's future regularization based on clinical trial evidence. Table 1 lists a few examples of ongoing clinical trials.

Table 1. Ongoing clinical trial examples involving phage therapy.

Dise	Pathogen(s)	Treatment	Sta	Referenc
Diabetic foot	Staphylococcus	Topical phage cocktail N	ot yet recruiting (expected start	NCT02664
Invasive infection in patients with	E. coli	Oral phage cocktail	Recruiting (estimated	NCT03808 103
Chronic airway infection in	P. aeruginosa	Nebulized phage	Recruiting (estimated	NCT04684

Diabetic foot ulcers	P. aeruginosa, S. aureus and/or Acinetobacter	Topical phage cocktail	Recruiting (estimated	NCT04803708
Prosthetic joint infections	Several pathogens	Combined antibiotic/personalized phage	Not yet recruiting (estimated	NCT04787250
Chronic airway infection in	P. aeruginosa	Nebulized phage	Not yet	NCT05010

3. General Aspects of the Use of Endolysins as Antimicrobials

Their lytic enzymes have also been investigated as potential antimicrobial agents in addition to phages [117]. In order to help with the injection of phage DNA or to facilitate the release of viral offspring by broad lysis of the bacterium, phage lysins are enzymes that mediate the enzymatic cleavage of peptidoglycan in the course of phage infection. A potential new family of antibacterial medicines called lysins may provide a solution to the issue of bacterial multidrug resistance. They quickly osmotically lyse Gram-positive bacteria when given exogenously by degrading peptidoglycan, which results in cell death. Protein engineering has expanded their effectiveness to Gram-negative bacteria, which have a protective outer membrane [118]. These endolysins, also known as "enzybiotics," have been demonstrated to be secure, efficient, quick-acting, and extremely specific [119,120]. They can be administered alone or in conjunction with conventional antibiotics, and they diminish biofilms and are unlikely to result in resistance [119,120]. Although they have some advantages, they have the same potential applications as phages. For instance, because enzymes cannot spread like phages do, their effect is directly dose-dependent and is therefore more controllable. Given the prior experience with the manufacture of heterologous therapeutic proteins, they should also be simpler to generate on an industrial scale because they cannot mobilize DNA, preventing horizontal gene transfer. The possibility of cytotoxic effects on people and animals is reduced because peptidoglycan is only found in

bacterial cells and not in mammalian ones. But because they are proteins, they might cause an immunological reaction. Indeed, research conducted in vitro and in vivo have demonstrated that repeated exposure to lysins might result in the production of neutralizing antibodies. Enzybiotics could potentially be used repeatedly to treat the same bacterial illness because these antibodies, while they do lessen the antibacterial action of the enzybiotics, do not totally neutralize them [122,123]. It can be a little bit different when using lysins to treat Gram-negative bacteria. The in vitro bactericidal activity of various non-engineered anti-Gram-negative lysins was entirely inhibited by antibodies when they were tested in vivo [125]. This implies that when bringing anti-Gram-negative enzyme antibiotics to the clinic, further optimization is required. However, in vivo infection models using engineered endolysins against Gram-negative bacteria have demonstrated encouraging outcomes [126]. The inactivity of many anti-Gram-negative enzybiotics in the presence of human serum, which is most likely caused by their high cationicity and may restrict their application for systemic treatment, is another significant issue [127]. However, some businesses and laboratories are already working on developing serum-active lysins against Gram-negative bacteria [126,128,129]. These advancements demonstrate that clinically useful lysins against Gram-negative bacteria will also be available in the medium future with adequate experimental development.

Enzybiotics that are administered systemically may also produce bacteria-lysed cellular debris, which could result in a pro-inflammatory reaction. Through membrane fragmentation, these bacterial cell fragments, which also comprise lipopolysaccharides, (lipo)teichoic acids, and peptidoglycan, may cause serious consequences such septic shock. Even in clinical studies involving human participants, allergic or severe inflammatory reactions have not yet been reported following administration of enzybiotics [130,131]. According to the findings of certain investigations, there should be an ideal dosage of enzybiotics that is sufficient to kill the bacterial pathogen without further peptidoglycan layer disintegration or the propagation of proinflammatory factors [122,123].

Recombinant lysins can now move into various stages of preclinical and clinical testing thanks to recent advancements. Even though some authors have noted that lysins may be able to enter the clinic in a shorter period of time than phages, their high technical feasibility and accelerated clinical advancements make them a good alternative therapy to replace or use in combination

with conventional antibiotics in the short term [132]. Phase II and III clinical trials using enzybiotic-based products are already being conducted by some businesses. Under the GladSkin brand, which is sold by Micreos, an enzybiotic for topical and cosmetic use is already available. Exebacase (CF-301) from ContraFect and iNtRON-N-Rephasin® SAL200 (Tonabacase), two endolysins, have recently been tested in human clinical trials (Phase I) for the treatment of S. aureus bacteremia without causing any negative side effects. Exebacase (CF-301) is currently in phase III to assess its action against S. aureus-caused bacteremia and endocarditis, while Tonabacase is currently in phase II [134].

4. Global Phage Therapy Market

As was mentioned in this review, there has been a considerable increase in the use of phage therapy recently. The market for phage therapy has grown to USD 567.9 million since 2017, and growth projections for the years 2018 to 2026 indicate a CAGR of 3.9 percent [136]. The largest market for the usage of phages in 2016 was the USA, which had a 37 percent share of the global market [137]. Due to the widespread use of phages in the food and environmental domains, Europe is the second-largest market. Georgia, Poland, and Belgium are three specific situations that are particularly noteworthy because they were among the first nations to move from basic research to the market. This market is most likely to grow in the near future due to significant investments in biotechnology infrastructures, changes in agricultural practices and the regulations that go along with them, and foreseen government initiatives that favor the use of these technologies (or at least discourage the widespread use of antibiotics) in a variety of sectors, including human health, in response to the antibiotic resistance crisis [138]. In this sense, treating illnesses brought on by MDR bacteria represents the most prospective market for phage therapy. By 2027, it is predicted that this will account for more than USD 13.8 billion annually worldwide [139]. Although patents for phage products have already been granted, there may still be some potential obstacles in the way of a successful market entry, such as the regulatory concerns, the quality of the outcomes from upcoming clinical trials of phage therapy , or

concerns about phage patentability [132,140]. Enzybiotics are more "canonical" than other therapeutic proteins, which is another reason to believe that they might enter the market sooner than complete phages by using the conventional route of molecule-level intellectual property protection and clinical trial administration. However, therapy with complete virions, particularly in its customized form, may be made available to patients under the condition that acceptable regulatory procedures are put in place.

Many scientific startups are concentrating on studying and characterizing phages and their products for potential therapeutic application in this context and given that giant pharmaceutical corporations have essentially stopped investing in new antibacterial chemicals. The majority of these new businesses are based in the USA, India, Korea, Canada, and a few European nations. Telum Therapeutics, the nation's first company devoted to the creation of endolysins, was just established [141]. If we take into account the most recent investments made in the field of antibiotic alternatives, like the BioNtech acquisition of PhagoMed [142], or the high investment rounds to support new endolysins search platforms, like in Micreos [143], we can also envision an optimistic short-term future for these developments.

5. The Challenge of Multidrug Resistant Bacteria

The World Health Organization produced a list of 12 bacterial species with a critical, high, and medium priority ranking based on the extent of resistance and available treatments in 2017 (Tacconelli et al., 2018). The current rate of antibiotic discovery and development far outpaces that of antibiotic resistance development, which poses a threat to global public health. Antimicrobial resistance may cause up to 10 million deaths annually by the year 2050, according to estimates (O'neill, 2014). Although this figure is debatable (De Kraker et al., 2016), it nonetheless emphasizes the grave issue with therapeutic options for multidrug resistant (MDR) bacterial infections that we currently confront (Bassetti et al., 2017). The bacterial viruses known as bacteriophages or phages are the bacteria's natural predators. These organisms are widely distributed and are thought to number in the trillions per sand grain on Earth (Keen, 2015).

6. SBIs in COVID-19 patients

Despite the fact that secondary bacterial infections (SBIs) are overrepresented in critically ill patients and present ongoing challenges, initial bacterial co-infections (2 days after admission) and secondary infections (>2 days after admission) are relatively uncommon among patients hospitalized with coronavirus disease 2019 (COVID-19) [1]. Well-known side effects of viral respiratory infections include secondary infections, which are primarily bacterial infections. Patients with SBIs on the COVID-19 were found to have significantly worse outcomes, longer hospital stays, and greater rates of ICU admission as compared to patients without SBIs [13,14]. Despite the generally low prevalence of laboratory-confirmed bacterial infections, a large-scale study in the United Kingdom found that the recorded positivity rates of cultures from patients admitted to critical care were high: 602 (42 %) of 1429 cultures from sputum, 207 (51 %) of 402 cultures from deep respiratory samples, and 500 (8 %) of 6157 cultures from blood. In contrast to the majority of studies, this one revealed no link between bacterial infections and patient death in the intensive care unit (ICU). Among 3028 hospitalized COVID-19 patients in New York City, USA, Kubin et al. identified 350 (12%) patients as having secondary infections that had been confirmed by a laboratory. They discovered that the hospital mortality rate for patients with secondary/co-infections was significantly higher than the hospital mortality rate for patients without (33 percent versus 19 percent) [15]. Zhou et al. reported observation in 191 hospitalized COVID-19 patients in Wuhan, China, and discovered that 27/28 patients with SBIs died despite the fact that 95% of patients got antibiotics [3]. This can be contrasted with research showing that secondary bacterial pneumonia most often caused mortality in recent influenza pandemics [16,17].

6.1 Why SBIs in COVID-19 patients is a challenge?

Problems associated to antibiotic resistance Antimicrobial resistance (AMR) was already a significant issue for public health prior to COVID-19. As a result, the prevalence of AMR bacteria in healthcare settings may help to explain why critically ill COVID-19 patients continue to experience high rates of SBIs after receiving intensive antibiotic therapy. According to local

epidemiology, ESKAPE pathogens (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, A. baumannii, Pseudomonas aeruginosa, and Enterobacter spp.) were the most common SBI agents isolated from respiratory and blood samples of COVID-19 patients. [1,15,18] For those "superbugs," the selection of antibiotics is limited, and to make matters worse, the use of some "last-resort" antibiotics, such colistin, is closely regulated due to their potential for organ toxicity, disruption of the normal flora, and the development of antimicrobial resistance.

6.2 COVID-19 related challenges

According to several investigations, SARS-CoV-2 pneumonia has a longer incubation period than pneumonia brought on by other viruses [4]. COVID-19 individuals who were critically unwell and had lengthy hospital stays were more likely to experience SBIs. Combination immunosuppress- sive medications, which are frequently used in COVID-19 patients and include corticosteroids, tocilizumab, and anakinra, may have considerably increased the incidence of blood-stream infection (BSI) in COVID-19 patients [13]. When compared to patients without BSIs, those with BSIs had considerably longer hospital stays, higher ICU admission rates, and higher fatality rates [13]. Additionally, COVID-19-induced pathological processes as mucus buildup, widespread alveolar injury, immunological signaling dysfunction, and immune cell depletion may have aided in the initiation of SBIs and decreased the effectiveness of medication therapy [7,19,20].

6.3 Initial phage therapy in COVID-19 related SBIs

In an ICU in Shanghai, China, devoted to COVID-19 patients at the beginning of March 2020, their team used phage therapy to successfully suppress a secondary CRAB infection outbreak [11]. The seriously ill patients in this ICU tended to be older and primarily male, which is consistent with prior data [1,13,21]. Four males with pulmonary CRAB infections that were

challenging to treat were found among the eight critically sick patients in the ICU throughout the research period. 18–40 days following the patients' admission to the ICU, Patient 4 was the first to develop pulmonary CRAB, followed by the other three patients in that order. The extracorporeal membrane oxygenation (ECMO) intubation site on Patient 2 also developed a topical infection. Regular antibiotic therapy had been used, but it had not been successful in containing CRAB infection. Phage susceptibility testing and multilocus-sequence typing (MLST) of the CRAB strains present in these individuals both exhibited identical patterns. In every case, treatment with a 2-phage combination led to lower CRAB loads. These findings suggested that phages may be used to quickly manage the SBI epidemic in COVID-19 patients [11].

An analogous conflict has started in the USA. The phage-based company APT said in September 2020 that it intended to use phages to treat eight COVID-19 patients who were also co-infected with CRAB as part of an urgent Investigational New Drug authorization. The U.S. Food and Drug Administration (FDA) granted APT an Expanded Access IND just two months later to make it easier for patients with COVID-19 to receive therapy across the country. A. baumannii, P. aeruginosa, or S. aureus-related pneumonia or bacteremia/septicemia were allowed instead of just CRAB pneumonia (NCT04636554). These findings have not yet been made public. The two trials stated above are the only two phage therapy studies with SBIs in COVID-19 patients, and both of them provide evidence that phage therapy may be useful in controlling outbreaks of hard-to-treat SBI pathogens.

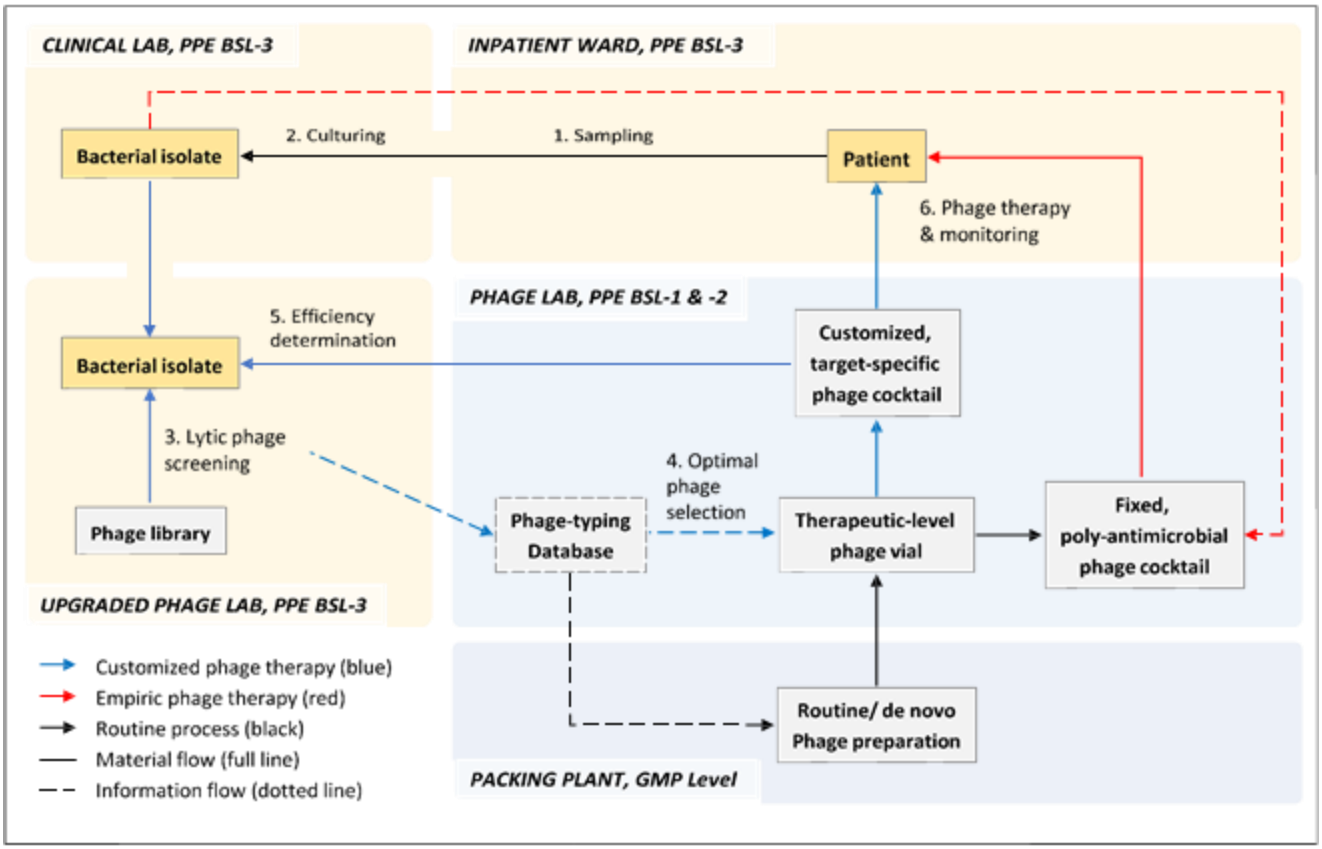
6.4 Challenges of phage therapy in COVID-19 related SBIs

The ongoing difficulties in this field may help to explain why phage therapy is rarely used in COVID-19. The challenges that phage therapy still faces were described in recently published reviews [22–24], including those related to safety, efficacy, accessibility, acceptability, and regulatory difficulties. Additionally, the current SARS-CoV-2 outbreak creates new challenges. First, because phages are not mobile, pathologic alterations brought on by COVID-19, such as airway obstruction and thrombus development, may obstruct the transport and dissemination of therapeutic phages to the sites of bacterial infection. The ability of bladder-irrigated phages to retrogradely reach their target microorganisms in the renal pelvis was demonstrated by Qin et al. [25]. Second, phage therapy may benefit from a synergy with a patient's immune responses in

order to be effective. Sadly, it has been discovered that COVID-19 alters immunological signaling and decreases immune cell levels in infection foci [19]. Additionally, the frequent use of combination immunosuppressive treatments in COVID-19 patients may considerably raise the risk of BSI [13]. Due to these circumstances, it is more difficult to eradicate bacteria, and as a result, a recurrence of phage-resistant bacteria may be a common side effect of phage therapy for COVID-19-related SBIs [11]. Third, the complicated nature of the entire phage therapy process, including pathogen isolation, phage screening, and efficacy evaluation, is undoubtedly due to the high biosafety level required in the management of patients infected with SARS-CoV-2 and the widespread distribution of the virus throughout the body. The direct binding of many enteric and respiratory viruses to the surfaces of different Gram-positive and Gram-negative bacteria has recently been described, despite the fact that bacteria do not sustain eukaryotic virus infection. influenza binding Numerous enteroviruses may become more stable when particular bacterial species are exposed to them, increasing their adhesion to respiratory epithelial cells in vitro [28–30]. SARS-CoV-2 isolation from COVID-19 patient samples and the subsequent steps (before inactivation) should be carried out in a designated laboratory by qualified personnel wearing biosafety level III personal protective equipment because it is unknown whether SARS-CoV-2 can bind to bacteria or survive in bacterial colonies (PPE BSL-3, Figure 1). When compared to usual circumstances, this will surely make things more difficult and expensive.

Clinical staff members working in the designated inpatient ward and clinical laboratory regularly provide patient care and conduct bacterial culture while outfitted in biosafety level III personal protection equipment (PPE BSL-3). In the typical phage laboratory, ready-to-use phage vials are regularly made using their original host bacteria and then packaged in a facility that has been approved for good manufacturing practices. An established library is moved to a special BSL-2 laboratory where phage screening and evaluation are carried out under BSL-3 PPE settings in order to provide tailored phage therapy. The phage(s) with the best lytic features are chosen once the phage-susceptibility result is obtained, and the corresponding vials are sent to the PPE BSL-3 phage laboratory for inspection of the killing-efficiency. The inpatient ward is then given qualified phage vials for phage therapy that have high titers against the target pathogens. Fixed-composition phage tails with broad-spectrum antibacterial capabilities against the epidemic strains can be employed for emergency usage in empirical phage therapy.

Figure 2



Workflow of phage therapy for SBIs in COVID-19 patients at Shanghai Institute of Phage (SIP).

6.5 COVID-19-related SBIs, SIP experience' of phage therapy

A workable process, the team at the Shanghai Institute of Phage (SIP) began to boost the storage of ready-to-use phage vials that target the most frequent hospital-acquired pathogens, such as the ESKAPE pathogens, as soon as the first wave of COVID-19 patients in Shanghai appeared. This method significantly hastened the delivery of phage to critically ill COVID-19 patients who needed treatment to start right away. In the meantime, they created a workflow based on the cooperation of five function zones that call for various levels of PPE. As shown in Figure 2, in a hospital designated under COVID-19, patient care and bacterial culture were carried out in the inpatient ward and clinical laboratory in accordance with the established protocols. Under BSL-3

PPE circumstances, a specific portion of the clinical laboratory was isolated for phage screening and efficiency analysis. Phage was frequently increased by growing in the original host bacterium in the typical microbiology lab to create ready-to-use phage vials. In a packaging facility with good manufacturing practices (GMP) certification, the vials were packaged. Qualified phage vials may be swiftly chosen and supplied to the inpatient ward for phage therapy by utilizing the material flow (from lower BSL zones to higher BSL zones) and the reverse information flow. Bacterial isolates were frequently phage-typed for epidemiological purposes, and this information is helpful for making appropriate therapeutic phages and putting together broad-spectrum, fixed-composition phage cocktails for use in emergencies (Figure 2) [31].

6.6 An in vitro strategy

In an ICU with critically ill COVID-19 patients, these phage therapy cases depict a typical nosocomial outbreak and care of secondary CRAB pneumo- nia [11]. The phage-resistant A. baumannii isolates that were in vitro-induced and in vivo-selected exhibited closely comparable phage susceptibility profiles when an A. baumannii strain was challenged with the same (first line) phage in both environments. As a result, we created a cocktail using the first line phage and a second phage that specifically targeted the in vitro produced first line phage-resistant A. baumannii isolate. The in vitro investigation showed that the first- and second-line phages worked in concert to prevent the target bacteria from reappearing within eight hours. However, only two of five cycles of phage therapy with the cocktail resulted in the eradication of CRAB; in the other three, the bacteria returned and proved resistant to the treatment [11]. This might have been caused in part by weakened host immune responses that prevented phage-mediated bacterial eradication [32]. The discovery of a more effective phage cocktail for reducing the anti-phage resistance connected with phage therapies may result from a prolonged phage-bacteria incubation and more thorough research of various phage combinations. Testing for phage-antibiotic synergy can provide additional information that can be useful, but it also adds additional workload and labor hours [33,34].

6.6 Phage-based vaccines

Phage-based vaccinations have a lot of potential benefits since they can be promptly switched in response to changes in the Coronavirus by using a staged approach. Vaccines based on phages are also self-fulfilling, which means that they automatically activate and increase immune response and have the capacity to display many antigens. Humans can employ phage for medicinal purposes, and there are good safety precautions in place. Recent research suggested regular booster vaccinations may be necessary to maintain protective levels of immunity and that the immunological response to SARS-CoV2 may be temporary [60, 61].

The potential benefits of phage-based vaccinations include adaptability to virus changes, rapid advancement, cost effectiveness, flexibility for method of administration (mucosal and intramuscular), and a potential oral drop. These advantages give promise as a potential weapon in containing this pandemic, along with the proven safety profile. Additionally, nations have the capacity to quickly boost productivity. In order to counteract the effects of this public health issue, researchers are totally devoted [60].

Conclusion

And

References

7. Conclusion

The discovery of all significant classes of antibiotics occurred during the period of antibiotic research, which ended more than 50 years ago. Since then, the primary antimicrobial medication developments have involved significant alterations to the already-existing natural chemicals, but this cannot guarantee against the rapid emergence of resistance to the newer antimicrobial derivatives. In addition to modifying the existing antimicrobials, there are a lot of prospective directions for research. There is an urgent need for antimicrobial alternatives, and phage use may be one of the more promising ones. Despite being used to treat a variety of illnesses for over a century, phages are still only used in a few nations for actual medical purposes. The implementation of phage therapy faces significant regulatory challenges because its efficacy must be established in accordance with current pharmaceutical guidelines. This necessitates the execution of adequately planned, randomized, placebo-controlled, and double-blind clinical trials. Alternative antimicrobial strategies are also urgently needed for existing or newly developed threat COVID-19 treatment. The COVID-19 (SARS-CoV-2) pandemic, which is steadily spreading throughout the world and putting the most attention on public health, education, travel, and financial situations right now. Due to the lack of a single therapy that can provide appropriate answers to COVID-19, enticing situations are without boundaries. Therefore, a global effort to make phage therapy available to everyone in the world is needed. It is clear that this calls for a concerted effort from countries to overcome logistical and administrative difficulties as well as from physicians and researchers to close the knowledge gap and promote advancements in the field.

7.1 Future prospective:

- How should we approach the present infection prevention and control approach, which is also effective after the epidemic?
- How might we respond if future contagious diseases are similar to this one? These are open-ended issues that need more thought and investigation. It is crucial to realize that there is no cure for the current pandemic, even though phages may have the ability to contribute to it.
- In order to maximize COVID-19 therapy, it is vital to follow clinical pharmacology, therapeutic, preventative, and diagnostic interventions. Whether intentional or not, the immediate and cell-free generation of artificial phages? Compared to conventionally make natural phages, this has a number of advantages.

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