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Clinical and experimental bacteriophage studies: Recommendations for possible approaches for standing against SARS-CoV-2

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ABSTRACT

In 2019, the world faced a serious health challenge, the rapid spreading of a life-threatening viral pneumonia, coronavirus disease 2019 (COVID-19) caused by a betacoronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). As of January 2022 WHO statistics shows more than 5.6 million death and about 350 million infection by SARS-CoV-2. One of the life threatening aspects of COVID-19 is secondary infections and reduced efficacy of antibiotics against them. Since the beginning of COVID-19 many researches have been done on identification, treatment, and vaccine development. Bacterial viruses (bacteriophages) could offer novel approaches to detect, treat and control COVID-19. Phage therapy and in particular using phage cocktails can be used to control or eliminate the bacterial pathogen as an alternative or complementary therapeutic agent. At the same time, phage interaction with the host immune system can regulate the inflammatory response. In addition, phage display and engineered synthetic phages can be utilized to develop new vaccines and antibodies, stimulate the immune system, and elicit a rapid and well-appropriate defense response. The emergence of SARS-CoV-2 new variants like delta and omicron has proved the urgent need for precise, efficient and novel approaches for vaccine development and virus detection techniques in which bacteriophages may be one of the plausible solutions. Therefore, phages with similar morphology and/or genetic content to that of coronaviruses can be used for ecological and epidemiological modeling of SARS-CoV-2 behavior and future generations of coronavirus, and in general new viral pathogens. This article is a comprehensive review/perspective of potential applications of bacteriophages in the fight against the present pandemic and the post-COVID era.

1. Introduction: emergence of threatening SARS-CoV viruses in the two last decades

The world witnessed a pneumonia outbreak of unknown etiology in Wuhan, China, in December 2019 [1]. The initial etiological studies disclosed that the disease causative agent was a virus of the *Coronaviridae* family named SARS-CoV-2. The substantial increase in detection of this virus in other parts of the world over a very short period of time led the World Health Organization (WHO) to declare a pandemic on March 1st, 2020. The clinical symptoms of this viral disease, which has become known as Coronavirus Disease 2019 (COVID-19), could range from mild respiratory disorders and fever to Acute Respiratory Distress Syndrome (ARDS), all potentially leading to death [2]. Based on the official WHO reports, the number of definitely infected and fatalities cases with

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COVID-19 reached 349,641,119 and 5,592,266 respectively, by January 25, 2021, worldwide (https://www.who.int/emergencies/diseases/novel-coronavirus-2019).

Many studies have been conducted on the supportive therapies, prevention, and management of this disease. Several promising vaccines, including BNT162B2 (Pfizer and BioNTech), mRNA-1273 (Moderna) and ChAdOx1 (AstraZeneca), have been introduced in the past two years [3]. The recommendations to prevent disease transmission or treat the patients are only based on the observed symptoms and the clinicians' point of view. When no antiviral or anti-inflammatory medication is effective, supportive management and antibiotic therapy are the only remaining options as the main pillars of therapy protocols for coping with secondary bacterial infections [4].

Phylogenetically, coronaviruses are classified in the subfamily *Orthocoronavirinae*, of the family *Coronaviridae*, order *Nidovirales* [5]. Coronaviruses are divided into four genera: *Alphacoronavirus* (α-CoV) and Betacoronavirus (β-CoV), which primarily infect mammals as their hosts, and Gammacoronavirus (γ-CoV) and Deltacoronavirus (δ-CoV), which mainly infect birds [6–8]. While coronaviruses are responsible for 20% of the common cold cases [9], they are the causative agents of some highly contagious and more severe respiratory infections over the last two decades. In addition to the current SARS-CoV-2 pandemic, two other viral pneumonia pandemics, namely severe acute respiratory syndrome and middle east respiratory syndrome, occurred in 2002 (first reported from China and rapidly spread throughout the world with the mortality rate of 11%) and in 2012 (first reported from Saudi Arabia and quickly spread to other countries with the mortality rate of 37%), respectively [10]. In all three cases, the viruses most likely originated from bats and were transmitted to humans through animal hosts [11,12]. While β-CoV has not been reported in aquatic organisms yet, α-CoV and γ-CoV have been reported in aquatic mammals including Harbor Seals (α-CoV) [13], Pacific Harbor Seals (γ-CoV) [14] and Bottlenose Dolphins (γ-CoV) [6]. There is a slight genetic homology between α-CoV and γ-CoV with SARS-CoV-2 however both are involved in pneumonia in seals and cetaceans [15]. Detection of the same coronaviruses in both coastal birds and aquatic mammals indicates potential transmission between hosts despite extreme differences in their habitats [15]. Therefore, considering the high mutation rate [16], zoonotic [10], highly contagious [11], and highly diverse, and omnipresence nature [17], the emergence of new strains and species and consequently new types of pneumonia or recurring epidemics by no means cannot be ruled out. Undoubtedly, research on the behavior of SARS-CoV2 in the environment significantly deepen our understanding of the coronaviruses life cycle, distribution and infection behaviors.

Moreover, when dealing with highly-contiguous viruses with high mutations rate, it is very important to understand their stability at different stresses or through disinfection. On the other hand, such studies come with a very high risk and requires expensive state of the art equipment and facilities which in results extremely restricts the studies. Providing the minimum standard for studies on a highly contagious pathogen such as SARS-CoV-2 is strictly limited to facilities with the highest biosafety level. Considering the high risk of working with this virus, phage-based models with similar structures, genome, and ecological or epidemiological behavior would play a critical role in facilitating the research [18].

The emergence of various types of resistance in bacteria, even to the newest and most potent antibiotics accompanied, the consequent side effects and complications of using standard or increased doses, necessitates finding alternative or complementary treatments [19]. Phages (bacteriophages) are viruses that attack bacterial cells as their host, making them a potential antibacterial agent [20–24]. Phage therapy will be successful if the number of pathogenic bacteria is reduced to an extent where the host defense system is competent enough to eliminate the remaining bacteria [25]. Many recent studies have shown the promising potential of phage therapy using only one bacteriophage or a phage cocktail or even in combination with either antibiotics or nanoparticles [26–31]. The scenario of pan-resistant secondary infections in SARS-CoV-2 portends a much more difficult time in post-COVID-19 era and even future pandemics. Hence, from both the public health and socio-economic points of views, there is an urgent need for reliable and accessible prevention, therapeutic or suppression strategies for SARS-CoV-2 epidemic.

This article reviews the value of bacteriophages in infectious disease research and proposes four approaches suitable to combat infection and pathogenesis of coronaviruses: (1) interaction between viral proteins and the host body or immune system; (2) phage-based surrogates; (3) inducing anti-inflammatory by delivery of viral proteins in hosts; and (4) providing phage therapy for secondary bacterial infections.

2. Phage display applications in COVID-19 and vaccines

Phage display and its applications in molecular biology were introduced by Smith et al. in 1985. Since then, the technique has increasingly attracted attention for two main reasons, expression and exposure of a diverse range of peptide ligands on the surface of phage, studying the interaction of the expresses ligands with target proteins or receptors [32,33]. The principle behind the technology is relatively simple; a desired corresponding DNA sequence is inserted in the nucleotide sequence of phages' coat proteins. Expression of the coat proteins will simultaneously express the guest sequence and will be "displayed" on the surface of the phage [32,33].

The proven potential of phage display can be used to treat novel strains of known pathogens or even newly emerged pathogens such as SARS-CoV-2 variants of concern, alpha, beta, delta and omicron [34]. Some of the main applications of phage display are evaluation of different antigen immunogenicity, epitope mapping, and vaccine and antibody development [35,36]. These approaches and how to be used to fight against SARS-CoV-2 are discussed below and are depicted in Fig. 1.

Phage display has been used to determine the main epitope efficiency and specificity of different viruses (Fig. 1). For example, as one of the major burdens on the health system, influenza virus undergoes antigenic shifts and drifts resulting in the emergence of new epitopes in a short period of time [37]. [38] constructed large libraries of H5N1 antigens to analyze monoclonal antibodies (MAbs) against them. The whole-genome-fragment phage display libraries were constructed to cover all of the expressed proteins of H5N1. These libraries were panned against the sera of individuals who recovered from H5N1 infection to screen the antigenic epitopes. They proved that MAbs content of convalescent sera identify hemagglutinin, neuraminidase catalytic site, M2 ectodomain, and for the first time PB1–F2 (a putative virulence factor) as one of the main antigenic epitopes of H5N1 influenza virus. This study shed light on the molecular interactions between MAbs and H5N1 epitopes that results in discovering new vaccines and novel serodiagnostic tools. The same methodology can be exploited for COVID-19 where phage display libraries can be generated for all of the SARS-CoV-2 epitopes and panned against the sera of convalescent individuals to discover the main antigenic epitopes of this virus to be used for vaccines development [39]. It is proved that envelope, nucleocapsid and spike proteins of SARS-CoV-2 have significant antigenic properties however, it is of great importance to discover the main epitopes in each of these proteins which can exert the strongest antigenic effect. In addition, SARS-CoV-2 undergoes.

Through displaying various epitopes of SARS-CoV-2 proteins it is feasible to determine these antigenic epitopes and use them in developing new vaccines and serodiagnostic tools.

Furthermore, studying the interactions between SARS-CoV-2 epitopes and human antibodies will result in a comprehensive understanding of the virus infection mechanism and the capability and capacity of human antibodies to overcome the antigen invasion (Fig. 1).

Another example of such application is epitope mapping of E32 and NS3 proteins of Hepatitis C virus through phage display. Pereboeva and Zhang et al. proved the antigenic properties of these proteins and

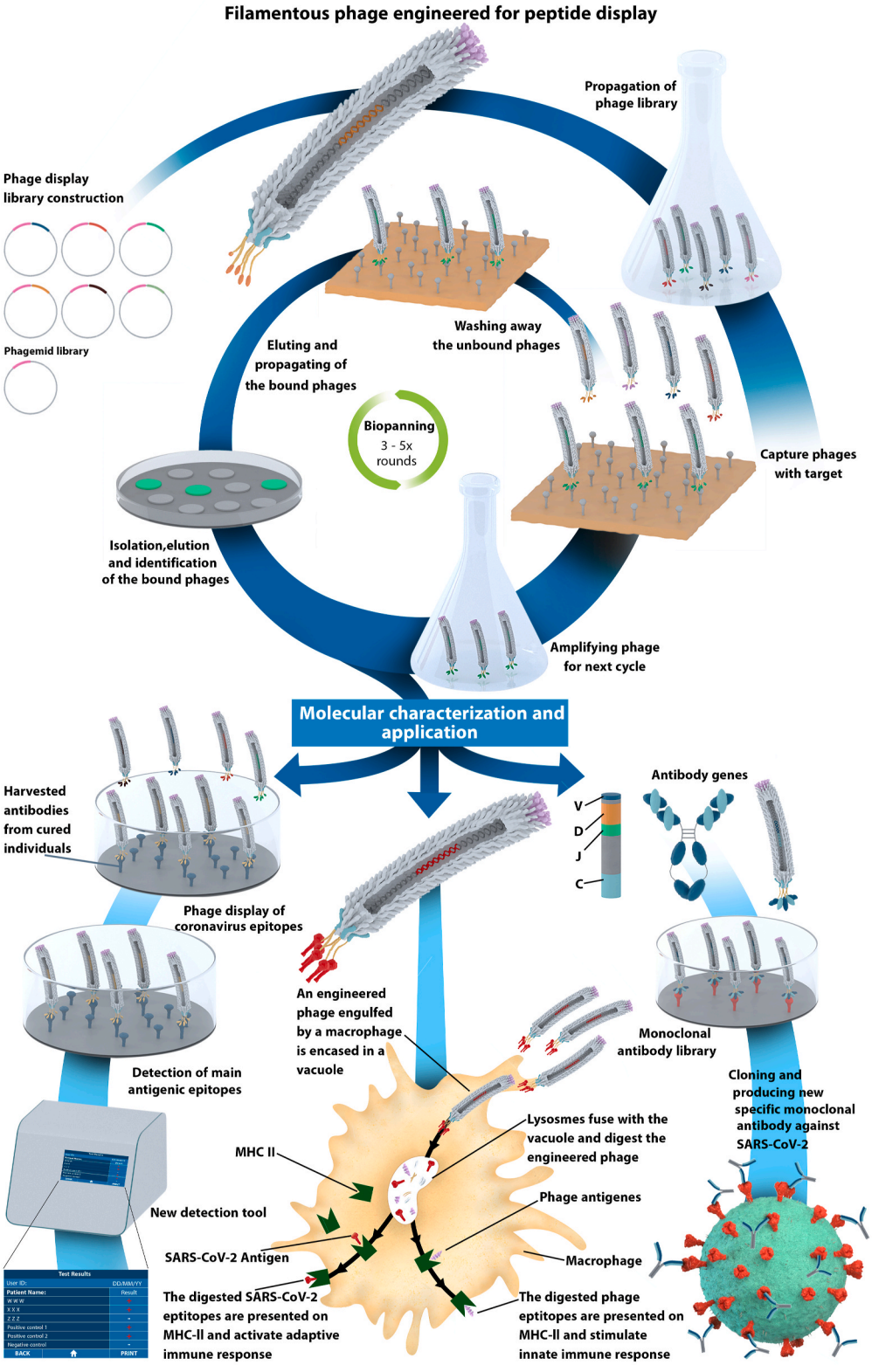

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Fig. 1. The schematic illustration of phage display and its applications. The upper panel depicts the step by step procedure of phage display technique. First, the nucleic acid sequences of the desired antigens are inserted into the phage genome. Over the phage propagation, the antigens will be displayed on the surface of phages. The propagated phages then enters a 3–5 cycles of biopanning where they are introduced to immobilized target molecules (other antigens, antibodies, etc.). Phages with the highest affinity will bind to the target while unspecific binders will be removed during washing steps. Repeating the panning cycles ensure selection of phages carrying highly specific antigens for the immobilized target molecule of interest. The eluted specific phages can be used for propagation in a specific host. The lower panels illustrate the promising applications of phage display in developing new strategies to combat COVID-19 pandemic. "Left panel": exploiting the specific antibodies produced by healed patients can be used to detect the main antigenic epitopes of SARS-CoV-2 and develop a new COVID-19 detection tool. Different antigenic epitopes of SARS-CoV-2 can be displayed on the surface of phages and be introduced to the harvested specific antibodies of cured patients. The highest affinity binders are collected as they displayed the main antigenic epitopes of SARS-CoV-2. These epitopes can be employed in ELISA-based techniques to differentiate between positive/negative individuals. "Middle panel": enhancing the effects of COVID-19 vaccines in two ways, displaying the main antigenic epitopes to the immune system, and vaccine adjuvant effect. Vaccines can be delivered through displaying the main antigenic epitopes on a phage. Macrophages engulf the phage-vaccine and present the SARS-CoV-2 antigens on its MHC-II to initiate the adaptive immune response to eliminate SARS-CoV-2 from infected cells. At the same time, the rest of the digested phages play an immunogenic role as an adjuvant by triggering the innate immune response which in turn boost the overall immune response of the host. "Right panel": construction of monoclonal antibody libraries. The cognate genes of antibody binding domain can be propagated in phages. The antigenic epitopes of SARS-CoV-2 are immobilized on a surface and are presented to the monoclonal antibody libraries. The phages displaying antibodies with the strongest affinity against SARS-CoV-2 epitopes are eluted and used as a novel promising pharmaceutical that can specifically detect and eliminate SARS-CoV-2 from infected individuals.

candidate human CD81 as their specific receptor [40,41]. Many other viruses such as Hepatitis A, B, E, and HIV-1 have also been epitope mapped. Once the antigenic epitopes and the corresponding recognition sites of the antibodies are discovered, the collected data can particularly be used in vaccine development [42,43]. In this context, the immune serum of convalescent individuals serves as the target of biopanning; therefore, potential vaccine candidates against a specific disease are isolated. For instance, novel epitopes of HIV-1 and HBV have been discovered by constructing and screening peptide phage display libraries on convalescent serum from individuals. Potential epitopes have immunized animal models to produce virus-neutralizing antibodies [44,45]. In another study, De Berardinis et al. identified epitopes of HIV-1 that can prime cytotoxic T-cell responses [46]. This approach was employed for other coronaviruses as well. Van Den Brink et al. constructed an antibody phage library. They panned against the immobilized envelope (E) and nucleocapsid (N) proteins of SARS-CoV. A total of six MAbs with significant bind strength to these proteins were discovered. Epitope mapping revealed a substantial role for N479 residue in binding to MAbs [47]. Identification of a dominant epitope in the S2 domain of SARS-CoV spike protein was also carried out using the M13 phage display library [48]. In a similar attempt to map the epitopes of SARS-CoV, Hua et al. identified TPEQQFT epitope that strongly binds to human MAbs and could be used as a new scaffold in developing promising vaccines [49].

Recent studies argued that N and E proteins of the SARS-CoV-2 have the most antigenic effect on the human body [50,51]. Therefore, phage display can be used for epitope mapping of SARS-CoV-2, and the sera of healed COVID-19 patients can be used to determine the main antigenic epitopes. Subsequently, the obtained data would be of extreme value in drug, vaccine or antibody development against SARS-CoV-2 (Fig. 1) [52,53].

Another application of phage display has been in MAbs development [33,35,36]. Large libraries of natural or synthetic antibodies have been constructed using V gene repertoires of the immune system [33]. In brief, these sequences are displayed on the surface of a phage. Then the interaction affinity and specificity of these MAbs are measured by presenting them to the main pathogenic antigens of interest (Fig. 1) [35,36]. It is noteworthy that the quality and size of such libraries are crucial in discovery of novel and more potent antibodies. The best approach is to construct large phage libraries using the full repertoire of human antibodies to generate full-length antibodies [54]. Hence, the complementarity determining region (CDR) located on antibodies that determine the binding of antigens demonstrate stronger affinity and ability to act against infectious antigens [55]. Marks and colleagues were the first to introduce a method to generate full-length antibodies using human antibody repertoire [56]. Mazor et al. established *E*-clonal approach, which enables isolation of full-length IgGs from phage displayed libraries [57]. Phage display antibody libraries can be derived from both immunized (donors who have been exposed to the antigens) and non-immunized donors. The genome of non-immunized libraries can be exploited to generate a wide range of antibodies that will lead to the discovery of novel antibodies for novel antigens. On the other hand, antibodies from immunized libraries have a stronger affinity towards the antigen as they have gone through *in vivo* affinity maturation process [58]. MedImmune, Raxibacumab and human antibody AT005 are just a few examples of phage display contributed MAbs against respiratory syncytial virus (RSV) anthrax and cancer, respectively [59–61].

Using the strategy, different SARS-CoV-2 antigens like N, M and E proteins can be panned and targeted for different MAb libraries. After several affinity steps, the strongest MAbs can be screened against SARS-CoV-2. The rational idea behind this approach has been successfully used in finding MAbs for SARS-CoV (also known as SARS-CoV-1), the closest known viral relatives of SARS-CoV-2 [62].

Taken all together, it is feasible to exploit the antibody repertoire of convalescent individuals to construct a large phage display library in which, after the screening procedures, several promising MAbs would be obtained. Furthermore, non-immunized phage display libraries can be used for developing novel antibodies for the upcoming SARS-CoV-2 variants which in turn may make phage display a beacon in the dark path of finding a perfect treatment for this burden on the health system. The proposed method is shown in Fig. 1.

Another application of phages and phage display in fighting against new pathogens is being used as inducer, booster or adjuvant of the immune system responses. In this concept, presenting the immunogenic antigen on a phage can lead to a more immediate and sophisticated immune response as discussed above [63,64] which is highly desired for vaccine development in general and SARS-CoV-2 in particular (Fig. 1).

To date, over 300 projects were carried out on exploring new vaccines for the disease and a handful of those showed promising results which are being used worldwide [65]. Different platforms were exploited to develop these vaccines. DNA, mRNA and proteins of SARS-CoV-2 were used as antigens to stimulate the immune system. Phage display could be implemented to produce protein vaccines such as NVX-CoV2373 [66] and Co-VLP [67] with a high yield, specificity and potential to express various peptides. Viral vectors have been used in ChAdOx1, SputnikV and Ad26COVs1 and proved to be an efficient approach to induce immune response against SARS-CoV-2 [65]. Similarly, bacteriophages may serve as new surrogates for the vaccine while playing an adjuvant role simultaneously.

Taken all together, phage display offers several exploitable advantages in developing new strategies to combat COVID-19 pandemic and its complications. Understanding the mechanisms of SARS-CoV-2 infection is of great importance, and phage display is an efficient means to study this virus's antigenic moiety and immunogenic properties.

3. Phage-based surrogates for coronaviruses

Many studies reported the presence of SARS-CoV-2 in wastewater which raised a crucial concern that COVID-19 may enter different habitats and even drinking water resources through urban or agricultural runoff or wastewater. For instance, recent studies reported SARS-CoV-2 in rivers that are the final destination of untreated human waste [68,69]. Although coronavirus load greatly decreases during primary and secondary wastewater treatment, there is still a potential risk that some viruses survive these stages and find their way into the treated wastewater [15]. Considering the similarities between the ACE2 receptor in mammals, if such a thing occurs, the survived viruses may infect both aquatic and terrestrial mammals such as harbor seals [70,71]. In that sense the contaminated aquatic aerosols can become another carrier of viruses into terrestrial habitats. Therefore, in addition to regular monitoring of coronavirus at the inflow and outflow points of wastewater facilities, the development of effective treatment methods to remove viruses seems vital to prevent future viral epidemics.

SARS-CoV-2 is classified as a risk group (RG) 3 biological agent by European Commission [72], therefore not every research center would be able to work on this virus. The same applies to other viruses and pathogens of RG 3 and 4. This will greatly restrict the number of research centers for such studies. To tackle the issue, it is possible to use characteristically similar phages to such pathogens including coronaviruses. In other words, the alternative solution would focus on using non-pathogenic surrogates like bacteriophages. In addition to being safe, phages can be propagated easier, faster, and at a lower cost (because no high-tech facilities or equipment is required), the purification process of phage particles is not very complicated and the proliferation rate is higher and faster compared to animal viruses.

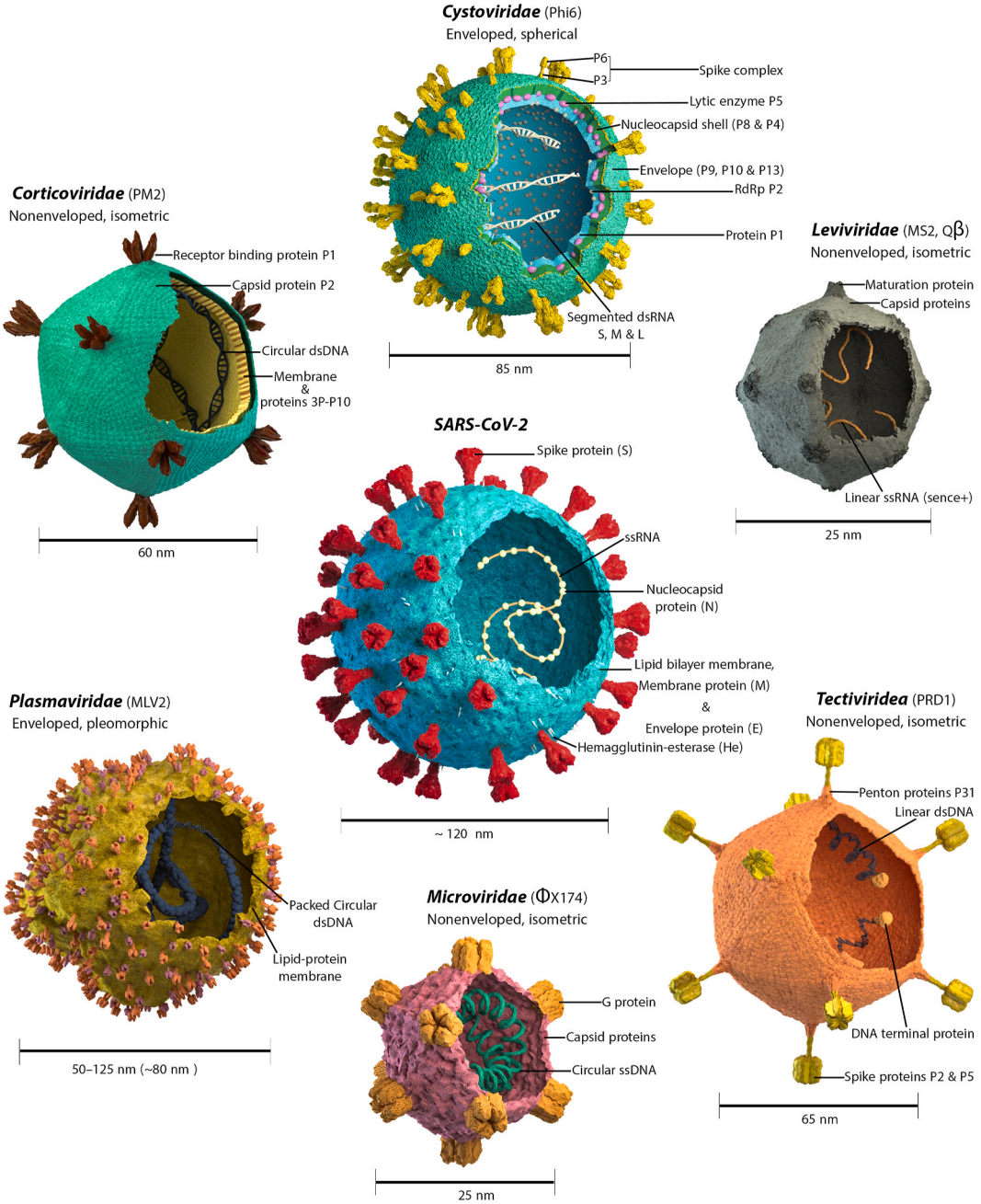

Fig. 2. Non-tailed phages with multifaceted capsid morphology (*Cystoviridae, Leviviridae, Corticoviridae, Tectiviridae*) and pleomorphic phages (*Plasmaviridae*). The morphological characteristics, type of genetic material and examples of each phage and other details are provided.

Table 1
Summary of the last studies using phage surrogates instead of viruses.

Phage (s)	Other viruses (if any)	Surrogate for	Note	Reference
MS2	Influenza A	ssRNA virus	UVC at 222 nm and 254 nm wavelengths significantly reduced the log concentration of both influenza and MS2 phage.	[74]
MS2	–	HEV	The majority (≥75%) of the MS2 was strongly bound to or entrapped in kaolinite and fiberglass colloids.	[75]
MS2	–	HEV	H_2FeO_4, $HFeO_4$, and IAOM, significantly inactivated MS2.	[76]
MS2	–	Replication and gene expression in ssRNA sense positive virus	MS2-based RNA-dependent transcription-translation reactions can control DNA-dependent gene expression by encoding a viral DNA-dependent RNA polymerase on an MS2 RNA template.	[77]
MS2	Adenovirus	HEV	Silver and copper significantly reduced the viable count of adenovirus and MS2.	[78]
MS2	–	Coronavirus	Coronavirus NC had a similar octahedral geometry structure to MS2, but its NC is bigger and has a membrane.	[79]
MS2	T4 and T7	HEV	While MS2 is sensitive to UV/H_2O_2, no inactivation was observed when exposed to wavelengths above 295 nm.	[80]
MS2 and Phi6	–	EBOV	Low concentration (25 ppm) of hydrogen peroxide vapor (HPV) was effective against MS2 and Phi6 in the absence of blood at 2 h, and in the presence of blood after 3 day contact time. Higher concentrations (>400 ppm) of HPV dramatically reduced (2–6 log) the phages counts in the presence of blood.	[81]
MS2 and Phi6		En and nEn viruses	Provided the comprehensive mechanistic understanding of En and nEn viruses' inactivation mechanism by free chlorine and UV254. Free chlorine is easily passed through the Phi6 membrane (surrogate for En viruses) and reacts with the NC and polymerase complex. Inactivation of Phi6 by free chlorine was approximately 30 times greater than MS2 (surrogate for NC virus). UV 254 inactivation kinetics of Phi6 and MS2 was similar.	[82]
Phi6 and Phi8	Avian influenza virus (H5N1)	Avian influenza virus (H5N1)	Phi6 could be a suitable indicator of disinfection due to its slightly higher chlorine resistance.	[83]
MS2	Poliovirus type 3	Poliovirus type 3	Ozone disinfection systems had more significant impacts on MS2 than poliovirus.	[84]
phi 6		Ebola Virus and Coronavirus	At the same absolute humidity conditions, Phi6 persisted longer than EBOV but not some of the tested coronaviruses.	[85]
MS2	Norwalk Virus, Poliovirus 1		The titer of all the tested viruses was reduced rapidly and extensively by the ozone disinfection method.	[86]
MS2 and Phi6 and phiX174		Coronaviruses	UV-C radiation partially reduced phage counts. Additionally, dry heat (70 °C for 30 min) was not fully effective for destruction of the bacteriophages.	[87]

IAOM, intracellular algal organic matter; NC, Nucleocapsid; CHIKV, Chikungunya virus; Hev, Human enteric viruses; EBOV, Ebola Virus; En, enveloped; nEn, non-enveloped.

In that sense, among the discovered phages so far, the morphologically classified as non-tailed phages have the greatest resemblance to coronaviruses. Only a small percentage of the known phages (about 4%) are non-tailed [73]. Some members of this group have almost identical geometrical capsid shapes and produce protein appendages that are similar to coronaviruses' spike to some extent. Besides the morphological similarity, the genome (RNA or DNA, single- or double-strand) of a surrogate bacteriophage is also of importance as it can considerably affect the viral stability at different environmental stresses (Fig. 2). Table 1 describes recent studies in which phage surrogates have replaced the pathogenic viruses.

Only a limited number of studies were conducted on the fate, transmission, inactivation, and eradication of enveloped viruses focused around human or animal models of coronaviruses or phage Phi6 (*Cystoviridae*) [88,89]. Although using coronaviruses will undoubtedly be more valuable for studying the current COVID-19 pandemic, using a diverse collection of other surrogate enveloped viruses can provide valuable data on the different aspects of SARS-CoV-2 s infection. Like other biological studies, future research on coronavirus must seek to describe and even standardize the conditions under which measurements are made. The environmental conditions, virus purification, and virus concentration in gene copies and infective units must be described. The known low-risk or risk-free viruses can considerably facilitate the research process as an alternative to the dangerous viral pathogens. For instance, MS2 phage is one of the oldest models in modern molecular biology [90] which had been fully characterized earlier. Recent studies reported its application in mimicking RNA viruses such as Ebola virus (EBOV), Hepatitis E virus (Dufour et al.), Norwalk Virus, Poliovirus and avian influenza virus (Table 1). Despite the fact that MS2 is an intrinsic naked virus [74–76,78] it is shown that it could be used as safe surrogate for the enveloped virus (influenza) and quasi-enveloped virus (HEV) as well. MS2 phage was successfully used to surrogate presence of EBOV in blood and it's successfully inactivation using hydrogen peroxide vapor [81]. Although MS2 is fundamentally classified as a non-enveloped isometric virus (Fig. 2) regarding to Ref. [81] report, is classified as a non-enveloped virus (Fig. 2), it can be a suitable candidate for research on environmental behavior, or the effects physicochemical stresses on filamentous enveloped virus of EBOV. In other words, the performance of MS2 was approved not only for enveloped viruses (influenza) but also was fully-conformed as surrogate for non-isomeric viruses like EBOV (filamentous enveloped). Coronavirus with regards to the extensive research that has been carried out on its genetic material, environmental behavior, or the effects physicochemical stresses [90]. Beside of the provided evidences above about MS2 phage, by considering morphological and genetic properties, the phages of *Leviviridae* family seems to be the best surrogates for coronavirus (Fig. 2) but this should be first tested *in vitro*.

4. Bacteriophages and the immune system responses

Studies on the effects of phages on immune cells are important for the applications of phages. Table 2 describes recent studies on the interactions between bacteriophages and immune cells. Bacteriophages can play a protective role for eukaryotic cells against other viral infections by competing with adsorption or penetration of other viruses or even by inhibiting their propagation (Table 2). Bacteriophages are also capable of inducing antiviral immunity through activating specific pattern recognition receptors (PPRs) such as TLR3 and TLR9 or regulating the immune system responses by inhibiting reactive oxygen species (ROS) production or modulation of immune responses to maintaining a balance (Table 2). It has been shown than lung infection with respiratory viruses is associated with inflammation and cell death

caused by excessive ROS production [106]. The harmful action of ROS on the functions of both pulmonary cells and red blood cells (RBCs) can be seen as a major contributor to the hypoxic respiratory failure observed in the most severe cases of COVID-19 [107]. Thus, most of the desirable effects of phages, like inhibiting the ROS, can play an important role in fighting against exciting or emerging COVID-19 infections.

4.1. Inhibitory effects of phages on viral adsorption & replication

Growing pieces of evidence do suggest that phages are inherently capable of binding specific cell-surface receptors. For example, phage adherence to mucosal surfaces may provide antimicrobial defenses where the rapid and directional transcytosis of diverse phages across lung epithelium can provide a protective barrier against other viral invasions [108]. Another surface receptor for bacteriophages is the cell surfaces integrin which are important in phage uptake. It was shown that T4 phage inhibits the adsorption and replication of human adenovirus (HAdV) in human lung and kidney epithelial cells (Table 2). Attachment of T4 phage to integrin impairs HAdV attachment to the target cells through competitive binding to epithelial cell surface integrin via its KGD sequence [94]. In T4 phage, the integrin KGD motif (Lys-Gly-Asp) is present in the capsid protein [109]. KGD motif located on the surface of gH/gL glycoprotein directs the EBV fusion of B cells and epithelial cells [110]. Thus, T4 phage can also interfere with EBV infection through a protein-protein binding competition [96]. Both *Escherichia coli* phage T4 and *staphylococcal* A5/80 phage significantly reduced the expression of HAdV-5 genes, while synthesis of HAdV-5 DNA was only inhibited by T4 [95]. The absence of KGD motif in A5/80 phage suggests an additional mediating mechanism involved in phage-mammalian cell interactions during viral infection.

Since the KGD motif is conserved and expressed in many the exposed structural protein of many virus, the same potential inhibitory mechanism can be considered for any virus that has this motif, such as SARS-CoV in protein S [111]. In other words, KGD + phages can act as a protective layer against infection of eukaryotic cells by SARS-CoV-2. Phage encapsulated in electrostatically stabilized positively charged liposomes may enhance delivery to the cell surface. It makes bacteriophage-encapsulated liposomes an appealing approach to treat antimicrobial infections.

4.2. Anti-inflammatory properties of phages

Direct interaction of bacteriophages with immune cells can induce certain cytokines with important role in antimicrobial immunity [96]. For example, Pf phage and phage RNA genome can induce production of type I interferon by triggering Toll-like receptor 3 (TLR3) [99]. Additionally, Interferon γ (IFN- γ) production is stimulated by *Lactobacillus*, *Escherichia* and *Bacteroides* phages and phage DNA genome through triggering TLR9 [102]. Both type I interferon and IFN- γ are potent antiviral cytokines [112,113].

Moreover, intraperitoneal application of T4 purified phages inhibited ROS production by cells exposed to endotoxin [114], which can reduce inflammation and inhibit cell death caused by ROX production in patients with a lung infection.

Bacteriophages can also regulate certain anti-inflammatory cytokines such as TNF production ([115]. *Staphylococcal* phages reduced bacterial lipopolysaccharide (LPS)-induced inflammatory cytokines *in vitro*, including TNF-α and IL-6 [97]. The immune response induced by *Staphylococcus aureus* and *Pseudomonas aeruginosa* phages was shown to be endotoxin-independent and predominantly anti-inflammatory [116]. Phage therapy of XDRAB bacteremia decreased the levels of inflammatory markers TNF-α and IL-6 [104] and significantly improved histologic damage of the infected lung cells in acute pneumonia model mice [105]. Clinical phage therapy regulates TNF production, reduces it when it is high, and increases it in low responders [115].

Notably, secondary bacterial infections in the respiratory tract could potentially contribute to the high mortality rate of COVID-19 or other respiratory viruses (i.e. influenza) especially in low-income countries where antibiotics are not always available. Antibiotic administration promotes a systemic inflammatory reaction requiring medical intervention after the excess release of high amounts of endotoxin (i.e., LPS and other PAMPs). Physicians are well aware of the role of the immune

Table 2
The effects of bacteriophages on the mammalian cell immune response.

Phages	Mammalian cell/ Eukaryotic virus	Notes	Reference
E79	Apoptosis	Presenting E79 to cells decreased the apoptosis ratio.	[91]
T4 and A3/R	Apoptosis (DEC-205+)	The number of DEC-205+ human myeloid cells was significantly reduced after treatment with T4 and A3/R phages.	[92]
T4 and M13	Hsp90	T4 and M13 down-regulated Hsp90 gene expression in PC-3 and is suggested as a potential anti-cancer bio-nanoparticle.	[93]
T4	Human AdV (HAdV)	Treatment with T4 significantly inhibited adsorption and replication of HAdV in a dose-dependent manner.	[94]
T4 and A5/80	HAdV	Phage treatment significantly reduced the HAdV-5 gene expression level. HAdV-5 Genome replication was only inhibited by T4.	[95]
T4	EBV	T4 phage interfered with EBV infection.	[96]
vB_SauM_JS25	NF kappa B	A *Staphylococcus* phage inhibited LPS-mediated NF kappa B activation.	[97]
Phage dsRNA	IFN-α	Phage dsRNA induced IFN-α production in human polymorphonuclear blood cells.	[98]
Pf phages and phage RNA	TLR3	Pf phages and phage RNA genome were endocytosed by triggering TLR3 and TIR domain-containing adapter-inducing interferon-β (TRIF)-dependent type I interferon production.	[99]
536_PI	IFN-α and IL-12	*E.C* phage 536_PI promoted IFN-α and IL-12 production in lung cells resulted in a healthy presence of an antiviral agent in the lungs of healthy uninfected mice.	[100]
Virome (including phages)	Respiratory bacterial and viral	Patients with viral pathogens (including CoV) had lower percentages of bacteriophages.	[101]
Lactobacillus, *Escherichia*, and *Bacteroides* phages as well as phage DNA	TLR9, IFN-γ	*Lactobacillus*, *Escherichia*, and *Bacteroides* phages and phage DNA genome stimulated IFN-γ production via the nucleotide-sensing receptor TLR9.	[102]
vB_SauM_JS25	murine norovirus (MNV)	the phage affects the innate response, such as the IFN-inducible GTPases and GBPs, and therefore exerts an antiviral effect *in vitro*	[103]
φkm18P phage	TNF-α and IL-6	Phage therapy in XDRAB bacteremia decreased the levels of inflammatory markers TNF-α and IL-6.	[104]
Bφ-R2096	Lung	Phage treatment significantly improved histologic damage of the infected lung cells of mouse acute pneumonia model.	[105]

XDRAB, extensively drug-resistant *Acinetobacter baumannii; DEC-205+*, lectin receptor recognizing ligands expressed during apoptosis and necrosis of different cell population; *E.C*, *Escherichia coli*.

system in the successful treatment of bacterial infections. Acute respiratory infections can be cured and prevented with phage, described by the immune-phage synergy model [117].

5. Phage therapy of pulmonary bacterial infections

Viral replication in pulmonary tissues could eventually results in a secondary bacterial infection by the opportunistic nosocomial pathogens [118]. In general, bacterial infections stimulate a stronger reaction by the innate immune system; hence, the invasion and growth of bacteria further stimulate the innate immune system, following which it secretes excess inflammatory material into the pulmonary alveoli. Add to this, additional bacterial load as a result of using artificial respiration which greatly facilitates entry and nesting of the invasive bacteria [119]. This process is accelerated by the continuation of the viral attack on lung cells. Therefore, more and more dead cell debris is provided for bacteria to feed on. Not only recurrence of this cycle causes increased accumulation of inflammatory fluids in the lungs but it also drastically reduces gas exchange; eventually, respiration without artificial ventilation becomes impossible and, in severe cases, will cause a drastic decline in gas exchange, lowered blood oxygen, and sepsis which finally leads to the patient's death [120]. Inhibiting bacterial growth and their elimination in any way can decrease synthesis and secretion of lung fluid. If accompanied by reduced viral load, it can significantly improve the clinical manifestations of the disease and patient's condition.

Development of such secondary bacterial infections and the emergence of their clinical complications vary from case to case, but may take an average of 17 days [120]. It is important to mention that bacteria start colonization of the respiratory tract before causing the acute respiratory distress syndrome [121,122]. In that sense, theoretically, commencing the antibacterial therapy before the occurrence of the acute respiratory distress syndrome can significantly decrease the disease intensity.

Co-infections are frequently reported as common consequences in patients suffering from respiratory diseases such as cystic fibrosis, viral influenza, and even those infected by coronaviruses such as SARS and the Middle East respiratory syndrome (Schofield et al.) [122]. However, there is no precise and comprehensive information on the prevalence of co-infection in COVID-19 patients. The very few studies addressing co-infection demonstrated various prevalence rates for secondary infections in COVID-19 patients. At least 50% of dead casualties had progressive bacterial infections (Table 3). The possibility of the disease becoming syndemic under health and social disparity conditions makes the matter worse [141,142]. This suggests the importance of paying attention to co-infection in the case of COVID-19. Generally, after identifying the possible pathogens causing co-infections in patients with COVID-19, it is possible to administer suitable antimicrobial agents. The infrequent reports on secondary bacterial infections in COVID-19 patients could be due to the high importance of the viral infection itself in the sense that it downplayed the role of secondary bacterial infection. Despite lacking comprehensive studies on COVID-19, the development of secondary infections (pneumonia) in patients suffering from other viral respiratory diseases such as influenza suggests a similar possibility in COVID-19 [121,122]. For example, the secondary infections caused by *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Aerococcus viridans*, *Haemophilus influenzae* and *Moraxella catarrhalis* are commonly found in patients with influenza [143]. Table 3 summarizes the reported secondary bacterial infections and sepsis in COVID-19 patients, including *Acinetobacter baumannii* and *Klebsiella pneumoniae* in ICUs-hospitalized patients, and infections associated with *S. pneumoniae*, *Pseudomonas aeruginosa*, *S. aureus*, *Chlamydia pneumoniae*, and *Legionella pneumophila*. There are repeated reports concerning occurrence of healthcare-associated pneumonia (HAP) and ventilator-associated pneumonia (VAP) caused by *P. aeruginosa*, *Escherichia coli*, *K. pneumoniae*, *Acinetobacter* spp., and *S. aureus* confirming the role of these pathogens in respiratory infections [144,145]. Therefore, undoubtedly a considerable portion of COVID-19 mortality can be due to bacterial co-infections of the respiratory system [146,147].

In case of secondary infections, prescription of broad-spectrum antibiotics at high doses and sometimes multidrug therapies for patients can limit common bacterial infections. Reports indicate that more than 70% of the patients in the current COVID-19 pandemic receive various antibiotics [148]. This further increases the risk of multidrug-resistant pathogens and the need to develop alternative strategies to fight bacterial infections in the post-COVID-19 era. Furthermore, as expected, antibiotics have a negligible effect because of the high frequency of resistant bacterial strains, formation of bacterial biofilm, and insufficient diffusion of antibiotics into the lungs [149]. In other words, adequate delivery of the effective doses of antibiotics to the infection site seems impossible; also, bacteria exhibit a wide range of resistance.

Contrary to antibiotics, phage therapy is much less susceptible to the emergence of resistance. One reason is the very high diversity of wild bacteriophages in nature (10^{31}). Another reason is their dynamic adaptation through mutation to new conditions such as emergence of resistance in the host. Moreover, phages can be easily engineered, which provide an inexhaustible source of antibacterial agents for researchers [150]. Furthermore, although the rate of resistance to phages is much lower than resistance to antibiotics, only a single bacterial strain develops resistance to a phage as the phage's host range (the efficacy range) is extremely narrow and even specific (contrary to antibiotics that influence the entire bacterial population.

Phage therapy of human pulmonary infections caused by *Staphylococci, Streptococci, Escherichia, Proteus, Klebsiella, Burkholderia* and *Pseudomonas* dates back to more than 50 years ago [151,152]. Over the past decades, inhaled phage therapy has been used against most of acute and chronic pulmonary bacterial infections [153–155]. Intranasal (pulmonary) delivery of phages using bacteriophage suspension nebulizer or dry powder inhalers seems to be the best method of administration due to easy delivery of the phages by inhalation in the respiratory tree from its uppermost (the nose) to its lowermost parts (pulmonary alveoli) [156,157].

Another important advantage of phage therapy is the phage activity influence on the immune response. An *in vivo* study has shown that rapid bacterial lysis by phages in a pneumonia case stimulates a much weaker inflammatory response than antibiotic therapy [100]. This study clearly indicates that there will be no serious concerns over the outcomes of inflammatory responses related to extremely rapid bacterial lysis if phages are used. In contrast, these outcomes are much more common in

Table 3
Secondary and co-infections reported in COVID-19 patients.

Bacterium	Region	
Acinetobacter baumannii	China	[123]
Acinetobacter baumannii	China	[124]
Gram-positive and gram-negative organisms (organism NR)	China	
Legionella pneumophila	China	[125]
Bacterial co-infection (organism NR)	China	[126]
Bacterial co-infection (organism NR)	USA	[127]
Bacterial co-infection (organism NR)	USA	[128]
Capnocytophagia spp., coagulase-negative *Staphylococci, Haemophilus influenzae, Moraxella* spp., *Escherichia coli, Streptococcus pneumoniae, Pseudomonas aeruginosa*	Canada	[129]
Bacterial co-infection (organism NR)	USA	[130]
Mycoplasma pneumoniae, Legionella pneumophila	China	[131]
Mycoplasma pneumoniae, Streptococcus pneumoniae	China	[132]
Mycoplasma pneumoniae	USA	[133]
Bacterial co-infection (organism NR)	Italy	[134]
Staphylococcus aureus	UK	[135]
Staphylococcus aureus	China	[136]
Mycoplasma pneumoniae	Singapore	[137]
Legionella pneumophila	Japan	[138]
Klebsiella pneumoniae Staphylococcus aureus	–	[139]
Acinetobacter baumannii	France	[140]

terms of antibiotic therapy. However, emergence of phage-resistant strains is still remained a main concern for a successful phage therapy which is required more careful investigation.

The turning point in using phage therapy for treating pulmonary infections can be found in prevalent chronic infections in people with cystic fibrosis (CF) as a result of increased multiple drug resistance bacteria associated with lungs disease in recent years [19].

Successful phage therapy for the respiratory system is strictly time-dependent and early administration significantly reduces bacterial load [153,155,158]. For example, *P. aeruginosa* was eliminated in all tested mice when treated with a single intranasal dose of phage shortly after infection (24/36 or 48/60 h). In comparison, by later administration of the phage therapy (144/156 h after the infection), only 70% of the infected mice had total pathogen elimination and partial reduction in Pseudomonas population was recorded in the remaining 30% [155]. [158] reported a similar results in which the survival rate increased significantly in mice receiving intranasal phage therapy only 2 h after infection. [153] reported prophylactic effects of phage therapy in laboratory models upon nasal administration of a high dose using a nebulizer. They reported a complete immunity (100% reduction of the pathogen) for up to 4 days in model animals.

Like any other treatment approach, *in vivo* efficacy is an important aspect of a commercial bacteriophage product. Several research addressed the *in vivo* intranasal use of phages to treat pulmonary bacterial infections in rodent models (Table 4), confirming the potential of phage therapy as a preventative or treatment strategy against secondary infections in COVID-19 pandemic. As mentioned above, in addition to studies on animal models, phage therapy of human pulmonary infections caused by *Staphylococci, Streptococci, Escherichia, Proteus, Klebsiella* and *Pseudomonas* has been employed for many years [151,152].

Undoubtedly, one of the advantages of phage therapy is the easy isolation of selective phages for local pulmonary infections in any region followed by a rapid genomic characteristic, *in vitro* and *in vivo* studies at a very low cost. After confirming the *in vitro* efficacy, *in vivo* experiments can be conducted using the available guidelines to introduce phage cocktails that are safe, efficient and compatible with the body's immunological conditions over a short period of time. Hence, in recent years numerous companies heavily invested on formulating diverse phage products for food safety, phage-enzyme, or medical and industrial applications [23]. These companies can play a critical and constructive role in speeding up the process involved in the development of lytic phages products.

6. Conclusions

Phages have a great promising potential in fighting COVID-19 pandemic. Previous research on phages reported their suitable and very effective performance in different fields of science. This article provided evidence that these biological entities can play a prominent role in improving secondary pulmonary infections by regulating immune responses or treating secondary or co-infections. Bacteriophages offer many potential applications in epitope mapping, antigen display, and surrogate agents in clinical, epidemiological or environmental studies. High abundance, inexpensive production on a large scale, compatibility with the environment, lack of known side effects as of now, morphological and genetic similarity to coronavirus, and ease of manipulation are some of the advantages of using bacteriophages in SARS-CoV-2 related research. Phage therapy may also be considered an effective solution for respiratory infections considering the post-COVID-19 era and the potential emergence of bacterial superbugs. Moreover, the need to detect coronavirus in public places and the limitations of using coronavirus in research confirm the need to have viral models. Furthermore, phage display is a highly effective platform for developing precise and easily accessible detection tools, novel vaccines and antibodies. Recent evidence demonstrated desirable regulating effects of phages on the host immune response, such as reducing inflammatory

Table 4
Summary of in-vivo phage therapy of bacterial pulmonary infections.

Bacterial infection	Phage (s)	Delivery method	Conditions and results	
Pseudomonas aeruginosa	PAK_P1, PAK_P2, PAK_P3, PAK_P4, PAK_P5, CHA_P1, LBL3, PhiKZ, LUZ19	Intranasal	100% survival in the mouse lung infection models receiving phages 2 h after infection.	[159]
	CHA_P3	Intranasal	A curative treatment (one single dose) administrated 2 h after the onset of the infection resulted in over 95% survival. A four-day preventive treatment (one single dose) resulted in a 100% survival.	[158]
	YH-6	Intranasal	100% survival in the mouse lung infection models receiving phages 2 h after infection	[160]
	PPA-ABTNL	Nebulization	Significant reduction in *P.A* in mink HP. Also, no significant difference was observed in the bacterial count of minks administered with phage of MOI 10 and MOI 100.	[161]
	φMR299-2 and φNH-4	Intranasal	Highly effective elimination of *P.A* (3–4 log units reduction in number) from murine lungs in 6 h.	[162]
Escherichia coli	536_P1, 536_P7 and adapted 536_P7	Intranasal	Intranasal administration of phage solution increased the survival rate of the animal model from 20% to 75%.	[163]
Klebsiella pneumoniae	1513	Intranasal	Intranasal administration of the phage 2 h after lethal pneumonia infection protected the mice model but weight loss was observed.	[164]
	SS	Intraperitoneal	A single dose of phage therapy (1010 PFU/ml) administered 3 h prior or immediately after bacterial infection was sufficient to rescue 100% of animal models from *K.P* -mediated respiratory infections while delaying the phage administration to 6 h after *K. pneumoniae* infection was found ineffective.	[165]
Staphylococcus aureus	S13′	Intraperitoneal	Survival of the mice model infected with hospital-acquired MRSA strains causing PAB due to administration of phage therapy 6 h after infection.	[166]
	Sb-1[a]	Inhalation	Sb-1 phage was added to the Pyophage cocktail and applied with a nebulizer. The amount of *S.A* and *P.A* in patients were drastically decreased.	[167]
Various lung infections	。[a]	Topical	Full recovery of the patients and complete elimination of bacteria (*E.C, K.P, Proteus, P.A* and *S.A*) reported for >80% infected cases.	[115]
Achromobacter xylosoxidans		Both inhalation using a compression nebulizer and orally	Patients' subjective conditions significantly improved, dyspnea resolved, and cough reduced.	[168]

[a] , in human; *P.A, P. aeruginosa, HP,* hemorrhagic pneumonia; MRSA, Methicillin-resistant *Staphylococcus aureus; S.A, S. aureus; E.C, E. coli;* PAB, pneumonia-associated bacteremia; *K.P, Klebsiella pneumoniae*. Pyophage cocktail, (a well-characterized phage cocktail).

responses, which can have a special role in improving the general condition of corona patients. Considering the high prevalence of secondary infections, the most significant advantage of phages is their capacity to cope with bacterial infections because they can increase the survival rate of patients and prevent the further emergence of antibiotic resistance.

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CRediT authorship contribution statement

Khashayar Shahin: Conceptualization, Funding acquisition, Project administration, Writing – original draft, Writing – review & editing. **Lili Zhang:** Writing – original draft, Data curation. **Mohammad Hossein Mehraban:** Methodology, Writing – original draft. **Jean-Marc Collard:** Writing – review & editing, Writing – original draft, Validation, Project administration, Investigation. **Abolghasem Hedayatkhah:** Writing – original draft, Visualization. **Mojtaba Mansoorianfar:** Resources, Validation, Investigation. **Abbas Soleimani-Delfan:** Writing – original draft, Visualization, Validation. **Ran Wang:** Conceptualization, Supervision.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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