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REVIEW

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Bacteriophages of *Klebsiella* spp., their diversity and potential therapeutic uses

Warren P. Herridge[1], Preetha Shibu[2], Jessica O'Shea[1], Thomas C. Brook[2] and Lesley Hoyles[1,*]

Abstract

Klebsiella spp. are commensals of the human microbiota, and a leading cause of opportunistic nosocomial infections. The incidence of multidrug resistant (MDR) strains of *Klebsiella pneumoniae* causing serious infections is increasing, and *Klebsiella oxytoca* is an emerging pathogen. Alternative strategies to tackle infections caused by these bacteria are required as strains become resistant to last-resort antibiotics such as colistin. Bacteriophages (phages) are viruses that can infect and kill bacteria. They and their gene products are now being considered as alternatives or adjuncts to antimicrobial therapies. Several *in vitro* and *in vivo* studies have shown the potential for lytic phages to combat MDR *K. pneumoniae* infections. Ready access to cheap sequencing technologies has led to a large increase in the number of genomes available for *Klebsiella*-infecting phages, with these phages being heterogeneous at the whole-genome level. This review summarizes our current knowledge on phages of *Klebsiella* spp. and highlights technological and biological issues relevant to the development of phage-based therapies targeting these bacteria.

INTRODUCTION

Klebsiella spp. belong to the family *Enterobacteriaceae* and are non-motile, capsulate, Gram-negative bacilli. *Klebsiella pneumoniae* is a commensal bacterium found in the gastrointestinal and respiratory tracts, and on the skin of healthy individuals. It is also ubiquitous in the environment. It is an opportunistic pathogen capable of causing a wide range of community-acquired and nosocomial infections, such as urinary tract infections (UTIs), respiratory tract infections and infections of wounds and soft tissue [1]. It has in recent years become one of the world's leading causes of nosocomial infections, with an increasing mortality rate, particularly in immunocompromised individuals, neonates and the elderly. It is also increasingly implicated in severe community-acquired infections such as pneumonia and meningitis [2].

Due to its widespread distribution and genetic make-up, *K. pneumoniae* has rapidly become a global threat to public health [3]. *K. pneumoniae* strains are frequently resistant to extended-spectrum beta-lactams such as penicillins and cephalosporins. Extended-spectrum beta-lactamase (ESBL)-producing *K. pneumoniae* strains remain susceptible to the carbapenem class of antibiotics, which includes imipenem and meropenem. However, there is increasing incidence of *K. pneumoniae* infections caused by strains that have become resistant to even carbapenems. These multidrug resistant (MDR) organisms are thought to have evolved in response to the increased use of carbapenems against ESBL-producing *K. pneumoniae*, with several independently evolved genetic elements conferring carbapenem resistance. *K. pneumoniae* carrying CTX-M-15 have spread throughout the world and are associated with a steadily increasing incidence of both nosocomial infections and, more recently, community-acquired infections, with an increasing mortality rate [4–7]. In Europe, *K. pneumoniae* carbapenemase (KPC) is the most common carbapenemase resistance gene in *K. pneumoniae* hospital-acquired infections (45%), followed by oxacillinase-48 (OXA-48-like) (37%), New Delhi metallo-beta-lactamase (NDM) (11%) and Verona integron-encoded metallo-beta-lactamase (VIM) (8%) [8]. In the UK, confirmed cases of KPC, OXA-48-like, NDM and VIM rose from 0 to 1 cases in 2007 to 661, 621, 439 and 86

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Author affiliations: [1]Department of Biosciences, Nottingham Trent University, Clifton Lane, Nottingham NG11 8NS, UK; [2]Life Sciences, University of Westminster, 115 Cavendish Street, London W1W 6UW, UK.
***Correspondence:** Lesley Hoyles, lesley.hoyles@ntu.ac.uk
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Abbreviations: BSI, bloodstream infection; ESBL, extended-spectrum beta lactamase; KPC, *Klebsiella pneumoniae* carbapenemase; LPS, lipopolysaccharide; MDR, multidrug resistant; NDM, New Delhi metallo-beta-lactamase; NGS, next-generation sequencing; UTI, urinary tract infection; VIM, Verona integron-encoded metallo-beta-lactamase.
Supplementary material is available with the online version of this article.

cases, respectively, in 2015 [9]. The spread of OXA-48-like *K. pneumoniae* strains has occurred mostly in the Mediterranean and northern Africa. They are primarily spread via ST101 strains as a result of travel in these regions, whereas ST395 is associated with clonal outbreaks throughout Europe [10]. NDM carbapenemase-producers originated in India, primarily in strains of *Escherichia coli* and *K. pneumoniae*, and they have spread throughout the world as a direct result of travel to and from the Indian subcontinent [11, 12]. Nordmann *et al.* [12] showed that more than half of NDM isolates from the UK were from patients with a history of travel to India or Pakistan. The UK appears to have the highest concentration of NDM isolates in Europe currently [13]. The contribution of *K. pneumoniae* to the antimicrobial resistance crisis is difficult to quantify. However, a recent population genomics study has shown that within- and between-hospital spread of carbapenem-resistant *K. pneumoniae* is the major driver of expansion of these bacteria within Europe, with carbapenemase-resistant isolates concentrated in clonal lineages ST11, ST15, ST11 and ST258/ST512 and their derivatives [14].

Similar to *K. pneumoniae*, *Klebsiella oxytoca* is an opportunistic pathogenic in humans, and it is becoming increasingly associated with nosocomial infections, particularly in immunocompromised patients [15]. It is also acquiring antimicrobial resistance genes and is detected throughout the UK [16, 17]. Consequently, it is now considered to be the second most clinically important pathogen of the genus *Klebsiella* [15].

Given the reduction in the effectiveness of antimicrobial therapeutics to treat *Klebsiella*-associated infections, alternative strategies must be developed in response. This literature review will focus on bacteriophages (phages) of *Klebsiella* spp. and their potential for use as alternative antimicrobial agents.

Risk factors for *Klebsiella* infections

Primarily an opportunistic pathogen prevalent in the hospital setting, *K. pneumoniae* has become a common cause of hospital-acquired infections, such as UTIs and bloodstream infections (BSIs), in which antibiotic-resistant strains are becoming more difficult to treat and are associated with an increased mortality rate. Perhaps the most ubiquitous risk factors for all forms of hospital-acquired *K. pneumoniae* colonization and infection are patient exposure to antibacterial agents and the length of hospital stay. Indeed, there consistently appears to be a positive correlation between the length of time a patient is required to stay in hospital and the chance of acquiring a *K. pneumoniae* infection, simply due to the increased exposure to healthcare-associated pathogens with time [18–20]. Moreover, a considerable number of studies aimed at identifying risk factors associated with such infections recognize previous antibiotic treatment as an important factor, particularly the widespread use of cephalosporins in the case of ESBL-producing *K. pneumoniae* infection [21], and carbapenems, fluoroquinolones, glycopeptides and aminoglycosides for infections caused by carbapenemase-producing *K. pneumoniae* [18].

Not surprisingly, invasive procedures such as surgical intervention and catheterization are also strongly associated with the acquisition of *K. pneumoniae* infection. Patients who are subject to invasive procedures such as the installation of a central venous catheter, for example, are likely to be immunocompromised individuals who have been hospitalized for a severe underlying health condition. These patients are, therefore, particularly susceptible to opportunistic infections that could lead to a BSI, in the aforementioned example, soft tissue and wound infections in patients subject to surgical procedures, or even severe cases of pneumonia or meningitis in neonates [22, 23].

Clinical features of disease may also be an important risk factor in the development of *K. pneumoniae* infection. Meatherall *et al.* [24] identified chronic liver disease and cancer as being the most significant factors involved in the development of *K. pneumoniae* bacteraemia; several studies have evidenced a link between diabetes mellitus and invasive *K. pneumoniae* infection as a result of poor glycaemic control and subsequent bacterial resistance to phagocytosis [21, 25, 26]. Nouvenne *et al.* [19] suggested an association between cardiovascular, respiratory, renal and neurological diseases, and colonization and infection by carbapenem-resistant *K. pneumoniae*.

K. oxytoca is the causative agent of paediatric antibiotic-associated haemorrhagic colitis, caused by overgrowth of the bacterium with the release of cytotoxin when the intestinal microbiota is disturbed by antibiotic treatment [27, 28]. Likely due to a combination of improved detection methods [17], increased international travel [16], contaminated hospital equipment [16], increasing numbers of immunocompromised patients and more complex treatment regimens, *K. oxytoca* is being isolated more frequently from neonatal intensive care units than in the past, and is now also being found in a range of clinical samples from adult patients admitted to critical care centres. *K. oxytoca* is showing multidrug resistance and appears to have higher drug resistance than *K. pneumoniae*, although this requires further study [29].

Virulence factors of *Klebsiella* spp.

K. pneumoniae, despite being considered to be an opportunistic pathogen, possesses an arsenal of virulence factors that enable the bacterium to both infect its host and resist the host immune response, allowing it to cause severe disease. The most studied virulence factors associated with *K. pneumoniae* are the capsule, lipopolysaccharide (LPS), fimbriae and siderophores.

The capsule is an extracellular matrix made up of strain-specific polysaccharides that surrounds the bacterium, forming a thick fibrous structure. The capsular polysaccharides produced by *K. pneumoniae* are called K antigens and, given that the polysaccharide produced depends on the strain of *K. pneumoniae*, they have traditionally been used to identify the strain using serological techniques [30]. The role

of the capsule in human disease has been studied extensively and it has been determined that is has a defensive function, providing protection against phagocytic immune cells, blocking complement-mediated lysis and reducing levels of proinflammatory cytokines [31, 32]. Indeed, the virulence of *K. pneumoniae* is greatly reduced in the absence of a capsule, as shown by infection of mice with acapsular mutants [33], and it is greatly increased in so-called hypervirulent strains, which produce more capsular material, resulting in a hypermucoviscous phenotype [2].

The LPS is composed of an O antigen, an oligosaccharide core and lipid A, and protrudes from the bacterial membrane [34]. The primary role of LPS in *K. pneumoniae* infection is to protect against the complement-mediated lysis of bacterial cells by binding of the complement component C3b away from the bacterial membrane, preventing the formation of the membrane attack complex C5b-9. This is carried out by the O antigen of the LPS, which, when absent, makes *K. pneumoniae* more susceptible to complement-mediated bacterial lysis [35].

K. pneumoniae expresses fimbriae, which are membrane-adhesive protrusions involved in the adhesion of the bacterium to host surfaces, facilitating its invasion. Two main types of fimbriae are exhibited by *K. pneumoniae*: type 1 fimbriae, which are filamentous, and type 3 fimbriae, which are helix-like in shape [36]. Moreover, the expression level of each type varies depending on the surface to which the bacteria attach. Type 1 fimbriae are expressed in the urinary tract and the bladder, but not in the gastrointestinal tract or the lungs [37]. Struve *et al*. [37, 38] speculate that the downregulation of type 1 fimbriae may occur because it reduces the ability of *K. pneumoniae* to penetrate the intestinal mucus layer in the gastrointestinal tract, as is seen with *E. coli*, whereas in the lungs, selection against fimbriated cells occurs due to rapid elimination by phagocytes. Type 3 fimbriae bind to extracellular matrices and medical devices, and are important for the development of biofilms [38].

Finally, *K. pneumoniae* must acquire iron from the environment to grow and multiply. There is very little free iron to be found in mammalian hosts, so the bacterium must express siderophores. These are molecules that have a higher affinity for iron than mammalian iron transport molecules, such as transferrin, enabling the bacterium to obtain iron for rapid growth and subsequent invasion. The primary siderophore expressed by *K. pneumoniae* is enterobactin, which is expressed in the majority of pathogenic strains; however, salmochelin, yersiniabactin, colibactin and aerobactin can also be expressed. Indeed, hypervirulent strains of *K. pneumoniae* are able to express multiple siderophores and are particularly associated with the expression of salmochelin, yersiniabactin, colibactin and/or aerobactin [39].

Genetic diversity of clinically relevant *Klebsiella* spp.

In keeping with the diversity of its virulence factors, antibiotic resistance mechanisms and clinical presentations, strains of *K. pneumoniae* also possess highly diverse and flexible genomes that are capable of producing considerable phenotypic variation. Indeed, the diversity of *K. pneumoniae* is such that the species is widely accepted to exist as four distinct phylogroups: KpI, KpII-A, KpII-B and KpIII, which are suggested to have diverged into three distinct species: *K. pneumoniae* (KpI), *Klebsiella quasipneumoniae* (KpII) and *Klebsiella variicola* (KpIII) [39]. Further genomic analyses have demonstrated that *K. pneumoniae* represents a complex of several species and subspecies: *K. pneumoniae*, *K. quasipneumoniae* subsp. *quasipneumoniae*, *K. quasipneumoniae* subsp. *similipneumoniae*, *K. variicola* subsp. *variicola*, *K. variicola* subsp. *tropica*, *Klebsiella quasivariicola* and *Klebsiella africana* [40].

In their whole-genome sequencing and pangenome-wide association study, Holt *et al*. [39] found that severe community-acquired infections were more often caused by phylogroup KpI that expressed siderophores and 'regulators of mucoid phenotype genes' *rmpA* and *rmpA2*, which regulate capsule production. Moreover, their study also confirmed the presence of SHV, OKP and LEN beta-lactamases as core chromosomal genes of all phylogroups, whereas acquired antibiotic resistance genes were more commonly found in KpI and KpII commensal isolates compared to either hospital-acquired or community-acquired infection isolates, suggesting that antibiotic resistance plays more of a role in opportunistic hospital-acquired infections caused by commensal *K. pneumoniae*, whereas more severe community-acquired infections are caused by strains enriched with virulence factors such as siderophores and increased capsular production.

Hypermucoviscous strains of *K. pneumoniae* – i.e. those that exhibit virulence genes such as yersiniabactin and *rmpA* – were first described in Southeast Asia and are commonly associated with community-acquired pyogenic liver abscess [41]. These hypervirulent strains very rarely exhibit the antibiotic resistance gene profiles commonly associated with opportunistic hospital-acquired infections, and until recently have remained treatable with antibiotics [42]. However, *K. pneumoniae* isolates with combined hypervirulence and antibiotic resistance are emerging. Given the highly diverse genome of the species, and the increasing selective pressures being applied to them in the form of antibiotics, hypervirulent antibiotic-resistant *K. pneumoniae* is threatening to become untreatable [39, 42].

Similar to *K. pneumoniae*, *K. oxytoca* has a highly diverse population structure, represented by different phylogroups (Ko1–Ko4, Ko6–Ko8) that encompass six species: *K. oxytoca* (Ko2), *Klebsiella michiganensis* (Ko1), *Klebsiella grimontii* (Ko6), *Klebsiella huaxiensis* (Ko8), '*Klebsiella pasteurii*' (Ko4) and '*Klebsiella spallanzanii*' (Ko3) [16, 43]. The complex shares numerous antimicrobial genes and mechanisms with *K. pneumoniae. K. oxytoca* has been studied far less than *K. pneumoniae*, and extensive studies of its global epidemiology are required [16].

Phages of *Klebsiella* spp.

Phages are viruses that infect bacteria and, as such, they are found in all environments where bacteria would normally

thrive. Viruses were initially suggested as a possible cause of clear zones on bacterial culture plates by William Twort in 1915, and in 1917 Felix d'Herelle confirmed this discovery, coining the term 'bacteriophage' [44, 45]. Prior to the discovery of the first antimicrobial agents, phages were considered to be the cure for bacterial infections and d'Herelle performed the first experimental phage therapy using an oral phage solution to treat dysentery [46]. However, after the discovery of antimicrobial compounds such as penicillin, the therapeutic uses of phages were largely disregarded due to the subsequent success of the antibiotic era. Phages remained useful, however, for scientific research as tools to improve our understanding of molecular biology, horizontal gene transfer and bacterial evolution, and as diagnostic tools [47]. More recently, though, given the rise in the number of MDR infections caused by bacteria such as *K. pneumoniae*, the use of phages has again come to the forefront as a potential alternative to current antimicrobial chemotherapies.

Life cycles

Phages primarily have two distinct life cycles they are able to adopt in order to reproduce: the lytic cycle and the lysogenic cycle. Both life cycles begin with the attachment of a phage to the surface of the bacterial host, followed by the subsequent injection of the phage's genetic material into the cell. In the lytic life cycle, the viral genome produces proteins that initiate the degradation of the bacterial genome, allowing the viral genetic material to take control of the host cellular machinery for the sole purpose of replicating the viral genome, synthesizing viral proteins and assembling those proteins into viable phage particles that are released from the bacterial cell in large numbers, destroying the host. The phages that are released are then able to continue infecting bacteria nearby. In the lysogenic life cycle, the viral genetic material is incorporated into the bacterial DNA, forming a prophage, and is replicated passively upon replication of the bacterial genome without destroying the host. Prophages in the lysogenic cycle are able to enter the lytic cycle under certain conditions (e.g. in the presence of environmental stressors), and begin actively replicating and producing viable phages at the expense of the host [48].

Although the lytic/lysogenic phage life cycle is a well-established concept in phage biology, we now know there are multiple phage life cycles. Pseudolysogeny is the process by which the phage genome enters a bacterial host but neither stably establishes itself as a prophage nor initiates a destructive replicative response, remaining inactive and possibly awaiting more desirable environmental conditions for viral replication [49]. Chronic infection, resulting in the shedding of phage particles over long periods of time without destruction of the host cell, can occur with the infection of filamentous phages in *Mycoplasma* [47]. Finally, the carrier state life cycle occurs when a heterogeneous population of bacteria, containing individuals that are both sensitive and resistant to a given lytic phage, leads to the destruction of sensitive bacteria and the survival of resistant bacteria, creating a stable equilibrium between viral and bacterial propagation [49].

In the context of using phages as a therapeutic alternative to antimicrobial chemotherapy, those that reliably employ the lytic life cycle to reproduce are most suitable, given that the end result is the destruction of bacterial host cells. Additionally, phages that are able to switch between multiple life cycles may not be reliable treatment options due to the possibility of dormancy and subsequent re-establishment of bacterial infection. This is just one aspect of comprehensive phage characterization that is an important consideration when choosing appropriate phage treatments.

Phage characterization

Phages of *K. pneumoniae* have been isolated from a variety of sources worldwide, including wastewater, sewage, seawater and human intestinal samples, and belong to four of the five families of the order *Caudovirales* (Table 1). These families make up the bulk of the order and are described as non-enveloped, tailed phages, with icosahedral heads containing double-stranded DNA: *Myoviridae* are characterized by long, straight, contractile tails; *Siphoviridae* by long, flexible, non-contractile tails; *Podoviridae* by short, non-contractile tails; and *Ackermannviridae* by contractile tails with up to four spikes present on each of six tail spike entities [50–52].

Genomic comparisons of lytic *K. pneumoniae* phages of the order *Caudovirales* highlight a variety of useful similarities and differences. The expression of polysaccharide depolymerases, for example, has been observed in several recently discovered phages of *K. pneumoniae* [53–55] and these enzymes have a role in the degradation of the capsule surrounding the exterior of the bacterium. The breakdown of the capsule by phage depolymerases has been purported to combat *K. pneumoniae* biofilms [56] and increase the susceptibility of the bacterium to antibiotics, phage infection and the immune system [55]. Additionally, phage depolymerase action can be observed in the laboratory with the production of 'haloes' around clear zones of lysis on bacterial culture plates after infection of *K. pneumoniae* with phage particles. This has become the basis for important laboratory methods used in the characterization of novel phages, revealing phage specificity and host range [57].

Moreover, differences observed among *Ackermannviridae*, *Myoviridae*, *Podoviridae* and *Siphoviridae* can be useful for preliminary identification. Restriction analysis, which uses bacterial restriction enzymes to digest phage DNA, can help to estimate the size of the phage genome in addition to identifying those that are already known to science prior to extensive characterization, and analysis by transmission electron microscope is able to reveal morphological characteristics such as phage tail structures [55]. Phylogenetic analyses have shown that several *Klebsiella* phages belong to accepted genera within the *Ackermannviridae*, *Siphoviridae*, *Podoviridae* and *Myoviridae* (Table 1), while others belong to new lineages with – as yet – no standing in viral taxonomy (Fig. 1 and https://doi.org/10.6084/m9.figshare.11635962.v1, available in the online version of this article).

Table 1. Known phages that infect one or more strains of *Klebsiella*

Phage	Family	RefSeq/GenBank accession no.	Genome size (bp)	Source	Reference
Magnus	*Ackermannviridae**	MN045230	157741	Wastewater plant	[107]
0507-KN2-1	*Ackermannviridae*	NC_022343	159991	Sewage	[108]
GH-K2	*Myoviridae*	Not available	Unknown	Sewage	[62]
Kpn1	*Myoviridae*	Not available	Unknown	Sewage	[78]
Kpn2	*Myoviridae*	Not available	Unknown	Sewage	[78]
Kpn3	*Myoviridae*	Not available	Unknown	Sewage	[78]
Kpn4	*Myoviridae*	Not available	Unknown	Sewage	[78]
PBKP05	*Myoviridae*	Not available	30240	Unknown	[109]
4 LV-2017	*Myoviridae*	KY271398	33540	Unknown	[110]
3 LV-2017	*Myoviridae*	KY271397	35100	Unknown	[110]
Kpn112	*Myoviridae*	KJ021043	35560	Unknown	Chandekar *et al*†
Mulock	*Myoviridae*	MN098327	43727	Wastewater sample	[111]
vB_KpnM_KpV52	*Myoviridae*	KX237516	47405	Unknown	Komisarova *et al.*†
vB_KpnM_KpV79	*Myoviridae*	MF663761	47760	Unknown	Komisarova *et al.*†
1611E-K2-1	*Myoviridae*	MG197810	47797	Unknown	Lin *et al.*†
JD001	*Myoviridae*	NC_020204	48814	Seawater	[112]
vB_KpnS_FZ14	*Myoviridae*	MK521906	49370	Sewage	[113]
vB_KpnM_KB57	*Myoviridae*	NC_028659	142987	Sewage	Volozhantsev *et al.*†
vB_KpnM_BIS47	*Myoviridae*	KY652726	147443	Sewage plant	[114]
ZCKP1	*Myoviridae*	MH252123	150925	Fresh water	[56]
Menlow	*Myoviridae*	MG428990	157281	Unknown	[115]
May	*Myoviridae*	MG428991	159631	Unknown	[116]
KP179	*Myoviridae*	MH729874	162630	Unknown	Kozlova *et al.*†
Mineola	*Myoviridae*	MH333064	166130	Unknown	[117]
JD18	*Myoviridae*	NC_028686	166313	Unknown	Fan *et al.*†
KPV15	*Myoviridae*	KY000080	167034	Wastewater	[118]
KP1	*Myoviridae*	MG751100	167989	Unknown	Kim.†
vB_KpnM_KpV477	*Myoviridae*	NC_031087	168272	Clinical sample	[119]
Marfa	*Myoviridae*	MN044033	168532	Swine faeces	[120]
PKO111	*Myoviridae*	NC_031095	168758	Sewage	[121]
vB_Kpn_F48	*Myoviridae*	MG746602	170764	Sewage	[122]
KP27	*Myoviridae*	NC_020080	174413	Wastewater plant	[55]
KP15	*Myoviridae*	NC_014036	174436	Irrigated fields	[55]
PMBT1	*Myoviridae*	LT607758	175206	Sewage	[123]
Miro	*Myoviridae*	KT001919	176055	Sewage	[124]
Matisse	*Myoviridae*	NC_028750	176081	Sewage	[125]
vB_KleM-RaK2	*Myoviridae*	NC_019526	345809	Unknown	[126]

Continued

Table 1. Continued

Phage	Family	RefSeq/GenBank accession no.	Genome size (bp)	Source	Reference
K64-1	*Myoviridae*	NC_027399	346602	Untreated water	[127]
Phage SS	*Podoviridae*	Not available	Unknown	Sewage	[72]
vB_Klp_5	*Podoviridae*	Not available	Unknown	Unknown	[128]
vB_Klp_6	*Podoviridae*	Not available	Unknown	Unknown	[128]
6 LV-2017	*Podoviridae*	KY271400	19260	Unknown	[110]
Kpn12	*Podoviridae*	Not available	~24000	Sewage	[70]
Kpn13	*Podoviridae*	Not available	~24000	Sewage	[70]
Kpn17	*Podoviridae*	Not available	~24000	Sewage	[70]
Kpn22	*Podoviridae*	Not available	~24000	Sewage	[70]
Kpn5	*Podoviridae*	Not available	~24000	Sewage	[70]
phiNK5	*Podoviridae*	Not available	~29000	Sewage	[67]
Patroon	*Podoviridae*	MK608335	39442	Wastewater plant	[129]
vB_KpnS_FZ12	*Podoviridae*	MK521905	39519	Sewage	[113]
vB_KpnP_IME321	*Podoviridae*	MH587638	39906	Unknown	[130]
2044–307 w	*Podoviridae*	MF285615	40048	Unknown	Zhao.†
vB_Kp1	*Podoviridae*	NC_028688	40114	Wastewater plant	Alvez *et al.*†
K5-4	*Podoviridae*	KY389316	40163	Sewage	[131]
KN1-1	*Podoviridae*	LC413193	40236	Unknown	[132]
Henu1	*Podoviridae*	MK203841.1	40352	Sewage	[133]
vB_KpnP_KpV767	*Podoviridae*	KX712070	40395	Sewage	[134]
kpssk3	*Podoviridae*	MK134560	40539	Unknown	[135]
SH-Kp 152234	*Podoviridae*	KY450753	40578	Unknown	Zhi *et al.*†
vB_KpnP_PRA33	*Podoviridae*	KY652723	40605	Sewage plant	[114]
vB_KpnP_KpV763	*Podoviridae*	KX591654	40765	Sewage	[134]
SH-Kp 152410	*Podoviridae*	MG835568	40945	Unknown	Xu *et al.*†
vB_KpnP_KpV289	*Podoviridae*	NC_028977	41054	Untreated sewage	[136]
KN3-1	*Podoviridae*	LC413194	41059	Unknown	[132]
K5-2	*Podoviridae*	KY389315	41116	Sewage	[131]
KP32	*Podoviridae*	NC_013647	41119	Roadside ditch	[55]
K11	*Podoviridae*	NC_011043	41181	Unknown	Savalia *et al.*†
KN4-1	*Podoviridae*	LC413195	41219	Unknown	[132]
vB_KpnP_KpV766	*Podoviridae*	KX712071	41283	Sewage	[134]
vB_KpnP_IME205	*Podoviridae*	KU183006	41310	Unknown	Bai *et al.*†
vB_KpnP_IL33	*Podoviridae*	KY652724	41335	Sewage plant	[114]
vB_KpnP_BIS33	*Podoviridae*	KY652725	41697	Sewage plant	[114]
K5	*Podoviridae*	NC_028800	41698	Wastewater	Schneider *et al.*†
KPO1K2	*Podoviridae*	Not available	~42000	Sewage	[60]

Continued

Table 1. Continued

Phage	Family	RefSeq/GenBank accession no.	Genome size (bp)	Source	Reference
vB_KpnP_KpV475	*Podoviridae*	NC_031025	42201	Clinical sample	[134]
KPV811	*Podoviridae*	KY000081	42641	Wastewater	[118]
AltoGao	*Podoviridae*	MF612071	43012	Wastewater plant	[137]
vB_KpnP_KpV71	*Podoviridae*	NC_031246	43267	Sewage	[134]
KP-Rio/2015	*Podoviridae*	KX856662	43557	Unknown	[138]
vB_KpnP_SU552A	*Podoviridae*	NC_028870	43595	Wastewater plant	[139]
F19	*Podoviridae*	NC_023567	43766	Unknown	Chen *et al.*†
KP34	*Podoviridae*	NC_013649	43809	Cesspool holding tank	[140]
vB_KpnP_SU503	*Podoviridae*	NC_028816	43809	Wastewater plant	[139]
phiBO1E	*Podoviridae*	KM576124	43865	Wastewater	[59]
NTUH-K2044	*Podoviridae*	NC_025418	43871	Untreated water	[141]
vB_Kp2	*Podoviridae*	NC_028664	43963	Wastewater plant	Alvez *et al.*†
phiKpS2	*Podoviridae*	KX587949	44024	Unknown	[142]
vB_KpnP_KpV74	*Podoviridae*	KY385423	44094	Clinical sample	[134]
vB_KpnP_KpV41	*Podoviridae*	NC_028670	44203	Sewage	[134]
vB_KpnP_KpV48	*Podoviridae*	KX237514	44623	Clinical sample	[134]
myPSH1235	*Podoviridae*	MG972768	45135	Unknown	[69]
P13	*Podoviridae*	Not available	45976	Sewage	[143]
SopranoGao	*Podoviridae*	MF612073	61644	Wastewater plant	[137]
Pylas	*Podoviridae*	MH899585	70408	Unknown	[144]
KpCHEMY26	*Podoviridae*	MN163281	70678	Environmental sample	[145]
KP8	*Podoviridae*	MG922974	73679	Wastewater sample	[146]
GH-K1	*Siphoviridae*	Not available	Unknown	Sewage	[62]
phage Z	*Siphoviridae*	Not available	Unknown	Wastewater	[54]
phiKp-lyy15	*Siphoviridae*	Not available	Unknown	Unknown	[147]
vB_Klp_1	*Siphoviridae*	Not available	Unknown	Unknown	[128]
vB_Klp_3	*Siphoviridae*	Not available	Unknown	Unknown	[128]
vB_Klp_4	*Siphoviridae*	Not available	Unknown	Unknown	[128]
1 LV-2017	*Siphoviridae*	KY271401	29880	Unknown	[110]
JY917	*Siphoviridae*	MG894052	37655	Unknown	Hao *et al.*†
KPP5665-2	*Siphoviridae*	MF695815	39241	Mastitis milk	[148]
vB_KpnS_IME279	*Siphoviridae*	MF614100	42518	Unknown	Zhao *et al.*†
2b LV-2017	*Siphoviridae*	KY271395	44279	Unknown	[110]
2 LV-2017	*Siphoviridae*	KY271396	44400	Unknown	[110]
5 LV-2017	*Siphoviridae*	KY271399	47014	Unknown	[110]
IME207	*Siphoviridae*	NC_031924	47564	Sewage	[149]
vB_Kp3	*Siphoviridae*	KT367887	48493	Unknown	Alvez *et al.*†

Continued

Table 1. Continued

Phage	Family	RefSeq/GenBank accession no.	Genome size (bp)	Source	Reference
Sushi	*Siphoviridae*	NC_028774	48754	Sewage	[150]
Sanco	*Siphoviridae*	MK618657	48790	Wastewater plant	[151]
KLPN1	*Siphoviridae*	NC_028760	49037	Human caecum	[152]
Shelby	*Siphoviridae*	MK931445	49045	Pond water	[153]
KPN N141	*Siphoviridae*	MF415412	49090	Unknown	Jeon *et al.*†
SH-Kp 160016	*Siphoviridae*	KY575286	49170	Unknown	Zhi *et al.*†
NJS1	*Siphoviridae*	MH445453	49292	Unknown	Zhu *et al.*†
TAH8	*Siphoviridae*	MH633484	49344	Unknown	Hao *et al.*†
NJS3	*Siphoviridae*	MH633486	49387	Unknown	Hao *et al.*†
vB_KpnS_GH-K3	*Siphoviridae*	MH844531.1	49427	Sewage	[62, 154]
1513	*Siphoviridae*	NC_028786	49462	Sewage	[66]
NJR15	*Siphoviridae*	MH633487	49468	Unknown	Hao *et al.*†
MezzoGao	*Siphoviridae*	MF612072	49807	Wastewater plant	[137]
KP36	*Siphoviridae*	NC_029099	49818	Wastewater plant	[55]
TSK1	*Siphoviridae*	MH688453	49861	Sewage	[79]
Sin4	*Siphoviridae*	MK931442	49916	Wastewater plant	[155]
Skenny	*Siphoviridae*	MK931444	49935	Activated sludge	[156]
NJS2	*Siphoviridae*	MH633485	50132	Unknown	Hao *et al.*†
Sweeny	*Siphoviridae*	MK931443	50241	Wastewater	[157]
vB_KpnS_FZ10	*Siphoviridae*	MK521904	50381	Sewage	[113]
KOX1	*Siphoviridae*	KY780482	50526	Wastewater	[158]
PKP126	*Siphoviridae*	NC_031053	50934	Sewage	[121]
vB_KpnS_KpV522	*Siphoviridae*	KX237515	51099	Sewage	Komisarova *et al.*†
phiKO2	*Siphoviridae*	NC_005857	51601	Unknown	[159]
48ST307	*Siphoviridae*	KY271402	52338	Unknown	[110]
Seifer	*Siphoviridae*	MH817999	58197	Unknown	[160]
YMC16/01/N133_KPN_BP	*Siphoviridae*	MF476925	58387	Unknown	Jeon *et al.*†
KPN U2874	*Siphoviridae*	MF415411	59087	Unknown	Jeon *et al.*†
KPN N137	*Siphoviridae*	MF415410	59100	Unknown	Jeon *et al.*†
KPN N54	*Siphoviridae*	MF415413	59100	Unknown	Jeon *et al.*†*
YMC15/11/N53_KPN_BP	*Siphoviridae*	MF476924	59100	Unknown	Jeon *et al.*†
KPN N98	*Siphoviridae*	MG835858	59214	Unknown	Jeon *et al.*†
vB_KpnS_FZ41	*Siphoviridae*	MK521907	106104	Sewage	[113]
Sugarland	*Siphoviridae*	MG459987	111103	Wastewater plant	[161]
KpGranit	*Siphoviridae*	MN163280	122710	Environmental sample	[145]
vB_Kpn_IME260	*Siphoviridae*	KX845404	123490	Sewage water	[162]
Kpp95	*Siphoviridae*	Not available	~175000	Unknown	[163]

Continued

Table 1. Continued

Phage	Family	RefSeq/GenBank accession no.	Genome size (bp)	Source	Reference

*Listed as *Ackermannviridae* but no evidence to support this affiliation via ViPTree. Clusters with halovirus HHTV-1 (NC_021322; unclassified DNA virus).
†No paper associated with the RefSeq/GenBank record(s).

Specificity and host range

To infect its host, a lytic phage must first attach itself to a susceptible bacterial cell. It achieves this by recognizing and binding a specific receptor on the surface of the host cell. This interaction between the phage tail structure and host receptor allows the phage to both identify susceptible bacteria and position itself for injecting its genetic material into the cell. Adsorption to the host can occur via any external structure depending on the phage and host, but in Gram-negative bacteria, such as *K. pneumoniae*, these can include the capsule, pili, outer-membrane proteins, sugar moieties or LPS [58]. This process, therefore, determines host range, i.e. the breadth of hosts that any given phage can infect.

D'Andrea *et al.* [59] showed that their newly discovered lytic phage ϕBO1E was able to specifically target KPC-producing *K. pneumoniae* of the pandemic clonal group 258 (CG258) clade II lineage, but not those of the closely related clade I lineage, due to the recognition and targeting of specific capsular polysaccharides present on strains belonging to clade II. In contrast, Verma *et al.* [60] found that the lytic phage KPO1K2, specific for *K. pneumoniae* B5055, could infect multiple strains of *K. pneumoniae*, as well as some strains of *E. coli* and, therefore, has a relatively broad host range compared to the clade-specific phage ϕBO1E.

It is generally considered, in the context of their therapeutic use, that lytic phages with a broad host range (e.g. at genus or species level) are more beneficial in combatting bacterial infection than those with a narrow host range (e.g. at strain level). Phages with a narrow host range are inappropriate for presumptive or prophylactic treatment, for example, and would rely on the identification of an infective agent prior to treatment. Additionally, even phages considered to have a broad host range would generally have a narrower spectrum of activity compared to antibiotics [61]. Therefore, efforts to increase the spectrum of activity of phage treatment has led to the development of phage cocktails, to increase the host range by using multiple phages in a single treatment [62], and even the hybridization of phage tail structures to increase the host range artificially [63].

Therapeutic potential of *K. pneumoniae* phages

There are a number of considerations to be made when selecting phages that are suitable for use as therapeutic antimicrobial agents. Firstly, phages must be effective in killing *K. pneumoniae*. During phage characterization, *in vitro* assessments of phage lysis and burst size are carried out on cultures of *K. pneumoniae*. Phages that produce rapid lysis of a bacterium and release large numbers of phage particles will produce large, clear plaques. Moreover, phages with a broad host range are generally considered to be more useful than those with narrow host range so that multiple strains may be targeted at once [64]. Secondly, lytic phages, due to the nature of their life cycle, clear bacteria quickly and efficiently compared to lysogenic phages, which integrate their genetic information into the host genome and remain dormant for an unspecified amount of time. In addition, lysogenic phages may transfer genes into the host that can confer toxin production and antibiotic resistance traits to the bacterium, thus making the infection more virulent and difficult to treat [64].

In vivo experimentation

Following *in vitro* investigations, the safety and effectiveness of any new therapeutic candidate must be measured in a suitable animal or insect model prior to human trials. In the case of *K. pneumoniae* phage research, mouse models have been used to investigate the effect of phage treatment against wound and soft tissue infections [65], pneumonia [66], liver abscesses [67] and bacteraemia [68], closely mirroring the spectrum of disease caused by the bacterium in humans. More recently, *Galleria mellonella* larvae have been used to test the efficacy of lytic phages and phage-encoded products to clear *K. pneumoniae* infections [69].

Kumari and colleagues have carried out a series of murine-based experiments aimed at identifying the therapeutic potential of the *K. pneumoniae* phage Kpn5. Isolated as one of five phage candidates (Kpn5, Kpn12, Kpn13, Kpn17 and Kpn22) from samples of sewage [70], Kpn5 was found to be the most effective, compared to the other four, when used to treat burn wound infections caused by *K. pneumoniae* B5055 in BALB/c mouse models [71]. When administered by intraperitoneal injection, Kpn5 produced an average 96.66% survival rate compared to the negative controls, which had a survival rate of 0% [72]. Additionally, when compared to topical treatments with both natural products (honey and aloe vera gel) [73] and antimicrobial agents (silver nitrate and gentamicin) [74], Kpn5 was found to be superior in both cases, providing a higher level of protection and reduced mortality rates. However, despite the promising results that this research group has produced, the authors note the possibility of *K. pneumoniae* forming resistance to Kpn5, as highlighted in their *in vitro* experiments, and provide no data on phage host range, having used only a single strain of *K. pneumoniae* throughout their studies.

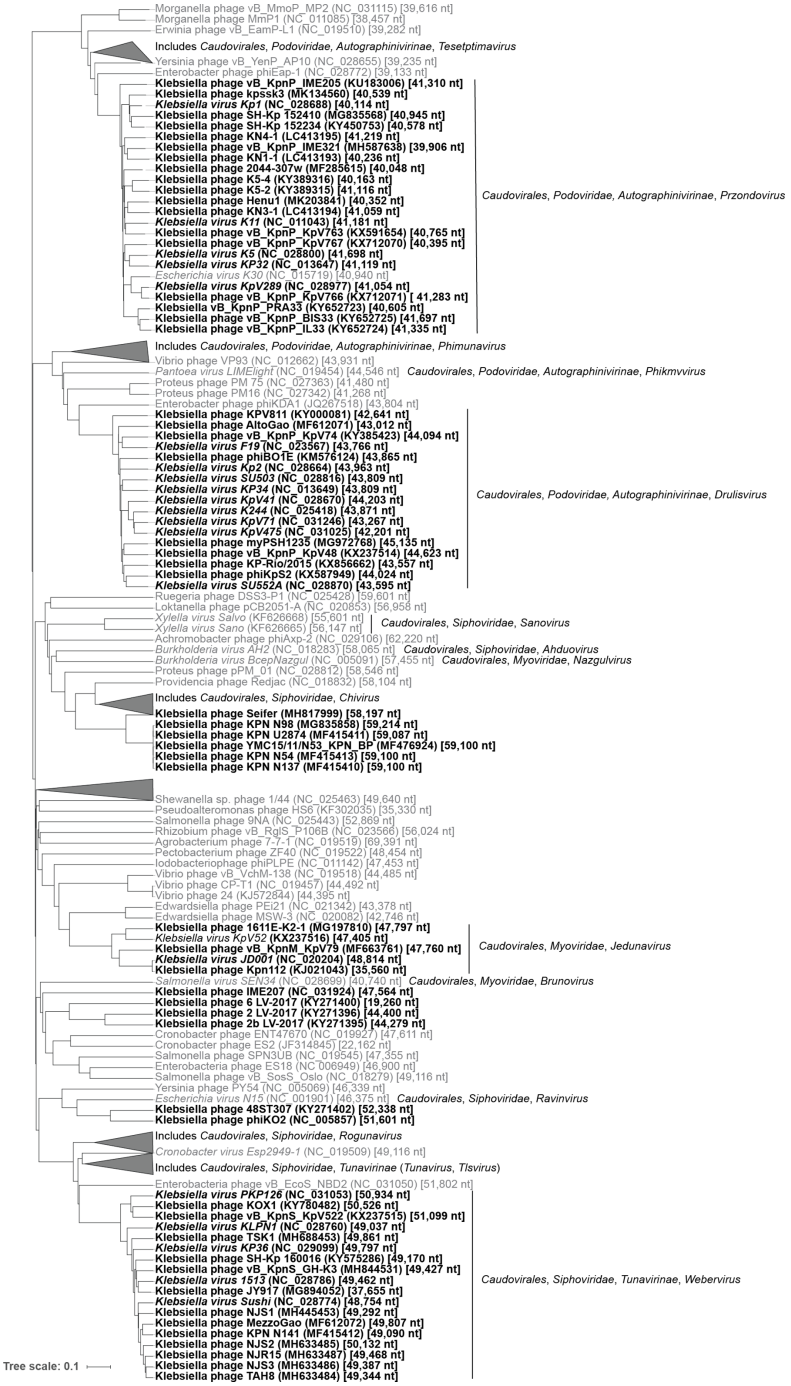

Fig. 1. Phylogenetic placement of dsDNA *Klebsiella* phages within the order *Caudovirales*. Placement of 109 genomes (Table 1) within ViPTree version 1.9 [164] was checked on 6 August 2019. Those sequences (*n*=84) that clustered together in groups of three or more were analysed with their nearest phylogenetic relatives using ViPTreeGen v1.1.2 (--ncpus 8 --method 'bioinj') and a non-redundant set of genomes (a fasta file of input sequences, https://doi.org/10.6084/m9.figshare.11635965.v1; newick-format file, https://doi.org/10.6084/m9.figshare.11635953.v1) to generate the tree shown (annotated using https://itol.embl.de and Adobe Illustrator). The taxonomy of the phages was checked via https://talk.ictvonline.org/taxonomy/ (release 2018b); accepted species names are written in italics. A phylogenetic tree showing the placement of the remaining 25 *Klebsiella* genomes within ViPTree version 1.9 is available (https://doi.org/10.6084/m9.figshare.11635962.v1; genome list, https://doi.org/10.6084/m9.figshare.11635950.v1; newick-format file, https://doi.org/10.6084/m9.figshare.11635971.v1) as Supplementary Material. Since the trees in this figure and the Supplementary Material were created, genomes for the following phages have been published: vB_KpnS_FZ10, Shelby, Sin4, Skenny, Sweeny and Sanco (*Webervirus*); vB_KpnP_FZ12 (*Przondovirus*); vB_KpnM_FZ14 (*Jedunavirus*); vB_KpnS_FZ41 and KpGranit (*Sugarlandvirus*); Patroon (*Teseptimavirus*); KpCHEMY26 (*Ithacavirus*); Magnus (genus unknown); Mulock (related to *Brunovirus*); Marfa (genus unknown). Additional information for these phages is available in Table 1.

The delivery method for phage treatment is also an important consideration. For example, intraperitoneal injection is rarely used in human treatment, given the relative ease of intravenous injection in most cases. In experiments carried out to treat murine lobar pneumonia, Cao *et al.* [66] determined that intranasal delivery of phage 1513 was able to produce a survival rate of 80% in the Swiss Webster mouse model, compared to 0% in the negative controls, 2 h after nasal inoculation of MDR *K. pneumoniae* 1513, as well as visibly reduced lung injury, in comparison to the negative controls. Chhibber *et al.* [72] demonstrated that intraperitoneal injection of phage SS administered immediately after intranasal inoculation of *K. pneumoniae* B5055 into BALB/c mice resulted in complete clearance of bacteria in 5 days, compared to 10 days in untreated mice, although the authors state that even a short delay of 6 h post-inoculation rendered treatment ineffective. However, Singla *et al.* [75] found that phage KPO1K2, encased in a liposome, was effective in treating lobar pneumonia induced in BALB/c mice by intranasal inoculation of *K. pneumoniae* B5055, even when phage treatment was delayed by up to 3 days.

Although there is a difference in the choice of phage in these published reports, and so studies cannot be compared directly, it does highlight the importance of investigating differing delivery methods for phage treatment, not only in a logistical sense but also in elucidating the most efficient method of delivery according to the type of infection and the length of incubation prior to treatment. Moreover, these studies only measured the *in vivo* effect of phage treatment against one strain of *K. pneumoniae*, providing no information regarding phage host range. Further experiments should, therefore, seek to determine whether the host range(s) of their respective phages are broad enough to be considered to be useful for therapeutic purposes.

While several studies have reported successful use of *K. pneumoniae* phages to clear infections in murine and *Galleria* models, the effects of phage infection on the microbiome (i.e. microbiota, metabolome) must now be considered when assessing phages (individually or as phage cocktails) as a viable treatment or patient decontamination measure. Hsu *et al.* [76] showed that infection with lytic phages caused an increase in phage resistance (28 to 68%) in a known bacterial population common to the human gut microbiota. Quantitative shifts in sensitive and non-sensitive strains were seen, highlighting the system-level effect of phage infection. Phage infection did not necessarily clear the target species but instead modulated the ecosystem towards a more stable gut environment. Phages inducing simultaneous knockdown of *Enterococcus faecalis* and *Bacteroides fragilis* populations had little effect on the microbiota compared with *E. coli* and *Clostridium sporogenes* phages, which caused significant decreases (10^6 g^{-1} stool) in *Bacteroides vulgatus*, *Proteus mirabilis* and *Parabacteroides distasonis* populations, and 10^8 g^{-1} stool decreases in *Akkermansia muciniphila* and *B. fragilis* populations. Perturbation of the microbiota by phages also affected the metabolome. The abundance of 17% of the examined compounds was altered significantly in the presence of phages. During initial phage infection, Hsu *et al.* observed a 10-, 17- and 2-fold reduction in tryptamine, a microbiome-associated metabolite known to play a role in accelerating gastrointestinal transit in mice [77]. This led them to suggest that phage infection could be used to modulate the microbiome in a targeted manner to influence systemic health.

Combination therapy

A number of *in vitro* experiments have identified the possibility of bacterial resistance arising as a result of phage therapy [62, 66, 70, 78, 79]. To reduce the emergence of phage-resistant strains of *K. pneumoniae* during treatment, research has begun to explore combination therapy either by using phage cocktails or combining phage treatment with antibacterial drugs.

Gu *et al.* [62] generated a phage cocktail (i.e. a combination of phages that have different but overlapping host specificities) made up of three lytic phages (GH-K1, GH-K2 and GH-K3) specific to *K. pneumoniae* strain K7. The authors found that co-culture of K7 with the phage cocktail produced fewer phage-resistant variants of K7 and a more efficient reduction in bacterial load compared to cultures treated with a single phage. Moreover, when treating bacteraemic mice, produced by intraperitoneal injection of K7, the phage cocktail produced a significantly lower blood bacterial count and enhanced mouse survival rates compared to mice treated with individual phages. A similar phenomenon was seen by Chadha *et al.* [78], who aimed to resolve *K. pneumoniae* B5055 burn-wound infections in BALB/c mice and found that their phage cocktail (made up of Kpn1, Kpn2, Kpn3, Kpn4 and Kpn5) induced a greater decrease in bacterial load compared to treatment with individual phages and a complete bacterial clearance in a shorter time.

Finally, in combining a lytic phage with ciprofloxacin against *K. pneumoniae* biofilms, Verma *et al.* [80] demonstrated a reduction in the development of both phage-resistant and ciprofloxacin-resistant *K. pneumoniae* strains, as well as having an enhanced effect against bacterial biofilms compared to individual treatments.

Human trials

The progression of phage research from *in vivo* experimentation to clinical trials involving humans has generated some friction among regulatory bodies in Western countries. However, countries in Eastern Europe and the former Soviet Union have routinely used phages in their healthcare systems for many years [81]. For example, the Eliava Institute of Bacteriophages, Microbiology and Virology in Georgia, and the Hirszfeld Institute of Immunology and Experimental Therapy in Poland both produce and supply phage therapeutic products specifically for routine human use [82].

In the West, regulatory issues surrounding the use of phages as therapeutic agents have hindered progress somewhat. It is not that there are specific regulations that prevent the use of phages in this way, but rather a lack of regulation that has placed limitations on progress. The unique nature of phages

compared to traditional therapeutic agents, as evolving and self-replicating biological entities, requires them to have new rules and regulations regarding their safety, production and use. It is this lack of regulation in the EU and the UK, combined with a lack of interest from pharmaceutical companies, and the concept of personalized medicine often associated with phage therapeutics, which in itself is a new method of infection control, that makes approval for human trials a lengthy and difficult process [83]. However, it should be noted that the Belgian government has introduced a pragmatic framework that facilitates tailored phage therapy (magistral phage regulatory framework), allowing non-authorized phage products to be prepared by a pharmacist for a given patient in line with a prescription from a physician and complying with relevant standards [84]. Phages are very occasionally and only under exceptional circumstances used therapeutically in the wider EU under the umbrella of Article 37 (Unproven Interventions in Clinical Practice) of the Declaration of Helsinki [84].

Despite these regulatory hurdles, a limited number of human trials have been carried out in relation to phage therapy, although none have specifically targeted *K. pneumoniae*. Rhoads *et al.* [85], based in the USA, carried out a phase I clinical trial on 42 patients with chronic venous leg ulcers to investigate the safety of a phage preparation specific to *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *E. coli*. The authors reported no adverse effects of phage treatment. In the same year, Wright *et al.* [86], based in the UK, carried out a phase I/II clinical trial to determine the safety and efficacy of their phage product targeting *P. aeruginosa* in chronic otitis. Their study involved 24 patients with chronic otitis and showed a reduction in *P. aeruginosa* counts and, again, no adverse effects of phage treatment. Although consisting of a small sample size, the apparent success of these first human trials did little to prompt changes to the regulatory obstacles currently associated with phage therapy.

Dutch clinicians reported successfully treating a renal transplant patient with a recurrent UTI caused by ESBL-producing *K. pneumoniae* with a combination of meropenem and phages after the patient turned to the Eliava Institute for phage therapy [87]. Several courses of meropenem alone had failed to treat the condition, but the patient remained infection-free 14 months after the combination phage–meropenem treatment. Italian clinicians reported using a custom-made lytic phage cocktail to decolonize the gut of a patient at high risk of recurrent invasive infections of an MDR, KPC-3-harbouring *K. pneumoniae* (ST307), without adverse effects [88]. A prospective study in India showed that single or cocktails of lytic phages could be used to treat and eradicate non-healing skin ulcers, in which bacterial biofilms were preventing antibiotics reaching their target(s) [89]. Patients were followed for 3 months after phage therapy to monitor wound size and healing. Wound size and depth decreased significantly between days 1 and 60, with more non-diabetics (19/21) cured compared with diabetics (20/27). Only 6 of the 48 patients harboured *K. pneumoniae* in their wounds (either in pure or mixed culture), and they had the slowest healing progress at the end of the follow-up. No information was provided as to how many different *K. pneumoniae*-infecting phages were included in the study or whether they had depolymerase activity that could facilitate biofilm breakdown and treatment of infections.

Future directions

Phage therapy shows promise as a potential response to the continued development and spread of MDR *K. pneumoniae*. *In vitro* and *in vivo* studies have confirmed the potential for phages to be used individually, as phage cocktails and in combination with current antimicrobial chemotherapeutic drugs. Moreover, the routine use of phage therapy in Eastern Europe, and the results from the small number of human trials that have been carried out in the West, suggest that phages are generally considered to be safe for use in humans. However, the lack of progress toward amending EU and UK regulations to account for phage therapy has hampered progress. The focus of future direction in the area of phage research must be to overcome this obstacle.

Using phage-derived gene products

Another avenue of phage research aimed at finding therapeutic solutions to MDR *K. pneumoniae* is the potential to use specific phage gene products rather than phages themselves to combat infection. This kind of treatment could be advantageous in that it would be easier and quicker to gain clinical approval for a recombinant protein product compared to the direct use of phages. Indeed, phage-derived recombinant proteins may be used to combat infections caused by bacteria such as *K. pneumoniae* directly, or as part of a combinatory approach to complement or enhance current antimicrobial regimes.

Phage proteins

In the lytic life cycle of an infecting phage particle, there are a number of proteins that the phage can use to ensure successful adsorption, infection, replication and release of progeny. In terms of potential antimicrobial agents against *K. pneumoniae*, there are a number of biologically interesting proteins to consider. Peptidoglycan hydrolases and polysaccharide depolymerases are normally present on the tail spikes of a phage particle and are involved in successfully infecting a bacterium after adsorption. Polysaccharide depolymerases degrade the macromolecular carbohydrates that make up the capsule surrounding the bacterial cell wall, whereas peptidoglycan hydrolases break down the peptidoglycan layer to penetrate the cell wall and access the cytoplasm to allow the phage to deposit its genetic material [90].

Holins, endolysins and spanins are proteins that are produced after the infection of a bacterium, and they are involved in the process of cell lysis whereby assembled phage particles 'burst' from the cell in order to spread and continue the infection cycle. Holins are hydrophobic transmembrane proteins that mediate the permeabilization of the inner cell membrane. This cannot independently cause cell lysis; however, it allows endolysins and spanins to translocate from the cytoplasm,

where endolysins degrade the peptidoglycan layer in-between the inner and outer cell membranes, and spanins disrupt the outer cell membrane present on Gram-negative bacteria. This is followed by bacterial cell lysis via osmolysis [90].

Polysaccharide depolymerases

The capsule of *K. pneumoniae* is an important virulence factor and allows the bacterium to avoid phagocytosis and complement-mediated lysis. It is, therefore, a prime target for recombinant phage-derived proteins and has been studied extensively. For example, tail tubular protein A (TTPA), a structural tail protein of phage KP32, was shown to have additional polysaccharide depolymerase activity. Pyra *et al.* [91] cloned and expressed TTPA in *E. coli* and determined its enzymatic activity by agar spot tests on lawns of *K. pneumoniae* PCM2713, which produced translucent zones of reduced growth. Subsequent microscopic analysis of treated and untreated *K. pneumoniae* revealed that cells treated with TTPA were stripped of their capsules. In a similar process of cloning, expression and agar spot testing, Pan *et al.* [92] discovered nine polysaccharide depolymerases expressed by phage ΦK64-1, each of which demonstrated activity against a specific capsular type of *K. pneumoniae*, which corresponded to the broad host range of the phage itself. This is interesting because not only does it confirm the role of enzymes such as polysaccharide depolymerases in the determination of phages' host specificity, but it also promotes the idea of artificially generated cocktails of recombinant enzymes that can target a wide range of *K. pneumoniae* strains.

A number of *in vivo* experiments have also been carried out investigating the effect of polysaccharide depolymerases on *K. pneumoniae* infection. Majkowska-Skrobek *et al.* [93] identified, cloned and expressed a KP36-derived capsule depolymerase, depoKP36, which produced haloes on lawns of *K. pneumoniae* in agar spot tests. The authors tested the ability of depoKP36 to treat infection caused by *K. pneumoniae* in *G. mellonella* and found that 100% of the larvae died without treatment, up to 40% survived when treated with depoKP36 post-infection, and depoKP36 treatment of bacteria prior to infection resulted in a death rate of only 23%. These results suggest that the decapsulating action of depoKP36 against *K. pneumoniae* led to a decreased ability of the bacterium to resist the host immune response. This was confirmed in subsequent research [94].

Endolysins

Endolysins have been studied extensively for use against Gram-positive bacteria, due to the absence of an outer cell membrane found in Gram-negative bacteria such as *K. pneumoniae*, which would normally hinder the action of the enzyme in the absence of spanins. However, recent research has also produced some promising results regarding the use of endolysins against Gram-negative bacteria. Maciejewska *et al.* [95] produced a recombinant endolysin from the *K. pneumoniae* phage KP27 and analysed its peptidoglycan-degrading activity on a range of Gram-negative bacteria, including strains of *K. pneumoniae*, *P. aeruginosa*, *Salmonella enterica* and *E. coli*, by co-incubation of bacteria and endolysin. The recombinant enzyme successfully lysed all strains of bacteria that were tested. However, the outer membrane of bacteria was permeabilized prior to endolysin treatment. This suggests that any potential endolysin-based infection control agents require mixing with outer-membrane-permeabilizing agents to be effective against *K. pneumoniae* [95].

To overcome the need for additional outer-membrane-permeabilizing agents during treatment of Gram-negative bacterial infections, artificial lysins (Artilysins) have been developed by the fusion of a phage endolysin with an outer membrane-destabilizing peptide [96]. Artilysins specific for *K. pneumoniae* have yet to be developed, but they have been successfully created for use against *P. aeruginosa* [97] and *Acinetobacter baumannii* [98]. This technology opens up the possibility of developing artificial endolysins for use in human therapy against not only MDR *K. pneumoniae*, but also MDR Gram-negative infections.

Further research

Recombinant polysaccharide depolymerases and artificial endolysins have the potential to be used as therapeutic agents in the fight against MDR *K. pneumoniae*. Polysaccharide depolymerases are able to degrade the capsule, an essential virulence factor of *K. pneumoniae*, which could find uses such as boosting the host immune response against the bacterium, and breaking down biofilms to allow current antibiotic regimes to access bacterial cells more easily. Artificial endolysins have the potential to work against infection as an independent antimicrobial agent. Further research is required in this area to fully realize the potential of such phage-derived recombinant proteins, and in doing so the mechanisms by which they are able to inhibit bacterial growth and/or eliminate infection may lead to new breakthroughs. Importantly, an obvious advantage over phage therapy is that recombinant protein products for use in humans have well-defined and established rules and regulations regarding their production, safety and use in the EU and UK, whereas phage therapy does not.

Concluding remarks

The increasing incidence of hospital-acquired and community-acquired infections caused by MDR *K. pneumoniae* and hypervirulent *K. pneumoniae*, respectively, is rapidly becoming a global threat to public health. The emergence of strains that are both MDR and hypervirulent is even more of a concern. *K. pneumoniae* is becoming as much of a threat today as its non-resistant counterparts were over a century ago prior to the discovery of antimicrobial compounds such as penicillin. In response, research efforts have begun to look back in time at a once-abandoned approach to bacterial infection, namely phage therapy. It is becoming increasingly clear that there is potential for phages and their gene products to become novel sources of antimicrobial strategies against MDR bacteria that current treatment regimens are simply becoming ineffective at countering. However, the field of phage therapy

is still very much in its infancy and is fraught with difficulties, both novel and familiar.

Safety

One of the major obstacles facing phage therapy is the novel safety implications regarding the use of self-replicating biological entities in humans. For example, it is evident that phages are capable of carrying antibiotic resistance [99] and toxin-encoding [100] genes that could be transferred to the target bacterium via the process of transduction. Proper characterization is, therefore, important when considering phages for therapeutic uses, and the presence of potentially harmful genes is commonly screened for during this process. However, the absence of harmful genes does not guarantee phage safety.

For example, the nature of a lytic phage is to increase its number at the expense of bacterial hosts. While this is the primary aim of phage therapy, little research has been conducted regarding the potential side-effects of this phenomenon. This is an important consideration because phages with a broad host range, or those within a phage cocktail, are often considered to be more appropriate for phage therapy. It is evident from the recent work of Hsu *et al*. [76] that the introduction of even a single phage into the mouse microbiota can have effects on the microbiome. What effect might therapeutic use of phages have on the normal microbiota of a human? Might it be safer to use individual phages, with a narrow host range, to minimize disruption of the commensal microbiota? If so, phage therapy will rely on very specific identification of infecting bacteria, and having the correct phage available for treatment. Or perhaps this particular side-effect may be deemed acceptable, as is the case with current antibiotic regimens. Additionally, the number of clinical trials that have assessed the safety of phage therapy in humans is limited, and those that have occurred have involved small sample sizes and have often relied on patient-generated data [82].

Practicality

The second barrier that must be overcome are the practical issues associated with phage therapy in the EU and UK. As discussed earlier, the regulations required to govern the safety, production and use of virus-based infection control mechanisms do not currently exist. The last attempt at tackling these regulatory hurdles came in the form of a phase II clinical trial funded by the European Commission. 'Launched in 2013 and achieved in 2017, PhagoBurn was the world first prospective multicentric, randomised, single blind and controlled clinical trial of phage therapy ever performed according to both Good Manufacturing (GMP) and Good Clinical Practices (GCP)' [101]. Although the project attempted to define appropriate practices for phage therapy during its assessment of he efficacy and tolerability of phage-treated burn-wound infections [102], only temporary allowances were made. While recommendations for subsequent clinical trials were made, no further regulatory improvements have been attempted.

Moreover, if regulations are updated to account for phage therapy, where would producers of phage products stand in relation to intellectual property? Can naturally occurring biological entities be patented and sold, or would this be reserved for phage cocktails and phage–drug combinations that exhibit 'unnatural' antimicrobial properties? Indeed, in terms of personalized medicine, phage cocktails may require production within the healthcare setting to suit a specific patient's needs. In this case, would the ingredients of a phage cocktail need to be individually patented and sold, or could cocktails be developed with the pliability for patient-specific modifications later? In the absence of profitable, patented technology, pharmaceutical companies may be reluctant to fund the research and development of such treatments.

Phage resistance

Finally, it could be argued that the issues surrounding phage therapy may be abrogated by using phage gene products instead. Being more akin to conventional antimicrobial therapeutics, they would be subjected to the well-established drug development processes and standards of production and safety that are currently in place. However, the use of both phages and their gene products against bacterial infection may still be subject to the age-old problem of bacterial resistance. Indeed, some of the studies outlined in this literature review suggest, or provide evidence of, the possibility of resistance against phage therapy, although this phenomenon has yet to be observed *in vivo*.

The first warnings regarding the development of antibiotic resistance [103, 104] went unheeded, resulting in the spread of MDR bacteria such as *K. pneumoniae*, and these are the grounds upon which phage therapy has become a renewed topic of research. The development of novel antimicrobial agents is, therefore, not sufficient to combat infection and bacterial resistance in the long term. Strategies regarding the use of any novel antimicrobial treatments must be developed to minimize the risk of the development of resistance. In terms of phage therapy, such strategies might involve using combination treatments, for example, phage–drug combinations or complex phage cocktails designed to minimize the selection pressures applied against bacteria during treatment.

Prevention should be the primary focus of healthcare-associated infection control procedures. The implementation or improvement of policies aimed at reducing the risk of patients developing bacterial infections must be concurrent with the development of novel antibacterial therapeutics to minimize the spread of resistance to treatment. Such procedures may include hand and environmental decontamination, safe installation and maintenance of medical devices, prompt removal of medical devices that are no longer needed, screening and decolonization programmes, and cautious use of antimicrobial agents.

Future research

The future of phage research is a promising one. Phages are perhaps the most numerous of all biological entities on the planet and as such could be the most valuable source of therapeutic solutions. As we further elucidate the interactions

between phage and bacterium, as predator and prey, advances in our understanding of the molecular mechanisms defining such interactions may afford us new information and ideas that can be applied to infection control. Indeed, phage research has already led to the development of artificial phage-derived antibacterial proteins – Artilysins [96] – and the artificial alteration of phage host range to infect a greater range of bacteria than is naturally possible is just beginning to come to fruition [63].

Furthermore, recent technological advances have seen next-generation sequencing (NGS) become increasingly used in phage research, providing a more robust platform from which to launch detailed phage characterization, screening of harmful genes and evaluation of potentially useful gene products [105]. Further technological advancements and categorization of information attained from methods such as NGS can only lead us onwards, providing new solutions to old problems.

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The authors declare that there are no conflicts of interest.

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