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

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Keywords: antimicrobial resistance, phage therapy, *Klebsiella oxytoca*, *Klebsiella pneumoniae*

Klebsiella spp. are commensals of the human microbiota, and a leading cause of opportunistic nosocomial infections. The incidence of multi-drug resistant (MDR) strains of _Klebsiella pneumoniae_ causing serious infections is increasing, and _K. 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 heterogeneous at the whole-genome level. This review summarises 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.

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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 (Podschun & Ullmann, 1998). 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 (Shon, Bajwa & Russo, 2013). Due to its widespread distribution and genetic make-up, *K. pneumoniae* has rapidly become a global threat to public health (World Health Organization, 2018). Similar to *K. pneumoniae*, *Klebsiella oxytoca* is an opportunistic pathogenic in humans, and is becoming increasingly associated with nosocomial infections, particularly in immunocompromised patients (Broberg, Palacios & Miller, 2014). It is also acquiring antimicrobial resistance genes and is detected throughout the UK (Eades et al., 2016; Moradigaravand et al., 2017b). Consequently, it is now considered the second most clinically important pathogen of the genus *Klebsiella* (Broberg, Palacios & Miller, 2014).

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.

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55 **Antibiotic resistance and *Klebsiella* spp.**

56 Antibiotic resistance is defined as the ability of a bacterium, such as *K. pneumoniae*, to resist the
57 effects of antimicrobial drugs that it was previously sensitive to. The development of antibiotic
58 resistance is a result of the evolutionary process of natural selection, by which pathogenic
59 bacteria are able to overcome the selection pressure applied to them during antimicrobial
60 treatment, rendering the drug less effective (Huang et al., 2017). The first antibacterial agents
61 were discovered between 1910 and 1935 (Ehrlich, 1910; Domagk, 1935; Fleming, 2001) with the
62 most famous being the discovery of penicillin by Alexander Fleming in 1929. Hailed as 'magic
63 bullets' in the fight against infection, these first antibacterial agents paved the way for the
64 discovery of almost all classes of antibiotics in use today (Aminov, 2010).

65

66 Despite early evidence of the possibility for future antibiotic resistance (Abraham & Chain,
67 1988) and warnings that unrestricted use could reduce their effectiveness (Fleming, 1945),
68 antimicrobials have been taken for granted. The continued overuse of antibiotics in both
69 healthcare and agricultural settings over the course of the last century has contributed to the
70 evolution and emergence of antibiotic-resistant strains of *Klebsiella* spp. and other Gram-
71 negative bacteria.

72

73 *K. pneumoniae* strains are frequently resistant to extended-spectrum beta-lactams such as
74 penicillins and cephalosporins. Extended-spectrum beta-lactamase (ESBL)-producing *K.*
75 *pneumoniae* are able to target the beta-lactam ring structure within antibiotic compounds,
76 neutralising their antimicrobial activity (Gürntke et al., 2014). Pathogenic ESBL-producing *K.*

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pneumoniae derive their antibiotic resistance enzymes most commonly from acquired genes, such as SHV-5 and CTX-M-15 (Gürntke et al., 2014; Moradigaravand et al., 2017a). Ever since ESBL-producing *K. pneumoniae* were first described by Knothe et al. (1983) in Germany, *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 (Khan et al., 2010; Chong et al., 2011; Mshana et al., 2013; Valenza et al., 2014).

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 multi-drug 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. *Klebsiella pneumoniae* carbapenemase (KPC) was first discovered in the United States (Yigit et al., 2001) and has since spread to all other parts of the world (Campos et al., 2016).

In Europe, KPC was found to be the most common carbapenemase resistance gene in *K. pneumoniae*-associated hospital-acquired infections (45%), followed by oxacillinase-48 (OXA-48-like) (37%), New Delhi metallo-beta-lactamase (NDM) (11%) and Verona integron-encoded metallography-beta-lactamase (VIM) (8%) (Grundmann et al., 2017). 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 cases, respectively, in 2015 (Public Health England, 2016). The spread of OXA-48-like

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K. pneumoniae strains has occurred mostly in the Mediterranean and Northern Africa and is primarily spread via ST101 strains as a result of travel in the regions, whereas ST395 is associated with clonal outbreaks throughout Europe (Potron et al., 2013).

NDM carbapenemase-producers originated in India, primarily in strains of *Escherichia coli* and *K. pneumoniae*, and have spread throughout the world as a direct result of travel to and from the Indian subcontinent (Nordmann et al., 2011; Johnson & Woodford, 2013). Nordmann et al. (2011) 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 (Glasner et al., 2013).

While MDR *K. pneumoniae* is itself a problem, its ubiquitous presence in both animal and human hosts, as well as in the environment, combined with its ability to acquire and maintain antimicrobial resistance plasmids and to pass those plasmids on to other Gram-negative bacteria, puts it into a relatively unique position to be able to amplify the spread of antimicrobial resistance genes throughout the world (Wyres & Holt, 2018). 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 (David et al., 2019). *K. pneumoniae* (and likely *K. oxytoca*) will continue to play a key role in the development of antimicrobial resistance and is, therefore, a prime target for novel antibacterial therapeutics (Wyres & Holt, 2018).

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123

124 **Risk factors for *Klebsiella* infections**

125 Primarily an opportunistic pathogen prevalent in the hospital setting, *K. pneumoniae* has become
126 a common cause of hospital-acquired infections, such as UTIs and bloodstream infections
127 (BSIs), in which antibiotic-resistant strains are becoming more difficult to treat and are
128 associated with an increased mortality rate. Perhaps the most ubiquitous risk factors for all forms
129 of hospital-acquired *K. pneumoniae* colonisation and infection are patient exposure to
130 antibacterial agents and the length of hospital stay. Indeed, there consistently appears to be a
131 positive correlation between the length of time a patient is required to stay in hospital, and the
132 chance of acquiring a *K. pneumoniae* infection simply due to the increased exposure to
133 healthcare-associated pathogens with time (Nouvenne et al., 2014; Shaikh et al., 2015; Liu et al.,
134 2018). Moreover, a considerable number of studies aimed at identifying risk factors associated
135 with such infections recognise previous antibiotic treatment as an important factor, particularly
136 the widespread use of cephalosporins in the case of ESBL-producing *K. pneumoniae* infection
137 (Tuon et al., 2011), and carbapenems, fluoroquinolones, glycopeptides and aminoglycosides for
138 infections caused by carbapenemase-producing *K. pneumoniae* (Liu et al., 2018).

139

140 Not surprisingly, invasive procedures such as surgical intervention and catheterisation are also
141 strongly associated with the acquisition of *K. pneumoniae* infection. Patients who are subject to
142 invasive procedures, such as the installation of a central venous catheter, for example, are likely
143 to be immunocompromised individuals who have been hospitalised for a severe underlying
144 health condition. These patients are, therefore, particularly susceptible to opportunistic infections
145 which could lead to a BSI, in the aforementioned example, soft tissue and wound infections in

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patients subject to surgical procedures, or even severe cases of pneumonia or meningitis in neonates (Tumbarello et al., 2006; Yu et al., 2016).

Clinical features of disease may also be an important risk factor in the development of *K. pneumoniae* infection. Meatherall et al. (2009) 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 (Silva et al., 2006; Tuon et al., 2011; Lee et al., 2016). Nouvenne et al. (2014) suggested an association between cardiovascular, respiratory, renal and neurological diseases, and colonisation 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 (Zollner-Schwetz et al., 2008; Herzog et al., 2014). Likely due to a combination of improved detection methods (Eades et al., 2016), increased international travel (Moradigaravand et al., 2017b), contaminated hospital equipment (Moradigaravand et al., 2017b), 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 compared with *K. pneumoniae*, though this requires further analyses (Singh, Cariappa & Kaur, 2016).

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169

170 **Virulence factors of *Klebsiella* spp.**

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

175

176 The capsule is an extracellular matrix made up of strain-specific polysaccharides that surrounds
177 the bacterium forming a thick fibrous structure. The capsular polysaccharides produced by *K.*
178 *pneumoniae* are called K antigens and, given that the polysaccharide produced depends on the
179 strain of *K. pneumoniae*, they have been traditionally used to identify the strain using serological
180 techniques (Wyres et al., 2016). The role of the capsule in human disease has been studied
181 extensively and it is thought to have a defensive role by providing protection against phagocytic
182 immune cells, blocking complement-mediated lysis and reducing levels of proinflammatory
183 cytokines (Yoshida et al., 2000; Cortés et al., 2002). Indeed, the virulence of *K. pneumoniae* is
184 greatly reduced in the absence of a capsule, as shown by infection of mice with acapsular
185 mutants (Lawlor, Handley & Miller, 2006), and greatly increased in so-called hypervirulent
186 strains which produce more capsular material resulting in a hypermucoviscous phenotype (Shon,
187 Bajwa & Russo, 2013).

188

189 The LPS is composed of an O antigen, an oligosaccharide core and lipid A, and protrudes from
190 the bacterial membrane (Follador et al., 2016). The primary role of LPS in *K. pneumoniae*
191 infection is protection from the complement-mediated lysis of bacterial cells by binding of the

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192 complement component C3b away from the bacterial membrane, preventing the formation of the
193 membrane attack complex C5b-9. This is carried out by the O antigen of the LPS which, when
194 absent, makes *K. pneumoniae* more susceptible to complement-mediated bacterial lysis (Merino
195 et al., 1992).
196
197 *K. pneumoniae* expresses fimbriae which are membrane-adhesive protrusions involved in the
198 adhesion of the bacterium to host surfaces facilitating its invasion. Two main types of fimbriae
199 are exhibited by *K. pneumoniae*: type 1 fimbriae which are filamentous, and type 3 fimbriae
200 which are helix-like in shape (Paczosa & Mecsas, 2016). Moreover, the expression level of each
201 type varies depending on the surface to which the bacteria attach. Type 1 fimbriae are expressed
202 in the urinary tract and the bladder, but not in the gastrointestinal tract or the lungs (Struve, Bojer
203 & Krogfelt, 2008). Struve et al. (2008, 2009) speculate that the downregulation of type 1
204 fimbriae may be because it reduces the ability of *K. pneumoniae* to penetrate the intestinal mucus
205 layer in the gastrointestinal tract, as is seen with *E. coli*, whereas in the lungs, selection against
206 fimbriated cells occurs due to rapid elimination by phagocytes. Type 3 fimbriae bind to
207 extracellular matrices and medical devices, and are important for the development of biofilms
208 (Struve, Bojer & Krogfelt, 2009).
209
210 Finally, *K. pneumoniae* must acquire iron from the environment to grow and multiply. There is
211 very little free iron to be found in mammalian hosts, so the bacterium must express siderophores.
212 These are molecules that have a higher affinity for iron than mammalian iron transport
213 molecules, such as transferrin, enabling the bacteria to obtain iron for rapid growth and
214 subsequent invasion. The primary siderophore expressed by *K. pneumoniae* is enterobactin,

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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 (Holt et al., 2015).

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 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 have been shown to have diverged into three distinct species: *K. pneumoniae* (KpI), *K. quasipneumoniae* (KpII) and *K. variicola* (KpIII) (Holt et al., 2015).

In their whole-genome sequencing and pangenome-wide association study, Holt et al. (2015) 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

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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 (Jun, 2018). 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 (Lee et al., 2017). 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 (Holt et al., 2015; Lee et al., 2017).

A pangenome study of *K. oxytoca* strains isolated from bloodstream infections in the UK and Ireland showed that *K. oxytoca* has a highly diverse population, composed of several distinct phylogroups (KoI, KoII, KoV, KoVI) (Moradigaravand et al., 2017b). It shares numerous antimicrobial genes and mechanisms with *K. pneumoniae*. *K. oxytoca* has been far less studied than *K. pneumoniae*, and extensive studies of its global epidemiology are required (Moradigaravand et al., 2017b).

PHAGES OF *KLEBSIELLA* SPP.

A phage is a virus that infects bacteria and, as such, is found in all environments where bacteria would normally thrive. Viruses were initially suggested as a possible cause of clear zones on

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bacterial culture plates by William Twort in 1915, and in 1917 Felix d'Herelle confirmed this discovery, coining the term ‘bacteriophage’ (Twort, 1915; Anonymous, 2011). Prior to the discovery of the first antimicrobial agents, phages were considered the cure for bacterial infections and d’Herelle performed the first experimental phage therapy using an oral phage solution to treat dysentery (d’Herelle, 1918). However, after the discovery of antimicrobial compounds such as penicillin, the therapeutic uses of phages were largely left alone 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 (Clokie et al., 2011). 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, synthesising 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

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283 incorporated into the bacterial DNA, forming a prophage, and is replicated passively upon
284 replication of the bacterial genome without destroying the host. Prophages in the lysogenic cycle
285 are able to enter the lytic cycle under certain conditions (e.g. in the presence of environmental
286 stressors), and begin actively replicating and producing viable phages at the expense of the host
287 (Wittebole, De Roock & Opal, 2014).
288
289 Although the lytic/lysogenic phage life cycle is a well-established concept in phage biology, we
290 now know there are multiple phage life cycles. Pseudolysogeny is the process by which the
291 phage genome enters a bacterial host but neither stably establishes itself as a prophage nor
292 initiates a destructive replicative response, remaining inactive and possibly awaiting more
293 desirable environmental conditions for viral replication (Siringan et al., 2014). Chronic infection,
294 resulting in the shedding of phage particles over long periods of time without destruction of the
295 host cell, can occur with infection of filamentous phages in *Mycoplasma* (Lee et al., 2017).
296 Finally, the carrier state life cycle occurs when a heterogeneous population of bacteria,
297 containing individuals both sensitive and resistant to a given lytic phage, leads to the destruction
298 of sensitive bacteria and the survival of resistant bacteria creating a stable equilibrium between
299 viral and bacterial propagation (Siringan et al., 2014).
300
301 In the context of using phages as a therapeutic alternative to antimicrobial chemotherapy, those
302 that reliably employ the lytic life cycle to reproduce are most suitable given that the end result is
303 the destruction of bacterial host cells. Additionally, phages that are able to switch between
304 multiple life cycles may not make reliable treatment options due to the possibility of dormancy
305 and subsequent re-establishment of bacterial infection. This is just one aspect of comprehensive

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306 phage characterisation that is an important consideration when choosing appropriate phage
307 treatments.
308
309 **Phage characterisation**
310 Phages of *K. pneumoniae* have been isolated from a variety of sources worldwide including
311 wastewater, sewage, seawater and human intestinal samples, and belong to one of three families
312 of the order *Caudovirales* (**Table 1**). These families make up the entirety of the order and are
313 described as non-enveloped, tailed phages, with icosahedral heads containing double-stranded
314 DNA: *Myoviridae* are characterised by long, straight, contractile tails; *Siphoviridae* by long,
315 flexible, non-contractile tails; and *Podoviridae* by short, non-contractile tails (Fokine &
316 Rossmann, 2014).
317
318 Genomic comparisons of lytic *K. pneumoniae* phages of the order *Caudovirales* highlight a
319 variety of useful similarities and differences. The expression of polysaccharide depolymerases,
320 for example, has been observed in several recently discovered phages of *K. pneumoniae* (Kęsik-
321 Szeloch et al., 2013; Chhibber, Nag & Bansal, 2013; Jamal et al., 2015) and these enzymes have
322 a role in the degradation of the capsule surrounding the exterior of the bacterium. The breakdown
323 of the capsule by phage depolymerases has been purported to combat *K. pneumoniae* biofilms
324 (Taha et al., 2018) and increase the susceptibility of the bacterium to antibiotics, phage infection
325 and the immune system (Kęsik-Szeloch et al., 2013). Additionally, phage depolymerase action
326 can be observed in the laboratory with the production of 'haloes' around clear zones of lysis on
327 bacterial culture plates after infection of *K. pneumoniae* with phage particles. This has become

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328 the basis for important laboratory methods used in the characterisation of novel phages, revealing
329 phage specificity and host range (Hughes et al., 1998).
330
331 Moreover, differences observed among *Myoviridae*, *Podoviridae* and *Siphoviridae* can be useful
332 for preliminary identification. For example, sequence analysis reveals that *Myoviridae* tend to
333 have a much larger genome size and a lower GC content compared to *Podoviridae* and
334 *Siphoviridae* (**Table 1**). Restriction analysis, which uses bacterial restriction enzymes to digest
335 phage DNA, can also help to estimate the size of the phage genome in addition to identifying
336 those that are already known to science prior to extensive characterisation, and analysis by
337 transmission electron microscope is able to reveal morphological characteristics such as phage
338 tail structures (Kęsik-Szeloch et al., 2013). Phylogenetic analyses show several *Klebsiella* phages
339 belong to accepted genera within the *Siphoviridae*, *Podoviridae* and *Myoviridae*, while others
340 belong to new lineages with – as yet – no standing in viral taxonomy (**Figure 1**, **Supplementary**
341 **Material**).
342
343 **Specificity and host range**
344 To infect its host, a lytic phage must first attach itself to a susceptible bacterial cell. It achieves
345 this by recognising and binding a specific receptor on the surface of the host cell. This
346 interaction between the phage tail structure and host receptor allows the phage to both identify
347 susceptible bacteria, and position itself for injecting its genetic material into the cell. Adsorption
348 to the host can occur via any external structure depending on the phage and host, but in Gram-
349 negative bacteria, such as *K. pneumoniae*, these can include the capsule, pili, outer membrane

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proteins, sugar moieties, or LPS (Bertozzi Silva, Storms & Sauvageau, 2016). This process, therefore, determines host range, i.e. the breadth of hosts that any given phage can infect.

D'Andrea et al. (2017) 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. (2009a) 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 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 (Loc-Carrillo & Abedon, 2011). 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 (Gu et al., 2012), and even the hybridisation of phage tail structures to increase the host range artificially (Yosef et al., 2017).

THERAPEUTIC POTENTIAL OF *KLEBSIELLA PNEUMONIAE* PHAGES

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There are a number of considerations to be made when selecting phages suitable for use as therapeutic antimicrobial agents. Firstly, phages must be effective in killing *K. pneumoniae*. During phage characterisation, *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 more useful than those with narrow host range so that multiple strains may be targeted at once (Harper, 2018). 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 (Harper, 2018).

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 (Kumari, Harjai & Chhibber, 2010b), pneumonia (Chhibber, Kaur & Kumari, 2008), liver abscesses (Hung et al., 2011) and bacteraemia (Vinodkumar, Neelagund & Kalsurmath, 2005), 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*

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infections (Majkowska-Skrobek et al., 2016; Manohar, Nachimuthu & Lopes, 2018; Thiry et al., 2019).

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 (Kumari, Harjai & Chhibber, 2010a), 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 (Kumari, Harjai & Chhibber, 2009). 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% (Chhibber, Kaur & Kumari, 2008). Additionally, when compared to topical treatments with both natural products (honey and aloe vera gel) (Kumari, Harjai & Chhibber, 2010c) and antimicrobial agents (silver nitrate and gentamicin) (Kumari, Harjai & Chhibber, 2011), 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.

The delivery method of 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. (2015) determined that intranasal delivery of phage 1513 was able to produce a survival rate of

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418 80% in the Swiss-Webster mouse model, compared to 0% of negative controls, 2 h after nasal
419 inoculation of MDR *K. pneumoniae* 1513 as well as visibly reduced lung injury, in comparison
420 to negative controls. Chhibber et al. (2008) demonstrated that intraperitoneal injection of phage
421 SS administered immediately after intranasal inoculation of *K. pneumoniae* B5055 into BALB/c
422 mice resulted in complete clearance of bacteria in 5 days, compared to 10 days in untreated mice,
423 although the authors state that even a short delay of 6 h post inoculation rendered treatment
424 ineffective. Singla et al. (2015) found that phage KPO1K2, encased in a liposome, was effective
425 in treating lobar pneumonia induced in BALB/c mice by intranasal inoculation of *K. pneumoniae*
426 B5055, even when phage treatment was delayed by up to 3 days.
427
428 Although there is a difference in the choice of phage in these published reports, and so studies
429 cannot be compared directly, it does highlight the importance of investigating differing delivery
430 methods of phage treatment, not only in a logistical sense, but also in elucidating the most
431 efficient method of delivery according to the type of infection and the length of incubation prior
432 to treatment. Moreover, these studies have each measured the *in vivo* effect of phage treatment
433 against only one strain of *K. pneumoniae*, providing no information regarding phage host range.
434 Further experiments should, therefore, seek to determine whether the host range of their
435 respective phages is broad enough to be considered useful for therapeutic purposes.
436
437 While several studies have reported successful use of *K. pneumoniae* phages to clear infections
438 in murine and *Galleria* models, the effects of phage infection on the microbiome (i.e. microbiota,
439 metabolome) must now be considered when assessing phages (individually or as phage cocktails)
440 as a viable treatment or patient decontamination measure. Hsu et al. (2019) showed that infection

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441 with lytic phages caused an increase in phage resistance (28% to 68%) in a known bacterial
442 population common to the human gut microbiota. Quantitative shifts in sensitive and non-
443 sensitive strains were seen, highlighting the system-level effect of phage infection. Phage
444 infection did not necessarily clear the target species but instead modulated the ecosystem
445 towards a more stable gut environment. Phages inducing simultaneous knockdown of
446 *Enterococcus faecalis* and *Bacteroides fragilis* populations had little effect on the microbiota
447 compared with *Escherichia coli* and *Clostridium sporogenes* phages, which caused significant
448 decreases (10^6 per gram stool) in *Bacteroides vulgatus*, *Proteus mirabilis* and *Parabacteroides*
449 *distasonis* populations, and 10^8 per gram stool decreases in *Akkermansia muciniphila* and
450 *Bacteroides fragilis* populations. Perturbation of the microbiota by phages also affected the
451 metabolome. Abundance of 17% of examined compounds was altered significantly in the
452 presence of phages. During initial phage infection, Hsu et al. observed a 10-, 17-, and 2-fold
453 reduction in tryptamine, a microbiome-associated metabolite known to play a role in accelerating
454 gastrointestinal transit in mice (Bhattarai et al., 2018). This led them to suggest phage infection
455 could be used to modulate the microbiome in a targeted manner to influence systemic health.
456
457 **Combination therapy**
458 A number of *in vitro* experiments have identified the possibility of bacterial resistance arising as
459 a result of phage therapy (Kumari, Harjai & Chhibber, 2010a; Gu et al., 2012; Cao et al., 2015;
460 Chadha, Katare & Chhibber, 2016; Tabassum et al., 2018). To reduce the emergence of phage-
461 resistant strains of *K. pneumoniae* during treatment, research has begun to explore combination
462 therapy either using phage cocktails, or by combining phage treatment with antibacterial drugs.
463

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Gu et al. (2012) 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. (2016), 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. (2009b) 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 (Kutter et al., 2010). For example, the Eliava Institute of

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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 (Furfaro, Payne & Chang, 2018).

In the West, regulatory issues surrounding the use of phages as therapeutic agents has 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 personalised 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 (Debarbieux et al., 2016). However, it should be noted that the Belgian government has introduced a pragmatic framework that facilitates tailored phage therapy (magistral phage regulatory framework) and allows 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 (Pirnay et al., 2018). 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 (Pirnay et al., 2018).

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.

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(2009), 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. (2009), 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.

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 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

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infection. This kind of treatment could be advantageous in that it would be easier and quicker to gain clinical approval of a recombinant protein product over the direct use of phages. Indeed, phage-derived recombinant proteins may be used to directly combat infections caused by bacteria such as *K. pneumoniae,* 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 (Drulis-Kawa, Majkowska-Skrobek & Maciejewska, 2015).

Holins, endolysins and spanins are proteins that are produced after the infection of a bacterium, and 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 permeabilisation of the inner cell membrane. This cannot independently cause cell lysis; however, it allows endolysins and spanins to translocate from the cytoplasm,

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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 (Drulis-Kawa, Majkowska-Skrobek & Maciejewska, 2015).

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. (2017) 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 cells treated with TTPA were stripped of their capsules. In a similar process of cloning, expression and agar spot-testing, Pan et al. (2017) 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 phage-host specificity, but it also lends the idea of artificially generated cocktails of recombinant enzymes that can target a wide range of *K. pneumoniae* strains.

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578 A number of *in vivo* experiments have also been carried out investigating the effect of
579 polysaccharide depolymerases on *K. pneumoniae* infection. Majkowska-Skrobek et al. (2016)
580 identified, cloned and expressed a KP36-derived capsule depolymerase, depoKP36, which
581 produced haloes on lawns of *K. pneumoniae* in agar spot-tests. The authors tested the ability of
582 depoKP36 to treat infection caused by *K. pneumoniae* in *Galleria mellonella* and found that
583 100% died without treatment, up to 40% survived when treated with depoKP36 post infection,
584 and depoKP36 treatment of bacteria prior to infection resulted in only a 23% death rate. These
585 results suggest that the decapsulating action of depoKP36 on *K. pneumoniae* produced a
586 decreased ability of the bacterium to resist the host immune response. This was confirmed in
587 subsequent research (Majkowska-Skrobek et al., 2018).
588
589 **Endolysins**
590 Endolysins have been studied extensively for use against Gram-positive bacteria, due to the
591 absence of an outer cell membrane found in Gram-negative bacteria such as *K. pneumoniae*
592 which would normally hinder the action of the enzyme in the absence of spanins. However,
593 recent research has produced some promising results regarding the use of endolysins against
594 Gram-negative bacteria also. Maciejewska et al. (2017) produced a recombinant endolysin from
595 the *K. pneumoniae* phage KP27 and analysed its peptidoglycan-degrading activity on a range of
596 Gram-negative bacteria, including strains of *K. pneumoniae*, *P. aeruginosa*, *Salmonella enterica*
597 and *E. coli*, by co-incubation of bacteria and endolysin. The recombinant enzyme successfully
598 lysed all strains of bacteria that were tested. However, the outer membrane of bacteria was
599 permeabilised prior to endolysin treatment. This suggests that any potential endolysin-based

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infection control agents require mixing with outer-membrane-permeabilising agents to be effective against *K. pneumoniae* (Maciejewska et al., 2017).

To overcome the need for additional outer-membrane-permeabilising 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-destabilising peptide (Briers et al., 2014b). Artilysins specific for *K. pneumoniae* have yet to be developed, but they have been successfully created for use against *P. aeruginosa* (Briers et al., 2014a) and *Acinetobacter baumannii* (Defraine et al., 2016). 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 antibiotics to more easily access bacterial cells. Artificial endolysins have the potential to work against infection as an independent antimicrobial agent. Further research is required in this area to fully realise 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-

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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 for which 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 are 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 (Colavecchio et al., 2017) and toxin-encoding (Strauch, Lurz & Beutin, 2001) genes that could be transferred to the target bacterium via the process of transduction. Proper characterisation is, therefore, important when considering phages for

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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.

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 more appropriate for phage therapy. It is evident from the recent work of Hsu et al. (2019) that 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 minimise 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 consisted of small sample sizes and often rely on patient-generated data (Furfaro, Payne & Chang, 2018).

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

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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)"* (European Commission, 2017). Although the project attempted to define appropriate practices for phage therapy during its assessment of efficacy and tolerability of phage-treated burn-wound infections (Jault et al., 2019), only temporary allowances were made. While recommendations for subsequent clinical trials were given, 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 personalised 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

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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 (Fleming, 1945; Abraham & Chain, 1988) went unheeded, resulting in the spread of MDR bacteria such as *K. pneumoniae*, and are the grounds upon which phage therapy has become a renewed topic of research. The development of novel antimicrobial agents is, therefore, not enough to combat infection and bacterial resistance in the long term. Strategies regarding the use of any novel antimicrobial treatments must be developed to minimise 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 minimise 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 minimise 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 decolonisation programmes, and cautious use of antimicrobial agents.

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714

715 **Future research**

716 The future of phage research is a promising one. Phages are perhaps the most numerous of all
717 biological entities on the planet and as such could be the most valuable source of therapeutic
718 solutions. As we further elucidate the interactions between phage and bacterium, as predator and
719 prey, advances in our understanding of the molecular mechanisms defining such interactions
720 may afford us new information and ideas that can be applied to infection control. Indeed, phage
721 research has already led to the development of artificial phage-derived antibacterial proteins –
722 Artilysins (Briers et al., 2014b) – and the artificial alteration of phage host range to infect a
723 greater range of bacteria than is naturally possible is just beginning to come to fruition (Yosef et
724 al., 2017).

725

726 Furthermore, recent technological advances have seen next-generation sequencing (NGS)
727 become increasingly used in phage research, providing a more robust platform from which to
728 launch detailed phage characterisation, screening of harmful genes and evaluating potentially
729 useful gene products (Philipson et al., 2018). Further technological advancements and
730 categorisation of information attained from methods such as NGS can only lead us onwards,
731 providing new solutions to old problems.

732

733 **ACKNOWLDGEMENTS**

734 TCB was funded by a University of Westminster PhD studentship. LH was funded by the
735 Medical Research Council (grant number MR/L01632X/1). LH is a member of the ESGHAMI

study group. All authors contributed to the writing of this article. This work used the computing resources of CLIMB (Connor et al., 2016).

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1236 **Table 1.** Known phages with that infect one or more strains of *Klebsiella*

Phage	Family	RefSeq/GenBank accession no.	Genome size (bp)	Source	Reference
vB_KpnM_KpV477	*Myoviridae*	NC_031087	168272	Clinical sample	Komisarova et al. (2017)
ZCKP1	*Myoviridae*	MH252123	150925	Freshwater	Taha et al. (2018)
KP15	*Myoviridae*	NC_014036	174436	Irrigated fields	Kęsik-Szeloch et al. (2013)
JD001	*Myoviridae*	NC_020204	48814	Seawater	Cui et al. (2012)
0507-KN2-1	*Myoviridae*	NC_022343	159991	Sewage	Hsu et al. (2013)
Matisse	*Myoviridae*	NC_028750	176081	Sewage	Provasek et al. (2015)
Miro	*Myoviridae*	KT001919	176055	Sewage	Mijalis et al. (2015)
PKO111	*Myoviridae*	NC_031095	168758	Sewage	Park et al. (2017)
PMBT1	*Myoviridae*	LT607758	175206	Sewage	Koberg et al. (2017)
vB_KpnM_KB57	*Myoviridae*	NC_028659	142987	Sewage	Volozhantsev et al.*
vB_Kpn_F48	*Myoviridae*	MG746602	170764	Sewage	Ciacci et al. (2018)
GH-K2	*Myoviridae*	Not Available	Unknown	Sewage	Gu et al. (2012)
Kpn1	*Myoviridae*	Not Available	Unknown	Sewage	Chadha, Katare & Chhibber (2016)
Kpn2	*Myoviridae*	Not Available	Unknown	Sewage	Chadha, Katare & Chhibber (2016)
Kpn3	*Myoviridae*	Not Available	Unknown	Sewage	Chadha, Katare & Chhibber (2016)
Kpn4	*Myoviridae*	Not Available	Unknown	Sewage	Chadha, Katare & Chhibber (2016)
vB_KpnM_BIS47	*Myoviridae*	KY652726	147443	Sewage plant	Labudda et al. (2017)
JD18	*Myoviridae*	NC_028686	166313	Unknown	Fan et al.*
vB_KleM-RaK2	*Myoviridae*	NC_019526	345809	Unknown	Šimoliūnas et al. (2012)
Mineola	*Myoviridae*	MH333064	166130	Unknown	Boeckman et al.*
May	*Myoviridae*	MG428991	159631	Unknown	Nguyen et al.*
Menlow	*Myoviridae*	MG428990	157281	Unknown	Newkirk et al.*
vB_KpnM_KpV79	*Myoviridae*	MF663761	47760	Unknown	Komisarova et al.*
vB_KpnM_KpV52	*Myoviridae*	KX237516	47405	Unknown	Komisarova et al.*
1611E-K2-1	*Myoviridae*	MG197810	47797	Unknown	Lin et al.*
KP179	*Myoviridae*	MH729874	162630	Unknown	Kozlova et al.*
KP1	*Myoviridae*	MG751100	167989	Unknown	Kim*
3 LV-2017	*Myoviridae*	KY271397	35100	Unknown	Villa et al. (2017)
4 LV-2017	*Myoviridae*	KY271398	33540	Unknown	Villa et al. (2017)
Kpn112	*Myoviridae*	KJ021043	35560	Unknown	Chandekar et al.*
K64-1	*Myoviridae*	NC_027399	346602	Untreated water	(Pan et al. (2015)
KPV15	*Myoviridae*	KY000080	167034	Wastewater	Aleshkin et al. (2016)
KP27	*Myoviridae*	NC_020080	174413	Wastewater plant	Kęsik-Szeloch et al. (2013)
KP34	*Podoviridae*	NC_013649	43809	Cesspool holding tank	Drulis-Kawa et al. (2011)
vB_KpnP_KpV475	*Podoviridae*	NC_031025	42201	Clinical sample	Solovieva et al. (2018)
vB_KpnP_KpV74	*Podoviridae*	KY385423	44094	Clinical sample	Solovieva et al. (2018)
vB_KpnP_KpV48	*Podoviridae*	KX237514	44623	Clinical sample	Solovieva et al. (2018)
KP32	*Podoviridae*	NC_013647	41119	Roadside ditch	Kęsik-Szeloch et al. (2013)
P13	*Podoviridae*	Not Available	45976	Sewage	Shang et al. (2015)
vB_KpnP_KpV41	*Podoviridae*	NC_028670	44203	Sewage	Solovieva et al. (2018)
vB_KpnP_KpV71	*Podoviridae*	NC_031246	43267	Sewage	Solovieva et al. (2018)
vB_KpnP_KpV766	*Podoviridae*	KX712071	41283	Sewage	Solovieva et al. (2018)
vB_KpnP_KpV767	*Podoviridae*	KX712070	40395	Sewage	Solovieva et al. (2018)
vB_KpnP_KpV763	*Podoviridae*	KX591654	40765	Sewage	Solovieva et al. (2018)
K5-2	*Podoviridae*	KY389315	41116	Sewage	Hsieh et al. (2017)
K5-4	*Podoviridae*	KY389316	40163	Sewage	Hsieh et al. (2017)
KPO1K2	*Podoviridae*	Not Available	~42000	Sewage	Verma, Harjai & Chhibber (2009a)
Kpn5	*Podoviridae*	Not Available	~24000	Sewage	Kumari, Harjai & Chhibber (2010a)
Kpn12	*Podoviridae*	Not Available	~24000	Sewage	Kumari, Harjai & Chhibber (2010a)
Kpn13	*Podoviridae*	Not Available	~24000	Sewage	Kumari, Harjai & Chhibber (2010a)
Kpn17	*Podoviridae*	Not Available	~24000	Sewage	Kumari, Harjai & Chhibber (2010a)
Kpn22	*Podoviridae*	Not Available	~24000	Sewage	Kumari, Harjai & Chhibber (2010a)
phiNK5	*Podoviridae*	Not Available	~29000	Sewage	Hung et al. (2011)
Phage SS	*Podoviridae*	Not Available	Unknown	Sewage	Chhibber, Kaur & Kumari (2008)
Henu1	*Podoviridae*	MK203841.1	40352	Sewage	Teng et al. (2019)
vB_KpnP_BIS33	*Podoviridae*	KY652725	41697	Sewage plant	Labudda et al. (2017)
vB_KpnP_IL33	*Podoviridae*	KY652724	41335	Sewage plant	Labudda et al. (2017)
vB_KpnP_PRA33	*Podoviridae*	KY652723	40605	Sewage plant	Labudda et al. (2017)
F19	*Podoviridae*	NC_023567	43766	Unknown	Chen et al.*
K11	*Podoviridae*	NC_011043	41181	Unknown	Savalia et al.*
Pylas	*Podoviridae*	MH899585	70408	Unknown	Powell et al.*
vB_KpnP_IME321	*Podoviridae*	MH587638	39906	Unknown	Wang et al. (2019)
SH-Kp 152234	*Podoviridae*	KY450753	40578	Unknown	Zhi et al.*
KP8	*Podoviridae*	MG922974	73679	Unknown	Bokovaya et al.*
SH-Kp 152410	*Podoviridae*	MG835568	40945	Unknown	Xu et al.*
KP-Rio/2015	*Podoviridae*	KX856662	43557	Unknown	Meira et al.*
KN4-1	*Podoviridae*	LC413195	41219	Unknown	Pan et al. (2019)
KN3-1	*Podoviridae*	LC413194	41059	Unknown	Pan et al. (2019)
KN1-1	*Podoviridae*	LC413193	40236	Unknown	Pan et al. (2019)
kpssk3	*Podoviridae*	MK134560	40539	Unknown	Shi et al.*

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Phage	Family	RefSeq/GenBank accession no.	Genome size (bp)	Source	Reference
phiKpS2	*Podoviridae*	KX587949	44024	Unknown	Shen et al. (2018)
myPSH1235	*Podoviridae*	MG972768	45135	Unknown	Manohar, Nachimuthu & Lopes (2018)
2044-307w	*Podoviridae*	MF285615	40048	Unknown	Zhao*
6 LV-2017	*Podoviridae*	KY271400	19260	Unknown	Villa et al. (2017)
vB_KpnP_IME205	*Podoviridae*	KU183006	41310	Unknown	Bai et al.*
vB_Klp_5	*Podoviridae*	Not Available	Unknown	Unknown	Karumidze et al. (2013)
vB_Klp_6	*Podoviridae*	Not Available	Unknown	Unknown	Karumidze et al. (2013)
vB_KpnP_KpV289	*Podoviridae*	NC_028977	41054	Untreated sewage	Volozhantsev et al. (2016)
NTUH-K2044	*Podoviridae*	NC_025418	43871	Untreated water	Lin et al. (2014)
K5	*Podoviridae*	NC_028800	41698	Wastewater	Schneider et al.*
phiBO1E	*Podoviridae*	KM576124	43865	Wastewater	D'Andrea et al. (2017)
KPV811	*Podoviridae*	KY000081	42641	Wastewater	Aleshkin et al. (2016)
vB_Kp1	*Podoviridae*	NC_028688	40114	Wastewater plant	Alvez et al.*
vB_Kp2	*Podoviridae*	NC_028664	43963	Wastewater plant	Alvez et al.*
vB_KpnP_SU503	*Podoviridae*	NC_028816	43809	Wastewater plant	Eriksson et al. (2015)
vB_KpnP_SU552A	*Podoviridae*	NC_028870	43595	Wastewater plant	Eriksson et al. (2015)
AltoGao	*Podoviridae*	MF612071	43012	Wastewater plant	Gao, Linden & Nelson (2017)
SopranoGao	*Podoviridae*	MF612073	61644	Wastewater plant	Gao, Linden & Nelson (2017)
KLPN1	*Siphoviridae*	NC_028760	49037	Human caecum	Hoyles et al. (2015)
KPP5665-2	*Siphoviridae*	MF695815	39241	Mastitis milk	Carl et al. (2017)
1513	*Siphoviridae*	NC_028786	49462	Sewage	Cao et al. (2015)
PKP126	*Siphoviridae*	NC_031053	50934	Sewage	Park et al. (2017)
Sushi	*Siphoviridae*	NC_028774	48754	Sewage	Nguyen et al. (2015)
vB_KpnS_KpV522	*Siphoviridae*	KX237515	51099	Sewage	Komisarova et al.*
TSK1	*Siphoviridae*	MH688453	49861	Sewage	Tabassum et al. (2018)
IME207	*Siphoviridae*	NC_031924	47564	Sewage	Liu et al. (2016)
vB_KpnS_GH-K3	*Siphoviridae*	MH844531.1	49427	Sewage	Gu et al. (2012); Cai et al. (2019)
48ST307	*Siphoviridae*	KY271402	52338	Unknown	Villa et al. (2017)
KPN N98	*Siphoviridae*	MG835858	59214	Unknown	Jeon et al.*
YMC16/01/N133_KPN_BP	*Siphoviridae*	MF476925	58387	Unknown	Jeon et al.*
YMC15/11/N53_KPN_BP	*Siphoviridae*	MF476924	59100	Unknown	Jeon et al.*
KPN N54	*Siphoviridae*	MF415413	59100	Unknown	Jeon et al.*
KPN N141	*Siphoviridae*	MF415412	49090	Unknown	Jeon et al.*
KPN U2874	*Siphoviridae*	MF415411	59087	Unknown	Jeon et al.*
KPN N137	*Siphoviridae*	MF415410	59100	Unknown	Jeon et al.*
Seifer	*Siphoviridae*	MH817999	58197	Unknown	Salazar et al.*
SH-Kp 160016	*Siphoviridae*	KY575286	49170	Unknown	Zhi et al.*
Sugarland	*Siphoviridae*	MG459987	111103	Unknown	Erickson et al.*
vB_Kpn_IME260	*Siphoviridae*	KX845404	123490	Unknown	Xing et al.*
NJR15	*Siphoviridae*	MH633487	49468	Unknown	Hao et al.*
NJS3	*Siphoviridae*	MH633486	49387	Unknown	Hao et al.*
NJS2	*Siphoviridae*	MH633485	50132	Unknown	Hao et al.*
TAH8	*Siphoviridae*	MH633484	49344	Unknown	Hao et al.*
phiKO2	*Siphoviridae*	NC_005857	51601	Unknown	Casjens et al. (2004)
NJS1	*Siphoviridae*	MH445453	49292	Unknown	Zhu et al.*
JY917	*Siphoviridae*	MG894052	37655	Unknown	Hao et al.*
vB_KpnS_IME279	*Siphoviridae*	MF614100	42518	Unknown	Zhao et al.*
1 LV-2017	*Siphoviridae*	KY271401	29880	Unknown	Villa et al. (2017)
2 LV-2017	*Siphoviridae*	KY271396	44400	Unknown	Villa et al. (2017)
2b LV-2017	*Siphoviridae*	KY271395	44279	Unknown	Villa et al. (2017)
5 LV-2017	*Siphoviridae*	KY271399	47014	Unknown	Villa et al. (2017)
vB_Kp3	*Siphoviridae*	KT367887	48493	Unknown	Alvez et al.*
phiKp-lyy15	*Siphoviridae*	Not Available	Unknown	Unknown	Lu et al. (2015)
vB_Klp_1	*Siphoviridae*	Not Available	Unknown	Unknown	Karumidze et al. (2013)
vB_Klp_3	*Siphoviridae*	Not Available	Unknown	Unknown	Karumidze et al. (2013)
vB_Klp_4	*Siphoviridae*	Not Available	Unknown	Unknown	Karumidze et al. (2013)
KOX1	*Siphoviridae*	KY780482	50526	Wastewater	Brown et al. (2017)
phage Z	*Siphoviridae*	Not Available	Unknown	Wastewater	Jamal et al. (2015)
KP36	*Siphoviridae*	NC_029099	49818	Wastewater plant	Kęsik-Szeloch et al. (2013)
MezzoGao	*Siphoviridae*	MF612072	49807	Wastewater plant	Gao, Linden & Nelson (2017)
GH-K1	Unknown	Not Available	Unknown	Sewage	Gu et al. (2012)
PBKP05	Unknown	Not Available	30240	Unknown	Oh et al. (2019)
Kpp95	Unknown	Not Available	~175000	Unknown	Wu et al. (2007)

*No paper associated with the RefSeq/GenBank record(s).

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Figure 1. Phylogenetic placement of dsDNA *Klebsiella* phages within the order *Caudovirales*. Placement of 109 genomes (**Table 1**) within ViPTree version 1.9 (Nishimura et al., 2017) 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 (fasta file of input sequences and newick-format file available in **Supplementary Material**) to generate the tree shown (annotated using https://itol.embl.de and Adobe Illustrator). Taxonomy of 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 in **Supplementary Material**.

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