In vitro Evaluation of Lytic Phages for Carbapenem-Resistant *Klebsiella pneumoniae* Isolates

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Abstract

Klebsiella pneumoniae is a dominate cause of sever community-acquired and nosocomial

infections, specifically among immunocompromised individuals. The increasing occurrence of

multidrug-resistant (MDR) isolates has significantly impacted the effectiveness of antimicrobial

agents. As we have entered the post antibiotic era, the use of bacteriophages to treat

pathogenic bacterial infections is becoming revitalized. Elucidating molecular details of phage-

bacteria interactions will provide insights into phage biology and better development of phage

therapies. In this study, a total of 22 non-duplicate *K. pneumoniae* isolates were assessed for

their genetic and phenotypic relatedness by multi-locus sequence typing (MLST), endonuclease

S1 nuclease pulsed-field gel electrophoresis (S1-PFGE) and *in vitro* antibiotic susceptibility

testing. Further, the beta-lactamase gene (bla_{KPC}) was characterized to determine the spread

and outbreak of *K. pneumoniae* carbapenemase (KPC)-producing enterobacterial pathogens.

Three phages were isolated and characterized to evaluate the application of lytic phages against

these 22 ST11 *K. pneumoniae* isolates. *In vitro* inhibition assays demonstrated the lytic potential

of three relatively broad host-range phages, imposing strong selections for phage-resistant

mutants. Comparative genomic sequence analyses revealed that phage-resistant mutants

harbored mutations in gene *wcaJ,* which is essential for the biosynthesis of colanic acid.

Together, this data suggests that *in vitro* characterization of phage-host interactions adds more

comprehensive knowledge to known anti-phage defense strategies and further emphasizes their

complexity and future challenges to overcome prior to using phages for controlling this

important MDR bacterium.

Keywords: Bacteriophage; Phage therapy; *Klebsiella pneumoniae*; Phage-host interactions;

Phage-resistance mechanisms

1. Introduction

The ongoing explosion of the antimicrobial resistance crisis has been accelerated by the overuse

and misuse of antibiotics, causing the emergence and increased prevalence of multidrug-

resistant (MDR) bacteria [1]. *Klebsiella pneumoniae*, a Gram-negative *Enterobacterales*, can be

found in the environment and has been associated with community-acquired infections [2]. The

increasingly common mechanism of carbapenem resistance in *K. pneumoniae* is mediated by

extended-spectrum β-lactamases (ESBLs), which are able to break the β-lactam structure in the

most commonly used antibiotics [3]. Thus, infections of *K. pneumoniae* carbapenemase (KPC)-

producing bacteria are consistently associated with poor clinical outcomes and represent a

significant and continued threat to the healthcare system. As this problem continues to grow,

there is a strong need to find an alternative means to conventional antimicrobials in order to

combat this clinical pathogen.

Bacteriophages (phages for short) are the most common and diverse biological entities in the

biosphere, playing an important role in promoting the bacterial diversity, structuring community

composition and being a key driver in nutrient turnover [4]. Lytic phages infect and lyse bacterial

hosts and are an attractive alternative towards combating the fast development of antibiotic-

resistant *K. pneumoniae* [5, 6]. The use of phages has gained interest over the past years and

phages that are specific for MDR bacterial pathogens, including *P. aeruginosa*, *Enterococcus*

spp., *Mycobacterium abscessus*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, and *E. coli* have been

identified and used to treat bacterial infections [7-14]. These studies demonstrated that lytic

phages can be harnessed to prevent and eradicate pathogenic bacteria, and in some cases,

alleviate current medical challenges posed by antibacterial resistance and thus further reduce

mortalities.

While previous applications of phage therapy have shown promising results, the technology is

still in a stage of development due to rapid evolution dynamics between phages and their hosts

[15]. For instance, phage specificity, known as precisely targeting the bacterial strain, is

regarded as a significant barrier to the successful application of phage therapies [16]. This

specificity is directly linked to the key step in recognition between phage receptor-binding

proteins (RBP) located on tail fibers, and phage receptors encoded on the susceptible bacterial

host cell surface [17]. A common form of developing phage resistance for bacterial hosts is

associated with loss or altered structure of the receptors to block the potential phage

adsorption in order to avoiding infection [18]. In general, the fitness costs (i.e., motility, nutrient

transport or antimicrobial susceptibility) make the mutants unable to compete with phage-

susceptible strains in both environmental samples and infection models, even in the presence of

phage predation [19, 20]. It is, therefore, crucial to characterize the phage-host interaction

regarding the phenotypic and genetic diversification in bacteria and phages prior to any

intended phage applications.

In this study, we analyzed the molecular epidemiological feature of a collection of 22 clinical

bla$_{KPC}$ producing *K. pneumoniae* isolates, specifically on the characteristics of ST, plasmid and

antibiotic susceptibility. Using these isolates, we isolated and characterize three phages native

to hospital sewage. Details of phage host range, genome, morphological characters and phage-

host interactions were established to assess the application of phage therapies against the

clinical isolates of *K. pneumoniae,* including carbapenem-resistant isolates.

2. Materials and methods

2.1 Bacterial strains

From December 2019 to January 2020 a total of 22 nonduplicate bacterial isolates of *K.*

pneumoniae were collected from Zhongshan Hospital, Shanghai, China. *Klebsiella pneumoniae*

and *E. coli* were routinely grown in either LB broth with aeration, or, on LB agar plates at 37°C.

The beta-lactamase gene (*bla*KPC) and undecaprenyl-phosphate glucose-1-phosphate transferase

gene (*wcaJ*) were detected by PCR and verified using Sanger sequencing (Sango Biotech,

Shanghai, China). Oligo sequences are available upon request. Antibiotics were added as

necessary to final concentrations, 100 µg/mL apramycin and 100 µg/mL spectinomycin.

2.2 Multi-locus sequence typing (MLST) and antibiotic susceptibility of bacterial isolates

In order to study population genetics and micro-evolution of this epidemic bacteria, MLST was

applied to characterize these 22 isolates using sequences of internal fragments of seven house-

keeping genes, according to the protocol available on the MLST Pasteur website

(https://bigsdb.pasteur.fr/klebsiella/klebsiella.html) [21]. The purified MLST amplicons were

sent for Sanger sequencing (Sango Biotech, Shanghai, China), followed by further comparison

with those available on the MLST database to determine the sequence type (ST). The VITEK2

compact automated system (bioMérieux, Lyon, France) was used to determine antibiotic

susceptibility profiles according to the manufacturer's instructions. The susceptibility

breakpoints were interpreted with reference to the latest documents from the Clinical and

Laboratory Standards Institute (CLSI).

2.3 S1-Pulsed Field Gel Electrophoresis (PFGE)

To characterize the plasmid content in each strain, S1 nuclease digestion analyzed with PFGE

was performed as previous described [22]. Briefly, the adjusted bacterial suspension mixed in a

1% agarose gel was digested with a proteinase K solution for 2 h at 54°C, then further treated

with S1 nuclease. The electrophoresis conditions used in this study were as follows: initial switch

time, 3 s; a final switch time, 36 s; gradient, 6 V/cm; angle, 120; temperature, 14°C; and time,

18.5 h. After the electrophoresis run was complete, the gel was stained with 1 mg/mL ethidium

bromide and visualized with the ChemiDoc XRS system (Bio-Rad Laboratories, CA, USA).

2.4 Isolation and proliferation of phage

Phages were isolated using an enrichment protocol as described previously [23]. Briefly,

infiltrated hospital sewage was mixed with the same volume of 2 × LB broth and incubated at

37°C for 6 h to stimulate proliferation of total abundances of specific phages targeting the *K.*

pneumoniae isolates above. Aliquots of enrichment samples were centrifuged (12,000×*g*, 10

min) and sterilized with 0.1% chloroform. The presence of *K. pneumoniae*-phages in the hospital

sewage samples were detected by double-layer agar assays with different fold dilutions of the

cell-free spent supernatant from the enrichment assay. In order to obtain individual phages,

single plaques were picked and purified three times with their corresponding hosts. Phage

stocks were enriched and titrated using double-layer agar assays as described previously, and

stored at 4°C before use [24].

2.5 Phage-host range assay

Phage host range were determined by spot assays. Aliquots of 2 μL phage lysate, approximately

10^9 PFU ml^{-1}, was spotted on lawns containing 4 mL top agar mixed with 300 μL mid-log phase

bacteria. After incubating at 37°C for 12 h, the spots were assessed by the turbidity of plaques

and characterized as strong infection (clear), weak infection (turbid) or no inhibition (no plaque).

2.6 Susceptibility of strain ZS15 to phage infection and isolation of phage-resistant mutants

Overnight (O/N) bacterial cultures were 1,000-fold diluted into LB broth and grown to an

exponential phase with an OD600 at 0.3 and infected with its corresponding phage at a MOI of

0.01. The suspension was incubated at 37°C with aeration and samples were harvested at 1-h

intervals. The phage lysis efficiency was determined by optical density measurements. The

experiment was done in triplicate along with parallel control cultures without phages. At

timepoint 8- h, phage lysates were streaked on to LB agar plates and individual colonies were

subsequentially purified to determine phage susceptibility by spot test assays as described

above.

2.7 Phage DNA extraction, genomic analysis, DNA manipulation and complementation assay

For the extraction of phage DNA, 2 mL aliquots of phage lysate with a titer of approximately 10^9

PFU mL^{-1} was digested with DNase I and RNase to remove any residual bacterial genomic DNA

and RNA according to the manufacturer's protocol [25]. After digesting for 1.5 h at 37°C, EDTA

was added to a final concentration of 20 mM to inactivate DNase I and RNase. Total phage DNA

was extracted using the Qiagen DNeasy Blood and Tissue kit (Qiagen, CA, USA). Genomic DNA

was sent to Sangon Biotech (Shanghai, China) and sequenced using an Illumina HiSeq sequencer.

Comparative genomic approaches were used for identifying phage-host interactions in *K.*

pneumoniae according to previous studies [26, 27].

For Δ*wcaJ* mutant construction and complementation assays, the pSGKP-spe-wcaJ, pCasKP and

pBAD33-wcaJ plasmids were constructed as described previously and by using the same oligos

as in previous studies [27-29]. *Klebsiella pneumoniae* cells were made electrocompetent as

follows. O/N cultures were diluted 1:1000 and grown to an OD600 nm between 0.3 and 0.6. A

volume of 1.5 mL of culture per transformation was pelleted by centrifugation at 8,000×g for 3

min at 4°C. Pellets were washed 3 times in 10% glycerol and finally resuspended in 150 μL 10%

glycerol. Plasmids were transferred using a Bio-Rad Micropulser (EC2). Mutants were selected

from LB plates containing the corresponding antibiotics, which were further verified by PCR and

DNA sequencing to check if they contained the desired mutations.

2.8 Bacteriophage morphology by TEM

Based on the genomic differences, phages were selected for examination by transmission

electron microscopy (TEM). Phage stocks were back-diluted in a SM buffer (50 mM Tris-Cl, pH

7.5, 99 mM NaCl, 8 mM $MgSO_4$, 0.01% gelatin) to a titer of 10^8 PFU/mL. Diluted phage lysates

were then placed on the surface of Formvar carbon-coated grids (Sigma-Aldrich, St. Louis, MO,

USA) for 5 min to allow adsorption. Grids were stained with 2% sodium phosphotungstate for 2

min prior to being washed in a drop of distilled water and air-dried before being examined using

a JEM-2100 microscope operated at 80 kV.

3. Results and Discussion

3.1 Isolation and characterization of *Klebsiella pneumoniae* strains

Emergence of carbapenem-resistant *K. pneumoniae* (CRKP) has become one major burden of

healthcare-associated infection in Chinese hospitals due to the dispersion of bacteria carrying

the bla_{KPC} gene [30]. KPC was first found in carbapenem-resistant *K. pneumoniae* strains in 2001

in North America, and now KPC-producing *K. pneumoniae* can be frequently isolated from

nosocomial samples. It is, therefore, essential to determine and characterize the nosocomial

spread of KPC clones and their corresponding phenotype and genotype. Together, a

phylogenetically diverse panel of 22 *K. pneumoniae* strains were recovered from various clinical

specimens at Zhongshan Hospital, Shanghai, China. According to the *K. pneumoniae* MLST

database, all of the 22 clinical isolates belonged to ST11, which is the predominant clone of KPC-

producing *K. pneumoniae* in China (Table 1). Despite more than 100 different sequence types

(STs) being reported worldwide, the mass dissemination of carbapenemase-producing *K.*

pneumoniae (KPC-Kp) has been restricted largely to ST258, ST11, ST340 and ST512 [31].

In addition, the bla_{KPC} gene was detected in 18 out of 22 (~81%) isolates, indicating the

prevalence of β-lactamase nosocomial pathogens (Table 1). The bla_{KPC} gene can be disseminated

between species by horizontal (lateral) gene transfer (HGT) via various means, such as plasmids,

transposons, prophages and integrative conjugative elements (ICEs), which play an important

effect in generating genotypic heterogeneity and phenotypic variation among bacterial

populations [32, 33]. For instance, transfer of interspecies KPC can occur through the

dissemination of mobile genetic elements between strains of *Staphylococcus aureus* [34].

Since pathogenicity and antimicrobial resistance genes are mainly coded by plasmids, in order to

identify the subtypes of *K. pneumoniae* ST11, pulsed-field gel electrophoresis with S1 nuclease

(S1-PFGE) were utilized to resolve the prevalence of plasmid fingerprints of all the 22 isolates,

particularly large ones, which clustered into seven primary groups (Fig. 1). All groups harbored

one to three plasmids, except cluster 7 that did not harbor any plasmid between 20 kbp to 500

kbp (strain ZS22). Cluster 1, cluster 2, cluster 5 and cluster 6 comprise the majority of bla_{KPC}

producing *K. pneumoniae* strains, while cluster 3, cluster4 and cluster 7 do not harbor any bla_{KPC}

gene, despite cluster 3 and 4 carrying multiple plasmids of varying sizes (Fig. 1). Thus, it appears

that S1-PFGE is a highly discriminative molecular typing method capable of effectively

discriminating heterogeneities of ST11 clones.

3.2 Imipenem and meropenem resistance involve production of β-lactamase

Individual antimicrobial data can be found in Table 1. For ticarcillin/clavulanic acid,

piperacillin/tazobactam, cefepime, aztreonam, ciprofloxacin and levofloxacin, drug resistance

was detected in all strains. In addition, low percentages of resistance to doxycycline,

minocycline, tigecycline, colistin and trimethoprim/sulfamethoxazole were also observed for

most strains. Of these, 18 (81.8%) out of the 22 isolates become resistant to both imipenem and

meropenem with a MIC of ≥16 μg/mL, while the other 4 (18.2%) were both sensitive to

imipenem and meropenem. For these four sensitive isolates, three of them were confirmed by

PCR and sequencing to have the bla_{KPC} gene. Interestingly, strain ZS14 harboring bla_{KPC} were

susceptible to imipenem and meropenem, perhaps indicating the resistance is associated with

changes in gene expression at the mRNA levels.

3.3 Isolation and characterization of phages

Three phages were isolated from local hospital sewage using the enrichment protocol to

evaluate the potential of phage therapy as an effective alternative against *K. pneumoniae*

strains. The host range of the three phages and their lytic efficiency against all of the 22

bacterial isolates were assessed using the spot test assay. All phage lysates formed transparent

plaques on strain ZS15, which are considered as an indicator of strong lysis.

According to the phage lytic profile, the phage isolates were able to infect all of the 22 bacterial

isolates, however, each phage displayed a unique profile in terms of host range patterns and

lytic efficiency (Fig. 2). Based on their host susceptibilities, phages were divided into two major

groups. Specifically, phage φ3 displayed the largest host range, while phage φ1 and φ2 showed

a relative narrow host range, infecting only 50% of the tested *K. pneumoniae* strains (Fig. 2). The

host range differences between the phages observed in this study is very likely due to the

different phage characterization, i.e., efficiency of adsorption and lytic efficiency in its potential

hosts. Due to these differences in phage susceptibility profiles and the limited sample numbers,

we do not know to what extent these isolated phages represent the true diversity and structure

of local hospital sewage phage communities. Further studies of viral metagenomics that are not

dependent on cell culture approaches are needed to help us uncover viral populations in

hospital sewage.

3.4 Morphology and genome sequence of phages

Based on transmission electron microscopy (TEM) analysis, all the phages belong to the family

Podoviridae of *Caudovirales* order based on date provided by the International Committee on

Taxonomy of Viruses (ICTV), having short, non-contractile tails and icosahedral heads (Fig. 3). In

addition, total genomic DNA was extracted from each of the three phages, and their genomes

were sequenced by Illumina MiSeq. The assembly sizes of these three phages ranged from 38 kb

to 40 kb, having a G+C content ranging between 50% to 53%, with 45 to 49 predicted protein-

coding genes (Table 2). All phage genomes (GenBank accession: phage 1, MT813140; phage 2,

MT813141; and phage 3, MT813142) were submitted to RAST (Rapid Annotation using

Subsystem Technology) and further annotated by the available pipeline. No identifiable virulent

genes, antibiotic resistance genes or genes involved in lysogenic cycles were detected.

Therefore, it appears that those phages isolated in this study can satisfy the safety assessment

based on their complete genome sequences.

3.5 Phage growth characteristics in LB broth

To examine the efficiency of phage infection, *K. pneumoniae* ZS15 was infection in liquid

cultures with individual phages (phage $\varphi1$, $\varphi2$ and $\varphi3$) at an MOI of 0.01, respectively, and their

inhibition was assessed by bacterial growth following the measurement of the optical density of

the bacterial cultures with and without phages. Phage-amended cultures showed similar lytic

potential of the 3 phages, with a drastic decline in optical density on the initial control of the

host population for 1.5 h followed by regrowth of the resistant population (Fig. 4). A similar

inhibitory effect of phage 1 and phage 2 was observed in the *in vitro* inhibition assay, with

significant difference of OD values among phage 1, phage 2 and phage 3-amended cultures (Fig.

4). Specifically, phage 3 was slightly more effective against the host than the rest of the phages

after 5 h. After 8 h, the OD600 nm of the phage-amended cultures of group 1 (ZS15+phage 1

and ZS15+phage 2) and group 2 (ZS15+phage 3) stabilized at 1.96 and 0.09, respectively,

corresponding to 52% to 96% of the final OD in the control group, respectively (Fig. 4).

Despite *K. pneumoniae* ZS15 displaying a strong susceptibility to infection by all three phages,

phage 3 was better at suppressing resistance to host strain ZS15 over phage 1 and 2. Bacteria

are known to rapidly develop phage-resistance when subjected to predation by lytic phages in

laboratory medium [18]. By screening phage resistance mutants, we attempted to better

understand the inhibition differences among each of the phage-amended cultures. Phage

insensitive mutants were isolated from phage lysate cultures during the incubation for the

analysis of changes in their susceptibility upon phage selection. Based on the results of spot test

assays, we observed that phage-amended cultures (ZS15+phage 1, ZS15+phage 2, and

ZS15+phage 3) were dominated by resistant non-mucoid clones after 8 h of incubation,

indicating conversion of mucoid *K. pneumoniae* strain ZS15 to non-mucoid variants by

consequence of selective pressure operated by phages. However, in the ZS15+phage 3 cultures,

heterogeneity in phage suspibilitiy was observed. Out of 30 phage lysate isolates, two (~6%)

were fully sensitive to phage 3 and displayed the hypermucoviscosity (HV) phenotype as the

parental strain ZS15. Moreover, a small fraction (~30%) of phage 3 resistant mutants reverted

back to the parental phenotype after three rounds of purification. Partially sensitive (i.e.,

reduced phage susceptibly when compared to the parental strain) subpopulations (~50%) were

isolated as well and did not revert back to the wild type phenotype despite three rounds of

purification. Only ~20% were fully resistant to phage 3, suggesting different anti-phage

mechanisms were allowing coexistence of phage sensitive mutants and that their lytic phages

could exist between bacterial host ZS15 and phage 3. Since phage susceptibility was only

measured at 8 h, we do not know the dynamics of phenotype heterogeneity over time or how

long such persistence can last before heritage mutations dominate the entire population.

3.6 Identification of genes promoting phage resistance and complementation analysis

To address the underlying mechanism providing bacterial protection against phage predation,

12 independently selected spontaneous phage-resistant mutants (four for each phage) and their

corresponding wild-type parental strain (ZS15) were subjected to genome extraction, whole

genome resequencing and mapped to the ZS15 reference genome (GenBank accession

GCA_014050205.1). High-probability mutations, such as high-frequent, non-synonymous

mutations within an open reading frame were subject to further screening.

Out of 12 phage-resistant mutants, nine harbored mutations in gene *wcaJ*. These nine

representative mutants were confirmed by PCR and Sanger sequencing, implying that *wcaJ*

mutation is a key contributor to phage resistance. This is consistent with our previous study

demonstrating that phage 117 used colanic acid as a phage receptor, mutating the *wcaJ* gene

which is the initial enzyme catalyzing the biosynthesis of colanic acid, affecting phage adsorption

processes [27, 35]. These results also parallel results for *K. pneumoniae* K7 isolates from Cai et

al., in which they found deletion of *wcaJ* could block phage GH-K3 infections [36].

Notably, we found that a small fraction (efficiency of plating, EOP<0.01%) of phage 3 still could

infect ZS15-3R and ZS15 Δ*wcaJ* cells, respectively, suggesting other mutations may play an

important role in providing phage φ3 resistance in addition to *wcaJ* (Fig. 5). These results open

the possibilities that phage 3 may use a primary receptor to initiate attachment but require a

secondary cell surface receptor to irreversibly bind to the bacterial host for injection. For

example, T4 uses its six, long tail fibers to recognize lipopolysaccharide and the outer membrane

protein C, present on the bacterial surface. This reversible attachment allows the phage to move

along the surface and find a suitable site to inject its DNA [37].

Based on our phage-host range spectrum analyses, we noticed 11 strains (50%) among the 22

isolates that were fully resistant to phage 1 and phage 2. Next, we determined if these 11

resistant isolates harbored a functional *wcaJ* gene or not. As expected, PCR results showed that

4 out of 11 resistant isolates did not contained a *wcaJ* gene, displaying full resistance to phage 1

and phage2, while being partially susceptible to phage 3 with a decreased EOP, again supporting

the previous data and being in line with the phage susceptibility of the Δ*wcaJ* mutant (Fig. 2).

Interestingly, we also found seven isolates contained an intact *wcaJ* gene while displaying full

resistance to phage 1 and phage 2, which was probably due to the complex nature of colanic

acid biosynthesis and mutations in other genes involved for phage adsorption or subsequent

steps of phage replication.

To test whether there are any other mutations involved in the phages (φ1, φ2, and φ3)

resistance, we cloned the wild-type *wcaJ* gene from strain ZS15 into the expression vector

pBAD33. The resulting construct was transformed into DH5α competent cells and further

electrophorized into *K. pneumoniae* ZS15- 1R, ZS15- 2R and ZS15- 3R to complement the

mutation *in trans*. Susceptibilities to the infections of the phages (φ1, φ2, and φ3) were

restored, confirming that *wcaJ* mutation was responsible for the phage resistance phenotype

(Fig. 5).

Taken together, we find that coevolution between the virulent phages and the dominant clone

of *K. pneumoniae* ST11 is an indication that perhaps most pervasive phages are extremely well

adapted to its host, specially, by using colanic acid as their own receptor and further driving

bacterial evolution by selecting phage-resistant mutants that leads to dynamics of an arm race.

Understanding the evolutionary implication of phage-colanic acid interactions will be beneficial

for predicting how phages interact with their host, as these colanic acid -related phage

resistance-mediated phenotypic variabilities may result in exopolysaccharide associated

antigenicity and virulence. Such characterization of phage resistance evolution will be used as a

guide for selecting proper phage targeting of specific receptors with a significant clinical

application. However, the isolated bacteria and phages may only represent a small fraction of

the true genomic diversity present in local environments.

3.7 Anti-phage defense-mediated altered sensitivity to antibiotics

Previous studies have shown that evolutionary phage-host interactions affect phage resistance

and antibiotic susceptibility both positively and negatively, and are highly genotype-dependent

in bacterial populations [38-40]. We started to question whether phage-resistant mutants

changed their antibiotic susceptibilities via antagonistic coevolution between hosts and

parasites in LB broth. To investigate this further, we performed Vitek 2 assays for antimicrobial

susceptibility, testing phage-resistant mutants (ZS15-3R) and their parental phage susceptible

strains (ZS15). Interestingly, for tigecycline susceptibility testing of carbapenem-resistant *K.*

pneumoniae ZS15 strain, we noticed that strain ZS15 evolved resistance to phage 3, while

simultaneously becoming more sensitive to tigecycline with the minimum inhibitory

concentration shifting from 8 µg/mL to 2 µg/mL. Tigecycline, known as a tetracycline antibiotic,

has shown the greatest antimicrobial activity against CRKP. However, genes e.g. *tet*(B), *tet*(A),

tet(K), *tet*(M) and *tet*(S) coding for multidrug efflux pumps are membrane-related proteins and

can efflux tetracyclines from the bacteria intracellularly, thus decreasing the MIC and

subsequently allowing protein synthesis without affecting cell viability [41]. Using membrane-

related proteins as a surface structure are one of the most common components functioning as

a phage receptor. Therefore, it makes sense that phage-resistant defense strategies would

confer a fitness cost for bacteria, i.e., becoming more sensitive to certain antibiotics. For

instance, in *P. aeruginosa*, phage OMKO1 evolved to become less resistant to antibiotics.

Likewise, in *E. coli*, the TolC protein evolved in a variety of diverse cellular functions, including

antibiotic efflux and phage TLS receptor [39]. However, unlike previous studies, the anti-phage

TLS mediated TolC mutation did not influence its role in antibiotic efflux. Overall, these results

reveal that a phage-host arm race can result in different outcomes in terms of antibiotic

susceptibilities, highlighting the diversity and complexity of host-interactions within bacterial

populations.

4. Conclusion

Successful application of phage therapy requires collaboration between microbiology and

preclinical development; however, little is known about their alignment to guide future

development. Since the discovery of phages by Frederick W. Twort in Great Britain (1915) and

Félix d'Hérelle in France (1917), many phages have been isolated and identified as potential

alternatives to antibiotics with different efficacy profiles. Phage specificity is both a benefit and

an obstacle for clinical trials, as phage receptor diversity affects phage-host interaction. When

the bacterial population is under phage attack, only a small fraction of cells can survive these

selective pressures, and those that survive determine the fate of the population. Therefore,

elucidating mechanistic details of phage-host interactions and how these would influence

bacterial physiological heterogeneity is fundamental to the development of phage therapies. In

this study, the three highlighted phages showed promising *in vitro* effects that perhaps these

phages can be tamed for our benefits without negatively affecting their natural balance. Further

clinical phage therapy trials are still needed to fill our knowledge gap between *in vitro* and *in*

vivo models.

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Conflict of interest

None declared.

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Table 1. MLST types and antibiotic susceptibility profiles of the 22 *Klebsiella pneumoniae* isolates

and their corresponding phage-resistant mutants. Antibiotic abbreviations: ticarcillin-clavulanic

acid (TIM), piperacillin-tazobactam (TZP), ceftazidime (CAZ), cefoperazone/sulbactam (CPZ/SBT),

cefepime (FEP), aztreonam (ATM), imipenem (IPM), meropenem (MEM), amikacin (AMK),

tobramycin (TOB), ciprofloxacin (CIP), levofloxacin (LVX), doxycycline (DOX), minocycline (MIN),

tigecycline (TGC), colistin (CST), trimethoprim-sulfamethoxazole (SXT).

Table 2. Morphological and genomic characterization of phages isolated from the local hospital

sewage.

Figure 1. Dendrogram generated by BioNumerics software (Version7.1, Applied Maths, St-

Martens-Latem, Belgium) showing cluster results of molecular epidemiology S1-PFGE analysis of

22 *Klebsiella pneumoniae* isolates. Band-based similarity coefficients were calculated using Dice

coefficient and unweighted pair group average method algorithm (UPGMA).

Figure 2. Phage-host range assay of the three phages (columns) against the 22 *Klebsiella*

pneumoniae isolates (rows). *Klebsiella pneumoniae* phages displayed broad and narrow host

ranges. Black, grey and white indicate infection, weak infection and no plaque formation,

respectively. The presence or absence of *wcaJ* gene of the 22 Klebsiella pneumoniae isolates.

Figure 3. Plaque images of phages and their corresponding TEM micrographs. Phages were

stained with 2% sodium phosphotungstate and were found to have short small podophage

morphology. Scale bar, 200 nm.

Figure 4. Inhibition assay of phage 1, phage 2 and phage 3 in the treatment at an MOI of 0.1 and

control without phage in cultures of *Klebsiella pneumoniae* ZS15. Optical density (OD600) was

measured at 1-h intervals over an 8-h incubation period. Error bars represent the standard

deviations from all experiments, carried out in triplicate.

Figure 5. Spot test assay of phage 1, phage 2 and phage 3 on the parental *Klebsiella pneuoniae*

ZS15 (A) and its derivative strains (B: Δ*wcaJ*, C: ZS15-1R, D: ZS15-1R pBAD33-wcaJ, E: ZS15-2R, F:

ZS15-2R pBAD33-wcaJ, G: ZS15-3R and H: ZS15-3R pBAD33-wcaJ)

									Minimum inhibitory concentration (µg/mL)											
Strain	MLST	bla_{KPC}	TIM	TZP	CAZ	CPZ	FEP	ATM	IMP	MEM	AMK	TOB	CIP	LVX	DOX	MIN	TGC	CST	SXT
ZS1	ST11	Yes	≥128	≥128	≥64	≥64	≥32	≥64	≥16	≥16	≥64	≥16	≥4	≥8	2	4	≤0.5	≤0.5	≤20
ZS2	ST11	Yes	≥128	≥128	≥64	32	≥32	≥64	≥16	≥16	4	8	≥4	≥8	2	8	1	≥16	≤20
ZS3	ST11	No	≥128	≥128	≥64	≥64	≥32	≥64	1	1	≥64	≥16	≥4	≥8	4	≥16	2	≤0.5	≤20
ZS4	ST11	Yes	≥128	≥128	32	≥64	≥32	≥64	0.5	≤0.25	≥64	≥16	≥4	≥8	2	8	1	≤0.5	≤20
ZS5	ST11	Yes	≥128	≥128	≥64	≥64	≥32	≥64	≥16	≥16	4	≤1	≥4	≥8	8	≥16	4	≤0.5	≤20
ZS6	ST11	Yes	≥128	≥128	≥64	≥64	≥32	≥64	≥16	≥16	≥64	≥16	≥4	≥8	1	2	≤0.5	≤0.5	≤20
ZS7	ST11	Yes	≥128	≥128	≥64	≥64	≥32	≥64	≥16	≥16	4	≤1	≥4	≥8	≥16	≥16	≥8	≤0.5	≤20
ZS8	ST11	Yes	≥128	≥128	≥64	≥64	≥32	≥64	≥16	≥16	≥64	≥16	≥4	≥8	4	8	2	≤0.5	≤20
ZS9	ST11	Yes	≥128	≥128	≥64	≥64	≥32	≥64	≥16	≥16	≥64	≥16	≥4	≥8	4	8	2	≤0.5	≤20
ZS10	ST11	Yes	≥128	≥128	≥64	≥64	≥32	≥64	≥16	≥16	≥64	≥16	≥4	≥8	≥16	≥16	4	4	≥320
ZS11	ST11	Yes	≥128	≥128	≥64	≥64	≥32	≥64	≥16	≥16	≥64	≥16	≥4	≥8	2	8	2	≤0.5	≤20
ZS12	ST11	Yes	≥128	≥128	≥64	≥64	≥32	≥64	≥16	≥16	≥64	≥16	≥4	≥8	2	8	1	≤0.5	≤20
ZS13	ST11	Yes	≥128	≥128	≥64	≥64	≥32	≥64	≥16	≥16	≥64	≥16	≥4	≥8	2	8	≤0.5	≤0.5	≤20
ZS14	ST11	No	≥128	≥128	32	≥64	≥32	≥64	1	0.5	≥64	≥16	≥4	≥8	2	8	≤0.5	≤0.5	≤20
ZS15	ST11	Yes	≥128	≥128	≥64	≥64	≥32	≥64	≥16	≥16	≥64	≥16	≥4	≥8	2	8	≥8	≤0.5	≤20
ZS16	ST11	Yes	≥128	≥128	≥64	≥64	≥32	≥64	≥16	≥16	≥64	≥16	≥4	≥8	4	≥16	2	≥16	≤20
ZS17	ST11	Yes	≥128	≥128	≥64	≥64	≥32	≥64	≥16	≥16	≥64	≥16	≥4	≥8	4	8	1	≤0.5	≤20
ZS18	ST11	Yes	≥128	≥128	≥64	32	≥32	≥64	≥16	8	8	≤1	≥4	≥8	≤0.5	2	≤0.5	≤0.5	≤20
ZS19	ST11	No	≥128	≥128	8	≥64	≥32	≥64	≤0.25	≤0.25	≥64	≥16	≥4	≥8	2	4	≤0.5	≤0.5	≤20
ZS20	ST11	Yes	≥128	≥128	≥64	≥64	≥32	≥64	≥16	≥16	≥64	≥16	≥4	≥8	1	4	≤0.5	≤0.5	≤20
ZS21	ST11	Yes	≥128	≥128	≥64	≥64	≥32	≥64	≥16	≥16	≥64	≥16	≥4	≥8	1	4	≤0.5	≤0.5	≤20
ZS22	ST11	Yes	≥128	≥128	≥64	≥64	≥32	≥64	≥16	≥16	≥64	≥16	≥4	≥8	1	2	≤0.5	≤0.5	≤20

Phage	Family	Host	Head diam (nm)	Head length (nm)	Tail diam (nm)	Tail length (nm)	Genome size (bp)	GC%	Genome type	Predicted genes	Phage receptor	Source
Φ1	Podoviridae	ZS15	63	58	13	14	40,428	53	dsDNA	45	Colanic acid	Sewage
Φ2	Podoviridae	ZS15	58	63	13	9	40,222	52.8	dsDNA	49	Colanic acid	Sewage
Φ3	Podoviridae	ZS15	63	63	13	11	38,741	50.7	dsDNA	46	Colanic acid	Sewage

Strain	Phage			wcaJ gene
	Φ1	Φ2	Φ3	
ZS1			▒ (light gray)	Yes
ZS2			▒ (light gray)	Yes
ZS3	▒ (light gray)	■ (dark gray)	■ (dark gray)	Yes
ZS4	■ (dark gray)	■ (dark gray)	■ (dark gray)	Yes
ZS5	▒ (light gray)	▒ (light gray)	■ (dark gray)	Yes
ZS6			▒ (light gray)	No
ZS7	■ (dark gray)	■ (dark gray)	■ (dark gray)	Yes
ZS8	▒ (light gray)	■ (dark gray)	■ (dark gray)	Yes
ZS9			■ (dark gray)	Yes
ZS10	■ (dark gray)	■ (dark gray)	■ (dark gray)	Yes
ZS11			▒ (light gray)	Yes
ZS12			▒ (light gray)	No
ZS13	▒ (light gray)	■ (dark gray)	■ (dark gray)	Yes
ZS14	▒ (light gray)	■ (dark gray)	■ (dark gray)	Yes
ZS15	■ (dark gray)	■ (dark gray)	■ (dark gray)	Yes
ZS16	■ (dark gray)	■ (dark gray)	■ (dark gray)	Yes
ZS17			▒ (light gray)	Yes
ZS18			■ (dark gray)	Yes
ZS19			▒ (light gray)	Yes
ZS20			▒ (light gray)	No
ZS21			▒ (light gray)	No
ZS22	▒ (light gray)	■ (dark gray)	■ (dark gray)	Yes