Supplementary Appendix

Table of Contents:

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Materials and Methods

K. pneumoniae identification, antibiogram, and phage susceptibility

Urine samples were plated on blood and MacConkey agar plates and incubated at 37°C. The resulting bacterial growth was quantified using semi-quantitative technique. Microbial identity of the bacteria was determined using MALDI-TOF mass spectrophotometry (Bruker). Antibiotic susceptibility was tested using the microbroth dilution technique by the Phoenix system (BD) at the UCSD clinical microbiology laboratory.

Phage susceptibility of the bacterial isolates was determined by spotting of 10 µl serially diluted phage suspensions onto bacterial lawns produced using the soft agar overlay method [1], and incubating the spotted plates at 37°C for 24 h to observe plaque formation. Efficiency of plating (EOP) was determined by comparing the phage titer on the clinical isolate against the titer on its original propagation host.

Determination of extended spectrum beta lactamase (ESBL) production was made using phenotypic assays for non-blood isolates and using genotypic assay for the CTX-M gene (Verigene) for blood isolates.

Genome sequencing and analysis of *Klebsiella* clinical isolates

Genomic DNA of the *Klebsiella* isolates were extracted using a Qiagen DNeasy Blood Tissue Kit as per the manufacturer's instruction. NanoDrop 2000 (Thermo Fisher Scientific, USA) and Qubit 3.0 (Thermo Fisher Scientific, USA) were used to quantify and validate the DNA quality. Sequencing libraries were prepared with the NEBNext Ultra DNA Library Prep kit and were sequenced to at least 300-fold coverage using an Illumina HiSeq 2500 (Azenta Life Sciences, Suzhou, China). Raw reads were trimmed using trimmomatic v0.38 [2] and assembled using SPAdes v3.15.3 [3]. Antibiotic resistance genes (ARGs) were identified using ABRicate v0.8.13 [4]. The capsule (K) locus was identified using Kaptive v0.5.1 [5]. The sequence type of the strains was determined by MLST v2.15 [6]. Core-SNP alignments were detected by comparing 722 genomes using Roary v3.11.2 (https://sanger-pathogens.github.io/Roary/). A phylogenetic tree based on genome-wide comparison was constructed from the core-SNP alignment with a GTR model and ascertainment bias correction using IQ-TREE 2 and then visualized using ggtree v3.6.2.

Phage propagation and genomic characterization

The isolation of phages Mineola and Metamorpho and their genome sequencing and annotations were reported previously [7, 8]. Phage pkp20 was isolated from raw sewage sample obtained in Melbourne, Australia; its genomic DNA was extracted using phenol-chloroform method and sequenced via Illumina HiSeq sequencing (150 bp pair-end), and the sequence reads were assembled using SPAdes v3.5.0 [3]. The isolation, propagation, and titering of all three phages were conducted using the soft agar overlay method [1]. All three phage genome sequences were completed with PCR primers to face off the ends of the assembled contig and Sanger sequencing of the resulting products. Genome analyses were carried out on the Center for Phage Technology (CPT) Galaxy and Apollo interfaces [9] with tools at default settings (https://cpt.tamu.edu/galaxy-pub). DNA similarity between the phage genomes was determined

using ProgressiveMauve [10]. ICTV taxonomy placement is based on nucleotide similarity determined by BLASTn against NCBI nt database.

Phage cross-resistance testing

Phage-resistant mutants against phages Mineola, Metamorpho, or pkp20 were generated by plating each undiluted phage lysates (~10^{10} pfu/ml, 100 µl) onto lawns of the clinical ESBL *K. pneumoniae* isolate C4023081 (isolated on 03/2021). Following overnight incubation at 37 °C, the emerged individual colonies were picked (eight colonies against each phage) and streaked onto fresh TSA plates to purify. The purified phage resistant mutants were tested for their susceptibility to phages Mineola, Metamorpho, and pkp20 by spotting 10 µl of serial diluted phage samples onto the bacterial lawns. EOP was calculated when phage susceptibility was observed.

Virulence assays using single and mixed phages

Fresh overnight culture of ESBL *K. pnuemoniae* isolate C5012365 (April 2021, the most recent host when the virulence assay was conducted) was adjusted with TSB to OD_{600} ~ 0.1 to achieve a concentration of ~10^8 cfu/ml. Phage lysates were tittered and adjusted to concentrations of 10^8 pfu/ml and 10^7 pfu/ml. For each assay, 180 µl of adjusted bacterial suspension in TSB was mixed with 20 µl of adjusted phage suspension in sterile, untreated Falcon (Corning) 96-well transparent plates to achieve final multiplicity of infection (MOI) of 0.1 and 0.01, respectively. The plate was incubated in a Tecan Spark 10 M plate reader (Tecan Group Ltd., Männedorf, Switzerland) at 37°C for 20 h with continuous shaking. The growth of the host (OD_{600}) was measured at 30-min intervals. Growth curves were obtained by plotting OD after baseline adjustment against time. The assays were performed with two biological replicates.

Phage purification for clinical administration

Propagation and purification of phages for clinical administration were carried out as described previously [11]. Briefly, phages were propagated on *K. pneumoniae* isolate C4023081 in TSB, and the harvested phage lysates were concentrated by centrifugation. The re-suspended phage pellets were purified using cesium chloride (CsCl) isopycnic gradient centrifugation, followed by dialysis. The dialyzed phages underwent further endotoxin removal using Biovendor Endotrap HD 5/1 columns, and endotoxin levels were quantified using the Biovendor EndoZyme II recombinant factor C assay. The final purified single phages were diluted and dialyzed in Lactated Ringer's to the final concentration of 5×10^{10} pfu/ml, and the phage suspensions were filtered through 0.22 µm filters and tested for sterility (USP71 test) at Nova Biologicals (Conroe, TX). The stability of individual phages and the cocktail stored in refrigerated conditions was tested weekly by phage titering. The three sterile single phage suspensions were mixed at equal volumes weekly to formulate enough phage cocktail for one-week's injection.

Phage pharmacokinetics on Day 1 of phage treatment

At the start of phage treatment (Day 1), patient serum samples were collected at different time points (baseline, 15, 30, 45, 60, and 120 min) following phage injection. Serial dilutions of the serum samples were spotted (10 µl / spot) on the soft agar bacterial lawn prepared using *K. pneumoniae* isolate C4023081. Phage inactivation half-life was determined using the non-compartmental analysis on PK Solver [12].

Neutralization of phage in patient samples

The neutralization of phages was tested by spiking the clinical patient samples (serum or urine) with phage stocks at 1:10 phage to serum ratio, to reach a starting phage level of ~10^9 pfu/ml in each sample. The spiked samples were incubated at room temperature, and dilutions were made from the spiked samples at different time points (0, 5, 10, 20, 25, 30, 40, 60, 90 min). From each dilution, 10 ul was spotted on the bacterial lawn prepared using *K. pneumoniae* isolate C4023081 and the spotted plates were incubated at 37 ºC for 24 h to observe plaques. Phage inactivation half-life was determined using the non-compartmental analysis on PK Solver [12].

Bacterial population in urine samples during phage treatment

Urine samples collected at the start of the phage administration, and several follow up samples were analyzed for total microbe counts, as well as the microbial identity. Serial dilutions of the urine samples were plated on TSA plates to enumerate the total microbe levels. From the plates, isolated colonies representing different morphologies were picked and were subjected to colony PCR to amplify the 16s rRNA genes using primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The obtained PCR products were sequenced via Sanger sequencing and results were analyzed by BLASTn against the NCBI nr database. The urine samples were also centrifuged (8,000 × g, 10 min) to pellet microbial cells. Total DNA was extracted from each pellet and was subjected to 16S metagenomic population sequencing at Microbial Genome Sequencing Center (Pittsburgh, PA). Briefly, the 16S DNA samples were prepared using Qiagen's QIAseq phased primers targeting the V3/V4 regions of the 16S gene and were sequenced on a V3 MiSeq 600cyc flowcell to generate 2x276bp PE reads. Reads QC was performed with the Illumina BCL Convert, and subsequent analysis were done using Qiime2 [13]. Operational taxonomic units (OTUs) were assigned using the Silva 138 99% OTUs full-length sequence database [14] and the VSEARCH [15] utility within Qiime2's feature-classifier plugin. OTUs were then collapsed to their lowest taxonomic units, and their counts were converted to reflect their relative frequency within a sample.

NCBI accession numbers

The complete genome sequences of *K. pneumoniae* phages Mineola, Metamorpho and pkp20 were deposited in GenBank under the accession numbers MH333064, MT701588 and OP331213, respectively.

Results

Characterization of phages and assembly of phage cocktail

In March-April 2021, a total of 111 *Klebsiella* phages (three from Monash University and 108 from Texas A&M) were screened against four ESBL *K. pneumoniae* clinical isolates from the patient (isolates from Sept 2020, Oct 2020, Jan 2021 and March 2021, see Table S1) in order to develop the phage treatment. Seventeen phages that showed activity against any of the tested isolates were further tested for their EOP on the clinical isolates compared to the phage's original propagation host. Three phages, Metamorpho, Mineola, and pkp20, showed no decrease in EOP on the most recent clinical isolates at the time of cocktail assembly (strains isolated in March and April 2021), and these phages were selected to assemble the treatment cocktail. Phages Metamorpho and Mineola were isolated from wastewater samples collected from different towns in Texas (Madisonville and Bryan, respectively) [7, 8], and phage pKp20 was isolated from sewage sample in Melbourne, Australia. Genomes of the three phages were sequenced to completion, and their genome features are summarized in Table 1. The three phages share significant DNA similarity with each other (75.9%-91.9%, Figure 1A), as well as with other T4-like phages such as *Klebsiella* phage JD18 (KT239446). Based on their sequence relationships to other phages and the criteria of the International Committee on Taxonomy of Viruses (ICTV) [16], these three phages can be assigned to species in the genus *Jiaodavirus* within subfamily *Tevenvirinae* (Table 1). The typical T4-like genomes of these three phages indicate their virulent lifestyle. No integrase genes indicating a temperate lifestyle, nor genes associated with antibiotic resistance or virulence were identified in any of these phages.

The ESBL *K. pneumoniae* isolate C4023081 (isolated in March 2021) was used to generate mutants resistant to each of these three phages. Eight resistant mutants were generated for each phage and these were tested to their sensitivity to the other two cocktail phages. All mutants resistant to pkp20 and Mineola were also resistant to Metamorpho, although Metamorpho was able to produce plaques at an extremely low frequency (10^{-8}~10^{-9}) on these mutants, suggesting an avenue for mutation to overcome resistance. Mutants resistant to Metamorpho were also resistant to pkp20 and Mineola with plaques observed at any dilution. When tested for virulence *in vitro*, the individual phages and phage cocktail demonstrated effective inhibition of the host growth at an MOI of 0.01, with regrowth of presumably phage-resistant bacteria after approximately 5 h, as is often observed in these types of assays [17] (Figure 1). A virulence assay run with an MOI of 0.1 generated similar results and the data is not shown.

Phages were propagated and purified individually to remove endotoxin and stored in lactated Ringer's (5×10^{10} pfu/ml) at 4°C, which demonstrated stability throughout treatment duration (data not shown). However, phage cocktail with mixed phages showed titer decrease (up to 85%) after one week and further decrease (up to 10-fold) after two weeks' storage at 4°C. For this reason, individual phages were mixed at equal volumes weekly to formulate the phage cocktail with an average endotoxin level of 3.87 EU/dose (5×10^{9} pfu/dose).

Table S1. Clinical episodes, clinical microbiology, and administered treatments over a 2-year period.

Date	Clinical episode	Treatment	Microbiology (urine culture)
March 2020	Post-operative cystitis	Cefpodoxime x 5d	*Citrobacter freuendii*
March 2020	Asymptomatic bacteriuria	Not treated	C. *freuendii* and vancomycin-resistant *Enterococcus faecium* (VRE)
May 2020	Asymptomatic bacteriuria	Not treated	*C. freuendii*
June 2020 (3 separate weekly cultures)	Asymptomatic bacteriuria	Not treated	*C. freuendii*
June 2020	Asymptomatic bacteriuria	Not treated	ESBL *Klebsiella pneumoniae* and *C. freuendii*
July 2020	#1Asymptomatic bacteriuria #2 Asymptomatic bacteriuria #3 Admitted with transplant pyelonephritis	#1 Not treated #2 Not treated #3 Initially meropenem and then ertapenem x 14d	#1 ESBL *K. pneumoniae* and *C. fruendii.* #2 ESBL *K. pneumoniae* and *C. fruendii.* #3 ESBL *K. pnuemoniae* in urine culture, blood culture negative
September 2020 (3 separate weekly urine cultures)	#1 Cystitis #2 Asymptomatic bacteriuria #3 Asymptomatic bacteriuria	#1 Fosfomycin x6d #2 not treated #3 not treated	*#1* ESBL *K. pnuemoniae* *#2* ESBL *K. pnuemoniae* *#3* ESBL *K. pnuemoniae*
October 2020 (2 separate weekly cultures	#1 Asymptomatic bacteriuria #2 Admitted with transplant pyelonephritis and bacteremia	#1 Not treated #2 Ertapenem x 14d followed by nitrofurantoin suppression	*#1* ESBL *K. pnuemoniae* *#2* ESBL *K. pnuemoniae* in urine and blood cultures
December 2020	#1 Cystitis leading to Emergency Room visit	#1 Ertapenem x 7d (previous nitrofurantoin discontinued)	*K. pnuemoniae*
January 2021	#1 Asymptomatic bacteriuria #2 Admitted with septic shock 5 days	#1 not treated #2 ceftriaxone x14d	#1 *K. pnuemoniae* #2 Blood and urine cultures negative

	after positive urine culture		
February 2021	Cystitis	Bactrim DS twice daily x7 days followed by once daily suppression	Culture not done
March 2021	#1 Cystitis #2 Asymptomatic bacteriuria #3 Cystitis	#1 Fosfomycin x6 days (2 doses). Bactrim suppression stopped #2 No treatment #3 Ertapenem x7d	#1 ESBL *K. pneumoniae* *#2* ESBL *K. pneumoniae* Culture not done
April 2021	Cystitis	Ertapenem x77d	ESBL *K. pneumoniae* x2 isolates
May 2021	Probable transplant pyelonephritis following kidney biopsy	Initially ertapenem x 6d followed by cefpodoxime x8d. Then placed on Bactrim DS three times a week suppression	*Escherichia coli*
August 2021	Cystitis	Ciprofloxacin x 5d; Bactrim suppression stopped.	ESBL *K. pneumoniae*
September 2021	Cystitis	Ciprofloxacin x5d	*K. pneumoniae* x 2 isolates
K. pneumoniae directed Phage Therapy			
Phage Day 1	Asymptomatic bacteriuria	Not treated	*E. faecalis*
Phage Day 8	No urinary symptoms	No antibiotics	Urine culture negative
Phage Day 15	No urinary symptoms	No antibiotics	Urine culture negative
Phage Day 22	Initially asymptomatic but then developed symptoms 2 days later with positive urinalysis and culture	Ciprofloxacin x 5d	*E. coli*
End of Phage therapy, Day 29	No urinary symptoms	No antibiotics	Urine culture negative
Nov 2021	No urinary symptoms	No antibiotics	Urine culture negative
Dec 2021	No urinary symptoms	No antibiotics	Urine culture negative
Jan 2022	No urinary symptoms	No antibiotics	Urine culture negative

Jan 2022	No urinary symptoms	No antibiotics	Urine culture negative
Feb 2022	No urinary symptoms	No antibiotics	Urine culture negative
Mar 2022	No urinary symptoms	No antibiotics	Urine culture negative
April 2022	Cystitis	Ceftriaxone -> Augmentin x5d	*K. pneumoniae*
May 2022	#1 Cystitis #2 Cystitis	#1 Keflex x 7d #2 ciprofloxacin x5	#1 *K pneumoniae* #2 *K pneumoniae*

Table S2. Results of antibiotic susceptibility testing of *K. pneumoniae* isolates as performed by the Phoenix system (BD) in the UCSD clinical microbiological laboratory.

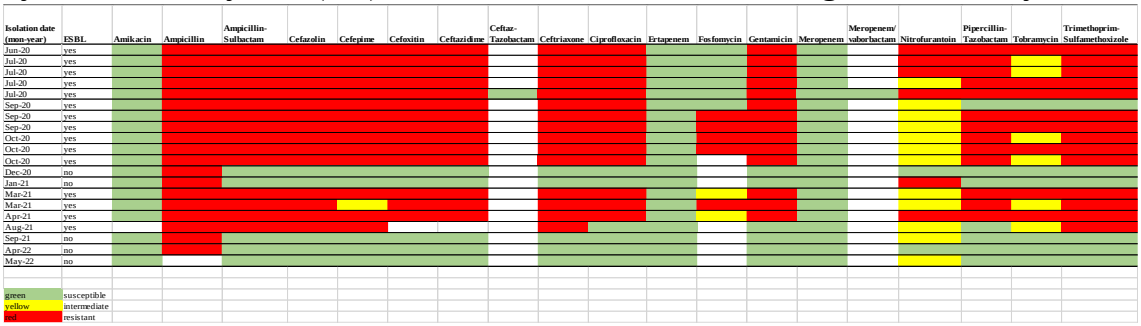


Table S3. Antimicrobial resistance genes identified in the sequenced *Klebsiella* isolate genomes.

Isolate ID	B8142334, B9236220	C2042673	C4023081	C5012365	20210909B1	D7091294
Date of isolation (mon-year)	Sep-20 & Oct-20	Jan-21	Mar-21	Apr-21	Sep-21	Apr-22
Total AMR genes	18	5	21	19	6	3
AMR genes	oqxA_1	oqxA_1	oqxA_1	oqxA_1	oqxA_1	mdf(A)_1
	oqxB_1	oqxB_1	oqxB_1	oqxB_1	oqxB_1	blaSHV-145_1
	blaSHV-106_1	blaSHV-187_1	blaSHV-187_1	blaSHV-187_1	blaSHV-27_1	fosA_3
	fosA6_1	fosA_5	fosA6_1	fosA6_1	fosA6_1	
	mdf(A)_1	mdf(A)_1	mdf(A)_1	mdf(A)_1	mdf(A)_1	
	sul2_2		sul2_2	sul2_2	fosA7_1	
	aph(3")-Ib_5		aph(3")-Ib_5	aph(3")-Ib_5		
	aph(6)-Id_1		aph(6)-Id_1	aph(6)-Id_1		
	blaTEM-1B_1		blaTEM-1B_1	blaTEM-1B_1		
	blaCTX-M-15_1		blaCTX-M-15_1	blaCTX-M-15_1		
	qnrB1_1		qnrB1_1	qnrB1_1		
	tet(A)_6		tet(A)_6	tet(A)_6		
	ant(3")-Ia_1		ant(3")-Ia_1	ant(3")-Ia_1		
	dfrA14_5		dfrA14_5	dfrA14_5		
	aac(3)-IIa_1		aac(3)-IIa_1	aac(3)-IIa_1		
	aac(6')-Ib-cr_1		aac(6')-Ib-cr_1	aac(6')-Ib-cr_1		
	blaOXA-1_1		blaOXA-1_1	blaOXA-1_1		
	catB3_1		catB3_1	catB3_1		
			fosA7_1	fosA7_1		
			aadA1_4			
			aph(3')-Ia_1			

Table S4. Bacterial counts and 16s DNA based identification of colonies representing different morphologies.

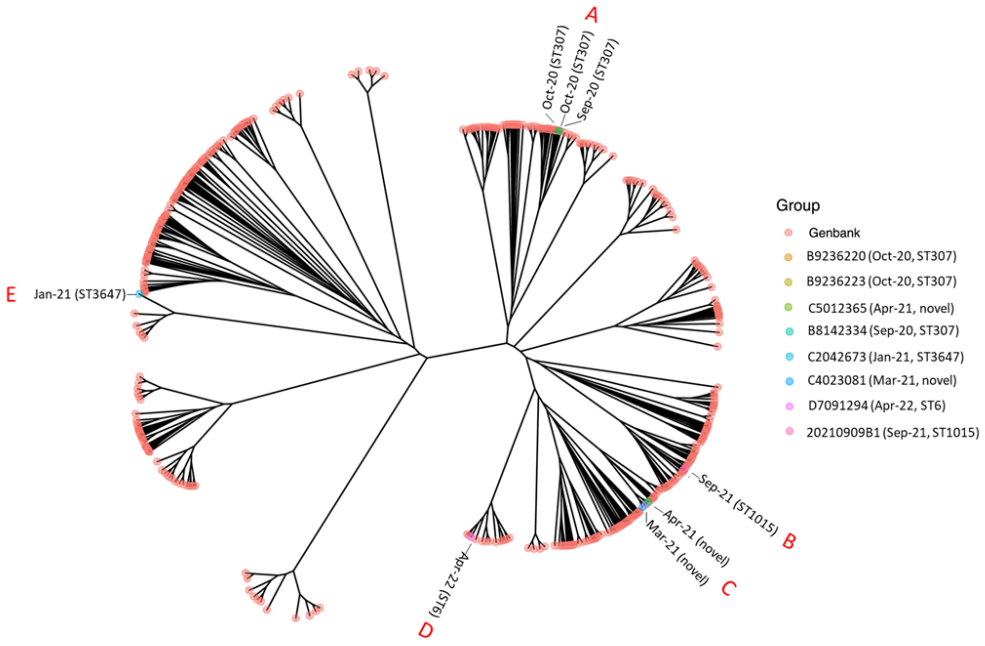
Urine sample timing	Bacterial counts in urine (CFU/mL)	Identification of representative colonies
One week prior to phage therapy)	NA	*Klebsiella pneumoniae*
		Klebsiella pneumoniae
		Klebsiella pneumoniae
		Klebsiella pneumoniae
		Klebsiella pneumoniae
		Klebsiella pneumoniae
Day 1 of phage therapy	1.00E+04	*NA (failed sequencing)*
		Staphylococcus epidermidis
		Staphylococcus epidermidis
Day 8 phage therapy	1.87E+04	*Staphylococcus epidermidis*
		Staphylococcus sp.
		Staphylococcus epidermidis
		Corynebacterium amycolatum
		Corynebacterium sp.
		Corynebacterium amycolatum
Day 15 phage therapy	1.30E+04	*Staphylococcus epidermidis*
		Staphylococcus sp.
		Staphylococcus epidermidis
		Staphylococcus epidermidis
		Staphylococcus epidermidis
		Staphylococcus epidermidis
Day 22 phage therapy	6.00E+04	*Corynebacterium amycolatum*

		Corynebacterium amycolatum
		Corynebacterium amycolatum
		Escherichia coli
Day 29 phage therapy	2.04E+03	*Staphylococcus epidermidis*
		Staphylococcus epidermidis
		Staphylococcus epidermidis
		Corynebacterium amycolatum
		Corynebacterium jeikeium
		Corynebacterium amycolatum
		Enterococcus faecium

Table S5. Microflora composition (represented as percentage breakdown) of the urine samples collected during phage treatment and determined by 16S metagenomic sequencing.

(See additional excel file due to table size).

Figure S1. The phylogenetic tree based on genome-wide comparison of the eight strains from this study in the context of 722 *Klebsiella* isolates.



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