Phages from Ganges River curtail in vitro biofilms and planktonic growth of drug resistant Klebsiella pneumoniae in a zebrafish infection model

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Abstract

Bacteriophages are a promising alternative for curtailing infections caused by multi drug resistant (MDR) bacteria. The objective of the present study is to evaluate phage populations from water bodies to inhibit planktonic and biofilm mode of growth of drug resistant *Klebsiella pneumoniae in vitro* and curtail planktonic growth *in vivo* in a zebrafish model. Phage specific to *K. pneumoniae* (MTCC 432) was isolated from Ganges river (designated as KpG). One-step growth curve, *in vitro* time kill curve study and *in vivo* infection model were performed to evaluate the efficacy of phage against planktonic growth. Crystal violet assay and colony biofilm assay was done to determine the action of phages on biofilms. KpG phages had a greater burst size, better bactericidal potential and enhanced inhibitory effect against biofilms formed at liquid air and solid air interfaces. *In vivo* injection of KpG phages revealed that it did not pose any toxicity to zebrafish as evidenced by liver/brain enzyme profiles and by histopathological analysis. *In vitro* time kill assay showed a 3 log decline and a 6 log decline in *K. pneumoniae* colony counts, when phages were administered individually and in combination with streptomycin, respectively. The muscle tissue of zebrafish, infected with *K. pneumoniae* and treated with KpG phages showed a significant 2 log decline in bacterial counts relative to untreated control. Our study reveals that KpG phages has the potential to curtail plantonic and biofilm mode of growth *in vivo* in higher animal models.

1. Introduction

Klebsiella pneumoniae is a gram negative nosocomial pathogen that can cause wide range of infections like pneumonia, upper respiratory tract infections, wound infections, diarrhea, urinary tract infection, bacteremia and septicemia (Jarvis et al. 1985). It is one of the leading causes of mortality and morbidity in bacterial sepsis (Vading et al. 2018). Roughly, 54% of *Klebsiella pneumoniae* strains that caused neonatal sepsis were observed to be multi drug resistant. As per WHO's antimicrobial resistant pathogens' list, carbapenem resistant *Enterobacteriaceae* falls under critical priority category (2017). Thus, there is an urgent need to devise new antimicrobials/resistance modulatory agents against carbapenem resistant *K. pneumoniae*. Due to evolutionary selection pressures, bacteria would invariably gain resistance to either existing or any novel antimicrobial agents/ even for resistance modulatory agents. In such a scenario, lytic phages would serve as a better choice, since; bacteriophages would serve as self-amplifying antimicrobial agent and development of resistance by bacteria against phages is a relatively manageable event than the development of antibiotic resistance, owing to compromise of antibiotic resistance in some cases and coevolution of phages (German and Misra 2001; Chan et al. 2016; Monferrer and Domingo-Calap 2019; Burmeister et al. 2020).

Phage therapy has been in vogue from 1930 until date as evidenced by works from Eliava Institute of Bacteriophages Microbiology Virology at Tbilisi, Georgia (Summers 2012). Commercial production of bacteriophages specific against *Staphylococcus spp*, *Streptococcus spp*, *Pseudomonas spp*, *Proteus spp*, *Shigella spp* were done till mid 1950's at EIBMV, HIEET, Poland (Sulakvelidze et al. 2001). Discovery of antibiotics, which exerted a broad spectrum of activity against bacteria and concern regarding use of bacterial viruses for therapy, led to decline in widespread use of phages for therapeutic purposes. Phages for human therapy to treat various ailments like skin infections, wound prophylaxis, burn wounds, respiratory infections and sepsis has been in practice for nearly 90 years (Abedon et al. 2011).

Phages specific for *K. pneumoniae* has been reported previously. Caecal filtrate of a healthy woman undergoing colonoscopy was shown to harbor lytic phage specific to *Klebsiella pneumoniae* sub *spp pneumoniae.* The phage belonged to *Siphoviridae* that harbored rosette like tail tip with depolymerase activity against capsular type K2 antigen and based on genome sequence analysis, it was classified under novel Kp36like virus (Hoyles et al. 2015). Cocktail of phages isolated from Ganges river and sewage water against *Escherichia coli*, *K. pneumoniae* and *Enterobacter* spp effectively reduced mixed bacterial load (resistant to carbapenem and colistin) (Manohar et al. 2019). Phage kpssk3 (*Podoviridae*) was isolated from sewage, had a very short latent period of 10 min and a larger burst size of 200 pfu/cell, which was able to lyse 25 clinical isolates of Carbapenem resistant *K. pneumoniae* (Shi et al. 2020). Genome analysis of phage kpssk3 revealed that it did not possess any resistance genes, virulence factors and had depolymerase activity towards exopolysaccharide (Shi et al. 2020). A lytic phage vB_KpnM-The.1 (*Myoviridae*), isolated from urban wastewater, was administered intraperitoneally to a BALB/c mice, immediately after an intranasal inoculation of *K. pneumoniae*, which resulted in a significant 7 log reduction of bacterial bioburden (Sasani and Fereshteh Eftekhar 2020). Another report showed that four lytic phages, belonging to the family *Podoviridae*, infecting *K. pneumoniae* capsular type K22, were isolated from environmental samples. They possessed narrow infectivity against a *K. pneumoniae* clinical isolates with K22 capsular type (Domingo-Calap et al. 2020). In addition, the plaque morphology of the phages showed a halo around the lysis area, implying presence of depolymerase that lyses the capsule (Domingo-Calap et al. 2020).

The objective of the present work is to study the efficiency of *Klebsiella spp* specific phages isolated from Ganges water, in curtailing drug resistant *K. pneumoniae*, both in planktonic and biofilm mode of growth, and also to test efficiency of lytic phages to mitigate *in vivo* infection using zebrafish as a model before proceeding to higher animal models. Towards this goal, in the present study, *K. pneumoniae* specific phage was isolated from Ganges water and its efficiency in curtailing planktonic and biofilm mode of growth in a drug resistant clinical isolate (MTCC 432) was evaluated both *in vitro* and *in vivo* in a zebrafish infection model.

2. Materials And Methods

2.1.Bacterial Strain and other materials:

Klebsiella pneumoniae subspp *pneumoniae* (MTCC 432), an isolate from human urinary tract, was obtained from MTCC, IMTECH Chandigarh. The strain was grown in nutrient broth at 37°C and preserved as glycerol stock at -80°C. Antimicrobial profiling of the strain was performed by microbroth dilution method (Andrews and Andrews 2001). All media used in the study was procured from HiMedia Labs, India. Antibiotics, salts and other chemicals were procured from Sigma-Aldrich, USA or Sisco Reaearch Labs, India.

2.2.Phage Isolation

Bacteriophages specific against *K. pneumoniae* was isolated by inoculating overnight culture of *K. pneumoniae* in the collected Ganges water, supplemented with 10X nutrient broth and incubated for 24 h at 37°C. The enriched water was centrifuged and the supernatant, containing phages, was collected. The supernatant was filtered through 0.45 µm filter (Whatman, GE Healthcare Life Sciences) and stored at 4°C until titer determination.

2.3.Agar Overlay Method

The bacteriophage titer was determined as described previously (Adams 1959). The phage containing lysate was serially diluted in SM buffer (100mM Sodium Chloride, 8mM Magnesium sulphate, 50mM Tris-cl (pH 7.5)) and 100 - 300 µl of each dilution was mixed with 100 µl of overnight culture. This was further mixed with 3 ml of soft agar (0.7% Luria Bertani Agar) and overlaid onto Nutrient agar plates. After incubation, the plates were observed for plaque formation and PFU/ml was determined. Plates showing 1–10 plaques was used to obtain homogenous plaque morphology by triple purification as mentioned earlier (Bonilla et al. 2016). Briefly, a single plaque was picked, resuspended in SM buffer, vortexed and centrifuged. The supernatant was serially diluted and plated as mentioned above for phage isolation. The process was repeated thrice. The resulting phage lysate from triple purification was ultracentrifuged at 4°C for 1 h at 30,000 rpm to obtain concentrated, high-titer phage lysate. The phage lysate was preserved as phage banks for further experiments.

2.4. pH Sensitivity and host specificity of the isolated phage:

The pH stability of the phage and host specificity was evaluated as mentioned previously (Anand et al. 2020). The phages (10^3 PFU/ml) were incubated in buffers with varying pH (3.0, 5.0, 7.0, 9.0 and 11.0) for 1 h. Post incubation, the phage titer was determined by agar overlay method and the percentage of phage survival was calculated.

In order to discern the host specificity of phage, the phage was tested against different clinical isolates of *K. pneumoniae* (BC936, E474, U2016, BC1415, BC1994) and *E. coli* (U3790, U3176, U1007, U3121, U2354) obtained from Sundaram Medical Foundation, Chennai, India. In addition, the phage was also tested against reference strains of *E. coli* (MG1655) *Acenitobacter baumannii, Enterobacter cloacae, Pseudomonas aeruginosa* and *Enterococcus faecalis.* Briefly, 500 µl of fresh bacterial culture was added to soft agar, overlaid on nutrient agar plate and allowed to solidify. 5 µl of the purified phage lysate were spotted on to the plate and incubated at 37°C.

2.5.One Step growth curve

A one-step growth curve of the phageswas performed as reported earlier (Pajunen et al. 2000). Early log phase cells of *K. pneumoniae* was mixed with phages at a multiplicity of infection (MOI) of 1 and was allowed to adsorb for 5-10 min. The cells were then centrifuged and pellet was resuspended in nutrient media. Samples were withdrawn for every 5 min and were plated to determine PFU/ml. The experiment was performed in triplicates, and values depict the mean of three observations ± standard deviation.

2.6.Transmission Electron Microscopy (TEM):

5 µl of high titer (10^{10} PFU/ml) phage suspension was deposited on a carbon-coated copper grid and were allowed to adsorb for 1 min. Phage particles were stained with 2% aqueous solution of uranyl acetate. Grids were examined with a FEI transmission electron microscope (Model JEM 2100F Jeol, Japan).

2.7.Time Kill Assay

Efficiency of the phages to curtail bacterial growth *in-vitro* over a time course of 24 h in broth culture was discerned using the time kill assay (Grillon et al. 2016). *Klebsiella pneumoniae* at a cell density of 10^6 CFU/ml was inoculated into LB broth and *Klebsiella spp* specific monophage was introduced into the culture. At different time points 0, 2, 4, 6 and 24h, samples were withdrawn and cells were plated on LB agar and plate counts were determined 24-48 h post incubation. Phage untreated culture was maintained as growth control. The experiment was performed in triplicates. Decline in bacterial counts relative untreated control would imply lytic potential of phages.

2.8.Crystal Violet Assay

Biofilms at liquid air interface is akin to biofilms on implantable medical devices (Christensen et al. 1985). Biofilms are formed with *K. pneumoniae* on 96-well micro titer plates with and without phages in Brain Heart Infusion (BHI) broth. Appropriate untreated control and broth controls were maintained. 24 h post treatment, biofilms were washed with PBS to remove unbound cells, crystal violet was added and unbound crystal violet was washed off. The bound crystal violet, which is an indirect measure of EPS formed was extracted with acetic acid and was quantified by measuring the absorbance at 595 nm.

2.9.Colony Biofilm Assay

The protocol for forming colony biofilms was essentially as described previously (Merritt et al. 2005). Briefly, 13 mm 0.2 µm membrane filters sterilized by UV radiation placed on sterile BHI agar and inoculated on the center with bacteria or bacteria along with phages. The ability of phages to decrease biofilm formation until 48 h was examined by visual observation.

2.10. Fish toxicity studies:

Zebrafish (*Danio rerio*) measuring ~ 4 to 5 cm in length, weighing approx. 300 mg, was purchased from a local aquarium. The protocols adopted were approved by the Institutional Animal Ethics Committee (CPCSEA-510/SASTRA/IAEC/RPP) of SASTRA Deemed to be University, India. Animal acclimatization was performed following previously established protocols (Phillips and Westerfield 2014). The study comprised of two groups: control and fish injected with phages. For toxicity evaluation, 6 fish were injected intramuscularly with 10^{10} PFU/ml of phages and mortality of the fish was monitored up to 48h. At the end of exposure (48 h), fish was sacrificed (anesthetized by 150mM MS-222 and euthanized by decapitation), liver/brain from two fish of the same group were pooled and homogenized in ice-cold buffer (Tris-HCl, 0.1M, pH 7.4). The homogenate was centrifuged (10,000g, 10 min, 4°C) to obtain supernatant, which was used for all analyses in duplicates. Brain acetyl-choline esterase and liver carboxyl esterase enzyme activities were determined as reported earlier (Christena et al. 2016). Acteylcholine iodide and α/β naphthyl acetate were used as substrates for determining brain and liver enzyme profiles, respectively. In order to evaluate if the phages induce inflammation or other immunological response, histopathological analysis was performed. Briefly, the muscle/liver tissue of the phages-injected fish were sectioned, subjected to Hematoxylin & Eosin staining, histopathology analysis was performed using a standard microscope (Nikon Eclipse Ni-U) in bright field mode and was compared with sham control.

2.11. Zebrafish infection:

Intramuscular infection of zebrafish with *K. pneumoniae* strain was performed as described earlier (Neely et al. 2002). 10ml (~ 1x 10^5 CFU/ml) of *K. pneumoniae* was injected intramuscularly to zebrafish (5/group) using a 3/10-cc U-100 insulin syringe with a 0.5-in.-long, 29-gauge needle. Similarly, sham control fish were injected with equal volume of sterile buffer. 2 h post infection, phages were administered intramuscularly, and both the groups were monitored for 48 h. After 48 h post treatment, fish from different groups were euthanized, injected muscle tissue was dissected, homogenized in sterile PBS. The homogenate was serially diluted and plated onto Luria Bertani agar plates and colony counts was determined after 24-48 h of incubation at 37°C.

2.12. Phage Identification by PCR

Consensus primers for Podoviridae infecting *Klebsiella* species were designed using the PhiSiGns tool (http://phisigns.sourceforge.net) (Dwivedi et al. 2012). To check the consistency of the primers, 60 different genomes of *Podoviridae* infecting *Klebsiella* spp were downloaded. The primers were checked for their ability to bind to the genomes by manually. The primer details are as follows:

Primer set 1 (CL1):

LEFT PRIMER: AAGGAGGGAATTACGGGATG-3' Tm: 60.15 GC%: 50.00
RIGHT PRIMER: AC(A/G)ATGGAGCCATTCTGGTC-3' Tm: 59.93 GC%: 50.00
PRODUCT SIZE: 193
RC degeneracy: GACCAGAATGGCTCCAT(T/C)GT
PCR Amplicon Sequence-1
AAGGAGGGAATTACGGGATGAATACACAAGGTTCAGGCTTATTGGTATTCGGCGAGAACGACGGTCGGTATACTGACCTGACTGCTCGTGGTATCTCAAAGGCGACATG
Reference Genome: >KU183006.1 *Klebsiella* phage vB_KpnP_IME205, complete genome

Primer set 2 (CL2):
LEFT PRIMER: 5'-GGAATCATGCCTGAAATGGT-3' Tm: 59.76 GC%: 45.00
RIGHT PRIMER: 5'-ATTACCCACTTCGGTTGCTG-3' Tm: 59.99 GC%: 50.00
PRODUCT SIZE: 125
PCR Amplicon Sequence
GGAATCATGCCTGAAATGGTGGAGGATGCAGAATGGAGCAAAGAGAAACAGCGGATAATCTTCCGTGTAGGAAGCGCTGCTCTGGGCCGTGCAGTGCATGCCCGACAG
Reference Genome: >NC_023567.2 *Klebsiella* phage F19, complete genome

50 ng of phage DNA isolated from cultured plaques was used as template for PCR. Non-template controls were maintained. The conditions for PCR amplification are as follows: 95°C – 1min, 30 cycles of 95°C – 35s; 54°C – 15s; 72°C – 1 min and final extension at 72°C – 7min. The resultant PCR amplicons was sequenced using Sanger sequencer and its identity specifically to viral family *Podoviridiae* was confirmed by BLAST analysis.

3. Results:

Ganges river water was sampled at two different locations Haridwar, and Rishikesh. Attempts to isolate phages from these water sources by agar overlay method showed that only Ganges water sampled from Rishikesh displayed plaques of uniform morphology against MTCC 432 and was designated as KpG (Fig. 1). Although other sample from Haridwar displayed a lysis zone, it did not display distinct plaque morphology that could be further purified. Lack of phages from different water sources, for a ubiquitous strain like *Klebsiella pneumonia*, was rather uncommon. Hence, we checked whether the strain used in the present study produced capsule. Staining revealed that MTCC strain indeed produced capsule (**Fig. S1**). Morphology of plaques from Ganges water showed a halo around the clear zone, which might indicate depolymerase activity of KpG that could target the capsule of the strain (Fig. 1) (Domingo-Calap et al. 2020). The phage displayed stability at the following pH 5.0, 7.0, 9.0, 11.0 with a survival rate of 86%, 100%, 91% and 77% respectively (Fig. 2). Interestingly, we observed that plaques from pH 11 group did not display halo around the lysis area, indicating the inability of these phages to produce depolymerase at pH 11.0. In order to check if KpG is specific to capsulated *K. pneumoniae*, KpG was spotted on other bacterial species – *E. coli, Acenitobacter baumannii, Pseudomonas aeruginosa, Enterococcus faecalis, Enterobacter cloacae* and uncapsulated *K. pneumoniae* strains. We did not observe any lysis in these different bacteria (data not shown), implying host tropism (specificity) of phages to its capsulated host strain (MTCC 432).

KpG was purified by triple plaque purification method and its PFU/ml were determined. One-step growth curve of KpG revealed **(Fig. S2)** a larger burst size of 224 PFU/cell and a latent period of 20 min. TEM images of the KpG suggested it to be belonging to *Podoviridae* (Fig. 3). We designed two PCRs for identification of *Podoviridae* by analyzing conserved regions of their genomes. We obtained 193 base and 125 base amplicons for PCR1 and 2 respectively (Fig. S3). The PCR amplicons were sequenced by sanger sequencing and was subjected to BLAST analysis against nucleotide database of NCBI and results from BLAST analysis revealed that both amplicons exhibited a high degree of similarity (97–98% similarity with e score $1.55e^{-59}$ and $5.88e^{-29}$ with CL1 and CL2 amplicons, respectively) with genome sequences of *Klebsiella phages* belonging to *Podoviridae.* This confirms that the KpG isolate belongs to *Podoviridae*.

Time kill studies performed with KpG phages at an MOI of 1, resulted in 0.5 log decline in the initial hours but, significant regrowth was obtained at 24 h (data not shown). Hence, time kill curve was performed with a MOI of 100, at which KpG phages caused an initial 3–4 log decline in bacterial cell counts by 4 h **(**Fig. 4**)**. However, by 24 h regrowth in bacterial cell counts were still observed, which might be due to differential expression of capsule, to avoid being infected by phages. A similar phenomenon was reported earlier (Holst Sørensen et al. 2012).

In order to overcome this, we tested the phages in combination with different antibiotics (Streptomycin, meropenem, colistin, erythromycin, ciprofloxacin and tobramycin) against *K. pneumoniae*. We found that the combination of streptomycin and phages were effective in inhibiting the growth of *K. pneumoniae*, in addition, phages reduced the MIC of streptomycin by 8-fold (from 64 µg/ml to 8 µg/ml). We further evaluated the combination *in vitro* in a time kill assay. As expected, the combination of streptomycin and KpG caused a 7-log decline relative to the untreated control within 6 h and by 24 h despite a slight regrowth, a decline of 6 log relative to the other groups was maintained **(**Fig. 4**)**. Streptomycin treatment alone did not show significant reduction in colony counts at 24 h.

Ability of phages to inhibit biofilm formed at liquid air interface was discerned by crystal violet assay, which showed that KpG phages caused a significant 5-fold decrease in biofilm formation at 10^{-8} dilution relative to biofilm formed by untreated bacteria (Fig. 5a). *K. pneumoniae* can also cause wound infections in immune compromised persons, wherein it typically forms biofilms at solid air interface, in order to mimic biofilms at solid air interface, colony biofilms of *K. pneumoniae* were formed on agar surface and ability of phages to inhibit colony biofilms were evaluated. The results revealed that treatment with KpG phages caused a substantial decline in colony biofilm formation at 24 and 48 h relative to the untreated control (Fig. 5b). Thus, both in time kill assay and in biofilm inhibition assay KpG showed better bactericidal and potential and biofilm inhibitory activity against *K. pneumoniae*.

Klebsiella pneumoniae were allowed to form biofilms on glass slides and were treated immediately with KpG phages and 24 h post treatment, the unbound cells were washed and slide was stained with live/dead imaging kit as per manufacturer’s protocol and was imaged using Nikon Fluorescent microscope (Nikon Eclipse Ni-U). Live/Dead imaging showed that treatment with KpG caused substantial proportion of dead cells as evidenced by yellow cells in merged image indicative of dead cells and only a small sub population were alive indicating lytic potential of phages (**Fig. S4**).

KpG phages were injected into zebrafish and toxicity, if any, due to phages were evaluated by assessing liver (alpha and beta naphthol) and brain (acetyl choline esterase) enzyme profiles of zebrafish. It did not cause any significant variation in liver enzyme profiles of zebrafish. But, discernible increase in brain acetyl choline esterase profiles were observed, which was not statistically significant ($P = 0.0529$) (**Fig. S5**). Histopathological analysis of hematoxylin and eosin stained muscle and liver tissue of KpG injected phages relative to untreated phages revealed that both muscle and liver tissue appeared similar to untreated control (**Fig. S6**) implying that phages did not induce either morphological/biochemical aberrations when injected *in vivo* in zebrafish.

Finally, to discern *in vivo* efficacy of phages, 10 µl of bacteria MTCC strain of *K. pneumoniae* at 0.1 OD (Corresponding to ~ 1×10^{6} CFU/ml) were injected into muscle tissue of zebrafish. 2 h post infection, KpG pahges (10^{8} PFU/ml) were administered intramuscularly and the ability of phages to curtail bacterial growth in infected muscle tissue relative to untreated control was evaluated. Our results showed that similar to *in vitro* trend, KpG phages caused a significant 2-log decline ($P = 0.0106$) in bacterial cell counts (Fig. 6). This reiterates the fact that lytic phages from Ganges were not only effective *in vitro* against planktonic and biofilm mode of growth, it was also effective *in vivo* in curtailing bacterial growth in infected muscle tissue of zebrafish and hence has the potential to mitigate *in vivo* infection by *Klebsiella pneumoniae* in higher animal models.

4. Discussion

Carbapenem resistant *Klebsiella pneumoniae* (CRKP) pose grave threat to public health especially in immune compromised patients and in neonates where mortality rate is very high (Investigators of the Delhi Neonatal Infection Study (DeNIS) collaboration 2016). CRKP classified as a critical priority pathogen by WHO, which severely limits therapeutic options available (Tumbarello et al. 2012). In this scenario, newer antibiotics are not the way out since bacteria will easily gain resistance to new antimicrobial agents due to evolutionary selection pressures. Although resistance modulators like betalactamase inhibitors, efflux pump inhibitors, quorum sensing inhibitors, cationic peptides are seen as a viable alternative, they were not as effective against MDR pathogens as expected. These bugs could circumvent these agents, for example, by producing betalactamases with metal co-factors that could not be inhibited by betalactamase inhibitors or by expressing redundant efflux transport proteins to extrude the antibiotics etc. Hence, to tackle AMR menace, biological control agents like lytic phages are considered as better alternatives (Aleshkin et al. 2016). The advantage of phage therapy is that it is highly specific and targeted hence it does not disturb commensal microbiota or lead to dysbiosis, given importance of commensal microbes in our health and well-being (Blander et al. 2017; Novince et al. 2017), whereas antibiotics could potentially harm commensal microbes resulting in dysbiosis.

Rivers like Ganges harbor a wide diversity of phages, which affords a rich source of targeted biological control agents against rising menace of drug resistant pathogens. The reason for thriving diversity of phages in Ganges river is the Himalayan permafrost (source of Ganges river) was previously a sea bed, when it melted, it released abiotic phages which were trapped long time ago contributing to unique diversity of bacteriophages (Khairnar 2016). This rich diversity of phages could be used as a viable source to curtail MDR pathogens in both planktonic and biofilm mode of growth.

In our study, the KpG from Ganges water was specific only for one clinical isolate obtained from urinary tract (MTCC 432 strain) and not towards other clinical isolates of *K. pneumoniae* or *E. coli* or other bacterial species like *Pseudomonas aeruginosa, Enterobacter cloacae* and *Acenitobacter baumannii*. Interestingly, MTCC strain produced capsule (Fig. S1), hence it is likely that KpG phages possess capsular depolymerases(Oliveira et al. 2019). pH stability assessment showed that KpG phages were active from pH 5.0 to pH 11.0. Depolymerase activity as indicated by halo around the plaques was observed till pH 9.0. Studies aimed at discerning the stability of the KpG depolymerase would be explored in future. Tropism of phages is a very well-established phenomenon. In an earlier study, a novel PhiKMV like virus infected only *K. pneumoniae* strains with K1 capsule but not the other capsular types. Capsule deleted K1 mutant strains could not be infected by this phage implying that capsule is essential for infection (Lin et al. 2014). On the other hand, broadly specific multi-host bacteriophage ΦK64-1 produced nine different capsular depolymerases, which enabled this phage to infect 10 different *K. pneumoniae* possessing distinct capsules. In this strain, capsule specific depolymerases were shown to be essential for infectivity, since mutants of these depolymerases failed to infect the corresponding strains (Pan et al. 2017). Capsular tropic phages are known in *Klebsiella spp*. Interestingly, phage FC3-1 trophic to core polysaccharide and O antigen of LPS of *K. pneumoniae* was reported in an earlier study (Tomás and Jofre 1985).

KpG phages had a larger burst size 224 PFU/cell with a short latent period of 25 min. A range of burst size has been reported with *Klebsiella* specific phages for example KPO1K2 displayed a burst size of 140 PFU/ infected cell (Verma et al. 2009). Another study had shown that Phage Z belonging to family *Siphoviridae* gave a burst size of 320 PFU/infected cell. Variety of phages from seawater specific for single strain of *Vibrio spp* displayed heterogeneous burst size (23–331 PFU/ infected cell) as reported in an earlier study (Yu et al. 2013).

TEM imaging revealed that the Ganges phages belong to family *Podoviridae*. Most of the reported phages fall under family *Siphoviridae, Podoviridae* or *Myoviridae* (Kęsik-Szeloch et al. 2013). KpG caused a 5 fold decrease in biofilm formation at liquid air interface and also caused a significant reduction of colony biofilms at solid air interface, which are akin to respiratory biofilms (Fig. 5a and 5b). Significant biofilm inhibitory effect despite having only a moderate bactericidal effect *in vitro* time kill assay (Fig. 4), coupled with the fact that MTCC strain produces capsule, imply that KpG phages might possess depolymerases, that in turn might be effective against extracellular polymeric substances of biofilms, which remains to be explored in further studies. On the other hand, live/dead imaging revealed that KpG phages caused significant lysis, although by Crystal violet assay and colony biofilm assay KpG appeared relatively better, comparable efficiency by live/dead imaging imply that timing of addition of phages is important. Concomitant addition of both bacteria and phages to a localized solid surface causes lysis, whereas if bacteria were initially allowed to attach and initiate biofilm formation, subsequent addition of KpG phages after 1–2 h of bacterial interaction with glass surface, may not cause significant bacterial lysis. Previous report has also shown that intranasal administration of phages 3 h prior infection or immediately after infection rescued mice from respiratory infection caused by *K. pneumoniae* strain whereas even 6 h delay in phage administration failed to rescue the mice highlighting importance of timing of phage administration (Chhibber et al. 2008). In an elegant study, to tackle mixed species biofilm formed by *K. pneumoniae* and *Pseudomonas aeruginosa*, wherein, *Pseudomonas spp* forms the bottom layer of the biofilm and is shielded by *K. pneumoniae. T*he authors have used a combination of phages especially one that produces *K. pneumoniae* depolymerases, which disrupts the top layer and allows *Pseudomonas aeruginosa* specific phage to access inner biofilm layer. *Klebsiella spp* specific phages lacking depolymerase activity were unable to provide access for *Pseudomonas spp* trophic phages. Lytic activity of phages was further enhanced in the presence of xylitol (Chhibber et al. 2015).

Among *in vivo* models, mice is commonly used to evaluate efficacy of bacteriophages in mitigating various infections ranging from burn wound, respiratory infections, sepsis and UTI (Capparelli et al. 2007; Malik and Chhibber 2009; Verma et al. 2009; Cao et al. 2015; Basu et al. 2015). To the best of our knowledge, zebrafish has not been evaluated as an animal model to evaluate bacteriophage therapy in infected fish. The advantages of zebrafish model is ease of availability and maintenance, optical clarity of embryo/larvae, short duration for study, a high degree of genetic homology with humans (Lieschke and Currie 2007). We have shown that injection of *Klebsiella spp* specific phages into adult zebrafish did not pose any toxicity as evidenced by liver and brain enzyme profiles and by histopathological analysis (Fig. S5 and Fig. S6). Earlier studies in mice have similarly shown lack of toxicity due to administration of phages and in addition, bioavailability of phages in various tissues were evident within 6 h post injection and half life of phages in mice was roughly 18 h (Verma et al. 2009). In our study, we observed a significant 2 log decline in bacterial cell counts in infected muscle tissue relative to untreated control due to lytic activity of KpG phages. Previous study showed that, in a mice full thickness wound model, when efficacy of phage therapy was compared with combination of gentamycin and silver nitrate to tackle infection by *K. pneumoniae*, it was observed that a single dose of phage as a topical application mitigated colonization of *K. pneumoniae*, whereas even multiple applications of silver nitrate and gentamycin failed to afford such protection (Kumari et al. 2011). In another study, same group showed that induced burn wounds in mice infected with MDR *K. pneumoniae* strain was successfully mitigated, with a significant decrease in bacterial load in blood and peritoneal lavage, when phages were administered either by subcutaneous or intraperitoneal route (Malik and Chhibber 2009). Significant biofilm inhibitory effect coupled with good *in vivo* effect in restricting bacterial growth in infected muscle tissue show that KpG phages curtail bacterial biofilms *in vitro* and restrict planktonic growth of *Klebsiella spp in vivo* in zebrafish. Lack of toxicity coupled with ease of performing the experiment indicates zebrafish can indeed serve as an initial *in vivo* model before evaluating efficiency of phages in mitigating bacterial infections in higher animal models.

Abbreviations

CFU/ml
colony forming units/ml
PFU/ml
Plaque forming units/ml
MOI
Multiplicity of infection
TEM
Transmission Electron Microscopy
PBS
Phosphate Buffered Saline
BHI
Brain Heart Infusion media

MIC
Minimum Inhibitory Concentration
MDR
Multidrug Resistant
CRKP
Carbapenem Resistant *Klebsiella pneumoniae*

Declarations

Ethical Approval:

All applicable international, national, and/or institutional guidelines for the

Consent to participate

Not applicable

Consent for publication:

Not applicable

Availability of data and material:

Almost all data generated or analyzed during this study are included in this published article (and its Supplementary Information files). The raw data would be available upon request.

Competing interests:

The authors declare that they have no competing interests.

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Authors Contributions:

NSS performed almost all the experiments and wrote the manuscript. ST carried out imaging studies. MMB participated in design of conserved primers, PCR amplification and sequence analysis.

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Figures

Figure 1

Plaque morphology of the Klebsiella pneumoniae specific phages isolated from Ganges river (KpG) The Ganges water from Rishikesh was enriched with the host and plated by agar overlay method. KpG shows depolymerase activity, which is evident from the halo around the plaques.

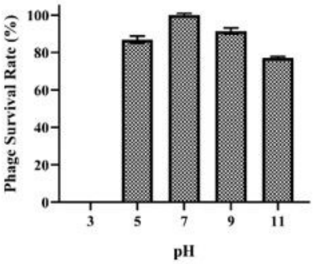

Figure 2

Stability of KpG at different pH. 103 PFU/ml of purified phage lysate was incubated in buffers of pH 3.0,5.0,7.0,9.0 and 11.0 for 1 h. Phage titer was estimated using agar overlay method and survival percentage was calculated.

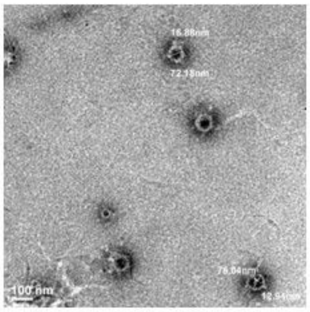

Figure 3

TEM analysis showed that KpG phages belonged to the family Podoviridae. Phage lysate of high titer was stained with 2% uranyl acetate and visualized under FEI transmission electron microscope (Model JEM 2100F Jeol, Japan). Image presented is representative of multiple images.

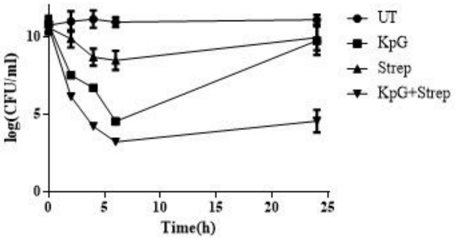

Figure 4

KpG phages + Streptomycin caused discernible reduction in colony counts in a time kill assay. 106 CFU/ml of K. pneumoniae was inoculated along with 108 PFU/ml and Streptomycin (individually and in combination). Samples were withdrawn at 0, 2, 4, 6 and 24 h serially diluted an plated on to LA plates to determine colony counts. Experiments were performed in triplicates and error bar represent standard error of the mean.

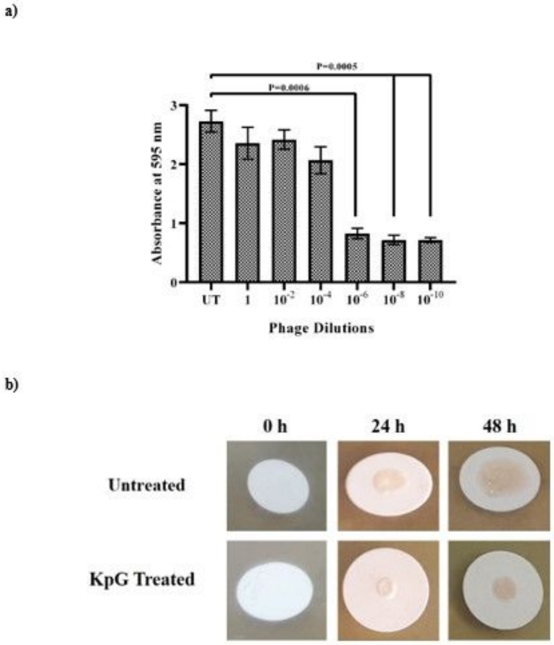

Figure 5

KpG phages efficiently inhibited biofilm formation of K. pneumoniae at liquid-air and at solid-air interface. a) Biofilms were formed in micro titer plates with or without KpG and washed with PBS 24 h post treatment. Crystal Violet was added and after 15 min, the unbound crystal violet was removed and the stain was extracted by acetic acid. The absorbance was measured at 595nm, which would be directly proportional to the biofilm formed. b) Membrane filters were placed on BHI agar and inoculated with bacteria with or without KpG. The ability of phages to decrease biofilm formation can be examined visually till 48 h. Images are representative of three independent experiments.

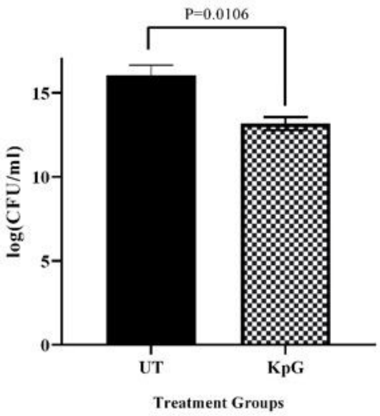

Figure 6

KpG phages was able to reduce bacterial bioburden in infected fish. 10μl of K. pneumoniae were injected intramuscularly in zebra fish and 2 h post infection, KpG was administered intramuscularly. 24 h post infection, the muscle was dissected, serially diluted and plated on to LA plates. CFU/ml was calculated. Experiment was performed in triplicates and error bar represent the standard error of the mean.

Supplementary Files

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- OSPSSupplementaryinformationPhages.pdf