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EVALUATION OF THE EFFICACY OF A PHAGE COCKTAIL AGAINST GENTAMICIN-RESISTANT *Klebsiella pneumoniae*

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Abstract: The bacteria *Klebsiella pneumoniae* is one of top aetiological agents associated with nosocomial infection, and it has gained its notoriety with the emergence of multidrug resistance strains. In this study, we evaluated the effect of lytic bacteriophage cocktail isolated from our local sewage as potential antimicrobial candidate against Gentamicin-resistant *Klebsiella pneumoniae*. A total of five clinical-acquired *K. pneumoniae* isolates including a carbapenem-resistant *K. pneumoniae* (CRKP) strain showed resistance towards gentamicin (GN). Phages were isolated using double-layer agar method against clinical- and community-acquired *K. pneumoniae* as host strains. Phage characterization using PCR partial sequencing of different viral genes; Lysin, Major Capsid Protein (g23) and Tail Fiber Protein has suggested that these phages possibly belonged to Myoviridae (ϕKPaV04, ϕKPaV08, ϕKPaV12) and Podoviridae (ϕKPaV03, ϕKPaV10). The characterized phages was selected for cocktail have exhibited high titer and broad host range with 22-44% lysis towards a panel of 18 *K. pneumoniae* strains. The antimicrobial efficacy of a single phage cocktail administration showed 80% growth suppression of GN-resistant *K. pneumoniae* after 18 h of incubation. Suggesting the possibility of phage cocktail to be used against nosocomial infections by multidrug resistant bacteria including being an alternative to antibiotic GN in the treatment of CRKP infections.

Keywords: Gentamicin-resistant, bacteriophage, *Klebsiella pneumoniae*.

Introduction

Nosocomial infections, also known as hospital-acquired infections refer to any systemic or localized conditions in patients that result from the reaction caused by an infectious agent or toxin contracted within a hospital environment (Kouchak & Askarian, 2012). The most common type of infections includes bloodstream infections (BSI), catheter-associated urinary tract infections (UTI), surgical site infections (SSI) and ventilator-associated pneumonia (VAP) (Khan *et al*., 2017). Pathogens referred to as the 'ESKAPE' bugs with growing multidrug resistance and virulence are responsible for the majority of nosocomial infections. These ESKAPE pathogens consist of *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. (Rice, 2008). Antimicrobial resistance in these pathogens causing high risk of mortality and morbidity are among the 12 bacteria listed by the World Health Organization (WHO) to urgently directed the research and development of new antibiotics (Tacconelli *et al*., 2018).

Klebsiella pneumoniae is a gram-negative encapsulated bacillus that belong to the Enterobacteriaceae family. *K. pneumoniae* is commonly found in the human gastrointestinal tract but instead, is frequently linked to lower respiratory tract infection and catheter-associated urinary tract infection (Navidinia, 2008). The emergence of *K. pneumoniae* exhibiting multidrug resistance phenotypes has made the nosocomial infections difficult to treat. K. *pneumoniae* is progressively resistant towards penicillin and ampicillin caused by genes classified as ESBL (extended-spectrum

beta lactamases). Often bacteria encoding for ESBL genes are also resistant towards wide-spectrum antibiotics such as cephalosporin and ceftazidime (Navidinia, 2008). Additionally, the emergence of carbapenem-resistant *K. pneumoniae* in recent years has affected many countries around the world and that it has now been regarded as a worldwide issue (Gaiarsa *et al*., 2015). The expression of enzyme carbapenemases by *K. pneumoniae,* renders bacteria resistant to almost all available β-lactams, including the carbapenems (Paczosa & Mecsas, 2016).

The first report of carbapenem-resistant *K. pneumoniae* (CRKP) in Malaysia was an imipenem resistant strain isolated from the blood culture of a 42-year old woman in 2004 (Palasubramaniam *et al*., 2007). The patient has undergone empirical treatment with intravenous imipenem, vancomycin and methylprednisolone, replaced by amikacin as a last resort. Unfortunately, the patient's health continued to deteriorate, and she died within 29 days of admission. Another case reported by Subramaniam & Muniandy (2005) on a nosocomial outbreak in the pediatric oncology unit of the University of Malaya Medical Centre (UMMC), Kuala Lumpur associated with ESBL SHV-5 *K. pneumoniae*. The strains were found to be resistant to ceftazidime, cefotaxime, ceftriaxone and aztreonam but susceptible to imipenem.

Aminoglycosides including gentamicin (GN) are active against ≥50% of CR *K. pneumoniae* isolates in vitro (Hirsch & Tam, 2010) and exhibit rapid bactericidal activity against susceptible strains during time-kill assays (Clancy *et al*., 2014). Similarly, combination therapy with β-lactams and aminoglycosides is well accepted for the treatment of bacteremia caused by ESBL-producing *Klebsiella pneumoniae* isolates (Paterson *et al*., 2004). However, Clancy and colleagues, (2014) have demonstrated that combination treatment with GN were ineffective against GN-resistant carbapenemases-producing *K. pneumoniae* strains. The overall number of multidrug-resistant *K. pneumoniae* is rising with declining active antibiotics. Therefore, it is imperative to find alternative ways to treat infections especially those caused by multidrug-resistance strains.

Bacteriophages also known as phages can be an ideal option to solve the emerging threat of multidrug-resistance pathogens such as *K. pneumoniae*. Phages are order of viruses that are able to infect bacteria, were independently discovered by British microbiologist Frederick Twort in 1915 and French-Canadian microbiologist Felix d'Hérelle in 1917 (Carlton, 1999). At the time of discovery, phages were regarded as a potential treatment for bacterial infections and have been developed to control bacterial diseases such as dysentery, cholera, and gangrene (Dublanchet & Bourne, 2007). Phages primarily use one of its two main lifecycles, either infecting the host cell resulting in propagative lysis (lytic cycle) or lysogenization (lysogenic cycle). However, lytic phages are preferable and suitable as candidates for phage therapy, because lytic phages subvert the host's biological function and utilize the host machinery for reproduction resulting in the rapid killing of their target host cell which leads to a great increase in phage production to which transduction is relatively rare. Self-replicating effect of lytic phages is a definite advantage, as it implies that phages are self-sustainable whenever bacteria are present (Mirzaei, 2016).

Recent reports highlighted the successful treatment of *Klebsiella pneumoniae* B5055–induced burn wound infection in BALB/c mice with a single dose of high titre *Klebsiella*-specific phage Kpn5 incorporated in a 3% hydrogel (Kumari *et al*., 2011). In another set of experiment, Chadha and colleagues (2016) evaluated the therapeutic efficacy of five mono-phages namely Kpn1, Kpn2, Kpn3, Kpn4 and Kpn5 in comparison to phage cocktail in resolving the course of *K. pneumoniae* B5055 induced-burn wound infection in mice. Although it was observed that mice receiving mono-phage therapy exhibited efficacy in resolving the course of infection, phage cocktail however,

managed to show maximum protection against burn wound infection caused by *K. pneumoniae* B5055.

The use of phages offers several benefits compared to conventional antibiotic treatment; firstly, phages have host cell specificity without disrupting the normal bacterial flora, which is able to reduce the risk of secondary infections often associated with antibiotic treatment (Nilsson, 2014). Secondly, phages are easily obtained from the environment, giving it a major lead in cases of sudden bacterial disease outbreaks. The convenience, easy availability and low cost to produce phage cocktails make it preferable over searching for new antimicrobial agents (Mohamed *et al*., 2018).

Therefore, the present study was conducted to isolate and characterize lytic phages from domestic sewage treatment plant using *K. pneumoniae* as host system and to evaluate the effect of lytic phage cocktail against GN-resistant *K. pneumoniae*.

Materials and Methods

Sample Collection

Clinical Strains of Klebsiella pneumoniae

A total of fourteen *Klebsiella pneumoniae* isolates were obtained from Borneo Medical Centre (BMC), Sarawak including three ESBL-producing strains and a CRKP strain. Antibiotic sensitivity test (AST) for *K. pneumoniae* clinical strains was performed according to BMC lab protocol.

Non-clinical Strains of Klebsiella pneumoniae

Additionally, four community-acquired *Klebsiella pneumoniae* were obtained from swab samples of students of Universiti Malaysia Sarawak (UNIMAS). *K. pneumoniae* ATCC 35657 and a non-*Klebsiella pneumoniae* strain, *K. aerogenes* were used as control strains throughout this study.

Ethical Consent

Both samples collected were approved by the UNIMAS Medical Ethics Committee with the reference numbers of UNIMAS/NC-21.02/03-02 Jld.2 (79) and UNIMAS/NC-21.02/03-02 (79), respectively.

Klebsiella pneumoniae Verification

Partial 16S rRNA gene sequences *of Klebsiella pneumoniae* isolates were extracted using the Wizard® Genomic DNA Purification Kit (Promega) and amplified by method described by Lean *et al*., (2014) using 27F forward primer (5'-AGAGTTTGATCMTGGCTCAG-3') and 152R reverse primer (5'-AGGA GGTGWTCCARCC-3'). All isolates were grown at 37 ^{o}C in Luria-Bertani (LB) media [Tryptone (10 g/L), yeast extract (5 g/L) and sodium chloride (10 g/L)]. The sequenced data of *K. pneumoniae* isolates were subjected to NCBI BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi.) and deposited in GenBank with the accession numbers tabulated in Table 1.

Antibiotic Susceptibility Testing (AST)

Gentamicin susceptibility of *K. pneumoniae* isolates was done on Mueller-Hinton agar plates (Oxoid, UK) in the presence of gentamicin disc (10 µg/disc) according to the standard of Kirby-Bauer disc diffusion method (Hudzicki, 2016). The diameter of the inhibition zone was measured according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.

Enrichment and Isolation of Bacteriophages

The enrichment method of Li & Zhang, (2014) was adapted for the isolation of *K. pneumoniae* phages with some modifications. Briefly, 20 ml of sewage sample collected from a residential college of University Malaysia Sarawak (Malaysia) was cleared of debris by centrifugation at 4,000 x g for 10 min at 4 ^{o}C and filtered through 0.45 µm membrane filter (Sartorius, Germany). Phages were amplified by culturing 1 ml of *K. pneumoniae* ATCC 35657 overnight culture with 30 ml of 5X LB fresh liquid medium and 20 ml of filtered sewage sample. The mixture was then incubated for 24 h at 37 ^{o}C with shaking at 55 rpm. After incubation, the mixture was centrifuged at 4000

x g for 15 mins, and the supernatant was filtered through a sterile 0.22 µm membrane filter (Sartorius, Germany).

Phage isolation was achieved by double-layer plaque assay using LB agar, as per methods described by Kropinski *et al*., (2009). The plates were incubated at 37 °C and presence of plaques was examined after 24 h. Single-plaque isolation was repeated three times to obtain a pure phage stock. The phage stocks were stored in SM buffer [NaCl (5.8 g/L), MgSO4 (1.2 g/L) and 50 mL 1M Tris-HCl (v/v)] at 4 °C with 1% (v/v) chloroform. Similarly, the titer of the phage stock lysate was accomplished by double-layer plaque assay and calculated using the following formula:

$$\frac{\text{PFU}}{\text{mL}} = \frac{\text{Number of plaques formed}}{\text{dilution x volume of diluted virus added}} \quad (1)$$

Host Range of Bacteriophages

The host range of the phage lysate was done by spot testing against all *K. pneumoniae* isolates including control strains *K. pneumoniae* ATCC 35657, *K. aerogenes* and *K. oxytoca*. All bacterial isolates were cultured on LB agar plate and was left to dry under room temperature for 15 minutes. After drying, 10 µl of high titre phage lysate (10^9 pfu/ml) was spotted on the surface of the plate and the plates were incubated at 37 ºC for 24 h. As a negative control, each bacterial strain was mock infected with sterile SM buffer. These plates were then examined for clear zone of bacterial lawn indicating the presence of phage lytic activity. All spot tests were repeated in duplicate.

Extraction of Bacteriophage DNA

Phage DNA extraction and purification methods were carried out using the Roche High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Germany) according to the manufacturer's protocol but the extracted DNA was eluted in 30µl of elution buffer.

PCR and Partial Sequencing of Bacteriophage Genomes

For confirmatory identification of the phages, the phage lysin gene (Mishra *et al*., 2014), and genes encoding for structural proteins such as major capsid protein gene (g23) (Filee *et al*., 2005) and tail fibre protein gene (JK primer set) (Lee, 2009) were amplified by PCR using specific primers as a potential marker for a specific viral family.

All PCR reactions were done in 30 µl reaction volumes containing 5 µl of 5x Green GoTaq® Flexi Buffer (Promega, WI, U.S.A.), 2 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTP mix (Promega, WI, U.S.A.), 1 µl of each 20 ρmol primer, 3µl GoTaq® DNA Polymerase (Promega, WI, U.S.A.) and 5 µl of DNA template. The sequenced genes were analysed using the NCBI BLASTn® search program https://blast.ncbi.nlm.nih.gov/Blast.cgi.

Evaluation of Phage Cocktail Infecting Gentamicin-resistant K. pneumoniae

Phage cocktail protocol was adapted from Gu *et al.,* (2012) to which the phage cocktail comprised an equal amount of each selected phage. The phage with broad host range, high titre and reproducibility were selected and combined based on the volume of one to one ratio into a cocktail and tested for their ability to lyse bacteria cultures of Gentamicin-resistant *K. pneumoniae*. The Gentamicin-resistant *K. pneumoniae* strains were grown to an early log-phase (OD_{600} of 0.1) in 250 mL flasks of LB liquid medium. The flasks were then inoculated with single administration of phage cocktail (volume 1:10) and incubated at 37 °C with shaking. The bacterial growth at OD_{600} was then analysed by using AgileSpec™ UV/Vis spectrophotometer (Thomas Scientific) at 0 h, 1 h, 2 h, 3 h and >18 h to determine the viability of the phages over time.

Results and Discussion

A need for other alternatives in eradicating the rise of multidrug-resistant pathogens has been

felt in the recent years as the development of new antibiotics decline. Our findings suggest that phages can potentially be used as a therapeutic agent against multidrug-resistant pathogens, in this case against *Klebsiella pneumoniae*. Since mono-phage treatment is more personalized and time consuming, therefore it gives a new perspective towards phage cocktail in treating *K. pneumoniae* infections. Results of previous studies have shown the successes of using phage cocktails against several bacterial infections such as MDR *A. baumannii* (Schooley *et al*., 2017), *Vibrio cholerae* (Yen *et al.,* 2017), *E. coli* O157:H7 (Mohamed *et al*., 2018; Ramirez *et al*., 2018), including *K. pneumoniae* infections (Chadha *et al*., 2016; Gu *et al*., 2012).

Sequencing of Klebsiella pneumoniae 16S rRNA gene

Rapid verification of *K. pneumoniae* was successfully determined by the amplification of 16S rRNA gene using a set of universal primers, 27F and 152R. A total of 17 bacterial strains exhibit 95-99% similar identities with the *K. pneumoniae* reference database in NCBI of which 14 were clinical isolates and the rest were community-acquired isolates. The amplification of 16S rRNA genes have also managed to amplify other *Klebsiella* spp. from the community-acquired isolates and were used as control strains throughout this study (Table 1).

Gentamicin-resistant Klebsiella pneumoniae identification

Klebsiella pneumoniae has become one of the most problematic pathogens due to the presence of thick polysaccharide capsule that acts as an antiphagocytic factor. Gentamicin is a clinically relevant antibiotic treatment against *K. pneumoniae*, and therefore, it was selected as a model antibiotic for this study (Gonzalez-Padilla *et al*., 2015; Rammaert *et al*., 2012; Shields *et al*., 2016). Based on the antibiotic susceptibility results, only five clinical isolates of *K. pneumoniae* specifically, CRKP-1, ESBL-KP2, ESBL-KP3, KP5 and KP9 have exhibited resistance towards Gentamicin and none of the community-acquired isolates *K. pneumoniae* were resistant (Table 1). Therefore, these five Gentamicin-resistant clinical isolates were subjected to further analysis.

Isolation of Bacteriophages

The large reservoir of pathogenic bacteria existing in sewage water due to fecal contamination makes it a relevant source for the isolation of various bacteriophages as phages commonly feed on the locally available organisms and cells (Sundar *et al*., 2009). For phage treatment against *K. pneumoniae*, lytic phages are preferred as they have notable antibacterial activity comparable to antibiotics (Sulakvelidze *et al*., 2001). About 33% (n=5/15) of the isolated phages were selected for further characterization, namely, ϕKPaV03, ϕKPaV04, ϕKPaV08, ϕKPaV10 and ϕKPaV12 as they displayed strong lytic activity, high titre count and clear-producing plaques. The titre count of each phage was 3.6×10^9, 4×10^8, 4.2×10^{10}, 1×10^9, 1.4×10^{12} pfu / ml, respectively.

Bacteriophages Host Range

The spectrum of activity of bacteriophage samples were tested against a panel of 21 *Klebsiella* spp. isolates, including *K. pneumoniae* ATCC 35657, *K. aerogenes* (KA1 and KA2) and *K. oxytoca* (KO1) as control strains (Table 1). All five selected phages were found to have different lytic activity towards different clinical- and community-acquired isolates, with each phage having 22-44% lysis towards a panel of *K. pneumoniae* covering 89% of the isolates including the CRKP and ESBL-producing *K. pneumoniae*. None of the non-KP isolates were lysed by these phages, and this is often a challenge for phage therapy because of the apparent host specificity towards its own bacterial species. However, this is a clear advantage over broad-spectrum treatment such as antibiotics because the non-target bacterial population will remain undisrupted (Koskella & Meaden, 2013).

While host range is often associated with the target bacteria possessing the correct receptor,

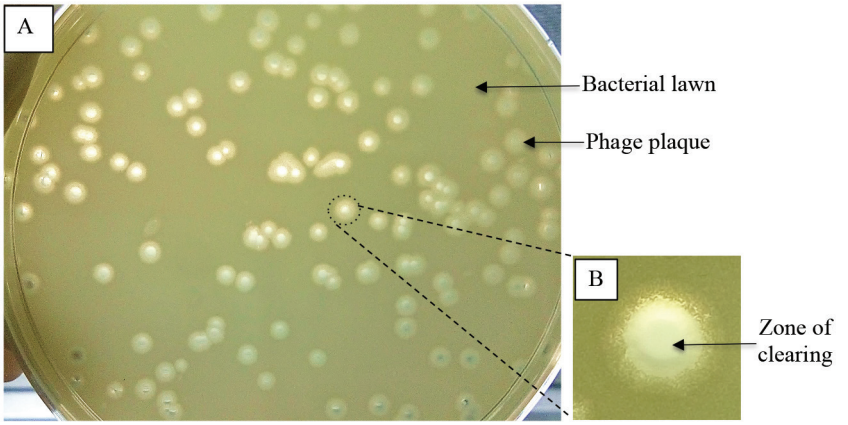


Figure 1: Clear and homogenous plaques (A) were formed by the lytic infection of ϕKPaV12 against its host *K. pneumoniae* KP15 in dilutions of 10^{-8}. Double-layer agar method and serial dilution were performed to enable a single plaque (B) to be obtained for plaque morphology studies and purification steps.

additional limitations on host range may include the microbial defense mechanisms against phage such as CRISPR (clustered regularly interspaced short palindromic repeats) loci together with their associated *cas* genes. (Stern & Sorek, 2011; Hyman, 2019). The CRISPR-cas system described as an adaptive resistance mechanism as new phage-genome segments were subsequently integrated and accumulated over time following the current and past phage infections and may therefore confer resistance to multiple phages (Hyman & Abedon, 2010; Vale & Little, 2010; Laanto *et al*., 2017). Nevertheless, phages can counter-adapt to host immunity by modifying the DNA sequences of protospacers or protospacer-adjacent motifs (PAMs) (Levin *et al.,* 2013) or by using anti-CRISPR proteins (Rauch *et al*., 2013; Laanto *et al*., 2017).

Holmfeldt *et al*., (2007) has proven in his study that phages with even minor variance in host range (differ in infection of one bacterial strain) were genetically different. Therefore, the isolated phages in this study were considered unique from each other even when isolated from the same sewage sample. Besides that, phage host range is important in therapeutic application as it outlines the potential scope of treatable bacterial strains for the coherent selection of phages towards personalized cocktail development (Moller *et al*., 2019).

Clear spots surrounded by translucent halos were observed in phage ϕKPaV03, ϕKPaV10 and ϕKPaV12, against host cell *K. pneumoniae* ATCC 35657 (Figure 2), indicating that host cell exopolysaccharides were depolymerized. This phenomenon suggested that the phage produced a depolymerase enzyme that could diffused through the agar layer to degrade bacterial exopolysaccharides into oligosaccharide units during infection (Lai *et al*., 2016). Mentioned phenomenon is observed with isolated phage vB_Pae575P-4 by Jurczak-Kurek *et al*., (2016) which produced plaques with a halo, was able to lyse the clinical, biofilm-forming *P. aeruginosa* strains, isolated from a patient with cystic fibrosis. Bacteria-producing biofilms causing chronic infections are often associated with the cause of failure of antibiotic therapy. Biofilm elimination takes time and requires the application of multi-phage cocktails or antibiotic supplementation (Maciejewska *et al*., 2018).

Partial Sequencing of Bacteriophage Genome

The use of viral conserved genes is to investigate the diversity of viral communities in different environments (Adriaenssens & Cowan, 2014). Either prokaryotic or eukaryotic viruses lack universal conserved gene, therefore, many different genes were evaluated as potential group-specific conserved genes (Table 2).

Table 1: High titre ($\geq 10^8$ pfu / ml) of each phage was spot tested on 21 different host bacterial strains including non-KP strains that belonged to *K. aerogenes* (KA) and *K. oxytoca* (KO). Susceptibility test towards Gentamicin is tabulated with **R** indicating resistance and **S** indicating Sensitive. Black zone indicates for lysis and white zone indicates for no lysis (N).

				Host bacteria for phage isolation				
Category	**Bacterial Strain**	**GenBank Accession No.**	**Gentamicin Resistant**	**ATCC KP**	**CRKP1**	**KP17**	**ATCC KP**	**KP15**
				Host Range				
				ϕKPaV03	**ϕKPaV04**	**ϕKPaV08**	**ϕKPaV10**	**ϕKPaV12**
Control strains	1. ATCC KP	N/A	**S**		N			
Clinical acquired strains	2. CRKP1	MK386770	**R**	N		N	N	N
	3. ESKP2	N/A	**R**		N	N	N	N
	4. ESKP3	MK386781	**R**	N	N		N	N
	5. ESKP4	N/A	**S**	N	N			
	6. KP5	MK386771	**R**	N	N		N	N
	7. KP6	MK386786	**S**	N	N	N	N	
	8. KP7	MK386785	**S**	N	N	N	N	N
	9. KP8	MK386783	**S**	N			N	N
	10. KP9	MK386782	**R**		N		N	N
	11. KP10	MK386784	**S**		N	N	N	N
	12. KP11	MK386779	**S**	N			N	N
	13. KP12	MK386778	**S**	N	N	N	N	N
	14. KP13	N/A	**S**		N	N		N
	15. KP14	MK386776	**S**		N	N		N
Community acquired strains	16. KP15	MK386773	**S**		N	N	N	
	17. KP16	MK386772	**S**				N	N
	18. KP17	MK386777	**S**	N	N		N	N
	Lysis efficiency of each phages (%)			44	22	44	28	28
	Total lysis (%)			16 out of 18 KP strains (89%)				
Non-KP	19. KA1	MK386775	**S**	N	N	N	N	N
	20. KA2	MK386774	**S**	N	N	N	N	N
	21. KO1	MK386769	**S**	N	N	N	N	N

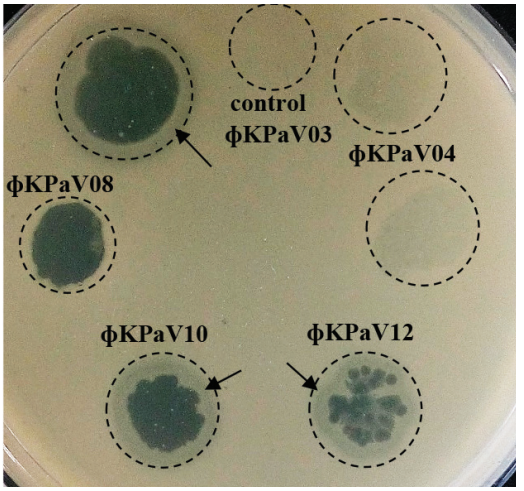


Figure 2: Phage ϕKPaV03, ϕKPaV04, ϕKPaV08 and ϕKPaV10 with high titre count (≥10^8 pfu / ml) was spotted against bacterial host *K. pneumoniae* ATCC 35657. Non homogenized dots formed by ϕKPaV12 were probably caused by low phage titre. Ten µl of sterile SM buffer was mock infected act as a negative control. Arrows indicate translucent halo observed surrounding the clear spot produced by ϕKPaV03, ϕKPaV10 and ϕKPaV12.

The major capsid gene of *Myoviridae* phage T4, g23, was one of the conserved virion genes used to distinguish between the subgroups of T4-type phages such as T-evens. Degenerate gp23 primers by Filee *et al*., (2005) have generated positive PCR products ranging from 380 to 600 bp among T-even phages from diverse marine locations. Similarly, PCR product size and BLASTn sequences of ϕKPaV08 and ϕKPaV12 have shown similar characteristics with 97% and 99% comparability, respectively to T4 g23 (Table 2). The g23 conserved gene appears to span a much greater diversity of bacteriophage groups within the *Myoviridae* family and making it a more desirable marker gene for investigating myovirus diversity (Adriaenssens & Cowan, 2014a).

Phage enzymes (lysins) possess different classes of peptidoglycan-degrading activities, including glucosaminidase, lysozyme-like, and endopeptidase activities (Antonova *et al*., 2019) function in breaking covalent bonds of peptidoglycan/murein layers of the host cell wall, causing the release of newly assembled phage progenies (Fischetti, 2005). The BLASTn analysis (Table 2) revealed that ϕKPaV03 shows high similarity with the putative peptidase of *Klebsiella* phage VB_KpnP_KpV48 (91% identity) belonging to family of *Podoviridae*. PCR amplification of ϕKPaV03 and ϕKPaV08 lysin gene using lysin specific primers have produced distinct bands size between 750bp and 1500bp. Likewise, Mishra *et al.,* (2014) have successfully amplified lysin gene of the *Staphylococcus aureus* phage with a single PCR product of 802 bp. The broad activity spectrum found in ϕKPaV03 and ϕKPaV08 (in Table 1) suggested that it could be due to the presence of lysin gene, with experimental evidenced from lysin of *E. coli* and *A. baumannii* phages that have shown a broad lytic activity against a wide range of Gram-negative bacteria (*K. pneumoniae, A. baumannii, P. aeruginosa, E. coli,* and *S. enterica*) (Antonova *et al.,* 2019).

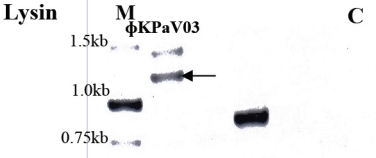
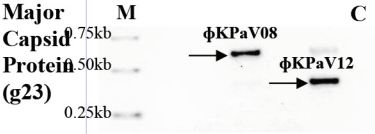
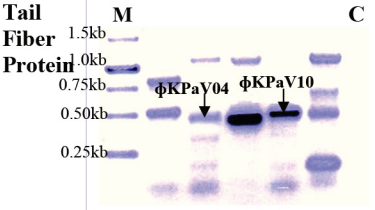
JK primer set used to target specific to tail fiber protein gene was derived from the work of Hee Suk Lee (2009) to optimize group-specific PCR detection of targeted *Siphoviridae* family that were predominantly found in fecal contaminated water settings. The JK primer set generates the size of 878bp of target amplicon. However, no distinct bands were produced of the similar size, noting that none of isolated phages belonged to the family of *Siphoviridae*. Nevertheless, PCR bands of phages ϕKPaV04 and ϕKPaV10 were selected for sequencing as they have only shown positive outcome from the amplification of Tail Fiber Protein gene. The BLASTn result for ϕKPaV04 and ϕKPaV10 have exhibit less than 50% of similarity towards the family of *Myoviridae* and *Podoviridae*, respectively. Correspondingly, Pieterse *et al*., (2018) have used similar primer set to represent diverse group of coliphages but only 35.4% of the coliphages were able to be identified and characterized by PCR analysis. Hence, Pieterse *et al*., (2018) correlates the used of inadequate primer sets to the discrepancy of the molecular identification of phages as the primer set only represents for its own specific group.

Overall, the use of viral conserved genes is relatively a more practical and economical way to identify and assess viral diversity especially when dealing with multiple samples. PCR-based analysis currently remained the best option for the identification of bacteriophages from various sources depending on the choice of conserved genes for a specific study (Adriaenssens & Cowan, 2014).

Single Administration of Phage Cocktail against GN-resistant Klebsiella pneumoniae

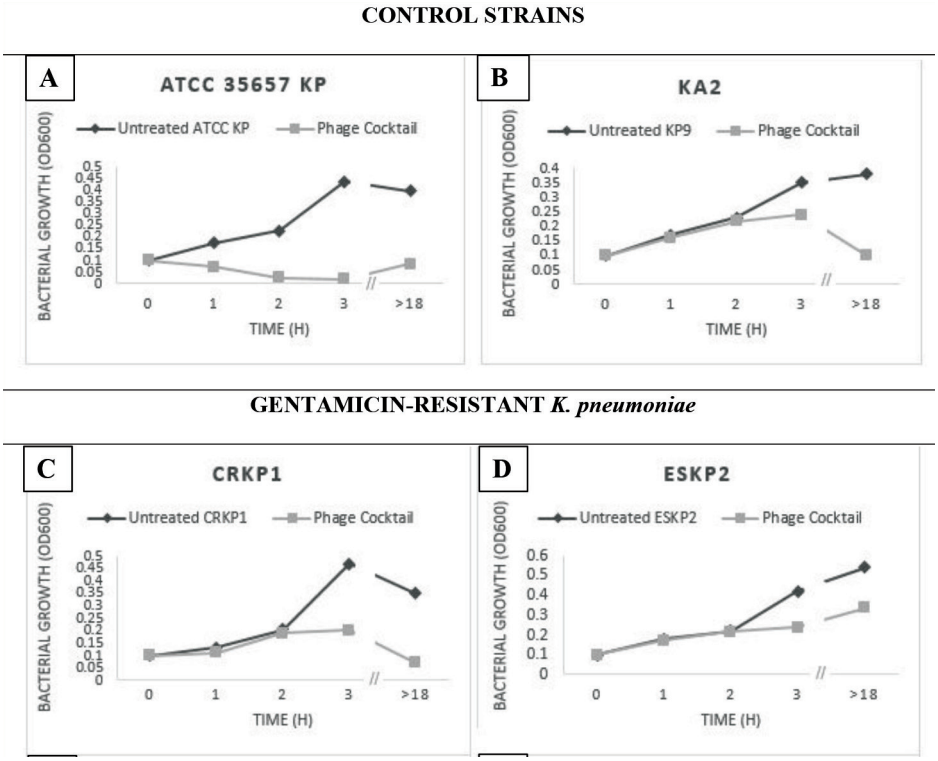
Previous studies on phage cocktails displayed promising results in significantly reducing the bacterial growth both in vitro and in vivo models (Chadha *et al*., 2016; Gu *et al*., 2012; Zhang *et al*., 2010). Therefore, a study was conducted by forming a phage cocktail using five high titre and broad host range bacteriophages to suppress the appearance of GN-resistant *K. pneumoniae*. When the mixture of those phages was added independently in single administration to the early log-phase of each GN-resistant *K. pneumoniae*, the declining of bacterial growth reading (OD_{600}) by the 2nd hour was observed. Noticed that (Figure 3) 100% of GN-resistant *K. pneumoniae* cell growth was reduced within the 3rd hour of incubation and 80% of bacterial cell growth remained suppressed even after more than 18 hours of incubation with only a single phage cocktail administration. As for GN susceptible strain *K. pneumoniae* ATCC

Table 2: Identification of phages using specific primers targeting gene; Lysin, Major Capsid Protein (g23) and Tail Fiber Protein. Selected phage genes with distinct bands (with arrow) were subjected for BLASTn sequencing to determine percentage of similarities with existing phage genomes in the database. 1kb DNA ladder used as marker and SM buffer acted as a negative control

Phage Gene	PCR Gel Electrophoresis	BLASTn results			
Lysin		Selected phage for sequencing	% identity	Sequence similarity	Family
		ϕKPaV03	91%	Putative peptidase of *Klebsiella* phage VB_KpnP_KpV48	dsDNA viruses, Podoviridae
Major Capsid Protein (g23)		ϕKPaV08	99%	Major capsid protein Klebsiella phage JD18	dsDNA viruses, Myoviridae
		ϕKPaV12	97%	Major capsid protein Klebsiella phage KPV15	dsDNA viruses, Myoviridae
Tail Fiber Protein		ϕKPaV04	48%	Ribonucleotide reductase, beta-subunit *Klebsiella* phage KP179	dsDNA viruses, Myoviridae
		ϕKPaV10	37%	Tail Protein *E. coli* phage	dsDNA viruses, Podoviridae

35657, the cell growth was eradicated as early as the first hour of incubation and continued to be suppressed after overnight incubation. *Klebsiella aerogenes* (KA2) was used as a control strain to evaluate the phage cocktail expansion of host range towards other *Klebsiella* spp. as no isolated phages were able to infect KA2 (Table 1). Positive outcome of phage cocktail against KA2 (Figure 3) were observed by the 3rd hour of incubation with more than twofold decrease in cell growth and remained stunted after 18 hours of incubation. This observation justified the formulation of phage cocktail is to create a therapeutic mixture with broader host specificity (Hyman, 2019).

Selecting suitable phage titre and determine the frequency of administration are key factors in controlling target bacteria (Tanji *et al.,* 2005). Rapid reduction of phage concentration after single administration of phage cocktail could occur against bacterial strains with fast reproduction rate, seen in ESKP3 and KP5 (Figure 3). This relates to the pharmacodynamics interaction between phages and host bacteria. A large dose of phages must be administered against a small bacteria population to allow the phage to replicate faster than bacteria (Levin & Bull, 2004). Under this condition, it would be necessary for a repeated administration of phage cocktail in order to sustain high titre of phage infecting fast growing bacteria such as ESKP3 and KP5.



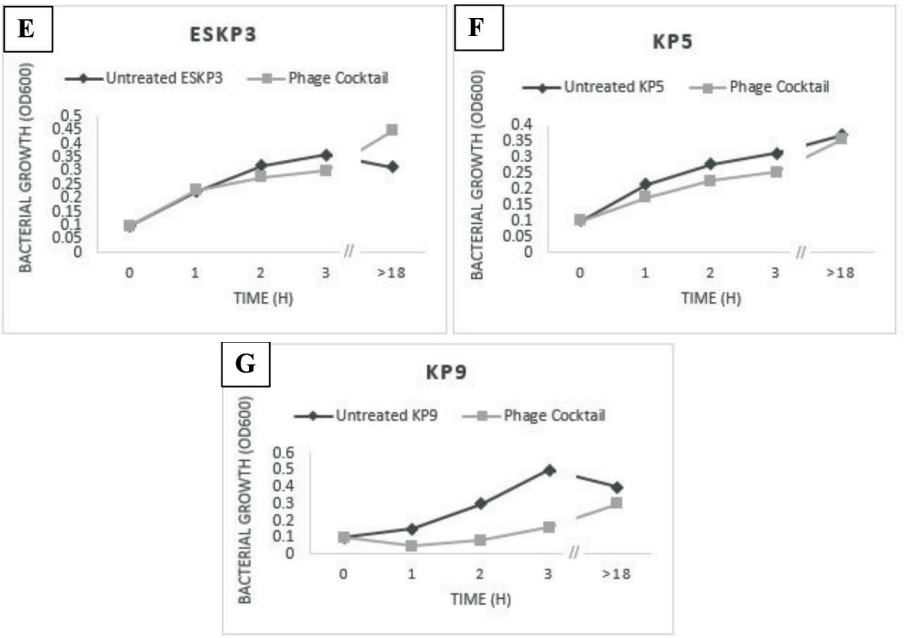


Figure 3: Time-kill curves of phage cocktail against GN-resistant *K. pneumoniae* (CRKP1 (C), ESKP2 (D), ESKP3 (E), KP5 (F) and KP9 (G)) and control strains (*K. pneumoniae* ATCC 35657 (A) and *K. aerogenes* (B)). Early log phase of bacteria (OD_{600} = 0.1) was infected by single administration of phage cocktail with the ratio volume of 1:10 and the bacterial growth concentration observed for >18 h.

Conclusion

The use of phages could be the best alternative solution towards the emergence of multidrug-resistant bacterial strains as they are readily available and easily isolated from the environment. In our study, five phages infecting *K. pneumoniae* were isolated from a domestic sewage. These phages namely ϕKPaV03, ϕKPaV04, ϕKPaV08, ϕKPaV10 and ϕKPaV12 have exhibited remarkable preliminary characterization with high titre and possessing broad host range, which are important in producing phage cocktail. A single administration of phage cocktail consisted of these individual phages have shown promising results against Gentamicin-resistant *K. pneumoniae.* Being aware of Gentamicin resistance in nosocomial infections is important since Gentamicin is often used as a last resort of antibiotic against carbapenem-resistant *K. pneumoniae* infections and in combination therapy with other antibiotics against ESBL-producing *K. pneumoniae* infections. Hence, our data supports the extended development of phage cocktail as a therapeutic and biocontrol agent in clinical applications treating wide range of *K. pneumoniae* infections including multi-drug resistant infections.

Abbreviations:

GN	Gentamicin
CRKP	carbapenem-resistant *Klebsiella pneumoniae*
ESBL	extended-spectrum beta-lactamases
PCR	polymerase chain reaction
SM	saline-magnesium

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