EVALUATION OF THE LYTIC KINETICS OF A COCKTAIL OF TWO WASTEWATER *SIPHOVIRIDAE* BACTERIOPHAGES AGAINST UROPATHOGENIC *KLEBSIELLA PNEUMONIAE*

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Abstract. In this study, two lytic phages were isolated which were designated as KPO1 and KPO2. These phages formed medium plaques with different size and had a head with a diameter of 54 ± 3.0 and 56 and ± 3.0 nm, respectively. They had a flexible, long noncontractile tail with a length of 85 ± 3.0 and 160 ± 3.0 nm, respectively. Both belong to order *Caudovirales* family *Siphoviridae*. They were stable at 4°C to 70°C upon thermal exposure and not viable at 80ºC and 90ºC, respectively. Concerning the pH stability, both retained a high titer (average: 8.1 $\log_{10}$ PFU/mL) from pH 4 to pH 10 for 1 h. No viable virions were detected at pH 12 and pH 2. The burst size of KPO1 and KPO2 computed as 98 and 110 virions per infected cells with the corresponding latent period of 23 min and 15 min, respectively. The *in vitro* lytic kinetics of the cocktail of KPO1 and KPO2 was evaluated against *Klebsiella pneumoniae* strain INF079. The highest reduction of *K. pneumoniae* were observed at 0.1MOI for both phages. According to the findings, the prepared phage cocktail significantly reduced the bacterial concentration from 0.4 to 0.12 at 38 h of post infection ($p < 0.05$). Hence, phages KPO1 and KPO2 could be a promising therapeutic agent for the urinary tract infections caused by *K. pneumoniae*.
Keywords: *bacteriophage, Caudovirales, multidrug resistance, lytic activity*

Abbreviations. AMR: Antimicrobial Resistance; OD: Optical Density; UTIs: Urinary Tract Infections; MOI: Multiplicity of Infection; PBS: Phosphate Buffer Saline; API: Analytical Profile Index; PFU: Plaque Forming Unit, CFU: Colony Forming Unit

Introduction

The genus *Klebsiella* causes urinary tract infection, pneumonia, and sepsis, mainly in susceptible populations, often resulting serious secondary complications in catheterized or ventilated patients, in both nosocomial and community settings (He et al., 2020; Dhesi et al., 2020). Moreover, the subclinical *Klebsiella* infection is associated with cardiovascular (Jameson et al., 2018) and inflammatory bowel disease (Read et al., 2021). Among the *Klebsiella* spp., *Klebsiella pneumoniae* is the most prevalent species giving rise to hypervirulent clones with diverse virulence factors (Atarashi et al., 2017).

Antimicrobial resistance (AMR) considered as the priority global health concern is driven by our miss use or overuse of antibiotics in agriculture and medicine. During the past 10 years, multidrug resistance of *K. pneumoniae*, particularly to carbapenems, has dramatically increased, making it a major burden on public health systems worldwide (Braun et al., 2016). In the Ambler classification system, *K. pneumoniae* classified under the clade A of β-lactamases, which contain serine at their active site and hydrolyze

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numerous cephalosporins, penicillin, and carbapenems (Rodriguez-Gómez et al., 2019). The World Health Organization considers *Klebsiella* as a priority pathogen because it can readily acquire and transmit AMR genes, particularly in health care settings (Tacconelli et al., 2018). There has been a rapid increase in the prevalence of multi-drug resistant (MDR) *K. pneumoniae* to most of the conventional therapeutic agents, and currently pandrug resistant *K. pneumoniae* are now common all over the world (Uzairue et al., 2022). MDR *Klebsiella* infections result in a rise in the risk of mortality (Poerio et al., 2022) are difficult to treat (Yang et al., 2022) and outbreaks can lead to economic crises associated with hospital stay and cost of treatment (Zhen et al., 2020). The incidence of urinary tract infections (UTIs) is second following respiratory tract infections in community and healthcare settings. Among uropathogens, *K. pneumoniae* ranks second next to *Escherichia coli*. Uropathogenic bacteria have some characteristics that favor the colonization of human cells, such as the production of toxins, siderophores and adhesins (Foxman, 2010). Similarly, these bacteria can form biofilms on medical devices which enable the agent to evade the host immune system and ultimately cause therapy failure (Dybowska-Sarapuk et al., 2017). For this reason, alternative therapy such as phage therapy is urgently needed. Bacteriophages (hereafter named as phages) offer one potential alternative treatment for wide range of multidrug resistant (MDR) pathogens including *K. pneumoniae* (Herridge et al., 2020).

Phage therapy is a promising weapon against MDR bacterial infections (Mousavi et al., 2021). Phage therapy relied on readiness, mainly in having a virtual or real "biobank" of lytic phages against major AMR bacterial pathogens. According to recent reports, due to the continuous increase in the phage resistant bacterial strains, an intense isolation and characterization of phages is needed to provide effective, timely treatment and mitigate side effects (Pirnay, 2020).

In this study, we amid to isolate lytic bacteriophages targeting the MDR uropathogenic *K. pneumoniae* as an indicator host. We assessed the morphology, lytic kinetics, host range and other characteristics of the isolated phages. Besides, we evaluated the lytic activities of a cocktail of two *K. pneumoniae* phages in comparison to the individual lytic activities.

Materials and methods

Host strain

One clinical strain of *K. pneumoniae* was used as host organism to isolate the phages from the wastewater system. The isolate was obtained from King Fahd Medical Research Center (KFMRC) bacterial culture collection. The clinical data of the isolate indicated that the isolate was generated from the urine sample of urinary tract infected patient. The molecular confirmation was made using 16s rRNA genome sequencing. The isolate preserved in 50% glycerol (v/v) at −20°C and revived in Nutrient Broth (NB) medium at 37°C overnight incubation.

Antibiotic susceptibility test

Drug sensitivity test was conducted using Kirby Bauer Disc Diffusion method on Mueller Hinton agar media according to the (Wayne, 2010) guideline using Hi-media antibiotic discs. The susceptibility results were interpreted using the standard breakpoints. The sensitivity test was performed in in three biological replicates.

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Wastewater sample

Domestic wastewater samples were collected from Jeddah Wastewater Treatment Plant, Jeddah, Saudi Arabia. The collected sample were transported to KFMRC microbiology laboratory in ice box and kept in the refrigerator for 2 h until the phage isolation process started.

Isolation of bacteriophage

The lytic wastewater phages were isolated according to the method described by Jurczak-Kurek et al. (2016). Wastewater samples were spun at 10,000 x g, 10 min. The supernatant was then filter using a 0.22 μm pore size syringe filter (Millipore, Billerica, MA, USA). The filtered samples were enriched by incubating it with equal volume of double strength nutrient broth supplemented with 2 mM $CaCl_2$ (TETRA Inc, Texas, United States) coupled with 1ml host bacteria at 37°C for 48 h with gentle shaking. Then after, the enriched samples were spun it and filtered similarly as mentioned above. The phage lysate was kept at 4°C until used.

Phage purification

The phage lysate was serially diluted in phosphate buffer saline (PBS) (pH 7.4) solution to attain a purified individual lysis plaque in the lawn of the host organism. For this, 100 μL of diluted phage lysate was inoculated into 100 μL of overnight bacterial culture (Optical density (OD): 0.4/600 nm), and the mixture was then transferred into 3 mL of soft agar and poured onto a solidified agar plate. The plates were incubated overnight at 37°C. Then, the positive plate for plaques was selected and single plaque was picked with pipette tips and immersed into 500 μL PBS. Then, double agar overlying technique was used to determine the titer of phages. This technique was repeated several times until we get pure plaques (Gencay et al., 2017).

Preparation of high titer phages stocks

The titer of the phage was concentrated by incubating the plate comprised high number of plaques covered with 10 mL PBS for 24 h at 4°C by swirling regularly. The aliquot was decanted into centrifuge tubes and the bacterial debris was removed by centrifugation at 1,000 x g for 15 min. The supernatant was then filtered (0.22 μm) and stored at 4°C. The titer of the phage was determined by the overlay method (Qadri et al., 2021; Teklemariam et al., 2022).

pH and thermal stability

The heat tolerance of the isolated phage was assessed by a standard protocol described by Jurczak-Kurek et al. (2016) with slight modification. A micro-centrifuge tubes containing one thousand microliter of the phage suspension (1 x 10^9 PFU/mL) was placed in the water bath arranged at different temperature (37-90°C) for 2 h. After heat treatment, the survival rate was determined by double agar overlying method. Similarly, the stability of our phage for various pH scales (i.e., 4, 5, 6, 7, 8, 9, 10, 11, 14) was carried out by the method described by Capra et al. (2006) with slight modifications. Briefly, 1,000 μL of the concentrated phage (1 x 10^9 PFU/mL) was introduced into 9 mL of sterile nutrient broth (at specific pH) and mixed thoroughly by up and down pipetting. Then, the suspension was incubated at convenient temperature (4^{o}C) for 2 h and the titer of the treated phage (its viability) was determined by double agar overlying method.

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One step growth

The latent period and the burst size of the phages were carried out as the formerly described method (Jamal et al., 2017). For this, 50 mL overnight bacterial culture (OD = 0.4–0.6) was spun and the pelleted cells were re-suspended in nutrient broth. Then, 500 µL of purified phage lysate (1 x 10^9 PFU/mL) was added into the suspension. The phage adsorption assay was carried out at this point. To remove unabsorbed phages, the mixture was spun (12,000 x g, 30 min). The pellet was mixed to nutrient broth (100 mL) and incubated with shaking (120 x g, 37°C). The suspension was taken out and diluted in PBS solution after an interval of every 3 min. Then, the titer of the phage was determined by double agar overlying method.

Killing assay

One hundreds microliter of the concentrated phage lysate (1 x 10^{10} PFU/mL) was added to an equal volume of broth culture (OD_{600} = 0.6; 1 × 10^7 CFU/mL) at a multiplicity of infection (MOI) of 100, 10, 1 and 0.1 in the wells of 96 well-microliter plate. Following overnight incubation at 37°C the rate of reduction of bacterial cell over time was assessed by measuring the OD at 600 nm, every 1 h intervals for 12 h period using a multimode microplate reader (Tecan Spark 10M, Switzerland). One well of the plate containing 200 µL of PBS and log phage bacterial culture (equal proportion) was used as the negative control (Pereira et al., 2011). This experiment was performed in triplicate.

Phage cocktail preparation

A two-phage containing cocktail was developed by combining the phages at equal proportion (1:1 ratio) with the same titer (10^9 PFU/mL), to a final titer of 10^9 PFU/ml. The phage cocktail was stored at 4°C until processed. Briefly, for each *in vitro* experiment, 100 µL of the phage cocktail (10^9 PFU/mL) was added to 1mL of host bacterium (1 × 10^7 CFU/mL) in a sterile cuvette and the mixture was incubated at 37°C. The OD_{600} was measured for 40 h using an UV/VIS spectrophotometer (Jenway, 6300, Hong Kong) to assess the killing effect against the respected bacterial strains. The phage free bacterial culture (100 µL) in a NB was used as a control (Pereira et al., 2011). Two independent experiments were conducted for each cocktail.

Host range determination

The host range of the phage was assessed using spot assay and double agar overlying method. For this, a panel of fifteen different pathogenic bacterial strains were used. Uninfected plate was used as a negative control (Chen et al., 2018).

Stability of phage cocktail

Stability of phage cocktail for different storage temperature were conducted. Briefly, 2 ml of concentrated phage lysates (1 x 10^{12} PFU/mL) were incubated at 37°C and 4°C for 6 months. At each month, 100 µL of phage suspension were taken to determine the phage titer using a double-layer agar method (Zurabov and Zhilenkov, 2021).

Transmission electron microscopy

A drop of the concentrated phage lysate (1 x 10^{10} PFU/mL) was placed on a 200-mesh grid and left for 3-5 min to adhere at which time the excess lysate was removed with filter

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paper and the grid given time to air dry. The negative staining was carried out with 6 µl of 2% phosphotungstic acid (aqueous, pH 6.5), for proper visualization of the agent under electron microscope (Hitachi H-600, Japan) at × 100,000 – 260,000 magnifications (Ackermann, 2009). The isolated phages were classified based on their morphological feature and the international guidelines (International Committee on Taxonomy of Viruses).

Statistical analysis

The data were recorded as means ± SE of triplicate experiments. Statistical analysis was carried out using GraphPad Prism software version 6 for Windows (GraphPad Software Inc., USA). The Kolmogorov–Smirnov and Levene's tests were conducted to check the normal distribution of the data and homoscedasticity, respectively. The difference between the phage treatment and the control group was analyzed using Student's *t*-test. The association between viral and bacterial concentrations in the *in vitro* assays, along with the sampling points, was computed using two-way analysis of variance (ANOVA) and the Bonferroni post-hoc test. The significance level was set at $p < 0.05$.

Results and discussion

Bacterial characteristics

The host *K. pneumoniae* was first confirmed by cultural, biochemical and at genomic level before used as host for phage isolation. Culturally, the bacterial isolate showed distinct colony morphology in Cystine Lactose Electrolyte Deficient (CLED) agar (yellowish, mucoid colony). Biochemically, the seven-digit profile number (5205773) obtained from the API-20E test indicated that the organism was 97% identical to *K. pneumoniae*. The partial 16S rRNA based genomic sequence revealed that the host *K. pneumoniae* was 100% identical to *K. pneumoniae* strain INF079 (Accession number - CP110765.1) (*Fig. 1*).

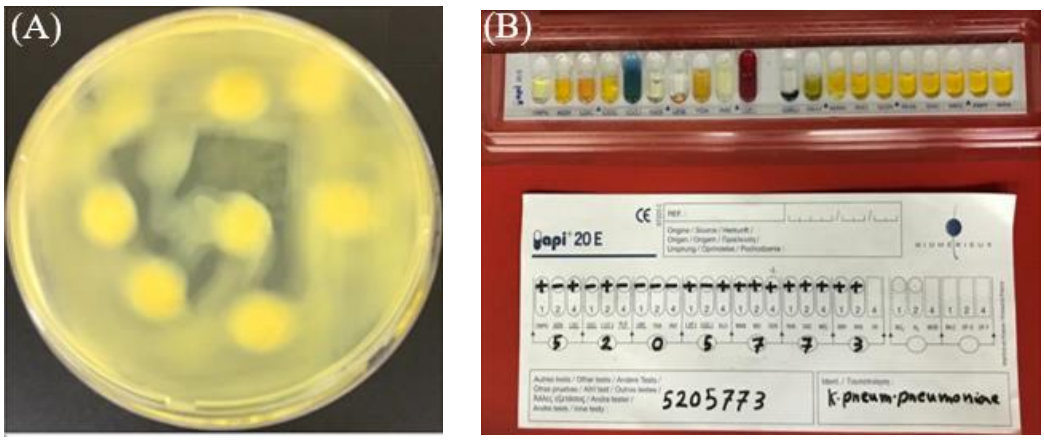

Figure 1. *(A) Cultural characteristic of K. pneumoniae on CLED agar; (B) API-20E test result of K. pneumoniae*

Antibiotic sensitivity test

Antibiotic resistance pattern of *K. pneumoniae* strain INF079 is shown in the radar plot (*Fig. 2*). The isolate was sensitive for 20% of antibiotics namely, ertapenem, meropenem

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and imipenem, while it was resistant for 80% of antibiotics tested (streptomycin, tobramycin, gentamicin, neomycin, ticarcillin, cefuroxime, cefotaxime, ceftriaxone, ceftazidime, ciprofloxacin, and levofloxacin).

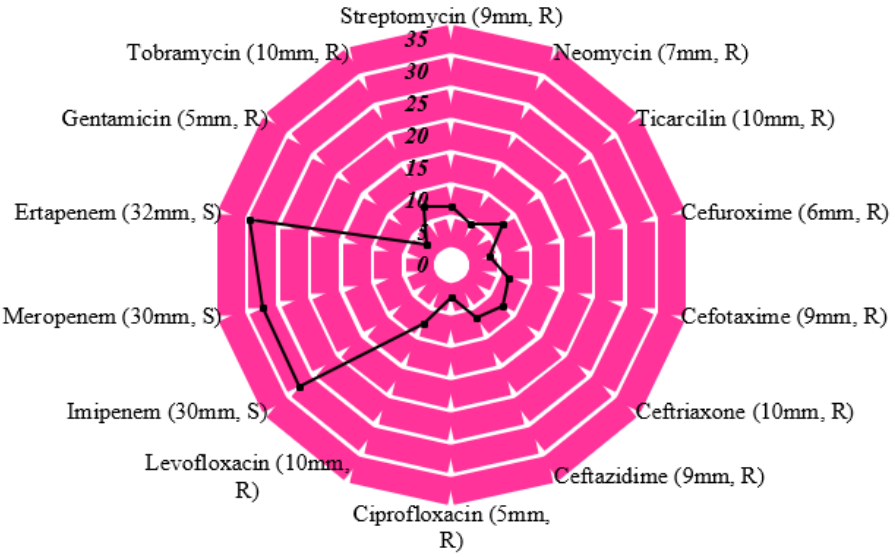

Figure 2. *Radar plot showing the antimicrobial sensitivity of K. pneumoniae strain INF079. R = resistant; S = sensitive; mm = millimeter*

Phage isolation and identification

Wastewater samples were collected from Jeddah Wastewater Treatment Plant which is the true representative of the collection site as it receives wastewater from different corners of the city. The purified samples were enriched and screened by spot assay on the lawn of *K. pneumoniae* strain INF079 (*Fig. 3*). According to the results both phages (KPO1 and KPO2) displayed clear (non-turbid) lytic zone on phage spotted area.

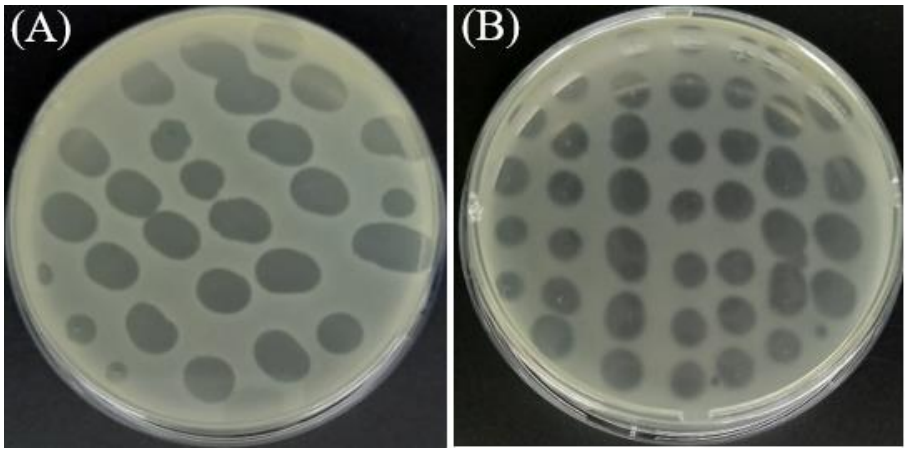

Figure 3. *Spot assay results of (A) KPO1; (2) KPO2. Clear lytic zones at the phage spotted area on the lawn of K. pneumoniae strain INF079*

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The lytic effect of these phages were further confrimed by DAL assay. According to this assay both phages formed different size plaques as showed in *Fig. 4*. The clearity of plaques indicated the phages are lytic (virulent) phages. The average size of the plaques were found to be 0.5 ± 0.2 mm and 1.5 ± 0.2 mm for KPO1 and KPO2, respectively (*Fig. 4A* and *B*). Based on the TEM results, KPO1 and KPO2 both had isometric type B1 capsids with a diameter of 54 ± 3.0 nm and 56 ± 3.0 nm, respectively. They had a flexible, long noncontractile tail with a length of 85 ± 3.0 nm and 160 ± 3.0 nm, respectively (*Fig. 4C* and *D*). According to the International Committee on Taxonomy of Viruses (ICTV) (Lefkowitz et al., 2018) KPO1 and KPO2 were belong to the order *Caudovirales* family *Siphoviridae*. A similar *Siphoviridae* virus, IME268, (Nazir et al., 2022) and Kpn31 (Balcão et al., 2022) were isolated from hospital waste targeting *K. pneumoniae* strain INF079. In addition, currently, one *Siphoviridae* (Qadri et al., 2021) and four lytic *K. pneumoniae* phages (vB_KpnP_FBKp16, vB_KpnP_FBKp27, vB_KpnM_FBKp34, and Jumbo phage vB_KpnM_FBKp24) have been isolated from wastewater and sewage waste, respectively (Estrada Bonilla et al., 2021). In general, wastewater is considered as a potential fertile environment for phage propagation as it carries different chemical constituents which support the growth of bacteria (phage host) (Gunathilaka et al., 2017).

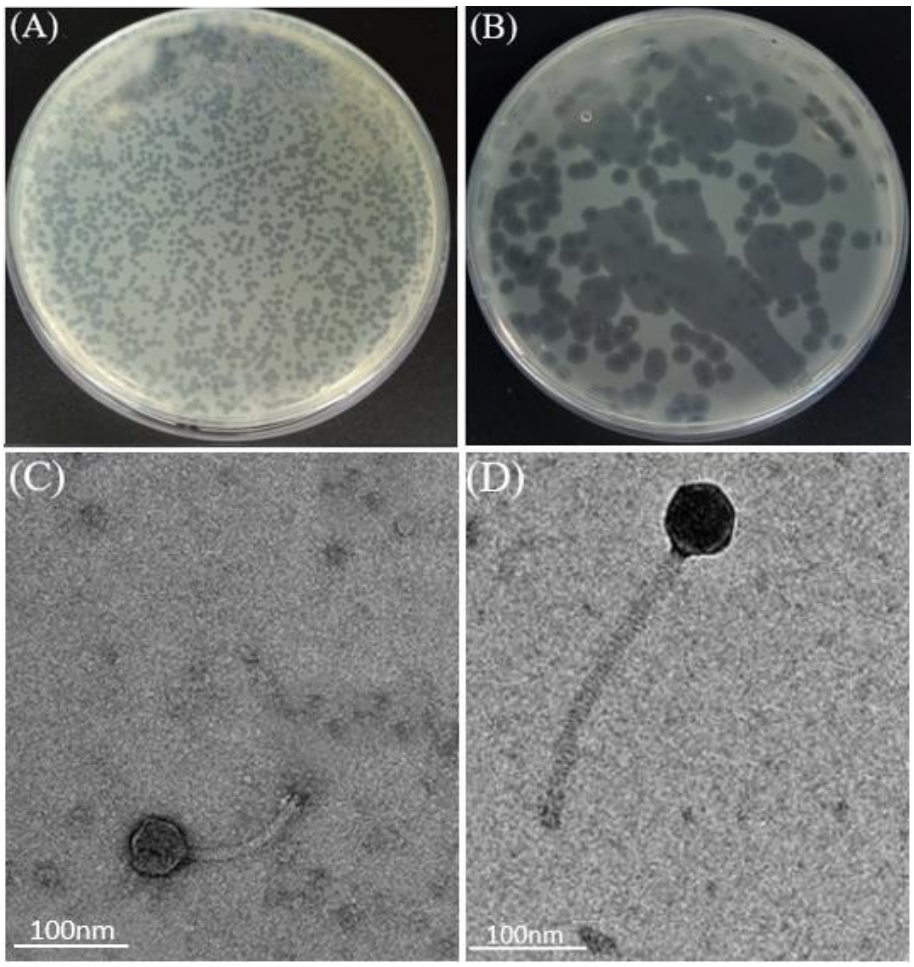

Figure 4. *(A) Plaque morphology of KPO1 (B) Plaque morphology of KPO2; (C) TEM of KPO1; (D) TEM of KPO2. Bar = 100 nm*

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Multiplicity of infection

The exponential phase culture of *K. pneumoniae* strain INF079 was infected with KPO1 and KPO2 at different phage titer to determine the MOI. The titer of these phages were measured at 2 h post infection. The results indicated that the optimal MOIs of both KPO1 and KPO2 phages were found to be 0.1 which gave the highest production of progeny virion 5 x 10^9 and 4.8 x 10^9 PFU/mL, respectively (*Table 1*).

Table 1. *MOI of KOP1 and KOP2 (* indicates the optimal MOI)*

Bacterial concentration (CFU/mL)	Phage titer (PFU/mL)	MOI	Titer of KPO1 at 12h (PFU/mL	Titer of KPO2 at 12h (PFU/mL)
1 x 10^8	1 x 10^6	0.01	5 x 10^7	4 x 10^7
1 x 10^8	1 x 10^7	0.1	5 x 10^9	4.8 x 10^{9*}
1 x 10^8	1 x 10^8	1	2.3 x 10^8	3.5 x 10^8
1 x 10^8	1 x 10^9	10	1.5 x 10^8	2.3 x 10^8
1 x 10^8	1 x 10^{10}	100	1.2 x 10^8	1.8 x 10^8

Key: MOI: multiplity of infection; PFU: plaque forming unit; CFU: colony forming unit; mL: mililiter; h: hour

Stability assay

As shown in *Tables 2* and *3*, phage KPO1 and phage KPO2 were stable from 4°C to 70°C upon thermal exposure and not viable at 80°C and 90°C, respectively. The mean titer of KPO1 and KPO2 were found to be 8.5 $\log_{10}$ PFU/mL and 8 $\log_{10}$ PFU/mL upon 1 h treatment at 4°C, 37°C, 40°C, or 60°C and no significant differences ($p > 0.05$) were recorded among them. Nevertheless, after 1 h incubation at 70°C and 80°C, the rate of survival of KPO2 reduced by 3 and 4 $\log_{10}$ PFU/mL, respectively ($p < 0.05$) (*Table 3*). Similarly, the viability of KPO1 phages was reduced by 3 $\log_{10}$ PFU/mL at 1 h post treatment (*Table 2*).

Table 2. *Thermal and pH stability of KPO1*

Temperature (OC)	Phage titer in log10 PFU/mL	P value	pH	Phage titer in log10 PFU/mL	P value
4	8.5	p > 0.05	2	0	p < 0.05
37	8.5		3	4.4	
50	8.5		4	7.5	p > 0.05
60	8.5		7	8.3	
70	4.5	p < 0.05	10	8.4	
80	0		11	5.5	p < 0.05
90	0		12	0	
100	0		13	0	

Key: log: logarithm; PFU: plaque forming unit. OC: degree Celsius

Concerning the pH stability, KPO1 and KPO2 retained a high titer (average: 8.1 $\log_{10}$ PFU/mL) from pH 4 to pH 10 for 1 h. However, they exhibited a significant decline ($p < 0.05$) in titer at pH 11 and reached to 5.5 and 4.5 $\log_{10}$ PFU/mL. No viable virions

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were detected at pH 12 and pH 2 suggesting that these phages did not resist strong alkaline and acidic condition, respectively ($p < 0.05$) (*Table 2* & *Table 3*). A comparable thermal and pH stability were observed with the phage PG14 that infect carbapenem-resistant *K. pneumoniae* G14 (Mulani et al., 2022). The high thermal and pH tolerance of the phage broadens the practical application condition of the phage (Peng et al., 2020).

Table 3. *Thermal and pH stability of KPO2*

Temperature (^{O}C)	Phage titer in log_{10} PFU/mL	P value	pH	Phage titer in log_{10} PFU/mL	P value
4	8.0	p > 0.05	2	0	p < 0.05
37	8.0		3	3.4	
50	8.0		4	7.7	p > 0.05
60	8.0		7	8.5	
70	5.5	p < 0.05	10	8.4	
80	4.0		11	4.5	p < 0.05
90	0		12	0	
100	0		13	0	

Key: log: logarithm; PFU: plaque forming unit. ^{O}C: degree Celsius

One step growth curve

A one-step growth curve was used to analyze the latency period and burst size of the phage (*Fig. 5 A* and *B*). The results showed that phage KPO1 and phage KPO2 had a latency period of 23and 15 min, respectively. The burst size of KPO1 and KPO2 computed as 98 and 110 virions per infected cells, respectively (*Fig. 5*). The burst size of these two phages is higher than burst size of lytic phage TUN1 (76 PFU/infected cells) specific for *K. pneumoniae* K64 clinical isolates (Eckstein et al., 2021) and a lytic phage, Kpn31 (9 PFU/infected cells) (Balcão et al., 2022). However, it is almost in harmony with the medium burst size (82 PFU/cell) and a short latent period (20 min) of a novel lytic phage, P545, isolated against carbapenem-resistant *K. pneumoniae* strain (He et al., 2020).

Bacterial reduction assay

The virulence of KPO1 and KPO2 were evaluated against *K. pneumoniae* strain INF079 at different MOIs. KPO1 phage and KPO2 phage reduced *K. pneumoniae* strain INF079 proliferation relative to the negative control (MOI 0) at all MOIs ($p < 0.05$). Data from the no-phage treated control (MOI:0) showed a typical sigmoid curve representing an uninhibited bacterial growth ($p > 0.05$), whereas experiments set up with different MOIs (100, 10, 1, 0.1, and 0.01) indicated inhibition of bacterial growth due to phage action. In comparison to the control (MOI: 0) the lysis kinetics of the phages KPO1 and KPO2 showed significant reduction of the concertation of the indictor host at 24 h of post infection with the OD value of 0.3, 0.55, 0.63, 0.65, and 0.7 at an MOI of 0.1, 0.1, 10, 100 and 0.01, respectively ($p < 0.05$). However, the reductions in the bacterial growth were lasted for 5-12 h except for 0.1MOI. There was no statistical significance was reported in the lytic kinetics among the four MOIs (MOI 0.01, MOI 1, MOI10 and MOI 100).

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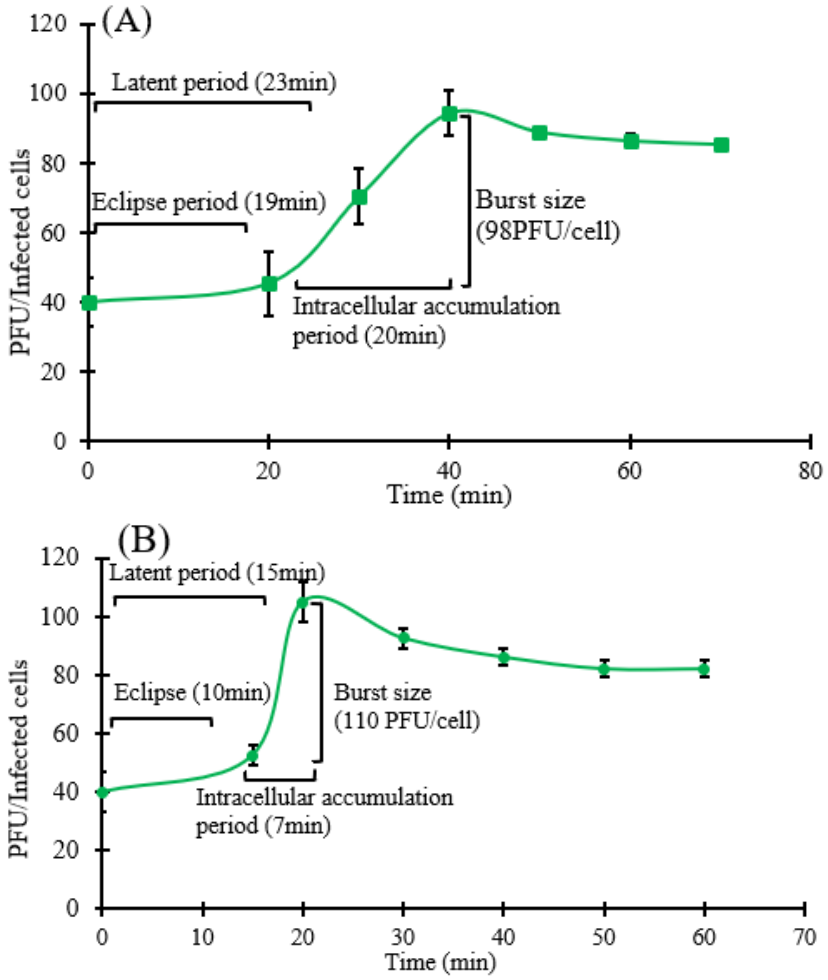

Figure 5. *One step growth curve of (A) KPO1 and (B) KPO2. Error bars represent mean value ± SD of triplicated experiments. Latent period (pre-infectious period): The period from the time of infection to the time of becoming infectious; Burst size: The average number of phage released per bacterium; Eclipse period: The time between infection by a bacteriophage and the appearance of mature virus within the cell*

The highest reduction of *K. pneumoniae* were observed at 0.1MOI for both phages and reached an OD value of 0.2 and 0.18 at 35 h following infection, respectively (*Fig. 6A* and *B*). A relatively lower level of reduction was recorded at 0.01 and 100 MOI, especially at the end of the infection pathway which may be due to re-growth of bacteria noticed at different time points. The instability of prolonged bacterial suppression generated by both phages at high or low MOI suggests evasion of phage resistant bacteria, a major obstacle in the biocontrol and/or therapeutic use of this phage (Garcia et al., 2008; Henrici De Angelis et al., 2021).

The lytic kinetics of a cocktail of KPO1 and KPO2

The *in vitro* lytic kinetics of the cocktail of two phages, KPO1 and KPO2, was evaluated against the host, *K. pneumoniae* strain INF079, at different time interval. According to the findings, the prepared phage cocktail significantly reduced the bacterial concentration from 0.4 to 0.12 at 38 h of post infection ($p < 0.05$) (*Fig. 7*).

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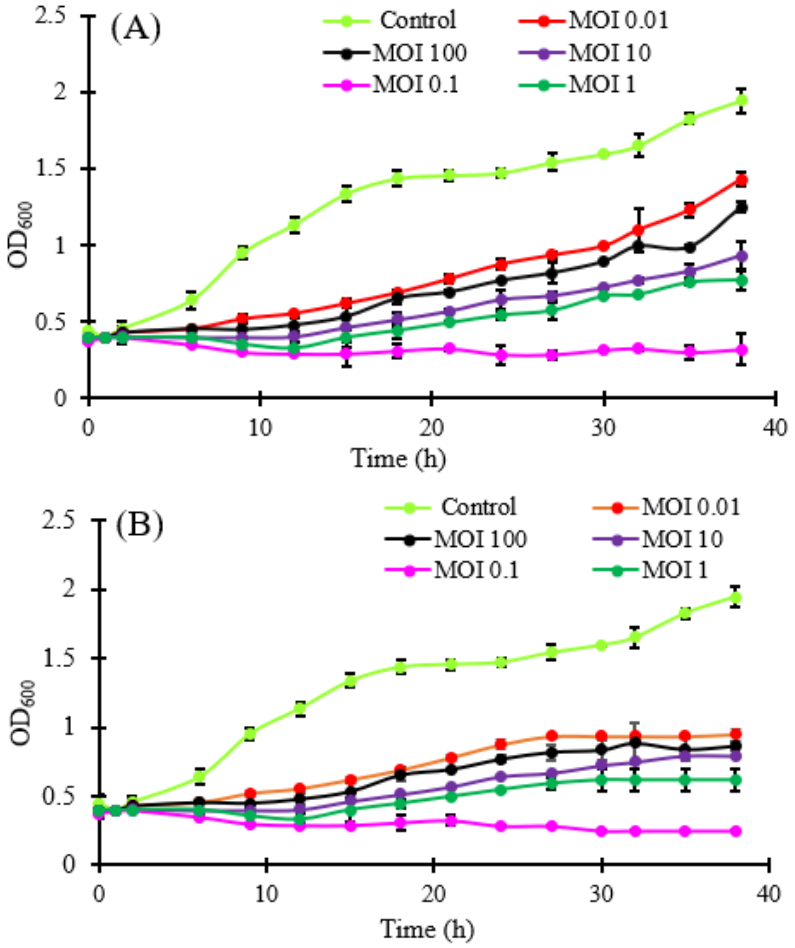

Figure 6. *The lytic kinetics of (A) KPO1 phage; (B) KPO2 phage. Error bars represent mean value ± SD of triplicated experiments. Two-way ANOVA and the Bonferroni post-hoc test were conducted using the GraphPad Prism software. Significant statistical differences were noted between the control and the respective phage-treated samples at different MOIs ($p < 0.05$)*

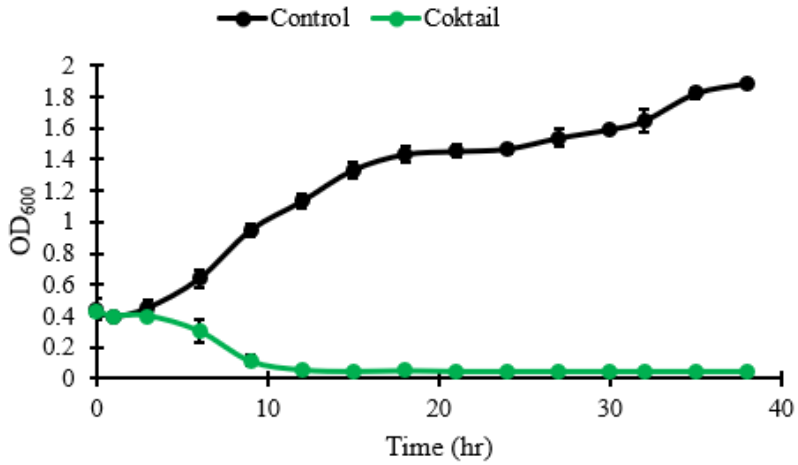

Figure 7. *The in vitro lytic kinetics of a cocktail of KPO1 and KPO2. Error bars represent mean value ± SD. Two-way ANOVA and the Bonferroni post-hoc test were conducted using the GraphPad Prism software. Significant statistical differences were noted between the control and the cocktail-treated samples ($p < 0.05$)*

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Host range

A total of 15 bacterial isolates were used to evaluate the host range of KPO1 and KPO2 (*Table 4*). The results showed that these phages lytic against for the tested *K. pneumoniae strains* (46.6%), while not for non- *K. pneumoniae* isolates. Hence, both phages displayed a relatively narrow host range compared to the previously isolated phages (*Table 4*). The narrow host spectrum of phages may be due to the limitations of the tested strains or the specificity of phage recognition sites. Our finding agrees with He et al. (2020).

Table 4. *Host range of KPO1 and KPO2*

Bacterial strain	Antimicrobial profile	Plaque assay results	
		KPO1	KPO2
K. pneumoniae (ATCC 700603)	MDR	+	+
K. pneumoniae (clinical isolate)	MDR	+	+
K. pneumoniae (clinical isolate)	MDR	+	+
K. pneumoniae (animal isolate)	MDR	+	+
Klebsiella oxytoca (ATCC 49131)	MDR	+	+
Klebsiella sp. strain Kp-8890 (OP102089.1)	MDR	+	+
Klebsiella sp. strain T36 (MW713441.1)	MDR	+	+
MRSA (ATCC 43330)	MDR	-	-
Enterococcus faecalis (ATCC 29212)	MDR	-	-
Shigella sonnei (ATCC 25931)	MDR	-	-
S. Enteritidis (CP016754.1)	MDR	-	-
S. Dublin (FJ997268.1)	MDR	-	-
S. Typhi (GU826683.1)	MDR	-	-
S. arizonae (CP000880.1)	NMDR	-	-
Pseudomonas aeruginosa ATCC 9027	NMDR	-	-
Total		**7/15(46.6%)**	**7/15(46.6%)**

Key: MDR: Multidrug resistant; NMDR: non-multidrug resistant; ATCC: American Type Culture Collection

Storage of phage cocktail

Stability of phage cocktail (original concentration = 1 x 10^{10} PFU/mL) at different storage temperature (37°C, 4°C, and -20°C) were evaluated for consecutive 6 months periods. Based on the results higher phage titer was recorded when the cocktail was stored at 4°C in comparison to the other two storage conditions (- 20°C and 25°C) but there was no statistical difference between them (*Fig. 8*). In comparison to the three storage conditions, at 6th month a lower phage titer (6 $\log_{10}$ PFU/mL) was recorded when the phage stored at -20°C. These findings were supported by the idea that storage condition is one of the factors which influence the titer of phages depending on the duration and the

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storage temperature implemented (Jończyk-Matysiak et al., 2019). According to Ackermann (2009) Caudovirales phages (phages which have tail) were the most stable to storage and exhibited the prolonged viability; some of them maintained stability even after 10–12 years at 4°C.

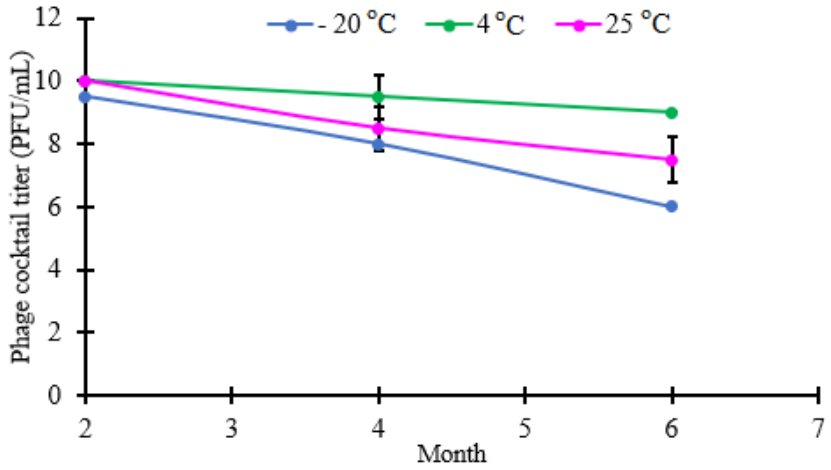

Figure 8. *Stability of a cocktail of KPO1 and KPO2 at different storage temperature for six months. Error bars represent mean value ± SD of three different experiments. Two-way ANOVA and the Bonferroni post-hoc test were conducted using the GraphPad Prism software. Significant statistical differences were not noted on the titer of the cocktail of the two phages at three different storage temperatures ($p > 0.05$)*

Conclusion

In this study, we successfully isolated a lytic phage from wastewater samples which showed clear inhibitory zones in the lawn of the host cell. They have showed good thermal and pH stability. Comparatively, phage KPO1 had good latent and burst size in comparison to phage KPO2. They have displayed a significant lytic activity against the host strain individually as well as in combination as a cocktail. However, the lytic potential of these phages should be evaluated in *in vitro* models. Besides, the isolated phages should be also tested for their lytic activity in combination with the conventional antibiotics.

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