Isolation and Characterization of Bacteriophage which Infected *Klebsiella pneumoniae*

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Abstract

Klebsiella pneumoniae is a clinically significant organism that has caused much public health concern. Due to the increasing prevalence of infections caused by multidrug-resistant *K. pneumoniae*, alternative therapies might be promising. The properties of host specificity, abundance in nature, and evolvability suggest bacteriophages might be a good candidate. In this study, we have isolated and characterized a lytic bacteriophage infecting *K. pneumoniae*, ØKN-2, which was isolated from wastewater. The transmission electron micrograph showed that ØKN-2 was classified as being in the *Myoviridae* family. Host-range determination revealed that ØKN-2 is specifically able to lyse 35.7% (5/14) of tested *K. pneumoniae*. Studies of the host range of different species showed that ØKN-2 unable to lyse other tested bacteria including *Acinetobacter baumannii*, *Peudomonas aeruginosa*, *Protues mirabilis*, *Enterococcus faecalis*, and *Escherichia coli*. Interestingly, ØKN-2 significantly reduced *K. pneumoniae* ATCC 700603 biofilm preformed in a dose dependent manner. Therefore, this study identified and tested bacteriophages that infect *K. pneumoniae* and proved the effectiveness of ØKN-2 against biofilm and host-specific characteristics which indicates that they are beneficial in the development of treatments against *K. pneumoniae* infections.

Keywords: *K. pneumoniae*, bacteriophages, biofilm, *Myoviridae*

Introduction

K. pneumoniae is an encapsulated Gram-negative bacterium that is a free-living organism in the environment, including in soil and surface waters and on medical devices (Rock et al., 2014). It can cause a wide range of diseases, including pneumonia, urinary tract infections (UTIs), bloodstream infections, and sepsis (Bengoechea & Pessoa, 2019). Nowadays, *K. pneumoniae* has been reported for increasing the prevalence of multidrug resistance which has led to the loss of effectiveness of antibiotics against infections (Paterson et al., 2004; Effah, Sun, Liu & Wu, 2020). Thus, the Centers for Disease Control and Prevention (CDC) of the USA designated *K. pneumoniae* as an urgent threat to public health (Solomon & Oliver, 2014). In addition, about 80% of *K. pneumoniae* nosocomial infections are multidrug-resistant strains for which the incidence of ESBL (extended-spectrum beta-lactamase) isolates ranges from 8% to 44% (Chhibber, Kaur & Kumari, 2008; Babapour, Haddadi, Mirnejad, Angaji & Amirmozafari, 2016). Moreover, it can form biofilms, an assemblage of surface-associated microbial cells that is enclosed in an extracellular polymeric substance matrix (Donlan et al., 2002). Several studies reported that biofilms can cause antibiotic resistance and are thus very difficult to treat (Breitbart, Wegley, Leeds, Schoenfeld & Rohwer, 2004; Nirwati et al., 2019). Therefore, there is a need for developing new strategies for the control of *K. pneumoniae* infection and biofilm prevention and removal.

Bacteriophages or phages are viruses that infect and replicate within specific bacteria. They are abundant on earth and play major roles in bacterial ecology, adaptation to novel environments, and in bacterial isolation and

pathogenesis (Breitbart et al., 2004; Vuotto, Longo, Balice, Donelli & Varaldo, 2014). Lytic or virulent phages infect the host bacterium, multiply, and kill their hosts. This property indicates that bacteriophages may be a good candidate to prevent bacterial infections. Many researchers have reported the effectiveness of using bacteriophages for the control of *K. pneumoniae* infection in animals (Chhibber et al., 2008; Cao et al., 2015). Taha, Connerton, Connerton and El-Shibiny (2018) demonstrated the efficiency of phage ZCKP1 to reduce bacterial counts and biofilm biomass and note that it shows potential as a therapeutic agent against *K. pneumoniae* infections of diabetics affected feet. Furthermore, phage 1513 has been shown to be a potential therapeutic agent in the treatment of pneumonia induced by multidrug resistance *K. pneumoniae* in mice by reducing the levels of *K. pneumoniae* and improving lesions in the lungs (Cao et al., 2015). Therefore, the objective of this study was to isolate *K. pneumoniae* bacteriophages and characterize the ability in various aspects such as morphology, bacterial host specificity, killing efficacy against the bacterial host, and biofilm degradation *in vitro*.

Methods and Materials

Bacterial strains and growth conditions

Bacterial strains used in this study included one strains of American Type Culture Collection (ATCC); *K. pneumoniae* ATCC 700603 (extended spectrum beta-lactamases (ESBL) producing *K. pneumoniae*), and thirteen clinical isolates of *K. pneumoniae* originated from the collection from the laboratory of Hua Chiew Hospital, Thailand and *K. pneumoniae* ATCC 700603 was obtained from the Faculty of Medical Technology, Rangsit University, Thailand. The other tested bacterial strains including *Acinetobacter baumannii*, *Peudomonas aeruginosa*, *Protues mirabilis*, *Enterococcus faecalis*, and *Escherichia coli* were also obtained from the laboratory of Hua Chiew Hospital, Thailand. All bacterial strains were grown under aerobic conditions in tryptic soy broth (TSB) or agar (TSA) at 37°C. The characteristics of all bacterial strains were described in Table 1.

Phage isolation and purification

K. pneumoniae specific bacteriophages were isolated from sewage samples from local wastewater treatment stations in Pathum Thani province, Thailand. Briefly, 100 µl overnight culture of *K. pneumoniae* 700603 was inoculated with a mixture of 20 ml sewage water and an equal volume of 2X TSB and incubated at 37 °C for 16-18 hr. Residual bacterial cells were removed with centrifugation at 4,000 × g for 20 min, and the supernatant was sterile using a 0.45 µm pore size filter (Millipore, USA) A spot test was performed for screening *K. pneumoniae* bacteriophages. Ten microliters of the filtrated sample were dropped onto *K. pneumoniae* ATCC 700603 cultures that were spread on TSA and incubated at 37 °C for 16-18 hr. A clear zone in the spotted area was selected for confirmation by a double-layer agar plaque assay. Briefly, 200 µl of selected filtrates was mixed with 50 µl *K. pneumoniae* ATCC700603 overnight culture in molten soft agar (0.4% TSA) and plated. Plaques were subjected to three rounds of purification according to standard procedures (Sambrook, Fritsch & Maniatis ,1989).

Transmission electron microscopy (TEM)

Bacteriophage morphology was determined by TEM at Vidyasirimedhi Institute of Science and Technology Wangchan Valley, Thailand. Briefly, one drop from the purified high titer 10^{14} pfu/ml bacteriophage stock was applied on copper grids and negatively stained with 1% uranyl acetate. Phage morphology was examined using

a J FEI Company/Netherlands. Based on their morphology, the phage was classified according to the guidelines of the International Committee on Taxonomy of Viruses (ICTV).

Phage host range testing

Purified bacteriophage at concentration of 10^8 pfu/ml was determined for their susceptibility and host range on the lawn of different *Klebsiella* spp. clinical isolates and other Gram-negative bacteria. Lytic characteristics were evaluated after 16-18 hr incubation at 37 °C; a clear zone in the spotted area indicated a positive result.

Killing efficacy of phages against *K. pneumoniae* 700603

The lytic ability of bacteriophage in different multiplicity of infections (MOIs) were assessed by growing *K. pneumoniae* ATCC 700603 until the optical density at 600 nm (OD600) = 0.2 with *circa* (ca.) 10^8 pfu/ml. Then, a bacteriophage with ca. 10^8 pfu/ml was added at MOI 0.1, 1.0 and 10. Bacterial growth in the bacteriophage-infected solution and the non-infected control was measured hourly using a spectrophotometer at OD 600 nm for 8 hr. The tests were determined in triplicate and the average OD 600 nm of the bacteriophage-infected solution and non-infected control was plotted in a graph.

Biofilm degradation assay

Biofilm degradation activity of bacteriophage on the *K. pneumoniae* ATCC 700603 biofilms was determined using the traditional biofilm assay as previously described with some modifications (Christensen,1989) Two hundred microliters of *K. pneumoniae* 700603 was incubated at 37 °C for biofilm formation at 24, 48 and 72 hr in 96-well polystyrene microtiter plates (MicroWell™ Plates, Nunc, Roskilde, Denmark). Unattached planktonic cells were then carefully removed and 10^9 pfu/ml bacteriophage stock was diluted in TSB and was added at MOI of 0.01,0.1, 1.0 and 10 and incubated for 24 hr. TSB without bacteria ws used as a negative control, while inoculated bacteria without phages served as a positive control. The biomass of preformed biofilm was quantified with 1% w/v crystal violet staining (Sigma, USA) for 15 min at room temperature. After removing the excess dye with PBS, the crystal violet was solubilized in 95% ethanol and left for 5 min to allow the stain to dissolve. The absorbance was measured using a plate reader at OD 620 nm. Media in each well were replaced every 24 ,48 and 72 hr. The experiments were performed in triplicate.

Statistical Analysis

In the killing efficacy of phages against *K. pneumoniae* 700603, each data point is presented as the mean from triplicate experiments, with standard deviations (± SD). Test and control sets at different MOIs of biofilm degradation assay at 24, 48 and 72 hr was compared using one way ANOVA with Dunnett's multiple comparisons test. Differences were considered statistically significant if p-value < 0.05. Analytical statistics were undertaken using GraphPad PRISM version 8.00 for Windows.

Results

Isolation of *K. pneumoniae* bacteriophages

One sample showed clear zones on the lawn of *K. pneumoniae* ATCC 700603. After confirmation by plaque assay and three rounds of purification, the isolated bacteriophage was named ØKN-2. ØKN-2 produced clear plaques on the lawn of *K. pneumoniae* 700603. It has a plaque size ranging from 2 to 3 mm in diameter (Figure 1)

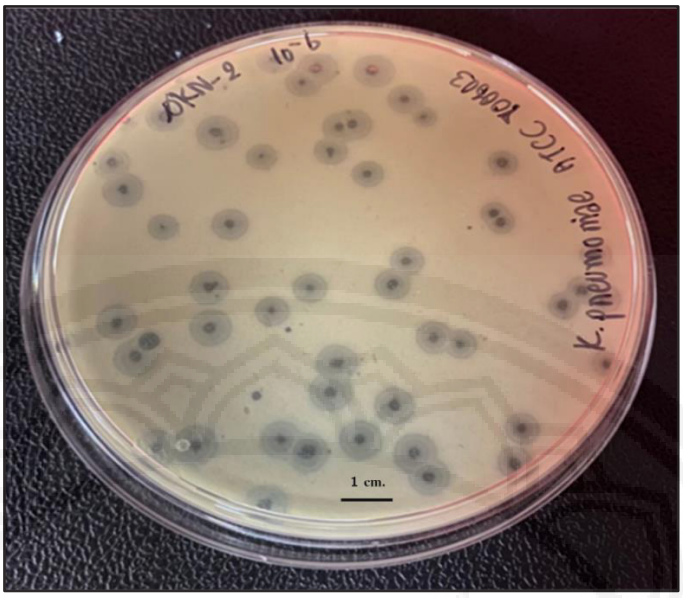

Figure 1 Plaque morphology of ØKN-2 on *K. pneumoniae* 700603 that were spread on TSA and incubated at 37 °C for 16-18 hr

Transmission electron microscopy (TEM)

Transmission electron microscopy showed that ØKN-2 had an icosahedral capsid with a diameter of ~90 nm and a contractile tail with collar and base plate which is typical of the morphology of phage in *Myoviridae* family (Figure 2). *Myoviridae* belong to the order of *Caudovirales* of tailed phages which contain dsDNA and have icosahedral heads (Ackermann, 2009).

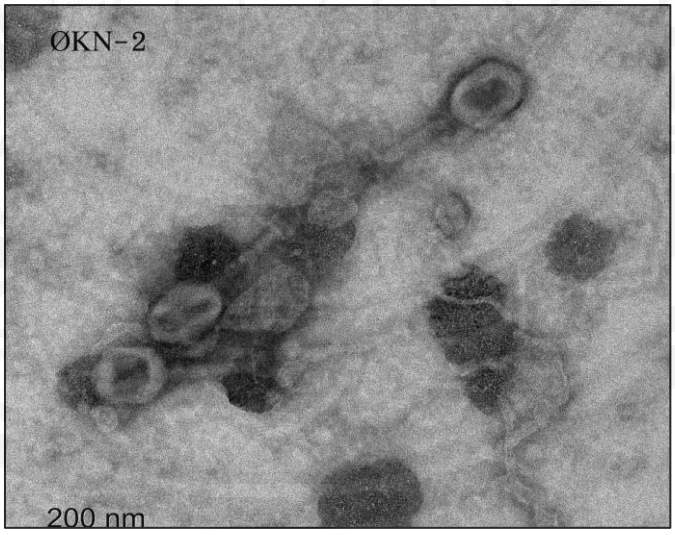

Figure 2 Transmission electron microscopy of ØKN-2. Bars, 200 nm

Phage host range testing

Host range tests of ØKN-2 were evaluated by a spot test with 14 *K. pneumoniae* clinical isolates from humans which showed that ØKN-2 had a narrow host range and was capable of lysing *K. pneumoniae* at 35.7%

(5/14) (Table 1). Other tested bacteria including *A. baumannii*, *P. aeruginosa*, *P. mirabilis*, *E. faecalis*, and *E. coli* were not lysed by ØKN-2, suggesting a specific capacity to infect *K. pneumoniae*.

Killing efficacy of phages against *K. pneumoniae* 700603

K. pneumoniae ATCC 700603 was treated with MOI 0.1, 1.0 and 10.0 of ØKN-2. The lytic activity of ØKN-2 showed the efficiency to clear lysis after 4 hr of infection at MOI of 10.0 with a reduction of the average OD 600 nm of infected culture observed to 0.000 until 8 hr, while the uninfected control was 0.8765 (Figure 3), suggesting the bacterium showed high susceptibility to ØKN-2.

Biofilm degradation assay

To determine the activity of ØKN-2 biofilm degradation, *K. pneumoniae* ATCC 700603 was grown in TSB in microtiter plates for 24, 48 and 72 hr, after which the biofilms were challenged with ØKN-2. Anti-biofilm activity of ØKN-2 was shown in both 24- and 48-hr biofilms. The OD 620 nm of the 24-hr biofilms showed significant reduction at MOI 1.0 (p=0.002) and 10.0 (p=0.002) while the 48-hr biofilm at MOI 10.0 also showed significant reduction (p=0.018) as compared to the untreated biofilm control (Figure 4). However, the reduction of OD 620 nm of 24-hr biofilms at MOI of 0.01 and 0.1, 48-hr biofilms at MOI of 0.1 and 1.0 and 72-hr biofilms at MOI of 1.0 and 10.0 were not statistically significantly different when compared to the untreated biofilm control. (Figure 4). In addition, ØKN-2 could not degrade 48-hr biofilm at MOI of 0.01 and 72-hr biofilms at MOI of 0.1.

Table 1 Host range of ØKN-2 tested on *Klebsiella* spp. and other bacterial species by spot test

Bacteria	Characteristics	Source	Number of isolates	Activity
K. pneumoniae (KN01)	Non-ESBL	Clinical isolation	1	-
K. pneumoniae (KN02)	Non-ESBL	Clinical isolation	1	-
K. pneumoniae (KN03)	Non-ESBL	Clinical isolation	1	-
K. pneumoniae (KN04)	Non-ESBL	Clinical isolation	1	-
K. pneumoniae (KN05)	Non-ESBL	Clinical isolation	1	-
K. pneumoniae (KN06)	Non-ESBL	Clinical isolation	1	-
K. pneumoniae (KN07)	Non-ESBL	Clinical isolation	1	-
K. pneumoniae (KN08)	ESBL	Clinical isolation	1	+
K. pneumoniae (KN09)	Non-ESBL	Clinical isolation	1	-
K. pneumoniae (KN10)	Non-ESBL	Clinical isolation	1	+
K. pneumoniae (KN11)	Non-ESBL	Clinical isolation	1	-
K. pneumoniae ATCC 700603	ESBL	ATCC	1	+
K. pneumoniae KN1C	Non-ESBL	Clinical isolation	1	+
K. pneumoniae KN2C	Non-ESBL	Clinical isolation	1	+
P. aeruginosa	-	Clinical isolation	1	-
P. mirabilis	-	Clinical isolation	1	-
A. baumannii	-	Clinical isolation	1	-
E. faecalis	-	Clinical isolation	1	-
E. coli ATCC 25923	-	ATCC	1	-

+: lysis; —: no lysis: ESBL: ESBL producing *K. pneumoniae*; Non-ESBL: Non-ESBL producing *K. pneumoniae*

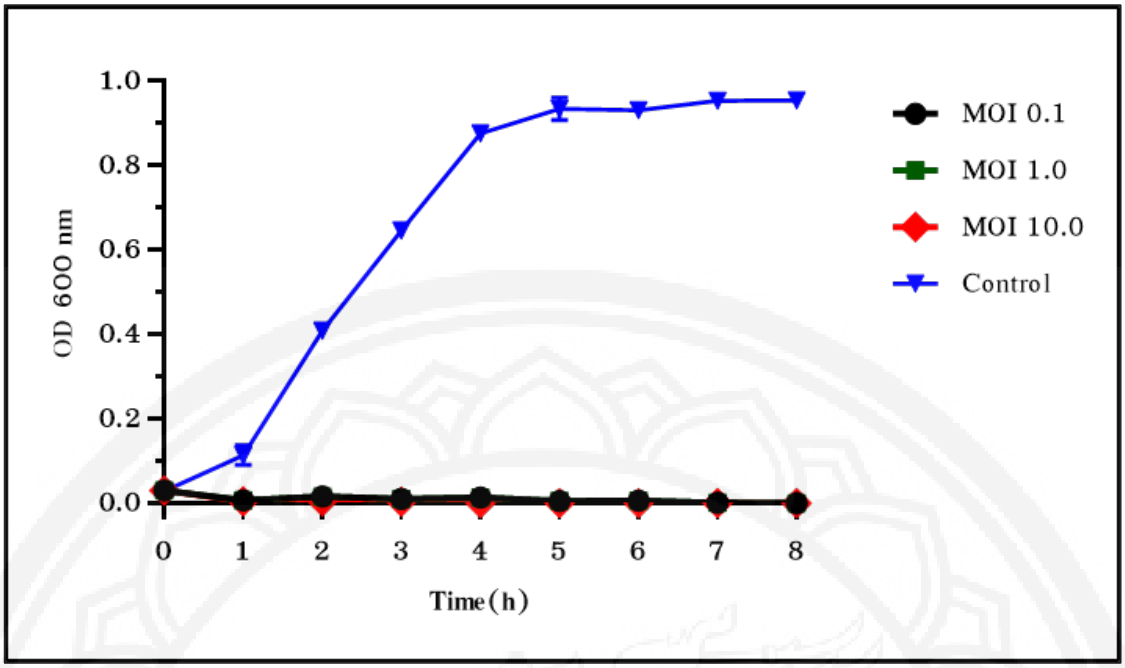

Figure 3 The efficiency of ØKN-2 against *K. pneumoniae* 700603 *in vitro* at MOI of 0.1 (●), 1.0 (■),10.0 (◆) and non-phage infected control (▼) was determined at OD 600 nm. Optical density at 600 nm was measured every hr up to 8 hr. All of the tests were performed in triplicate

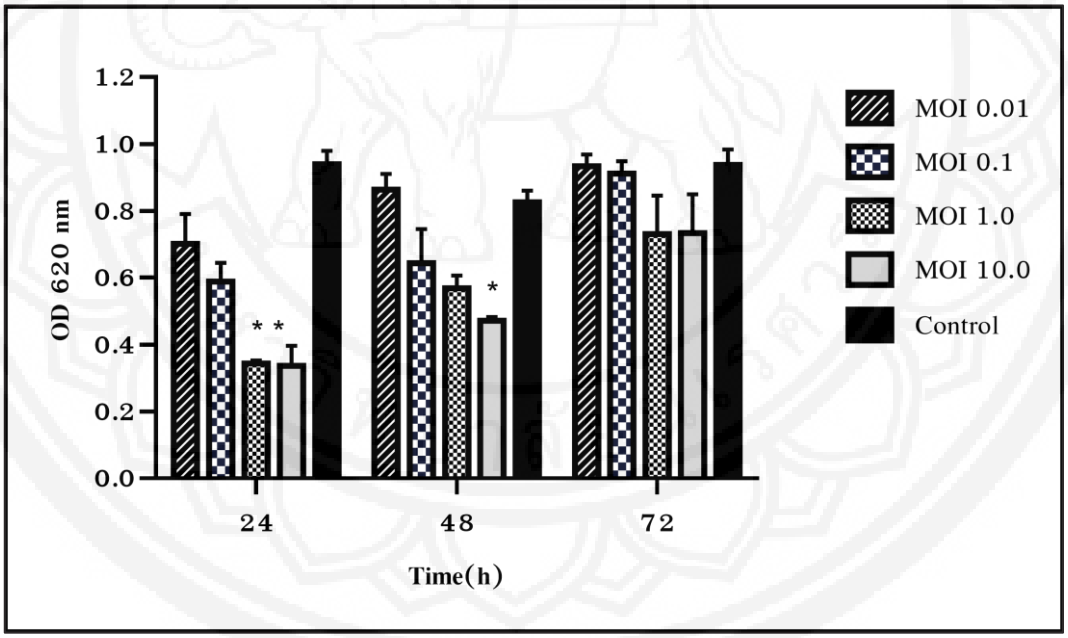

Figure 4 Effect of ØKN-2 on *K. pneumoniae* 700603-produced biofilm degradation. The established biofilm was infected with ØKN-2 at MOI of 0.01, 0.1, 1.0 and 10.0. Non-phage treated biofilm was used as a positive control. OD at 620 nm was measured at 24, 48 and 72h. Each data point is the mean from 3 experiments. Error bars represent standard deviation. The asterisk (*) on the top of the bar is an indication of statistically significant biofilm reduction compared with non-phage treated control

Discussion

K. pneumoniae is a clinically important bacterial pathogen that is most commonly isolated from biofilms (Donlan, 2001; Donlan & Costerton, 2002; Parasion, Kwiatek, Gryko, Mizak & Malm, 2014). Recently, the development of alternative approaches to treat *K. pneumoniae* infections has gained increasing attention due to the high prevalence of multi-drugs resistant *K. pneumoniae* and the frequent failure of antibiotic therapy. The application of lytic bacteriophage appears to be one of the promising treatment strategies for *K. pneumoniae* infection (Lin et al., 2014; Pan et al., 2019; Wang et al., 2019).

In this study, *K. pneumoniae* bacteriophage, ØKN-2 was isolated from sewage water at local wastewater treatment stations in Pathum Thani province, Thailand. ØKN-2 is belonging to the *Myoviridae* family which is similar to previous isolated *K. pneumoniae* phage (Kpp95) isolated from hospital samples (WU, Chang, Yen, Yang & Tseng, 2007). Host- range determination revealed that ØKN-2 is able to lyse 35.7% (5/14) of the tested both ESBL and non-ESBL *K. pneumoniae* isolated. This characteristic is also observed in *K. pneumoniae* phage, phage KP1801, which showed a host range with infecting 50% (10/20) of the tested *K. pneumoniae* ESBL strains (Wintachai, Naknaen, Thammaphet, Pomwised, Phaonakrop, Roytrakul & Thammaphet, 2020). Similarly, Bhetwal, Maharjan, Shakya, Satyal, Ghimire, Khanal and Parajuli (2017) demonstrated that *K. pneumoniae* phages, ØKN-1, ØKN-2, ØKN-3, ØKN-4, and ØKN-5, had a narrow host range and show ability to lyse the tested *K. pneumoniae* ESBL and *Klebsiella* spp. In addition, ØKN-2 could not lyse *K. pneumoniae* clinical isolates KN01, KN02, KN03, KN04, KN05, KN06, KN07, KN09, and KN11, or *P. aeruginosa*, *P. mirabilis*, *A. baumannii*, *E. faecalis*, and *E. coli*. The possible explanation of this characteristic may be that these bacteria do not have surface receptors as the recognition attachment site of ØKN-2 (Stone, Campbell, Grant and McAuliffe, 2019). ØKN-2 also showed the efficiency to eradicate the host bacterium *in vitro* by clear lysis at MOI of 0.1, 1.0 and 1.0. Interestingly, after 4 hr of infection at MOI 10 show a marked reduction of OD600 nm of ØKN-2 infected culture to zero until 8 hr, suggesting it has the potential to be used as a bacterial treatment tool. ØKN-2 at MOI 10.0 has the potential to inhibit biofilm formation at 24 and 48 hr with a statistically significant reduction ($p = 0.002$ and $p = 0.018$, respectively) when compared to the untreated biofilm control, suggesting this bacteriophage could be applied to control and inhibit *K. pneumoniae* biofilm formation. This activity was similar to previous study. *K. pneumoniae* phage (Kpp95) has the efficiency to remove ESBL- *K. pneumoniae* biofilm with significant reduction at 24 and 48 hr post treatment (Wintachai et al.,2020). The mechanism for phages to inhibit *K. pneumoniae* biofilm formation might be due to phages clearing the bacterial biofilm and lysing the cells by encoding lyase (Bruessow, 2013). Another possibility is that phages expressing enzymes that degrade extracellular polymers, destroying the polysaccharide matrix and proteins in the biofilm that encapsulate the bacteria, clearing the bacterial protective barrier, and then entering the biofilm to destroy the bacteria (Tian, Li, Nazir & Tong, 2021). However, anti-biofilm activity of ØKN-2 at 72 hr did not show significant inhibition of biofilm formation (p-value ≥ 0.05), suggesting it has the efficacy to inhibit biofilm formation with range from 24-48 hr. The use of phages for biofilm control has been studied for a wide range of organisms and applications. For example, Chhibber et al. found that mixed-species biofilms of *K. pneumoniae* and *P. aeruginosa* could be disrupted by a cocktail of phages specific to each host (Chhibber, Bansal & Kaur, 2015). The same researchers also demonstrated that the combination of the *K. pneumoniae* bacteriophage B5055 together with antibiotic could enhanced biofilm removal capacity than with bacteriophage

alone (Chhibber, Nag & Bansal, 2013). Other researchers applied and investigated the effect of pretreating hydrogel-coated catheters with *P. aeruginosa* phages on biofilm formation by *P. aeruginosa* in an *in vitro* model. The results showed that the pretreatment of catheters with cocktail *P. aeruginosa* phages reduced the 48-hr mean biofilm cell density by 99.9% (Fu et al., 2010). These results suggest the potential of applying phages, especially phage cocktails, to the surfaces of indwelling medical devices for mitigating biofilm formation by clinically relevant bacteria. Therefore, the use of ØKN-2 in combination with antibiotics or in combination with other *K. pneumoniae* phages (phage cocktails) might be possible for biofilm removal. The use of phage cocktails allows more bacteria to be controlled, so more *K. pneumoniae* phages could be used for the treatment of *K. pneumoniae* infections. Further experiment should be conducted to assess this activity and characterized the genome of this bacteriophage to establish its potential for *K. pneumoniae* application treatment.

Conclusion and Suggestions

In our present study, we isolated and characterized the *Myoviridae* family bacteriophage, ØKN-2, which infects *K. pneumoniae* and demonstrated its significance as a treatment against *K. pneumoniae* infections and demonstrating the ability of ØKN-2 against *K. pneumoniae* in both planktonic and biofilm, and their host-specific characteristics.

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