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Isolation and characterization Phage UPM1705 against multi-drug resistant *K. pneumoniae* 1705

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Abstract. The rise in in the number of drug-resistant bacteria that can resist almost all kinds of antibiotics is due to the overuse of these antibiotics (e.g., carbapenems). Thus, there is a need to find an alternative to antibiotic treatment such as the use of phages. In this study, phage UPM1705 was isolated from a polluted lake which can lyse its host *Klebsiella pneumoniae* ATCC BAA-1705. Based on morphological appearance from transmission electron microscopy, UPM1705 belongs to *Caudovirales* (*Myoviridae*). UPM1705 can reach a titer of 10^7 PFU/ml based on the double-layer method. It has a burst size of 298 PFU/bacteria cell and a latent period of 80 min, a rise period of 75 min, and adsorption time of 20 min based on a one-step growth curve assay using an MOI of 0.02. It was stable from 4°C to 80°C and retained its functionality at pH between 4 to 11, with pH of 7 being the optimum pH for the phage growth. The efficiency of UPM1705 was tested via a turbidity assay at MOI of 0.02, 0.2, and 2. UPM1705 was able to clear the turbidity of the host bacteria culture at all of these three MOIs. Thus, UPM1705 has the potential to be used for phage therapy.

Keywords: *Klebsiella pneumoniae*, bacteriophage, phage therapy, multi-drug resistance, *Caudovirales*

INTRODUCTION

Overuse of antibiotics in hospitals, agriculture and other settings has led to an increase in the number of drug-resistant bacteria (Ventola, 2015). In addition, it was predicted that 10 million people would die annually by 2050 due to the increase in drug-resistance bacteria (O'Neill, 2014). Interestingly, the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) are responsible for hospital-acquired infections (Santajit & Indrawattana, 2016; Shallcross & Davies, 2014). ATCC strain *K. pneumoniae* 1705 which belongs to this group of pathogens is a Gram-negative bacterium that has developed antibiotic resistance even to carbapenem due to the presence of the bla_{KPC} gene (DeLeo *et al.*, 2014). According to Jacob *et al.* (2013), the percentage of carbapenem resistant *K. pneumoniae* had increased from 1.6% to 10.4% in USA in 2012, while in India, the number of carbapenem resistant *K. pneumoniae* has risen from 9% in 2008 to 44% in 2010 (Veeraraghavan *et al.*, 2017). Moreover, according to the European Centre for Disease Prevention and Control (ECDC) (2019) the number of carbapenem resistant *K. pneumoniae* among the European population in 2017 was 7.2%. In India for instance, an increase in the mortality rate observed

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in hospitals from October 2014 until May 2015 was due to infection by *E. coli*, *K. pneumoniae*, *P. aeruginosa and A. baumannii* resulting in pneumonia, sepsis, meningitis, otitis, and gonorrhea (Chandy & Bhattacharya, 2015). Interestingly, it appears that the use of phage therapy as complementary to treatment with antibiotics was able to extend the effectiveness of the antibiotics to treat these diseases (Torres-Barceló & Hochberg, 2016).

Bacteriophages or phages are viruses that have unique properties such as being highly selective toward their hosts, auto-dosing capability, low toxicity, and have the ability to release capsule depolymerases that can break up the Gram-negative bacterial capsule (Principi *et al.,* 2019) compared to antibiotics (Romero-Calle *et al.,* 2019). Furthermore, this will allow the phage to be used as an alternative to antibiotics without harming the normal flora and beneficial microbes of the host due to its high specificity (Essa *et al.,* 2020). Many studies were conducted in testing the efficiency of the phages (e.g. vB_KpnM-Teh.1, vB_KpnS_Kp13, VTCCBPA43, and Phage 1513) (Sasani & Eftekhar, 2020; Horváth *et al.,* 2020; Anand *et al.,* 2019; Cao *et al.,* 2015). However, due to the high specificity of phages which is usually strain specific, a cocktail of phages is usually needed to ensure efficient killing of different strains of bacteria, thus leading to the need for more and more phages to be isolated and characterised. Therefore, in the present study, phage UPM1705 was isolated against *K. pneumoniae* 1705, characterised, and tested for its efficiency to lyse the bacteria *in vitro*.

MATERIALS AND METHODS

Isolation and growth of phage

Phage UPM1705 was isolated from polluted lake water (3°00'19.3"N 101°42'52.6"E) and tested against *K. pneumoniae* 1705 as host. Approximately 50 ml of the lake water was centrifuged for 10 min at 6000 ×g at 4°C. The supernatant was filtered using a 0.45 µm membrane filter (MILLEX-HN, nylon, 33 mm). In order to enrich the phages, 100 µl of *K. pneumoniae* 1705 (OD_{600nm}=0.6) in 0.2 M $CaCl_2$ was added to 10 ml of the filtered supernatant before being made up to 30 ml with 5 × LB broth (final concentration of 3.33× LB) (Merck, Germany) and shaken overnight at 37°C (Jordan *et al.*, 2011). The mixture was centrifuged for 10 min at 8000 ×g at 4°C and the supernatant filtered again. The presence of phages was confirmed through spotting onto a soft overlay agar containing the host bacteria. Phages resulting with a positive spot test were then subjected to dilution and plated using the double layer agar method (Kropinski *et al.*, 2009). Single plagues formed on plates were cut from the double layer agar and placed into SM buffer [100 mM NaCl, 8 mM $MgSO_4 \cdot 7\ H_2O$, 50 mM Tris-Cl (1 M, pH 7.5), and 0.01 g of gelatin)] overnight at 4°C, filtered and subjected to propagation on double layer agar again the next day. This was repeated three times to ensure purified phages were obtained which showed uniformed distribution of plaques per plate. High-titered phages was prepared from plates flooded with SM buffer, which was centrifuged and filtered. The filtrate was precipitated using 1.5 mM of NaCl and 12% of PEG-8000 to phage lysate and left at 4°C overnight. The next day, the lysate was centrifuged at 12,000 ×g for 10 min and the pellet was resuspended in 1 ml of sterile SM buffer with 1 ml of 1 M KCl. After being left on ice for 1 h, the mixture was re-centrifuged at the same speed and temperature as mentioned previously. The supernatant which consisted of purified concentrated phage was stored at 4°C for use while the pellet which consisted of KCl bound to PEG was discarded. The phage titer was measured using the double layer agar method.

Morphological appearance examination of UPM1705 using Transmission Electron Microscopy

Transmission electron microscopy (TEM) was used to determine the order and family of phage belonging in accordance to the International Committee on Taxonomy of Viruses (ICTV) which is responsible for classification of viruses. Sample preparation was done via purifying UPM1705 on 38% sucrose gradient in Beckman Coulter Class S ultracentrifuge using SW 40 Ti rotor at a speed of 288 000 ×g for 4 h at 4°C (Hurwitz *et al.*, 2012). Then, thin carbon support films were prepared by sublimation of a carbon thread onto a freshly cleaved mica surface. Phage was adsorbed onto the carbon film and negatively stained with 2% (w/v) aqueous uranyl acetate, pH

5.0. The sample was visualized using JEM-2100F field emission electron microscope, 200 kV FE (Field Emission). Morphology of phage was identified based on Goodridge *et al.* (2003) and Harrach *et al.* (2011) and in accordance to International Committee on Taxonomy of Viruses (ICTV).

Bacteriophage one-step growth curve

The one-step growth curve was performed to determine the adsorption rate, burst size, latent period, and time required to reach its burst size for one life cycle by the phage within the host. UPM1705 was used to infect the host bacteria at MOI of 0.02 (Kutter & Sulakvelidze, 2004) and grown at 37°C. Aliquots of the mixture were then assayed quantitively at every 15 min over 185 min. The plaques were counted via the double-overlay agar method according to Kutter & Sulakvelidze (2004).

Temperature and pH stability assays

The thermal stability of UPM1705 was determined by incubating 1 ml of UPM1705 at 10^3 PFU/ml in SM buffer from 4°C till 80°C, and then the titer of the phage was measured via the double-overlay agar method. Similarly, for pH stability, 1 ml of UPM1705 at 10^3 PFU/ml in LB was incubated at pH of 2, 4, 7, 11, and 14 for 1 h at 37°C before being titered using the double-layer agar overlay method (Taj *et al.*, 2014).

Host range of phage UPM1705

The host range of UPM1705 was determined according to D'Andrea *et al.* (2017). The phage was tested against multiple sub-species of *K. pneumoniae* (IMR-1 non-drug resistant strain, ATCC BAA-2146, ATCC BAA-1705, A01 until A18, and A20), multi-drug resistant *Staphylococcus aureus* 10, and *Escherichia coli* (ATCC 25922 and Top 10). Ten µl of the phage were spotted onto an overlay agar containing the bacterial host. The agar plates were incubated overnight at 37°C.

Effectiveness of UPM1705 against Klebsiella pneumoniae 1705 in vitro

The effectiveness of UPM1705 to kill *K. pneumoniae* 1705 was determined *in vitro*. Fifty µl of *K. pneumoniae* 1705 at the log phase was mixed with the same amount of phage UPM1705 using three different MOIs of 0.02, 0.2, and 2, followed by incubation by shaking at 37°C and 180 rpm. An aliquoted amount (1 ml) of sample was removed after every 30 min for measurement at OD_{600nm} using spectrophotometry (Rajnovic *et al.*, 2019).

DNA isolation of UPM1705 and determining the genomic nature of the phage

The phage genomic DNA was extracted using a modified conventional method (Sambrook & Russell, 2006). The purity and concentration of the phage DNA were determined using NanoDrop spectrophotometer (Implen GmbH, Schatzbogen 52, D-81829, Munich, Germany) (Zeman *et al.*, 2017). The genomes were then treated with DNAse I and RNase A to determine whether the genome is DNA or RNA. The DNA isolated was run through 0.7% agarose gel through electrophoresis at 95V for 30 min to determine the approximate size of the DNA.

Statistical analysis

The descriptive statistical analysis was conducted via SPSS v.23 Chicago: SPSS Inc. One-Way ANOVA was performed followed by post-hoc multiple comparison using Tukey HSD test to determine differences in the phage titer between different treatments. Statistical significance was assumed when $p<0.05$. The actual measured data were used as points to draw the graph and the error bars standard deviations (n=3).

RESULTS

Morphological appearance examination of UPM1705 using Electron Microscopy

The TEM micrographs revealed that UPM1705 had tails which were contractile with a length of 128.1±0.8 nm, and a width of 19.1±4.1 nm (Figure 1). Based on these observations, it was concluded that UPM1705 belonged to *Myoviridae* consistent with the T4-Like viruses' family. UPM1705 also had a large and elongated head with a size of 78.88±1.6 nm which is a distinguishing feature for the *Myoviridae* (King *et al.*, 2012).

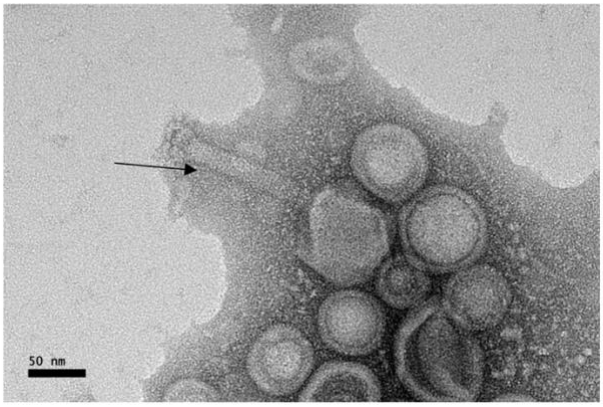

Figure 1. TEM micrograph of Phage UPM1705, scale bar, 50 nm that indicates belonging to *Myoviridae* which as shown by the arrow head is due to presence of a contractile tail.

One-step growth curve of UPM1705

The results were obtained at MOI of 0.02. As shown in Figure 2, UPM1705 has a burst size of 298 PFU/bacteria cell, a latent time of 80 min, a rise period of 75 min, and an adsorption time of 20 min.

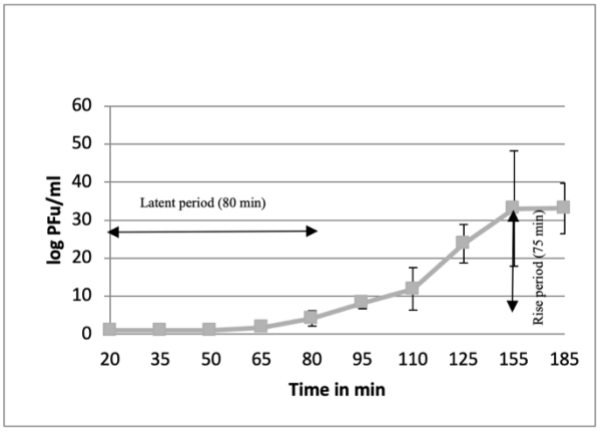

Figure 2. One-step growth curve of UPM1705. The phage has a latent period of 80 min, rise period of 75 min, and 155 min while releasing 298 virus/bacterial cell. The experiment was done in triplicates. Error bars are standard deviations (n=3).

Temperature, pH stability test, and host range of UPM1705

The survivability and stability of phage were assessed at different temperatures. The experiment revealed that UPM1705 was stable from 4°C till 80°C (Figure 3a). The optimum pH for UPM1705 was 7 although it was still able to survive between pH of 4 to 11 (Figure 3b). Phage UPM1705 was shown to be very specific to its host since none of the other tested strains showed the presence of plaque formation.

Effectiveness of UPM1705 against Klebsiella pneumoniae 1705 in vitro

The result showed the efficiency of the UPM1705 in lysing the host bacteria by turbidity reduction as measured spectrophotometrically at OD_{600nm}. The host bacterium growth was monitored when treated with the phage. In all MOIs used in the assay, the time required for UPM1705 to start lysing its host was 180 min while the OD_{600nm} reading was almost zero after 210 min at MOI of 0.2 and 240 min for the rest of the MOIs (Figure 4).

Nature of phage UPM1705 genome

The *Caudovirales* possess DNA genomes. In order to validate that UPM1705 belongs to this order, the genome of UPM1705 was extracted and treated with DNAse I and RNase A. Degradation of the genome with DNase A showed that UPM1705possess DNA genomic material as shown in Figure 5.

DISCUSSION

Phage therapy is one of the alternatives that can combat drug-resistant bacteria, especially when antibiotics have failed in the treatment (Lin *et al.,* 2017; Chan *et al.,* 2013). This potential alternative treatment has attracted more scientists to discover, isolate, and characterise phages that are suitable for therapeutic purposes (Rios *et al.,* 2016). In order for phages to be used for therapeutic purposes, there are some guidelines required to determine their suitability for therapeutic purposes (Hyman, 2019).

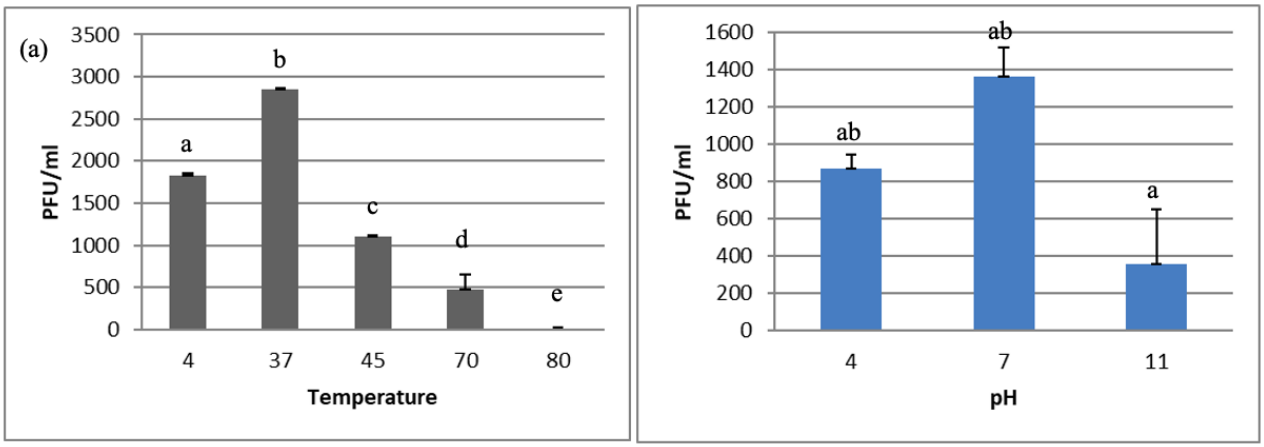

Figure 3. Growth characteristic of UPM1705 at different temperatures and pH. (a) The optimum temperature of UPM1705 was 37°C while able to form plaques at 80°C and (b) the optimum pH for UPM1705 was 7. The experiment was done in triplicates. Bars with different letters indicate significant difference at $p<0.05$ (Tukey HSD post-hoc test)Error bars are standard deviations (n=3).

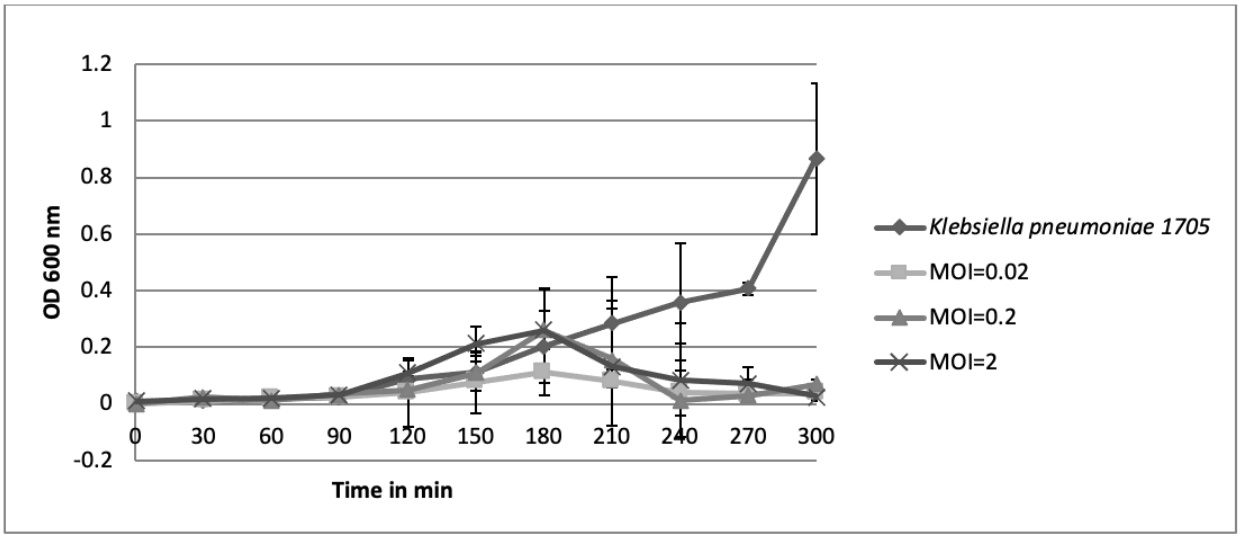

Figure 4. Turbidity assay of UPM1705 against *K. pneumoniae* 1705. UPM1705 was co-cultured with *K. pneumoniae* 1705 at MOI of 0.02, 0.2, and 2, respectively at 37°C. *K. pneumoniae* 1705 was grown as control. UPM1705 was able to lyse its host at 180 min in all MOIs. The experiment was done in triplicates. Error bars are standard deviations (n=3).

In the present study, UPM1705 was isolated from a polluted lake and was shown to be able to lyse *K. pneumoniae* 1705. *Myoviridae* family belongs to *Caudovirales* order, most of the phages belong to the order *Caudovirales* (ds-DNA) which consist of 96% of all known phages that possess a tail. *Myoviridae* family is distinguished from the other family due to the presence of a contractile tail. Based on ICTV, UPM1705 belongs to *Myoviridae* based on its DNA genome and the presence of the contractile tail presented in the myograph along with the presence of an elongated head (King *et al.*, 2012; Duda, 2020). Phages that can be used for therapeutic purposes ideally has a fast one-step growth curve Unfortunately, the time required for UPM1705 to be released from its host was 155 min; indicating that UMP1705 has a slow-release (Hyman, 2019). Nevertheless, this criterion does not exclude this phage to be used for therapeutic purposes since UPM1705 could efficiently lyse its host within 180 min via turbidity assay (Necel *et al.*, 2020). UPM1705 has an optimum temperature of 37°C and pH of 7 while there was a 35.79% drop in PFU count at 4°C, 61.4% at 45°C, 83.12% at 70°C, and highest lost is at 99.99% at 80°C in comparison to the optimum

temperature (37°C). These again emphasizes the potential therapeutic capability of UPM1705 since *K. pneumoniae* infects the lungs and causes pneumonia at the body temperature (37°C) and at pH 7, respectively (Cao *et al.*, 2015). Interestingly, UPM1705 was still viable at 70°C and 80°C; this might be perhaps due to the formation of cross-links within the phage capsid proteins or due to mutations at the gene G protein sequences presented in some phages (φ X174 Mutant) (Caldeira & Peabody, 2007; Kadowaki *et al.*, 1987). Phage was shown to be very specific (narrow host range) to its host; therefore, UPM1705 must be used within a phage cocktail to be used for therapeutic purposes. In addition, UPM1705 was specific to its host bacteria and does not harm normal flora unlike antibiotics which kill both pathogenic and normal flora bacteria (Loc-Carrillo *et al.*, 2011). For further consideration of UPM1705 to be used for therapeutic purposes, additional criteria such as genome sequence analysis for the presence of toxin and drug resistance genes need to be tested (Hyman, 2019). Likewise, cytotoxic test on zebrafish larvae should also be carried out (Fazry *et al.*, 2018).

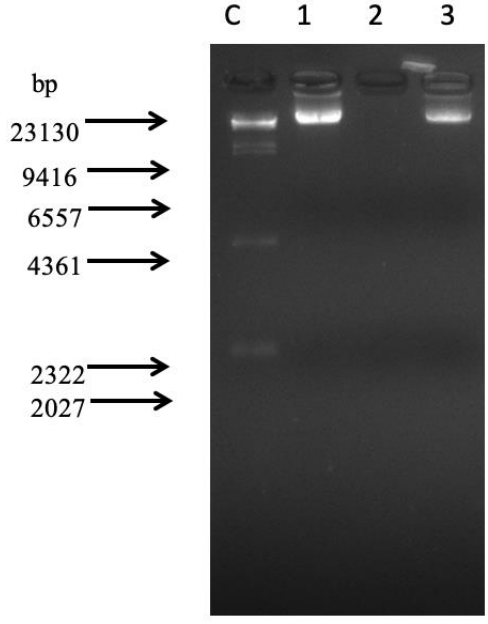

Figure 5. Agarose gel electrophoresis of phage UPM1705 genomes digested with DNase 1 and RNase A. Lane C: λ DNA-*Hind*III molecular markers, lane 1: UPM1705 DNA (control); lane 2: UPM1705 DNA with DNase I; lane 3: UPM1705 with RNase A; Agarose gel electrophoresis (0.7% gel) was performed at 95 V for 30 min. The absence of the DNA bands in lane 2 confirms that the genome of UPM1705 is a DNA virus.

CONCLUSION

In conclusion, the continuous increase in multi-drug resistance had led us to search for an alternative treatment that includes phage therapy. In the present study, the phage UPM1705 that belong to the *Myoviridae* family has shown to lyse *K. pneumoniae* 1705 *in vitro* and was resilient at high temperatures. This shows that UPM1705 has therapeutic potential against multidrug resistance pathogen such as the *K. pneumoniae*.

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