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Isolation and characterisation of phages targeting clinical *Pseudomonas aeruginosa* carrying virulence genes

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ABSTRACT

Aims: The aim of the study was to isolate and characterise bacteriophages specific to *Pseudomonas aeruginosa* carrying virulence genes.
Methodology and results: Four clinical strains of *P. aeruginosa* CL1, CL2, CL3 and CL4 were obtained from Queen Elizabeth Hospital, Kota Kinabalu, Sabah. The bacterial strains were screened for virulence genes *exoS*, *toxA* and *oprI* and biofilm production. Six *P. aeruginosa* specific bacteriophages, namely PAtk1, PAtk2, PAtk3, PAtk4, PAtk5 and PAtk6, were isolated from Tasik Kejuruteraan, Universiti Kebangsaan Malaysia. These bacteriophages were screened for lytic spectrum against *P. aeruginosa* and two species of Enterobacteriaceae (*Escherechia coli* and *Salmonella* Typhi). PCR results showed that all strains possessed *exoS*, *toxA* and *oprI* genes except CL2 that lacked *exoS*. Nevertheless, it was CL2 that produced the highest biofilm density. Further, based on Transmission Electron Microscopy, PAtk15 and PAtk6 were classified into the family Myoviridae and Siphoviridae, respectively. Among all six isolated phages, only PAtk4 and PAtk6 showed the broadest lytic spectrum in which lytic activity was observed against all clinical *P. aeruginosa* strains.
Conclusion, significance and impact of study: In this study we reported the isolation of six bacteriophages from Myoviridae and Siphoviridae that are specific to *P. aeruginosa* possessing *exoS*, *toxA* and *oprI* genes. Bacteriophages Patk4 and PAtk6 were able to infect all four strains of *P. aeruginosa*, making these phages potential agents in combating infections by the bacterium.

Keywords: Myoviridae, Siphoviridae, *exoS*, *toxA*, *oprI*

INTRODUCTION

Pseudomonas aeruginosa causes various nosocomial infections, including urinary tract infections, pneumonia, infections of burns and bacteremia (Holguín *et al*., 2015). Infections by this pathogen are frequent in patients with immune deficiency or diabetes. It is the primary pathogen responsible for a high percentage of death due to sepsis (Young, 1984). The bacterium is the most common hospital-acquired infecting agent and leading cause of respiratory infection in cystic fibrosis patients (Bendiak and Ratjen, 2009). Infections tend to become chronic when the infecting strain converts to colonisation mode, which includes production of biofilms that are hindered by attacks from antibodies, host phagocytes and some antibiotics, promoting development of resistant variants (Breidenstein *et al*., 2011). A recent study mentioned the close relationship between biofilm formation and the bacterium drug resistance pattern in which Extended Spectrum Beta-Lactamases (ESBL)-producing *P. aeruginosa* that form biofilms tend to be more pathogenic compared to its non-ESBL producing counterpart (Ullah *et al*., 2017). The resistant pathogen may gain access to the bloodstream and subsequently cause death due to the production of endo- and exotoxins (Liu, 1974). In addition to their intrinsic resilience towards antibiotic treatment, *P. aeruginosa* are increasingly found to contain multidrug-resistant plasmids (Nordmann *et al.*, 2007). There is also a variety of virulence factors produced by *P. aeruginosa*, including enzymes that promote tissue invasion and extracellular polymers that form biofilms (Pollack, 1984).

In this study, we focused on the most significant virulence factors encoded by *exoS*, *toxA* and *oprI* in *P. aeruginosa*. *exoS* gene encodes for an ADP-ribosyltransferase, namely exoenzyme S, which is secreted directly through a type-III secretion system into the cytosol of host epithelial cells (Galle *et al*., 2012). Furthermore, this gene was found to be frequently

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present in drug resistant isolates (Fazeli and Momtaz, 2014). *toxA*, considered as one of the major extracellular virulence factors produced by *P. aeruginosa*, encodes for exotoxin A protein which is also an ADP-ribosyl transferase. Expression of this gene permanently inhibits protein synthesis in eukaryotic cells, which results in cell mortality (Iglewski *et al.*, 1975). Further, *oprI* encodes for one of the major outer membrane proteins in *P. aeruginosa* that is crucial for bacterial interactions with the surrounding environment (Khan and Cerniglia, 1994).

It is suggested that antibiotic resistant bacteria will kill 10 million people per year by 2050, more than the number of mortalities caused by cancer (O'Neill, 2014). Thus, this serves as a signal to develop new classes of antimicrobial therapies. Bacteriophages have been explored as a biological alternative or supplement to the current antibiotic therapy for *Pseudomonas* infections (Viertel *et al*., 2014). Here, we describe the detection of virulence genes e*xoS*, *toxA* and *oprI* in *P. aeruginosa* and the isolation and characterisation of freshwater lake-derived bacteriophages specific against the bacterium.

MATERIALS AND METHODS

Bacterial strains

Clinical isolates of *P. aeruginosa* CL1, CL2, CL3 and CL4 were obtained from the Pathology Laboratory, Queen Elizabeth Hospital, Kota Kinabalu, Sabah, Malaysia. In addition, *Escherichia coli* UKMCC 1006 and *Salmonella* Typhi UKMCC 1013 (obtained from Universiti Kebangsaan Malaysia Culture Collection) were used to observe the infection spectrum by the isolated bacteriophages.

Bacterial genomic DNA extraction

DNA from *P. aeruginosa* CL1, CL2, CL3 and CL4 was extracted through boiling method (Holmes and Quigley, 1981). One or two bacterial colonies grown on an agar plate was collected and suspended into a 1.5 mL microcentrifuge tube containing sterile 0.25 mL DNase/RNase-free water. The bacterial suspension was then boiled for 10 to 15 min and centrifuged at 10,000 × *g* at 4 °C for 10 min. The obtained supernatant containing DNA was collected and stored at –20 °C prior to PCR amplification.

Detection of virulence genes in *P. aeruginosa* CL1, CL2, CL3 and CL4 by PCR

Polymerase chain reaction (PCR) was performed by using specific primers to detect *exoS*, *toxA* and *oprI* virulence genes (Al-Kaaby, 2015) (Table 1). Following optimisation, the PCR mixture was set up as follows: GoTaq® Reaction Buffer 1 (10 µL), PCR Nucleotide Mix, 10 mM each (1.0 µL), upstream primer (1.0 µL), downstream primer (1.0 µL), GoTaq® DNA Polymerase (0.25 µL), template DNA (5 µL of < 0.5 µg/50 µL) and nuclease-free water (Promega Corp., USA) for a 50 µL final volume. The reaction mixtures were subject to the following empirically optimised thermal cycling parameters: initial denaturation temperature of 95 °C (5 min); followed by 30 cycles of denaturation at 95 °C (30 sec), annealing 58 °C (30 s) and extension 72 °C (1 min) and then final extension at 72 °C (10 min). Negative control (water) was included in every set of PCR reaction.

Table 1: List of primer sequences used in PCR to detect virulence genes *exoS*, *toxA* and *oprI*.

Primer	Size (bp)	Sequence	Gene Bank code
exoS	684	F: GCTTCAGCAGAGTCC GTCTT R: GCCGATACTCTGCTG ACCTC	L27629.1
toxA	487	F: GGCTATGTGTTCGTC GGCTA R: TGATCGCCTGTTCCTT GTCG	AF227424.1
oprI	202	F: CGGCTGGGAGATTGC TGTTA R: CCTTGCGATAGGCTT CGTCA	X58714.1

Detection of amplicons

Following amplification, aliquots (5 µL) were removed from each reaction mixture and examined by gel electrophoresis (80 V, 45 min) in gels composed of 1% (v/w) agarose containing gel red dye in TAE (1×) buffer. Gels were visualised under UV illumination and all images obtained were archived. The result was considered positive for *Pseudomonas* if the three genes resulted in an amplicon of the expected size via electrophoresis.

P. aeruginosa biofilm biomass quantification

Biofilm biomass quantification of *P. aeruginosa* strains was conducted using colorimetric micro-titer plate assay. Each *P. aeruginosa* strain was grown overnight at 37 °C in Luria Bertani (LB) broth. The overnight culture (50 µL) was inoculated into 5 mL of LB broth and grown at 37 ºC until it reached an approximate Optical Density (OD_{600}) of 0.5 – 0.7. The culture (10 µL) was then transferred into a well (of a 96-well plate) containing 190 µL LB broth and incubated at 37 °C overnight. Cells were washed with phosphate saline buffer (PBS) twice and air dried before adding crystal violet (200 µL, 1% (w/v) and left at room temperature (25 °C) for 15 min. Excess crystal violet was removed and the well plate was washed twice with PBS and air dried. The bound crystal violet stain was dissolved by using 33% (v/v) glacial acetic acid and left to stand for 5 min before transfer to clean well. Absorbance of the

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biofilm biomass was measured at 570 nm using ELISA reader. All experiments were conducted in triplicates with sterile LB broth as negative control.

Phage isolation

Bacteriophages were isolated using the double agar overlay method with 0.75% (w/v) LB agar where *P. aeruginosa* UKMCC 1011 was used as the indicator strain. Water samples (obtained from Tasik Kejuruteraan, Universiti Kebangsaan Malaysia, Bangi, Malaysia) was centrifuged at 6000 rpm for 10 min before filtering the supernatant through a 0.22 µm sterile syringe filter. For phage propagation, a mixture of 100 µL of water sample and 200 µL of overnight bacterial culture were added to 5 mL of 0.75% (w/v) top LB agar and poured onto 1.5% (w/v) bottom LB agar and incubated at 37 °C overnight. Formation of plaques was examined the following day. For negative control, Salt-Magnesium (SM) buffer was used as a mock bacteriophage and added to the molten agar. The harvested bacteriophages were selected according to their plaque morphology. Several rounds of plaque purification were performed to obtain pure bacteriophage suspensions. Purified plaques were then tested against *P. aeruginosa* CL1, CL2, CL3 and Cl4.

Preparation of concentrated phage stock

Purified bacteriophages were serially diluted in SM buffer and plated with host bacteria before overnight incubation at 37 °C. Plates with the highest number of plaques were chosen. After adding SM buffer (5 mL), each plate was left at room temperature (25 °C) and swirled every 10 minutes for at least 1 h. The liquid and top agar layer were transferred into a sterile tube, vortexed and centrifuged at 1300 × *g* (10 min) to remove agar residues. The obtained supernatant was filtered through 0.22 µm sterile membrane filter and stored in 50% (v/v) glycerol at –80 °C and in SM buffer at 4 °C. Stock titres were determined by double-layer agar method (Swanstrom and Adams*,* 1951).

Host spectrum determination for bacteriophages

P. aeruginosa CL1, CL2, CL3, CL4, *E. coli* UKMCC 1006 and *S.* Typhi UKMCC 1013 cultures were each (24-hour grown, 200 µL) inoculated into 5 mL of 0.75% (w/v) top LB agar and overlaid on 1.5% bottom LB agar. Each overlay was allowed to solidify for 15 min before 5 µL of phages were dropped onto the solidified 0.75% top LB agar. The agar was dried prior to incubation at 37 °C overnight. Negative control consists of each bacterial strain in sterile SM buffer without bacteriophages. A positive result was indicated by the formation of clear zones on bacterial lawns.

Transmission Electron Microscopy (TEM)

Bacteriophage particles were negatively stained and examined by Electron Microscopy at Electron Microcsopy Laboratory of Universiti Kebangsaan Malaysia. Staining was performed by firstly depositing phage suspension on copper grids with carbon-coated Formvar film and subsequent staining with 2% (v/v) uranyl acetate (pH 4.0). Phage morphologies were examined using Transmission Electron Microscope (Philips CM12 TEM). Each phage was characterised based on their morphology according to Ackermann (2011).

RESULTS

Detection of virulence genes *exoS*, *toxA* and *oprI* in and biofilm biomass quantification of *P. aeruginosa* CL1, CL2, CL3 and CL4

The presence or absence of *P. aeruginosa* virulence genes *exoS*, *toxA* and *oprI* in *P. aeruginosa* CL1, CL2, CL3 and CL4 strains are shown through electrophoresis gel images in Figures 1-3, respectively. Based on the images, all virulence genes were detected in all four strains except for *exoS* that was not observed in CL2. This shows that CL2 was unable to produce exoenzyme S encoded by the *exoS* gene.

Overnight biofilm formation by the *P. aeruginosa* strains was quantified through crystal violet assay. Amongst all strains, CL2 had the highest amount of absorbance (OD_{570}) (1.93 ± 0.27) followed by CL1, CL3 and CL4 (1.06 ± 0.32, 0.35 ± 0.05, 0.25 ± 0.14, respectively) (Table 2).

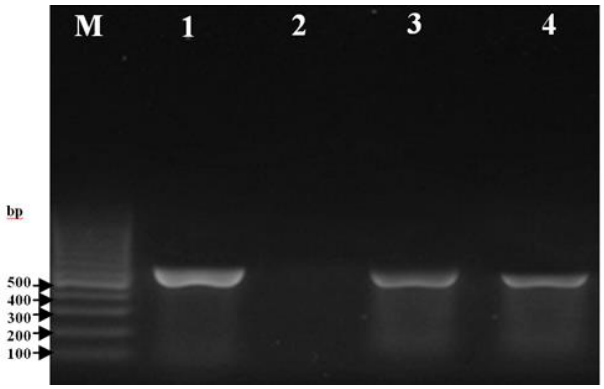

Figure 1: PCR amplification of *exoS* gene (684 bp) separated by electrophoresis on a 1% agarose gel, stained with red dye at 80 volts/cm for 1 h. Photographed under UV light. 1000 bp DNA marker (M); (1) *P. aeruginosa* CL1; (2) *P. aeruginosa* CL2; (3) *P. aeruginosa* CL3; (4) *P. aeruginosa* CL4.

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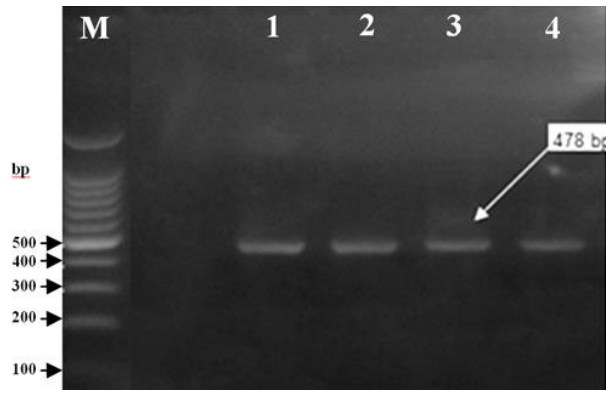

Figure 2: PCR amplification of *toxA* gene (478 bp) separated by electrophoresis on a 1% agarose gel, stained with red dye at 80 volts/cm for 1 hour. Photographed under UV light. 1000 bp DNA marker (M); (1) *P. aeruginosa* CL1; (2) *P. aeruginosa* CL2; (3) *P. aeruginosa* CL3; (4) *P. aeruginosa* CL4.

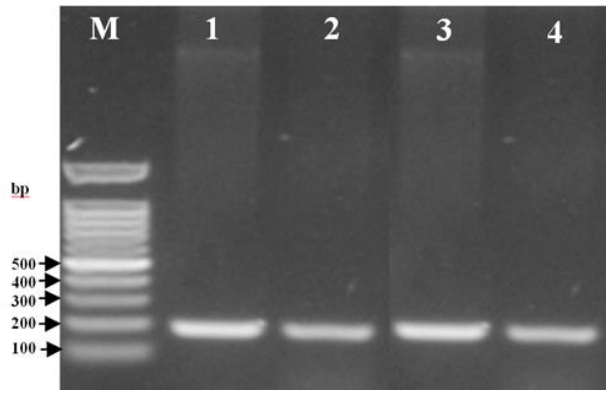

Figure 3: PCR amplification of *oprI* gene (202 bp), separated by electrophoresis on a 1% agarose gel, stained with red dye at 80 volts/cm for 1 hour. Photographed under UV light. 1000 bp DNA marker (M); (1) *P. aeruginosa* CL1; (2) *P. aeruginosa* CL2; (3) *P. aeruginosa* CL3; (4) *P. aeruginosa* CL4.

Table 2: Comparison of biofilm biomass (OD_{550}) by *P. aeruginosa* CL1, CL2, CL3 and CL4.

P. aeruginosa strain	CL1	CL2	CL3	CL4	Negative control
Absorbance (OD_{550})	1.06 ± 0.32	1.93 ± 0.27	0.35 ± 0.05	0.25 ± 0.14	0.13±0.08

Phage characteristics

We screened water samples for phages infecting *P. aeruginosa* by using *P. aeruginosa* UKMCC 1011 strain as the host strain using double layer agar method. Six *P. aeruginosa* specific phages were isolated and labelled PAtk1, PAtk2, PAtk3, PAtk4, PAtk5 and PAtk6. PAtk2 formed clear plaques after 24 h of incubation at 37 °C, whilst the rest produced plaques with a smaller clear centre surrounded by halos.

The isolated phages were then observed through TEM (Philips CM12 TEM) with their morphological characteristics summarised in Table 3. Based on the micrographs, PAtk1-5 was classified into the family Myoviridae due to the presence of contractile sheaths, meanwhile PAtk6 was classified into the family Siphoviridae due to the presence of long tails without contractile sheaths. Phages that were classified under Myoviridae have icosahedral heads and contractile sheaths. Basal plates for PAtk2, PAtk3, PAtk4 and PAtk5 were observed with diameters ranging from 49 nm to 77 nm. PAtk3 had the longest size in length among the isolated Myoviridaes with total length of 300.63 nm, whilst PAtk6 (family Siphoviridae) possessed long flexible tails with no contractile sheath and basal plate. Collar structure characteristics were observed in PAtk1, PAtk2, PAtk3 and PAtk4. All six phages were further tested against *P. aeruginosa* CL1, CL2, CL3 and CL4.

Bacteriophage host spectrum

Table 4 shows a summary of the host spectrum for the isolated phages. Amongst all six phages, PAtk4 and PAtk 6 showed the broadest lytic spectrum by showing lytic activity against all clinical *P. aeruginosa* strains. However, PAtk1 and PAtk2, alongside PAtk5 were unable to show lytic activity against *P. aeruginosa* CL4 and CL1, respectively. PAtk3 had the narrowest host range against the *P. aeruginosa* strains by exhibiting lytic activity on CL2 and CL3 only. Phage lytic ability was also screened against *E. coli* UKMCC 1006 and *S.* Typhi UKMCC 1013 but no lytic activity was observed, indicating the specificity of these phages against *P. aeruginosa.*

DISCUSSION

The increasing reports on worldwide multidrug resistance have raised concerns that led to the development of alternative therapeutical methods including phage therapy. *P. aeruginosa* is a major opportunistic pathogen that causes nosocomial and respiratory infections including those in cystic fibrosis (CF) patients (Pier, 1998). Thus, in this study, we isolated bacteriophages possessing lytic activity against clinical *P. aeruginosa* strains and investigated genotypic (*exoS*, *toxA* and *oprI*) and phenotypic (biofilm) virulence properties of the bacterial strains. The presence of *toxA* has been found to correlate with antimicrobial resistance in *Pseudomonas* sp. (Amirmozafari *et al.*, 2016), whilst *exoS* is highly important for invasion and colonisation (Bradbury *et al*., 2010) and *oprI* is associated with an outer membrane protein tethering to peptidoglycan in *P. aeruginosa* species (Wessel *et al.*, 2013).

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Table 3: Morphological properties and family classification of isolated phages.

Phage	Particle size (nm)					Family
	Head	Tail length	Tail width	Base plate	Total length	
PAtk1	105.44	164.68	31.83	Absent	270.12	Myoviridae
PAtk2	104.64	128.09	25.03	51.87	232.73	Myoviridae
PAtk3	113.64	186.99	26.40	77.51	300.63	Myoviridae
PAtk4	106.64	126.58	25.06	58.66	233.22	Myoviridae
PAtk5	108.72	137.38	24.60	49.20	246.1	Myoviridae
PAtk6	98.00	179.62	33.67	Absent	277.62	Siphoviridae

Table 4: Phage host spectrum on *P. aeruginosa*, *E. coli* and *S. Typhi*.

Bacteria	Type	Phage					
		PAtk1	PAtk2	PAtk3	PAtk4	PAtk5	PAtk6
Pseudomonas aeruginosa	CL1	+	+	—	+	—	+
	CL2	+	+	+	+	+	+
	CL3	+	+	+	+	+	+
	CL4	—	—	—	+	+	+
Escherichia coli	UKMCC 1006	—	—	—	—	—	—
Salmonella Typhi	UKMCC 1013	—	—	—	—	—	—

+, lysis; —, no lysis

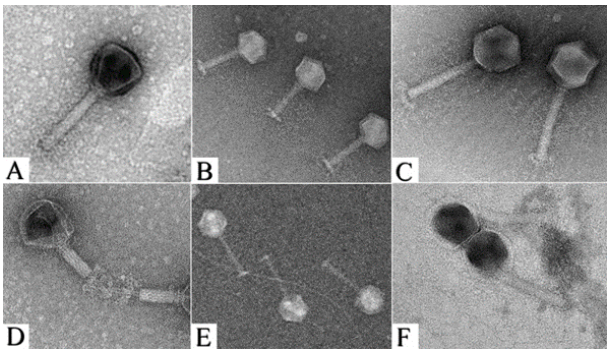

Figure 4: Electron micrographs demonstrating myoviral and siphoviral morphotypes for the isolated *P. aeruginosa* phages. The bar represents 200 nm length. A: PAtk1; B: PAtk2; C: PAtk3; D: PAtk4; E: PAtk5 and F: PAtk6.

Our investigation on clinical *P. aeruginosa* isolates showed that *exoS*, *toxA* and *oprI* were detected in all strains except for *exoS* in CL2. When comparing the biofilm production of these bacteria, CL2 produced the highest amount of biofilm biomass as shown from the absorbance readings. A study led by Azimi *et al*. (2016) discovered that *exoS* was only found in 2.5% of biofilm-producing *P. aeruginosa* isolates, implying that the gene is more prevalent in non-biofilm producing strains. Given that the exoenzyme S regulon is activated by eukaryotic cell contact, *P. aeruginosa* biofilms lacking *exoS* should not be producing much of type III effectors encoded by the gene (Azimi *et al*., 2016).

Biofilm production is regarded as committed action and also an act of self-defence by bacterial communities that usually hinders the effectiveness of treatment towards bacteria (Drenkard, 2003; Weitere *et al*., 2005). In *P. aeruginosa*, the *PA01* gene was observed to have an effect on its biofilm formation; however, it does not totally hinder formation but causes defective biofilm structure (Murphy *et al.*, 2014). Therefore, instead of screening for biofilm-related genes, we investigated the status of these strains in forming biofilms. Hypothethically, strains with *exoS*, *toxA* and *oprI* with high efficiency of biofilm formation will be those that are highly virulent and resistant to antibiotics. Thus, in this study, we have chosen several clinical strains from a hospital, screened these strains for virulence genes, biofilm production and used these isolates as target for bacteriophage screening. This has enabled us to isolate bacteriophages that are highly virulent against antibiotic resistant *Pseudomonas aeruginosa*.

Bacteriophage morphology is one of the most important aspects of phage classification and therefore the characteristics of isolated phages were observed in this study. Six phages belong to the family Myoviridae and Siphoviridae under the order Caudovirales, where most *P. aeruginosa* bacteriophages are classified. Members of Caudovirales mostly consist of the typical head-and-tail morphology and contain double-stranded DNA (dsDNA) (Ackermann, 2011). Phages under Myoviridae have contractile tails while Siphoviridae consist of long, flexible ends (Aksyuk *et al*., 2009), that were observed in PAtk1 – PAtk5 and PAtk6, respectively. Nevertheless, PAtk1, PAtk2, PAtk3, PAtk4 and PAtk5 had slight differences in the size of their icosahedral head that could be due to errors in replicating viral DNA in these phages. Alterations in DNA due to mutations can result in

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errors during protein synthesis that may lead to a larger number of capsid proteins synthesised during gene expression. This can increase the phage head size even when they have similar genomes sequence and size. Further work on comparing genomes of phage with similar morphology is necessary to further address this possibility.

Amongst all isolated phages, PAtk4 and PAtk6 exhibited the broadest host range. The variety in lytic activity of these isolates against different *P. aeruginosa* strains could be attributed to the specificity of interaction between phage attachment structures and receptors on host cell surface (Yoon *et al*., 2007). The mechanism of host invasion by phage depends on a number of factors, including phage types, host cell structural material (e.g. glycoproteins and lipopolysaccharides), transport machinery and interactions between host cells through F pili. Since PAtk4 and PAtk6 were the most virulent amongst the phages isolated, they should be further examined to determine their properties as potential antimicrobial agents against virulent strains of *P. aeruginosa*. Molecules that can be recognised by phages are essential components of the host cell surface (Sulakvelidze and Morris, 2001). Further characterisation is necessary to understand the intraspecific relationships among different types of *P. aeruginosa* and its phages. Some changes and alteration of host cell wall and membrane may affect the interaction between phage capsids and host membrane proteins, thus resulting in various degrees of successful infections. Lytic function of phage is the most crucial factor in phage therapy that advocates towards killing of pathogen (Woznica *et al.*, 2015; Cisek *et al.*, 2017). Lysogenic phages are unsuitable as it increases the resistance capability of target bacterium against antibiotics due to genetic material transfer (Balcazar, 2014).

CONCLUSION

In this study, we report the isolation of six bacteriophages specific to *P. aeruginosa* possessing *exoS*, *toxA* and *oprI* genes. Five of the phages came from the family Myoviridae whilst one is from Siphoviridae. Phages PAtk4 and PAtk6 were the most virulent amongst all phages isolated, warranting further studies to determine and characterise their properties as potential antimicrobial agents against virulent strains of *P. aeruginosa in vitro* and *in vivo*.

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