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Isolation and characterization of MDR *Pseudomonas aeruginosa* lytic bacteriophages as a potential therapeutic alternative to traditional antibiotics

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Abstract

The emerging resistance of *pseudomonas aeruginosa* to several antibiotic groups has resulted in difficulty in treating infected patients. As a result, bacteriophage therapy is now considered a promising treatment substitute. In this study, a novel bacteriophage, PaMAsh, with lytic activity against *P. aeruginosa* was isolated from wastewater. PaMAsh exhibited a moderate host range against the four tested bacterial strains, as well as high stability over a wide range of pH and temperature. Electron microscopy revealed that the PaMAsh phage belongs to the family Podoviridae.

Keywords: *Bacteriophage, Pseudomonas aeruginosa, PaMAsh, Multidrug-resistance*

1. Introduction

Interest in phage therapy has been renewed in recent years as bacteriophages are being used to treat bacterial infections (Golkar *et al*., 2014; and AaEK, 2005) (Rahmani *et al*., 2015), without harmful effects on the human body (Dubey *et al*., 2016). Moreover, bacteriophages are ubiquitous, and their isolation is relatively inexpensive (De Vos and Pirnay, 2015).

Sewage water is well-known as a rich source of bacteriophages owing to the fact that most sewage water is rich in organic material and ammonia (Lobocka *et al*., 2014). Thus, it is an excellent environment for the growth of both bacteriophages and their bacterial hosts (Shende *et al*., 2017). The lack of well-organized urban wastewater management and poor disinfection treatment before wastewater is released into the sewerage system may increase the distribution of pathogenic bacteria, especially multi-drug resistant species, from individual households into the environment (Shende *et al*., 2017).

In this study, we aimed to isolate lytic phages specific for *Pseudomonas aeruginosa* and morphologically identify the phages by transmission electron microscopy. *P. aeruginosa* is an opportunistic gram-negative bacterium, which greatly contributes to their diversity of infections, varying from skin infections to septicemia and pneumonia (Schroeter, 1872; and Migula, 1900). The most commonly used antibiotics against *P. aeruginosa* are classified under three antibiotic classes: aminoglycosides, beta-lactams, and fluoroquinolones (Bassetti *et al*., 2018). They are administered depending on the infection site and severity of infection (Subedi *et al*., 2018).

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However, *P. aeruginosa* can develop resistance to these three essential antibiotic classes by acquiring antibiotic resistance genes (Ashish *et al*., 2012). Antibiotic resistance is progressively increasing in a vital group of *P. aeruginosa* strains called the Liverpool Epidemic Strain (LES); among which, all of the isolates have been isolated from cystic fibrosis patients (Ashish *et al*., 2012).

The World Health Organization (WHO) has categorized carbapenem-resistant *P. aeruginosa* as a "critical" pathogen; thus, novel antimicrobials to treat such bacterial infections are urgently needed (Organization, 2017). Isolating additional bacteriophages specific for *P. aeruginosa* is a promising solution to address the increasing antibiotic resistance rates. Some (Fukuda *et al*., 2012), , (Yuan *et al*., 2019), (Klai and Sellamuthu, 2020). Furthermore, *Pseudomonas* phages have been used in *in vivo* studies in animal and human trials. For example, dogs with otitis caused by *P. aeruginosa* infection successfully recovered after treatment with a phage cocktail consisting of six phages (Wright *et al*., 2009). Additionally, an *in vivo* study of 24 patients suffering from otitis caused by antibiotic-resistant *P. aeruginosa* showed a major reduction in bacterial counts after treatment with bacteriophages over a period of two weeks (Wright *et al*., 2009). Therefore, the isolation of more phages with activity against *P. aeruginosa* is crucial to counter antibiotic resistance and advance bacteriophage applications.

2. Materials and methods

2.1. Phage host

P. aeruginosa strain (A.J. D 2) was gifted from King Abdelaziz hospital and used as host for bacteriophage isolation in our study. Refreshed bacterial strain was inoculated into slant nutrient agar at 4°C. The identification of this strain was confirmed based on morphology, biochemistry, and molecular aspects (Glasgow et al. 1999)). Antibiotic sensitivity testing was accomplished using the disc diffusion method according to Bauer (1966) after adjusting the turbidity of the bacterial suspension to the 0.5 McFarland standard. All bacterial strains were sub-cultured in slants, nutrient broth, and 20% glycerol stocks at -80°C (Kronvall *et al.*, 1971).

2.2. Molecular characterization of bacterial isolates

Genomic DNA was extracted from *P. aeruginosa* using the QIAamp® DNA Mini Kit in Europe following the manufacturer's instructions, with slight modifications as proposed by (Atashpaz *et al*., 2010).

2.3. Molecular identification using 16 S rRNA sequencing

Amplification of the 16S rRNA gene was performed using an MJ Research Peltier Thermal Cycler with forward primer 5'-AGAGTTTGATCMTGGCTCAG-3' and reverse primer 1492R 5'-TACGGYTACCTTGTTACGACTT-3'. The products were cleaned up using a kit (Millipore in Europe) and sent to the Macrogen sequencing company in South Korea.

2.4. Bacteriophage isolation

2.4.1. Sample collection and transportation

The study was conducted from October 2020 to December 2020. Wastewater sample collection locations were purposively selected based on the degree of contamination of the water sample and the proximity of the site to our laboratory. All samples were collected aseptically in screw-cap bottles and transported to the KFMRC in an icebox, where they were stored in a refrigerator until they were processed within 24 h of collection.

2.5. Bacteriophage spotting assay for lytic activity

To determine the lytic activity of phages against the host strains, a spot assay was performed following the protocol of (Pereira *et al*., 2011). Thereafter, the plates were examined for the presence of any plaques where the lysate was added; phages that produced a clear plaque were considered virulent phages.

2.6. Purification and titration of bacteriophage

Phage purification was conducted using the top agar (soft agar) overlay technique described by (Gencay *et al*., 2017). Three successive experiments were conducted until single plaques with similar morphologies were obtained. Purified phage filtrate was stored at 4°C. Double-layered technique was used to determine phage titers, and each bacteriophage concentration was expressed as plaque-forming units (PFU) / mL https: / / barricklab.org

$$\text{Titration: } \frac{PFU}{mL} = \frac{Number\ of\ plaques}{D \times V}$$

D = Phage concentration (10^8)

V= Volume of purified phage for example 100 μL

2.7. Stability of phage under physical conditions

2.7.1. Thermal stability

Thermal stability of the isolated phages was tested using the protocol described by Jurczak-Kurek *et al*. (2016). Phage lysates (100 μL; 108 PFU / mL) were collected in sterile Eppendorf tubes and incubated in a water bath over a range of temperatures from 30°C to 90°C for 120 min. The bacteriophage titer after treatment was determined using the double-layer agar assay method. The percent stability was estimated by the proportion of surviving phage titers after treatment relative to the total phage titer before treatment. The thermal stability curve (mean of triplicate experiments vs. time) was plotted using Excel 2010.

2.7.2. Phage pH Stability Test

The phage pH stability test was conducted as described by Verma *et al*. (2009) with minor modifications. To adjust the required acidic and basic pH (pH 2–14), 1 M HCl or 1 N NaOH was added drop wise into fresh nutrient broth, and the pH measured using a pH meter Designed and Manufactured in the UK by Bibby Scientific Ltd, brand JENWAY. The pH-adjusted medium (9 mL) was mixed with phage suspension (1 mL) and incubated for 3 h at 37 ºC. Again, the phage titer for each suspension was determined by the double-layer agar method, and the ratio of surviving phage was calculated. The pH stability curve (mean of triplicate experiments vs. time) was plotted using Excel 2010.

2.8. Determination of phage host range

The host range spectrum of the lytic phages isolated during the study period was determined by a spot test according to (Jurczak-Kurek *et al*., 2016) using a panel of 12 strains classified under different genera. In brief, a lawn of selected host bacterium was produced by mixing 1 mL of overnight bacterial culture with 0.7% molten soft agar; thereafter, the solution was poured over the surface of a modified nutrient agar plate. Subsequently, 5 μL of purified lytic phage lysate (108 PFU / mL) was spotted onto the bacterial lawn. After overnight incubation, the lytic ability of the phage lysate was determined based on the clarity of the plaques produced and recorded as (-) no plaques, (+) turbid plaques, or (++) clear plaques.

2.9. Transmission Electron Microscopy (TEM)

Electron micrographs of phage lysates were obtained using the method described by van Regenmortel *et al*. (2000). Briefly, purified phage lysate (108 PFU / ml) was dropped (20–30 μL) onto 400 mesh carbon-coated copper grids, allowed to stand for 2 min, and then negatively stained with 2% (w / v) phosphotungstic acid (pH 7.0). Excess sample was removed by blotting the side of a grid with soft paper and then air dried for examination by TEM (Hitachi, H-7000 Tokyo, Japan; located at the KFMRC), at 80 kV and 15,000–25,000 × magnication. Based on the microscopic images, the phages were classified using the International Committee on Taxonomy of Viruses (ICTV) guidelines (https: / / talk.ictvonline.org / **)**

3. Results

Biochemical test results for bacteria isolated from wastewater samples are shown in (Table 1).

Table 1: Biochemical test results for bacteria isolated from patient from hospital

Biochemical tests	Mnemonic	Results
1 Ala-Phe-Pro-ARYLAMIDASE	(APPA)	–
2 H2S PRODUCTION	(H2S)	–
3 BETA-GLUCOSIDASE	(BGLU)	–
4 L-Proline ARYLAMIDASE	PROA	+
5 SACCHAROSE/SUCRALOSE	SAC	–
6 L-LACTATE alkalinization	ILATK	+
7 Glycine Arylamidase	GLY A	–
8 O/129 RESISTANCE	O129R	–
9 ADONITOL	ADO	–
10 BETA-N-ACETYL-GLUCOSAMINIDASE	BNAG	–
11 D-MALTOSE	DMAL	–
12 LIPASE	LIP	+
13 d-TAGATOSE	dTAG	–
14 ALPHA-GLUCOSIDASE	AGLU	–
15 ORNITHINE DECARBOXYLASE	ODC	–
16 Glu-Gly-Arg-ARYLAMIDASE	GGAA	–
17 L-Pyrrolydonyl-ARILAMIDASE	PYR A	–
18 GlutamylArylamidase pNA	AGLTP	–
19 D-MANNITOL	dMAN	–
20 PALATINOSE	PLE	–
21 D-TREHALOSE	DTRE	–
22 SUCCINATE alkalinization	SUCT	+
23 LYSINE DECARBOXYLASE	LDC	–
24 L-MALATE assimilation	IMLTA	–
25 L-Arabitol	IARL	–
26 D-Glucose	dGLU	+
27 D-MANNOSE	Dmne	+
28 Tyrosine ARYLAMIDASE	TyrA	–

Table 1 (Cont.)

Biochemical tests	Mnemonic	Results
29 CITRATE (SODIUM)	CIT	+
30 Beta-N-ACETYL-GALACTOSAMINIDASE	NAGA	–
31 L-HISTIDINE assimilation	IHISa	–
32 ELLMAN	ELLM	–
33 D-CELLOBIOSE	dCEL	–
34 GAMMA-GLUTAMYL-TRANSFERASE	GGT	+
35 BETA-XYLOSIDASE	BXYL	–
36 UREASE	URE	–
37 MALONATE	MNT	+
38 ALPHA-galactosidase	AGAL	–
39 COUMARATE	CMT	–
40 L-LACTATE assimilation	ILATa	–
41 BETA-GALACTOSIDASE	BGAL	–
42 FERMENTATION-GLUCOSE	OFF	–
43 BETA-ALANINEARYLAMIDASE pNA	BAlap	+
44 D-SORBITOL	dSOR	–
45 5-KETO-D-GLUCONATE	5KG	–
46 PHOSPHATASE	PHOS	–
47 BETA-GLUCORONIDASE	BGUR	–

3.1. Antibiotic sensitivity of P. aeruginosa strain

Antibiotic testing showed that *P. aeruginosa* A.J. D 2 was resistant to multiple antibiotics such as amoxicillin, ciprofloxacin, amikacin, and sulfamethoxazole**,** whereas it was susceptible to three types of the following antibiotic groups: aminoglycosides (gentamicin), fluoroquinolones **(**norfloxacin), and polymycin (colistin sulfate) (Table 2).

Table 2: Antibiotic resistance profiling of the clinical strain of *P. aeruginosa* strain A.J. D2

	Antibiotics	Result	MIC
ViTEK2 2	Ampicillin	R	-
	#Piperacillin/Tazobactam	R	>= 128
	Ceftazidime	R	>= 64
	Cefepime	R	64=<

Table 2 (Cont.)

	Antibiotics	Result	MIC
	Amikacin	R	16
	Imipenem	R	>= 16
	Meropenem	R	16=<
	Gentamicin	R	8
	Tigecycline	R	>= 8
	Ciprofloxacin	R	2
Disc-diffusion method	Nitrofurantoin	R	-
	Norfloxacin	S	12 mm
	Cotrimoxazole	R	-
	Cephalothin	R	-
	Nalidixic acid	R	-
	Sulphamethoxazole	R	-
	Gentamicin	S	12 mm
	Colistin sulphate	S	15 mm
	Amoxycillin	R	-
	Cefoxitin	R	-

3.2. Bacteriophage isolation and morphology

One lytic bacteriophage was isolated from the collected raw sewage samples using of *P. aeruginosa* (Figure 1.2). Titration of the bacteriophage was examined in the range of 10^6 to 10^9PFU / mL following 48 h of incubation at 37ºC with the host bacteria. Based on this analysis, *P. aeruginosa* was inhibited by 109 PFU / mL (Table 3). The isolated bacteriophages produced clear plaques ranging from 2–3 to mm with clear edges (Figure 4).

Table 3: Titration of bacteriophage at 10^{-8}

Type of phage	The equation	Total of PFU
PaMAsh titer	$\frac{109 PFU/ml}{0.1\times10^{-8}}$	$=1.09\times10^9$ PFU \ ml

The isolated bacteriophage produced clear plaques ranging from 2-3 mm with well-defined boundaries (Figure 4).

3.3. Phage Host Range

The isolated phage showed various activities against several bacterial hosts using the spot test assay. Lytic activity was observed against four of the six bacteria tested, including *Shigella Sonni* (ATCC 25931), *Klebsiella oxytoca* (ATCC49131), *Proteus vulgaris* (ATTC49132), and *Salmonella enterica* (ATCC 14028) (Table 4). The PaMAsh phage showed lytic activity against four species: *Shigella sonni* (ATCC 25931), *Klebsiella oxytoca* (ATCC49131), *E. coli* (ATCC25922), and *Salmonella enterica* (ATCC 14028) (Figure 5).

Table 4: Phage host range against six bacterial isolates (Source of isolates: (ATCC) using spot assay technique

Bacteria strains	*Enterococcus faecalis* (ATCC 29212)	*Shigella sonni* (ATCC 25931)	*Klebsiella Oxytoca* (ATCC49131)	*Proteus Vulgaris* (ATTC49132)	*E. coli* (ATCC25922)	*Salmonella enterica ATCC 14028*
Results	–	+	+	–	+	+

Note: * (+) complete lysis, (–) no lysis

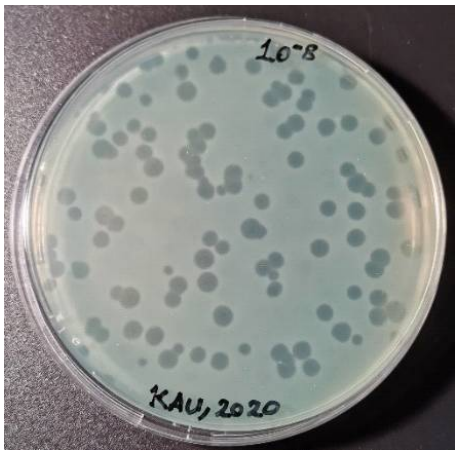

Figure 4: The isolated bacteriophage produced clear plaques ranging from 2-3 mm

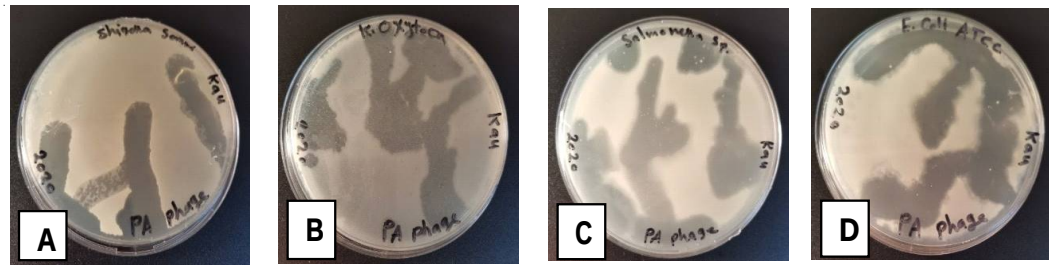

Figure 5: PaMAsh phage host range against six bacterial isolates by using spot assay technique

3.4. Phage stability testing

Phage stability remained at 100% at 25°C and continued to show stable activity even after 1 h of incubation at 37-80°C. However, the PaMAsh phage was not activated when the temperature reached 100°C or above

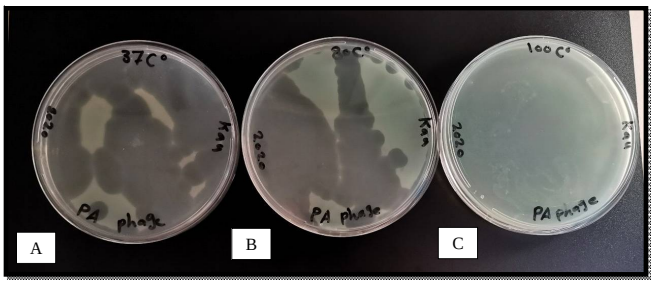

Figure 6: Spot test for PaMAsh phage at (A) 37°C, (B) 65°C and (C)100°C

(Figures 6 and 8). The PaMAsh phage was less susceptible to alkaline pH than acidic pH, remaining stable over the pH range of 6-8, but decreasing in viability between pH 1-2 (inactivated at pH 14) (Figure 7).

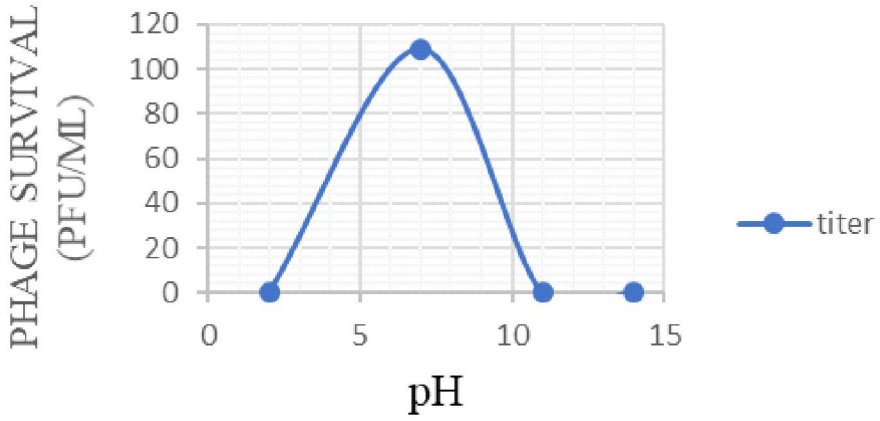

Figure 7: pH stability for PaMash phage

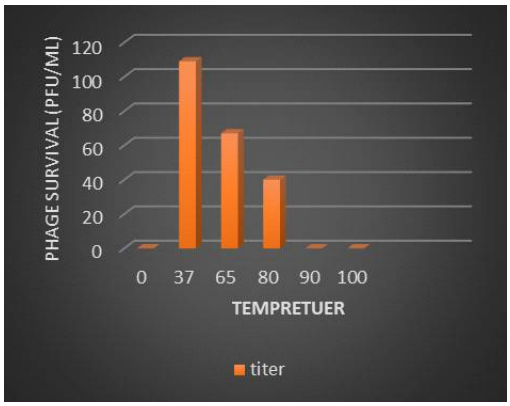

Figure 8: Thermal stability for PaMash phage

3.5. Virion morphology

TEM analysis revealed that the phage virion consisted of a head and a very short, noncontractile tails. Thus, the repurified PaMAsh phage was classified under the Podoviridae family (Figure 9).

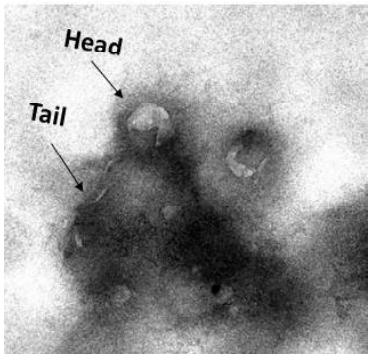

Figure 9: Transmission electron microscopy analysis of the purified PaMAsh phage belongs to the

4. Discussion

P. aeruginosa in the current study was resistant to 17 different antibiotics representing all seven common groups of antibiotics; it is thus considered a multidrug-resistant bacterium. This bacterium has been previously used as a host for phages isolated from wastewater samples. One phage isolated in this study, PaMAsh, efficiently lysed *P. aeruginosa* cells and produced clear plaques in a spot test.

The PaMAsh phage has a high plaque measurement size (2–3 mm). Similar results were reported by Menon *et al*. (2021), who isolated a novel bacteriophage, AM.P2, from a wastewater source in India, which was effective against clinical *P. aeruginosa* strain PAO1. The AM.P2 plaque was large with a diameter of ~5 mm with a clear center. Larger plaques are closely related to smaller phage head sizes, which can thus diffuse throughout agar more readily (Jurczak-Kurek *et al*., 2016). Adnan *et al*. (2020) also found a phage that was active against *P. aeruginosa* from a wastewater sample in Pakistan; their *P. aeruginosa* strain was resistant to seven antibiotics and thus considered antibiotic resistant. The isolated phage, named MA-1, exhibited plaques ranging from 1.5-3.0 mm diameter.

The isolated PaMAsh phage in the current study was stable at temperatures from 37°C to 80°C for 2 h. However, the viability steadily declined above 80°C. Krasowska *et al*. (2015) found a similar temperature susceptibility for phages they isolated, exhibiting resistance to temperatures as high as 80°C for 1 min. Similar to our findings, Ruchi *et al*. (2010) found that phages remained viable when exposed to a temperature of 70°C. In contrast, Imam *et al.* (2019) reported that Phage MIJ3, which was isolated from an equine livery yard in Leicestershire, United Kingdom, showed high stability at 60°C but decreased in titer when the temperature increased above 60°C for 1 h

In the current study, PaMAsh exhibited lytic activity against four strains of pathogenic bacteria. de Melo *et al*. (2019a) reported a similar result for a phage isolated from sewage and tested against 36 *P. aeruginosa* strains isolated from domestic animals. The PaMAsh phage was able to lyse the host completely, indicating that the phage possesses a wide range of activity against the tested bacterial strains, as summarized in Table 4.10. The wide host range and phage types reported in our study agree with those reported by Yarmolinsky (2004) and Bielke *et al*. (2007), who indicated that phage host range is not always restricted by genus. In contrast, Carey-Carey-Smith *et al*. (2006) described narrow-range phages that were restricted to a maximum of two bacterial species.

5. Conclusion

Our study showed that the bacterial isolate *P. aeruginosa*, had significantly higher antibiotic resistance rates, which can lead to sharing of resistance with other environmental bacterial pathogens. Here, we isolated and characterized phages from wastewater using the wastewater bacterial isolate *P. aeruginosa* as the host system. Recovered phage lysates had a broad host range, indicating their potential to be used as alternative therapeutics in combating infectious diseases caused by multidrug-resistant bacterial pathogens. There is a need, however, to understand *in vivo* phage-mediated selection; the current study was conducted for only a limited period of time, which is not representative of wastewater discharge for the duration of all seasons in a year. Therefore, the findings of this study may not reflect the actual situation in the study area throughout the year. A large-scale, year-long study is required to obtain comprehensive conclusions.

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