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Isolation and characterization of novel phage (*Podoviridae* ϕ PauNE1) and its efficacy against multi-drug-resistant *Pseudomonas aeruginosa* planktonic cells and biofilm

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Abstract

Background: Bacterial pathogen (*Pseudomonas aeruginosa*) could form biofilm that conveys multi-drug resistance. Bacteriophage as an alternative to antibacterial resistance is useful against biofilm complications. This study evaluated antibacterial and biofilm removal activities of lytic phage, specific against multi-drug-resistant clinical *P. aeruginosa*.

Results: The phage showed a wide range of pH (5–10) and heat (7–44 °C) stability. Electron microscopy showed ϕ PauNE1 phage head (60 nm in diameter) and non-contractile tail (12 nm in length by 8 nm in width); hence, the family *Podoviridae* and the order *Caudovirales*. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed structured protein of 55 kDa and double-stranded DNA of 45 kb. The phage was species specific and had broad host range activity. It inhibited bacterial growth at multiplicity of infection (MOI) 1–0.000001 pfu/ml. Inhibition was maximal at both low (1×10^5) and high (1×10^9) bacterial CFU/ml. Biofilm removal test showed that the phage removed more than 60% cell biomass within CFU/ml of 1.5×10^8 , 6.0×10^8 and 1.0×10^9 .

Conclusion: Phage (ϕ PauNE1) was unique and had broad host range activity. The phage exhibited strong bacteriolytic activity against biofilm forming multi-drug-resistant strains. It had no lytic effect on the heterogeneous strains and so a promising bioagent.

Keywords: Multi-antibacterial-resistance *P. aeruginosa*, Biofilm formation, Phage bio-agent

1 Background

Pseudomonas aeruginosa is a pathogen frequently implicated in healthcare-associated infections (HAIs), especially in critically ill and patients with low immunity [1]. It is a versatile pathogen with the ability to cause diverse range of infections such as superficial infections, systemic and life-threatening conditions with mortality rate of 40–60% [2]. Various studies reported biofilm formation

of this organism as complication of human disease [3, 4]. Biofilm-associated *P. aeruginosa* morbidity and mortality were reported mostly in hospitals, where they are one of the leading pathogens in cases like cystic fibrosis and infections within nursing homes [5]. The organism is notorious as a multiple antibacterial-resistant pathogen difficult to treat globally [6]. According to World Health Organization's "priority pathogens," *P. aeruginosa* made first list among the catalogue of 12 bacterial species that pose the greatest threat to public health [7]. The implication of the WHO's report was that these bacteria have become resistant to a number of antibiotics, including carbapenems and the third-generation cephalosporin [7].

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In fact, the organism has been reported to be intrinsically resistant to many antibiotics including newer β -lactam [8, 9]. Consequently, scholars have severally attempted to describe the relative severity and complexity, many scholars have described it as “Extremely Drug Resistant” (XDR) [6]. Available clinical data suggest that high morbidity and mortality rates associated with antibacterial-resistant *P. aeruginosa* infections are mainly nosocomial and in particular surgical wounds [1]. Usually, patients with this complication have prolonged hospital stay [1].

Globally, there is intensive search for newer antibacterial agents or alternative to the existing classes of antibiotics available for containment of public health pathogens. Phage technology appears to be one of such promising alternatives. Phage therapy is considered more beneficial than antibiotics because it is safer and more specific in activity [10, 11]. Application of bacteriophages to treat/control bacterial infection is known as phage therapy [12]. The advantage of the use of phage over antibiotics arises from the fact that the mechanism by which phage cause death of the host (bacteria) cells is entirely different from the mechanisms of antimicrobials [12]. Another advantage of this technique is that the earth biosphere contains a very large number ($\approx 10^{31}$) of bacteriophages [13], although only few of these have been characterized [14]. Therefore, in an effort to find alternative to failing antibacterial agents, this study isolated and characterized novel lytic phage that could eradicate multi-drug-resistant clinical *P. aeruginosa* and evaluated their specificity and biofilm removal activities.

2 Methods

2.1 Isolation and identification of bacterial strain

The multi-drug-resistant *P. aeruginosa* isolate from teaching hospital was identified using conventional standard methods [15]. It was further identified with VITEK[®]-2 automated system using Gram negative identification (GN ID) card according to the manufacturer’s instruction (BioMerieux, France) [16]. The strain was cultivated and maintained in Luria–Bertani broth and agar (Miller’s, USA), respectively, at 37 °C. Bacterial growth was monitored using turbidimeter at optical density of 600 nm (OD_{600}). An OD_{600} of 0.6 corresponded to 1×10^9 cells/ml was used in the study.

2.2 Antibiotic sensitivity test

Antibiogram was conducted using VITEK[®]-2 system (BioMerieux, France) [17]. The antibiotic susceptibility test packs (AST GN590 card) of VITEK[®]-2 system for Gram negative organisms were used. The antimicrobial test card contained 19 (ampicillin, ampicillin/sulbactam, cefotaxime, cefpodoxime, ceftazidime, cefuroxime, cefuroxime/axetil, ciprofloxacin,

gentamycin, imipenem, levofloxacin, meropenem, nitrofurantoin, piperacillin, piperacillin /tazobactam, tetracycline, tobramycin and trimethoprim /sulfamethoxazole) antibiotics for Gram negative bacteria in each panel. *Pseudomonas aeruginosa* CLI-22 was characterized as multi-drug resistant (MDR) according to CLSI guidelines [18].

2.3 Phage isolation

The multi-drug-resistant *P. aeruginosa* was used to isolate and propagate specific phage. The PAO1 and Pa ATCC-27853 (Phage lab-Texas A&M, USA) were used for quality control. Bacteriophage was isolated from sewage (collected from sewage treatment center, College Station, Texas, USA) following a simple enrichment procedure [12]. Aliquot of sewage water aseptically collected from a sewage plant was centrifuged for 10 min at 8000 rpm in cold condition (4 °C) and then filter sterilized with 0.22- μ m pore-size membrane filters.

2.3.1 Phage enrichment

A 30 ml of double-strength tryptone soya broth (TSB), 30 ml of filter-sterilized sewage water and 100 μ l of 24-h culture of test organisms (MDR clinical *P. aeruginosa*) were mixed in a sterile 250-ml conical flasks and incubated at 37 °C on 200 rpm thermo-shaker incubator (Dubuque, Iowa 52,001, USA) for 24 h. After incubation, the phage-host mixtures were centrifuged at 8000 rpm for 5 min at 4 °C and filtered through 0.22- μ m pore-size membrane filter [12].

2.3.2 Spot titration

The phage was extracted from the filtrate by spotting 10 μ l of the phage suspension onto the test bacterial lawn within aseptic zone. Top-agar plate was produced by mixing a 100 μ l of 4-h old culture of test bacteria strain (MDR clinical *P. aeruginosa*: 1×10^9) with 4 ml of molten tryptone soya agar (TSA), and the suspension was dispensed uniformly over the surface of 20 ml of TSA plate. The inoculated top-agar was allowed to solidify. After incubation at 37 °C for 24 h, zones of lysis were monitored [13].

2.3.3 Production of microplaques

The isolated lysed phage from the spot plate was enumerated using double agar layer plaque assay technique [13]. Briefly, A 100 μ l of tenfold serial diluted phage suspension and 100 μ l of host bacteria (1×10^9 CFU/ml) were mixed with 4 ml of molten TSA. The suspension was dispensed uniformly over the surface of 20 ml of hard TSA in 96-mm-diameter plate. The plate was allowed to set and then incubated at 37 °C for 12 to 16 h. After

incubation, the presence of plaques was observed by comparison with a negative control plate [13].

2.3.4 Phage purification and amplification

The phage was purified by three successive single-plaque subculture method until homogenous plaque was obtained [12]. The purified phage was amplified using 100 μ l of 10^{-3} phage lowest dilution which produced 10^4 – 10^5 PFU/ml on agar layer plate. After incubation, the phage was harvested with phage buffer solution (SM: 100 mM NaCl, 25 mM TrisHCl pH7.5, 8 mM $MgSO_4$, 0.01% w/v gelatin). The lysate was centrifuged at 8000 rpm for 10 min and then filter sterilized with 0.22- μ m pore-size membrane filter. The phage lysate was stored at 4 °C [12].

2.4 Examination of phage morphology by Transition Electron Microscope (TEM)

The morphology of the phage was detected by negative staining with uranyl acetate; sample was examined by transmission electron microscopy at an acceleration voltage of 100 kV and at calibrated magnifications. Images were recorded digitally with a slow-scan CCD-Camera (ProScan, 1024 \times 1024, Scheuring, Germany) with ITEM Software (Olympus Soft Imaging Solutions, Münster, Germany) [14].

2.5 Phage-host range determination

Fifteen clinical isolates of MDR *P. aeruginosa*, a laboratory strain of *P. putida* and control strain PA01, were used to determine phage-host range, using standard spot test. The sensitivity of bacterial strains was confirmed by appearance of lysis zones at the site where the phage suspension was spotted. The phage was tested against each bacterial strain in three independent replicates. The lysis was categorized as clear (+), turbid (c) or no reaction (–) [19].

2.6 Isolation of bacteriophage DNA

Purified phage (10^{10} – 10^{11} PFU/ml) was treated with 10 μ g/ml of DNase (New England Biolabs) at 37 °C for 30 min and precipitated with 5 ml of 3 mM NaCl in 30% polyethylene glycol (PEG). The pellet was re-suspended in 500 μ l of buffer (5 mM $MgSO_4$). The mixture was treated with 1-ml purification resin in a mini column (Promega Wizard DNA purification kit, USA) and finally washed by adding 2 ml of 80% isopropanol. The DNA was eluted from the resin by adding 100 μ l of sterile water at 80 °C and was centrifuged at 13,000 rpm for 1 min. The DNA was stored at –20 °C [20].

2.6.1 Restriction endonuclease enzyme digestion patterns

Specific volume (1 μ l of 200–400 ng/ μ L) of DNA was digested with 0.5 μ l of EcoR1, EcoRv, Bam H1, Pst1, Hind III, Bgl II restriction endonucleases (Fermenters, USA). The solution was incubated at 37 °C for 24 h. The DNA fragments generated were subjected to electrophoresis at 100 V for 1 h 30 min in TBS buffer and in a 1% agarose gel stained with 7.5 μ l of ethidium bromide. The DNA in the gel was visualized under UV trans-illuminator. One hundred base pair DNA ladder markers were used as standard to measure the size of DNA fragment [21]. The phage structural protein was determined by separating purified phage preparation on a sodium dodecyl sulfate polyacrylamide gel as previously described [22].

2.7 Stability of the Phage under different (pH) values

The phage stability in the pH was determined by mixing 100 μ l volume of phage suspensions (1×10^5 pfu/ml) in a series of tubes containing 900 μ l of SM buffer (100 mM NaCl, 25 mM Tris HCl, 8 mM $MgSO_4$, 0.01% w/v gelatine) with p^H ranging from 1 to 10 (1.5, 3, 5, 7, 9 and 10). The mixture was incubated at 37 °C for 1 h, after which the phage suspension was immediately serially diluted in SM buffer (pH 7.5), and phage titer was determined by double-layer agar plate method for every treated sample. The phage survival rates were expressed as percentages of viable phage in suspensions [23].

2.8 Phage binding rate at specific temperatures

The binding rate of phage was estimated using the protocol previously described [22, 23] with some modification. Three replicate experiments were conducted to estimate adsorption rates. The cell concentrations at the beginning and the end of each experiment were determined and used separately for the estimation of each replicate adsorption rate.

The influence of temperature on the adsorption rates of phage on the host cell was determined by incubating the phage infected culture at 7, 15, 25, 37 and 44 °C for 30 min in SM buffer (100 mM NaCl, 25 mM TrisHCl, 8 mM $MgSO_4$, 0.01% w/v gelatine) at multiplicity of infection 0.01. After incubation, mixture was centrifuged, the supernatant was assayed for unabsorbed phage (double-layer plaque titration) and the count was compared with the titer of a control without cells. Phage adsorption rates were expressed as percentage of adsorbed phage in relation to the initial phage counts.

2.8.1 One-step growth curve

The growth of phage was investigated by determining the phage latent period, burst time and burst size as previously described [24]. Twenty milliliters of the host cell

(MDR clinical *P. aeruginosa*) were incubated at 37 °C until the optical density (OD_{600 nm}) was 0.6; the culture was concentrated by centrifugation at 12,000 rpm for 5 min. The concentrate was mixed with equal volume of 500 µl LB broth and 500 µl phage (3.5×10^8 pfu/ml). The phage adsorption rate was determined a minute after the mixture. The mixture was centrifuged at 12,000 rpm for 2 min to remove non-adsorbing phage. The resulting pellet was suspended in 100 ml LB broth and kept at 37 °C. Samples were taken at five-minute intervals up to 90 min, and immediately tenfold serial dilution was carried out, and the phage titer was estimated through double-layer soft agar method.

2.9 Phage bacteriolytic activity in vitro experiment

An optical density (OD₆₀₀) of 0.4 (2×10^9 CFU/ml) broth culture of test bacteria (MDR clinical *P. aeruginosa*) strain was inoculated into double-strength LB broth supplemented with 1% of glucose. A sterile 96-well micro-titer plate with flat bottom (Greiner bio one, Germany) was filled with 100 µl of inoculated medium in such a way to get 1×10^9 CFU/well (MOI 1) in the columns 1–3, 1×10^7 CFU/well (MOI 0.1) in columns 4–6, 1×10^5 CFU/well (MOI 0.01) in columns 7–9, and 1×10^3 CFU/well (MOI 0.001) in columns 10–12. However, the columns in row H were the control, and the wells in H1, H4, H7 and H11 were alternatively filled with 100 µl of sterile double-strength LB broth with glucose and 100 µl of sterile phage buffer. The remaining wells in row H were filled with 100 µl of sterile phage buffer solution and appropriate bacterial CFU/well without phage. Phage dilution in SM buffer solution was added (100 µL) into the wells, from row A–G to obtain 1×10^7 – 1×10^1 PFU/well (MOI: 1–0.00001). The plate was incubated in 120 rpm thermo-shaker at 37 °C for 18 h. After incubation, 50 µl of 0.1% filter-sterile 2,3,5-triphenyltetrazolium chloride (TTC) (Alfa Johnson Matthey Company, Lancaster, USA) was added into each well (final concentration 200 µg/ml, 50 µg/well) and incubated for additional 3 h. Absorbance was read at 540 nm, using a micro-titer plate reader (PerkinElmer Wallac EnVision 2104 Multilabel Reader, USA) [22].

2.9.1 In vitro determination of phage removal of biofilm

The efficacy of phage to remove biofilm formed by MDR clinical *P. aeruginosa* was investigated using the protocol as previously demonstrated with modification. The test bacteria were cultured overnight in three different media (LB, Muller Hinton broth, and TSB) at 37 °C. The strain of *P. aeruginosa* known as PA ATCC was used as control. The host and the control bacteria were diluted to OD₆₀₀ 0.1, 0.3 and 0.5. The test was determined as previously described [22].

3 Results

3.1 Antibiotic sensitivity of host cell

The *P. aeruginosa* studied was 100% resistant to some β-lactam (ampicillin and ampicillin/sulbactam) and cephalosporin (cefepodoxime, cefuroxime and cefuroxime/axetil) tested. It was 97% resistant to cefoxitin and 95% to cefotaxime, tetracycline and trimethoprim/sulfamethoxazole, respectively. However, carbapenem was the most effective antibacterial agent being 98% susceptible. The susceptibility pattern of the tested strain to antibiotics is shown in Table 1.

3.2 Isolation and host range of ϕParuNE1 phage

The phage specific to MDR clinical *P. aeruginosa* was isolated from sewage water. The phage was designated ϕParuNE1. It exhibited potent lytic activity against MDR clinical *P. aeruginosa* isolate with clear large to medium plaques that measure 1–2.5 mm in diameter (Fig. 1). The titer was 1×10^{11} PFU/ml. The phage was stable during long-term storage at 4 °C in phage buffer solution (100 mM NaCl, 25 mM TrisHCl pH7.5, 8 mM MgSO₄, 0.01% w/v gelatine).

3.3 Phage morphology and family affiliation

The appearance of the phage by transmission electron microscope is shown in Fig. 2. The nature of the nucleic acid and the morphology of the phage showed icosahedral capsid of 60 nm in diameter, and a stubby, non-contractile tail measuring 8 nm. The phage was assigned to the family *Podoviridae* on the basis of its morphology.

3.4 Phage genome isolation and protein analysis

The genome of the ϕParuNE1 phage as detected on 1% agarose gel was a double stranded DNA due to its sensitivity to restriction endonucleases EcoRI, EcoRV and Hind III. The DNA size was 45 kb (Fig. 3A) and was sensitive to digestion by EcoRI, EcoRV and Hind III except PstI enzymes. Distinct fragments were obtained when the DNA was digested with these enzymes. The EcoRV enzyme yielded six fragments, while EcoRI and Hind III enzymes yielded four fragments, respectively (Fig. 3B). The SDS-PAGE revealed one predominant polypeptide with a molecular mass of 43 kDa (Fig. 3C).

3.5 Phage host range

The host range test had nine MDR *P. aeruginosa* susceptible to ϕParuNE1 phage, while others including laboratory strain of *P. putida* and control strain of PA01 were

Table 1 Antibacterial sensitivity profile of the MDR clinical *P. aeruginosa*

Antibiotic	LSI resistance breakpoint (mm)	Sensitivity (%)	Intermediate sensitivity (%)	Resistance (%)
1 Ampicillin	≤ 13	0	0	100
2 Ampicillin/Sulbactam	≤ 11	0	0	100
3 Cefotaxime	≤ 14	0	5	95
4 Cefoxitin	≤ 14	0	3	97
5 Cefpodoxime	≤ 17	0	0	100
6 Ceftazidime	≤ 14	91	4	5
7 Cefuroxime	≤ 14	0	0	100
8 Cefuroxime/Axetil	≤ 14	0	0	100
9 Ciprofloxacin	≤ 15	83	0	17
10 Gentamycin	≤ 15	38	43	19
11 Imipenem	≤ 13	95	5	0
12 Levofloxacin	≤ 13	82	0	18
13 Meropenem	≤ 15	98	2	0
14 Nitrofurantoin	≤ 13	95	0	5
15 Piperacillin	≤ 14	20	67	13
16 Piperacillin/Tazobactam	≤ 14	17	70	13
17 Tetracycline	≤ 11	5	0	95
18 Tobramycin	≤ 12	85	0	15
19 Trimethoprim/sulfamethoxazole	≤ 10	5	0	95

CLSI Clinical Laboratory Standard Institute

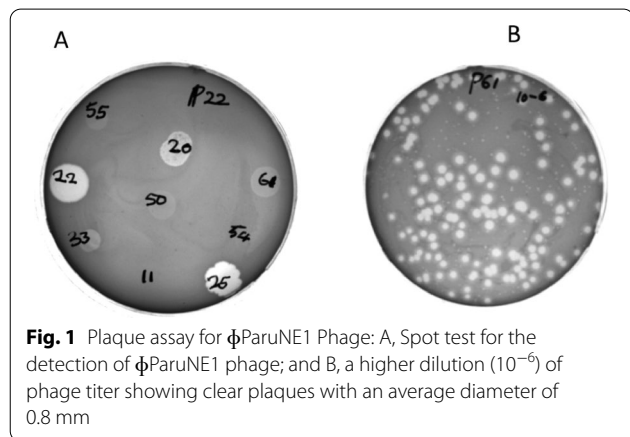


Fig. 1 Plaque assay for ϕ ParuNE1 Phage: A, Spot test for the detection of ϕ ParuNE1 phage; and B, a higher dilution (10^{-6}) of phage titer showing clear plaques with an average diameter of 0.8 mm

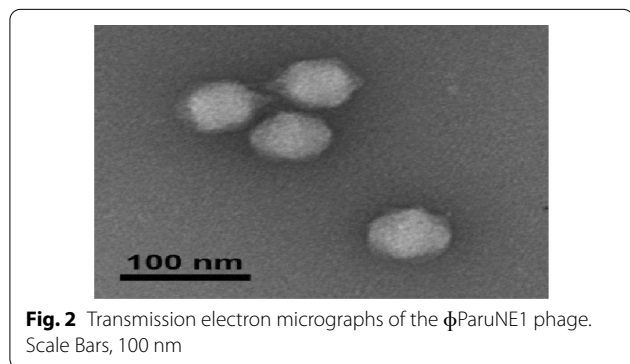


Fig. 2 Transmission electron micrographs of the ϕ ParuNE1 phage. Scale Bars, 100 nm

resistant. The characteristic clear zone of inhibition is shown in Table 2.

3.6 Phage binding rate at various temperatures

The phage showed varied adsorption rate at different temperature ranges. A higher number (57%) of ϕ PauNE1 phage adsorbed at 44 °C, 47% at 15 °C, 42% at 37 °C, while 40% adsorbed at 7 °C; however, a lowest number (37%) adsorbed at 25 °C. Phage was able to adsorb within the temperature range of 7–44 °C, their adsorption rates were considerably higher at 15 °C, reaching maximum at 44 °C (Fig. 4).

3.7 pH stability

The ϕ PauNE1 phage stability was maximal at pH 10 (160% survival) and was also relatively stable at other pH values of 5 (86%), 9 (97%) and 7 (100%). A low pH values of 3 showed 65% phage survival, while 1.5 pH values was lethal and totally inactivated the phage (Fig. 5).

3.8 Growth characteristic

The latent phase for ϕ ParuNE1 was longer (Fig. 6) and estimated to be 28 min. The number of phages in the culture increased suddenly as the phage released the progeny. The ϕ ParuNE1 had burst size of 300 phage per infected cell.

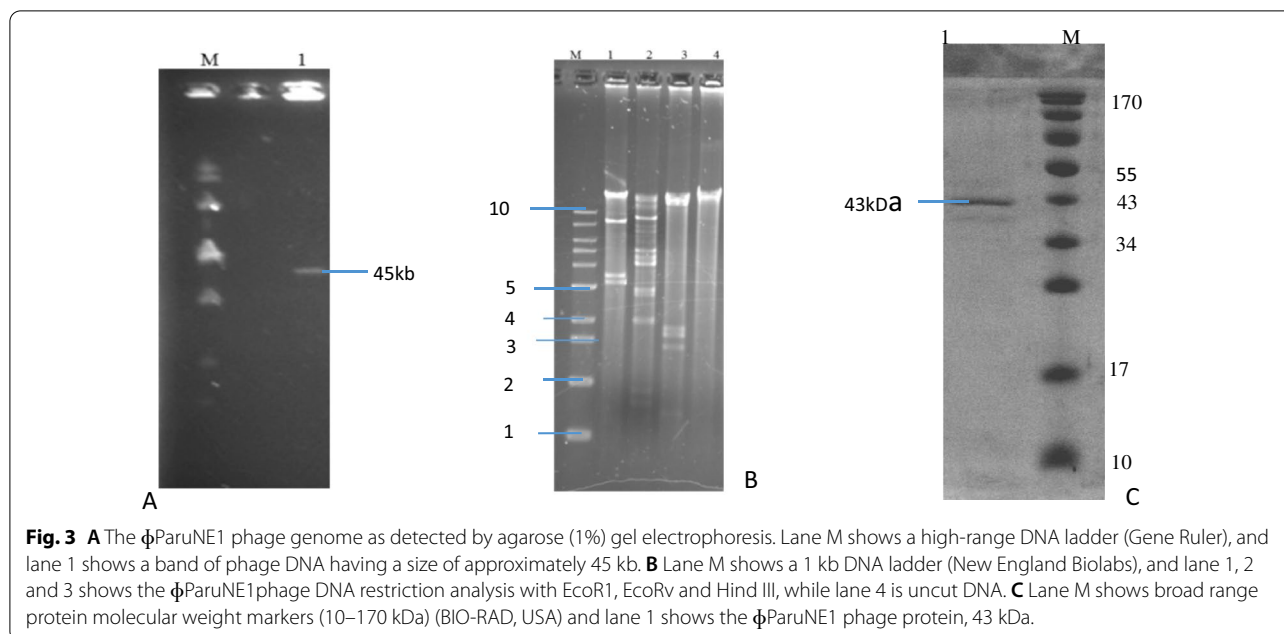


Fig. 3 **A** The ϕ ParuNE1 phage genome as detected by agarose (1%) gel electrophoresis. Lane M shows a high-range DNA ladder (Gene Ruler), and lane 1 shows a band of phage DNA having a size of approximately 45 kb. **B** Lane M shows a 1 kb DNA ladder (New England Biolabs), and lane 1, 2 and 3 shows the ϕ ParuNE1 phage DNA restriction analysis with EcoRI, EcoRV and Hind III, while lane 4 is uncut DNA. **C** Lane M shows broad range protein molecular weight markers (10–170 kDa) (BIO-RAD, USA) and lane 1 shows the ϕ ParuNE1 phage protein, 43 kDa.

Table 2 Bacterial strains used in the study for ϕ ParuNE1 phage host range

<i>P. aeruginosa</i> strains	Lysis
PA 22-MDR CLI	+
PA 61	+
PA 20	+
PA 55	–
PA 54	–
PA 33	+
PA 64	+
PA 25	+
PACS	–
PA 46	–
PA 24	C
PA 52	–
PA 40	C
PA 59	+
<i>P. putida</i>	–
PA 01	–
PA 14	+
PA 44	+
	60%

3.9 Bacteria growth and biofilm formation inhibition

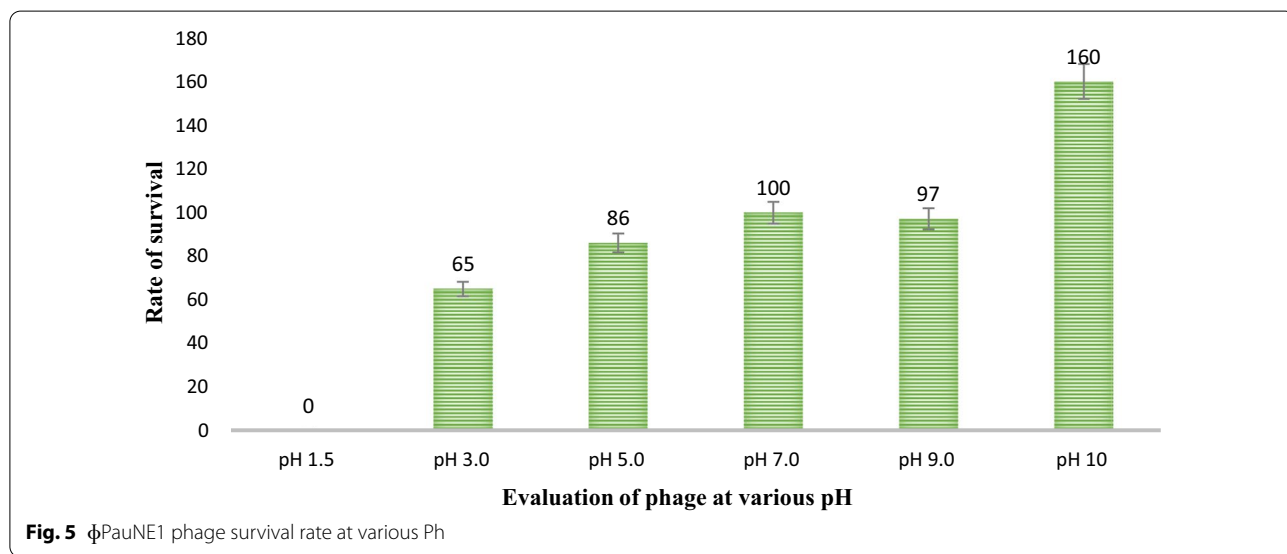
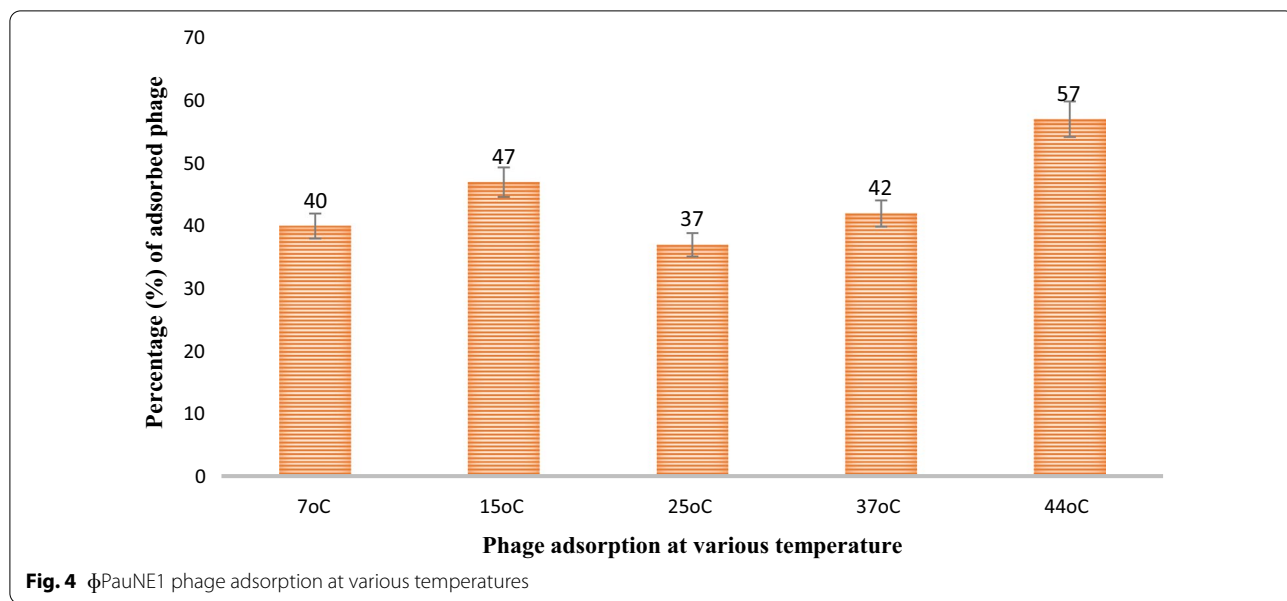
The phage inhibited bacterial growth at all multiplicity of infection (MOI) (1, 0.1, 0.01, and 0.001). Inhibition was higher at both high and low CFU/ml of 10^9 , 10^7 and 10^5 . The ϕ ParuNE1 phage inhibited 40%, 57% and 58% of MDR clinical *P. aeruginosa* strain at MOI (1×10^2 PFU/

ml [MOI 0.00001]) with CFU/ml 10^9 , 10^5 and 10^3 , respectively. The results are shown in Fig. 7. The ϕ ParuNE1 phage removed the biofilm formed by host cell, as well as the cell biomass by more than 60% when the host cell counts was 1.5×10^8 cfu/ml, 6.0×10^8 cfu/m and 1.0×10^9 cfu/ml, respectively (Fig. 8).

4 Discussion

Multi-drug resistance is a global public health challenge. The rates of resistance in *P. aeruginosa* are increasing worldwide [25, 26]. Besides, the remarkable ability of *P. aeruginosa* to form biofilms in many environments renders antibiotic treatments ineffective, thereby promoting chronic infectious diseases and mortality [27–29]. Consequently, public health experts and researchers are in search of newer antibacterial agents including phage therapy as demonstrated in this study and so some other reports [30].

Bacteriophages are in abundance in aquatic environments and play useful role in controlling their host populations [31]. Hence, it could be used to eliminate microbial contaminants that pose threats to public health. Phages have been successfully demonstrated for therapy in some bacterial diseases for example, cases of chronic otitis [32], burn wounds [33], chronic lung infection and biofilm-associated cystic fibrosis [34]. This report presents species specific phage which is likely one of the few reported cases of phage lytic to this strain as there were no reports of lytic phage to all *P. aeruginosa* species. Thus, the underlying aim is to identify and characterize novel bio-phage that show a high specificity and

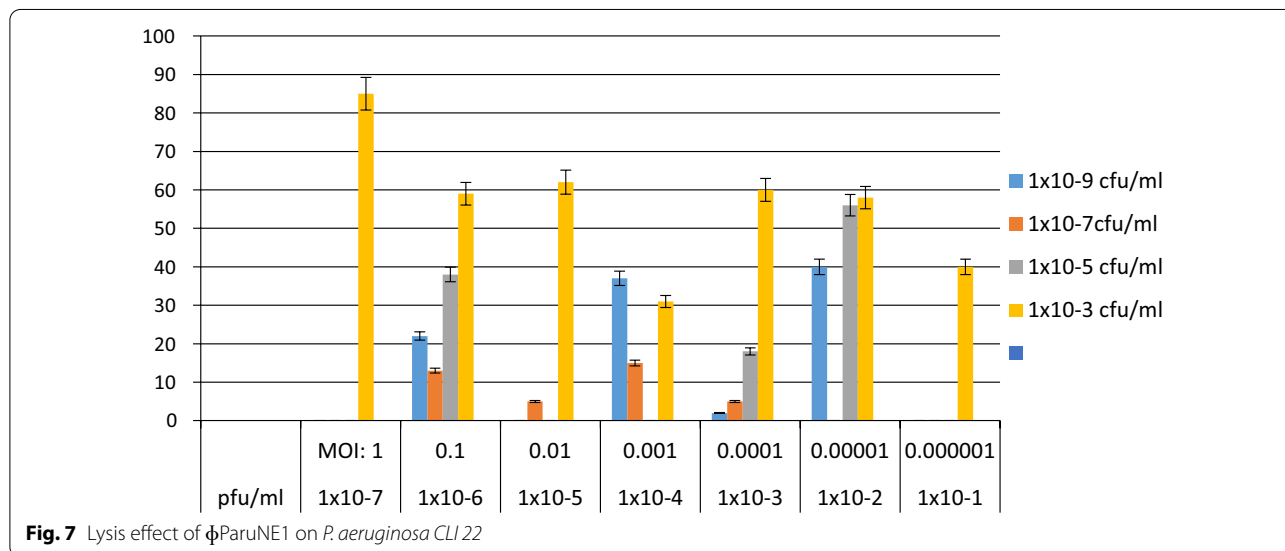
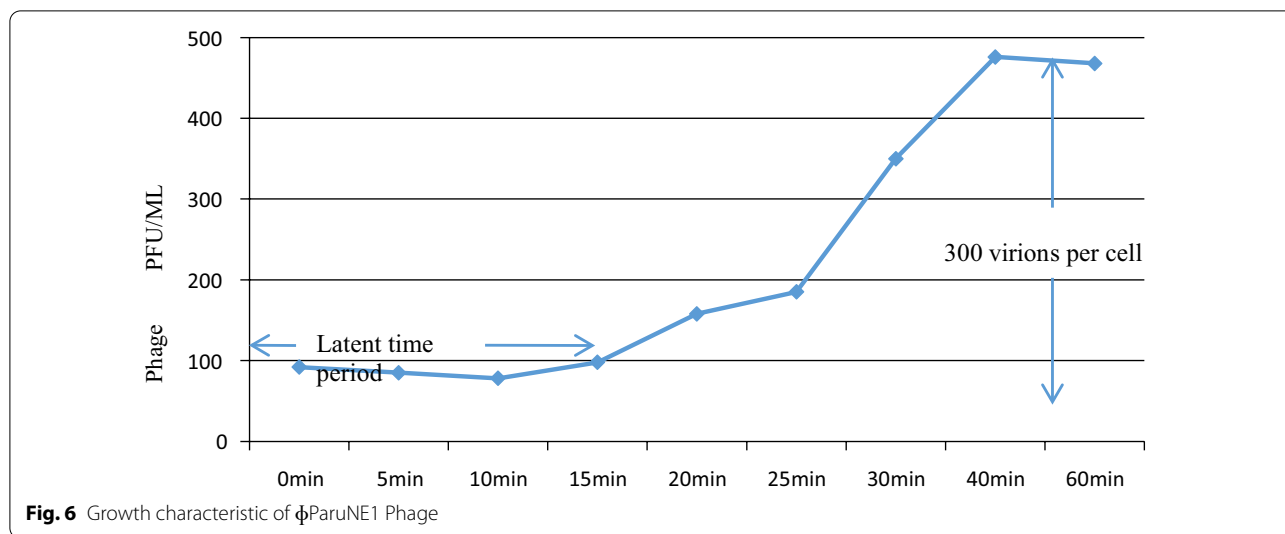


a narrow range against newly emerging and re-emerging MDR *P. aeruginosa* strains.

The ϕ ParuNE1 phage was isolated from sewage water. This supports earlier report that sewage is quite rich in nutrient for the survival of diverse microorganisms and phages are usually found in association with their host bacterial species [35, 36]. The ϕ ParuNE1 phage was highly lytic, giving clear plaques of an average diameter of 1–2 mm. It showed a very narrow host range, infecting only *pseudomonas* species among the bacteria tested. These include *P. aeruginosa* CLI 22, *P. aeruginosa* 61, *P. aeruginosa* 20, *P. aeruginosa* 25, *P. aeruginosa* 33 and *P.*

aeruginosa 64. This is in line with the previous report that phages are very specific to their host cell receptors [37].

Pseudomonas aeruginosa grows in temperature range from 10 to 44 °C, with optimal temperature around 35 °C [42]. Accordingly, it is expected that the highest adsorption rates of its phage should be within this range and this agrees with this report. Previous findings have reported that bacteriophages may vary in both their thermal and pH stability [36]. This phage (ϕ ParuNE1), therefore, showed very high and almost identical adsorption at both 7 and 37 °C; being considerably higher at 15 °C, reaching



maximum at 44 °C. This has demonstrated high tolerance to a wide range of temperature (7–44 °C). This finding was in agreement with earlier observation [24]. Similarly, our data on ϕParuNE1 phage viability at various pH values showed good stability over a wide (pH 3 to 10) pH range. The phage was inactivated at lower pH values of 1.5 but showed maximum stability at pH 10. This finding is in agreement with earlier report that most phage thrive well at pH range of 5 to 9 under physiological conditions [36]. Survival of phages at neutral and alkaline pH in contrast with acidic pH has been reported [38]. They are said to be less stable in acidic environments since it denatures their proteins [39]. Accordingly, the examined ϕParuNE1 phage could be considered for topical application for skin

and soft tissue wound treatment and intravenous application, with skin and blood, pH being around 5.5 and 7.4, respectively. Oral administration of this phage formulation may not be efficient since the stomach pH is usually acidic.

The growth curve of ϕParuNE1 phage showed a latent period of 28 min and a burst size of 300 virions per cell. Similarly, a latent time of 21 min and a burst size of 320 virions per cell was reported for the Myoviridae MJ1 phage against *E. coli* [40], while a latent time of 28 min and a burst size of 300 virions per cell was reported for Myoviridae WZ1 phage against *Shigella dysenteriae* [41]. In contrast, a previous report [39] showed a small burst size of 13 virions and high latent

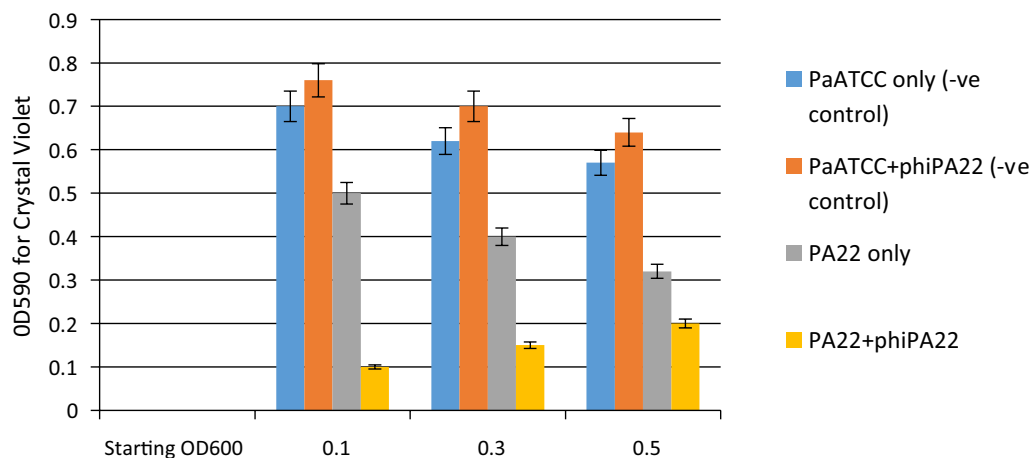


Fig. 8 Effect of ϕ ParuNE1 phage on biofilms produced by *P. aeruginosa* CLI 22

time period of 31 min, indicating that both latent time and burst size vary among different bacteriophages; the differences may be associated with their size and nature of the envelope. The later therefore, underscore the importance of certain parameters such as inoculation timing, phage absorption rate to the host, and burst size as critical factors to be considered for the success of formulation of bio-phage therapy.

According to electron micrograph, ϕ ParuNE1 phage is placed within the family *Podoviridae* since the genome is double stranded and also sensitive to restriction endonucleases that cut only dsDNA. This observation agrees with earlier report that all *Podoviridae* possessed dsDNA as genetic material [40]. The examination of in vitro phage lytic efficacy showed that ϕ ParuNE1 phage attained more than 60% of bacterial reduction at MOI 1, 0.1, 0.01, and 0.001, respectively, for both bacterial planktonic growth and growth in the form of biofilm. The maximal bacterial lysis was observed during 16-h time period. Inhibition occur at both higher and lower MDR clinical *P. aeruginosa* CFU/ml of 10^9 , 10^5 and 10^3 . The MDR clinical *Pseudomonas aeruginosa* formed biofilms in all the media used. The phage almost eliminated biofilm in all MOI and reduced biofilms for more than 60% with MOI 0.1 at high cfu/ml of host cell strain, hence indicating the impressive potential of this phage for use in phage therapy. Accordingly, the phage shows very good potential as *P. aeruginosa* biofilm formation control agent. Few studies have demonstrated the ability of phage to reduce cell density of *P. aeruginosa* biofilms in line with this report [27]. However, source of bacterial isolate may vary. This study may have confirmed the potentials of phage as a possible bioagent to control *P. aeruginosa* biofilm that presently constituting public health threat.

5 Conclusion

ϕ ParuNE1 virulent phage is effective in the eradication of MDR clinical *P. aeruginosa* planktonic culture and biofilms with some remarkable properties. It possessed a latent time period and burst size suitable for bacteriophage therapy. With these potential characteristics, the ϕ ParuNE1 phage promising candidate for phage therapy against *P. aeruginosa* infection and contamination and therefore recommend that this potential be further explored.

Abbreviations

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; MDR: Multi-drug resistant; LBB: Luria-Bertani broth; TSB: Tryptone soya broth.

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Authors' contributions

NV conceived and designed the study, carried out the experiments and also the major contributor in writing the manuscript. JJ designed and supervised the phage isolation procedures. KP co-supervised the experiment, also analyzed and interpreted the data regarding phage inhibition of bacterial growth and biofilm formation. CA contributed in bacteria isolation, identification and writing of manuscript. RY performed electron microscopy of the phage. AO is the principal academic supervisor, co-designed the experiments and co-reviewed the manuscript. JD hosted my training, co-designed the experiments, supervised the molecular experiment of the phage and co-reviewed the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All the data generated and analyzed during the study are included in the main manuscript.

Declarations

Ethics approval and consent to participate

The study approval was obtained from the Institutional Review Board (IRB) of the College of Medicine, University of Lagos, Nigeria (ref. No: CM/COM/8/VOL. XIX). Between June 2007 and April 2009, clinical samples were collected from Lagos University Teaching Hospital (LUTH), Idi-araba and National Orthopaedic Hospital, Igbobi (NOHI). All participants consented and filled the informed consent form. The consent obtained from study participants was written.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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