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# Isolation and characterization of a lytic bacteriophage (VB_PAnP_PADP4) against MDR- *Pseudomonas aeruginosa* isolated from septic wound infections

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**Resistance to multiple drugs by pathogens has become the sweltering problem for the treatment of bacterial infections. To overcome this problem of drug resistance, several diversified bacteriophages can be used as one of the alternatives and attractive approaches. The present study aimed to detect, isolate and partially characterize the bacteriophage used against MDR-*Pseudomonas aeruginosa* isolated from wound infections. A total number of 130 pus samples were collected and processed for the bacterial isolates from which a potential pathogen (*P. aeruginosa*) was identified and characterized using morphological, biochemical and molecular techniques (16S r-DNA technique). Among the Pseudomonas isolates, 26 multidrug-resistant strains were detected by Kirby-Bauer disc diffusion method. Bacteriophages (n=15) were collected against the MDR-*P. aeruginosa* and one of the efficient lytic phage (PADP4) was selected for this study. The transmission electron microscopic studies revealed that the selected phage PADP4 morphological features resemble the *Podoviridae* family. Phage PADP4 had the highest stability in a broad range of pH, temperatures, and high burst size (270 phages/ infected bacterial cells). Further, the potential phage, PADP4 was co-cultured with MDR-*Pseudomonas aeruginosa* and showed a significant growth reduction at 12 h of incubation and at multiplicity of infection of 1. Irrespective of the drug resistant bacteria, phage PA DP4 showed perfect lytic activity against the MDR- *P. aeruginosa*. It has been clarified that PADP4 could be a future promising agent for controlling MDR-*P. aeruginosa*.**

**Key words:** Septic wounds, MDR-*P. aeruginosa*, bacteriophage, transmission electron microscope (TEM).

## INTRODUCTION

Pseudo*monas aeruginosa* (*P. aeruginosa*) is a gram-negative bacillus, motile, a facultative anaerobe, and non-spore former; it is responsible for a wide variety of diseases in both humans and animals. Generally, *P. aeruginosa* is an opportunistic and nosocomial infectious organism that can could develop infections in severe

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burns, injured and surgical wounds and as well as in immunocompromised subjects. It is also responsible for the development of pneumonia, upper respiratory tract infections, bacteremia, endocarditis and urinary tract infections (UTI) (Kumari, 2009; Garbe et al., 2010; Kwiatek et al., 2015).

Development of natural drug resistance by bacterial pathogens is one of the greatest problems to the human health. During the clinical manifestations, *P. aeruginosa* acquired resistance to most of the antibiotics which were precisely available. During the infestation, all the MDR-pathogens formed biofilm which facilitated infections such as catheter associated urinary tract infection (UTI), and ventilator-associated pneumonia. In general, most of the bacterial pathogens were encapsulated with polysaccharide matrix, which is difficult to treat with conventional therapeutics. However, these signs contribute to a significant increase in mortality and morbidity rate particularly during sepsis. Therefore, there is an urgent need for the development of alternative therapies, in general and MDR-Pseudomonas, in particular. Mehta et al. (2007) and Yakha et al. (2015) reported that treatment with broad-spectrum antibiotics may eradicate the planktonic cells unlike biofilms; hence, an alternative strategy is required globally to protect the clinical community.

Bacteriophage therapy is a good old method in ancient times where there was no awareness of chemotherapies. Due to greatest achievement and production of antibiotics, the application of bacteriophages was overwhelmed. So far so good the MDR-bacterial strains renewed the interest and recapped the application of phages as therapeutic agents (Azizian et al., 2013). Specific bacteriophage selectively kills bacteria and this phenomenon may be an excellent alternative method to solve the global problem of multidrug resistance. However, the use of bacteriophages against several pathological abnormalities is referred to as phage therapy. Instead of whole phages, phage lysins were the primitive factors in this regard. Usually, bacteriophage therapy is used on the basis of lytic state of art against MDR-strains and has several advantages than antibiotics. The major advantages of the phage therapy include host specificity, self-replication and environmental safety of bacteriophages (Azizian et al., 2013; Chan et al., 2013).

The basic essence of bacteriophages is that they attack and destroy specific pathogens where unspecified bacteria are not affected, making them to live freely in the environment. Feasibility bacteriophage multiplies very rapidly in the host bacteria, increases the phage titer and is far more effective to control pathogens. Exclusively when the concentration of the pathogen decreases sufficiently, the phage titer also declines. This is one of the remarkable indicators of phage therapy, which has also been confirmed successful in aquaculture to protect one from experimentally induced bacterial infections. Therefore, it has also been approved by the USA research groups and they continue to work towards the development of the whole phage to be used as an alternative therapeutics (Kutter et al., 2010). This process is very much similar with optimization and purification of phage components as well as antibacterial substances opening up new opportunity against various vital infections (Piracha et al., 2014; Sulakvelidze et al., 2001).

In the present study, we have reported the detection and partial characterization of bacteriophage PADP4, isolated against MDR-*P.aeruginosa* (National Center for Biotechnology Information (NCBI); the accession number allotted to *P.aeruginosa* in this study is *P.aeruginosa* KY018605.

## MATERIALS AND METHODS

### Bacterial isolate

Different *P. aeruginosa* strains were isolated from pus samples of septic wounds and identified by different cultural methods (cetrimide agar media). They were further identified by using morphological and physiological test (gram staining, oxidase, IMVIC test, nitrate reduction test and catalase, carbohydrate fermentation test for glucose, maltose, lactose, galactose and sucrose). After confirmation by 16S r-DNA sequencing, *P.aeruginosa* culture was preserved for further analysis.

### Screening method for MDR-pathogen

*P. aeruginosa* was the most predominant isolate from the septic wounds, tested for antibiotic susceptibility to identify MDR-bacteria. An MDR-bacterium must show resistance to different antibiotics with different concentrations. The following lists of antibiotics were used for the selection of MDR strains and their susceptibility patterns were recognized exclusively according to the Clinical and Laboratory Standards Institute (CLSI) guidelines; (benzylpenicillin (30 µg), ampicillin (30 µg), amoxicillin (30 µg), kanamycin (20 µg), tobramycin (20 µg), streptomycin (20 µg), cefotaxime (20 µg), vancomycin (10µg) and tetracycline (10 µg) (Towns et al., 2010; Jaafar et al., 2014). The most potent MDR-*P. aeruginosa* was selected for these studies and were submitted to NCBI for the allotment of accession number.

### Molecular identification of MDR-*P. aeruginosa*

One among the *P. aeruginosa* isolates was noticed to have the highest drug resistance to a wide range of antibiotic regimen; therefore, it was selected for the molecular confirmation by 16S r-DNA sequencing. The selected and confirmed strain was inoculated with 100 mL of Luria Bertani (LB) broth and incubated at 42°C and 120 rpm for 24 h. After incubation, the culture was subjected to centrifugation at 8000 rpm /10 min. Further, the pellet was collected for DNA extraction using DNA extraction kit (Thermo fisher scientific company, USA). Meanwhile, the culture suspension was measured by UV spectrophotometer for the culture density component and the primers were used for its molecular diagnosis by PCR. Primer information is shown in Table 1. Therefore, the PCR amplicon was purified and further processed for sequencing. Forward and Reverse DNA sequencing reaction of PCR amplicon was carried out with 704F and 907R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. A consensus sequence of 1512 bps

**Table 1.** Sequence information of universal primer of *Pseudomonas aeruginosa*

| S/N | Universal 16S rRNA | Primer sequence (5' to 3') | Product length (bp) |
|---|---|---|---|
| 1 | Forward primer | GGCGACCACCTGGACTGATACTGACACTGAGG TGCGAAAGCGTGGGGAGCAGAC | 785 |
| 2 | Reverse primer | ACCTGGACTGATACTGACACTGAGGTGCGAA AGCGTGCGAAGCAAAC | 876 |

16S r-DNA was generated from forward and reverse sequence data using multiple alignment software program Clustal W. The 16S r-DNA sequence was used for BLAST alignment search tool of the NCBI Genbank for the accession number (Table 1).

**Bacteriophage isolation**

Sewage samples were collected from nearby RIMS hospital, Kadapa, Andhra Pradesh; they were used to isolate selective bacteriophages against MDR-*P. aeruginosa* strain. The collected sewage samples centrifuged at 8000 rpm for 10 min to debris were removed and the filtrate was filtered through 0.45 µm syringe driven filters (Millipore filters, Himedia, Mumbai) mixed with LB broth containing a selected strain of MDR -pathogen. This mixture was incubated at 37°C for 15 h in a shaking incubator and later centrifuged at 10,000 rpm per 10 min. It was then filtered through a 0.45 µm membrane. So the isolated phage filtrates were screened for host-specific phages by spot test. Subsequently, the positive culture from the spot test was further used for plaque forming assay by double layer agar method, which indicates titer levels of the phages. The same experiments were repeated successively at time interval to obtain single and pure plaques or phage stocks. These lysates were filtered (50 mM Tris-CL, 99 mM NaCl, 8mM $MgSO_4$, 0.01 gelatin (pH 7.5) at 4°C with 1% chloroform (Khairnar et al., 2013; Pallavali et al., 2017).

**Plaque assay**

A plaques assay or agar overlay method or double layer agar method was used to quantify the titer of lytic bacteriophages as plaque forming units (PFU). $10^{-3}$ of bacteriophage (PADP4) dilutions (1000 fold serial dilution in SM buffer) was mixed with 18 h old culture of MDR-*P. aeruginosa* and incubated at 37°C for 30 min to allow the phages to be absorbed by the bacterial cell. To this mixer, a total of 5 mL of 45°C top LB agar was added and then poured on to the previously solidified bottom LB agar. The plates were incubated at room temperature for 18-24 h and counted to estimate the abundance of lytic bacteriophages (Santos et al., 2009).

**Latent period, burst size of phage PA DP4**

One step growth curve experiment was implemented to determine the latent period and burst size of the phages. The single step growth curve experiment was performed as described by Pajunen et al. (2000). 50 mL of MDR-*Pseudomonas* KY018605 culture was incubated to mid-exponential phase (reaching to O.D600 0.6); then cells were harvested and centrifuged (10000 rpm per 30 s at 4°C). The pellet was re-suspended in 0.5 mL of LB broth and mixed with 0.5 mL of the phage solution having plaque forming unit (PFU) of $1x10^9$ PFU/mL. This mixture was allowed to stand for 3 min at 37°C so that the phages were adsorbed by the host cells. The mixture was then centrifuged at 13,000 rpm for 2 min to remove the free phage particles. The pellet was re-suspended in 100 ml of LB broth and culture was incubated at 37°C with shaking at 150 rpm. Samples were examined for every 3 minutes up to 45 min and after centrifugation at 13,000 rpm for 1 minute, the phage titrations were obtained by double layer agar method (triplicates). The latent period was defined as the time interval difference between the adsorption and beginning of the initial rise in the phage count. The burst size of phage PADP4 was calculated as the ratio of the final phage titer to the initial count of infected bacterial cells during the latent period (Bolger-Munro et al., 2013; Piracha et al., 2014).

**Analysis of calcium and magnesium ion effect on phage adsorption**

In order to measure the effect of divalent metal ions on phage adsorption rate $CaCl_2$, $MgSO_4$ was used (Suárez et al., 2008). The 100 ml of *P. aeruginosa* overnight culture having O.D 600 0.6 was divided into four autoclaved flasks of 25 ml each. In one set of the flasks was inoculated with 500 µL phage having $1x10^9$ $PFU^{-1}$(control), while the second set of flasks was inoculated both with 500 µL phage and 250 µL of 10 mM $CaCl_2$, $MgSO_4$ and incubated with constant shaking at 120 rpm and 37°C. Samples were taken from both sets of flasks at different time intervals of 0, 5, 10, 15, 20, 25, 30 and 35 min. Samples were centrifuged at 13,000 rpm for 3 minutes to sediment the phage adsorbed bacteria. The supernatant was assayed for unabsorbed phages via double layer plaque assay method and counts were compared with the titer of control (Piracha et al., 2014; Suárez et al., 2008).

**Thermal and pH stability of phage PADP4**

Thermal stability tests were used to describe the impact of environment factors on phage growth, and their stability is required to maintain as lytic phage. The optimization studies (temperature, pH, metal ion effects) as obtained showed the level of integrity and exclusions of phage in an appropriate concentration (Suárez et al., 2008). Phage filtrates ($1 \times 10^9$ PFU/ml) were taken in micro-centrifuged tubes and treated under different temperatures: 37°C (control), 40, 50, and 60°C for 30, 60 and 90 min. After incubation, double layer agar method was performed for each treated sample to evaluate the lytic ability of phages with respective temperatures compared to the control (37°C) (Kęsik-Szeloch et al., 2013; Piracha et al., 2014). For the pH stability assay, a phage suspension ($1\times 10^9$ PFU/ml) was inoculated in a series of tubes containing SM buffer at pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0 and incubated at 37°C for 4 h. After incubation double layer agar method was performed for each treated sample to evaluate the lytic ability of phages with respective PH compared to the control $P^H$ 7. All assays were performed in duplicate (Suárez et al., 2008).

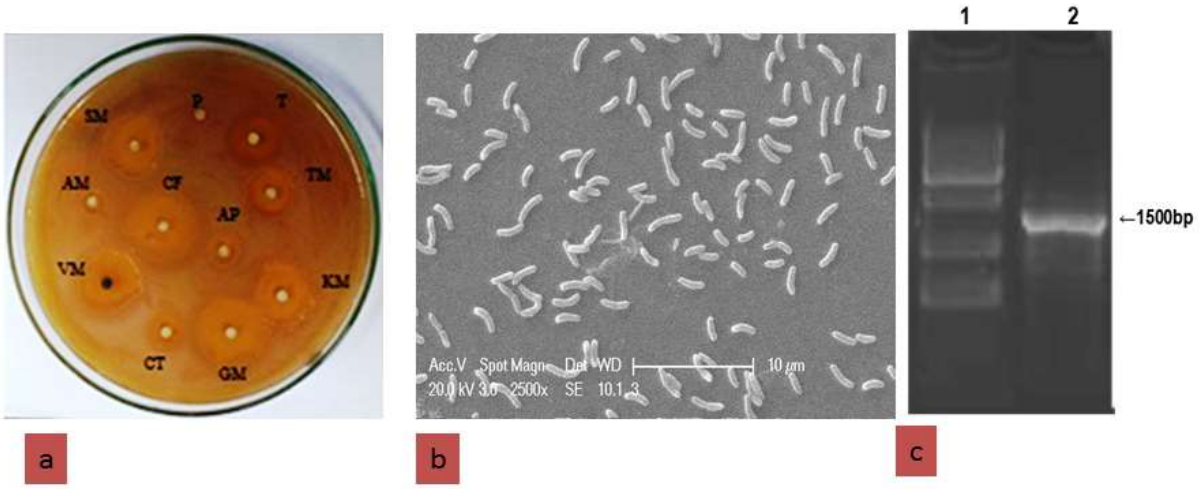


**Figure 1.** Antibiotic susceptibility pattern and SEM image of *Pseudomonas KY01860.* **a)** *P. aeruginosa KY018605* on Muller-Hinton agar media was sensitive to ciprofloxacin (CF), gentamycin (GM), vancomycin (VM), intermediate sensitive to kanamycin (KM), tetracycline (T), and streptomycin(SM), and resisted benzyl penicillin (P), amoxicillin (AM), ampicillin (AP), cefotaxime (CT). **b)** Scanning electron microscopy (SEM). *P. aeruginosa* grown in LB agar media for 12 h, fixed in paraformaldehyde on coverslip, dehydrated in increasing concentrations of ethanol (30-100%), and sputter coated before SEM. Bacterial cells were arranged as slight curved rods. **c)** 0.8% Agarose gel showing single 1.5 kb of 16S r DNA amplicon. Lane 1, 1 Kb DNA ladder; Lane 2, 16Sr DNA amplicon.

### Host range

The selection of PADP4 bacteriophage host range was confirmed based on clinical significance such as *P. aeruginosa, S. aureus, Escherichia coli* and *Klebsiella pneumoniae* isolated from wound sepsis. The susceptibility range of bacteriophage was tested via spot assay technique along with the control (Armon and Kott, 1993).

### Bacterial reduction assay

*P. aeruginosa* pure culture was maintained spectrophotometrically ($OD_{600}$ at 0.6) and 1 mL of culture suspension was inoculated in 2 flasks having 150 mL of LB broth. The phage supernatant 500 µL was introduced in one flask and another flask was treated as control; both flasks were incubated in shaking incubator at 37°C and 120 rpm. The $O.D_{600}$ readings were taken after every 2 hours for 24 h using spectrophotometer. The values were compared with control and triplicates were maintained for analysis of results (Pallavali et al., 2017; Piracha et al., 2014).

### Phage confirmation study by transmission electron microscope

TEM analysis was conducted to determine the characterization of bacteriophage including its morphology. The confirmed and partially characterized bacteriophage was used to treat the MDR-pathogen isolated from septic wounds. TEM analysis was carried out with FEI Tecnai G2 S-Twin, Department of Nano Technology, and University of Hyderabad, India. Further, the purified bacteriophage filtrate was filtered through 0.45 µm filters (0.45 µm PVDF syringe driven filters, Hi Media, Mumbai) with consent monitoring of centrifugation at 30,000 rpm for 60 min (Beckmann Ultracentrifuge, Proteomics laboratory, University of Hyderabad, Hyderabad). The pellet was mixed with 5 mL of SM buffer. 5 µL of phage suspension was overlaid on the formvar coated 200X200 copper grid; excess phage solution was removed with the pieces of filter paper from the edges of the grid and we waited for 3 min. 5 µL of 0.5% uranyl acetate was then applied to the grids, excess solution was immediately removed, and grids were air dried. Samples were viewed with the FEI Tecnai G2 S-Twin Transmission Electron at operated voltage of 80 KV (Kwiatek et al., 2015).

## RESULTS

### Isolation and identification of MDR- *Pseudomonas* KY018605

*P. aeruginosa* is the predominant isolate of septic wounds from 130 pus samples. Of the 26 *P. aeruginosa* isolates PA 13 was selected for further studies because it showed resistance to cefotaxime (20 µg), benzyl penicillin (30 µg), ampicillin (30 µg), tobramycin (20 µg) and amoxillin (30 µg). The drug resistance pattern, scanning electron microscopic image (SEM) and 16S r- DNA sequencing information is shown in Figure 1a, b and c.

In Figure 1a, it is seen that *P. aeruginosa* PA 13 showed resistance to these antibiotics of three different classes. Strongly, there is a fascinated multidrug resistance activity constantly identified as a specific host for isolation of bacteriophages. Scanning electron microscopy studies revealed the morphology of *P. aeruginosa* (Figure 1b); *P. aeruginosa* PA13 strain had the highest drug resistance, so it was treated and selected as the best strain for our study. Further it was characterized by 16 S r-DNA (1500bp) sequencing and substantially submitted to NCBI database, which provided its accession number as *Pseudomonas KY01860* (Figure 1c).

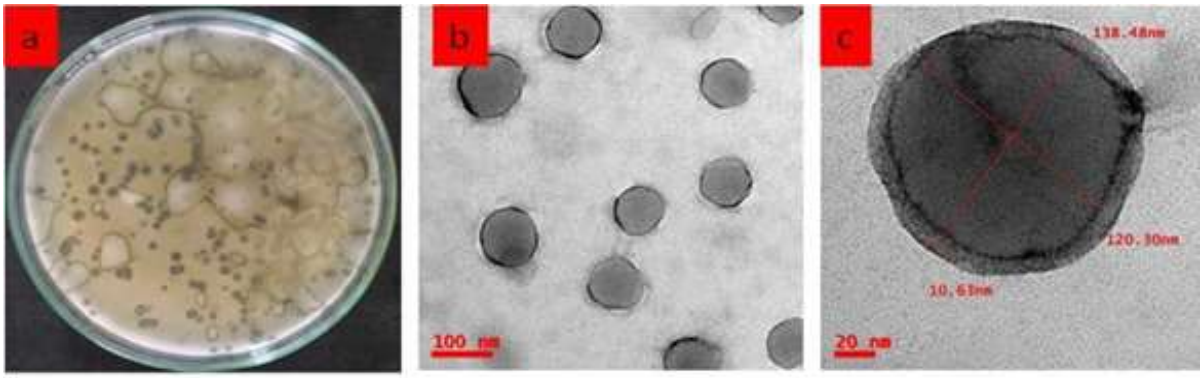


**Figure 2.** Phage PA DP4 bacteriolytic activities against *Pseudomonas KY018605.* **(a)** Phage $V_B$_PAnP_PADP4 forms plaques on the bacterial lawn by double layer agar method. **(b)** Phage PADP4 belonging to *Podoviridae* family and was characterized morphologically with Transmission Electron Microscopy (TEM) by negative staining with 2% uranyl acetate. Scale bar = 100 nm. **(c)** Enlarged image of Phage $V_B$_PAnP_PADP4 with scale bar = 20 nm.

### Isolation of bacteriophage against *Pseudomonas KY01860*

The respective bacteriophages were detected and isolated by double layer agar method. Amongst these isolates, phage PADP4 was selected and partially characterized. Phage PADP4 showed specific lytic activity against MDR-*P. aeruginosa* (Figure 2). Transmission electron microscope is one of the most precious and widely used methods for identifying the morphological features of bacteriophages as well as their classification. Phage PA DP4 showed icosahedral symmetrical head without tail. Based on these features, it was confirmed that PADP4 belongs to *Podoviridae* family (Figure 2b, 2c). This phage isolate was named according to the newly proposed naming system vBPanP/M/S KPno, where vB = bacterial virus; Pan = REBASE abbreviation for genus/species of the host; P = Podovirus; M=Myovirus; S = Siphovirus; KPno = name and number of phage. So the name of PADP4 was given as Phage $V_B$_PAnP_PADP4 (Kęsik-Szeloch et al., 2013).

### Biophysical characterization of Phage PA DP4

Later on, one step growth curve of PADP4 was observed at prominent latent period for 25 min, and an average burst size of PADP4 was calculated as 270 PFU/infected bacterial cell (Figure 3a). One step growth curve of bacteriophages was influenced by the host, selective medium and external factors. The bacterial reduction probability by bacteriophages was monitored from 2 to 24 h and also bacteriophage PADP4 *in vitro* assay (Figure 3b). From the figure, it shown that PADP4 has the highest lytic activity against MDR- *P. aeruginosa*, which reflects on the optical density of the infected organism and sometimes develops the biostatic condition. Hence the reduction of optical density of bacterial growth is intensified due to development of phage resistance. Various external factors were also tested for temperature, pH, effect of metal ions on the adsorption rate of bacteriophages (Figure 4). From the figure, it is seen that the most important external factor affecting bacteriophage stability is pH and temperature affects the lytic activity of PADP4. They were exclusively fluxuvated with different pH ranges that is more stable phages were obtained with a pH range of 6 to 8, but there was no appropriate activity detected at pH 2.0, 3.0, 10.0 and pH 12. But during the constant 4 h of incubation, it was observed that PADP4 showed traces of infectivity at pH 4 and 9 (Figure 4a). Thereafter thermal stability results were noticed, that temperature is one of the critical factors affecting bacteriophage activity during the phage therapy. The phage PADP4 survived at 0, 20 and 37°C, with no significant loss in plaque formation (PFU). The percentage of survival of PADP4 fluctuated randomly for every milliliter i.e. by one order of magnitude over 30, 60 min at 50 and 60°C, respectively (Figure 4b). Hence, these results suggest that extreme temperatures and pH conditions favorably affect the stability of phage PADP4.

Effects of divalent metal ions ($MgSO_4$ and $CaCl_2$) on the adsorption rate of bacteriophages were well studied and reported in Figures 5a and b respectively. From the figures, it seen that $MgSO_4$ and $CaCl_2$ ions induced the infection rate of MDR- *P. aeruginosa* PA13 by PADP4, which tremendously enhances the adsorption rate of bacteriophages reflected on PFU observed in regular time intervals of 10, 20 and 30 min. These results suggest that metal ion impact increases the rate of infectivity as well as burst size.

## DISCUSSION

*P. aeruginosa* is one of the emerging multi drug resistant, nosocomial pathogen and causes health care complications. Development of drug resistance in *P.*

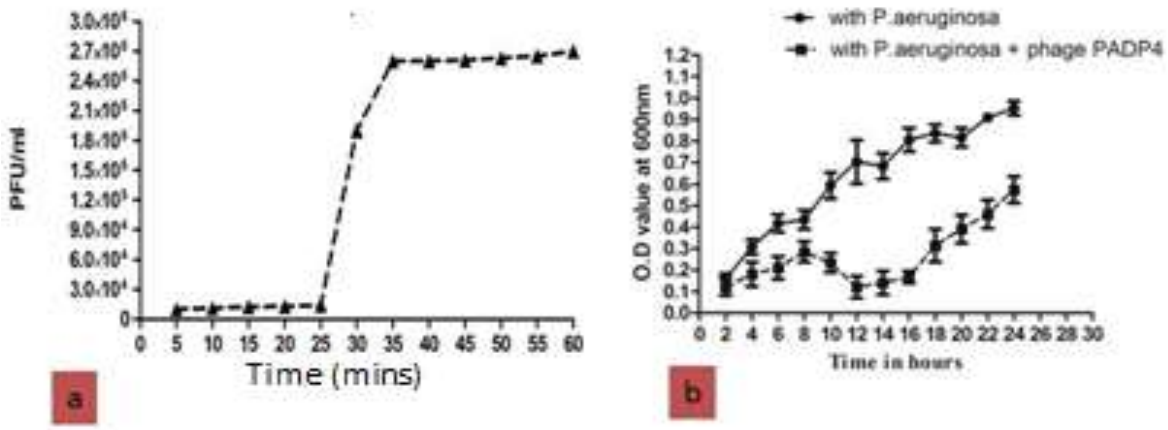


**Figure 3.** One step growth curve of Phage $V_B$_PAnP_PADP4. a) Single step growth curve experiment of phage PADP4 showed the latent period is about 25 min (the time interval between the phage adsorption and initiation of the first burst of the bacteriophage) and average burst size is 270 viral particles per single infected bacterial cell. Latent period and burst size of phage were concluded from the curve with a tri phasic arrangement. **b)** Lysis of *P. aeruginosa KY018605* strain by the phage PADP4 in LB broth medium. OD development of a control flask having uninfected *P. aeruginosa* and test flask having the *P. aeruginosa* cultures infected with its specific phage PADP4. Phage infection leads to a drastic decrease of the *P. aeruginosa* culture at 12-14 h of incubation.

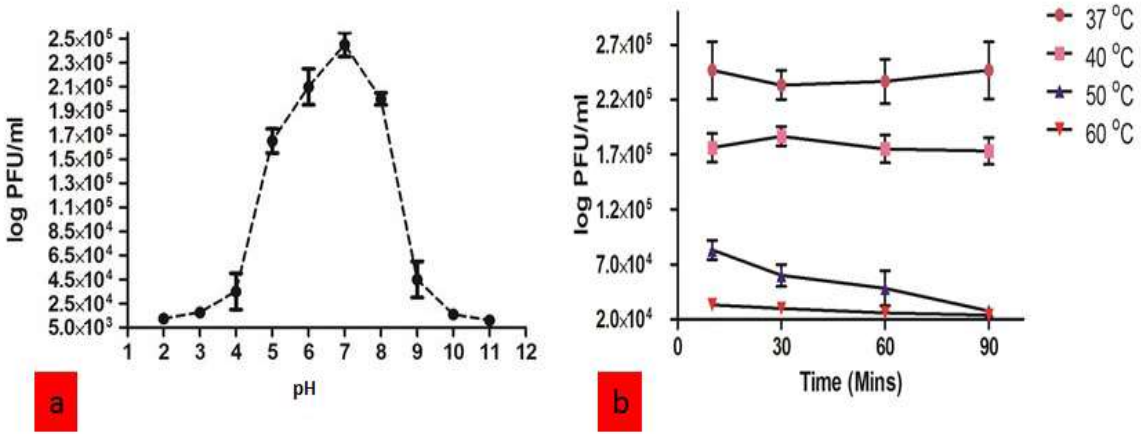


**Figure 4.** Effect of $p^H$ and temperature on stability of Phage PADP4. **(a)** Influence of pH on the stability of phage infection. Phage PA DP4 suspension was incubated for 4 hat the different pH values indicated by dark circles. **(b)** Effect of temperature on the infection ability of PADP4. Results obtained with different temperatures, and times of incubations are shown. In both cases, the data showed here the mean of two independent experiments.

*aeruginosa* is due to the modification of genetic material, transferred drug resistance from R-plasmids, and transposons. In general, the development of drug resistance is responsible for outer membrane proteins which are able to construct biofilms as well as increase the rate of infection (Kwiatek et al., 2015). There are a number of reports available on the isolation of bacteriophaes against *P. aeruginosa* in general but the present investigation mainly focuses on the specificity of the lytic nature of the host, bacteriophage type and their partial characterization for the development of new isolate having more clinical significane. Therefore, based on these concepts, we highlighted the isolation and partial chracterization of bacteriophages such as metal requirements for the stability and infection of bacteriophages, temparture and pH and bacterial reduction assay in *invitro* method. Many reports described that receptor based integrity on host, adsorption

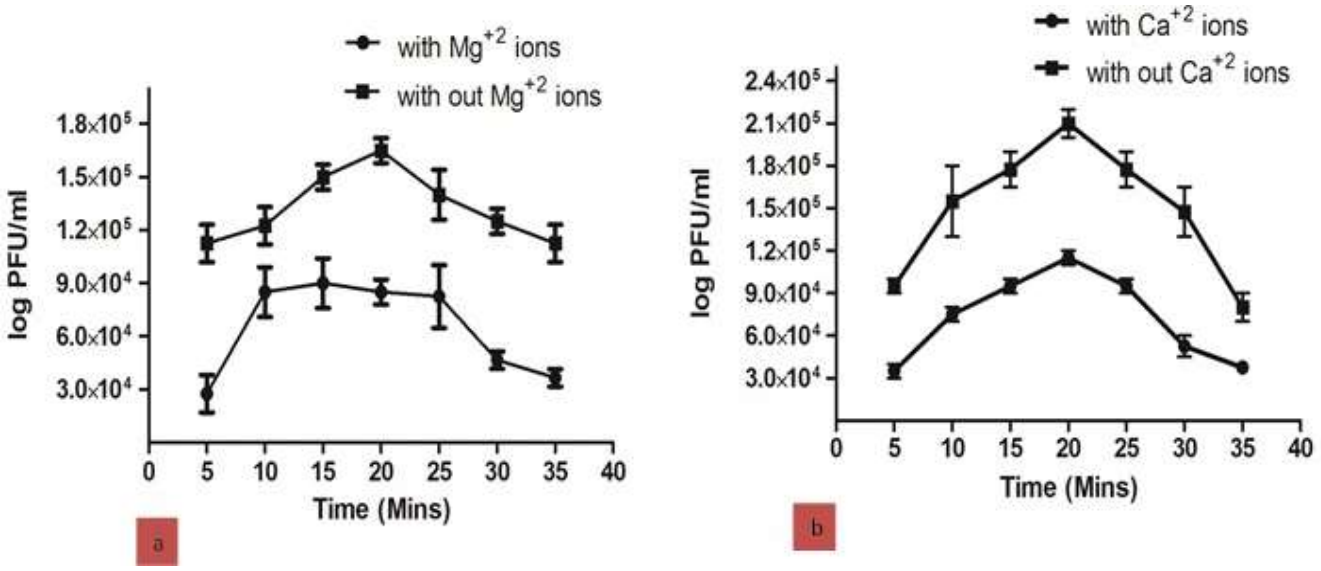


**Figure 5.** Effect of calcium and magnesium ions on the absorption rate of Phage PADP4. PADP4 suspensions were incubated at different time intervals (5, 10, 15, 20, 25, 30, and 35 min) in a 10 mM $MgSO_4$ and $CaCl_2$ in SM buffer. The rates of adsorption ability of PADP4 in the presence and absence of divalent metal ions were assayed by using the double layer agar method. In both cases, the data showed here the mean of three independent experiments.

fluctations leading to not only establishment of lysis but also these bacteriophages would be used as alternative drugs and theraupatics for various systemic infections (Pallavali et al., 2017; Pavlenishvili and Tsertsvadze, 1993; Rhoads et al., 2009; Sulakvelidze et al., 2001; Vandersteegen et al., 2013).

According to Bolger-Munro et al. (2013), the single step growth curve experiment illustrated that the convaluted steps in multiplication of bacteriophages target the growth curve, latency as well as burst levels. They also noticed that latent or incubation period is the right time where the bacteriophages are located inside their host and excel within 20 min; present results noticed that phage PADP4 has the 25 min as latent period.The high burst size would be the preferable feature for the phages which has theraupatic role; burst size explain the average number of phages produced from the single bacterial cell. The other physiological factors (temparature, pH, metal ion effect) play an important role in the mulptilication of phages, phage-bacterial intergration. Shafia and Thompson (1964) noticed that 0.1 M calcium ions were nessacery for the adsorption of bacteriophages (øμ-4) to Bacillus sterothermophilus NU-10 bacteria (Shafia and Thompson, 1964). Pajunen et al. (2000) noticed that latent period and burst size by one step growth curve of the phage YeO3-12 on bacteria *Y.enterocolitica* is 25 min and 100 to 140 PFU/Cell; these results is similar to our results but we noticed higher burst size (290 PFU/cell). Piracha et al. (2014) disclosed the role of calcium ions (1 mM) in the stabilization, adsorption and also infectivity rate is increased in presence of 1mM calcium ions in the solution. These metal ions plays a role in between phage and the host receptors for their accessibility to enhances the biological communication between them (Piracha et al., 2014). From this study, it was noticed that the phage PADP4 shown maximum adsorption at 10 mM concentration and at remaining concentartions the minimum range of adsorption were noticed (Data not shown).

Suárez et al. (2008) demonstrated that the study of bacteriophage QF9 is not required calcium ions and showed the same percentage of adsorption even with or without calcium ions but our results contradict with our results of phage PADP4 showed three fold higher adsorption were noticed in presece of calcium ions and magnisium ions. Fauquet et al. (2005) proven that 5 mM $cacl_2$ is required for the stability of phage PM2 and phage PM2 is stable at pH 6 to 8 (Fauquet et al., 2005). Temparature is one the cretical parameter to determine the stability and infectivity of bacteriophages (Olson et al., 2004). During the course of the analysis our finding showed that the existence of PADP4 in SM buffer at 4°C could be maintanined for two years and would exist up to 60˚C for 30 mins, but usually the maximum infectivity was observed at 37 and 40˚C with highest affinity towards their host and is companion to study of Suarez. Thorne and Holt (1974) observed phage CP-51 have optimum stability at 15°C and even survive at room temperature but at high temperature phage CP-51 lost its lytic capacity (Thorne and Holt, 1974). pH is another important factor for the stability and also for the infectivity of phages. Phage PADP4 showed stability at pH range 5 to 9 and maximum stability were noticed at pH 7. Our results are similar with those of Sharp et al. (1946) phage

T2 that showed that stability at pH range 5 to 9, but maximum stability was noticed at pH 5 to 6 (Sharp et al., 1946). Kłak et al. (2010) study revealed that Phage T4 showed the stable at pH 6–7.4 and unstable at the pH 5 and 9.2 (Kłak et al., 2010).

## Conclusion

In conclusion, a highly virulent bacteriophage $V_B$_PAnP_PADP4 were isolated and partially characterized from sewage samples. It has therapeutic potential to treat septic wounds caused by MDR-*P.aeruginosa*. Analysis of TEM studies revealed that $V_B$_PAnP_PADP4 resembles *Podoviridae* family and has a strong lytic activity against *P.aeruginosa.* Thus the isolated phage will be effectively proposed to treat wound infections in near future. However, further studies about antibacterial activity of this phage on other bacterial pathogens or *invivo* host cells are required to expand the use of phages.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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