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Research Article

Isolation and Characterization of Bacteriophage for Controlling *Pseudomonas aeruginosa,* Isolated From Northern Border Region, Kingdom of Saudi Arabia.

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ABSTRACT

In this study, we isolated and characterized of a bacteriophage designated as PhiPSA1 active against multi-drug resistant bacteria *Pseudomonas aeruginosa* isolated from sewage samples collected inNorthern Border Region, Kingdom of Saudi Arabia. Morphological analysis by Transmission Electron Microscopy revealed that PhiPSA1 belongs to the *Myoviridae* Family, with a head diameter of about 55±5 nm in diameter and with a short tail 18±2 nm in length. The host range showed that PhiPSA1 able to infect different *P. aeruginosa* strains and has no effect on other tested bacteria. One-step growth curves of PhiPSA1 revealed eclipse and latent periods of 20 min, with burst sizes of about 85 per infected cell. Phage treatment prevented the growth of *P. aeruginosa* for up to 40 h with multiplicity of infection ratios of 1. These results suggest that this phage has a high potential for phage application to control *P. aeruginosa*.

Keywords: Phage, Multi-drug Resistant, *P. aeruginosa*,*myovirus*

INTRODUCTION

The multi-drug resistant bacteria are considered a fatal and a very dangerous threat to the life of people and animals not only in the Northern Border Region, Saudi Arabia; but also all over the world. Thus, the treatment of infections caused by multi-drug resistant pathogenic bacteria has become a major problem all over the world. Recently, the phage or bacteriophage therapy becomes a good, new and safe tool for overcoming on the bacterial threat instead of the antibiotics.So, the use of bacteriophages is an attractive and a new approach and tool to overcome this problem of drug resistance in several pathogenic bacteria that cause fatal and dangerous diseases for human, animals and plants [1-4]. Nowadays, many groups of antibiotics currently in use are becoming ineffective to control bacterial pathogens because many bacteria

have attained multi-drug resistance. The indiscriminate and wide spread use of antibiotics has caused drug resistance in bacteria because of the over administration, self-medication, random prescription of improper drugs and prolonged use of antibiotics [5]. The emergence of multi-drugresistant bacteria and the dearth of new classes of antibiotics have prompted the exploration of new agents with antimicrobial activity. One possible approach is the application of lytic bacteriophages as antibacterial agents, owing to their ability to self-replicate, their high specificity against target bacteria and their ubiquity within the environment [2-6]. Bacteriophages constitute a majority of biological organisms on the Earth and have crucial influences on the evolution of bacteria [8]. With an estimated 10^{31} bacteriophages [8], an appreciation of the diversity and complexity of these organisms can only be gauged by the current body of knowledge produced by their study. The study of (bacteriophages or short phages) was historically a major driving force in the development of molecular biology. Several studies have shown the potential of use of phages to treat infectious diseases in animals [10-15], plants [16-18], and humans [19, 20]. Bacteriophage treatment is another emerging method in biofilm control and removal [15]. Almost all studies concerning *P. aeruginosa* phage species have been done with sewage water samples collected from environmental and hospital surroundings in European, Australian, and Asian [22-23], leaving a void regarding the species found in unexplored locations. Phages can be used effectively as part of integrated disease management strategies. The relative simplicity of get ready phage medications and minimal effort of creation of these specialists make them great possibility for broad use. In any case, the viability of phages, as is valid for some natural control specialists, depends enormously on winning ecological components and in addition on vulnerability of the target pathogenic bacteria. Awesome care is vital amid advancement, creation and use of phage medicines. Phage-based illness control administration is a dynamic procedure with a requirement for nonstop modification of the phage readiness so as to successfully battle possibly adapting pathogenic organisms[24-25]. The use of bacteriophages as antimicrobial agents controlling pathogenic bacteria has appeared as a promising new strategy and it seems that phage therapy may provide a good alternative solution to antibiotics. The abundance of phages in the environment highlights their potential use for control of pathogenic bacteria in food and animals. For an effective treatment, bacteriophages should be present in high concentrations, stable over time and in in vivo conditions, able to meet the bacteria without any restriction [25-26]. To our knowledge, to date there is no study on the isolation and characterization of bacteriophages and used it as alternative therapy against multi-drug resistant bacteria in the Northern Border Region, Saudi Arabia. Therefore, we carried out this project to find new phages that attack some common multi-drug resistant bacteria*Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions:

Different strains of *P. aeruginosa*, were originally isolated from sewage samples collected inNorthern Border Region, Kingdom of Saudi Arabia. The strains were identified and confirmed as *P. aeruginosa* according to traditional and molecular methods. Stock cultures were stored in LB broth containing 20% glycerol at -20^{o}C.

Phage Isolation:

The bacteriophage was isolated from sewage water samples from different locations and stations at Northern Border Region,Kingdom of Saudi Arabia,using the enrichment technique of Adam[27]. Briefly, a sewage sample wascentrifuged at 10,000 xg for 15 min, and thenfiltered using a sterile 0.45 µm Millipore filter. Fifty milliliters of the filtrate was added to anequal volume of Nutrient broth, which was inoculated with 1ml of *Pseudomonas*, then

subjected to incubation with shaker at 120 rpm at a temperature of 37ºC/24 hrs. The centrifugation of bacterial cultures was performed at 10,000 xg/10 min. The supernatant was filtered using a sterile 0.45 µm Millipore filter, then used as a phage source to be found on thepropagative host.

Purification &Propagation of Phage:

The bacteriophage was purified by three single plaque isolation assays using sterilepasture pipette as described by Adam [27]. In brief, a single plaque was picked up, then placed in 0.5 ml of nutrient broth harboring 100 µl of ***P. aeruginosa***, then subjected to incubation at37ºC with shaking at 1200 rpm. Afterwards, centrifugation of phage-bacteria mixture was done at 10,000xg for 10 min. The supernatant was filtered via a sterile 0.45 µm Milliporefilter to exclude any bacterial cells. Storage of purified phages was done at 4ºC.

Determination of Phage Host Range:

The isolated bacteriophage was tested against different isolates of pathogenic bacteriato detect the host range as previously explained. The propagation of bacterial lawns was achieved on nutrient agar plates, followed by addition of 10 µl of phage droplets (1 X 10^7 plague forming unit, PFU/ ml). These plates were incubated at 37ºC/ 24 hrs then tested for existence of plaques (lytic zones). Efficient phage was selected on the bases of lysis profile, clarity of plaque and size.

Transmission Electron Microscopy Analysis:

A drop of phage suspension (10^7PFU/ml) was located on 200 mesh copper grids with the carbon-coat films, and the excess was removed with a filter paper. The uranyl acetate as asaturated solution was placed on grids, and the excess was removed by filter paper. The purified phage particle was stained with uranyl acetate, and then was examined using anElectron Microscope as previously described [28].

One-Step Growth Experiment:

The single-step growth curve of the isolated bacteriophage was determined as explained by Pajunen[29]. The phage was added at a multiplicity of infection (MOI) = 1.0 to *P. aeruginosa*cells then the phage was allowed to be adsorbed on host cells at 37ºC/10 min. The phage-host mixture was centrifuged at 10,000 xg for 10 min, and thepellet harboring infected cells was suspended in 10 ml of nutrient broth then incubated at room temperature. The specimens were takenin duplicate at 15 min interval for a period of 150 min. The titer of phage was estimated by the double-layer plague assay. The first sample set was subjected to dilution before titration; while, 1% chloroform (V/V) was used to treatthe second sample set for the release of intracellular phages and determination of theeclipse period.

Effect of phiPSA1 on the growth of P. aeruginosa

The effect of phiPSA1 on the growth of MDR *P. aeruginosa*:

This experiment was evaluated invitro in a liquid media over a time period up to 40 hrs. The optical density at OD_{600} was measured each 4 hrs for the control (bacterial culture alone) and for the treated bacterial broth (bacteria treated with phiPSA1 at MOI 1 phage) as previously described.

RESULTS

Identification of clinical *P. aeruginosa*:

Ten strains of *P. aeruginosa*, were originally isolated from a variety of sewage samples collected inNorthern Border Region, Kingdom of Saudi Arabia. The strains were identified as *P. aeruginosa* on the basis of the morphology, a positive oxidase reaction, growth at 42^{o}C in CetrimideAgar Medium for 72h, and conventional biochemical tests. The majority of *P. aeruginosa* isolates showed resistance to antibiotics testedall strains of *P. aeruginosa* were tested for their ability to phage infection by using them individually as an indicator.

Isolation and morphology of PhiPSA1phage:

The bacteriophage against MDR *P. aeruginosa*was detected from Northern Border Region sewage water by spot test and double over layer agar technique. We picked up single plaque for propagation and characterization (Figure 1 A and B). Phage particles were observed during Electron Micrograph. The isolated phage was

designated as PhiPSA1.Under electron microscopy; the phage had an icosahedral head (55±5 nm) and long contractile tail with length of (18±5 nm). The phage PhiPSA1 appeared to be a member of *Myoviridae*family based on its morphological features (Figure 1C).

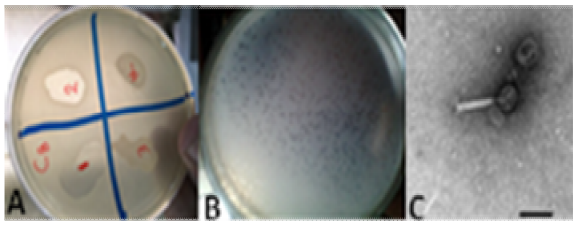

Fig. 1:Plaques of PhiPSA1formed on *E. coli*.(A) Spot Test Technique. **(B)** Single plaques by overlayer agar technique. **(C)** Electron Micrograph of **PhiPSA1** phage particles under TEM. The virions were negatively stained with uranyl acetate. Scale represents 100 nm.

Host Range of PhiPSA1Phage:

Our results showed that PhiPSA1could infect all MDR strains of *S* ***P. aeruginosa***; and could not infect other Gram-positivebacteria as shown in Table (1).

Characteristics of PhiPSA1phage growth:

The phage growth parameters, latent period, rise period and burst size, were determined. The one step growth curve of PhiPSA1phage on *Proteus* as a host was determined (Fig. 2). The growth characteristics of PhiPSA1phage revealed a latent period of 20min, followed by a rise period of 100 min. The average burst size was 85 per infected cell (Fig.2).

Table 1. Host Range of PhiPSA1*P. aeruginosa*

Bacteria	PhiPSA1
*P. aeruginosa*NB1	+
*P. aeruginosa*NB2	+
*P. aeruginosa*NB3	+
*P. aeruginosa*NB4	+
*P. aeruginosa*NB5	-
*P. aeruginosa*NB6	+
*P. aeruginosa*NB7	-
*P. aeruginosa*NB8	+
*P. aeruginosa*NB9	+
E. coli	-
S. aureus	-

(+) Indicates that the strain is susceptible to the phage andproduces plaques, with titers in parenthesis,while (−) indicates that no plaques were observed.

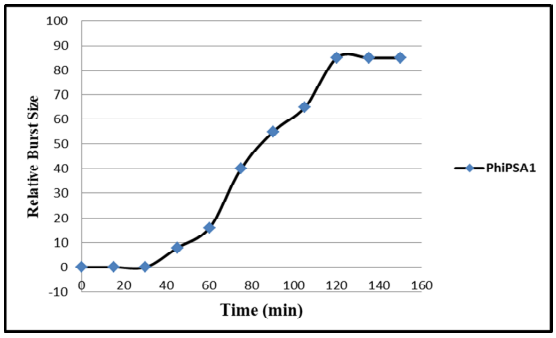

Fig. 2: Single-Step Growth Curve for PhiPSA1 *P. aeruginosa*phage.The plaque forming units (PFUs) per infected cell in cultures of *P. aeruginosa*at different time post infection are shown. Samples were taken at intervals (every 20 min up to 160 min).

Stability of PhiPSA1 phageat Different pH and Temperatures:

The stability of isolated phage PhiPSA1 wasassayed by spot test and plaque assay. Data in Table 2 showed that PhiPSA1phage was extremely heat stable which it still remained active after 10 minutes exposure at 75°C; Furthermore, the results showed that phage loss its infectivity at 80°C (Table 2).

Table (2): Effect of Temperature on PhiPSA1 *P. aeruginosa*phage.

Temp (C°)	PhiPSA1
	Pfu/ml
Control	$4.8x10^{10}$
30	$4.4x18^{8}$
40	$7.3x10^{7}$
60	$5.7x10^{5}$
70	$3.9x10^{2}$
75	$1.9x10^{1}$
80	0.0
90	0.0

Different pH degrees on the phage particles stability was examinedat range of 2-10. The results in Table15 showedthat phage maintained its infectivity when incubated in a pH range between 4 and 9. However, PhiPSA1 could not be

detected at less than pH 4 and more than 9 (Table3).

Table (3): Effect of pH values on PhiPSA1 *P.aeruginosa*phage.

pH Value	PhiPSA1
	Pfu/ml
2	0.0
3	0.0
4	$3.1x10^4$
5	$3.8x10^4$
6	$6.7x10^6$
7	$4.8x10^{10}$
8	$4.0x10^8$
9	$2.1x10^8$
10	0.0
11	0.0
12	0.0
13	0.0

Effects of PhiPSA1phage on the growth of MDR *P. aeruginosa*strains:

The lytic effect of PhiPSA1 on the growth of *P. aeruginosa*were assessed by measuring OD_{600} of the bacterial culture infected with the phage at MOI of 1.0 and incubated at 37 ºC (Figure 3).

The growth of MDR *P. aeruginosa* was inhibited by PhiPSA1 infection and the bacterial populations were significantly reduced 24 hrs post treatment ($P<0.05$) and reached to complete lysis at 40 hrs incubation (Figure 3).

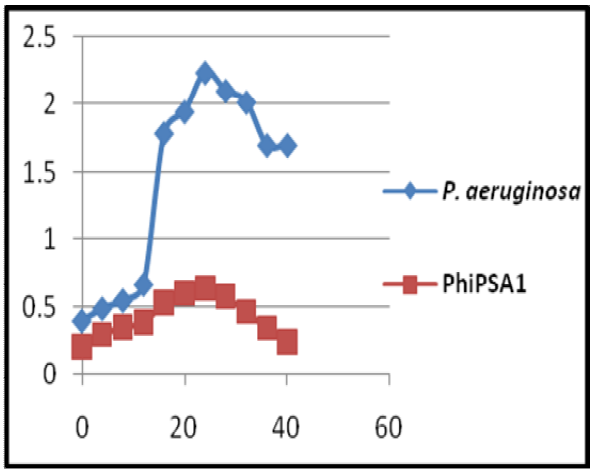

Fig.3: Effects of phage PhiPSA1 on the growth of *P.aeruginosa* at a MOI= 1.Growth of *P. aeruginosa*is represented by measuring optical density at (OD_{600}).

DISCUSSION

The main aim of our study was the isolation and characterization of lytic a bacteriophage specific to *P. aeruginosa* isolated from different locations in the Northern Border Region, Kingdom of Saudi Arabia.So that, in this study, amyovirus infecting strains of *P. aeruginosa*was isolated from sewage samples collected inNorthern Border Region, Kingdom of Saudi Arabia.Electron micrograph of the isolated phage was characterized as myovirus and. The dimension of the myovirus was resembled that have previously podoviruses isolated from*P. aeruginosa*[30].The incubation temperature of bacteriophage is the most important factor, which determines phage activity.The results in Table (2) showed that PhiPSA1phage wasthermostable,between temperature ranges of 30–50°C, with no significant loss in phage particle number, which is a very important parameter for phages considered for therapeutic application and it still remained active after 15 minutes exposure at 75°C.A priority objective of our future research will be to identify the nucleotide sequence and protein of the isolated phage. Several investigations have shown the potential use of phages to treat *P. aeruginosa* infections [31]. Since approximately all of strains tested were susceptible to PhiPSA1 (Table 1), we explored the utilization of this phage as a possible alternative to antibiotics, we assessed for *in vitro* susceptibility *P. aeruginosa*to PhiPSA1 phage. The turbidity of bacteria was measured as an optical density at 600nm. Obtained results indicated that prevention of multi-drug resistant growth was more efficient when it was treated with PhiPSA1. The results in Fig. (3)showed a quick lysis of host cells after treatment with phage. These results suggest that it may be a realistic alternative to use phage like these, after further characterization, to control *P. aeruginosa*, however, more phages should be isolated to allow for the preparation of phage cocktails which might be an even more successful method for the application of phages in this situation, due to a significantly less probability of bacterial resistance to all of the

phages. In summary, here we describe the isolation and characterization of a myovirus against multi-drug resistant *P. aeruginosa* strains. The results suggested that PhiPSA1 has promising effect and their application can be applicable to combat these resistant pathogens.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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