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CHARACTERIZATION AND IN VITRO EFFICACY STUDIES OF WIDE HOST RANGE LYTIC BACTERIOPHAGE ΦDMPA1 INFECTING PSEUDOMONAS AERUGINOSA ISOLATED FROM PYOGENIC SKIN INFECTIONS

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ABSTRACT

Aims: To characterize the bacteria *Pseudomonas aeruginosa* isolated from patients infected with pyogenic skin infections and the bacteriophage isolated from cocktail of sewage samples. To evaluate in vitro efficacy of Bacteriophage lytic activity against *Pseudomonas aeruginosa* and its stability.

Methods and Results: A total of 114 samples from the patients of pyogenic skin infections were analyzed over a period of twelve months of three major hospitals by standard conventional microbiological methods. Among the four major causative multidrug resistant bacterial isolates *Staphylococcus aureus, Pseudomonas aeruginosa, Proteus mirabilis and Escherichia coli*, *S.aureus* was found to be most predominant. We isolated 05 *P.aeruginosa* strains and tested for multidrug resistance by microbroth dilution and disc diffusion method. The taxonomical identification by conventional microbiological studies of *P.aeruginosa* was confirmed by 16S rRNA gene sequencing and phylogenetic analysis. Among the tested *P. aerugeinosa* isolates one was resistant to all the antibiotics tested and other strains to multiple antibiotics. The mixture of sewage originated from hospital, municipal and domestic waste was used as source for the isolation of potent bacteriophage ΦDMPA-1 having lytic activity against multidrug resistant *P. aerugeinosa* (DMPA-1). The phage was identified as belonging to genus PB1 of the family *Myoviridae* and characterized for host range and stability in terms of lytic after different time intervals of storage. The phage retained lytic activity against the host for three months period under 4-8^{0}C, -20^{0}C and -40^{0}C without significant loss in the titre.

Conclusion: The outcome of the study is evident that pyogenic skin infections are one of the sources of multidrug resistant *P.aeruginosa*. Highly potent and stable Bacteriophage ΦDMPA.1 isolated from the sewage cocktail showing good lytic activity on DMPA.1 and have broad host range on other *P. aeruginosa* strains isolated.

Significance and Impact of the Study: The present study shows the prevalence of multidrug resistant *P.aeruginosa* in pyogenic skin infections and further insisting to investigate other multidrug resistant bacteria. The study also has an impact on need based isolation of bacteriophages on various drug resistant bacteria as an alternative to antibiotics to reduce the upcoming danger level of multidrug resistance in bacteria.

KEY WORDS: Antibiotics, bacteriophage, *Pseudomonas aeruginosa*, resistance, skin infection.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic human pathogen that is ubiquitously found in various biotic and abiotic environments. It has the ability to express a variety of virulence determinants which cause a wide range of infections in plants, nematodes, insects and animals. As a typical opportunist, it infects injured, burned and immunodeficient humans and causes persistent respiratory infections in individuals suffering from cystic fibrosis (Lyczak *et al*., 2000) and life-threatening infections in burn wound patients (Pruitt *et al*., 1998). It is frequently isolated from human patients afflicted with cystic fibrosis, otitis media, keratitis, and burn wound infections as an etiological agent of septicemia in immunocompromised individuals. Furthermore, *P. aeruginosa* is also commonly found in peritonitis-sepsis cases

secondary to ruptured appendices in otherwise healthy children (Aronoff *et al*., 1987). Infection with *P. aeruginosa* forms a major health problem because of its inherent resistance to multiple drugs and the ability to gain resistance to all available treatments through mutation (Livermore, 2002). Multi-drug resistance (MDR) in *P. aerugenosa* is attributed to a combination of resistance mechanisms, mainly including multi-drug efflux pumps, aminoglycoside-modifying enzymes, Beta lactamases and target site modifications. Especially the efflux system is considered to be a very effective mechanisms of resistance due to the fact that it can mediate resistance to many classes of drugs (Tenover, 2006). Comprehensive studies from 23 countries conducted by European Antimicrobial Resistance Surveillance System has shown that 18% of *P.aerugenosa* isolates as MDR (Souli *et al*., 2008). Because the pathophysiology caused by *P. aeruginosa* infections is quite complicated, more and more relevant infection models need to be tested for the efficacy and relevancy of the antibacterial therapies. Prior to the discovery and widespread use of antibiotics, it was suggested that bacterial infections could be prevented and/or treated by the administration of bacteriophages (Sulakvelidze *et.al*, 2001). Local delivery (skin, ears, teeth) of the phage has proved extremely successful in the treatment of topical infections, as has the inhalation of phages for the treatment of lung infections (Ryan *et al*, 2011). In the present study, we have characterized multidrug resistant *P.aeruginosa* isolated from patients with pyogenic skin infections. Lytic phage ΦDMPA-1 against multi drug resistant *P.aeruginosa* from the domestic and hospital sewage was characterized for morphology, host range, and stability at various conditions. In vitro lytic activity of the potent bacteriophage against multidrug resistant *P. aeruginosa* was evaluated.

MATERIALS AND METHODS

Media and Chemicals

Luria Bertani (LB) Agar (Hi-media, India), LB broth, LB soft agarose (0.7%),Cetrimide agar, Pseudomonas agar, SM buffer with gelatin (50mL/L of 1M Tris of pH 7.5, 5.8 g NaCl, 2g $MgSO_4.7H_2O$, 5mL/L of 2% gelatin in distilled water) were prepared and used (Sambrook *et al.,* 1989), Agarose (Hi-media, India), Chloroform (Merck, India), Glycerol (Merck, India), Ethidium bromide (Hi-media, India).

Clinical *P.aeruginosa* isolates from pyogenic skin infection patients

A total of 5 *P. aerugeinosa* and other potent bacterial isolates from the patients of pyogenic skin infections of three major hospitals were used in the study and the data has been previously published (Manjunath *et al.*, 2013). The isolates were characterized and screened for antibiotic susceptibility test by standard microbiological methods and the drug resistant organisms were identified in accordance with CLSI guidelines (NCCLS, 2005).

Characterization and antibiotic susceptibility of *P. aeruginosa*

Antibiotic sensitivity test

The clinical isolates were screened for antibiotic susceptibility by Kirby Bauer's disc diffusion method (Bauer *et al.*, 1966) using the antibiotic discs (Hi-media Ltd,India) on Mueller-Hinton agar according to Clinical and Laboratory Standard Institute (CLSI) guidelines (NCCLS, 2005) and as per the hospital antibiotic policy. Bacterial suspension of 0.5 Mc Farland standard was used for the test. Antibiotic discs Amikacin (30mcg), Amoxicillin (30mcg), Ceftazidime (30mcg), Cephotaxime (30mcg),Ciprofloxacin (5mcg), Cefoparazone (75mcg), Levofloxacin (5mcg) and Tobramycin (10mcg) were used for Gram negative bacteria including *P.aeruginosa*. Finally the results were recorded by measuring the diameter of zone of inhibition of each antibiotic disc and scored as sensitive or resistant. *P.aeruginosa* (MTCC 4676) was used as a control strain.

16S rRNA gene amplification and phylogenetic analysis

Extraction of DNA

The genomic DNA was extracted by following the method of Daniel *et al.,* (1994). A 0.25-ml aliquot of the organism suspended in nutrient broth was centrifuged at 12,000 x g for 5 min. The supernatant was discarded, and the pellet was resuspended in 100 µl of cell lysis solution (250 U of lysozyme (Sigma Chemical Co., St. Louis, Mo.) per ml, 25 U of lysostaphin (Sigma) per ml, 10 mM EDTA, 10 mM Tris (pH 8, in deionized H20). This suspension was incubated for 30 min at 35°C, placed in a 95°C heat block for 10 min, cooled to room temperature, and then diluted in 900 µl of sterile water.

Polymerase chain Reaction:

The identification of the test strains was carried out at molecular level employing the 16S rRNA gene amplification and sequencing. The 16S primers and conditions used in PCR amplification of 16S rRNA gene are as described previously

(Sauer *et al*., 2005) with slight modifications. The primers used were as follows UNI_IL forward primer (5'-GGTGGAGCATGTGGTTTA-3'), UNI_IR reverse primer (5'-CCATTGTAGCACGTGTGT-3') (synthesized at Sigma Aldrich, Bangalore). The master mix of total volume of 25 µl was composed of the following; 1 µl of each primer (10 µM concentration) 0.2 mM dNTPs (Bangalore GeNei). 1 X PCR buffer (Sigma Aldrich, USA), 2.5 mM $MgCl_2$ (Bangalore GeNei), 4 µl containing 10 ng of genomic DNA as template and 1 U of *Taq* DNA polymerase (Sigma Aldrich, USA). PCR was performed with following temperature conditions: Initial denaturation at 94^0C for 5 min followed by 35 cycles of 94^0C for 45 s, the annealing temperature of 44^0C for 30 s and synthesis at 72^0C for 40 s. The final extension was carried out at 72^0C for 5 min. The PCR amplifications were performed in a Thermocycler Gene Amp PCR system 9700 (Applied biosystems, USA) in 0.2 ml reaction tubes. Expected amplicon of 287 bp was visualized with Ethidium bromide after gel electrophoresis in 1 % agarose gel.

Gene sequencing and phylogenetic tree construction

For determining the nucleotide sequences of 16S rDNA, the PCR amplified products were purified using PCR purification kit (Sigma Aldrich), DNA sequencing of the purified product was performed by dideoxy chain termination method using gene specific primers at the sequencing facility of Vimta Labs (Hyderabad, India). The gene sequences obtained were analyzed by using the BLAST search programme (Altschul *et al*, 1997). The sequence of the PCR product was compared with known 16S rRNA gene sequences in the Gene Bank by multiple sequence alignment using the CLUSTAL W Program (Thompson *et al*., 1994) and the phylogenetic tree was constructed using Mega 5 program (Tamura *et al*., 2011).

Isolation and characterization of Bacteriophage

Multidrug resistant *P.aeruginosa* strain (DMPA-1) isolated from the pyogenic skin infections and the cocktail of sewage originated from hospital, municipal and domestic waste was used as a source for the isolation of phage ΦDMPA-1. Initially, sewage sample was pre-treated with 200µl of chloroform per 25ml of sample for 15 minutes at room temperature (Sambrook *et al*., 1989). LB broth (2 ml of 10X concentrated, Hi-media), 2 ml of 16-18 hours grown host bacterial suspension containing 10^8 cfu in LB broth and 18 ml of sewage were mixed in a 100ml capacity sterile cotton plugged conical flask. The flask was incubated overnight at 37^0C for 15-16 hours. Subsequently, 200 µl of chloroform (Merck, India) was added and the flask was further incubated at 4^0C for 1 h. The lysate was centrifuged in 1.5 ml eppendorf tubes at 4000 x g for 10 minutes and the supernatant was aspirated with a sterile 5 ml syringe and passed through a 0.22 µm membrane filter (Minisart, Sartorius, Germany). The filtrate stock was assessed for bacteriophage titre by double-agar layer overlay method (Adams, 1959) with modification (Merabishvili *et.al*, 2009). Phage stock was serially diluted 10-folds (10^{-1} to 10^{-4}) using SM buffer with gelatin (50mL/L of 1M Tris pH 7.5, 5.8 g NaCl, 2g $MgSO_4.7H_2O$, 5mL/L of 2% gelatine in distilled water) (Jamalludeen *et al.*, 2007) and from each 100µl of sample was aspirated and dispensed into sterile 15ml screw capped tubes, then mixed with 100µl of host bacterial suspension of 0.5 Mac Farland standards. The content was mixed and incubated at 37^0C for 20 minutes with gentle shaking. Luria bertani agarose (0.7%) of 3ml maintained at 47^0C in water bath was dispensed into each 15ml tubes, mixed gently and poured on dried LB agar plates of 90 mm as top layer to spread uniformly (Sambrook *et.al*., 1989). A control tube containing host bacterial suspension and 4ml of top agarose without filtrate was cultured on LB agarose plates (Jamalludeen *et al.*, 2007). Plates were allowed to set and incubated at 37^0C for 15-16 hours. A sterile gel cutter with a rubber bulb was used to cut the gel along with the underlying agar. A few prominent and well isolated plaques were taken through suction and transferred into an eppendorf tube containing 1ml SM buffer, to which 1 drop of chloroform was added. The contents were held at room temperature for 1-2 hours to allow the bacteriophage particles to diffuse out of Agarose (Sambrook *et. al.*, 1989). The phage showing potent lytic activity against *Psuedomonas aerugenosa* was designated as Φ DMPA-1 and phage preparations were stored at 4^0C for further use.

Preparation of Phage stock

A phage plate lysate stock was prepared by mixing 100µL of bacteriophage suspension with 100 µL of the host bacterium suspension of 0.5 Mac Farland standard in 15ml screw capped tube (Sambrook *et al*. 1989) and incubated for 20 min at 37^0C. The top LB agarose (0.7%) of 3 mL maintained in molten (47^0C) condition was added, mixed and poured onto LB agar plates, allowed to set and incubated for 6–8 h at 37^0C. The phage was recovered by adding 3 mL of SM buffer to each plate and the plates were stored at 4-8^0C for several hours with gentle shaking on rocking platform to allow bacteriophage to diffuse from the agarose. SM buffer containing phage particles was transferred to a polypropylene tube, 0.2 mL of chloroform was added and again 2ml of SM buffer was also added. The lysate was drained to recover the maximum phage particles. The mixture was gently vortexed, then centrifuged at 4000 x g for 15 min at 4-8^0C (REMI, CPR-24 Centrifuge, India). The supernatant was aspirated, a drop of chloroform was added, and

the phage suspension was stored at 4- 8^0C. The titres of the phage stocks were determined by plaquing 10-fold dilutions by the soft agarose overlay method.

Electron Microscopy

A high titre bacteriophage lysates of 10^8 pfu/mL previously filtered through 0.22µm Minisart Sartorius filter was purified by centrifugation and treated with a fixative 1% glutaraldehyde. A drop of purified and fixed phage lysate was deposited on Formvar carbon-coated copper grids and negatively stained with 1% phosphotungstic acid (PTA). The grids were examined in Tecnai G^2 Biotwin (Philips-Netherland) transmission electron microscope. The images of phages were captured and measured the size of head and tail.

Determination of Phage host range (Spot test)

All the *P. aeruginosa* isolates were screened for the susceptibility on phage ΦDMPA-1 by spot test. The host bacteria were subcultured in Luria bertani broth and incubated for 6-8h. The susceptibility of host bacteria maintained at log phase to phage ΦDMPA-1 was assessed by double agar overlay method. The suspensions of bacteria were taken in 1.5ml eppendorf tubes and centrifuged at 4000xg (Eppendorf miniplus) for 10 minutes, supernatants were discarded and the pellets were resuspended in 1ml of 10mM $MgSO_4.7H_2O$ to attain the suspensions of 0.5 Mac Farland standard. The bacterial suspensions of 100µl of each strain were added to 15ml sterile tubes with molten 3ml LB agarose maintained at 47^0C. The contents were mixed and overlaid on pre-dried LB agar plates. The dried plates having bacterial layer, on which 5 µl of phage suspension (10^8 pfu/ml) was dropped and incubated at 37^0C without inverting the plates. Plates were observed for phage mediated lysis in the form of clear and turbid spots after 6h, 18h and 24 h. The results were scored as a clear zone of complete lysis (++), partial lysis with turbidity (+) and no lysis (0).

Storage stability of phage ΦDMPA-1

The storage stability studies were conducted at temperature conditions -40^0C, -20 ^{0}C, 4^0C, and 20^0C and also in presence of 20% (v/v) glycerol at -40^0C and -20^0C. Phage titres were determined after 3, 6, 9 and 12 months of storage by double agar layer technique (Adams, 1959).

Stability of the phage ΦDMPA-1 to pH, Temperature and Chloroform

A purified phage filtered through 0.22µm Minisart Sartorius filter of 10^8pfu/ml strength was incubated at room temperature and 60^0C with shaking. The preparations were also incubated at pH 2, 4, 5, 6, 7, 8, 9 and 10 for 1 h and 4 hrs at room temperature and 37^0C with shaking. An equal volume of phage (10^8pfu/ml) was mixed with chloroform and incubated for 2 and 24 h at room temperature with shaking (Zuanna *et al*., 2011). After exposing the phage sample at various conditions, phage titre was assessed by double agar layer technique (Adams, 1959).

RESULTS

Characterization of multidrug Resistant *P. aeruginosa*

The antibiotic susceptibility data of *P. aeruginosa* isolates are presented in **table 1**. Among the *P. aerugenosa* strains, 4 isolates were found to be resistant to most of the antibiotics tested and the isolate DMPA-1 was resistant to all the antibiotics tested.

Table 1. Antibiotic susceptibility profile of *P.aeruginosa* isolates

Isolates No.	Antibiotic discs							
	Ak	Ac	Ca	Ce	Cf	Cs	Le	Tb
DMPA-1	R	R	R	R	R	R	R	R
DMPA-2	S	R	S	R	S	R	S	S
DMPA-3	S	R	S	R	S	R	S	S
DMPA-4	S	R	R	R	S	R	S	S
DMPA-5	R	R	R	R	S	R	S	R

Antibiotic Discs: Ak-Amikacin, Ac- Amoxycillin, Ca- Ceftazidime, Ce- Cephotaxime, Cf- Ciprofloxacin,Cs- Cefoperazone, Le-Levofloxacin, Tb- Tobramycin., **Pattern : R-** Resistant, **S**-Sensitive

16S rRNA gene sequencing and phylogenetic analysis:

The 16S rRNA gene 287 bp amplicons base sequences of the DMPA-1 were blasted and phylogenetic tree was constructed with closely matched aligned sequence. The phylogenetic tree reveals that isolate closely relates to *P.aeruginosa* strains – Z992, Z91, Z401, FARP72 and AEBBITS.1. The phylogenetic tree of 16S rRNA gene of isolate is presented in **Figure 1**. The phylogenetic tree confirmed the isolate as *P. aeruginosa*.

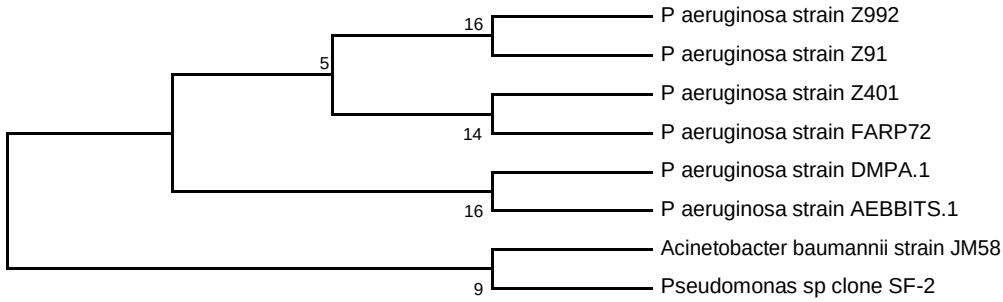

Figure 1 Phylogenetic tree of 16S rRNA gene of DMPA-1

Isolation and morphological features of ΦDMPA-1

The cocktail of sewages originated from hospital, domestic, municipal waste and the water from water treatment plant were used as source to isolate the phage ΦDMPA-1. The samples were screened using the host strain multidrug resistant *P. aeruginosa* (DMPA.1) which has produced clear plaques of 2 mm diameter in the double-agar layer technique. Phage ΦDMPA-1 was purified by picking a single prominent plaque, negatively stained with 1% phosphotungstic acid (PTA) and observed by transmission electron microscopy (Figure 2). Transmission electron microscopy has provided the basis for the recognition and establishment of bacteriophage families and is one of the essential criteria to classify novel viruses into families. It allows for instant diagnosis and is thus the fastest diagnostic technique in virology (Ackermann, 2012). Since 1959, nearly 6300 prokaryote viruses have been described morphologically including 6196 bacterial and 88 archeal viruses. As in previous counts, the vast majority (96.3%) are tailed and only 230 (3.7%) are polyhedral, filamentous or pleomorphic. In all surveys, over 96% of phages were tailed and belonged to the families *Myoviridae*, *Siphoviridae*, and *Podoviridae* (Ackermann and Prangishvili, 2012). The tails of the Myoviridae members consists of a neck, a contractile sheath and a central tube. Myoviruses tend to be larger than other groups and include some of the largest and most highly evolved tailed phages (~1300 observations,25% of tailed phages) (Ackermann, 2009). The phage ΦDMPA-1 possesses an isometric icosahedral capsid of approximately 136.37 nm diameter connected to contractile tails (217.19 nm) consisting of sheath and a central tube belongs to genus PB1 members of the family *Myoviridae* (Ackermann and Dubow, 1987; Ackermann, 2009). The morphological characteristics of phage was examined by Virologist, Hans-W Ackermann and confirmed as phage belongs to genus PB1 of the family *Myoviridae*.

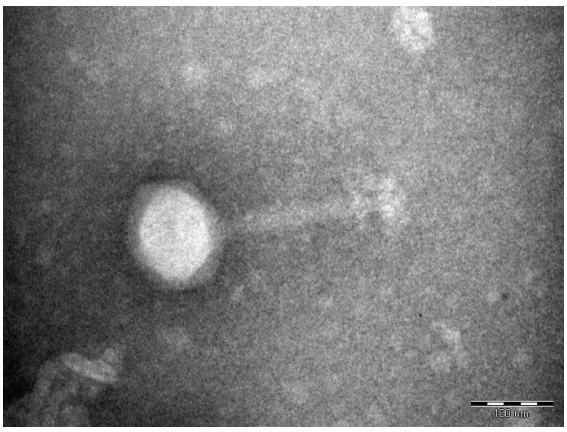

Figure 2 Electron micrograph of Φ DMPA-1

Host range

Bacteriophage ΦDMPA-1 has an ability to lyse the host and also have broad host range on, among the *P.aeruginosa* isolates obtained from pyogenic skin infections. The lytic activity of the phage was assessed on *P. aeruginosa* isolates by spot test. Among 5 isolates, 3 isolates (60%), which produced completely cleared zones in spot tests; thus, these isolates were considered to be susceptible to infection with ΦDMPA-1. Among the remaining 2 *P. aeruginosa* isolates, 1 (20%) has developed turbid plaques and 1 (20%) developed no plaques in spot tests which was resistant to ΦDMPA-1 infection. The inference of the results is that, ΦDMPA-1 has a broad host range among *P. aerugenosa* strains isolated from pyogenic skin infections.

Stability of the phage ΦDMPA-1

Stability of the phage was examined under different parameters by exposing at varied conditions. Phage lysates with a titre of 10^8pfu/mL were stored at room temperature, -40^{0}C, -20^{0}C, 4^{0}C, and 20^{0}C and also in presence of 20% (v/v) glycerol at -40^{0}C and -20^{0}C. Phage titres maintained at various conditions were examined after 3, 6, 9 and 12 months of storage by double agar layer technique (Adams, 1959). There was no significant decrease in the level of phage titre after 3 month storage at all of the tested temperatures, except at room temperature, where the titre was reduced by 30%. ΦDMPA-1 was highly sensitive to higher temperature (60^{0}C) for 15 minutes, with a 100-fold decrease in titre. There was no decrease in the titre in chloroform treated sample after 4 and 24 h. Phage ΦDMPA-1 was completely lysed at pH 2 and 4 after 1 h of incubation at both temperatures. Incubation at pH 4 and 37°C caused a 30% and 80% decrease in phage titre after 1 and 5 h of incubation, respectively. At the same pH condition, 10-fold reduction was observed in plaque-forming units per ml at room temperature after 5 hours. ΦDMPA-1 was stable within a pH range 5–9.

DISCUSSION

The emergence of pathogenic bacteria resistant to most, if not all, currently available antimicrobial agents has become a critical problem in modern medicine, particularly because of the concomitant increase in immunosuppressed patients. Mounting concerns about drug-resistant pathogenic bacteria (Cohen, 1992) have rekindled interest in alternative treatments of bacterial infections. Prominent among these alternatives is phage therapy, the use of bacteriophages to kill or otherwise control the bacterial populations in infected hosts. The rapid emergence of new *P.aeruginosa* strains as well as the persistence of the existing antibiotic-resistant clinical isolates has led to an urgent need to explore more sustainable alternative strategies such as phage therapy to manage *P.aeruginosa*-mediated infections. Many research groups turned their attention to the phage treatment of *P.aeruginosa* infections in mice, guinea pigs and pet dogs (Soothill *et al*., 2004). Recently, the therapeutic mixture of six lytic phages was shown to be effective in clinical ear infections caused by *P. aeruginosa* (Soothill *et al*., 2004). The efficacy of phage therapy using a genetically modified filamentous phage (Pf3R) (Hagens *et al*., 2004), lytic phage isolates, or phage cocktails has been also investigated against various experimental mouse infection models with *P. aeruginosa* that include burn wound infection (McVay *et al*., 2007). A report of the treatment of single cases of human burn wounds indicates that bacteriophage multiplication is associated with clinical improvement (Marza *et al*., 2006). A first randomized, double-blind and placebo-controlled Phase I/II clinical trial was performed on 24 patients suffering from chronic otitis caused by MDR.

P. aeruginosa, showing efficacy and safety in treatment of this infection (Wright *et al*., 2009). Nevertheless, a crucial condition towards practical application of phage therapy will be the availability of a large library of well-characterized phages (Denou *et al*., 2009). In the present study, ΦDMPA-1 is a *P.aeruginosa*-specific virulent lytic phage exhibiting strong bacteriolytic activity against MDR clinical isolates of *P. aerugenosa* and also have storage stability for three months at lower temperatures without any significant decrease in phage titre.

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