Efficacy of Bacteriophages Against Multi Drug Resistant *Pseudomonas Aeruginosa* Isolated From Cystic Fibrosis Patients

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EFFICACY OF BACTERIOPHAGES AGAINST MULTI DRUG RESISTANT *PSEUDOMONAS AERUGINOSA* ISOLATED FROM CYSTIC FIBROSIS PATIENTS

By

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ABSTRACT

Bacteriophage viruses also known as bacterial eaters that infect and eventually kill specific bacteria as their hosts. To date, however very few comprehensive studies of human phage therapy have been performed. Multi drug resistance .*Pseudomonas aeruginosa* is a pathogen of clinical concern, often associated with cystic fibrosis. Over time it is getting difficult to treat *Pseudomonas aeruginosa* induced infection using antibiotics and it is necessary to identify new approach. *Pseudomonas aeruginosa* specific bacteriophage PK was therefore isolated in this study and characterized, in order to check its efficacy as therapeutic agent. By performing spot test phage presence is confirmed and then purified. Optical density of bacterial culture treated with isolated phage was checked, in order to evaluate the reduction in bacterial growth. Phage genomic was extracted and analyzed by gel electrophoresis. pH optimization of phage at different pH (5,6,7,8,9) was noted. Stability of phages under varying temperature (25, 40,60 and 37) was determined. The isolated phage PK was found to be best stable at 37- degree Celsius. PK phage was also stable under pH 7. PK phage genome is more than 10 kb. Studies on its biological characteristics may provide useful information and knowledge in establishing potential therapeutic agent against *Pseudomonas aeruginosa* infection.

Key words: Bacteriophage, Drug Resistance, Pseudomonas, cystic fibrosis

Introduction

Phages or bacteriophages are viruses that infects specific bacteria, (Chan and Abedon 2012). They are most abundant and genetically diverse biological entities on the planet with a large number of global population, (Hemminga, Vos *et al.* 2010). It is estimated that there are 5 to 10 viruses per bacterium (Weinbauer 2004). Bacteriophages are found in all corners of nature and they are found in all environments that support multiplication of bacteria (Kęsik-Szeloch, Drulis-Kawa *et al.* 2013). Bacteriophages multiply within the bacterial cells and can be detected almost in all places where live microbes are present. These are isolated from all environments and environment friendly niches like waste waters, sea water, and soil (Zhan, Buchan *et al.* 2015). With estimated 10^7 phages per gram in ecosystem and 10^{11} in soil (Guttmann, Raya *et al.*, 2005). Bacteriophages contribute significant job in the regulation of microbial stability in each ecosystem owing high specificity, lasting vitality and capacity to reproduce quickly in the appropriate host (Guttman, Raya *et al.* 2005). More than 25000 phage nucleotide sequence has been submitted in International Nucleotide Sequence Database Consortium (INSDC) (Adriaenssens and Brister 2017).

Phage consists of a double stranded DNA either RNA molecule enclosed in a protein or lipoprotein layer. Historically, the ICTV International Committee on Taxonomy of Viruses has used virions and nucleic acid as a basis for dividing phages into 13 families (Ackermann 2007).

The replication strategies following the successful penetration of phage genetic material dictate the virulent (lytic) or temperate nature of bacteriophage. In lytic cycle, injected phage genome control on the bacterial replication machinery and manufacture necessary components for new progeny phage. (Kutter and Sulakvelidze 2004).

In humans different acute and chronic infections such as skin infections and abscess, in addition to various systemic infections in patients with hereditary diseases such as cystic fibrosis are more complicated by *Pseudomonas aeruginosa*, (Al-Wrafy, Brzozowska *et al.* 2017). In particular, among patients with MDR *P. aeruginosa* infection, highest mortality rate have been recorded in hospital stays, (Oliver, Mulet *et al.* 2015), (Nathwani, Raman *et al.* 2014). There are several factors that may be inherent or acquired in the emergence of *P. aeruginosa* strains,

pneumonia, and mechanical heart valves associated infections, caused by *P. aeruginosa*, (Poole 2011; Cole, Records *et al.* 2014).

Phage-based therapies appears to be promising innovative treatments that deserve attention, (Hurley, Cámara *et al.* 2012). Many active phages have been identified against *P. aeruginosa*, (Harper and Enright 2011) and many of these phages are effective against cytic fibrosis patients with *P. aeruginosa* strains, (Saussereau, Vachier *et al.* 2014).

Lytic Bacteriophages may be an alternative to antibiotics for treating bacterial infection that do not response the treatment with normal antibacterial drugs, (O'flynn, Ross *et al.* 2004). According to the National Institute of Health, phages are innovative elements that can be used to combat bacterial resistance, (Fauci and Marston 2014). In addition, the genetics of lytic phages do not contain complete genes, so they cannot coexist with the host, and not able to transfer toxic genes from one host to another (Guttman, Raya *et al.* 2005). Unlike broad spectrum antibiotics, bacteriophages are specific to prokaryotic host cells and show less common illegal resistance to non-host cells (Sulakvelidze and Kutter 2004).

Therefor this study was executed with the aim to identify and characterise the *Pseudomonas aeruginosa* bacteriophage from different sewage water samples.

1. To characterize the lytic bacteriophage that can be apply to control the growth of pathogenic multi-drug resistant bacterial specie.

MATERIALS AND METHODS

STUDY AREA AND DURATION:

The study was conducted at Department of Medical Lab Technology, The University of Haripur from February 2019 to August 2019.

SAMPLE COLLECTION:

Waste water samples were collected from different hospitals and laboratories of Abbottabad. Waste water samples *(n=06*) were collected in 15 ml of sterile falcon tubes. *Pseudomonas aeruginosa* strains isolated from cystic fibrosis patients were obtained from Department of Microbiology and Molecular genetics, Punjab University, Lahore.

MORPHOLOGICAL CHARACTARISTICS:

The bacterial strains were identified morphologically based on their shape and color.

GRAM STAINING:

Gram staining of smears was performed using standard protocols and observed under an optical microscope with 40X and 100X magnification,

BIOCHEMICAL TESTS:

Purified colonies of bacteria were further subjected to biochemical tests like oxidase and catalase.

OXIDASE TEST:

The oxidase test is a significant differential method to identify all Gram-negative bacteria quickly. By using a sterile loop, a well-isolated pure colony was put on the filter paper impregnated .with oxidase reagent. A color change was observed after 30 seconds where colonies were streaked.

CATALASE TEST:

All isolated colonies were analyzed for catalase production. A freshly prepared H_2O_2 with the concentration of 3% was placed on the clean glass slide. Fresh bacterial culture (24 hour) was emulsified using a sterile wire loop. Bubbles were observed within 45 seconds.

ANTIBIOTIC SUCESPIBILITY PROFILING:

The method used by Kirby Beaur to detect antimicrobial susceptibility of *P. aeruginosa* clinical isolates.When the organism was resistant to all the agents studied (ceftazidime, cefepime, aztreonam, ciprofloxacin, piperacillin and gentamicin*) P. aeruginosa* was classified as MDR, (Gould 1994). Purified bacterial cultures were taken and added to saline buffer, and then this bacterial suspension was spread on Muller Hinton agar with the help of sterile glass spreader and kept for drying. Antibiotics discs (Imipenem, meropenem, gentamicin, amikacin, Cefotaxime, Ceftadizime, Ciprofloxacin and Ampicillin) were placed on inoculated plates with the help of sterile forcep and incubate at 37ºC for 24 hours. Results were then examined by measuring the inhibition zone.

ENRICHMENT OF PHAGE:

For enrichment of phage method, (Cerveny, DePaola *et al.* 2002) the isolation of phage method was adopted. Waste water samples were collected from different hospitals (Ayub teaching hospital, Benazir Bhutto Shaheed hospital, Women and children hospital, Al Maida Hospitals and Abbott Laboratory) and transferred to the laboratory quickly for the phage isolation. Remove the sediment particles from the sewage water sample. Centrifuge the water sample at 4000 rpm for 5 minutes. Add 10ml of autoclaved 5X LB broth to the flask and supplement with 4 ml of centrifugal sewage water sample. Inoculate the same medium with 1 ml of 24 hours fresh bacterial culture. Place it at 37°C for 24 hours with continuous shaking at 120 rpm. Then remove the culture from the incubator and add chloroform to the final concentration of 1%. Then filter it by using syringe filter (0.22 µm, Finetech research and innovation corporation (Cat# 2208/22). The filtrate obtained was used for determination of plaques on bacteria culture plates. A single plaque was picked with the help of a sterile micropipette tip. Phage were purified by picking the bacteriophage and propagation by the double layer agar technique and expressed as plaque forming units per milliliter (*pfu*/ml) (Yang, Liang *et al.* 2010).

SPOT TEST AND PLAQUE ASSAY:

The spot check was carried out in order to detect the presence of phages in supernatant described by (Chang, Chen *et al.* 2005). After the confirmation by spot test, plaque assay was performed, dilute the phage suspension in series and mix with 100 µl of 24 hours fresh bacterial culture. Leave the mixture unchanged at 37°C for 10-15 minutes. After this, pour the mixture onto the LB agar plates and add 3-5 ml of semi-solid agar. Then mix the soft agar with the mixture of phage bacteria by swirling the agar plates and allow them to solidify. After incubation at 37^{o}C, look for small different clear plaques.

PURIFICATION OF PHAGE:

All the isolated phages were purified by successive single-plaque isolation until the standard procedure obtained homogenous plaques defined by (Sambrook 1989).

After the plaque formation, select an isolated plaque with a sterile surgical blade. After receiving the plaque, mix it with 25ml of LB broth containing 1ml of fresh bacterial culture;

incubate it for 24 hours at 37°C. Then add 1% of chloroform and left it undisturbed for 30 minutes, centrifuge it for 10 minutes at 10,000 rpm. Then syringe filter the supernatant and label it as a phage suspension.

To verify the capability to overlap the lytic capacity, repeat this procedure 6-8 times to completely purify the phages. The plaques of similar morphology are placed at 4°C or -80°C by the addition of glycerol at the final concentration of 5%.

PLAQUE FORMING UNIT (pfu) CALCULATION:

Plaque forming units are quantitative measures of phage activity expressed per ml. The agar overlay technique was used to calculate pfu/ml. Serial dilutions of up to 10^{-15} phage suspensions were prepared using a nourishing broth. Eight dilutions were performed, namely 10^{-1}, 10^{-3}, 10^{-5}, 10^{-7}, 10^{-9} and $10^{-11}10^{-13}$ 10^{-15}. 100µl of phage suspension dilution were incubated with bacterial suspension culture for 5 minutes at 37°C with continuous shaking.

Then each of the phage bacterial suspension was poured onto labeled agar plates, respectively. These plates were poured with 5ml of Luria-Bertani broth. Then incubate at 37ºC for 24 hours.

Plaque forming unit was calculated by following formula:

$$\frac{\text{Average no. of plaques}}{\text{Volume X dilution factors}} = \text{pfu/ml}$$

REDUCTION ASSAY:

Overnight grown host bacterial cultures were each added to 24 tubes containing LB-broth. Twelve tubes were inoculated with phage PK while the other twelve contained no phages as control. Tubes were kept in shaking incubator at 37°C for 24 hours. Optical density (OD600) was taken for 24 hours at 2 hour interval. The reduction rate in bacterial growth was evaluated by measuring the OD (Haq *et al.* 2012).

OPTIMIZATION OF PHAGE AVTIVITY UNDER VARIYING pH:

As different phages live in different pH environments, optimal pH is an essential requirement for phage development. pH stability test was done according to (Verma, Ning *et al.* 2009) with a slight modification.

The currently known titer and the phage suspensions are placed in the nutrient broth at pH 4, 5, 6, 7, 8, and 9 at 37°C for one hour. After incubation and adjusting pH to 7, plaque assay was applied. The difference in the titer was tested.

OPTOMIZATION OF PHAGE AVTIVITY UNDER VARIYING TEMPRATURE:

To evaluate stability of the phages at various temperatures, the phages aliquots were incubated at 25°C, 37°C, 45°C and 60°C for 24 hours. After the specified interval, the double layer is drawn before the agar technique and brought to room temperature. Then plaque assay is applied. The differences in the titers are tested.

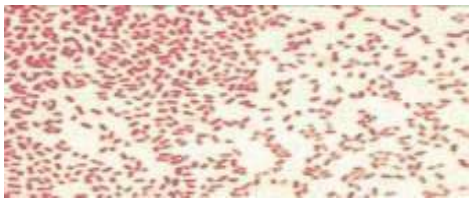
GENOMIC CHARACTERIZATION:

Nucleic acid extraction was carried out by using (Sambrook and Russell 2001), process with minor modification. The Nucleic acid was extracted from phage particles by mixing 500µl of purified phage with 500 µl of phenol, chloroform, isoamyl alcohol, with ratio of, (25:24:1). In order to confirm genome nature, that is either it is DNA or RNA, extracted genome was treated with DNAase 1 and RNAase A in two separate reactions and incubated at 37°C for four hours. Isolated sample was electrophoresed on 1 % agarose gel and visualize under UV light (Khwaja *et al.* 2016).

RESULTS AND DISCUSSION

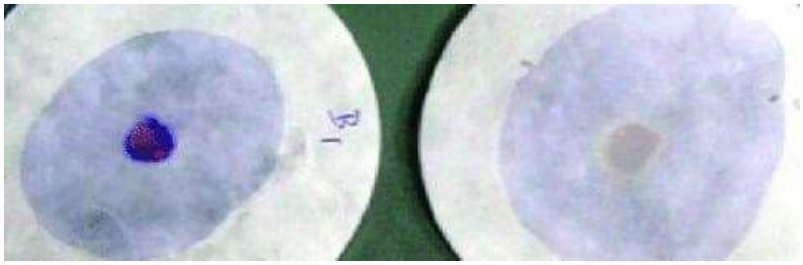
BACTERIAL ISOLATION:

Bacteria isolates from cystic fibrosis patients were identified by their morphology and biochemical testing at Microbiology and Molecular Genetics Department, University of Punjab. All isolated bacteria showed gram negative rods by gram staining (Figure 4.1a). Oxidase and catalase showed positive results as shown in figure 4.1b and 4.1c.



Gram staining

Catalase test showing positive results



Oxidase test showing purplish blue color (positive result) for *Pseudomonas aeruginosa.*

ANTIBIOTIC SUCESPIBILITY PROFILING

Figure 4.2 Antibiotic sensitivity pattern of *Pseudomonas aeruginosa* by Kirbybauer Method on Muller Hinton medium.

The isolated bacterial sample is confirmed multi drug resistance according to criteria declared by WHO (Organization, 2014) (Figure 4.2). According to (Vincent H. Tam *et al.*, 2010) 14 % isolates were found to be multi drug resistance *Pseudomonas aeruginosa.* All isolates were carbapenems and quinolones resistant, 91% were penicillin's / cephalosporins resistant, and 21% were aminoglycosides resistant. Continuous monitoring of multidrug-resistant *P. aeruginosa* prevalence and resistance mechanism would allow us to formulate rational treatment strategies to combat nosocomial infections, (Ciofu *et al.*, 2005).

PHAGES ISOLATION AND SPOT TEST:

Figure 4.3: Spot Test of phage lysate PK showing clear large plaque on *P. aeruginosa* lawn.

Out of 6 samples two samples showed clearing zones on *P. aeruginosa* lawn. Waste waters of hospitals and clinics are known to contain different kind of infectious bacteria therefore there is great likelihood of phages presence against different bacteria.

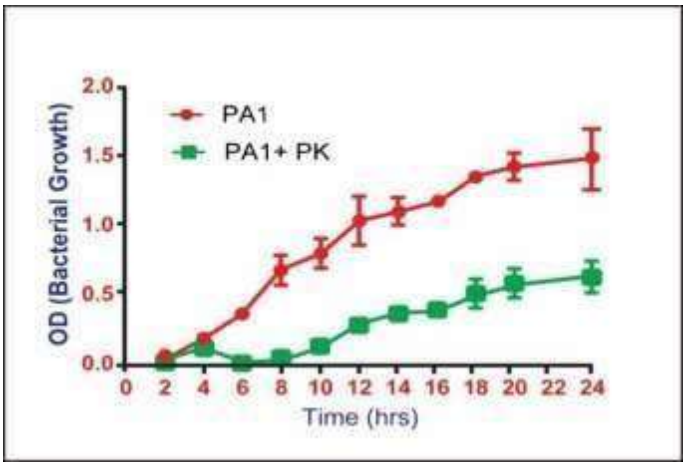
Spot test is very efficient approach for initially detecting the bacteriophages in any sample and many recent studies. Bibi et al in 2016, Kumari et al 2009 and Hyman and Abedon, 2010 also used spot assay for rapid detection of bacteriophage against *E.coli* form sewage water.

PLAQUE ASSAY:

Figure 4.4: Plaques formation by phage PK after double layer agar assay.

Plaque assay by double layer agar method has been used by many researchers for bacteriophage titer (Haq, Chaudhry *et al.* 2012; Chaudhry, Haq *et al.* 2014; Mirzaei and Nilsson 2015; Bibi, Abbas *et al.* 2016; Kwiatek, Parasion *et al.* 2017). The hazy ring suggests that bacteriophages isolated have produced depolymerase enzyme as free enzyme produced during lytic cycle (Kimura and Itoh 2003). In an another study in which bacteriophages isolated against *P. aeruginosa*, bacteriophages showed clear plaques surrounded by small halos (Zhao, Wang *et al.* 2009).

REDUCTION ASSAY:



Growth curve showing treated with PK phage and without PK phage

The results of the current study revealed that bacterial culture with an isolated phage (PK) can prevent the growth of MDR *P. aeruginosa* for up to 8 hours, then that the OD gradually

increases after ten hours while constant increment in OD of control culture (bacteria without phage) indicated increasing in bacterial growth.

An increase in bacterial OD indicates that MDR *P. aeruginosa* may have developed resistance to phage (PK), and a similar type of result has been reported because the phage lost their capacity to lyse bacteria, 10 hours after incubation. The optical density (OD600) was taken for 24 hours at 2 hour interval (Haq *et al.* 2012).

OPTIMIZATION OF PHAGE PK AVTIVITY UNDER VARIYING pH:

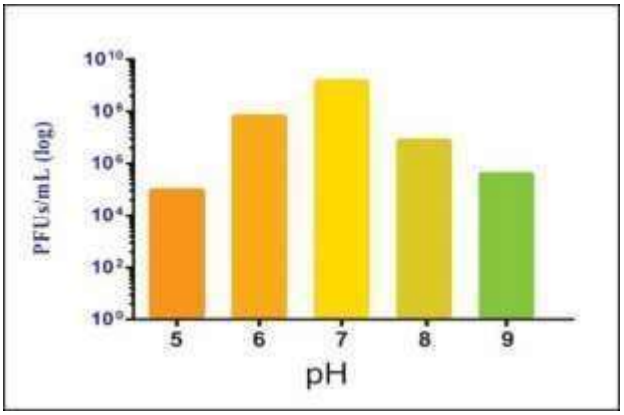


Figure 4.6: Effect of pH on stability of PK bacteriophage.

Highest titer of PK phage was recorded after incubation at pH 7.0. The titer of the phage drops down when pH reduced to 5.0, similarly phage titer continued to drop dramatically when pH raised to pH 8.0 and onwards. It has been found in many studies that pH 7 is favorable for maximum phage activity (Haq *et al.* 2012, Bibi *et al.* 2016) (Lu, Breidt Jr *et al.* 2003; Capra, Quiberoni *et al.* 2006). High acidic and alkaline pH denature viral protein and thus subsequently phage viability and activity that's how activity of phages were affected by acidic and alkaline pH (Hazem 2002). Therefore, isolated phage is not suitable for direct oral therapeutic purpose without some kind of protective layer such as alginate and chitosan (Malik, Sharma *et al.* 2017) since the baseline pH of 1.5 in human stomach (Travis, Brambilla *et al.* 2015) will deactivate the phage.

OPTIMIZATION OF PHAGE PK AVTIVITY VARIYING TEMPRATURE:

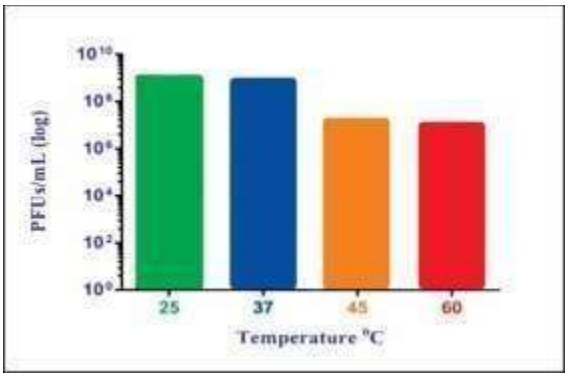


Figure 4.6 Thermal stability of PK bacteriophage at various temperatures.

The PK phage was found to be unstable at 45ºC and 60ºC with significant reduction in titer. Whereas, increment in phage titer at 37ºC followed by 25ºC indicate that the phage is resistant to extreme temperature. As PK phage is best stable at both 37ºC it can be said that the average human body temperature of 37ºC will not thermally deactivate its lytic activity if PK was considered as a therapeutic agent against *Pseudomonas aeruginosa* in Cystic Fibrosis patients.

It was found that the maximum lytic potential of *Pseudomonas aeruginosa phage* is at 25°C and 37°C. while, activity of the phage decreases with increasing temperature**.** Temperature influences the whole phage replication process including attachment, penetration, and multiplication (Olson 2004). Additionally, temperature regulates the viability, occurrence, and storage of bacteriophage ((Jończyk, Kłak *et al.* 2011).

GENOMIC CHARACTERIZATION OF PHAGE PK:

Genome of the phage PK was extracted and found as DNA. As the mixture treated with DNases showed no band while mixture treated with RNases showed clear band on gel electrophoresis (Figure 4.8). Genome size of phage was more than 10 kb. The band in lane 2 appeared very clear in gel image that is indication of high concentration and purity of extracted phage DNA.

Research studies also reported DNA phages against *Pseudomonas aeruginosa* when genome nature was explored (Wlodarczyk *et al.*, 2015; Kwiatek *et al.*, 2017).

Fig 4.7: Electrophoresis of Phage genome.

Lane 1: Completely digested genome with DNAase, Lane 2: 10 Kb ladder; Lane 3-4: PK phage treated with RNAase.

CONCLUSIONS AND RECOMMENDATIONS:

This study led to the isolation of bacteriophage PK from a water sample which was further characterized to identify its host range, stability under the different adverse condition, and a couple of important parameter of its growth dynamics. Due to PK phage stability under temperature and pH fluctuation along with its depolymerase production ensures its suitability for the treatment of *P. aeruginosa* related infections in synergy with antibiotics.

Protein characterization of this phage should be done so that lytic proteins genes should be cloned and express in *E. coli* for therapeutic use in future. Effects and side effects of these phages in cystic fibrosis patients should be evaluated for therapeutic use against MDR *P. aeruginosa*.

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