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Isolation And Characterization of Lytic Bacteriophage from Sewage Water Against *Pseudomonas Aeruginosa*

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Abstract

Background: Multidrug resistance is the significant threat for treating the bacterial infections around the globe. *Pseudomonas aeruginosa* is one of the antibiotic-resistant pathogens, associated with nosocomial infections. Biofilm growth and inherent resistance make *Pseudomonas aeruginosa* more resistant to multidrug, it has acquired resistance to almost all antibiotics. Increasing emergence of antibiotic-resistant bacteria has develop a growing interest in bacteriophages as an alternative antimicrobial agent.

Results: In current study, bacteriophage SS1 was isolated from hospital sewage and domestic waste water with lytic activity against *Pseudomonas aeruginosa* strains. Isolated phage maximum lysis was preserved by phage at 7 p^H while its viability is lost at extreme p^H (1,3, 11 and 13). Isolated phage has excellent thermal stability at 40°C but lost it on extreme temperatures ranging from 60°C and above, under UV their viability is lost by increasing the UV duration and completely lost after 60 minutes of exposure. Phage shows narrow host range as revealed by the results only involve in the lysis of *Pseudomonas aeruginosa*

and few *Salmonella* and *Escherichia coli* species.
Conclusion: It could be concluded from the present study that the isolated phage has narrow host range and exhibits the potential to be used as drug substitute tool against Pseudomonas *aeruginosa.*

Introduction

Pseudomonas aeruginosa is a free-living, gram negative, rod-shaped pathogen with high degree of antibiotic resistance (Jamal *et al*., 2017). *Pseudomonas aeruginosa* is ubiquitous organism, located in many diverse settings such as soil, water as well as animals and human bodies, also found in plants, sewage and in hospitals (Chibeu *et al., 2009).* It is an opportunistic pathogen of humans, because it rarely causes infection among healthy individuals. Instead, it mostly infectious to immunocompromised patients such as those suffering with cystic fibrosis and other immune deficiency diseases like cancer and AIDS while it also complicates the patients with cystic fibrosis in addition it is included in top three-gram negative pathogens associated with worst visual diseases (Kowiatek *et al*., 2014) (Debarbieux *et al*, 2010). *Pseudomonas aeruginosa* has a prominent role as an etiological agent for serious infections in patients suffering from burn wounds (Catherine et al., 2017). Emergence of multidrug resistant bacterial strains increase the health hazard around the globe, due to over exposure to broad spectrum antibiotics pathogens have many resistance mechanisms (Nivas *et al*., 2015). Multidrug resistance becomes one of the major barrier in the way of treating microbial infections (Pallavali, *et al*., 2019). Frequent use of antibiotics increases the resistance in few bacterial strains, important with respect to human infections, one of them is *Pseudomonas aeruginosa*, it cause biofilm mediated infections in humans (Chan *et al*., 2016). *Pseudomonas aeruginosa* show 73%, 94%, 88%, 73% and 3% resistance to chloramphenicol, colistin, tetra cycline and Imipenem respectively (Piracha *et al*., 2014) . Multi drug resistance enhances health hazards (Nivas *et al*., 2015). This multidrug resistance leads to the failure of conventional treatment ways of bacterial infections (Jamal *et al*., 2017). Drug resistance makes more challenging to treat and diagnose the infections caused by resistant pathogens (Pachori *et al*., 2019). Food and drug administration proved nine drugs in 1998 to 2005, out of which only two were effective against resistant pathogens while currently there is no class of effective drug against them (Nivas *et al*., 2015). Ultimate failure of drug resistance results in high rate of mortality and morbidity (Pachori *et al*., 2019). Due to emergence of bacterial strains with multidrug resistance, new therapeutic as well as prophylactic techniques are demanding to control their infections (Pruitt *et al.,* 1998). For combating against these pathogens, bacteriophages are used in various countries modality is called phage therapy. Phages are used even as antibacterial agents before the discovery of penicillin, the first antibiotic (Summers, 2001).

Pseudomonas aeruginosa is the therapeutic challenge for the medical world, it is resistant to multiple classes of antibiotics even more complicated during the course of therapy, it is also has reported resistant mechanism such as fluroquinolone resistance among them, results of chromosomal mutation (Lister *et al*., 2009). *Pseudomonas aeruginosa* show high tendency to resist against Beta lactam (Jamal *et al*., 2017). *Pseudomonas aeruginosa* chromosome encoded a variety of resistance mechanisms including aminoglycoside inactivating enzymes, also exhibit mutation in QRDR of both gyrA and topoisomerase iv gene (Akasaka *et al*., 2001).

One of the novel treatments against drug resistant pathogen is phage therapy, using bacteriophages is a good choice against multidrug resistant bacteria as natural predator on them (Jamal *et al*., 2017). Phage therapy is much better with respect to antibiotics in certain mode of actions (Bondin *et al.,* 2014). A British pathologist first discovered phages in 1915 (Toth *et al.*, 2003), and in 1917 phages were found to be involve in lysis of shigella specie in broth (Delbrück, 1945). Phages are basically having two types on the basis of host range; the one called polyvalent can attack on many species while

the others are monovalent; only specific for a particular specie (Demerec and Fano, 1945). Phages are very specific for particular specie depends on recognitions system of phages (Kutter and Sulakvelidze, 2004). Phages have proteins on their surfaces by the help of which they recognize the receptors on the host body (Naster *et al.,* 2004).

Materials and methods

Bacterial Strains and Growth Conditions

The bacterial strains used in this study were obtained from Fuji foundation hospital Rawalpindi. Confirmed strain of *Pseudomonas aeruginosa* was obtained as freezing stock and then streaked on the cetrimide agar in a sterile petri dish after that it kept on incubation at 37°C. In this way pure culture of *Pseudomonas aeruginosa* was obtained.

Antibiotic sensitivity test

Pseudomonas aeruginosa antibiotic resistance was verified by the process of disc diffusion. Cefixime, gentamycin, doxycycline, cefazoline, and linezolid have been used.

Table 1. Antibiotic sensitivity test

S.no	Antibiotics	Zone of inhibition (mm)	Results resistant
1	cefixime	0	+
2	gentamycin	0	+
3	doxycycline	0	+
4	linezolid	15	+
5	cefazoline	0	+

Sample collection

A total of 60 sewage samples were collected from DHQ hospital Haripur and domestic sewage; the collected samples were in sterilized falcon tubes and then transported to laboratory immediately for the further process.

Phage isolation and purification

Phage were isolated from a sewage sample by following standard enrichment protocol (Jamal *et al.,* 2017). Samples were collected from sewage and left in a flask for 1 hour. Then supernatant was collected from flask and centrifuged for 10 min on 10,000 rpm. Centrifuged supernatant was then transferred to a sterile 50mL falcon tube and mixed with overnight culture of bacteria in nutrient broth with log phase bacterium and kept on incubator for overnight at 37°C. Next day chloroform was added (2% final concentration) and left for 20 minutes for the purpose to lyse the bacterial wall, it was followed by centrifugation at 10,000 rpm for 10 minutes and filtered with 0.2 µm syringe filter syringe filter, after that filtrate was stored at 4°C for used further.

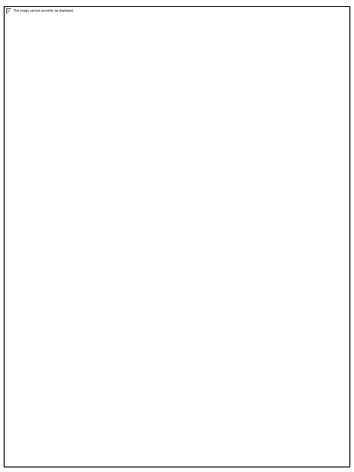

Fig. 1. Individual clear plaques produced by phage SS1 on double layer agar assay plate.

Titration of phage suspension

Serial dilutions of the phage suspension from 10^{-1} to 10^{-6} was performed. Phage titer was determined by using the formula PFU/mL = number of plaques/ (dilution factor) (volume spread on plate). Titer of the dilution 10^{-2} was determined as ($3.15x10^5$ pfu/mL) 10^{-3} was ($2.70x10^5$ pfu/mL) 10^{-4} was ($1.10x10^5$ pfu/mL) while dilution 10^{-5} produced ($0.90x10^5$ pfu/mL) ad 10^{-6} were Plaques were ($0.70x10^5$ pfu/mL) counted two times and both time same results were obtained.

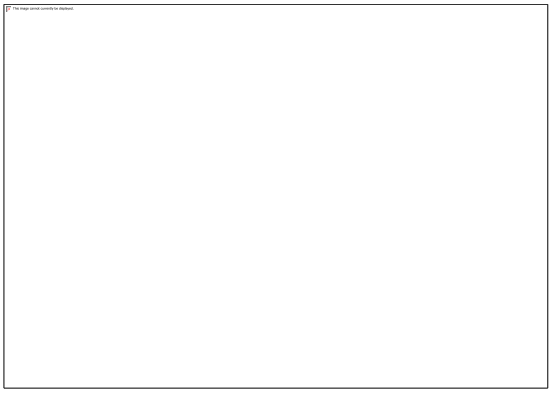

Fig. 2: Titration of phage suspension (10^4)

Determination of phage host range

Isolated phages against *pseudomonas aeruginosa* was tested against different disease-causing bacteria including *Salmonella typhae*, *staphylococcus aureus*, *Escherichia coli*. *Pseudomonas aeruginosa* and *Escherichia coli* was susceptible while none of other were infected by isolated phages.so, it means it has narrow range as shown by the results.

pH susceptibility test

Phages have the property to survive in extreme conditions. For the purpose to check the viability of the phage various pH (3,5,7,9,11) were used. Results show that phages maintain its viability from (5,7,9) and lost it on extreme pH (3 and 11). Lytic potential was only maintained at 7 (neutral pH), as well as they highest viability was also observed on this pH. So, results show that their viability is affected by extreme acidic and alkaline pH.

Fig. 3. pH susceptibility test**,** Y-axis represents average number of plaques while, X-axis represents pH (3 to 11)

Influence of a range of temperatures on phage stability

Heat stability test was performed in order to check the dynamics of isolated phages. Phages were incubated at different phages ranging from 40°C,50°C, 60°C, 70°C, and 80°C for 1 hour.

It was observed that phages maintained its viability at 40°C (210 plaques) to 50°C (190), phage viability gradually decreased at 60°C) and completely lost at 70°C and 80°C.

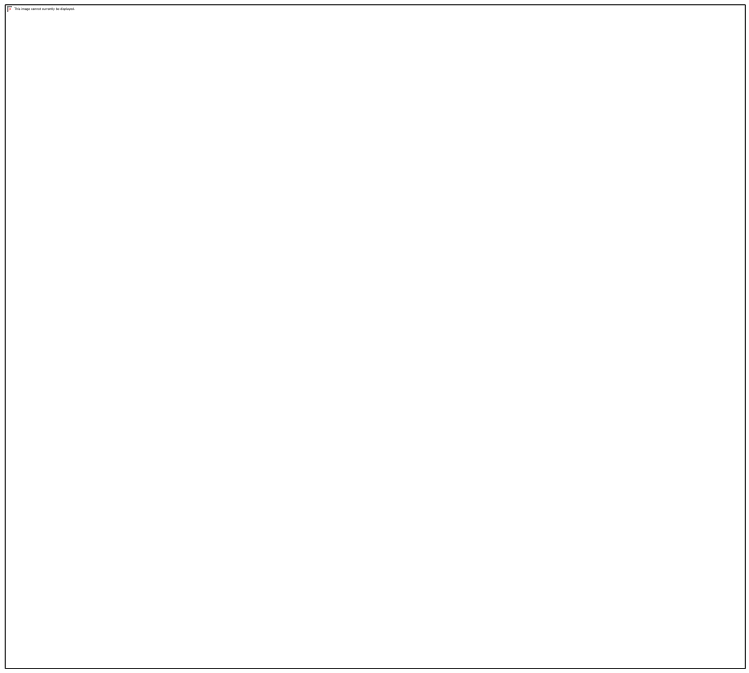

Fig. 4. Heat stability test, Y-axis represents average number of plaques while X- axis represents different temperatures

Effect of ultraviolet (UV) radiations on phages

For the purpose to find out either UV effect the phages are not, phages were exposed to uv radiations for different time periods (10, 20, 30, 40, 50, 60 minutes) and in this way found the effect of ultraviolet radiations on the performance of phages.

Phages maintain their viability and stability up to 30 minutes but later as time increased, they lost their viability to infect the *Pseudomonas aeruginosa.*

Fig. 5. UV test , Treatment of phage under UV radiations for different intervals of time, Y-axis represents numbers of plaques while X-axis illustrate time

Bacterial reduction assay

For checking that phage has potential to restrict the growth of *pseudomonas aeruginosa* while in vitro, bacterial reduction assay was performed for up to 13 hours. And it was observed that phage can restrict the growth of *pseudomonas aeruginosa* for 7 to 8 hours compared to control group, results show sudden fall in optical density (0.121 to 0.061) from first hour of incubation. After seven hours increase in the optical density was observed.

One-step growth curve of phage

Burst size and latent time period was determined by following the growth experiment, in the result tri-phase curve was obtained. Curve represents the latent, log and stationary phase. Curve showed the latent time period of 33 minutes where burst size was 300 phages per cell.

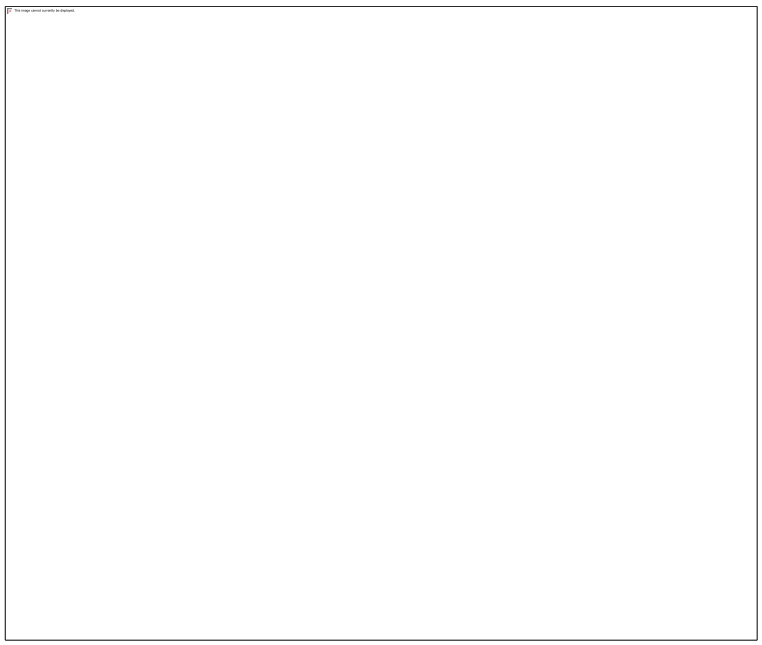

Fig. 6. One- step growth curve test One step growth curve. Y-axis represents numbers of plaques while X-axis indicates time (Latent period 33 minutes and burst size 300/infected cell).

Organic solvent sensitivity test

Organic solvents sensitivity of phages was checked by using chloroform and ethanol. Lysate was incubated for 60 minutes with chloroform and ethanol. Titer of the phages which incubated with chloroform show not much significant change while the phages incubated with ethanol was completely inactive, time of incubation for both was same.

Fig. 7. Organic solvent sensitivity test: First column represents the control, second chloroform and third ethanol, chloroform show no significant change while ethanol completely inactive the phages.

Discussion

Multiple-drug resistant pseudomonas aeruginosa is one of the leading causes of nosocomial infections specifically related to developing countries. Over exposure to antibiotics made *pseudomonas aeruginosa* more resistant to many clinically available antibiotics including carbapenem. *Pseudomonas aeruginosa* has resistance property by having chromosomally encoded efflux, outer membrane permeability and ability to

form biofilm, these all are barriers in the way to treat its pathogenicity. In these alarming conditions need to find out alternate to antibiotics is demand of the day.

For this purpose, bacteriophages are the excellent alternate for controlling the bacterial infections, which cannot be treat with antibiotics. Phages are found in nature with abundance and in diverse environment, could be a great choice to choose as antibacterial agent. Although phage therapy was also used in past but recently as antibiotic resistance is increased, phage therapy regains its importance. Phages are very specific to their target specie, strains.

In this study phages were isolated from sewage water, as sewage is highly diverse place, have a huge quantity of microbes, with great genetic diversity. Sewage water of hospital and household was taken for isolation of phages with high lytic property and short host range, infect only few species used in studies. The phage tolerates the temperature between 37°C to 60°C and being killed at 70°C by high temperature. pH test show that they are stable at pH from 3 to 11, with high stability rate.

Antibiotics activity resistance was checked and it was observed that pseudomonas aeruginosa is highly resistant to antibiotics. Therefore, this study is significant as it isolated and characterize the phages from sewage water and used against MDR *pseudomonas aeruginosa*.

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