Original Research

Journal Home page : www.jeb.co.in ★ E-mail : editor@jeb.co.in

Journal of Environmental Biology

JEB™

ISSN: 0254-8704 (Print)
ISSN: 2394-0379 (Online)
CODEN: JEBIDP

Molecular characterization of lytic phages specific to antibiotic resistant isolates of *Pseudomonas fluorescens* infecting *Labeo rohita* and *Clarias batrachus* of Sub Himalayan region

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Key words

Aquaculture
Genomic
Phage
Pseudomonas fluorescens

Publication Info

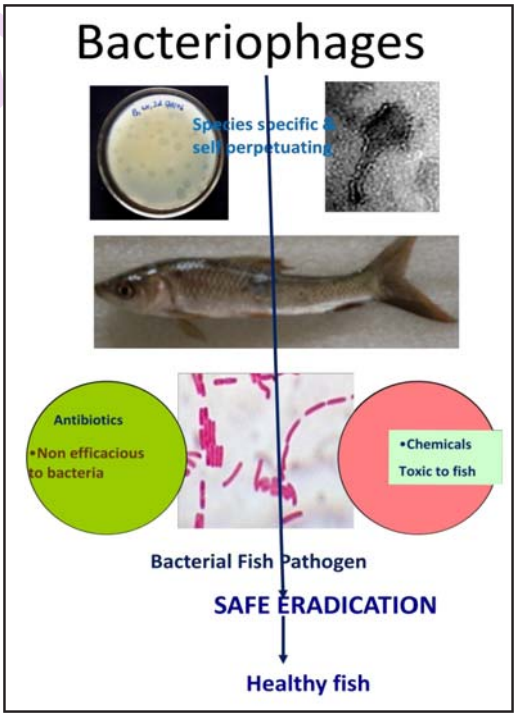
Paper received : 30.05.2016
Revised received : 29.09.2016
Re-revised received : 10.10.2016
Accepted : 16.12.2016

Abstract

Aim : This investigation was aimed to develop antibacterial strategy to circumvent the problem of antibiotic resistant *P. fluorescens* infecting *L. rohita* and Indian walking catfish, *C. batrachus* in Himalayan and Sub Himalayan regions.

Methodology : Four lytic phages (PFPD, PFPK, PFPN and PFPC) were isolated against ten isolates of pathogenic *P. fluorescens* following overlay method using water and bottom sediments of Himalayan and Sub Himalayan regions. One step growth experiment was carried out to know the eclipse, latent periods and burst size of phages. Phage genomic DNA was extracted following chloroform: phenol: isoamyl method and digested using restriction enzymes (EcoRI and Hind III) (Bangalore genei, Bangalore) and isolation of phage protein was made as per Laemmli (1970) with slight modification.

Results : Phages were identified as a member of family *Siphoviridae* (isometric head of 236.42nm and non-contractile long tail of 521.22 nm) with having ds DNA of 20-21 kbp and structural viral proteins of 22 - 102 kDa. Phages exhibited minimum eclipse period (10 - 15 min), latent period (20 min) and highest burst size of 130. These phages conferred clear lytic plaques in the lawn of 8 of 10 (80%) host bacterium at 0.01 MOI, tolerated wide range of temperature (25 to 35°C), pH (6.5 to 8.0), yielded highest titer (10^8 pfu) at 30 ±5 °C and exhibited highest (99%) *in vitro* lytic activities. The host bacterium did not develop resistance to all the phages studied during the entire period of the study.



Interpretation : The profound *in vitro* lytic attributes of PFPD and PFPK phages and their tolerance to temperature and pH at par with aquaculture activities reveals that they may be a suitable option to alleviate transmission of even systematic *P. fluorescens* infection in hatchery and culture system. This information will provide more insight into the potential use of phage to mitigate incidences of Bacterial Haemorrhagic Septicaemia in aquaculture.

Introduction

Pseudomonas fluorescens is a Gram-negative rod shaped bacterial fish pathogen that has been found to be associated with Bacterial Haemorrhagic Septicaemia (BHS) in cultivable Indian Major Carps - IMC (*L. rohita, Catla catla, Cirrhinus mrigala*), common carps, *Cyprinus carpio communis* (Prasad and Qureshi, 1994), ulcered *C. batrachus* (Prasad and Qureshi, 1995) and other aquatic animals (Parvez *and* Mudarris, 2014) and causes a great economic loss to aquaculture. Its persistent occurrence with several ulcerative diseases of carp (*L. rohita* and *C. carpio*), catfish, *C. batrachus* (Prasad *et al*., 2011) and Epizootic Ulcerative Syndrome (EUS) infected fish (Qureshi *et al*., 2000; Darak and Barde, 2015) that has virtually devastated the Indian fisheries industries during 1990 has attracted the attention for its remedial measures.

The common control methods of *P. fluorescens* infection are based on the application of quick lime in case of seasonal ponds and $KMnO_4$ and antibiotics in hatchery and culture ponds (Prasad *et al*., 2010). Moreover, various isolates of *P. fluorescens* have been found to be resistant (*in vitro*) to commonly used antibiotics (Prasad, 2008; Bekta and Yıldırım, 2013). Concerns of antibiotic resistance have also brought attention due to the large amount of antibiotics used in animal production, also in aquaculture (Hollis and Ahmed, 2013). Exposure of environmental microbes, including bacterial pathogens to antibiotics used in aquaculture through fish feed (Cabello *et al.,* 2013) has enabled them to develop resistance (Di Cesare *et al*., 2013). Moreover, attempts have been made to search out effective control methods for mitigation of *P. fluorescens* infection in aquaculture using phages as remedial measures (Prasad *et al.,* 2010).

Since the discovery of phages, they have been used as remedial measures to combat with the antimicrobial resistance. Phages are viruses that subsist on bacteria and generally lead a lytic life in which survival of the host bacterium becomes extremely difficult. This nature of phage has attracted its use as therapeutic agent. Moreover, a good number of phages has been characterized against a number of freshwater bacterial fish pathogens *viz. P. aeruginosa* (Kumari *et al*., 2009; Khairnar *et al*., 2013), *Flavobacterium columnare* (Prasad *et al.,* 2010, 2011; Laanto *et al*., 2014, 2015), *F. psychrophilum* (Christiansen *et al*., 2016; Muziasari *et al*., 2016) and *P. fluorescens,* the causative agent of BHS in fish (Prasad *et al*., 2010; Radhakrishnan and Subramanian, 2012).

The phages identified so far are made of hexagonal head consisting DNA within a protein capsid and a tail comprising of lipid or protein (Taddei and De Paepe, 2006). Most of the phages identified generally contain double-stranded DNA as their genetic material (Radhakrishnan and Subramanian, 2012). More than 95 % of known phages belong to the order Caudovirales (Maniloff and Ackermann, 1998) with main families *viz.* Myoviridae (long double-layered contractile tail), Siphoviridae (long non flexible tail) and Podoviridae (short stubby tail) and are distinct based on morphological characteristic, in particular, tail-shape (Deveau *et al*., 2006). Phages are species specific, they attack their targeted bacteria, inject their DNA into the host which replicates there in and leads to death of bacterial cell without disturbing the non - targeted bacteria. Hence, phages are relatively safe bactericidal candidates as they have no harmful effect against animal or plant cells (Kutter and Sulakvelidze, 2004) and do not affect other beneficial microorganisms. In addition, they can be easily isolated from the native host bacterial environment.

In view of the above, the present study was carried out to investigate an appropriate strategy to circumvent the problem of antibiotic resistant *P. fluorescens,* which would provide more insight into the potential use of phage to mitigate incidences of Bacterial Haemorrhagic Septicaemia in aquaculture.

Materials and Methods

Bacterial isolates : A total of ten *P. fluorescens* (PF1 - PF10) isolates, used in the present study, were isolated from different water bodies of Himalayan (Nainital Lake) and Sub - Himalayan, Ganga river at Kanpur and fish ponds (Delapeer Talab, Choudhary fish culture Talab) of Rohilkhand region using Brain Heart infusion broth / *Pseudomonas* isolation agar (BHI / PIA Hi-Media, Mumbai). All *P. fluorescens* isolates used in the study were identified according to Austin and Austin (2012) as *Pseudomonas* on the basis of scoring selective biochemical tests (catalase, gelatin degradation, nitrate, oxidase, fluoricin pigmentation, MR –VP reaction, H_2S production) and compared with ATCC 13525 using phage typing (Chinthala and Gundala, 2013), which were distinctive for *P. fluorescens*. To determine the optimal temperature and pH requirements, *P. fluorescens* (PF) isolates were subjected to different temperature (5 - 40^{0}C) and pH (5.0-8.5) value adjusting by 1N NaOH /1N HCl and incubation period (24- 96hr) following Buchanan and Gibbons (1974).

Phage isolation and purification : Four lytic phages were isolated against ten isolates of *P. fluorescens* following Sambrook and Russell (2001), with slight modification using water and bottom sediments of Nainital Lake Uttrakhand, Ganga river at Kanpur and Fish ponds (Delapeer Talab, Choudhary fish culture Talab) of Rohilkhand region.

Propagation of phage : Amplification of polyvalent phages (PFPD, PFPK, PFPN and PFPC) was accomplished through liquid culture technique and streak plate method. The Pseudomonas Isolation Agar (PIA) medium containing 1% agar was poured in Petri-dishes and overlaid with 5ml soft medium (BHI) containing 0.5% agarose, 50 µl $MgSO_4$ and 500 µl of fresh culture of *P. fluorescens*. After solidification inoculating loop was touched on the surface of plaques and streaked on overlaid plate, incubated at 30 ± 5 ^{0}C in BOD incubator (REMI) for 16-18hr and examined for the appearance of inhibitory zone at streak line in the bacterial lawn.

Plaques appeared in bacterial lawn were scrubbed using sterilized inoculating loop, filled with SM medium and kept for 4 hr in refrigerator. Later, the suspension was eluted, centrifuged (8000 rpm), filtered (0.22µm) and stored in glass tubes at 4^0C until use.

Determination of multiplicity of infection (MOI) pfu: cfu: To determine accuracy of bacteria (cfu) verses phages (pfu) for getting exact dose, an experiment was conducted *in vitro* using broth culture challenging PF10 bacterium with PFPD phage (pfu : cfu) @ 1 : 10^1, 1 : 10^2, 1 : 10^3 and 1 : 10^4 following the method of Prasad *et al*. (2011) and dilution in which maximum reduction recorded in mean cfu value was determined as multiplicity of infection (MOI).

One step growth experiment : In order to know the eclipse, latent periods and burst size one step growth experiment of phages was conducted. For this, 100 ml suspension of phage (PFPD) and bacterium (PF4) was prepared at an MOI of 0.01, vortexed and incubated at 30 ± 2ºC. Sample were collected (1 ml) at 0, 10, 20, 30, 40, 50 and 60 min to check the pfu. Fifty percent of the sample was centrifuged at 10,000 rpm, filtered (0.22 µm) and rest was used as non-filter sample. 100 µl of filtered and non-filtered samples were overlaid separately (in triplicate) on hard PIA medium to enumerate pfu.

Stability test : To know phage stability at various temperatures, phage aliquots (10^8 PFU ml^{-1}) in SM buffer were incubated at 5, 10, 15, 20, 25, 30, 35 and 40^0C for 24 min. Similarly for pH stability, phage aliquots were added to SM buffer adjusted with 1N HCl or 1N NaOH to a pH range of 4.5 to 11.0 and incubated at room temperature for 1 hr followed by phage titer using a plaque assay.

Morphology of phage : A drop of suspension containing high concentration of phage (10^{6-8} pfu ml^{-1}) fixed in 2.5% of β-glutaraldehyde for 1 hr and washed with 0.1 M Disodium phosphate buffer (pH – 7.2) was put on the carbon-coated grid, left for 2 min and excess aliquot was removed with the help of a filter paper. Thin film developed on carbon coated grid was negatively stained with 2% aqueous Urenyl acetate (pH 4.0), examined using transmission electron microscope (TEM; JEOL, Japan) and images were scanned at 80 kv at All India Institute of Medical Science (AIIMS), Delhi. The phages were classified according to the guidelines of the International Committee on Taxonomy of Viruses (ICTV) (Fauquet *et al.,* 2005).

Extraction of phage genomic DNA and restriction enzyme digestion : Phage genomic DNA was extracted using chloroform: phenol: isoamyl following Sillankorva *et al. (*2008) with slight modification. Briefly, 500µl phage sample (10^8 pfu ml^{-1}) was added with 10 µl of 0.5 % SDS and proteinase K (Bangalore genei, Bangalore) at a final concentration of (0.05) mg ml^{-1} and incubated at 65^0C for 15-30 min in a water bath. Thereafter, equal volume of Phenol: chloroform: Isoamyl was added to remove proteinaceous material and extracted (3X). After Phenol: chloroform: Isoamyl treatment nucleic acid was precipitated with 70 % chilled ethanol and suspended with 20 µl nuclease free water (Millipore) and electrophoresed for confirmation of phage DNA. The phage DNA was digested with restriction enzymes (EcoRI and Hind III), following standard restriction digestion assay as per manufacturer's instructions (Bangalore genei, Bangalore) and electrophoresed using 0.7 percent agarose gel.

Phage protein isolation : The procedure developed by Laemmli (1970) was followed with slight modification for the isolation of phage protein. Briefly, to isolate phage proteins 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using filtered (0.22µm) PFPD, PFPN and PFPK phages mixed with (20:20) sample buffer (0.0625 M Tris HCL, pH 6.8, 1% SDS, 15% glycerol and 1% beta mercaptoethanol bromophenol blue). Before running gel mixture of phage, sample buffer was heated for 5 min at 90 - 95^0C in a water bath. Samples were loaded in gel along with protein marker (NEX- GEN Pink ADD pre stained protein Ladder). The protein bands developed were visualized after staining the gel with silver stain.

Results and Discussion

Ten of twenty isolates *of Pseudomonas* spp. isolated from diseased *L. rohita* and *C. batrachus* conferred positive response to oxidative, oxidase, catalase, gelatin degradation, nitrate, lysine, citrate utilization and fluoricin pigmentation tests and negative to H_2S production, indole and MR-VP reactions at par with *P. fluorescens* (ATCC 13525) were numbered as PF1 - PF10. They formed well developed colonies at optimal temperature of 30 ± 5^0C and at pH 7.2 ± 0.2, but failed to proliferate at 45^0 C and pH below 6.0 and above pH 9.0. Four lytic phages *viz.* PFPD, PFPK, PFPN and PFPC isolated using water samples of Himalayan (Nainital lake) and Sub- Himalayan region - Ganga river at Kanpur and fish ponds (Choudhary Talab, Delapeer Talab)

Table 1 : Lytic phages isolated from different water bodies of Himalayan and Sub- Himalayan regions

Sl. No.	Name of phage	Sampling site	Place	Type of plaque	Size of plaque	Test org. PF
1	PFPD	Delapeer Talab	Bareilly	Discriminate	6.5mm	PF4/PF10
2	PFPK	Kanpur river	Kanpur	Discriminate	5.2mm	PF4/PF10
3	PFPN	Naini lake	Nainital	Discriminate	7.4mm	PF4/PF10
4	PFPC	Chaudhari Talab	Bareilly	Indiscriminate	<3mm	PF4/PF10

Table 2 : Eclipse and latent periods of filtered and non filtered phages of *P. fluorescens* isolated from diseased fish

Phages	Filter sample		Non- filter sample	
	Eclipse period(min)	Latent period(min)	Eclipse period(min)	Latent period(min)
PFPD	3.13±0.15	3.43 ±0.12	3.14 ±0.07	3.04 ± 0.07
PFPN	2.99 ±0.01	3.28 ±0.02	3.09 ±0.08	3.11 ±0.05
PFPK	3.02 ± 0.03	3.24 ±0.01	3.14 ±0.04	3.10 ±0.04
PFPC	2.19 ± 0.10	2.71 ±0.04	2.80 ± 0.06	2.73 ± 0.01

Table 3 : Host range sensitivity of four lytic bacteriophages against ten isolates of *P. fluroscense*

I No.	PF Phage	PF1	PF2	PF3	PF4	PF5	PF6	PF7	PF8	PF9	PF 10	Host Range
1	PFPD	+	-	+	+	+	+	+	+	-	+	8
2	PFPK	-	-	+	+	-	+	+	-	-	+	5
3	PFPN	+	-	-	+	+	-	-	+	-	+	5
4	PFPC	-	-	+	+	+	+	-	-	-	+	5

of Rohilkhand region against 10 *P. fluorescens* isolates obtained from diseased fish. The phages developed in the form of indiscriminate micro plaques (Fig. 1A) of <3 mm in the samples of Choudhary Talab, Bareilly (fed with sewage water) and discriminate macro plaques (Fig. 1B) of 5.2 to 7.4 mm in the samples of fish ponds and riverine water within 18-24hr. Phages isolated from Delapeer Talab, Ganga river at Kanpur, Nainital lake and Choudhary Talab were designated as PFPD, PFPK, PFPN and PFPC (Table 1), respectively. The population of indiscriminable and discriminable plaques was enumerated to 10^8 pfu ml^{-1} in sewage water and bottom sediment of fish ponds. It explicates the ubiquitous distribution of *P. fluorescens* phages in fish pond and reverine system. The occurrence of phages against *F. psychrophilum* which causes rainbow trout fry syndrome and cold water disease (Stenholm *et al.,*2008;) (Castillo *et al*. 2014) reported from water and bottom sediments.The host range sensitiveness of four phages (PFPD, PFPK, PFPN and PFPC) determined against ten strains (PF1- PF10) of *P. fluorescens* revealed that the phages of present isolates were effective against all the isolates of *P. fluorescens.* But PFPD and PFPN phages were highly effective against PF4 and PF10 bacterial isolates. Result of phages PFPD, PFPN, PFPK and PFPC amplification accomplished through streak plate revealed the highest inhibitory zones (Fig. 2A and B) which were eluted in sterilized S.M. medium diluents with a yield of 10^{12} - 10^{14} pfu ml^{-1}. Diverge plaque sizes of *P. fluorescens* phages were also recorded (Prasad *et al*., 2011).The virulent phages make the survival of host bacterium extremely difficult, and hence is considered as an important therapeutic agent to mitigate the antibiotic resistance of bacterial pathogens (Prasad *et al*., 2010; 2011; Castillo *et al*., 2014).The capability of phage to increase in number during the infectious process makes phages excellent potential diagnostic and therapeutic agents to combat bacterial pathogens. It advocates that they have a high range of infectivity and bear profound virulent attributes. It strongly supports that lytic phages (PFPD and PFPK) would be a potential candidate to mitigate the population of *P. fluorescens* below threshold level in aquaculture. Recent findings made in *P. fluorescens* (Radhakrishnan and Subramanian, 2012), *F. columnare* (Prasad *et al*., 2011; Laanto *et al*., 2014, 2015), *A. salmonicida* (Imbeault *et al*., 2006), *F. psychrophilum (*Stenholm *et al*., 2008; Castillo *et al*., 2014; Christiansen *et al*., 2016; Muziasari *et al*., 2016) and *P. aeruginosa* (Kumari *et al*., 2009*;* Khairnar *et al*., 2013) phages are in conformity with the present findings. Electron microscopy of two *P. fluorescens* phages (PFPD and PFPK) revealed the presence of a large isometric hexagonal head of 236.42 nm with a long non-contractile tail of 521.22 nm (Fig. 3) and were similar in their morphologies. The head and tail dimensions of these phages closely resembled to Siphoviridae family (Ackerman, 2005) and their persistent infection confirmed that they were DNA virus, however, in the present study molecular evaluation also explicated that they were DNA virus.

Response of four phages (PFPD, PFPK, PFPN and PFPC) to diverse temperature (5^0- 40^0C) and pH (4.5-11.0) explicated that they could proliferate very well at 25^0- 35^0C, but failed to produce plaques below 25^0C and above 40^0C. The mean pfu values were brought down to the minimum at 40^0C and were completely inactivated at 45^0 C. A decrease in viability in terms of pfu count varied depending on the specific phage. All phages were found to be stable at pH 6.5 to 9.0 but were inactivated at pH 10. Highest lytic activity of phages of *P. fluorescens* (Prasad *et al*., 2010), *S. aureus* (Gupta and Prasad, 2011) and *F. columnare* (Prasad *et al*., 2011; Laanto *et al*., 2014, 2015) was reported at 10 to 45, 37.5 and 5 to 40^0C and pH 6.5 to 8.0 and < 6.5 to > 10.5, respectively, on which host bacterium prefers to proliferate.

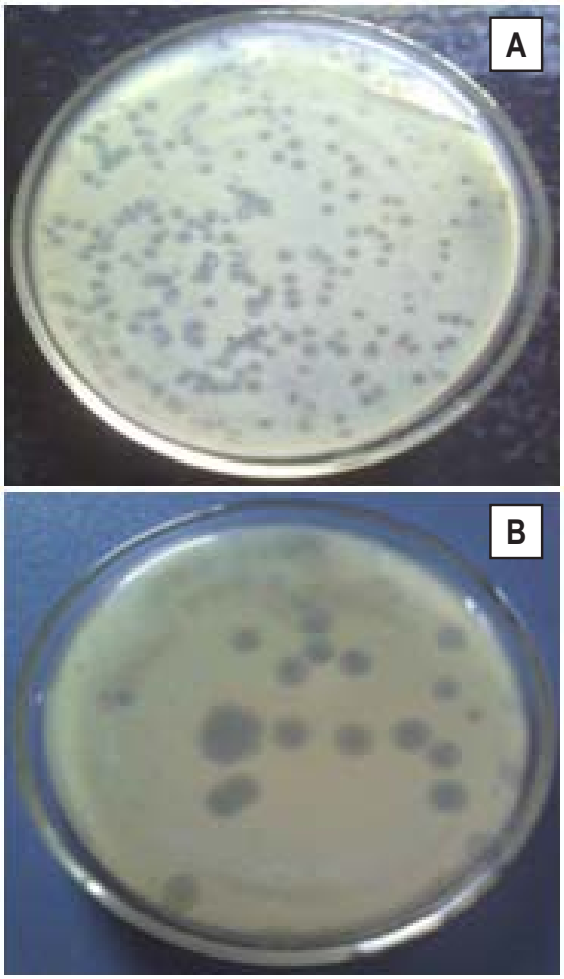


Fig.1 : Well developed Micro <3mm (A) and Macro (B) of 5- 7mm of PFPD phage plaques marked with bald spots in the lawns of *P. fluorescens* (PF10) developed on PIA medium

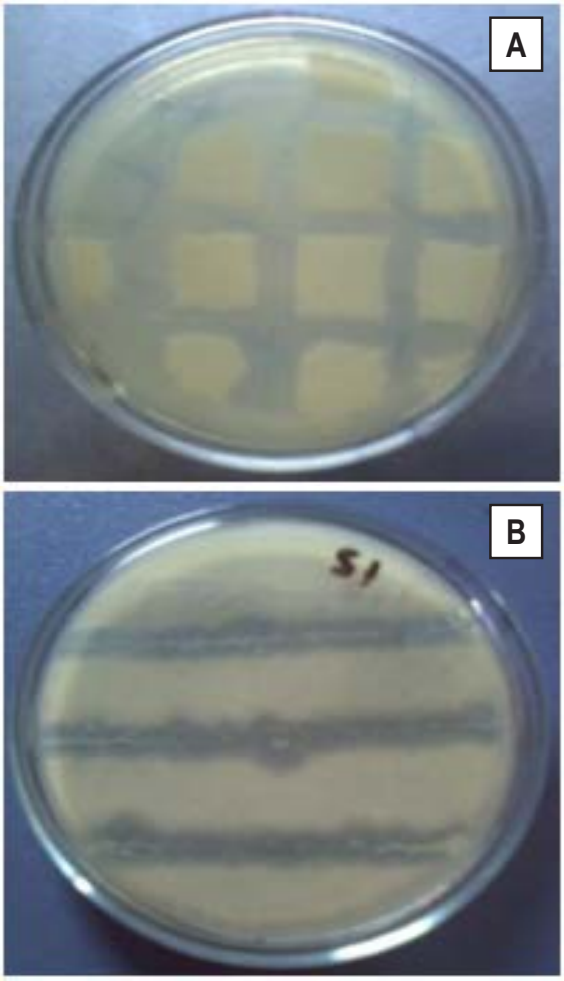


Fig. 2 : Horizontal and vertical eroded zones (A) and horizontal eroded zones (B) as an indication of inhibitory lines of PFPD phage streaked over the surface of *P. fluorescens* (PF10) lawn grown on PIA medium

There were variations in the latency of filtered phages tested as the initial mean log10 pfu value of PFPD, PFPK, PFPN and PFPC was noted to 3.13 ± 0.15, 2.99 ± 0.01, 3.02 ± 0.03 and 2.19 ± 0.10 at 0 min (Table 2). There was nonoccurrence of plaques up to 10 to 15 min and recurrence of phage plaques with slight increase in mean log10 value at 20 min. This value augmented by more than 1 to 2 logs at 40, 50 and 60 min (Fig. 4). On the contrary, in non – filtered phage the initial mean log10 pfu count of PFPD, PFPK, PFPN and PFPC was noted to 3.14 ± 0.07, 3.09 ± 0.08, 3.14 ± 0.04, 2.80 ± 0.06 at 0 min which exhibited nonoccurrence of plaques up to 20 min. There was recurrence of plaques after 20 min with a concomitant elicitation of 1 to 2.5 logs at 30, 40, 50 and 60 min. It revealed that the eclipse period of PFPD phage would be 10 - 15 min because there was loss of plaques during this period. The latent period was 20 min because there was reappearance of plaques more than its initial value. The burst size of PFPD phage was enumerated to 130. The result of one step experiment suggested that PFPD phage had minimum eclipse period and maximum burst size at temperature suitable for host bacterium (25^0- 35^0C) and aquaculture. Variation in adsorption rate, eclipse period (10-20 min) and burst size per bacterial (180 -370) cell has also been recorded in case of *P. aeruginosa* (Kumari *et al.,* 2009) and *F. columnare* phages (Prasad *et al.,* 2010).

Lytic activity of phages is one of the most important factors for their application as therapeutic candidate to combat with the spread of antibiotic resistant bacterial pathogens. Result

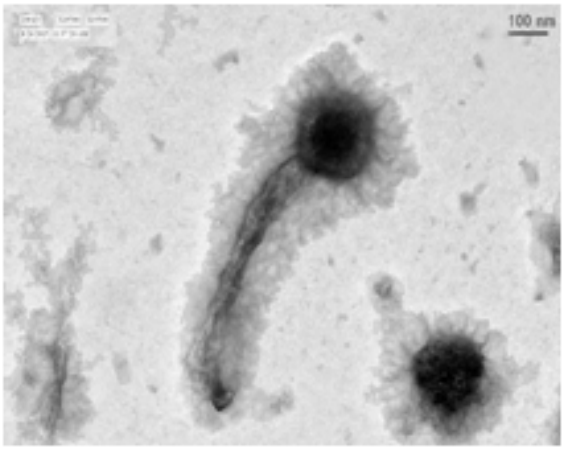


Fig. 3 : *PFPD phage* specific to *P. fluorescens (PF10)* marked with large isometric hexagonal head (*236.42 nm)* with a long non-contractile tail (521.24 nm) *a member of Siphoviridae* (TEM)

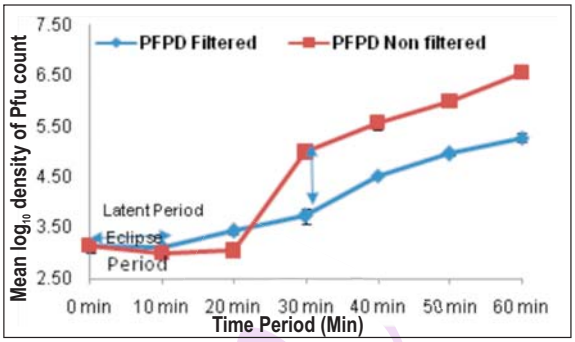


Fig. 4 : Mean log_{10} pfu of PFPD phage grown in broth culture of PF10 bacterium PI with filtered and non-filtered phages

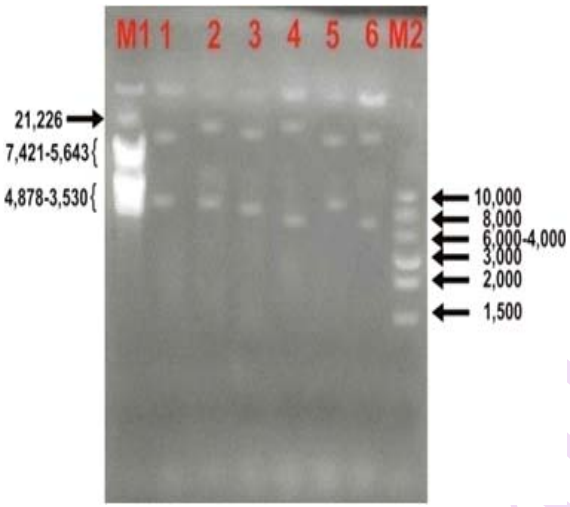


Fig. 5 : Restriction enzyme digestion of DNA isolated from different phage sample (PFPD, PFPN, PFPK).Lane 1 M1, λ EcoRI; Lane 2, PFPD digested with λ EcoRI; Lane 3, PFPN digested with λ EcoRI; Lane 4, PFPK digested with λ EcoRI; Lane 5, PFPD digested with Hind III; Lane 6, PFPN digested with Hind III; Lane 7, PFPK digested with Hind III; Lane 8,M2 1 Kb

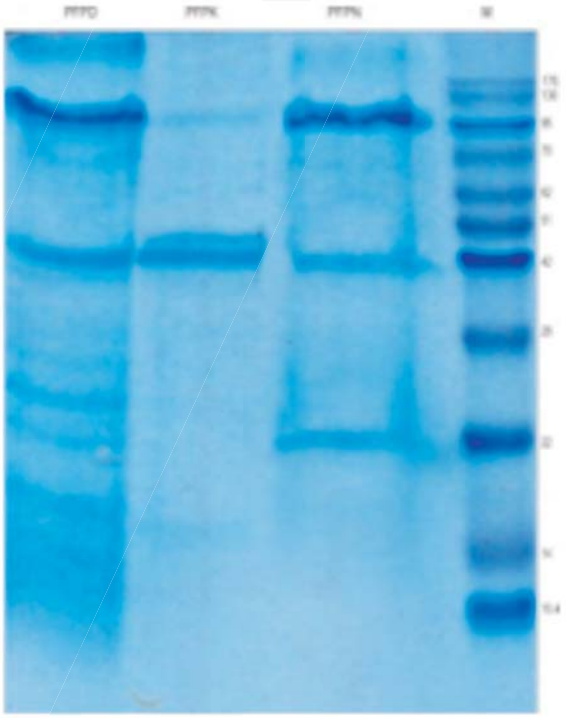


Fig. 6 : Three major structural protein bands of approximately 102, 45 and 42kDa of PFPD, PFPK and PFPN phages of *P. fluorescents* respectively belonging to Siphoviridae assessed through SDS-PAGE (Lane M, protein molecular weight marker (kilobases), lane A, PFPD, lane B, PFPK, lane C, PFPN)

of sensitiveness of different isolates of *P. fluorescens* (PF1 - PF10) to four selected phages (PFPD, PFPK, PFPN and PFPC) revealed that PF 4 and PF10 *P. fluorescens* isolates were lysed by all the four phages used. The PFPD phage lysed 8 of 10 (80%) of host bacterium, hence it was selected for further experimentation while rest of the phages (PFPK, PFPN and PFPC) lysed only 50% of *P. fluorescens* isolates (Table 3). The results of comparative lytic activity of selected phages checked in different water samples (Brain Heart Infusion broth -BHI, distilled water and pond water) explicated a decrease in mean log_{10} cfu value of BHI broth after 6hr of phage inoculation. This value further declined to minimum to its original concentration at 48 hr period. Similar trend was also noticed in distilled water except fishpond water where it was delayed.

The genomic DNA of three purified phages (PFPD, PFPK and PFPN) extracted using Phenol: Chloroform: Isoamyl and digested with EcoRI and Hind III restriction enzymes and subjected to restriction enzyme digestion patterns explicated that all these phages had genome of 20-21kbp size (Fig. 5) with slight variation in their size pattern. The *P. fluorescens* phages were found to exhibit different banding pattern with Hind III and EcoRI restriction enzymes which confirmed that all *Pseudomonas* phages carry ds DNA with slight genomic diversity. However, *P. fluorescens* phages of distinct genomic diversity of 26-82 kbp have been isolated from sewage water (Radhakrishnan and Subramanian, 2012). Variations in DNA sizes have been recorded: 37 kb of *P. aeruginosa* phage MP22 (Goszczynska *et al*., 2000), 58 kb of D3 phage (Kwan *et al*., 2006) and 24 kb of phages of *P. aeruginosa* belonging to family *Podoviridae* (Kumari *et al*., 2009).

Protein analysis of three *P. fluorescens* phages *(*PFPD, PFPK and PFPN) belonging to *Siphoviridae* family showed three major structural protein bands of approximately 102, 45 and 42kDa and 1 minor structural protein band of molecular weight approximately 22kDa (Fig. 6). There were variations in their structural protein distribution. The proteins of molecular weight of 102 kDa and 22kDa were present in two phages (PFPD and PFPN), while protein of molecular weight of 42 kDa was present in all three phages. It suggested that there were variations in molecular weight of major proteins which varied among phage lysate specific to same bacterial strain. It has conformity to the proteins of phages specific to *P. aeruginosa* (Kumari *et al*., 2009) and *P. fluorescens* isolated from sewage water (Radhakrishnan and Subramanian, 2012).

In conclusion, the result of the present study provides evidence of profound lytic impact of *P. fluorescens* phages (PFPD, PFPN, PFPK and PFPC) and represents their interesting therapeutic importance to combat with the problems caused by this pathogen in fish. It also signifies that PFPD and PFPK phages may be highly suitable to alleviate transmission of even systematic PF infection in hatcheries and culture systems.

Acknowledgments

The authors are thankful to DBT, Government of India, New Delhi for financial assistance. Thanks are also due to Prof. T. A. Qureshi for his constructive criticism and valuable suggestions rendered during the preparation of this manuscript.

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