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Lytic bacteriophages specific to *Flavobacterium columnare* rescue catfish, *Clarias batrachus* (Linn.) from columnaris disease

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Abstract

This investigation was aimed to find out appropriate strategy against antibiotic resistant bacterial fish pathogen, *F. columnare*. This pathogen was found persistently associated with fishes causing columnaris disease and ensuing mass mortality in hatchery and culture system of Sub - Himalayan region. Nine lytic *F. columnare* phages (FCP1 – FCP9) specific to its fifteen isolates were isolated from the water and bottom sediments of various geo-climatic regions of North India. The *F. columnare* phage FCP1 (made of hexagonal head and non contractile long tail belonging to family Podovariedae, a member of DNA virus) exhibited broader host range to lyse 9 out of 15 isolates of *F. columnare*. Therapeutic ability of FCP1 phage was assessed in *C. batrachus* inoculated intramuscularly (im) with virulent bacterial isolate FC8 and post inoculated (PI) with FCP1phage (@ 10^8 : 10^6 :: cfu : pfu) through intramuscular (im), immersion (bath) and oral (phage impregnated feed) treatment. Significant ($p<0.001$) reduction (less than 10^{-3} cfu ml^{-1}) in host bacterium in the sera, gill, liver and kidney of challenged fishes was noted after 6 hr of phage treatment. Quantum of phage played a significant role in bringing down bacterial population as in the sera of dose 1 (@ 4.55 X 10^6 pfu ml^{-1}) and dose 2 (@ 9.15 X 10^6 pfu ml^{-1}) treated fishes mean $\log_{10}$ cfu value reduced by 3 logs (58.39%) and 5 logs (73.77%) at 96 hr, respectively. Phage treatment led to disappearance of gross symptoms, negative bacteriological test, detectable phage and 100% survival in experimentally infected *C. batrachus*. Result of this study provides evidence of profound lytic impact of FCP1 phage and represents its interesting therapeutic importance against antibiotic resistant *F. columnare*.

Key words

Bacteriophage, *C. batrachus*, Columnaris disease, *Flavobacterium columnare*

Introduction

Flavobacterium columnare (FC) has emerged as serious problem by being persistently associated with fish hatchery and culture systems of Himalayan and Sub - Himalayan regions of India. This bacterium has caused significant loss to aquaculture industry in terms of mass mortality of fry/fingerlings (Verma *et al*., 2007 and Prasad, 2009). In freshwater aquaculture, *F. columnare* has been recognized as an aetiological agent of columnaris disease of culturable and wild species of fishes (Figueiredo *et al.,* 2005; Kubilay *et al.,* 2008). Prevalence of *F. columnare* has been noted in the liver, gill and serum of infected fishes (Welker *et al.,* 2005 and Verma and Prasad, 2008) indicating it's invasive nature. The conventional control methods for this bacterial pathogen are mainly based on the use of chemicals or antibiotics which have proved to be toxic to fish. The use of antibiotics in hatchery, fish feed and sometimes in culture system is very common, however, this bacterium has found to be resistant (*in vitro*) to commonly used antibiotic (Prasad *et al.,* 2005). In view of the increasing trend of multiple drug resistance (MDR) development in fish pathogenic bacteria, an alternative control measures have become highly imperative. In

this contest application of highly virulent bacteriophages specific to bacterial fish pathogens are being attempted against good numbers of bacterial pathogens (Nakai and Park, 2003; Stenholm *et al*., 2008 and Prasad, 2007).

In progress of development of phage therapy, nine lytic isolates (FCP1-FCP9) of bacteriophages have been isolated against 15 isolates of *F. columnare* from water and bottom sediments of fish farm, reservoirs and river system. Of these FCP1 phage exhibited good promise owing to its *in vitro* (in broth culture) broad lytic spectrum and proliferation at 25 ± 2°C and 7.0 to 10.0 pH (Prasad, 2008), the optimal condition of aquaculture. In India, though substantial work has been carried out on the use of bacteriophage in marine aquaculture (Vinod *et al*., 2006; Karunasagar *et al.,* 2007 and Shivu *et al.,* 2007) but application of phage therapy in freshwater aquaculture is in infancy stage. Not much is known about the efficacy of *F. columnare* bacteriophages in our country. Keeping this in mind, present investigation was planned to assess the efficacy of phage against antibiotic resistant pathogenic isolate of *F. columnare* in catfish experimentally infected with FC8 bacterium and PI with it's respective polyvalent phage (FCP1) to develop phage therapy in aquaculture.

Materials and Methods

15 isolates of *F. columnare* (FC1-FC15) isolated from naturally infected fishes of Sub-Himalayan region were grown in *Flavobacterium* isolation agar (FIA) at 25 (± 2)°C and compared with ATCC 51823. Following LD_{50} (Reed and Muench, 1938) in *C. batrachus* , highly virulent one FC8 was amplified in FIB (broth) as per Malik *et al.* (1996). Bacterial population was determined by colony forming unit (cfu) through spread plate and desired concentrations (10^{6-8} cfu ml^{-1}) of FC8 were prepared by serial dilution.

Nine lytic phages specific to fifteen isolates of *F. columnare* were isolated from the water bodies (Himalayan region - Lakes of Nainital; Sub Himalayan region- water and bottom sediment of Sharda and Nanaksagar reservoirs, UK and fish ponds of Rohilkhand region, Bareilly) grown and their efficacy was evaluated at 25 (± 2) °C as per Sambrook and Russell (2001). Amplification of polyvalent phage (FCP1) was accomplished (Park *et al.,* 2000), harvested, centrifuged (0.22 µm) and preserved at 4°C. Accuracy of pfu dosage was determined by *in vitro* (in broth culture) challenging FC8 with FCP1 phage (pfu ::cfu) @ 1::10^1; 10::10^2; 1::10^3; 1::10^4 and maximum reduction in mean cfu count obtained was determined as multiplicity of infection (MOI) of phage.

Experimental design: Three hundred and fifty healthy Indian catfish, *C. batrachus* measuring about 20-25 cm in length and weighing about 25 - 30 ± 5gm selected for this study were collected from different water bodies of Bareilly. Fish were firstly immersed in 0.01% $KMnO_4$ solution for 5 min to remove parasitic infection, if any. They were kept @ 25 fish/tank in plastic pools (1 X 1m) filled with 300 lit. of non-chlorinated freshwater and acclimatized for 10 d. They were fed twice morning and evening (@ 5% of body weight of fish) with laboratory prepared pelleted feed containing 35% protein as per Pearson's square method. During acclimatization 25% (75 lit.) water of plastic pools was exchanged daily to maintain physico-chemical parameters (pH and dissolved oxygen).

100 fish were divided in four groups (G1 to G4) with triplicate comprising 10 (n = 10) *C. batrachus* in each replicate. They were transferred to glass aquaria (12 X 12 X 36") filled with non chlorinated tap water (70 lit.) in which temperature was maintained at 20 to 25°C by using thermostat (Sobo, China). Each fish of G2, G3 and G4 was challenged intramuscularly with 0.5 ml of FC8 (@ 10^8 cfu ml^{-1}) and fish of G1 group received 0.5 ml of sterilized PBS only and served as control. After 24 hr of bacterial inoculation, fish of G3 and G4 groups were post inoculated (PI) separately with single intra muscular (im) injection of FCP1 phage with dose1 (4.55 X 10^6 pfu ml^{-1}) and dose 2 (9.15 X 10^6 pfu ml^{-1}), respectively. G2 group remained as infected untreated positive control. Water of each aquarium was also fed with 10 ml FIB and experiment was conducted for 96 hr. Samples of sera, gill, liver and kidney all groups (G1, G2, G3 and G4) were obtained by sacrificing the fishes at 0, 6, 12, 24, 48 and 96 hr after post inoculation (PI) of phage for the determination of colony forming unit (cfu) and plaque forming unit (pfu) in them by spread and overlay plate method (Sambrook and Russell, 2001).

Similarly in a separate trial for immersion treatment, 100 fish were divided in four groups (G1 to G4) with triplicate comprising 10 (n = 10) *C. batrachus* in each replicate and kept in glass aquaria (12 X 12 X 36") filled with non chlorinated tap water (70 lit.) maintaining a temperature (using thermostat, Sobo, China) of 20 to 25°C. Following 24 hr of bacterial inoculation, fish of G3 and G4 groups were subjected to bath treatment by immersing them in a suspension of 0.2 ml l^{-1} (15 ml 70 l^{-1} $aquarium^{-1}$) of FCP1 phage comprising 4.55 X 10^6 pfu ml^{-1} (dose 1) and 9.15 X 10^6 pfu ml^{-1} (dose 2) and kept for 96 hr. Water of each aquarium was also fed with 10 ml FIB. Samples for cfu and pfu count were obtained at 0, 6, 12, 24, 48 and 96 hr after post inoculation (PI) as depicted above.

Further in another trial for oral treatment, 100 fish were divided in four groups (G1 to G4) with triplicate comprising 10 (n = 10) *C. batrachus* in each replicate and kept in glass aquaria (12 X 12 X 36") filled with tap water (70 lit.) maintaining a temperature (using thermostat, Sobo, China) of 20 to 25°C. Following 24 hr of bacterial inoculation, fish of G3 and G4 groups were fed twice a day (@ 5% total body weight per day) with laboratory prepared phage impregnated feeds by taking a basal component (as per Pearson's square method) and bacteriophage suspension of FCP1phage @ 4.55 X 10^6 pfu $ml^{-1}g^{-1}$ (dose1) and 9.15 X 10^6 pfu ml^{-1} g^{-1} of feed (dose2). Water of each aquarium was also fed with 10 ml FIB. Samples for cfu and pfu count were obtained at 0, 6, 12, 24, 48 and 96 hr after post inoculation (PI) as depicted above.

Statistical analysis: Significant differences in mean log_{10} cfu and pfu values of different organs of challenged fish generated as a result of intramuscular (im), immersion and feeding methods of phage

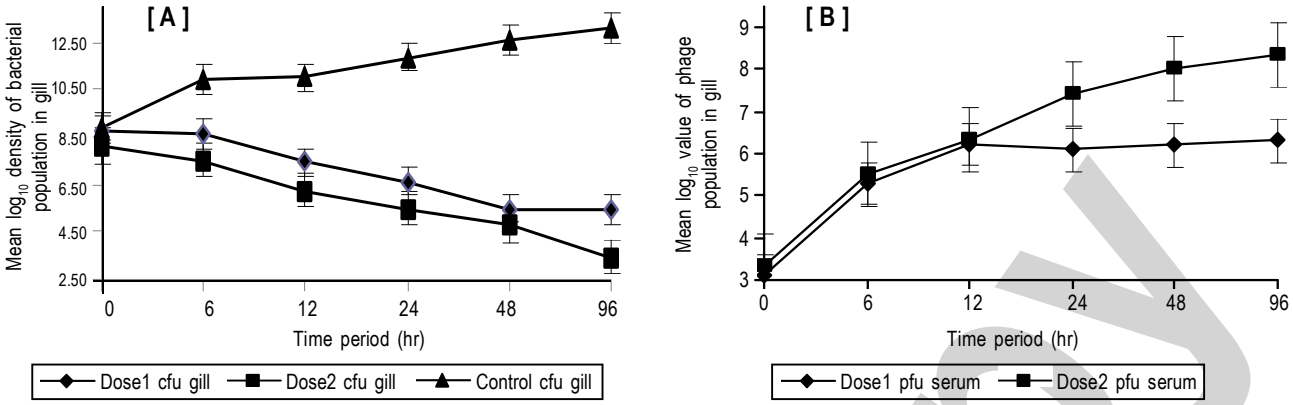

Fig. 1: Mean $\log_{10}$ density (cfu) of *F. columnare* (A) and mean pfu $\log_{10}$ density of phage (B) in the serum of *C. batrachus* experimentally inoculated (im) with FC8 bacterium and challenged (im) with phage FCP1

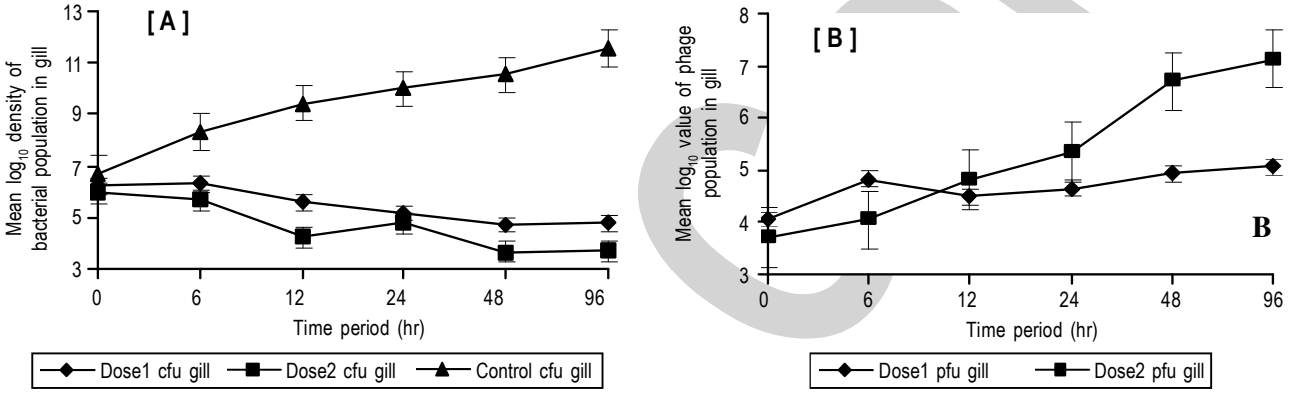

Fig. 2: Mean $\log_{10}$ density (cfu) of *F. columnare* (A) and mean pfu $\log_{10}$ density of phage (B) in the gill of *C. batrachus* experimentally inoculated (im) with FC8 bacterium and challenged (im) with FCP1 (phage)

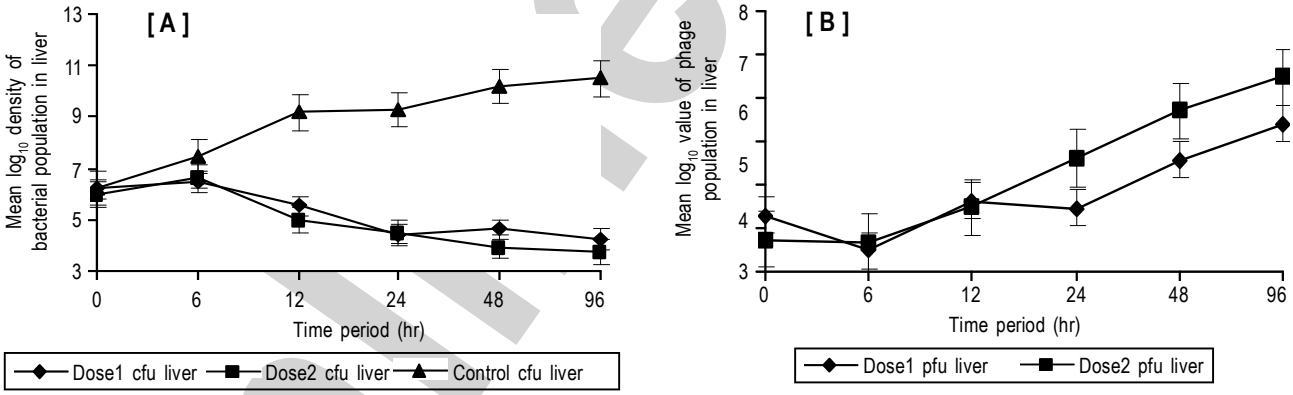

Fig. 3: Mean $\log_{10}$ density (cfu) of *F. columnare* (A) and mean pfu $\log_{10}$ density of phage (B) in the liver of *C. batrachus* experimentally inoculated (im) with FC8 bacterium and challenged (im) with FCP1 phage

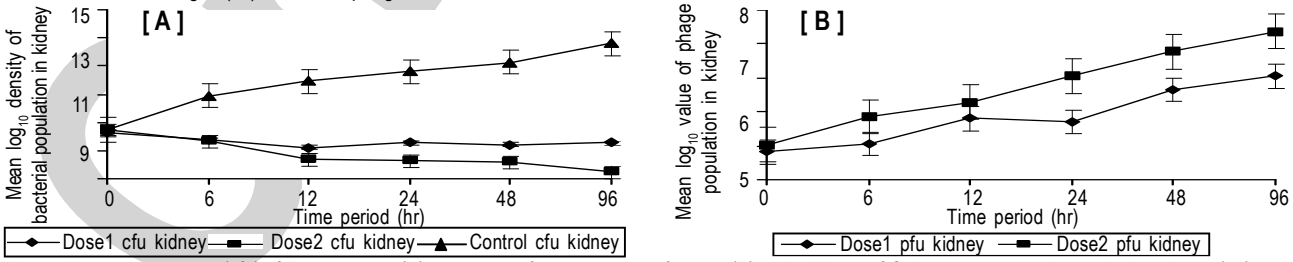

Fig. 4: Mean $\log_{10}$ density (cfu) of *F. columnare* (A) and mean pfu $\log_{10}$ density of phage (B) in the kidney of *C. batrachus* experimentally inoculated (im) with FC8 bacterium and challenged (im) with FCP1 phage

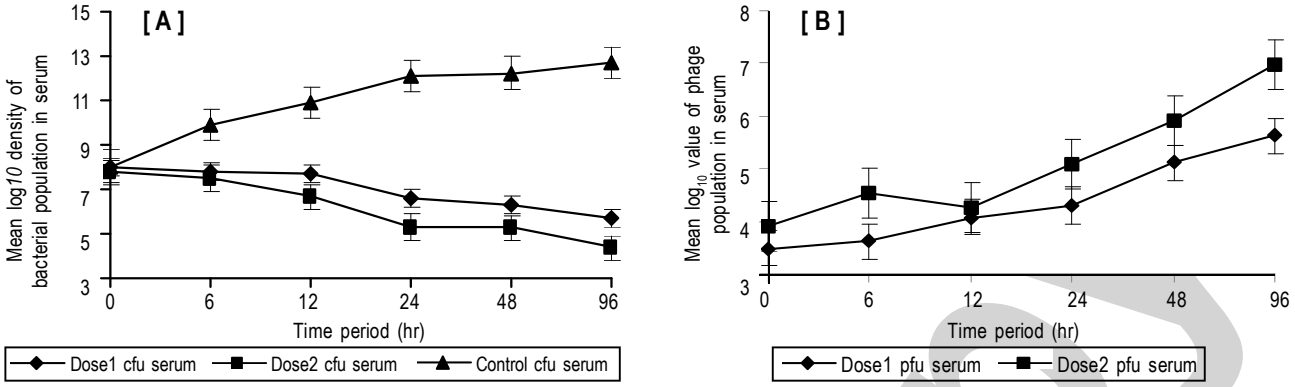

Fig. 5: Mean $\log_{10}$ density (cfu) of *F. columnare* (A) and mean pfu $\log_{10}$ density of phage (B) in the serum of *C. batrachus* experimentally inoculated (im) with FC8 bacterium and challenged (immersion) with FCP1 phage

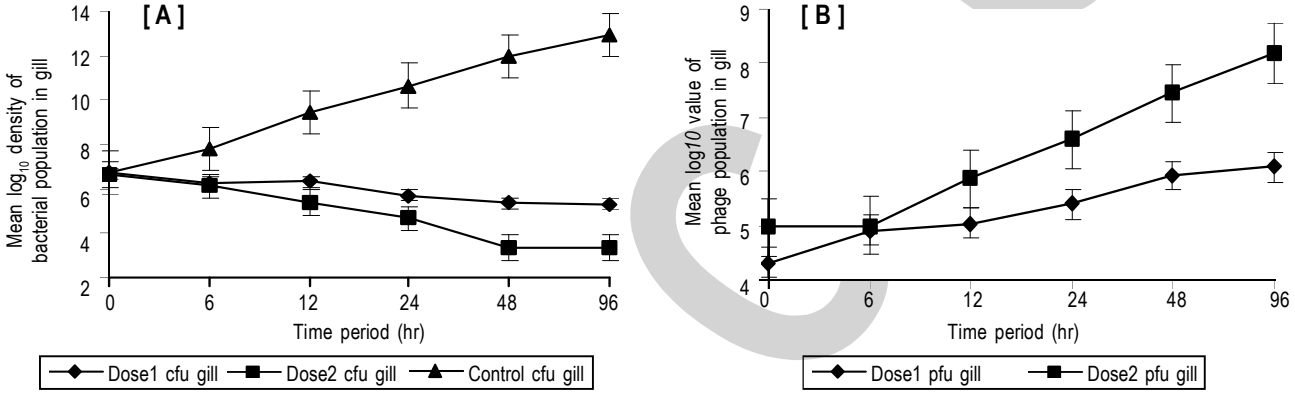

Fig. 6: Mean $\log_{10}$ density (cfu) of *F. columnare* (A) and mean pfu $\log_{10}$ density of phage (B) in the gill of *C. batrachus* experimentally inoculated (im) with FC8 bacterium and challenged (immersion) with FCP1 phage

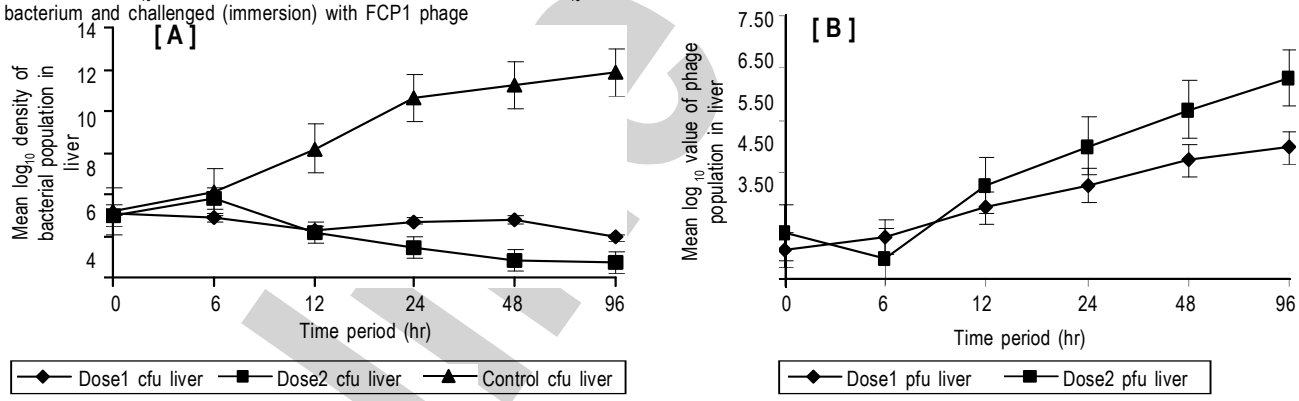

Fig. 7: Mean $\log_{10}$ density (cfu) of *F. columnare* (A) and mean pfu $\log_{10}$ density of phage (B) in the liver of *C. batrachus* experimentally inoculated (im) with FC8 bacterium and challenged (immersion) with FCP1 phage

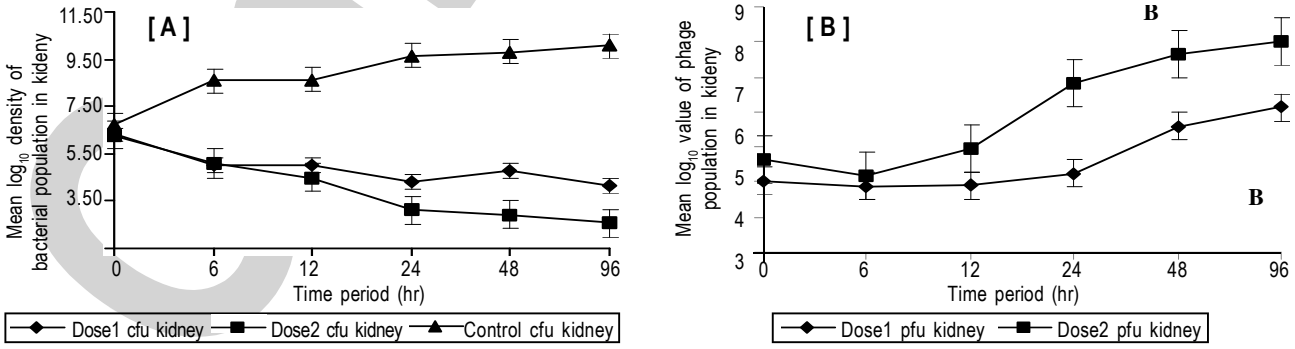

Fig. 8: Mean $\log_{10}$ density (cfu) of *F. columnare* (A) and mean pfu $\log_{10}$ density of phage (B) in the kidney of *C. batrachus* experimentally inoculated (im) with FC8 bacterium and challenged (immersion) with FCP1 phage

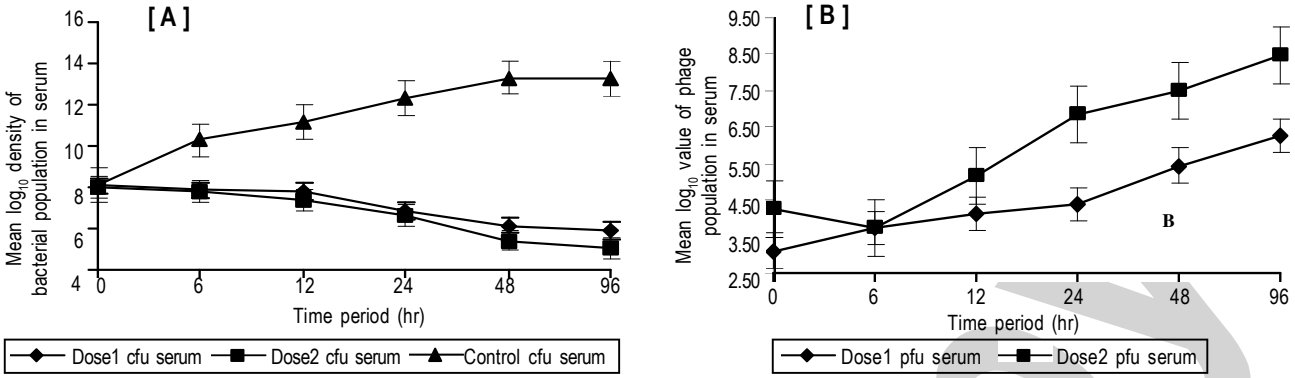

Fig. 9: Mean $\log_{10}$ density (cfu) of *F. columnare* (A) and mean pfu $\log_{10}$ density of phage (B) in the serum of *C. batrachus* experimentally inoculated (im) with FC8 bacterium and challenged (orally) with FCP1 phage impregnated feed

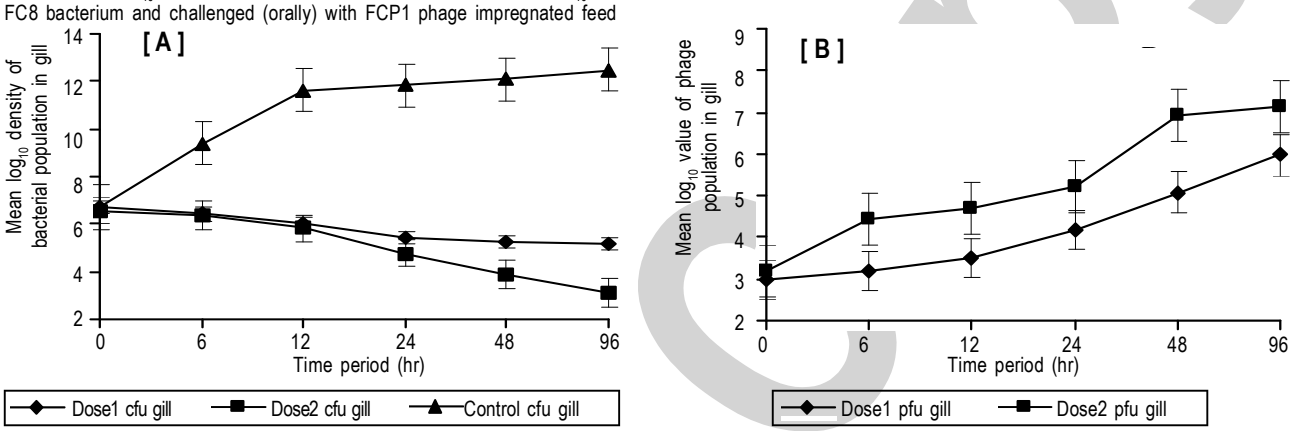

Fig. 10: Mean $\log_{10}$ density (cfu) of *F. columnare* (A) and mean pfu $\log_{10}$ density of phage (B) in the gill of *C. batrachus* experimentally inoculated (im) with FC8 bacterium and challenged (orally) with FCP1 phage impregnated feed

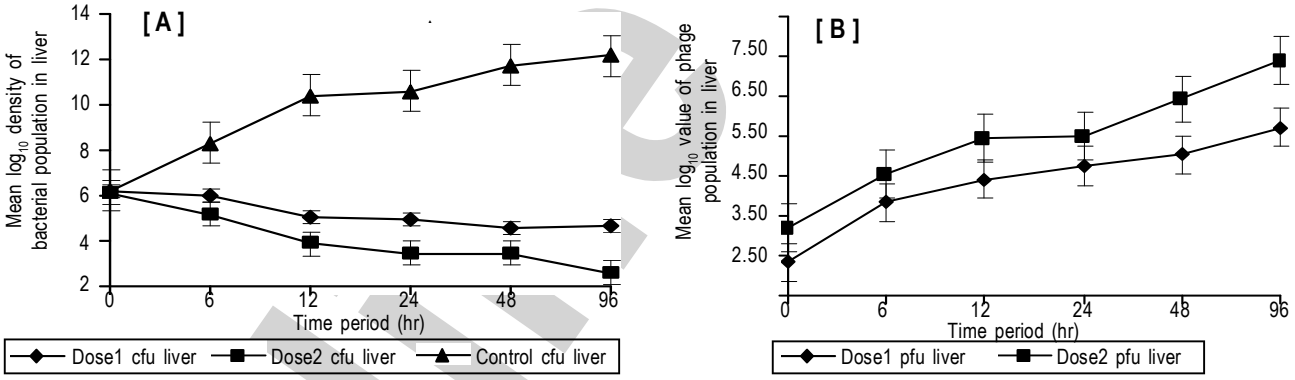

Fig. 11: Mean $\log_{10}$ density (cfu) of *F. columnare* (A) and mean pfu $\log_{10}$ density of phage (B) in the liver of *C. batrachus* experimentally inoculated (im) with FC8 bacterium and challenged (orally) with FCP1 phage impregnated feed

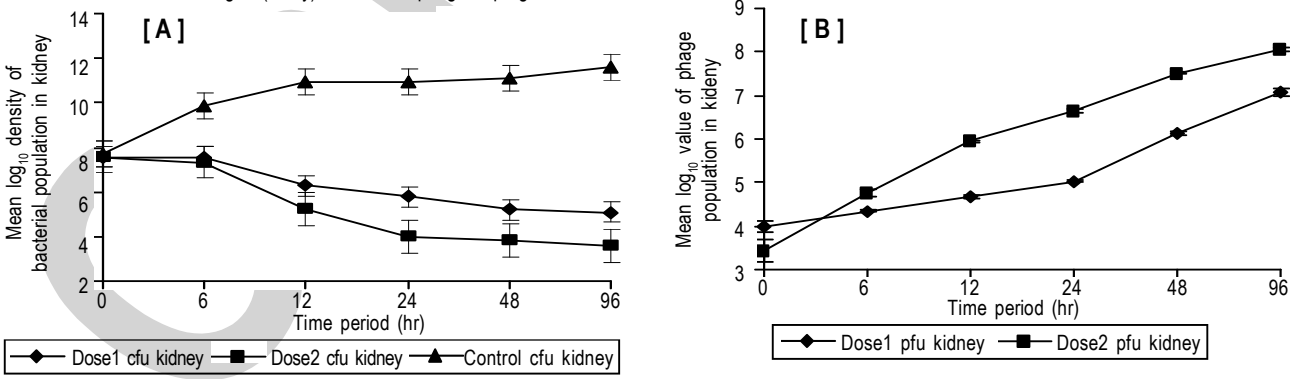

Fig. 12: Mean $\log_{10}$ density (cfu) of *F. columnare* (A) and mean pfu $\log_{10}$ density of phage (B) in the kidney of *C. batrachus* experimentally inoculated (im) with FC8 bacterium and challenged (orally) with FCP1 phage impregnated feed

persistently associated with columnaris disease in various fish of Sub-Himalayan region (Prasad, 2009) and conferred (*in vitro*) resistance introduction into fish were expressed in terms of $\log_{10}$ mean ± SE and levels of significance were performed using SPSS (2001) version 11.

Results and Discussion

***In vivo* sensitivity:** The *C. batrachus* inoculated intramuscularly (im) with FC8 bacterium @~10^8 cfu ml^{-1} $fish^{-1}$ (positive control) exhibited tediousness at 24 hr of incubation onwards and showed the development of grayish to white or yellow lesions usually surrounded by reddish hyperemic zone on the general body surface especially at caudal region and ventral side of the fish and led to 60% mortality. Small to large ulcers were present on the general body surface more at the site of injection in experimentally infected fish. Foregoing result explicates the development of explicit symptoms of columnaris disease in experimentally infected *C. batrachus* and suggests the virulent nature of FC8 bacterial isolate. This bacterium has been found persistently associated with columnaris disease in various fish of Sub-Himalayan region (Prasad, 2009) and conferred (*in vitro*) resistance to most of the commonly used antibiotics (Prasad *et al*., 2005). The *C. batrachus* experimentally infected with FC8 and post challenged with two dosages (dose 1 and dose 2) of FCP1 phage after 24 hr of bacterial inoculation, showed normal behaviour after 6-12 hr of PI and mortality did not occur up to 96 hr in both the treated groups. Similarly fish of negative control which received PBS only displayed normal appearance in terms of overall performance without any mortality.

Prevalence of *F. columnare* (FC8) and FCP1phage in intramuscularly treated fish: The multiplicity of infection (MOI) of FCP1 phage verses FC8 bacterium was calculated to 0.01 (*i.e.* 10^6 : 10^8:: pfu : cfu). The lytic relationship of FCP1 phage and FC8 bacterium assessed in terms of colony forming unit (cfu) and plaque forming unit (pfu) count in the samples of serum, gill, liver and kidney of *C. batrachus* intramuscularly (im) infected with FC8 bacterium and PI (after 24 hr of bacterial inoculation) with two doses (@ 4.55 X 10^6 pfu ml^{-1} and @ 9.15 X 10^6 pfu ml^{-1}) of FCP1 phage through im, immersion and oral (phage impregnated feed) advocated that there was significant ($p<0.001$) reduction in the mean $\log_{10}$ cfu count in phage treated fishes in comparison to positive control (pc).

In the sera of positive controlled *C. batrachus*, the mean $\log_{10}$ cfu count of FC8 increased by 4 logs at 96 hr. Conversely, there was reduction in this value by 3 and 5 logs at 96 hr in dose 1 and 2 treated groups (Fig. 1A). The experimentally infected and phage treated *C. batrachus* did not exhibit any symptoms of columnaris disease. There was spontaneous elicitation in mean $\log_{10}$ pfu values in dose 1 and dose 2 treated fish and accounted to 4 and 5 logs, respectively at 96 hr (Fig. 1B). In gill, the mean $\log_{10}$ value of cfu count increased by 5 logs at 96 hr with an augmentation of 24.61-73.67% at 6 to 96 hr in group 3. This value brought down by 2 logs in dose 2 treated fish (Fig. 2A). The mean $\log_{10}$ pfu value increased by 1 and 3 logs in dose 1 and 2 treated fish at 6 to 96 hr (Fig. 2B). In liver, the mean $\log_{10}$ cfu reduced by 2 and ~ 3 logs in dose1 and 2 treated ones with a loss of 59.50 and 64.35% at 96 hr, respectively (Fig. 3A). There was concurrent elicitation in mean $\log_{10}$ pfu count by 2 and 4 logs at 96 hr in dose 1 and 2 treated fish (Fig. 3B). In kidney, maximum declination of 1 and 3 logs was noted at 96 hr in dose 1 and 2 treated groups and percentage wise declination was 37.89-94.89% at 6 and 96 hr, respectively (Fig. 4A). The mean $\log_{10}$ pfu increased by 2 and ~ 4 logs in dose 1 and 2 treated fish at 96 hr (Fig. 4B).

Enhancement in mean $\log_{10}$ cfu count of *F. columnare* (FC8) in the sera of intramuscularly (im) challenged positive control group (G2) fish after 96 hr elucidated that this bacterium proliferated readily in experimentally infected fish in the absence of infectious agent (virus) throughout the experiment. Conversely, concomitant reduction (3 -5 logs) in dose 1 and dose 2 treated (PI with phage) groups of fish at 96 hr advocated that FCP1phage gets amplified exponentially and confers rapid lysis of targeted bacterium therefore the cfu value got reduced significantly. Comprehensive account of doses of FCP1phage envisages that higher quantum of phage titer alleviates bacterial population more rapidly than lower dose, otherwise severe infection with high mortality would have been occurred in challenged fish. The substantial accretion in mean $\log_{10}$ pfu count of FCP1 in sera in dose 1 and 2 treated groups connotes the profound lytic nature of this phage. Higgins *et al*. (2005) have reported sharp declination in *Salmonella* challenged with respective phage and suggested that using 10^8 pfu ml^{-1} of bacteriophage resulted in significant less effectiveness than higher titers. Sillankorva *et al.* (2008) reported that lytic phages have an increased potential for lysing a variety of bacterial strains. Protective potential of bacteriophages has been evaluated in controlling diseases caused by *P. plecoglossicida* in ayu, *Plecoglossus altivelis* (Park *et al*., 2000) and *V. harveyii* in shrimp, *Penaeus monodon* (Shivu *et al.,* 2007) and found to be efficacious.

Bath treatment (immersion): In bath treatment, there was significance ($p<0.001$) elicitation of ~ 4 - 5 logs in mean $\log_{10}$ cfu count in the sera of positive control fish at 96 hr. Conversely, declination of 3 and 4 logs was noted in dose 1and 2 treated fish at 96 hr (Fig. 5A). There was concomitant enhancement in mean $\log_{10}$ density of pfu count in sera of phage treated fish which accounted to 2 and 3 logs in dose 1 and 2 treated fish, respectively (Fig. 5B). The concurrent reduction of about 2 and 3 logs in the gill of challenged fishes was recorded in dose 1 and 2 treated groups at 96 hr. Substantial augmentation (about 2 logs) in mean $\log_{10}$ values (pfu) was accounted in dose 1 treated fish with an enhancement of 40.68% at 96 hr period. In dose 2 treated groups, this value (pfu) got enhanced by 3 logs with an augmentation of 64.55% at 96 hr (Figs. 6A,B). In liver, the mean $\log_{10}$ cfu values declined by 1 and 3 logs at 96 hr in dose 1 and 2 treated groups. The concurrent rise in mean $\log_{10}$ density (pfu) in the liver of phage treated fishes was accounted to 2 and 3 logs in dose 1 and 2 treated groups (Fig. 7A,B). In kidney, there was declination in mean $\log_{10}$ values of cfu and accounted to 2 and 4 logs in dose 1 and 2 treated groups at 96

hr with a mark reduction of 58.39 and 74.33%, respectively (Fig. 8A). Initially there was reduction in mean $\log_{10}$ pfu values which accounted to 4.04 ± 0.23 to 3.89 ± 0.07 at 6 and 12 hr, thereafter an elicitation of 5.131 to 51.78% (2 logs) and 6.13 to 71.91% (4 logs) was noted during 12 to 96 hr in the kidney of dose 1 and 2 treated fish, respectively (Fig. 8B).

Reduction in mean $\log_{10}$ cfu count in the different organs of bath (immersion) treated fishes elucidated that in dose 1 and dose 2 treated groups, phage overcome the population of the host bacterium within 48-96 hr. and 24-48 hr, respectively and alleviates it entity below threshold level (less than 10^{3-4} cfu ml^{-1}). They expressed lytic activity similar to that of sera as higher titer of phages lysed (2-3 logs) the targeted bacterium in relatively shorter period of time (10 hr) in comparison to lower quantum and implies that lytic activity of phages is dose dependent in gill too. Wagenaar *et al.* (2005) also reported that cfu and pfu displayed opposing high and low over time, indicative of alternative shifts in amplification of bacteria and phage. Stenholm *et al.* (2008) suggested that declination in the bacterial population of *F. psychrophilum* with its respective phage depends on lytic relationship between the phage and the host bacterium. Skurnik and Strauch (2006) and Skurnik *et al*. (2007) and Hanlon (2007) reported the use of bacteriophages that parasitize and kill the bacteria, as a novel and most effective approach for treating infections caused by multidrug – resistant bacteria.

Oral treatment (through phage impregnated feed): Pattern of elicitation in mean $\log_{10}$ cfu count in positive control fish and its declination in the sera of *C. batrachus* experimentally infected with FC8 and treated with FCP1 phage was similar to that of bath treatment with slight variations. In dose 1and 2 treated group, this value reduced by 2 and 3 logs at 96 hr (Fig. 9A). The mean $\log_{10}$ pfu count in phage treated fish increased by 3 and 4 logs at 96 hr (Fig. 9B), respectively. In gill, this value declined by 2 logs in dose 1 treated group with a concurrent loss of 58.75% at 96 hr. Reduction in mean $\log_{10}$ cfu count of dose 2 treated fish was 3 logs (75.05%) at 96 hr (Fig. 10A). The mean $\log_{10}$ pfu count in the gill of phage treated fish augmented during 6 to 96 hr in both the treated groups (Fig. 10B). The subsequent declination in the mean $\log_{10}$ cfu count in liver of phage treated fish was ~ 2 logs (61.39%) in dose 1 and ~ 4 logs (78.69%) in dose 2 treated groups at 96h (Fig. 11A). The mean $\log_{10}$ pfu count increased up to 96h and accounted to 3 logs (115.64%) in dose 1 and 4 logs (131.75%) in dose 2 treated groups (Fig. 11B). There was reduction of 2 logs (56.07%) in the mean $\log_{10}$ cfu count in the kidney of dose 1 treated fish. In dose 2 treated groups, this value declined by 4 logs (65.47%) at 96 hr (Fig. 12A). The mean $\log_{10}$ pfu count concurrently increased during 6 to 96 hr and accounted to 3 logs in dose 1 and ~ 5 logs in dose 2 treated groups at 96 hr (Fig. 12B).

The significant ($p<0.001$) reduction in mean $\log_{10}$ cfu count at 96 hr in gills of fish treated with dose 1 and dose 2, respectively connotes that lytic FCP1 phage remains virulent even in feed. They expressed high lytic activity similar to that of sera as the higher titer of phages lysed (2-3 logs) host bacterium in relatively shorter period of time (10 hr) in comparison to lower quantum. Substantial declination in mean $\log_{10}$ cfu count in the liver of intramuscularly (im) treated groups at 96 hr explicates that phages have been involved in their own amplification and as a result more numbers of host bacterium got infected and lysed. It caused recovery in the symptoms of columnaris disease of experimentally infected phage treated *C. batrachus*. Involvement of phage in concomitant lysis of the host bacterium has been reported (Holmfeldt *et al.*, 2007) and resulting bacterial abundance in several orders of magnitudes below threshold level. The concurrent reduction (1-4 logs) in mean $\log_{10}$ cfu in the kidney of *C. batrachus* in intramuscularly bath (immersion) and oral (phage impregnated feed) treated groups at 96 hr, respectively envisages that efficacy of bacteriophage has clearly dose dependent. Karunasagar *et al.* (2007) had suggested that treatment with 1 µl (~ 10^8 pfu ml^{-1}) phage was almost in effective in bringing down bacterial (*V. harveyi*) numbers while treatment with 10 µl bacteriophage brought about 1 log reduction in cfu count at 18 hr. Treatment with 100 µl led to about 2 log reduction at 18 hr.

Foregoing results advocate that phage introduction in fish through intramuscularly route have a better lytic impact on host bacterium as evidenced by disappearance of gross clinical symptoms, negative bacteriological tests and detectable phage in comparison to bath and feed methods. It seems that there would be a fast and direct introduction of phage to host bacterium in the blood circulation and their spread throughout the system therefore we could get the phage in serum, gills, liver and kidney of challenged fish. Nakai and Park (2003) and Huff *et al.* (2003 and 2006) have also noted the declination in host bacterium in the different organ in challenged animals treated with its respective phage. In present investigation, phage inoculation was made at 24 hr after bacterial challenge and incubated at 25 (±2)°C, which covers the entire range of rearing water temperature for catfish aquaculture in India.

There was absolute survival in *C. batrachus* experimentally infected with FC8 and treated with two dosages (dose 1 @ 4.55 X 10^8 pfu ml^{-1} and dose 2 @ 9.15 X 10^8 pfu ml^{-1}) of FCP1 phage also supports the above view. Vinod *et al.* (2006) in case of *V. harveyi* phages, have reported the endurance of shrimp larvae infected with pathogenic *V. harveyi* increased by 45-55% and decreased luminescent bacterial counts by 2-3 log units after two days. They suggested that in the hatchery trail, addition of phage increased shrimp survival from 17 to 86% after 17 days. In present study targeted bacterium (FC8) could not be recovered from the challenged fish (dead and alive) but phages were recovered from these fishes at 96 hr. Similarly phages were also not recovered from the control fish which suggests that phages need specific carrier for *in vivo* propagation also.

Results signify that FCP1 phage would be a best prophylactic and therapeutic candidate to prevent transmission of even systematic *F. columnare* infection in hatchery and culture system and hence

application of FCP1 phage would be highly suitable to circumvent the problem of such infection in aquaculture.

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