Potential of Bacteriophage as Therapeutic Agent against Experimentally *Streptococcus agalactiae* Infection in Tilapia (*Oreochromis niloticus*)

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Abstract

A lytic bacteriophage, PUB 01-1 was isolated from tilapia rearing pond water using Streptococcus agalactiae UB 01 as a host bacterium. The genome of the phage was double stranded DNA with a size of about 58 kb. Transmission electron microscopy revealed that the phage had an icosahedral head of 65 nm in diameter and a long non-contractile tail of about 270 nm long and 12 nm wide. It was classified as a member of the family Siphoviridae. All 6 strains of S. agalactiae examined were sensitive to the phage but the rest of the tested bacteria were not.

The median lethal dose (LD_{50}) of S. agalactiae UB 01 for tilapia (Oreochromis niloticus) by intraperitoneal injection was 1.58 x 10^4 CFU/fish. The titer and retention time of the phage in kidney of uninfected tilapia receiving diet supplemented with 10^3 PFU/g of diet (diet 1) were lesser than those of uninfected fish receiving diet supplemented with 10^8 PFU/g of diet (diet 2). The presence of S. agalactiae UB 01 in fish increased the titer and retention time of the phage in fish kidneys. Diets 1 and 2 could reduce mortality of tilapia infected with S. agalactiae UB01 at a LD_{50}. Diet 2 resulted in no mortality while 30% mortality was observed in the group receiving diet 1. These results indicate that phage PUB 01-1 has the potential as a therapeutic agent for controlling fish disease caused by S. agalactiae.

Keywords: Bacteriophage, Phage therapy, *Stretococcus agalactiae*, Tilapia.

Introduction

Streptococcus agalactiae is characterized as a group B *Streptococcus* due to the presence of Lancefield group B specific carbohydrate antigen in its cell wall[6]. This bacterium is a Gram-positive, oxidase negative, catalase negative and CAMP (Christie, Atkin and Munch-Petersen) positive coccus. It has a broad host range and is pathogenic to mammals, reptiles, amphibians and fish[15,23,24,31]. It has been isolated from numerous fish species in natural disease outbreaks and has been shown to be pathogenic in experimental trials using different routes of infections[5,8,30]. The clinical signs of *S. agalactiae* infected fish include loss of appetite, swimmimg abnormalities, unilateral or bilateral exophthalmia, corneal opacity and external hemorrhages. The internal organs of the affected fish show many changes such as spleen enlargement, ascites as well as pale liver discoloration[21]. The *S. agalactiae* infection has been reported to cause significant mortality in cultured freshwater and saltwater fish, leading to high economical loss to fish farmers worldwide. Recently, the pathogen has caused serious damage to tilapia *Oreochromis niloticus* farming in Thailand. The cultured tilapia in earthen ponds and cages has been affected throughout the country[29].

S. agalactiae infection in aquaculture has been mainly controlled by antibiotics. However, adverse effects resulting from the use of antibiotics are evident. It can lead to the development of drug resistance in the fish pathogen[1,2] and the accumulation of drugs in the treated fish[7]. These problems have become a global concern because they are harmful to environment and human health. Therefore, there is a need to develop alternative therapies for *S. agalactiae* infection in aquaculture. One of the promising approaches is bacteriophage therapy.

Baceriophages (or phages) can be used to treat infectious diseases caused by bacteria because they can specifically infect and lyse bacteria. The method using phage for controlling bacterial infections is called bacteriophage therapy or phage therapy. It has been successfully used for controlling bacterial infections in both human and animals[3,12]. Its potential use in aquaculture has also been demonstrated. A number of phages against fish pathogenic bacteria have been identified and shown to be able to reduce mortality rates of the infected fish. These include phages against *Aeromonas hydrophila*[20], *Aeromonas salmonicida*[9], *Flavobacterium psychrophilum*[4], *Lactococcus garvieae*[14], *Pseudomonas plecglossicida*[16] and *Streptococcus agalactiae*[18,19].

The considerable phenotypic and genotypic diversity within populations of both phages and bacteria and the high specificity between phages and their hosts cause the complication in using phages to control certain bacterial pathogens. The previously isolated phages specific to particular bacterial strains may not be active against other bacterial strains. Therefore, the different phages may be required for controlling the bacterial strains having the same species and causing the same diseases.

The purpose of the present study was to isolate and characterize a lytic phage specific to *S. agalactiae*. This work also demonstrated the potential use of fish diets

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supplemented with the phage to control *S. agalactiae* infection in tilapia which is one of the most cultured fish species in Thailand with a high incidence of *S. agalactiae* infection.

Material and Methods

Bacteria, bacteriophage and culture conditions: The fish pathogenic bacteria used in this study and their culturing conditions are presented in table 1. *Streptococcus agalactiae* UB 01, a fish pathogen isolated from kidneys of tilapia suffering from streptococcosis in a fish farm in Ubon Ratchathani province, Thailand was used as a host bacterial strain for phage detection. The other bacterial strains were used as test organisms in the host range study. Bacterial stock cultures were stored as frozen cultures at -80°C in appropriate culturing media supplemented with 15% (v/v) glycerol. Throughout the experiments, strains were subcultured every 2 weeks on appropriate agar media and kept at 4°C. Before use, liquid cultures prepared from a single colony were transferred twice into appropriate liquid media and incubated under the conditions presented in table 1.

Bacteriophage PUB 01-1, a bacteriophage specific to *S. agalactiae* UB 01, was obtained in this study. It was stored at 4°C within 6 months in BHI broth containing 0.1% (v/v) chloroform. For a long term storage, the phage suspension in BHI broth supplemented with 15% (v/v) glycerol was frozen at -80°C.

Detection of phage and host range study: For phage detection, 100 mL of five culture pond water samples were collected from each of the five tilapia farms in Ubon Ratchathani province, Thailand. Each sample was filtered through a 0.45-µm-pore-size membrane filter and enriched by mixing with an equal volume of double-strength BHI broth containing *S. agalactiae* UB 01 at the concentration of 10^6 CFU/ml. After 24 h of incubation at 25°C, the culture was centrifuged at 5,000 xg for 10 min and filtered. The resulting filtrate was subjected to the spot-on-lawn assay to observe lytic activity of phage. Five ml of BHI soft agar (0.7% agar, prewarmed to 60°C) was seeded with 0.1 mL of a host culture containing *S. agalactiae* UB 01 cells at the concentration of 10^6 CFU/mL, mixed thoroughly and poured onto a BHI agar plate.

After solidification, 10 µL of each filtrate was spotted onto the top agar layer. The plate was left at room temperature for 30 min to allow the filtrate to be well absorbed through the agar and then incubated at 25°C for 24 h. A clear zone on the plate, resulting from the lysis of host cells, indicated the presence of phage. The spot-on-lawn assay was also used to examine the lyitc activity of the phage PUB 01-1 against various strains of fish pathogenic bacteria listed in table 1.

Purification of phage and phage titer determination: The filtrate confirmed to contain phage specific to *S. agalactiae* UB 01 by the spot-on-lawn assay was subjected to the agar overlay method to purify the phage. After suitable dilutions of phage containing filtrate, 0.1 mL aliquots were added to 0.1 mL of log phase culture containing *S. agalactiae* UB 01 cells at the concentration of 10^6 CFU/mL. The mixture was incubated for 15 min at 25°C, mixed with 5 mL of soft BHI agar (0.7% agar, prewarmed to 60°C) and poured onto the surface of a BHI agar plate. The plate was incubated at 25°C for 24 h to allow plaques to be formed. A single plaque was picked and added into 5 ml of log phase culture containing *S. agalactiae* UB 01 cells at the concentration of 10^6 CFU/ml. After 24 h of incubation, the culture was centrifuged at 5,000 xg for 10 min and filtered.

Table 1
Bacterial strains used in this study and their sensitivity to phage PUB 01-1

Bacteria	Culturing condition	Lysis[a]
Aeromonas hydrophila ATCC 14174	BHI medium, 25°C	-
Edwardsiella tarda ATCC 23693	BHI medium, 25°C	-
Flavobacterium columnare ATCC 49512	Shieh medium supplemented with tobramycin (1 µg/mL), 25°C	-
Lactococcus garvieae ATCC 43921	BHI medium, 25°C	-
Pseudomonas anguilliseptica ATCC 33660	BHI medium, 25°C	-
Streptococcus iniae ATCC 29178 *Streptococcus shiloi* ATCC 51499 *Streptococcus phocae* ATCC 51937	BHI medium, 25°C BHI medium, 25°C BHI medium, 25°C	- - -
Streptococcus agalactiae Strains ATCC 12386 ATCC 12403 ATCC 51487 ATCC 13813 ATCC 27956 UB 01	BHI medium, 25°C	 + + + + + +

[a] + = clear zone formed, - = no clear zone formed

The filtrate was subjected to the agar overlay method. A randomly selected single plaque at this step followed the same protocol as mentioned above but the resulting filtrate was kept as a phage stock instead of undergoing the agar overlay method. The agar overlay method was also used to determine phage titer. Plaques formed on bacterial lawns were counted and the numbers were used to calculate the phage titer in plaque forming units per ml (PFU/mL).

Electron microscopy of phage: Phage particles were sedimented from 5 ml of the phage stock at 75,000 xg for 2 h using J2-21 centrifuge (Beckman, Palo Alto, CA, USA). The pellet was re-suspended in 50 µL of SM buffer (10 mM $MgSO_4$, 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.01% gelatin). 5-µl aliquote of phage suspension was spotted on top of a Formvar-coated copper grid (Proscitech, Brisbane, Queenland, Australia) and allowed to adsorb for 5 min at room temperature. The phage was stained by addition of 5 µL of 2% sodium phosphotingstate (pH 7.2). After 4 min, excess stain was removed and the grid was allowed to air dry for 10 min. The stained phage was examined in a Hitachi H-300 electron microscope (Hitachi, Tokyo, Japan) at 80 kV. The phage size was determined from the average of five independent measurements.

Analysis of phage nucleic acids: Phage nucleic acid was extracted by using PureLink Viral RNA/DNA Mini Kit (Invitrogen, Carlsbad, CA, USA) according to the protocol provided by the manufacturer. The purified ucleic acid was tested for sensitivity to Ribonuclease A, Nuclease S1 and restriction enzyme *Pst*I (all from Sigma-Aldrich, St. Louis, MO, USA) according to the supplier's recommendations. The results were analyzed by 0.8% agarose gel electrophoresis. The genome size of the phage PUB 01-1 was determined by pulse-field gel electrophoresis (PFGE) of the purified phage nucleic acid. The electrophoresis was carried out with 0.8% agarose gel in 0.5x Tris-borate-EDT buffer at 15°C for 15 h, using switch time ramped from 1 to 12 s and a voltage of 6 V/cm. The PFGE size standard used was low range PFG marker (New England Biolabs, Ipswich, MA, USA).

Preparation of fish: Tilapia were purchased from "Nong Khon" Farm in Ubon Ratchathani, Thailand. They were maintained in 150-L plastic tanks for two weeks to acclimatize to laboratory conditions prior to the experiments. Unless stated otherwise, the fish were held at 25°C with a 12-h light/12-h dark photoperiod and fed at 5% body weight twice a day with C. P. CLASSIC commercial fish diet (S. W. T. Co., Bangkok, Thailand). All experiments were conducted with fish weighing 10 ± 1 g. They were stocked in aquaria 24 h prior to the experiments. The aquaria were cleaned daily by siphoning off two thirds of the water and replacing it with fresh water. Water quality was monitored every 2 d and was maintained within the following ranges: dissolved oxygen concentration at 6.0 ± 0.6 mg/l, pH at 7.0 ± 0.5, total hardness at 34.5 ± 1.8 mg/L as $CaCO_3$, alkalinity at 29.5 ± 2.0 mg/l as $CaCO_3$, ammonia at less than 0.5 mg/L.

Determination of Median lethal dose (LD_{50}): Groups of 10 fish with an average weight of 10 ± 1 g were used for determining LD_{50} of *S. agalactiae* UB 01. Ten-fold serial dilutions of *S. agalactiae* UB 01 culture with initial concentration of 10^8 CFU/mL were prepared. One hundred µL of each dilution was injected intraperitoneally into each fish. For control, the same volume of physiological saline was used instead of the bacterial suspension. Each dilution trial was performed in duplicate.

Mortalities were recorded daily for 2 weeks. Dead fish were removed from the aquaria daily. Their kidneys were aseptically streaked on BHI agar. After incubation at 25ºC for 24 h, the bacterial grown on the agar were confirmed to be *S. agalactiae* by using the API 20 Strep test kit (bioMerieux Industry, Hazelwood, MO, USA). The median lethal dose (LD_{50}) was calculated by the method of Reed and Muench[25].

Preparation of fish diets: Fish diets supplemented with phage PUB 01-1 were prepared. Diets 1 and 2 were commercial fish diets supplemented with the phage at the final concentrations of 10^8 and 10^3 PFU/g of diet respectively. Each fish diet mixture was mixed with distilled deionized water (1 mL/g) until a homogenous mixture was obtained. The mixture was passed through a minced-meat processing machine, producing extruded strings, which were dried at 30°C for 24 h and then broken down to about 2-mm-long pellets. The control fish diet (Diet 3) was prepared using the same process as the other fish diets except adding no phage.

Fate of PUB 01-1 in fish: Two experiments were designed to examine the fate of phage PUB 01-1 in fish, each of which was performed in duplicate. The experiment I was conducted to examine the presence of the phage in kidneys of uninfected fish receiving fish diets supplemented with the phage. In this experiment, three groups of 25 tilapia were fed diets 1, 2 and 3 separately once. These groups were reared for another 4 days with the control diet without the phage. Fish in the groups were sacrificed in order to detect the phage in their kidneys 3 to 96 h after feeding the tested diets. The experiment II was conducted to examine the presence of the phage in kidneys of infected fish receiving diets 1, 2 and 3. In this experiment, three groups of 25 tilapia were infected with *S. agalactiae* UB 01 by intraperitoneal injection of 100 µL of bacterial suspension, at a LD_{50}.

Immediately after bacterial infection, they were fed diets 1, 2 and 3 separately once. These groups were reared for another 4 days with the control diet. Fish in the groups were sacrificed in order to detect the phage in their kidneys 3 to 96 h after inoculation. To determine the concentration of phage particles (in PFU/g) in collected fish kidneys, the organs were weighed and homogenized with 9 volumes of physiological saline. After centrifugation at 7,000 xg for 10 min, the supernatants were subjected to plaque assay as mentioned above.

Therapeutic effect of phage supplemented fish diets: Three groups of 10 tilapia were used to study the therapeutic effect of phage supplemented fish diets against *S. agalactiae* UB 01 infection in fish. They were infected with *S. agalactiae* UB 01 by intraperitoneal injection of 100 µL of bacterial suspension at LD_{50}. Immediately after bacterial infection, the infected fish were fed dites 1, 2 and 3 once, each diet for one group. The fish were reared for another 2 weeks with the control diet without the phage. Mortality of the fish was observed daily. To confirm the cause of death, kidneys of the dead fish were examined for the presence of the pathogenic bacterium.

To isolate the bacterium in collected fish kidneys, the organs were weighed and homogenized with 9 volumes of physiological saline. After centrifugation at 2,500 xg for 3 min, the supernatants were subjected to bacterial isolation on BHI agar. The bacterial colonies grown on the agar were identified using Gram staining, biochemical dentification anf the API 20 Strep test kit (bioMerieux Industry, Hazelwood, MO, USA). This experiment was performed in duplicate.

Results and Discussion

Detection of phage: Five pond water samples were screened for the presence of phage active against *S. agalactiae* UB 01. Only one sample gave the positive plate containing a clear zone of the spot test. After the sample underwent plaque purification, the phage formed small, clear, round plaques of about 1.5 mm in diameter on the *S. agalactiae* UB 01 lawn (Fig. 1). The production of a clear inhibition zone in spot-on-lawn assay and clear plaques in plaque assay by the phage PUB 01-1 indicated that it was a lytic phage. Since the phage was isolated using *S. agalactiae* UB 01 as a host organism, it was designated PUB 01-1.

Phage therapy is among the interesting choices because it has many advantages over antibiotic-based treatment. Due to their high specificity to hosts, phages affect only their bacterial hosts without any harm to fish normal flora, famers, consumers and environmental organisms. Phage residues in fish are uncommon because after host cells are killed, the titer of phages in fish decrease almost immediately and are eventually discarded from animal bodies very rapidly[13].

Furthermore, much evidence in support of the effectiveness of phage therapy against bacterial infectious diseases in human and animals has been accumulated since the 1980s. These reports indicated that appropriate administration of phages could be used to treat lethal diseases caused by both Gram-negative and Gram-positive bacteria[13].

The use of phage to control diseases in fish has recently become the therapeutic approach of interest and the number of reports describing the success of this approach in fish is increasing. In this study, a phage PUB 01-1, specific to *S. aglactiae* UB 01, was isolated from a pond water sample, characterized and tested for its potential to be used as a therapeutic agent to control the fish disease caused by the bacterium.

Host range study: The host range of PUB 01-1 was determined with 14 strains from 9 species of fish pathogenic bacteria. The phage was lytic against all 6 strains of *S. agalactiae* but not against any strain of *Shigella* including *S. iniae, S. shiloi* and *S. phocae*. Of the remaining strains tested, none was sensitive to the phage (Table 1). For phage therapy, phages with a broad host range spectrum within a species are desired because they can be effective against a large number of members of a particular species. The narrow host specificity of phages can cause complexity in preparation of therapeutic phages. In some cases, combination of several phages is required for therapeutic application. This study showed that the phage PUB 36 was lytic against all of the tested *S. agalactiae* isolates with different sources but not any other tested bacteria.

Therefore, this phage has potential application as a therapeutic agent to control *S. agalactiae* infectious disease. Finding bacteriophages with a broad host range is not uncommon.

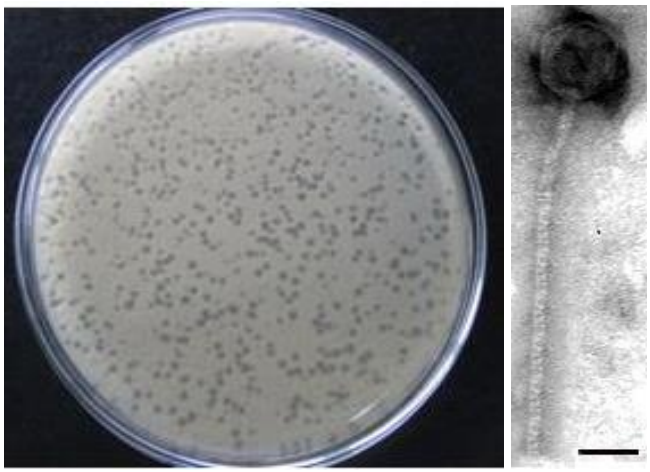

Figure 1: Plaques on *S.agalactiae* UB 01 lawn (a) and transmission electron micrograph (b) of phage PUB 01-1. Bar = 50 nm

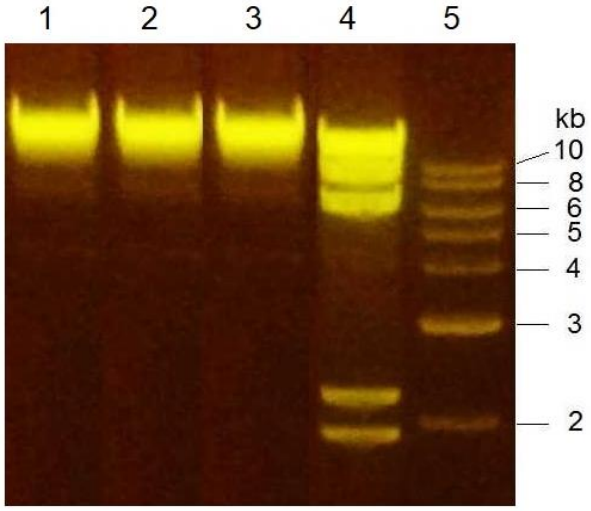

Figure 2: Agarose gel electrophoresis of phage PUB 01-1 nucleic acid. Lane 1 uncut PUB 01-1 nucleic acid; lane 2, PUB 01-1 nucleic acid digested with Ribonuclease A; lane 3, PUB 01-1 nucleic acid digested with Nuclease S1; lane 4, PUB 01-1 nucleic acid digested with *Pst*I and lane 5, 1 kb DNA marker (New England Biolabs)

Vinod et al[32] isolated a *Vibrio harveyi* phage from shrimp farm water. It was found to be virulent against all of the 50 strains of *V. harveyi* tested, isolated from different sources such as seawater, hatchery water, shrimp farm water and sediment.

Electron microscopy of phage: The ultrastructure of the phage was examined by transmission electron microscopy as shown in fig. 1. The phage had an icosahedral head of 65 nm in diameter and a long non-contractile tail of about 270 nm long and 12 nm wide. The virion did not have other additional structures such as a baseplate, a collar or fibers.

Analysis of phage nucleic acids: The nucleic acid isolated from phage PUB 01-1 was examined for its sensitivity to different nucleic acid digesting enzymes. It was found that the nucleic acid could not be digested by Ribonuclease A and Nuclease S1. However, it was sensitive to the restriction enzyme *Pst*I (Fig. 2). The size of the nucleic acid as revealed by the pulsed-filed gel electrophoresis was about 58 kb.

One of the criteria for classification of phages to a particular group is their genetic and morphological characteristics. The genome of phage PUB 01-1 was double stranded DNA because it was resistant to Nuclease S1, an single stranded DNA digesting enzyme and Ribonuclease A, a RNA digesting enzyme, but sensitive to the restriction enzyme *Pst*I, a double stranded DNA digesting enzyme. Transmission electron microscopy revealed that the phage was a tailed phage with an icosahedral head and a long noncontractile tail. Based on these characteristics, phage PUB 01-1 can be classified as a member f the family *Siphoviridae* according to the International Committee on Taxonomy of Viruses[11].

Variations have been observed among tailed phages specific to fish pathogenic bacteria. They have been placed in all families of tailed phages including *Siphoviridae*, *Myoviridae* and *Podoviridae*[10,22]. Besides phage PUB 01-1, several other phages specific to fish pathogens have been classified as *Siphoviridae* phages including *Yersinia ruckeri* phage Yer2AT[28], *Lactococcus garvieae* phage PLgY[17] and *Flavobacterium psychrophilum* phages FpV-5, FpV-7, FpV-9 and FpV-14[27].

Determination of Median lethal dose (LD_{50}): The cumulative mortality of tilapia was observed for 14 days after the fish was intraperitoneally infected with different doses of *S. agalactiae* UB 01 and the results are shown in fig. 3. With the dose of 10^7 CFU/mL, the first death was observed two days after infection while the death caused by other doses began at day 3 of the infection. All deaths occurred within 6 days after bacterial infection, irrespective of injected dose and the pathogenic bacterium was found in the kidneys of all dead fish. Based on the mortality, the calculated LD_{50} of *S. agalactiae* UB 01 for tilapia was $10^{2.8}$, this being equivalent to 1.58×10^4 CFU/fish.

Fate of PUB 01-1 in fish: The fate of PUB 01-1 in both uninfected and infected tilapia receiving diets 1, 2 and 3 was examined and the results are shown in table 2. No phage could be detected in fish fed control diet (diet 3). For both uninfected and infected fish receiving phage supplemented fish diets 1 and 2, the phage was first detected in fish kidneys 3 h after feeding and the titer of phage and the retention time of phage in fish reared with diet 1 were greater than that in fish reared with diet 2. The phage was discarded from kidneys of fish receiving diet 2 very rapidly; no phage could be detected in kidneys of uninfected and infected fish 12 and 24 h after feeding respectively.

The infected fish had higher titer of phage in kidneys and longer retention time of phage in kidneys than the uninfected fish, irrespective of the type of administrated diet. In the case of infected fish, phage PUB 01-1 was found to be able to exist in kidneys of fish receiving diet 1 for 72 h after feeding.

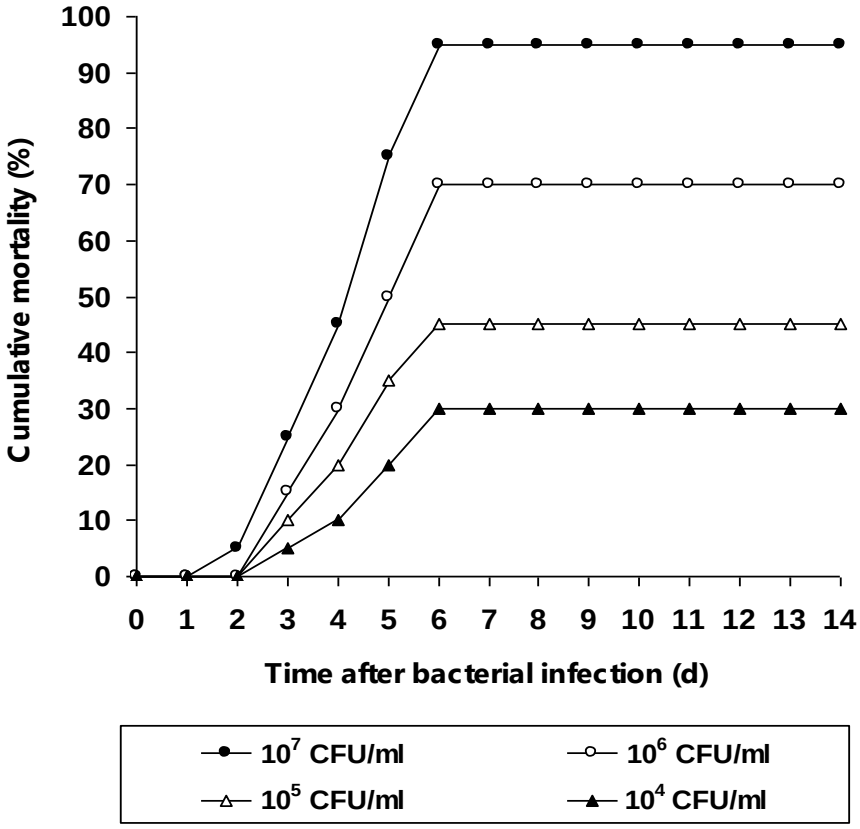

Figure 3: Cumulative mortality of tilapia experimentally infected with *S. agalactiae* UB 01

Table 2
The titers of phage PUB 36 (PFU/g) in kidneys of uninfected and infected tilapia fed diets 1, 2 and 3 at different time after feeding

Time after feeding (h)	Uninfected fish			Infected fish		
	Diet 1	Diet 2	Diet 3	Diet 1	Diet 2	Diet 3
0	ud	Ud	ud[a]	ud	ud	ud[a]
3	2.8×10^2	4.1×10^7	ud	9.6×10^4	5.7×10^{10}	ud
6	7.4×10^2	2.6×10^6	ud	5.4×10^3	3.2×10^9	ud
12	ud	8.3×10^4	ud	1.1×10^2	4.6×10^7	ud
24	ud	5.7×10^2	ud	ud	7.3×10^5	ud
48	ud	Ud	ud	ud	2.5×10^3	ud
72	ud	Ud	ud	ud	4.4×10^2	ud
96	ud	Ud	ud	ud	ud	ud

[a] Undetectable (detection limit = 10 PFU/g)

The presence of phage PUB 01-1 in kidneys of tilapia fed diets 1 and 2 indicated that oral administration was an effective way to transfer the phage into fish bodies. Interestingly, phage that was administrated orally appeared in the kidneys of uninfected fish without host bacterial cells as a transport vehicle, although the time that the phage remained in the organ was relatively short (6 and 12 h after feeding diets 1 and 2 respectively). Movement of phage from the alimentary tract to the blood circulation system was also observed during human phage therapy[26].

However, the presence of *S. agalactiae* UB 01 in fish increased the titer and retention time of the phage in fish kidneys, irrespective of amount of phage administrated. This may be due to the multiplication (using lytic cycle) of the phage in fish bodies. The appearance of the phage in kidneys of both uninfected and infected fish receiving phage supplemented diets suggested that orally administrated phage can be expected to kill bacterial cells in internal organs, which means that phage therapy can be effective at the systemic infection stage.

Therapeutic effect of phage supplemented fish diets: The effect resulting from the oral administration of the phage supplemented fish diets on the mortality of fish experimentally infected with *S. agalactiae* UB 01 is shown in fig. 4.

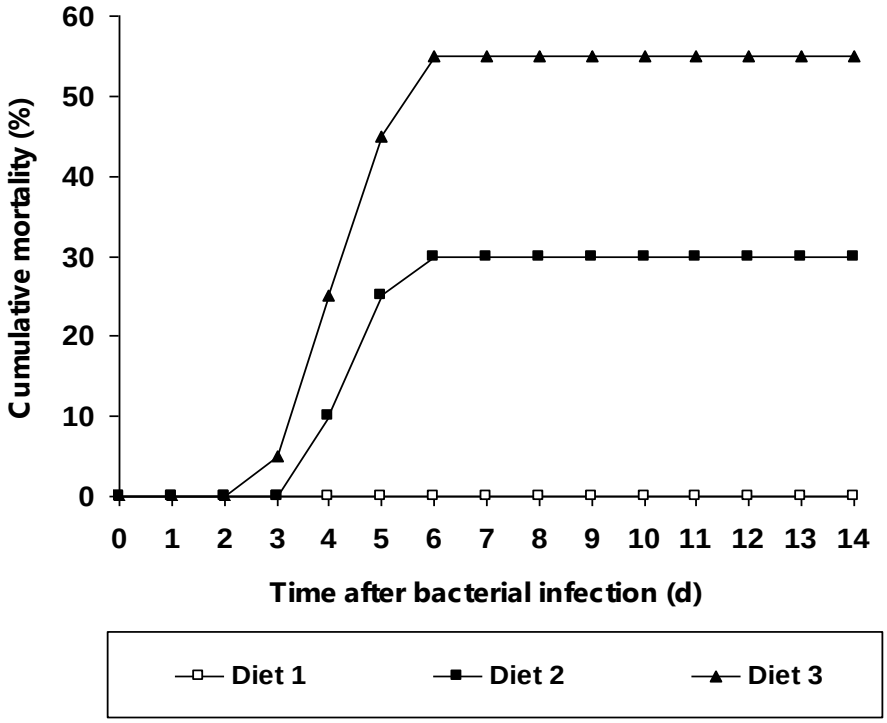

Figure 4: Effect of fish diets supplemented with different concentrations of phage PUB 01-1 on the cumulative mortality of tilapia experimentally infected with *S. agalactiae* UB 01

In the group receiving the control diet with no phage (diet 3), the cumulative mortality of the infected fish was 55%. A dose-dependent reduction in mortality of the infected fish was obtained when the fish were fed the diets containing phage PUB 01-1 once immediately after the bacterial infection. No mortality was apparent in the fish receiving the fish diet supplemented with 10^8 PFU/g of diet (diet 1) while 30% cumulative mortality was observed in the group treated with the fish diet supplemented with 10^3 PFU/g of diet (diet 2).

Although many phages against fish pathogens have been isolated, a small fraction of them has been tested *in vivo* for their ability to control diseases in fish. Therefore, it is of interest to examine the potential of phage PUB 01-1 as a therapeutic agent against *S. agalactiae* UB 01 infection in tilapia. Furthermore, the ability of phage PUB 01-1 to undergo lytic cycle in tilapia encouraged us to perform the study. This present study demonstrated that the orally administrated phage PUB 01-1 could destroy intraperitoneally injected *S. agalactiae* UB 01 in fish. The phage supplemented diets 1 and 2 reduced the cumulative mortality of the infected fish, though with different degree of effectiveness. No mortality was observed in the group fed diet 1 while the diet 2 resulted in 30% cumulative mortality. It is unclear that the less effectiveness of diet 1 in controlling *S. agalactiae* infection in tilapia was due to the low dose of the phage given to fish or the rapid clearance of the phage from fish or the combination of both.

Our preliminary study showed that the successive 3 day oral administration of diet 2 gave the same result as a single dose administration of diet 1 (data not shown). However, further well-designed studies should be performed to examine the advantages and disadvantages of both therapeutic approaches, the treatment by using a single high dose of phage and the treatment with multiple low doses of phage.

Conclusion

In conclusion, this study presents the use of a phage to control experimental *S. agalactiae* infection in fish. Our successful phage treatment of experimentally induced *S. agalactiae* infection in tilapia suggests that phage PUB 01-1 could be used as a therapeutic agent to control this disease in fish. Experiments to determine the effectiveness of phage against natural infections should be performed in order to develop a phage control treatment for the disease.

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(Received 08th August 2024, accepted 15th October 2024)