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Research Article
Use of Bacteriophage to Control Experimental *Aeromonas hydrophila* Infection in Tilapia (*Oreochromis niloticus*)

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Abstract

Background and Objective: Antibiotics have been used to treat *Aeromonas hydrophila* infections in fish farming. However, their extensive uses can cause many negative effects including the development of drug-resistant bacterial strains. The main objective of this study was to find an alternative to antibiotics to inhibit *A. hydrophila* both *in vitro* and *in vivo*. **Materials and Methods:** A bacteriophage infecting *A. hydrophila* was isolated from a fish a pond water sample. It was classified based on its genome type studied by enzymatic digestion and morphology investigated by transmission electron microscopy. Its ability to control experimental *A. hydrophila* infection in tilapia (*Oreochromis niloticus*) was examined by feeding tilapia with fish diets supplemented with different titers of the bacteriophage. **Results:** A bacteriophage specific to *Aeromonas hydrophila* UR1 designated PAh4 was isolated and classified as a member of the family *Myoviridae*. When tilapia experimentally infected with *A. hydrophila* at the median lethal dose (3.16×10^5 CFU per fish) were fed the fish diets supplemented with the bacteriophage PAh4 at doses ranging from 10^5-10^8 PFU g^{-1} of diet, the diets could reduce the mortality rate of infected tilapia in a dose-dependent manner. **Conclusion:** The bacteriophage PAh4 can be used as an alternative to antibiotics to control *A. hydrophila* infection in tilapia.

Key words: *Aeromonas hydrophila*, bacteriophage, motile aeromonas septicemia, *Myoviridae*, *Oreochromis niloticus*, phage therapy, tilapia

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Aeromonas hydrophila is a rod-shaped, gram-negative, motile, facultative anaerobic bacterium. It is known to infect fish, reptiles and amphibians and human[1]. *Aeromonas hydrophila* causes disease in fish known as Motile Aeromonas Septicemia (MAS), Haemorrhagic Septicemia, Ulcer Disease, or Red-Sore Disease. The disease is characterized by the presence of small surface lesions leading to sloughing off the scales, hemorrhaging in the gills and anus, ulcers, exophthalmia, dropsy, the presence of ascitic fluid in the peritoneal cavity and swelling of the kidney and liver[2]. The occurrence of the disease relates to stress conditions of fish which can be arisen when fish are mishandled, overcrowded, transported under poor conditions, grown in poor water quality and reared with poor nutritional status. The disease primarily affects freshwater fish such as channel catfish (*Ictalurus punctatus*) and tilapia (*Oreochromis niloticus*), several species of bass including striped bass (*Morone saxatilis*) and largemouth bass (*Micropterus salmoides*) and many species of tropical and ornamental fish[3]. In Thailand, *A. hydrophila* infection is one of the major causes of economical damage to fish producers, especially tilapia farmers.

The most common approach for the treatment of *A. hydrophila* infection in fish farming is the use of chemotherapeutic agents, especially antibiotics. Many of them are used to control fish disease including oxytetracycline, chloramphenicol, furanace, florfenicol, oxolinic acid, piromidic acid, thiamphenicol, sulphonamide, nitrofuran derivatives and pyridine carboxylic acids[4,5]. However, the use of antibiotics in aquaculture has many negative impacts. It can result in the development of drug-resistant bacteria and therefore to reduce the efficacy of the drugs. The accumulation of antibiotics in the environment and the fish can cause potential risks to consumers and the environment[6,7]. Due to the adverse effects of antibiotics, the search for a safe and environmentally friendly strategy to control the fish disease caused by *A. hydrophila* infection has become a major issue of study for many research groups.

Bacteriophages (or phages) are viruses infecting bacteria. They have very narrow target spectra and some phages may be active against only a specific strain[8]. This high degree of specificity allows phages to be used against targeted microorganisms in a mixed population without disturbing the microbial ecosystem. Based on their characteristics, phages are interesting choices to replace antibiotics for treating *A. hydrophila* infection in fish. There have been many reports describing phages of fish pathogenic bacteria suggested that phages can be useful for controlling bacterial infections in fish[9-11].

In this study, a phage infecting *A. hydrophila* was isolated and characterized. The isolated phage was examined for the ability to control experimental *A. hydrophila* infection in tilapia (*Oreochromis niloticus*) which is one of the most cultured fish species in Thailand with a high incidence of *A. hydrophila* infection.

MATERIALS AND METHODS

Study area: The study was carried out at the Department of Biological Science, Faculty of Science, Ubon Ratchathani University, Thailand from January, 2018-December, 2019.

Bacterial strains and culture conditions: Eleven strains of *Aeromonas hydrophila* were used in this study. Ten of them designates as UR1-UR10, were isolated from kidneys of diseased tilapia collected from 6 different fish farms in Ubon Ratchathani province, Thailand. The other strain of *A. hydrophila*, *A. hydrophila* ATCC 700183, was obtained from the American Type Culture Collection (ATCC). The other twelve fish pathogenic bacteria listed in Table 1 were used to determine the phage host range. Trypticase Soy Broth (TSB) and Trypticase Soy Agar (TSA) were used for culturing the bacterial strains. Bacterial stock cultures were stored as frozen cultures at -80°C in TSB containing 20% glycerol (v/v).

Table 1: Host range of phage PAh4

Bacterial host	Source[a]	Lysis[b]
Aeromonas hydrophila UR1	Farm 1	+
Aeromonas hydrophila UR2	Farm 2	+
Aeromonas hydrophila UR3	Farm 2	+
Aeromonas hydrophila UR4	Farm 2	+
Aeromonas hydrophila UR5	Farm 3	+
Aeromonas hydrophila UR6	Farm 3	+
Aeromonas hydrophila UR7	Farm 4	+
Aeromonas hydrophila UR8	Farm 5	+
Aeromonas hydrophila UR9	Farm 6	+
Aeromonas hydrophila UR10	Farm 6	+
Aeromonas hydrophila ATCC 700183	ATCC	+
Aeromonas bestiarum ATCC 51108	ATCC	-
Aeromonas eucrenophila ATCC 23309	ATCC	-
Aeromonas salmonicida subsp. *Salmonicida* ATCC 14174	ATCC	-
Aeromonas sobria ATCC 43979	ATCC	-
Flavobacterium hydatis ATCC 29551	ATCC	-
Lactococcus garvieae ATCC 49156	ATCC	-
Pseudomonas plecoglossicida ATCC 700383	ATCC	-
Staphylococcus piscifermentans ATCC 51137	ATCC	-
Streptococcus agalactiae ATCC 51487	ATCC	-
Streptococcus difficilis ATCC 700208	ATCC	-
Vagococcus salmonicida ATCC 51200	ATCC	-
Yersinia ruckeri ATCC 29473	ATCC	-

[a]ATCC: American type culture collection, [b]+: Lytic activity against bacterial host, -: No lytic activity against bacterial host

Phage isolation and enrichment: In this experiment, *A. hydrophila* UR1 was used as a bacterial host for phage isolation. Phage was isolated from water collected from ponds where the diseased fish used in this study were obtained. Phage isolation and enrichment were performed as previously described[12].

Phage detection and host range: Phage enriched samples were initially tested for the presence of phage by using the spot-on-lawn method[12]. Four milliliter of soft TBA (0.4% agar) was inoculated with 100 µL of a log phase culture of *A. hydrophila* UR1, mixed gently and poured onto a TSA plate. Ten microliter of each phage enriched sample was spotted on the solidified soft agar. The plate was incubated at 30°C for 24 hrs before checking for a clear zone at the position on which the phage enriched sample was spotted. A clear zone in the plate, resulting from the lysis of host cells, indicated the presence of phage. The spot-on-lawn assay was also used to determine the phage host range against all fish pathogenic bacteria listed in Table 1.

Phage titer determination: The phage enriched sample producing a clear zone against *A. hydrophila* UR1 was subjected to phage titer determination by using plaque assay[12]. The phage titer was expressed as plaque-forming unit per milliliter (PFU mL^{-1}).

Phage purification: The phage of interest was purified from a phage enriched sample by using the following protocol described previously[12]. The resulting purified phage was called phage suspension.

Analysis of phage morphology and phage genome: Phage morphology was studied by transmission electron microscopy as mentioned in our previous report[12]. Phage genome was analyzed by enzymatic digestion with S1 nuclease, RNase A and *PvuI*[12].

Fish preparation: Tilapias of mixed sexes were obtained from Nong Khon Farm (Ubon Ratchathani, Thailand). They were maintained in 500 L plastic containers at 30°C, subjected to a 12 hrs light/12 hrs dark cycle and fed a commercial fish diet (Thai Spring Fish Co. Ltd., Rayong, Thailand) for 2 weeks before experiments. To verify that the fish were free of bacterial infection, they were randomly sampled and their livers and kidneys were aseptically streaked on TSA and incubated at 30°C for 24 hrs.

All experiments with the fish were conducted in 45 L aquaria at 30°C. Fish weighing 10±1 g were stocked in the aquaria (10 fish per aquarium) 24 hrs before the experiments. The commercial tilapia diet was supplied twice daily at the rate of 5% of fish body weight per day.

Examination of Pathogenicity of *A. hydrophila*: The pathogenicity of *A. hydrophila* UR1 was examined using the method previously described by Phumkhachorn and Rattanachaikunsopon[13]. The median lethal dose (LD_{50}) was calculated by the method of Reed-Muench[14] using the following Eq.:

$$\log LD_{50} = [\alpha \log b]+c$$

where, α is the mortality rate >50%-50%/mortality rate >50%-mortality rate <50%, b is the dilution rate (10^{-1}) and c is the log of minimum dilution rate in which the mortality rate was >50%.

Fish diets preparation: Fish diets supplemented with a phage suspension and oxytetracycline were prepared. Diets 1-5 were prepared by mixing a phage suspension with the commercial fish diet. The final concentrations of the phage in Diet 1, 2, 3 and 4 were 10^5, 10^6, 10^7 and 10^8 PFU g^{-1} of diet, respectively. Diet 5 was the fish diet supplemented with oxytetracycline at the concentration of 0.5% (w/w). The control diet (Diet 6) was prepared using the same process as the other fish diets except for no added phage or oxytetracycline.

Fish feeding experiment: To study the effect of phage supplemented fish diets on *A. hydrophila* UR1 infection *in vivo*, the following experiment was conducted. Groups of 10 uninfected fish were fed Diets 1, 2, 3, 4, 5 and 6 separately for 10 days. On the eleventh day, the fish were infected with *A. hydrophila* UR1 by intraperitoneal injection at a dose causing 50% mortality (LD_{50}). The fish continued to be fed the assigned diets for 10 days. Mortality was observed daily from the day of bacterial injection which was considered as day zero. Dead fish were removed from the aquaria daily and their livers and kidneys were subjected to bacterial isolation on TSA to examine the presence of *A. hydrophila*. Bacterial isolation was also performed with livers and kidneys of surviving fish to confirm that they were free of *A. hydrophila* infection. The experiment was conducted in five replicates.

RESULTS

Phage detection and characteristics of plaques: From the initial screening of pond water samples for a phage infecting *Aeromonas hydrophila* UR1 using spot-on-lawn assay, one sample gave the positive result with a clear inhibition zone indicating that the detected phage was a lytic phage. The phage was designated PAh4. When the phage was subjected to the plaque assay, it formed small, clear round plaques (about 1.5 mm in diameter) on the lawn of *A. hydrophila* UR1.

Phage host range: Spot-on-lawn assay was used to examine the ability of the phage PAh4 to infect various strains of fish pathogenic bacteria listed in Table 1. All of the strains of *A. hydrophila* used in this study were lysed by the phage. However, other strains of *Aeromonas* and the rest of the fish pathogenic bacteria were not susceptible to the phage.

Phage morphology: The ultrastructure of phage PAh4 examined by transmission electron microscopy revealed that it was a tailed phage (Fig. 1). The phage had an isometric head (about 102 nm in diameter) and a short contractile tail (about 246 nm long and 18 nm wide) with a base plate at the end of the tail.

Analysis of phage genome: The phage PAh4's genome was tested for its sensitivity to several enzymes digesting nucleic acid and checked by agarose gel electrophoresis. It was found that the genome was digested by *Pvu*I but not by S1 nuclease and RNase A (Fig. 2). Sizes of the bands resulting from digesting the genome with *Pvu*I were approximately 10, 5.5, 3.5, 2.5 and 1.2 kb.

Examination of pathogenicity of *A. hydrophila* UR1: Pathogenicity of *A. hydrophila* UR1 for tilapia is shown in Table 2. All of the dead fish died within 5 days after bacterial injections and the pathogen was found in their livers and kidneys. In Table 2, mortality rates obtained from injecting tilapia with *A. hydrophila* UR1 at the doses of 10^5 and 10^6 CFU per fish were 40 and 60%, respectively. The median lethal dose (LD_{50}) of *A. hydrophila* UR1 for tilapia calculated from these results was $10^{5.5}$ CFU per fish or 3.16×10^5 CFU per fish.

Fish feeding experiment: Before testing the effects of fish diets supplemented with the phage PAH4 and oxytetracycline on tilapia infected with *A. hydrophila*, all of the diets (Diet 1-6) were fed separately on uninfected fish

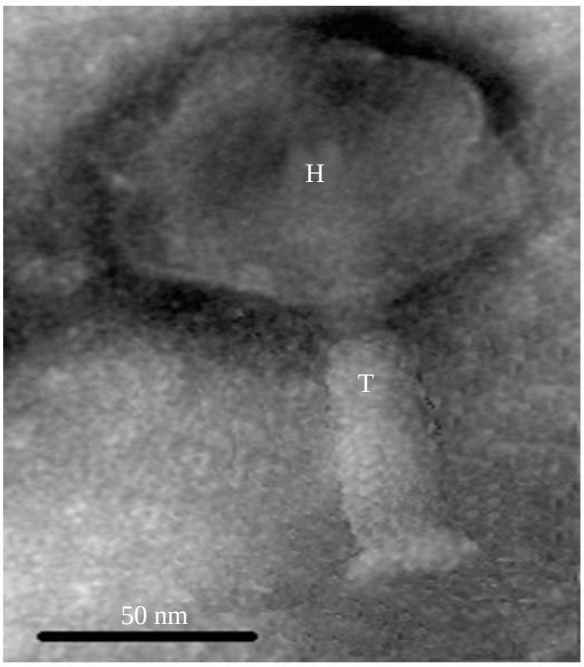

Fig. 1: Transmission electron micrograph of phage PAh4 showing its head (H) and tail (T)
Bar = 50 nm

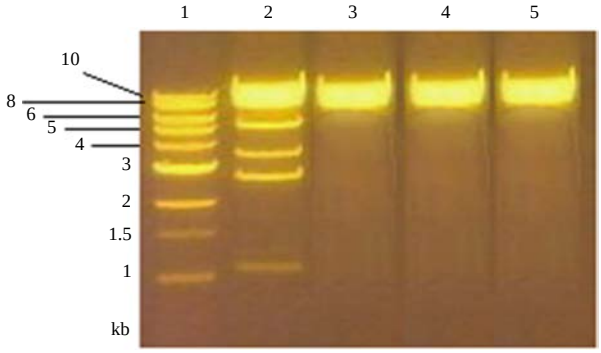

Fig. 2: Analysis of genome extracted from phage PAh4 using agarose gel electrophoresis. Lane 1: DNA standard (1 kb ladder, New England Labs), Lane 2: Cut with *Pvu*I, Lane 3: Cut with S1 nuclease, Lane 4: Cut with RNase A and Lane 5: Uncut

twice a day for 10 days. It was found that the diets had no adverse effect on the fish based on mortality, appearance, feeding response and behavioral alterations of the fished which were observed daily. When *A. hydrophila* infected tilapia fed diets supplemented with the phage (Diet 1-4), reduction in mortality of the fish was observed in a dose-dependent manner (Fig. 3). Moreover, the mortality of

Table 2: Mortality of tilapia intraperitoneally injected with different dilutions of *A. hydrophila* UR1 suspension

Aeromonas hydrophila (CFU/fish)	Number of dead fish/no. of tested fish[a]					Mortality (%)
	1	2	3	4	5	
10^8	10/10	9/10	10/10	9/10	10/10	96
10^7	9/10	9/10	9/10	8/10	9/10	88
10^6	6/10	7/10	6/10	6/10	5/10	60
10^5	4/10	5/10	4/10	4/10	3/10	40
10^4	2/10	2/10	3/10	2/10	3/10	24
10^3	1/10	1/10	1/10	2/10	1/10	12
10^2	1/10	0/10	0/10	0/10	1/10	4

[a]Results from all replicates (replicate 1-5)

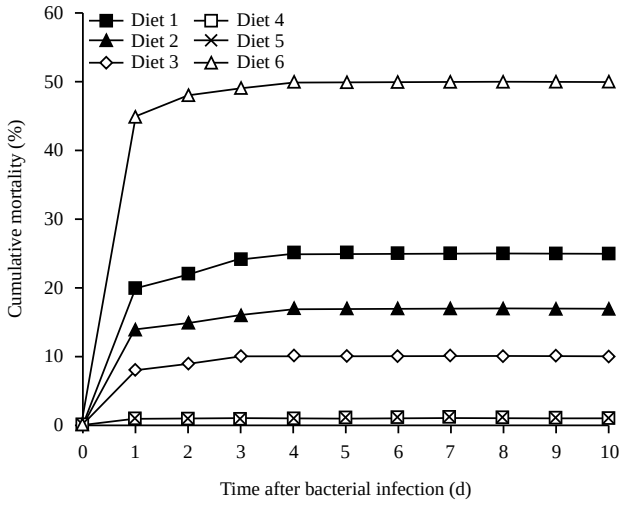

Fig. 3: Effect of fish diets supplemented with phage PAh4 at doses of 10^5 (Diet 1), 10^6 (Diet 2), 10^7 (Diet 3), 10^8 (Diet 4) PFU g^{-1} of diet and oxytetracycline (Diet 5) on the mortality rate of *A. hydrophila* UR1 infected tilapia compared with the control fish diet (Diet 6)

the infected fish treated with Diet 4 (containing the phage at the concentration of 10^8 CFU g^{-1} of diet) was not different from that treated with Diet 5 (containing 0.5% (w/w) of oxytetracycline).

DISCUSSION

Recently, the use of antibiotics to control diseases in fish aquaculture has raised a major concern on their safety to the environment, farmers and consumers[15]. Phage therapy for the fish disease has come to our attention because bacteriophages have several characteristics suitable to replace antibiotics. Bacteriophages are generally present everywhere including foods and water we consumed. Since they infect only a specific host, the use of them to control pathogenic bacteria in food animals does not disturb normal flora normally residing in animals and consumers. Phage therapy has been successfully used to control diseases in a wide range of animals including mice[16], cattle, poultry, pigs[17] and fish[18].

The pathogenic bacteria used in this study were *A. hydrophila* isolated from tilapia suffered by Motile Aeromonas Septicemia cultured in 6 different fish farms in Ubon Ratchathani Provinces, Thailand. *Aeromonas hydrophila* UR1 was selected to be a representative of all isolated pathogenic bacteria to be used as a major host throughout this study. Its pathogenicity was confirmed by the determination of its median lethal dose (LD_{50}) for tilapia which was 3.16×10^5 CFU per fish. Because bacteriophages and their hosts are generally present in the same environment, we decided to use water from ponds where all *A. hydrophila* used in this study were isolated as samples for searching a phage specific to the bacteria. By using *A. hydrophila* UR1 as a host, the phage PAh4 was isolated and found to be able to infect the host bacterium.

The phage PAh4 had a narrow host range. It specifically infected *A. hydrophila*. *Aeromonas* in different species and the bacteria in different genus used in this study were not susceptible to the phage. Phages with narrow host ranges might be more suitable as biocontrol agents than those with broad host ranges because they are likely to cause less harm to normal flora. Although phages with narrow host range sometimes cause limitations in their use, this problem can be overcome by using cocktails or combinations of several phages. For example, Mateus *et al*.[19] reported the success of using phage cocktails (containing two or three phages of VP-1, VP-2 and VP-3 phages) to control fish pathogen *Vibrio parahaemolyticus*.

Information on the phage genome and morphology are necessary for phage classification. The sensitivity of phage PAh4's genome to *Pvu*I (but not to S1 nuclease and RNase A) suggested that its genome was double-stranded DNA. Transmission electron microscopy revealed that the phage PAh4 was a tailed phage with an isometric head and a noncontractile tail. According to the International Committee

on Taxonomy of Viruses[20], tailed phages with double-stranded DNA are classified in the *Caudovirales* order. This order contains three families including the *Myoviridae* (with long, contractile tail), the *Siphoviridae* (with long, noncontractile tail) and the *Podoviridae* (with short tail). Based on its nucleic acid and morphological characteristics, the phage PAh4 was tentatively classified as a member of the *Myoviridae* family. Phages specific to fish pathogenic bacteria previously reported did not only exist in the *Myoviridae* family but also in the other two families of the *Caudovirales* order[10,21,22].

In vivo experiments were conducted by feeding tilapia with fish diets supplemented with phage at doses ranging from 10^5-10^8 PFU per fish. The phage supplemented fish diets were shown to be able to reduce the mortality rate of tilapia experimentally infected with *A. hydrophila* in a dose-dependent manner. Besides, all of the tested fish diets had no adverse effect on the fish. No difference was found in mortalities between the fish treated with phage at the dose of 10^8 PFU per fish and those treated with oxytetracycline. The results suggest that there is therapeutic potential to phage PAh4. It could be used to replace antibiotics to control the disease of tilapia. Experiments to study the use of phage PAh4 supplemented fish diets in tilapia farm against natural *A. hydrophila* infections is underway in order to develop a control treatment for the disease in aquaculture of tilapia.

CONCLUSION

It can be concluded that there is therapeutic potential to phage PAh4. It could be used to replace antibiotics to control the disease of tilapia. Experiments to study the use of phage PAh4 supplemented fish diets in tilapia farm against natural *A. hydrophila* infections are underway to develop a control treatment for the disease in aquaculture of tilapia.

SIGNIFICANCE STATEMENT

This study discovers a new phage designated as PAh4 that can be beneficial for control infection of *Aeromonas hydrophila* in aquaculture. This study will help the researcher to develop environmentally-friendly aquaculture by using the phage as an alternative to antibiotics that many researchers were not able to accomplish. Thus, antibiotic-free aquaculture is proven to be possible.

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