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Thesis for the Degree of Master of Fisheries Science

**Isolation and Characterization a New
Bacteriophage Infecting Fish Pathogenic
*Aeromonas hydrophila***

by

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KOICA-PKNU International Graduate Program of Fisheries Science

Graduate School of Global Fisheries

Pukyong National University

February 2019

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어류 병원균인 *Aeromonas hydrophila* 을
감염시키는 새로운 박테리오파지의 분리
및 특성 규명

Advisor: Prof. Tae Jin Choi

by

Muhammad Akmal

A thesis submitted in partial fulfilment of the requirement
for the degree of Master of Fisheries Science
in KOICA-PKNU International Graduate Program of Fisheries Science,
Graduate School of Global Fisheries,
Pukyong National University

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**Isolation and Characterization a New Bacteriophage Infecting
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A dissertation

by

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February 22, 2019



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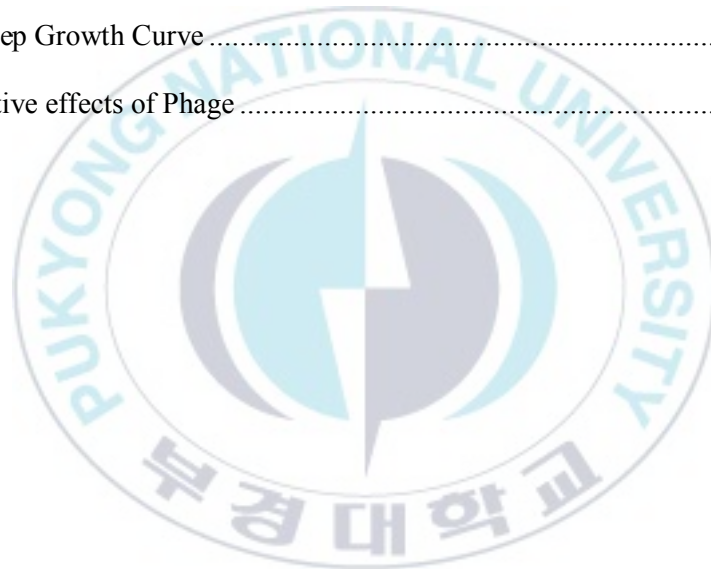
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Abstract

The aquaculture industry is suffering from severe losses due to *Aeromonas* infection. Due to recent increase in the multidrug resistance (MDR) *Aeromonas* Sp. ineffectiveness of different treatment methods scientist are exploring more biological control methods. Bacteriophage are promising candidate in this regard as they can even kill MDR bacteria with almost no negative effects towards the environment and organism

The present study was designed to isolate and characterize the lytic bacteriophage, and to determine its protective effect against the *Aeromonas* infection in Loach fish

Water and soil samples were collected from different location of Korea. Phage isolation was performed by the enrichment method. It was characterized by TEM, one step growth

curve, pH and temperature stability. Next generation sequencing (NGS) was performed for genome analysis.

We isolated a phage Akh-2 from, Korea, found to inhibit the growth of 4 out of 8 tested *A. hydrophila* strains. TEM result showed that Akh-2 most probably belongs to the Siphoviridae family, having the head and tail size 50 ± 5 nm and 170 ± 5 nm, respectively. One- step growth curve of phage Akh-2 showed that it has a latent period of 50 ± 5 minutes and with a burst size of 139 ± 5 PFU per infected cell. The phage is stable in pH range from 6 to 8 and the temperature range from -80 °C to 46 °C. NGS data confirms that the size of genome is about 114901bp with G + C content of 44.22 % and 254 open reading frames. The Loach that received phage therapy had shown increase in survival rate and delayed mean death time when compared to the control.

Keywords: *Aeromonas hydrophila*, Phage, Disease, Biocontrol

Introduction

Aquaculture is the only fastest growing food providing sector all around the world (Defoirdt et al., 2011). Its global production is expected to reach 79 million tons by the end of 2021, and fish will beat beef, poultry and pork in terms of production (Gon et al., 2017).

Disease outbreaks are recognized as a constant threat for this huge production level, resulting in billions dollars loss per year. High level stocking density, poor water quality, over feeding are the main influencing factors in this context. A variety of bacterial, fungal and viral diseases are easily found in different fishes (Nandi et al., 2017; Defoirdt et al., 2011). Among various disease-causing bacterial genera Gram positive bacterial genera (*Chromobacterium*, *Flavobacterium*, *Pseudomonas*, *Proteus* and *Aeromonas*) and Gram negative bacterial genera (*Staphylococcus*, *Streptococcus* and *Micococus*) have been proven pathogenic for fishes and resulted in the heavy economic losses in aquaculture sector.

Aeromonas spp. are considered as the most common bacteria in different water bodies around the world and are frequently responsible for several diseases among the cultured and feral fish species (Baird et al., 2009; Jun et al., 2013). *Aeromonas*

hydrophila, is a Gram negative and rod shaped bacterium is the main culprit for a disease called Motile Aeromonas Hydrophila septicemia (MAS), also known as tail rot, infecting seriously various fresh water fish species like Loach (*Misgurnus anguillicaudatus*), channel cat fish (*Ictalurus punctatus*), common carp (*Cyprinus carpio*) and lesser to marine fish species. Symptoms of these infections include, abdominal distension, anemia, accumulation of fluid, presence of ulcer hemorrhage and damage to the different organs, resulting in the mass mortality of the fishes around the globe (DA & Didamony, 2016; Easwaran et al., 2017)

Several types of antimicrobials agents e.g. oxolonic acid, sulphonamides, furazolidone, chloramphenol, amoxicillin and oxytetracycline are used to counter or prevent disease outbreak. Use of these drugs without any recommendation has created major problems like severe disturbance in aquatic ecosystem, changing the gut microflora of organism and birth of multi drug resistance bacteria. (van der Aa Kühle et al., 2005; Jaime Romero, 2018).

There are several other solutions like vaccines, oils and probiotics, commonly practiced around the world having their own pros and cons. Vaccination seems to be an ideal solution for the prevention of the disease but it fails to treat the existing infections and the commercial availability of these vaccines in the market is still limited. Essential oils are also used to cure the infections but their use is very limited. The use of different types of probiotics are also gaining popularity, but some may interact with the healthy

flora of organism to create more troubles.so there is a dire need to search alternative methods to overcome these issues (Jaime Romero, 2018).

Application of bacteriophage in aquaculture seems to be a promising solution to deal these pathogens. Phages are bacterial viruses having most abundant forms of life on the earth, with the ability to infect more than 70 % of the marine bacteria (Wommack & Colwell, 2000). Phages can infect the bacteria either with their lytic or lysogenic cycle, while some can infect both cycles. In their lytic cycle phage infects host bacteria through attachment, penetration, controlling the host DNA synthesis machinery and production of its own progeny. While in lysogenic cycle phage incorporates its DNA into the host cell, host cell can replicate normally for long time, and phage can start its production at any stage of life (Hoai et al., 2016; Richards, 2014).

The use of phage is environment friendly, efficient, inexpensive and have almost no side effect as the phages are more target oriented toward their host. Based on their unique features phages have been used to control various fish and shell fish pathogens viz, *Vibrio parahaemolyticus*, *V. harveyi*, *V. anguillarum*, *Aeromonas salomonicida*, *Lactococcus garvieae*, *Pseudomonas plecoglossicida*, *L. garvieae* (Gon et al., 2017)

Different phages have been used to control the Motile Aeromonas Septicemia, (Wu et al., 1981) was probably the earliest one using the phage treatment in loaches against the *A. hydrophila*, by isolating the AH1, a very strong lytic phage. Recently (Jun et al., 2013) has isolated 2 phages belonging to Myoviridae family, named as pAh1-C and

pAh6-C against *A. hydrophila*. They determine the efficacy of the entry route for the phages by adding them in the feed against the intra peritoneal injection. (Imbeault et al., 2006) also used phage HER 1107 for the treatment of antibiotic resistant strain of *A. salomonicida* in the brook trout.

A very few extensive attempts are found for the characterization and determination the efficiency of phages to determine against the bio control of disease. Therefore the present study was designed to isolate and characterize the phage infecting pathogenic *A. hydrophila* with a main objective to counter the disease causing pathogens in different culture systems.



Materials and Methods

2.1. Bacterial Strain and Culture

A total of 7 different strains (Table. 1) of *A. hydrophila* was used in the study. All these strains were cultured on the Nutrient Agar at 37°C and stored at -80°C with the addition of 80% glycerol solution for its long term use.

Table 1. Description of different strains of *A. hydrophila* used in the study

No.	Bacteria	Strain ID	Source
1	<i>Aeromonas hydrophila</i>	KTCC 2358	1*
2	<i>Aeromonas hydrophila</i>	KCCM 32586	2**
3	<i>Aeromonas hydrophila</i>	AH-A6	3***
4	<i>Aeromonas hydrophila</i>	AH-A8	3
5	<i>Aeromonas hydrophila</i>	AH-20	3
6	<i>Aeromonas hydrophila</i>	AH-21	3
7	<i>Aeromonas hydrophila</i>	AH-Juwah	3

*Korean collection for type culture
**Korean culture Centre for microorganism
***Seoul National University

2.2. Sample collection, Phage Isolation and Purification

A total of 300 samples of soil and water was collected from different locations (Figure. 1) from the sea water and fish farms near Geoje Island, during the months of January to February, 2018 to isolate the phage.



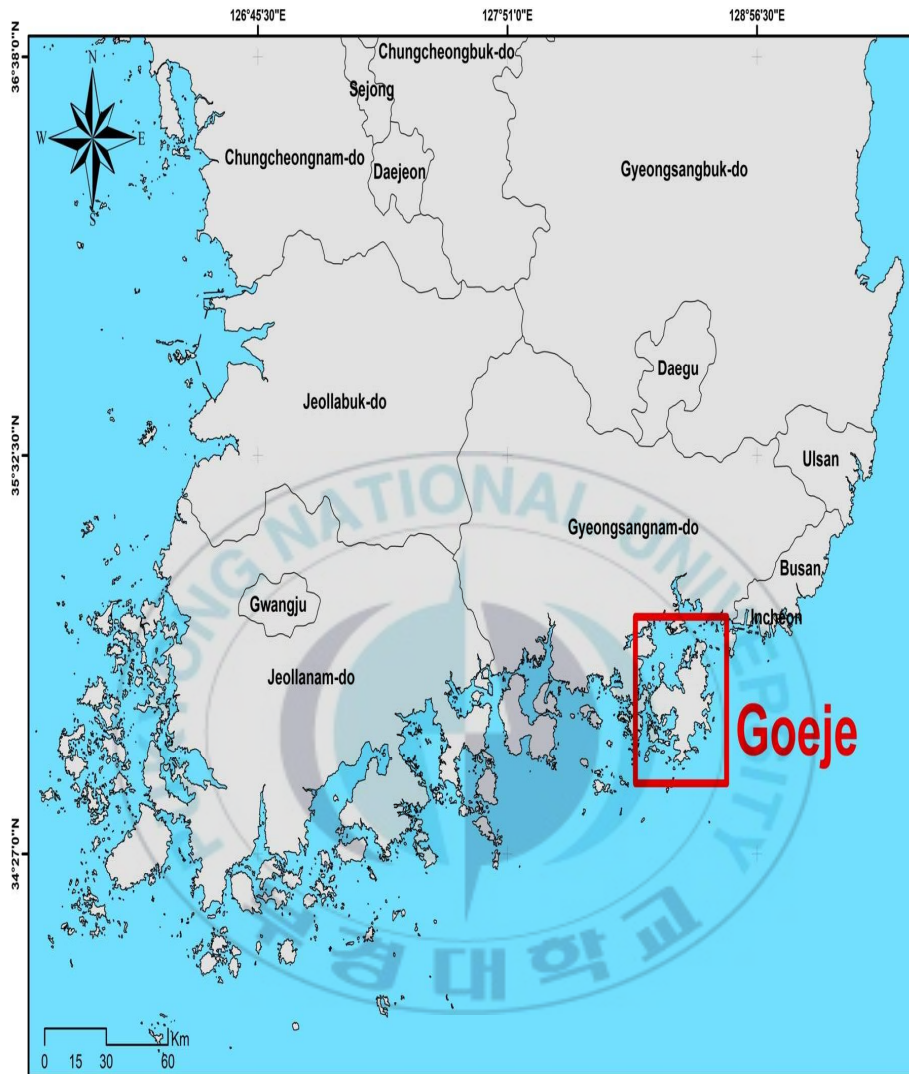


Fig. 1. Location of Goeje Island from where samples were collected

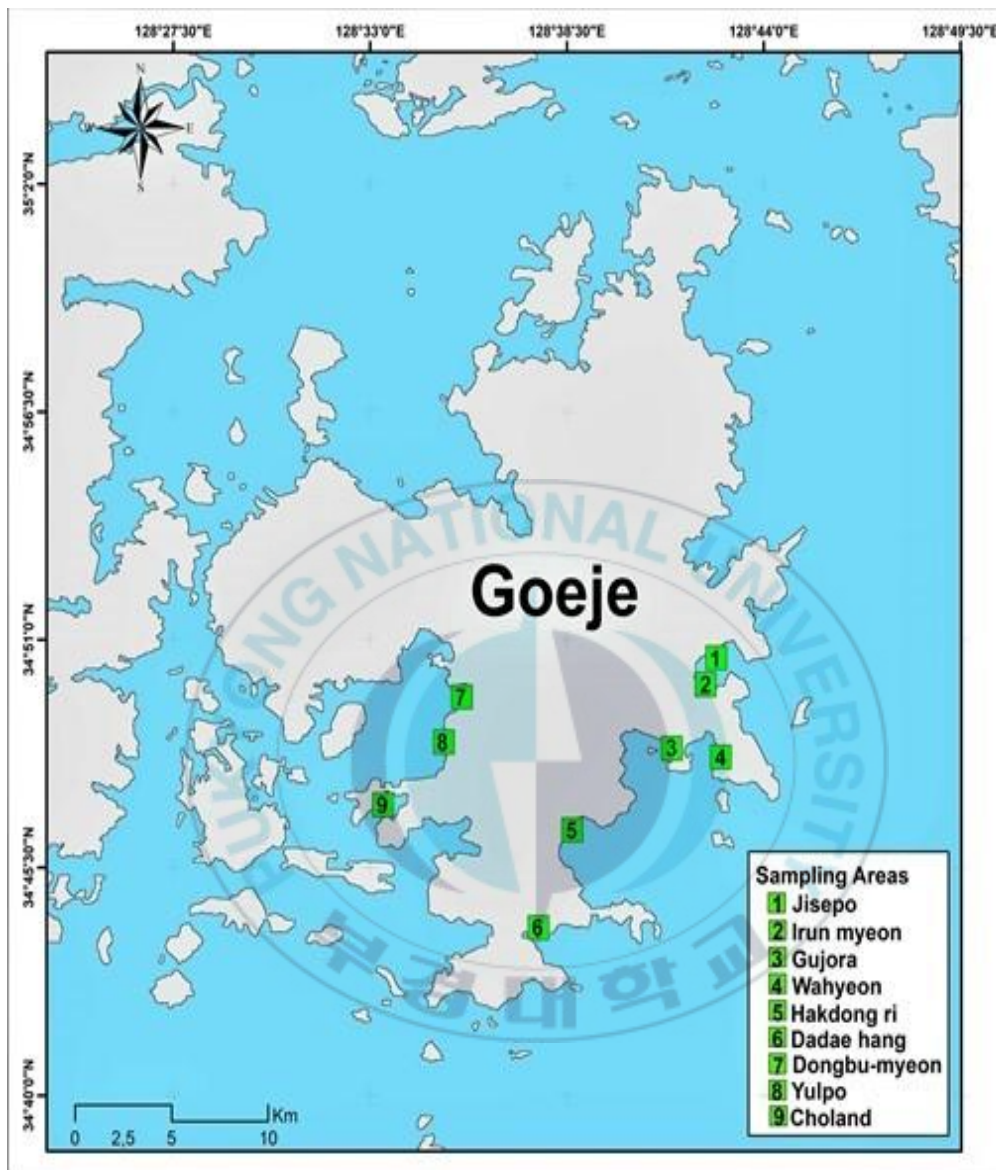


Fig. 2. Description of sampling site

For the isolation of phage, water samples were centrifuged at 3500 rpm at 4°C for 45 min, followed by the filtration through a filter (having mesh size 0.02µm). These samples were preserved at 4°C for the further use. In the case of soil samples enrichment was conducted directly prior to any filtration or centrifugation.

Enrichment of phage was performed by mixing 3 ml of liquid culture of bacteria, 4 ml of the media NA (Nutrient Agar) and 300 µl of the filtered sample mixed together in 50 ml tube and incubated at 37°C in shaking incubator for 24 hrs. The samples were centrifuged at 3500 rpm at 4°C for 45 min, followed by the filtration through a filter of 0.02µm size to obtain the pure phage.

For the confirmation about the presence of lytic phage in the filtrate, spot assay was performed. Briefly, 300 µl of liquid culture and 4 ml of top agar (0.75 % agar) were mixed together and spread in the petri plate prepared with normal agar and NA. After hardening of TA, 3µl of filtrate was spotted and presence was confirmed by the appearance of clear plaque after 24 h at 37°C.

For the purification of the lytic phage, A single plaque was picked up with sterile end-cut disposable pipette tip cut tip and suspended in 1ml of sodium chloride magnesium sulphate (SM) buffer (100 mM NaCl, 10 mM MgSO₄.7H₂O and 50mM Tris HCl, pH 7.5) followed by sterilization. The same process was repeated 5 times until the consistent

plaque morphology was obtained. The phage isolated by this process was named as Akh-2.

For the amplification of the initial titer of phage, 5ml of bacteria O.D (0.500) at 600 was added with 500ul of purified phage and cultured overnight in shaking incubator at 37⁰C. After filtration, plaque assay was performed, petri plates showing complete lysis by phage were washed with 5ml of SM buffer and titer was determined by the double assay. Same procedure was repeated until the titer up 10⁹ was obtained. This was stored at 4⁰C for the further use in the experiment.



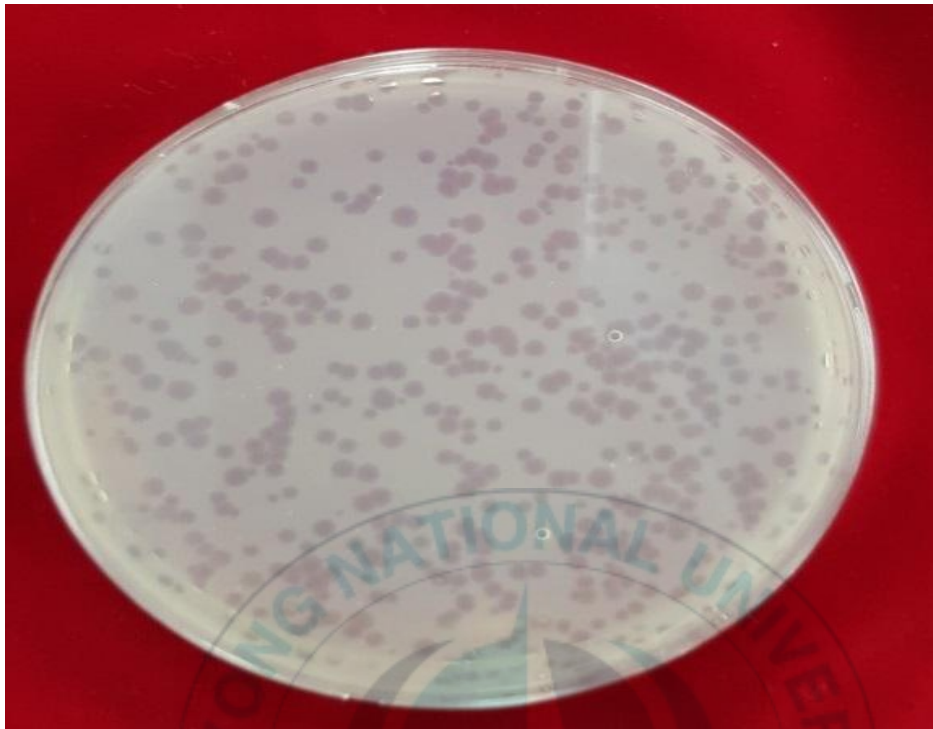
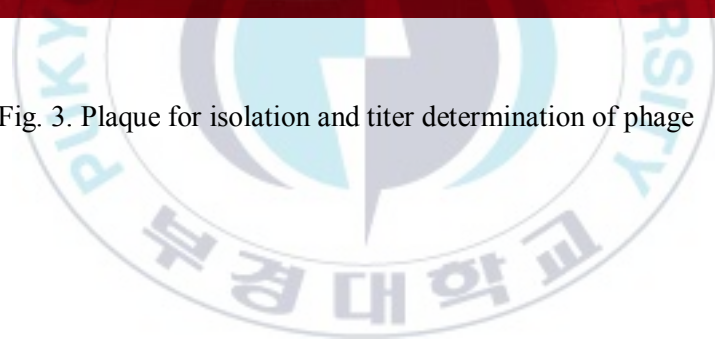


Fig. 3. Plaque for isolation and titer determination of phage



2.3. Electron Microscopy of Phage

The high titer phage having 10^{11} PFU/ml suspended in SM buffer were adsorbed on the carbon coated grids. 2% uranyl acetate was used to negatively stain the phage particle. After drying at room temperature, the grids were examined by electron micrographs at NICEM of Seoul National University.

2.4. Host Range Determination

A total of 5 different type of bacteria with 28 different strains was used (Table. 2). The host range experiment was conducted to determine the lytic range of phage against the each bacteria. It was accumulated by spotting 5ul of lytic phage on the bacterial host prepared with their media, the presence of clear plaque showed that phage can kill the respective bacteria.

Table 2. Bacterial species used for determination of host range

Bacteria	Strain ID	Culture Media	Temperature °C
<i>Aeromonas hydrophila</i>	KTCC 2358, KCCM, 32586, AH-A6, AH-A8, AH-20, AH-21, AH-Juwah	Nutrient Agar	37
<i>Pectobacterium</i>	35, 48, 63, 92, E42, E44	Tryptic soy broth	27
<i>Streptococcus aureus</i>	S75, S86, S103, S106	Tryptic soy broth	27
<i>Bacillus</i>	B1, B2, B3, B4, B5, B6, B8, B11, B12, B13, B87, B97	Tryptic soy broth	27
<i>E. coli</i>	CJY H7	Tryptic soy broth	27

2.5. Stability at different pH and Temperature

Phage stability was determined according to (Verma et al., 2009) with some modifications. For the evaluation of stability against different temperatures (-80 °C, -20 °C, 4 °C, 25 °C, 37 °C, 45 °C, 50 °C and 55 °C) the phage (10^7 PFU/ml) in SM buffer was incubated at the respective temperature for 3 days and sample were taken after each day to determine the titer by plaque assay.

Phage stability against the different pH (4, 5, 6, 7, 8, 9, 10, 11 and 12) was determined by preparing the solution of respective pH with Tris-chloride. Phage lysate (10^7 PFU/ml) in different pH solution and incubated at 37 °C and sample were taken for 3 continuous days and sample were taken after each day to determine the titer by plaque assay.

2.6. One-Step Growth Curve o

Growth pattern was calculated according to (Verma et al., 2009) with some modification. Briefly the 1ml of bacteriophage 10^9 PFU/ml was added in the 20 ml liquid OD 600= 0.4-0.5 to maintain MOI (0.001). The mixture was allowed to stand at 37 °C for 5 min. After the adsorption of phage, mixture was centrifuged at 35000 rpm for 30 minutes, the supernatant was removed carefully and pellet was suspended in 20 ml of

liquid culture. The mixture was incubated at 37°C for one hour and samples were taken after 5 min, plaque assay was performed to determine to change in titer with time.

2.7. Phage DNA Isolation

Phage lysate (10^8 PFU/ml) was centrifuges for 4 times at 6000 g for 10 min. It was further centrifuge for 2 h at 10 °C. The supernatant was discarded totally and the remaining pellet was dissolved in the 500 ul of SM buffer. The suspension was treated with DNase and Ranse to remove the any contamination from free nucleic acid. The DNA extraction was carried out as described by (Griffiths et al., 2000) with some changes. Briefly 0.5 ml of phenol –chloroform –isoamyl alcohol and 0.5 ml of hexadecyltrimethylammonium was used for extraction from 1ml phage solution. The sample was centrifuged (16,000 g) for 5 min at 4°C. The supernatant was added to a new vial and mixed with chloroform isoamyl alcohol and centrifuged (16,000 g) for 5 min at 4°C. The supernatant was removed and a new clean tube and DNA was precipitated two fold volume of 30% (wt./v) polyethylene glycol for 2 hours at 25°C. the mixture was centrifuged again at 18,000 g for 10 min at 4°C. The pellet was washed with 70 % (v/v) ethanol, centrifuged again 18,000 g for 10 min at 4°C, the pellet was air dried and re-suspended in 30ul of EDTA for the further use.

2.8. Whole genome sequence analysis

The WSG was executed by Next generation sequencing (NGS) technology with Illumina Hiseq sequencer by THRAGEN ETEX Bio institute, Korea. The genomic DNA sample of Akh-2 processed further for library preparation under sample ID V2 TN1809D0396. The sequence reads were assembled de novo using Platanus (1.2.2). Open reading frames (ORFs) were forecasted with GeneMark.hmm, while their functions were annotated with RAST and BLAST software at NCBI. The other homologous sequences obtained from Gene bank data base were used for the construction of phylogenetic tree by MEGA 5.05.

2.9. Challenge test and estimation of lethal dose

For the provision of more natural and similar infection route, identical to the aquatic environment, we adopted the methodology described by (Zhang et al., 2016a). A total of 40 healthy adipose fin clipped (Af-clipped) loach with average body weight of 12 g were distributed into four 30-L tanks filled with 15-L of water, labeled as control, T1, T2 and T3.

For the determination of optimal challenge dose of KCTC 2358, T1, T2 and T3 were inoculated with *A. hydrophila* to maintain a final concentration of 1.0×10^5 CFU/ml, 1.0×10^6 CFU/ml and 1.0×10^7 CFU/ml respectively, while there was no bacterial

inoculation in control group. After the 30 min immersion in respective treatment, fishes were transferred experimental tanks with normal water and observed for 1 week for determination of clinical signs /mortality.

2.10. Phage treatment of infected fish

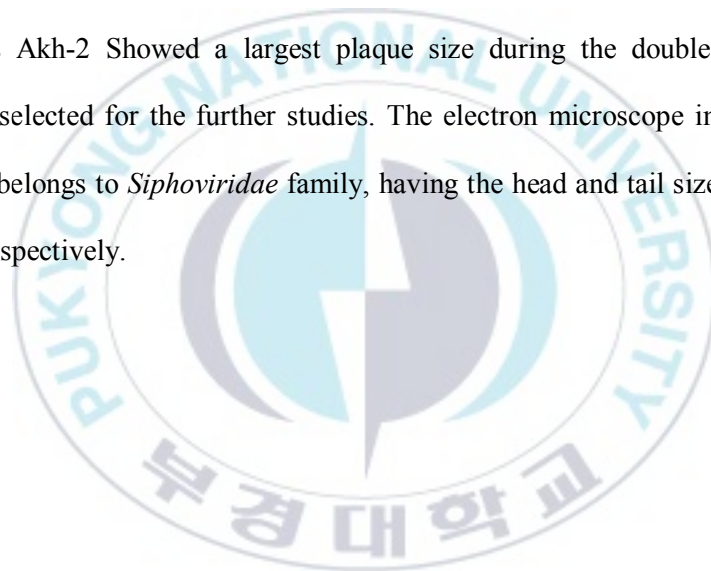
A total of 160 healthy loach with average body weight of 10 g were divided into 4 groups and placed in 30-L tanks filled with 15-L of water. Af-clipping and immersion method as described earlier was used for the inoculation of bacteria and phage.

Group I was bacterial infection group, in which loach were immersed in 1.0×10^7 CFU/ml for 30 min. Group II was phage therapy group in which loach was immersed in 1.0×10^7 CFU/ml for 30 min followed by immediate immersion in 1.0×10^8 PFU/ml for 30 min at MOI 1. Group III was phage control group, in loach was immersed in 1.0×10^8 PFU/ml for 30 min. Group IV was controlled group in which loach did not receive any bacteria or phage. After the each immersion loach were raised in independent 15-L water filled experiments tanks and observed for one week without any feeding. The experiments was repeated three different times on separate occasions. The water temperature was kept constant (28 ± 2 °C) throughout the experiment. All the experiments were performed under the guidelines of Animal Ethical Committee of Pukyong National University, Korea.

Results

3.1. Isolation of Bacteriophage

For the isolation of phage a total of 300 water and soil samples were collected from 9 different sites around the Geoje Island (Fig .1). All of these samples were tested against 5 different types of bacteria (Table. 1). We have isolated phage from water sample designated as Akh-2. It showed a largest plaque size during the double agar overlay method and was selected for further studies. The electron microscope image of phages shows that it belongs to *Siphoviridae* family, having the head and tail size 50 ± 5 nm and 170 ± 5 nm, respectively.



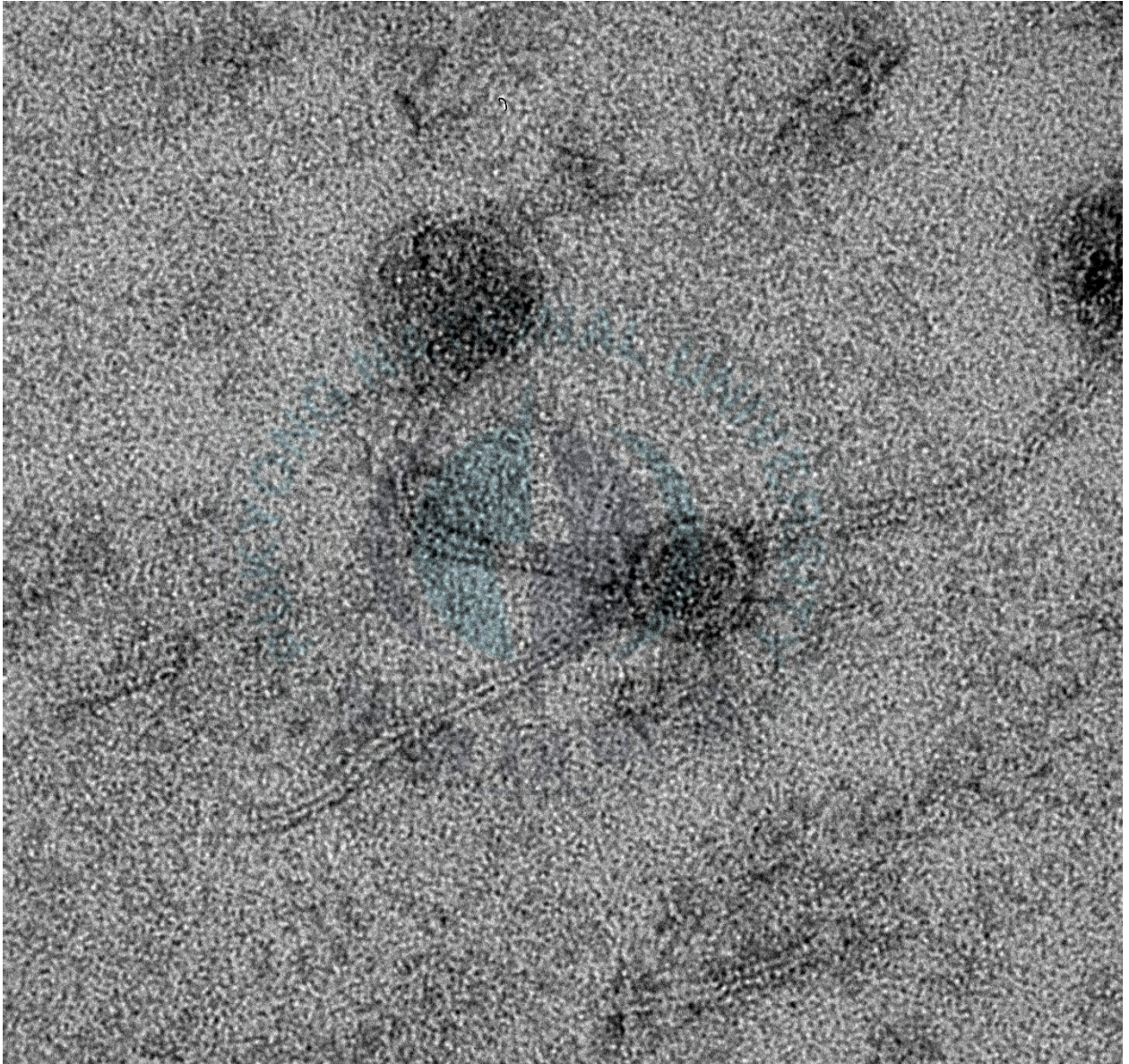


Fig. 4. Electron micrograph of Akh-2

3.2. Host Range

A total of 5 different type of bacteria with 28 different strains was used. (Table. 3). Akh-2 can affect the four strains of *A. hydrophila*. While it cannot affect any other tested bacterial strains making it more host specific.



Table 2. Host Range of bacteria

Sr.	Bacteria	Strain ID	Lytic activity
1	<i>Aeromonas Hydrophila</i>	KTCC 2358	+
		KCCM 32586	-
		AH-A6	+
		AH-A8	-
		AH-20	+
		AH-21	-
		AH-Juwah	+
2	<i>Pectobacterium</i>	35	-
		48	-
		63	-
		92	-
		E42	-
		E44	-
3	<i>Streptococcus aureus</i>	S75	-
		S86	-

		S103	-
		S106	-
4	<i>Bacillus</i>	B1	-
		B2	-
		B3	-
		B4	-
		B5	-
		B6	-
		B8	-
		B11	-
		B12	-
		B13	-
		B87	-
B97	-		
5	<i>E.coli</i>	CJY H7	-

+ Shows Lytic Activity -No Lytic Activity

3.3. Temperature and pH stability

Phage can survive at temperature ranging from -80°C to 45°C , its titer starts decreasing from 46°C and it cannot survive at 60°C . While its can stored at -80°C to 4°C , for long time without any loss in its titer. (Fig 6)

For the pH range, Akh-2 can survive between 5 to 11 ph. While the best one is 7 ph. (Fig 7)



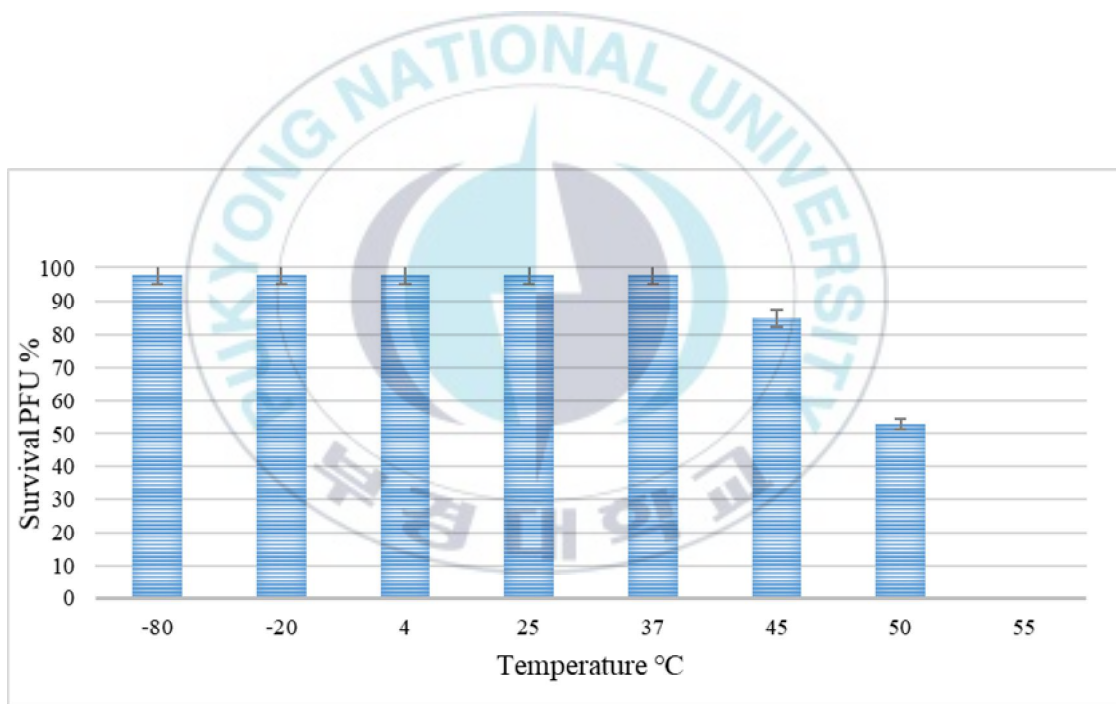


Fig. 5. Temperature stability of Akh-2

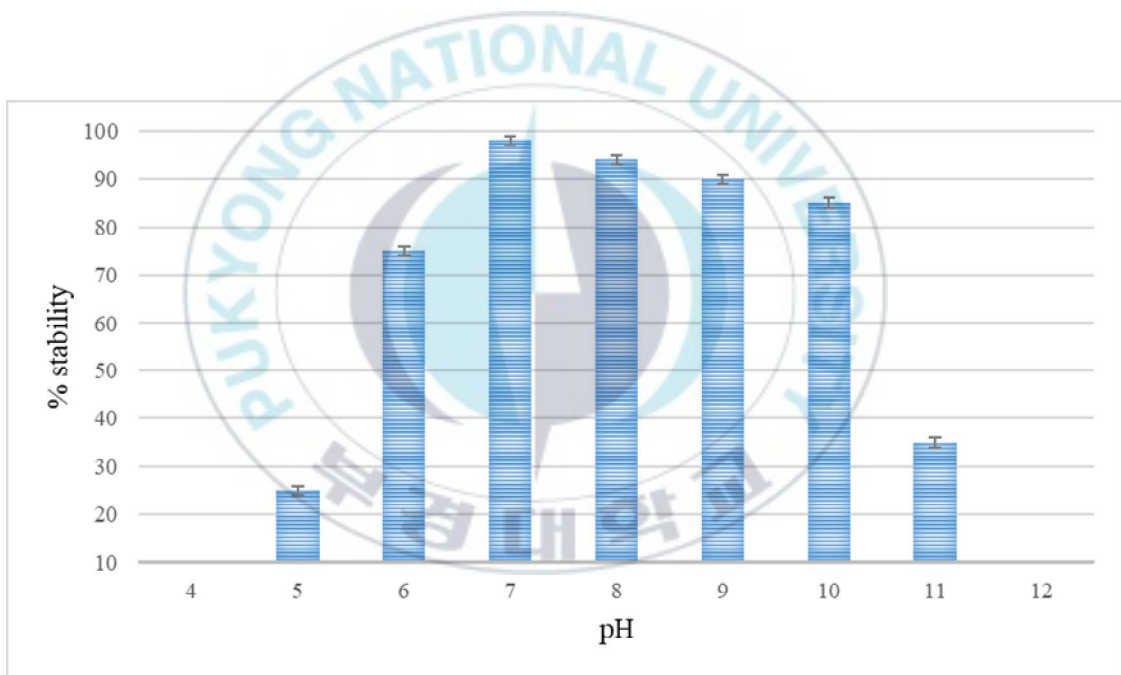


Fig. 6. pH stability of Akh-2

3.4. One step growth curve

Results from one step growth curve shows that Akh-2 has a latent period about 50 ± 5 min.

While the burst size was $145 \pm$ PFU/ml.

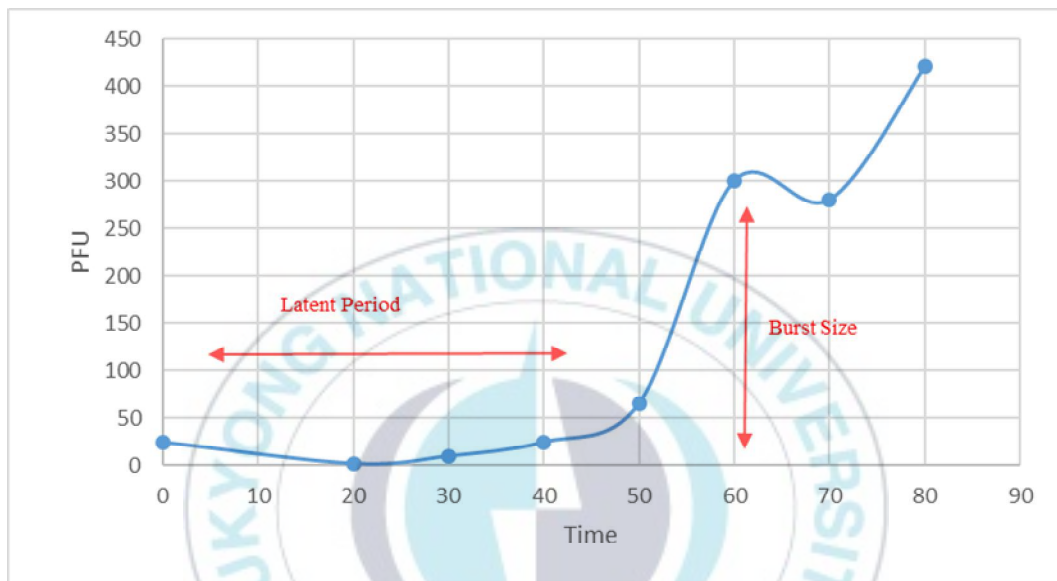


Fig. 7. One Step Growth Curve

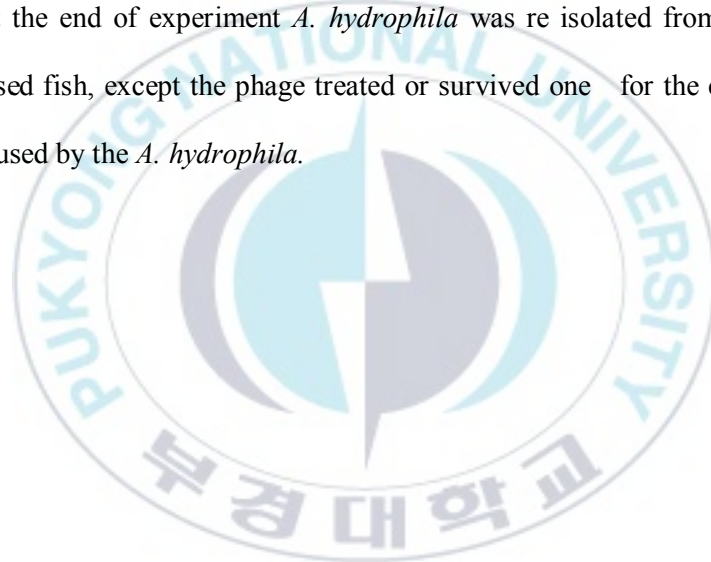
3.5. Genome analysis

The genome of Akh-2 was sequenced and characterized, which confirmed the presence of double strand DNA. The statistic of sequencing by MiSeq revealed the total reads, total bases (Gbp) and G+C percent about 16,820,356, 1.070 and 45.22, respectively. The size of assembled genome as revealed by De novo assembly using Platanus was about 114,901 bp. A total of 254 ORFs were generated. 114 were encoded by plus strand and 141 by negative strand. By using RAST and BLAST in NCBI data base 187 were assigned as hypothetical protein, 31 as a tRNA and 36 were predicted for different functions. Most of predictions corresponded for different structural protein i.e. (tail fiber, major capsid and phage protein), DNA ligase (repairing of new DNA), and protein involved in virus replication.

3.6. Protective effect of AKh-2 in infected loach

According to the results of challenge test, experimental loach (T2 and T3) challenged with 1.0×10^6 CFU/ml and 1.0×10^7 CFU/ml for 30 min, showed 100 % mortality within 2 days. While 1- 2 days delayed in 100 % mortality was observed in loach (T1) immersed in 1.0×10^5 CFU/ml.

The results for the Akh-2 treatment of infected fish are shown in Tab. 4. At the end of 7 days experiment, Group 1, challenged with *A. hydrophila* (1.0×10^7 CFU/ml) showed 95 ± 3 % cumulative mortality with abdominal haemorrhage and presence of red spots on the body. Group II, phage therapy (MOI 1) showed 30.12 ± 5.2 % of cumulative mortalities, 7 days of post infection. The survival rate of fish were increased 60.32 ± 4.21 % when treated with phage confirming the protective effect of phage against the infection. Group III, that received only phage (1.0×10^8 PFU/ml) and group IV (neither phage nor bacteria) showed 100 ± 0 % survival rate without any symptoms of disease confirming the safety of phages. At the end of experiment *A. hydrophila* was re isolated from the kidney of dead or diseased fish, except the phage treated or survived one for the confirmation of mortalities caused by the *A. hydrophila*.



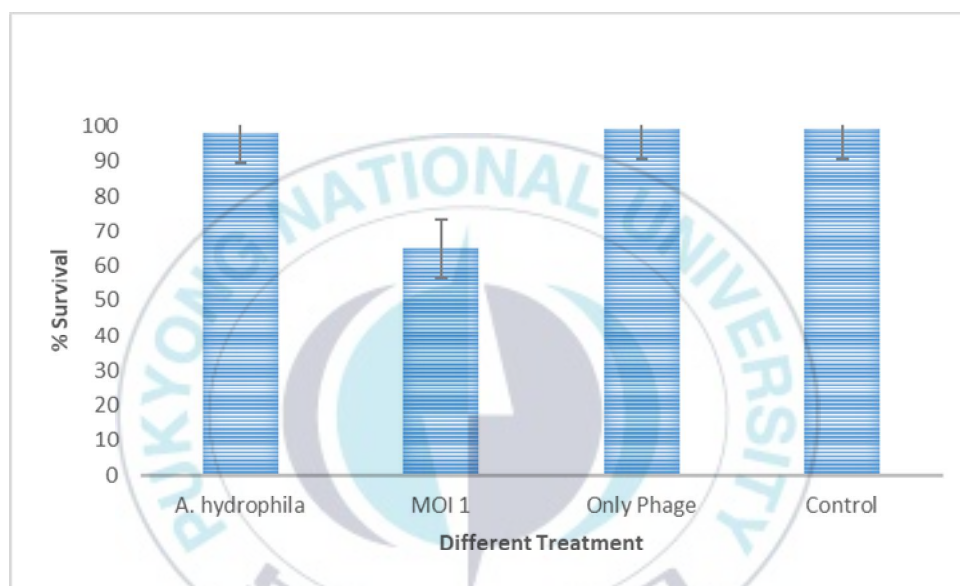


Fig. 8. Protective effects of Phage

Discussion

By 2050, the world greatest challenge would be the provision of the food to its 9 billion peoples. Aquaculture is the only fastest growing sector that can provide fish as a healthy and cheap source of food to the masses. Further expansion in aquaculture operations required for this massive production is under the constant threat of heavy production and financial losses due to bacterial disease (Nations, 2000; Giraud, 2004).

The excess use of antibiotics as chemotherapeutic agent has produced serious challenges, i.e. emergence of drug resistance bacteria, manipulation of organism's natural intestinal fauna and harmful effects towards the natural environment. Different studies recommended phage therapy as potential biocontrol approach to overcome described challenges (Chen et al., 2018; Kim & Park, 2018; Kwon et al., 2017).

A. hydrophila was described as leading cause of Motile Aeromonas septicemia all around the world in fresh water fish culture. Its infections in Loach and presence of multidrug resistance bacteria was previously reported by (Jun et al., 2013) in Korea.

In present study we isolated bacteriophage Akh-2 infecting *A. hydrophila* (KTCT 2358) from the sea water collected from Geoje Island, Korea. The abundance of phage is linked with their host population. The presence of highest bacterial count in sea water labels it as

a richest source of phage with an estimated population of 2×10^{35} . During the disease, water samples taken from site of infections can also be used for the isolation of specific phage. The chances are higher to find the specific phage due to abundance of their host pathogen (Housby & Mann, 2009; Phumkhachorn & P, 2018). Isolation of different phages from the sea, riverine and pond water was reported by (Easwaran et al., 2017; Haq et al., 2012; Le et al., 2018).

TEM image suggested that Akh-2 belonged to *Siphoviridae* family having icosahedral head and long non contractile tail, which is, in accordance with the previous observation made by (Ackermann, 2007). (Park et al., 2000) reported that *Siphoviridae* family phage can be used for the treatment in bacterial infection in fish, which promotes Akh-2 as a potential candidate for the phage therapy.

Bacteriophage infection can be initiated in accordance with the specific receptor molecule located on the surface of host cell. Most of the bacteriophages are found to be very specific for these receptors required for the attachment and penetration in host cell (Haq et al., 2011). Akh-2 host ranged result showed that it can effect only 4 out of 8 tested strains of *Aeromonas*, while no lytic effect were observed against the other non-*aeromonads* species. Same specific host range results were described by (Kim et al., 2012). The high specificity of Akh-2 can be described in beneficial way against the normal flora of aquatic organism.

One-step growth curve describe all the different stages involved in multiplication of bacteriophage. It is noticed that for every environmental conditions there is strong relationship between the latent period and burst size i.e. high level of phage fitness is associated with optimal latent time (Shivu et al., 2007). Phage Akh-2 has latent period 50 ± 5 min, which is almost same as phage AhSzq-1 and AhSzw-1 with 50 and 60 min respectively. The burst size of a phage mainly depends upon its plaque size and the latent period, while latent period can be affected by the type of phage, host and environmental conditions (Giraud, 2004; Weinbauer & Rassoulzadegan, 2003). The burst size of Akh-2 is about 139 ± 5 PFU/infected cell which is higher than phage AhSzq-1 and ahSzw-1 with 45 PFU/infected cell.(Yuan et al., 2018). The higher burst size accelerate the propagation of the phage to overcome the infection in short time. The shorter latent period and high burst size makes Akh-2 more suitable candidate for the phage therapy.

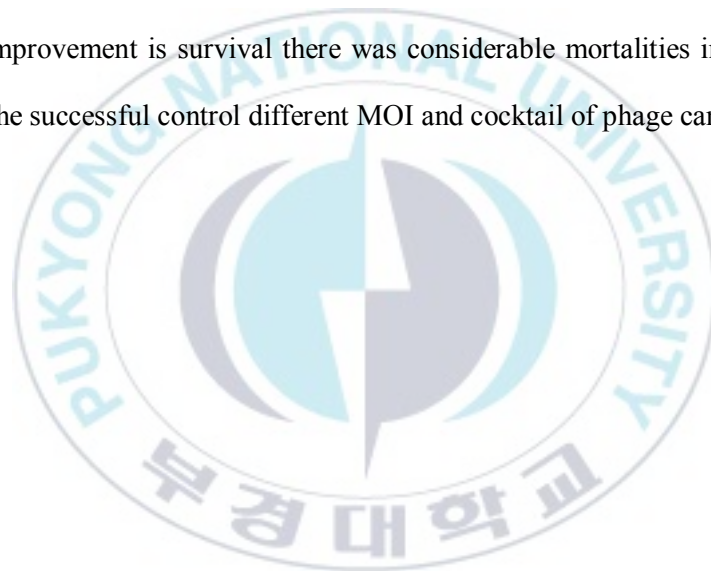
There are many factors that can affect the normal process of phage infection i.e. attachment, penetration and the multiplication, but temperature and pH play a vital role (Jończyk et al., 2011). During the farming of warm water fish species, optimum range is for temperature and pH is 25°C to 37°C , 7-9 respectively, to obtain best results phage must survive during these conditions. Akh-2 stability result against the tem and pH confirmed within the optimum range for the warm water fish farming. Phage pAh-1 can also tolerate optimum range of pH and temp for warm water farming operations as reported by (Easwaran et al., 2017). Akh-2 has an 114,901-bp double strand DNA with G±C content of 45.22 %, while other *Aeromonas* phage AhSzq-1 and AhSzw-1 has

112,586-bp and 115,739-bp double strand DNA. The genome size of Akh-2 is higher than AhSzq-1 and its amount is comparatively low as compare to AhSzq-1. The G±C % content of Akh-2 is higher than AhSzq-1 and AhSzw-1, which is only 43.96 and 43.82 %, respectively. A total of 141 and 431 ORFs were predicted for AhSzq-1 and AhSzw-1, which are comparatively less than Akh-2 as it predicted ORFs were about 254. While there were found 31 tRNA in Akh-2 that is greater than AhSzq-1. The tRNA are responsible for the provision of fitness to by recognizing high frequency codons (Yuan et al., 2018; Bailly-Bechet et al., 2007).

Aeromonas species had been reported as causative agent for the mass mortality in loaches in Korea. In some cases 50% mortality rate was observed even with the use of antibiotics, confirming the presence of antibiotic resistant strain of *Aeromonas* species (Jun et al., 2010; Garcia-Rodríguez et al., 2017). Alternatives to antibiotics are currently needed for effective and biological control of these bacteria. Bacteriophage had been using in controlling the bacterial infection, since their discovery. Their therapeutic effects have been confirmed in animals, human, crops and aquaculture (D'Herelle & Smith, 1926; Allan & Stevenson, 1981). Several studies reported isolation of *Aeromonas* phages (Wu et al., 1981; Easwaran et al., 2017; Vincent et al., 2017; Kim et al., 2012). Several researchers reported the genome analysis of phages as reported by (Yuan et al., 2018; Laanto et al., 2015), while other reported partial characterization of phage with determination of their protective effect in fishes (Le et al., 2018).

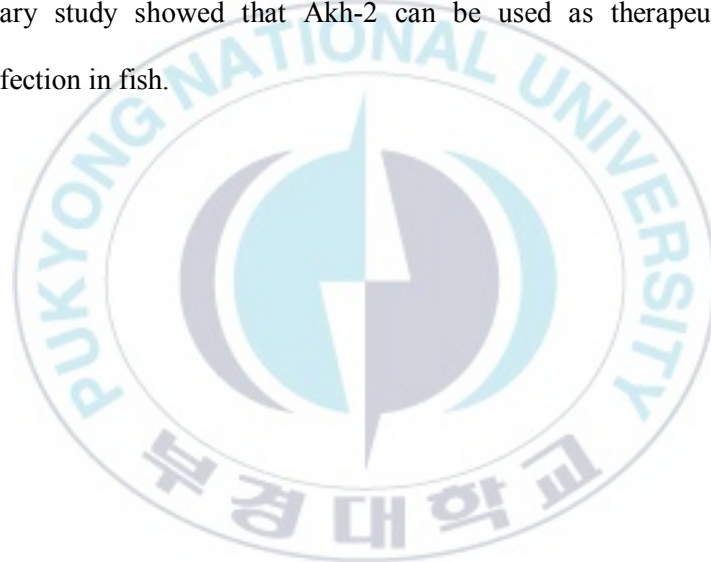
In present study we characterize and analyze the genome of newly isolated Akh-2 and determined it's protective in loach. For the pathogenicity trial, adipose fin clip methods followed by immersion in bacteria was used , as described by (Zhang et al., 2016b) , with a same lethal calculated by (Jun et al., 2010). Based upon on our preliminary experiment results, phage treated fish showed 60 % more survival and delay time when treated with MOI 1. Our result strongly suggest that phage can be used as therapeutic agent for the prevention of disease. These results are in line with those previous MOI 1 showed around 60 % survival rate as mentioned by(Jun et al., 2013).

Despite the improvement is survival there was considerable mortalities in phage treated loaches. For the successful control different MOI and cocktail of phage can be utilized.



Conclusion

In the present study we have isolated phages Akh-2 the fish pathogenic *A. hydrophila*. These phages were isolated from the water samples collected from the Geoje Island, South Korea. Akh-2 can survive at different ranges of temperature and pH, with a short latent period and highest size making it a more suitable candidate for the control of MAS. Our Preliminary study showed that Akh-2 can be used as therapeutic against the *Aeromonas* infection in fish.



References

- van der Aa Kühle, A., Skovgaard, K. & Jespersen, L. 2005. In vitro screening of probiotic properties of *Saccharomyces cerevisiae* var. *boulardii* and food-borne *Saccharomyces cerevisiae* strains. *International journal of food microbiology*.
- Ackermann, H.W. 2007. 5500 Phages examined in the electron microscope. *Archives of Virology*, 152(2): 227–243.
- Allan, B.J. & Stevenson, R.M. 1981. Extracellular virulence factors of *Aeromonas hydrophila* in fish infections. *Canadian journal of microbiology*, 27(10): 1114–1122.
- Bailly-Bechet, M., Vergassola, M. & E Rocha. 2007. Causes for the intriguing presence of tRNAs in phages. *genome.cshlp.org*.
- Baird, A.H., Guest, J.R. & Willis, B.L. 2009. Systematic and Biogeographical Patterns in the Reproductive Biology of Scleractinian Corals. *Annual Review of Ecology, Evolution, and Systematics*, 40(1): 551–571.
- Chen, L., Yuan, S., Liu, Q., Mai, G., Yang, J., Deng, D., Zhang, B., Liu, C. & Ma, Y. 2018. In Vitro design and evaluation of phage cocktails against *Aeromonas salmonicida*. *Frontiers in Microbiology*, 9(JUL): 1–13.

- D'Herelle, F. & Smith, G. 1926. *The bacteriophage and its behavior*.
- DA, E.A. & Didamony, G. El. 2016. New Approach to Use Phage Therapy against *Aeromonas hydrophila* Induced Motile *Aeromonas* Septicemia in Nile Tilapia. *Journal of Marine Science: Research & Development*, 6(3): 6–11.
- Defoirdt, T., Sorgeloos, P. & Bossier, P. 2011. Alternatives to antibiotics for the control of bacterial disease in aquaculture. *Current Opinion in Microbiology*, 14(3): 251–258.
- Easwaran, M., Dananjaya, S.H.S., Park, S.C., Lee, J., Shin, H.-J. & De Zoysa, M. 2017. Characterization of bacteriophage pAh-1 and its protective effects on experimental infection of *Aeromonas hydrophila* in Zebrafish (*Danio rerio*). *Journal of Fish Diseases*, 40(6): 841–846.
- Hoai, T.D., Nishiki, I. & Yoshida, T. 2016. Properties and genomic analysis of *Lactococcus garvieae* lysogenic bacteriophage PLgT-1, a new member of Siphoviridae, with homology to *Lactococcus lactis* phages. *Virus Research*, 222: 13–23.
- Housby, J.N. & Mann, N.H. 2009. Phage therapy. *Drug Discovery Today*, 14(11–12): 536–540.
- Imbeault, S., Parent, S., Lagacé, M., Uhland, C.F. & Blais, J.-F. 2006. Using Bacteriophages to Prevent Furunculosis Caused by *Aeromonas salmonicida* in

- Farmed Brook Trout. *Journal of Aquatic Animal Health*, 18(3): 203–214.
- Jaime Romero, C.G.F. and P.N. 2018. Antibiotics in Aquaculture – Use, Abuse and Alternatives. *Health and Environment in Aquaculture in Aquaculture*, 2: 64.
- Jończyk, E., Kłak, M., Międzybrodzki, R. & Górski, A. 2011. The influence of external factors on bacteriophages—review. *Folia Microbiologica*, 56(3): 191–200.
- Jun, J., Kim, J., Gomez, D., Jr, C.H.C., Han, J.E., Shin, S.P. & Park, S.C. 2010. Occurrence of tetracycline-resistant *Aeromonas hydrophila* infection in Korean cyprinid loach (*Misgurnus anguillicaudatus*). *African Journal of ...*, 4(9): 849–855.
- Jun, J.W., Kim, J.H., Shin, S.P., Han, J.E., Chai, J.Y. & Park, S.C. 2013. Protective effects of the *Aeromonas* phages pAh1-C and pAh6-C against mass mortality of the cyprinid loach (*Misgurnus anguillicaudatus*) caused by *Aeromonas hydrophila*. *Aquaculture*, 416–417: 289–295.
- Kim, J.H. & Park, S.C. 2018. Biological control of *Flavobacterium psychrophilum* infection in ayu (*Plecoglossus altivelis altivelis*) using a bacteriophage PFpW-3. *Korean Journal of Veterinary Research*, 58(1): 39–43.
- Kim, J.H., Son, J.S., Choi, Y.J., Choresca, C.H., Shin, S.P., Han, J.E., Jun, J.W., Kang, D.H., Oh, C., Heo, S.J. & Park, S.C. 2012. Isolation and characterization of a lytic myoviridae bacteriophage PAS-1 with broad infectivity in *Aeromonas salmonicida*. *Current Microbiology*, 64(5): 418–426.

- Kwon, A.S., Kang, B.J., Jun, S.Y., Yoon, S.J., Lee, J.H. & Kang, S.H. 2017. Evaluating the effectiveness of *Streptococcus parauberis* bacteriophage Str-PAP-1 as an environmentally friendly alternative to antibiotics for aquaculture. *Aquaculture*, 468: 464–470.
- Laanto, E., Bamford, J.K.H., Ravantti, J.J. & Sundberg, L.-R. 2015. The use of phage FCL-2 as an alternative to chemotherapy against columnaris disease in aquaculture. *Frontiers in Microbiology*, 6: 829.
- Le, T.S., Nguyen, T.H., Vo, H.P., Doan, V.C., Nguyen, H.L., Tran, M.T., Tran, T.T., Southgate, P.C. & Kurtböke, D.İ. 2018. Protective Effects of Bacteriophages against *Aeromonas hydrophila* Causing Motile *Aeromonas* Septicemia (MAS) in Striped Catfish. *Antibiotics*, 7(1): 16.
- Nandi, A., Banerjee, G., Dan, S.K., Ghosh, K. & Ray, A.K. 2017. Probiotic efficiency of *Bacillus* sp. in *Labeo rohita* challenged by *Aeromonas hydrophila*: assessment of stress profile, haemato-biochemical parameters and immune responses. *Aquaculture Research*, 48(8): 4334–4345.
- Nations, A.O. of the U. 2000. *The State of World Fisheries and Aquaculture, 2000*.
- Park, S.C., Shimamura, I., Fukunaga, M., Mori, K.-I. & Nakai, T. 2000. Isolation of bacteriophages specific to a fish pathogen, *Pseudomonas plecoglossicida*, as a candidate for disease control. *Applied and Environmental Microbiology*, 66(4):

1416–1422.

Phumkhachorn, P. & P, R. 2018. A LYTIC PODOPHAGE SPECIFIC TO FISH PATHOGENIC EDWARDSIELLA TARDA. *Pakistan journal of biotechnology*, 15(1): 117–121.

Richards, G.P. 2014. Bacteriophage remediation of bacterial pathogens in aquaculture: a review of the technology. *Bacteriophage*, 4(4): e975540.

Shivu, M., C.R, B., Girisha, S.K., Karunasagar, I., Krohne, G. & Karunasagar, I. 2007. Molecular characterization of *Vibrio harveyi* bacteriophages isolated from aquaculture environments along the coast of India. *Wiley Online Library*, 9(2): 322–331.

Verma, V., Harjai, K. & Chhibber, S. 2009. Characterization of a T7-Like lytic bacteriophage of *Klebsiella pneumoniae* B5055: A potential therapeutic agent. *Current Microbiology*, 59: 274–281.

Vincent, A.T., Paquet, V.E., Bernatchez, A., Tremblay, D.M., Moineau, S. & Charette, S.J. 2017. Characterization and diversity of phages infecting *Aeromonas salmonicida* subsp. *salmonicida*. *Scientific Reports*, 7(1): 1–10.

Weinbauer, M.G. & Rassoulzadegan, F. 2003. Are viruses driving microbial diversification and diversity? *Environmental Microbiology*, 6(1): 1–11.

Wommack, K.E. & Colwell, R.R. 2000. *Virioplankton: Viruses in Aquatic Ecosystems*.

Microbiology and Molecular Biology Reviews.

Wu, J., LIN, H., JAN, L. & Hsu, Y. 1981. Biological control of fish bacterial pathogen, *Aeromonas hydrophila*, by bacteriophage AH 1. *Fish Pathology*, 15(3): 271–276.

Yuan, S., Chen, L., Liu, Q., Zhou, Y., Yang, J., Deng, D., Li, H. & Ma, Y. 2018. Characterization and genomic analyses of *Aeromonas hydrophila* phages AhSzc-1 and AhSzw-1, isolates representing new species within the T5virus genus. *Archives of Virology*, 163(7): 1985–1988.

Zhang, D., Xu, D.H. & Shoemaker, C. 2016a. Experimental induction of motile *Aeromonas septicemia* in channel catfish (*Ictalurus punctatus*) by waterborne challenge with virulent *Aeromonas hydrophila*. *Aquaculture Reports*.

Zhang, D., Xu, D.H. & Shoemaker, C. 2016b. Experimental induction of motile *Aeromonas septicemia* in channel catfish (*Ictalurus punctatus*) by waterborne challenge with virulent *Aeromonas hydrophila*. *Aquaculture Reports*, 3: 18–23.