Characterization and protective effects of lytic bacteriophage pAh6.2TG against a pathogenic multidrug-resistant Aeromonas hydrophila in Nile tilapia (Oreochromis niloticus)

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Abstract

Bacteriophage is considered an alternative to antibiotics and environmentally friendly approach to tackle antimicrobial resistance (AMR) in aquaculture. Here, we reported isolation, morphology and genomic characterizations of a newly isolated lytic bacteriophage, designated pAh6.2TG. Host range and stability of pAh6.2TG in different environmental conditions, and protective efficacy against a pathogenic multidrug-resistant (MDR) *Aeromonas hydrophila* in Nile tilapia were subsequently evaluated. The results showed that pAh6.2TG is a member of the family *Myoviridae* which has genome size of 51,780 bp, encoding 65 putative open reading frames (ORFs), and is most closely related to *Aeromonas* phage PVN02 (99.33% nucleotide identity). The pAh6.2TG was highly specific to *A. hydrophila* and infected 83.3% tested strains of MDR *A. hydrophila* (10 out of 12) with relative stability at pH 7 9, temperature 0 40 $^{\circ}$C and salinity 0 40 ppt. In experimental challenge, pAh6.2TG treatments significantly improved survivability of Nile tilapia exposed to a lethal dose of the pathogenic MDR *A. hydrophila*, with relative percent survival (RPS) of 73.3% and 50% for phage multiplicity of infection (MOI) 1.0 and 0.1, respectively. Significant reduction of bacterial counts in rearing water at 3 h (6.7 $\pm$ 0.5 to 18.1 $\pm$ 6.98 folds) and in fish liver at 48 h post-treatment (2.7 $\pm$ 0.24 to 34.08 $\pm$ 26.4 folds) was observed in phage treatment groups while opposite pattern for bacterial counts was observed in untreated control. Interestingly, the surviving fish provoked specific antibody (IgM) against the challenged *A. hydrophila*. These results might explain the higher survival in phage treatment groups. In summary, the findings suggested that the lytic bacteriophage pAh6.2TG is an effective alternative to antibiotics to control MDR *A. hydrophila* in tilapia and possibly other freshwater fish.

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Highlights

- A lytic phage pAh6.2TG specific to multidrug-resistant (MDR)*Aeromonas hydrophila* isolates was isolated, identified and characterized in this study.
- pAh6.2TG was classified as a member of the family *Myoviridae*which has genome size of 51,780 bp, encoding 65 putative open reading frames (ORFs)
- pAh6.2TG was highly stable at pH = 7 9, temperature from 4 to 40°C, and salinity from 0 to 40 ppt.
- Phage pAh6.2TG significantly improved survivability of Nile tilapia challenged with the pathogenic MDR *A. hydrophila* with RPS of 50 73.3%

Abstract

Bacteriophage is considered an alternative to antibiotics and environmentally friendly approach to tackle antimicrobial resistance (AMR) in aquaculture. Here, we reported isolation, morphology and genomic characterizations of a newly isolated lytic bacteriophage, designated pAh6.2TG. Host range and stability of pAh6.2TG in different environmental conditions, and protective efficacy against a pathogenic multidrug-resistant (MDR) *Aeromonas hydrophila* in Nile tilapia were subsequently evaluated. The results showed that pAh6.2TG is a member of the family *Myoviridae* which has genome size of 51,780 bp, encoding 65 putative open reading frames (ORFs), and is most closely related to *Aeromonas* phage PVN02 (99.33% nucleotide identity). The pAh6.2TG was highly specific to *A. hydrophila* and infected 83.3% tested strains of MDR *A. hydrophila* (10 out of 12) with relative stability at pH 7 9, temperature 0 40 °C and salinity 0 40 ppt. In experimental challenge, pAh6.2TG treatments significantly improved survivability of Nile tilapia exposed to a lethal dose of the pathogenic MDR *A. hydrophila* , with relative percent survival (RPS) of 73.3% and 50% for phage multiplicity of infection (MOI) 1.0 and 0.1, respectively. Significant reduction of bacterial counts in rearing water at 3 h (6.7 ± 0.5 to 18.1 ± 6.98 folds) and in fish liver at 48 h post-treatment (2.7 ± 0.24 to 34.08 ± 26.4 folds) was observed in phage treatment groups while opposite pattern for bacterial counts was observed in untreated control. Interestingly, the surviving fish provoked specific antibody (IgM) against the challenged *A. hydrophila* . These results might explain the higher survival in phage treatment groups. In summary, the findings suggested that the lytic bacteriophage pAh6.2TG is an effective alternative to antibiotics to control MDR *A. hydrophila* in tilapia and possibly other freshwater fish.

Keywords: *Aeromonas hydrophila* , alternative to antibiotics, antimicrobial resistance, aquaculture, bacteriophage, multidrug resistance

INTRODUCTION

The farming of carps, tilapias, and catfishes accounts for 35.84% of world aquaculture production with revenue of 83 billion dollars in 2018. They contribute not only great economic value but also food and global nutrition security (FAO, 2020; Naylor et al., 2021). One of the challenges for sustainable aquaculture is production loss due to infectious diseases (Stentiford et al., 2020; Stentiford et al., 2017). *Aeromonas hydrophila* infection is considered one of the most important bacterial diseases responsible for the loss of millions of dollars in the global freshwater aquaculture industry (da Silva et al., 2012; Hossain et al., 2014; Peterman & Posadas, 2019; Pridgeon & Klesius, 2012). The control of this disease still heavily relies on antibiotics, especially in low-middle income countries (LMICs). Consequently, a global issue of concern of multidrug-resistant (MDR) *A. hydrophila* is becoming increasingly ubiquitous (Guz & Kozinska, 2004; Patil et al., 2016; Stratev & Odeyemi, 2016). Non-antibiotic approaches can minimize the requirement for antimicrobials to tackle infectious diseases in both animals and human health (Hoelzer et al., 2018). In the battle to combat *A. hydrophila* infection in aquaculture system, bacteriophage is one of the environmentally friendly approaches which replace or complement chemotherapy to reduce the hazard of bacterial disease and antimicrobial resistance in aquatic animals.

Lytic bacteriophages (also called phages) are unique viruses that can infect and kill bacterial cells (Kutateladze & Adamia, 2010). Phage therapy is a viable option to control bacterial infections due to their unique advantages, including high host specificity, rapid self-proliferation, and low intrinsic toxicity (Cao et al., 2021). For instance, Luo et al. (2018) injected phage HN48 with multiplicity of infection (MOI) = 1 (MOI represents the ratio of the numbers of virus particles to the numbers of the host cells) against *Streptococcus agalactiae* infection in Nile tilapia (*Oreochromis niloticus*) with relative percent survival (RPS) of 60%. Feeding phage cocktails of PVHp5 and PVHp8 showed protective effectiveness in turbot (*Scophthalmus maximus*) against *Vibrio harveyi* infection with RPS from 38.6 to 79.5% (Cui et al., 2021). In addition, intraperitoneal injection of phages FpV4 and FPSV-D22 showed protection of rainbow trout (*Oncorhynchus mykiss*) to *Flavobacterium psychrophilum* with RPS of 53.8%, while feed-based and bath administrations were not effective (Donati et al., 2021). Previous studies have demonstrated that phages can be applied in aquaculture to combat *A. hydrophila* infection (Anand et al., 2016; Cao et al., 2020; Dang et al., 2021; Jun et al., 2013; Le et al., 2018). Hence, strategy using phages for biocontrol of *A. hydrophila* has become increasingly attractive. The earlier studies have analyzed phenotypic and genotypic characterization, and evaluated protective effect of phages against *A. hydrophila* , including *Myoviridae* pAh1-C and pAh6-C (Jun et al., 2013); *Podoviridae* Ahp1 (Wang et al., 2016); *Myoviridae* pAh-1 (Easwaran et al., 2017);*Myoviridae* CT45P and TG25P (Hoang et al., 2019);*Podoviridae* MJG (Cao et al., 2020), *Myoviridae* AHP-1 (Chandrarathna et al., 2020); *Siphoviridae* Akh-2 (Akmal et al., 2020), *Podoviridae* LAh1-LAh6, *Siphoviridae* LAh7, and*Myoviridae* LAh10 (Kabwe et al., 2020); *Myoviridae* PVN-02 (Tu et al., 2020); *Myoviridae* AhyVDH1 (Cheng et al., 2021). In this study, we isolated and characterized specific an *A. hydrophila* phage from water sources in Mekong Delta, Vietnam. Subsequently, we evaluated its protective effects for juvenile Nile tilapia challenged with a pathogenic MDR *A. hydrophila* .

MATERIALS AND METHODS

Bacterial isolates

All bacterial strains used in this study are listed in Table 1. The isolates of *Aeromonas* , *Streptococcus* , and*Edwardsiella* were cultured in Tryptic Soy Broth (TSB; Becton Dickerson, USA) at 28 ˚C while *Lactobacillus*isolates were cultured in De man, Rogosa, and Sharpe (MRS, HiMedia, India) broth at 37 °C. All laboratory isolates of*Aeromonas* were previously isolated from diseased fish using selective medium, Rimler-Shotts agar (RS, HiMedia, India) supplemented with Novobiocin (Oxoid, UK), identified by PCR and sequencing of*gyr* B housekeeping gene (Navarro & Martínez-Murcia, 2018). Multidrug-resistant strains of *A. hydrophila* (Table S1) were identified based on the method proposed by Magiorakos et al. (2012).

Phage isolation and morphology

Preparation of host strain

The MDR *A. hydrophila* BT09 (Tables 1 and S1) was chosen as a bacterial host for phage isolation. Prior to phage isolation, prophage induction using Mitomycin C (Sigma-Aldrich, USA) was carried out as described by Walker et al. (2009) to ensure that the host cells do not contain prophage. Briefly, 100 µL of bacterial cells suspended in normal saline solution (OD_{600} = 0.6) was added into each of 10 mL of TSB supplemented with 250, 500, and 1,000 ng/mL of Mitomycin C. All cultures were incubated at 28 °C for 8 h. The induced phage production using Mitomycin C was evaluated by the Plaque Drop Assay (Adams, 1959).

Phage isolation

Water samples were collected from striped catfish culture ponds in Tien Giang Province, Vietnam. The samples were enriched to increase phage concentration according to Van Twest and Kropinski (2009) and isolated by Plaque Assay method described by Jun et al. (2013). Briefly, the samples were centrifuged at 4,500 *x g* , 4 °C for 30 min, and the supernatant was filtered through a 0.2 µm filter (Merck Millipore, USA) to remove residual bacteria cells. Then, 10 mL filtrate was mixed with 10 mL of *A. hydrophila* BT09 in TSB supplemented with 1.0 mM $CaCl_2$ and 0.5 mM $MgSO_4$(MTSB). The mixture was cultured at 28 °C for 24 h with 50 rpm shaking. The mixture was then centrifuged at 10,000 *x g* , 4°C for 15 min, and the collected supernatant was serially diluted (10^{-1} to 10^{-4}). A volume of 100 µL of each dilution was transferred to a tube containing 3.0 mL of TSA 0.5% agar supplemented with 1.0 mM $CaCl_2$and 0.5 mM $MgSO_4$ (MTSA), together with 100 µL of*A. hydrophila* . The mixture was vortexed lightly and poured onto a plate of TSA 1.5% agar. The plates were incubated at 28°C for 16 h and the growth of phages was observed (clear plaque on the plate). The individual clear plaque was picked and aseptically transferred to 200 µL of SM buffer (100 mM NaCl, 10 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.5). The mixture was vortexed vigorously and kept in 4 °C refrigerator overnight. The phages in SM buffer were obtained by filtering the supernatant through a 0.2 µm filter after centrifugation at 10,000 *x g* for 10 min. The filtrate was propagated four times continuously using the same protocol mentioned above for purification of the obtained phages. The isolated phages were stored in SM buffer supplemented with 30% glycerol at -80 °C until used.

Examination of phage morphology

The structure and size of the phage were determined by Transmission Electron Microscope (TEM). The specific procedure was as follows; the phage solution (3 mL) was centrifuged twice at 200,000 *x g* for 90 min. The pellets were resuspended in sterile distilled water. A volume of 50 µL of 1% glutaraldehyde (g/vol) was then added to immobilize the sample and rinsed with 0.1 M of cacodylate before proceeding with the dye. The samples were coated with 0.1% Poly-Lysine solution onto the surface of the 200-mesh carbon-coated grids to increase the adhesion of phages on the mesh. A volume of 10 µL of the phages was added to the grid and allowed to dry naturally for 5 min. The samples were dyed with 1% uranyl acetate sterilized with a 0.2 µm filter. The samples were washed with distilled water, allowed to dry for 5 min and imaged with a TEM-JEOL 1010 (Japan) with light projected through the grid for about 5 s at 80 kV. Phage morphology was classified according to the guideline of International Committee on Taxonomy of Viruses (ICTV) and Ackermann (2007).

2.3. Host range and specificity

The host range of phage pAh6.2TG isolated in this study was conducted on the collection of 17 *A. hydrophila* isolates from diseased fish (Tables 1 and S1). In this study, the Plaque Drop Assay was performed as described by Adams (1959) with minor modifications. Briefly, double-layer agar plates containing tested bacterial cells were prepared. Then, 5 µL of phages (10^8 PFU/mL) was dropped on the surface of each plate, kept without moving for 30 min and incubated at 28 °C for 16 h. Normal saline solution was used as negative control. Phage susceptibility was indicated by a clear zone appearing at the location of the drops while no clear zone indicated unsusceptible host. Specificity test of phage pAh6.2TG to other common aquatic pathogens (*Aeromonas veronii* ,*Aeromonas schubertii* , *Edwardsiella ictaluri* ,*Streptococcus agalactiae*) and probiotic

bacteria (*Lactobacillus fermentum* , *Lactobacillus plantarum*) (Table 1) was done in the same manner. All tests for host range and specificity were done in triplicates.

2.4. Phage stability in different environmental conditions

Stability of phage pAh6.2TG at different temperature (4, 25, 30, 35, and 40°C), pH (3, 5, 7, 9 and 11), salinity (0, 5, 10, 20, 40These tests were carried out by incubating 100 µL of phage culture (approx. 10^9 PFU/mL) at the respective temperatures, pH, and salinity for 1 and 24 h in 10 mL of SM buffer. All the experiments were conducted in triplicates. The stability of phages in rearing water was performed in duplicates by adding 2 mL of phage pAh6.2TG (approx. 8.5×10^{10} PFU/mL) into 50 L of water (pH = 7.0 ± 1.0, 0% NaCl) containing 20 of Nile tilapia and maintained at 30 ± 1.0 °C. The concentration of viable phages was enumerated by plaque assay (Jun et al., 2013). Phage concentration (logPFU/mL) before incubation in different conditions was set to be 100%.

2.5. Genome characterization

Phage genome extraction and next-generation sequencing

The phage particles prepared by liquid propagation in TSBM were desalted using Millipore Amicon ultra-centrifugal filter 10,000 NMWL (Merck, United States) at 10,000 *x g* , 4 °C for 15 min and concentrated by ultracentrifugation at 300,000 *x g* , 4°C for 3 h (Beckman Coulter, German). The pellets were resuspended in SM buffer. Phage genomic DNA was extracted using Phage DNA Extraction Kit (Cat. 46800, Norgen Biotek, Canada) following the manufacturer's protocol. Quality and concentration of DNA were measured by Nanodrop (Colibri, German) and Quibit 4.0 (Thermo Scientific, United States). Purified genomic DNA (3.15 ng/µL) was subjected to library preparation and sequencing using Next Generation Sequencing System with Illumina Novaseq 6000 platform (Pair-end, 150; library construction size, 350 bp; data output, 1.0 GB, data quality, Q30 > 80) at KTEST company, Vietnam.

Phage genome assembly and annotation

Raw reads were filtered using Fastp *v* 0.20.1 with the qualified phred score [?] Q25 and 8 bases trimming from 5'/3' end (Chen et al., 2018). Host associated sequences were filtered out by mapping trimmed reads to the genome of *A. hydrophila* type strain (accession no. NZ_CP016990.1) using Bowtie2 *v* 2.3.4.3 (Langmead & Salzberg, 2012). Only unaligned reads were subjected to genome assembly using Unicycler *v* 0.4.8 (Wick et al., 2017) on the Galaxy web platform at usegalaxy.org (Afgan et al., 2016). Potential phage sequence was identified by submitting the assembled contigs to PHASTER web server (Arndt et al., 2016). The predicted phage sequence (assigned as 'pAh6.2TG' in this study) was annotated using Prokka *v* 1.14.6 with Viruses annotation mode (Seemann, 2014). The annotated phage genome was visualized using DNAplotter (Carver et al., 2009).

Phage taxonomic identification and phylogenetic reconstruction

Identification of phage species was carried using VICTOR web service (Meier-Kolthoff & Göker, 2017). VICTOR is a tool that perform pairwise genome comparison of prokaryotic viruses and automatically constructs phylogenomic trees using Genome-BLAST Distance Phylogeny method (GBDP) with the formula D0. This tool also classifies the virus at the species, genus and family level with the taxon boundaries estimating by OPTSIL program (Göker et al., 2009). Herein, only the genomes of the viruses belonging to the family *Myoviridae* (n = 91) were included in this genome comparison since pAh6.2TG was predicted as an unknown*Myoviridae* by PHASTER tool described in the above section.

In addition to genome comparison, the phylogenetic analyses based on the terminase large subunit (terL) and major capsid protein (MCP) amino acid sequences of pAh6.2TG and other related species (predicted by VICTOR) were also performed via PhyloSuite v1.2.2 (Zhang et al., 2020). Amino acid sequences were aligned using MAFFT (Katoh & Standley, 2013) and the maximum-likelihood trees were constructed using IQ-TREE (Nguyen et al., 2015) with 5,000 ultrafast bootstraps and best-fit model (LG+G4) estimated by ModelFinder. Phylogenomic tree, terL- and MCP-based trees were visualized using Phandango (Hadfield

et al., 2018) and iTOL web tools (Letunic & Bork, 2019). Lastly, the protein sequence similarities between pAh6.2TG and the closest viral taxa were determined using CoreGenes3.5 web server with Blastp threshold score at 75 (Turner et al., 2013).

Effect of phage on Nile tilapia challenged with MDR *A. hydrophila*

Experimental fish

Healthy Nile tilapia (10.5 ± 4.7 g) obtained from a commercial tilapia hatchery in Thailand were acclimated for 2 weeks in dechlorinated tap water with aeration at 28 ± 1.0 °C before the experiments. The fish were fed with commercial tilapia feed (crude-protein 30%) at rate of about 3% of fish weight twice daily. Before starting the experiments, ten fish were randomly selected for bacterial isolation and found to be free of *A. hydrophila* . The experimental animal protocols were approved by Chulalongkorn University (Approval no. CU-IACUC 2031006).

Fish survivability and sample collection

This experiment aimed to investigate whether lytic phage treatment improves survivability of Nile tilapia challenged with a pathogenic MDR*A. hydrophila* BT14. A total of 258 fish were randomly divided into six groups with 2 replicate tanks per each group (Figure S1): Group 1 was exposed to culture medium without phage (no Ah + no phage); Group 2 was exposed to bacteria without phage (Ah + no phage); Group 3 was exposed to culture medium and phage pAh6.2TG at multiplicity of infection (MOI) = 0.1 (no Ah + phage 0.1); Group 4 was exposed to culture medium and treated with phage at MOI = 1.0 (no Ah + phage 1.0); Group 5 was challenged with *A. hydrophila* and treated with phage at MOI = 0.1 (Ah + phage 0.1); Group 6 was challenged with *A. hydrophila* and treated with phage at MOI = 1.0 (Ah + phage 1.0).

In bacterial challenge groups (2, 5 and 6), 1 L of MDR *A. hydrophila* BT14 (approx. 8×10^8 CFU/mL) was added to 50 L water to reach a final concentration of approx. 2×10^7 CFU/mL. Groups 5 and 6 tanks had 2 and 20 mL of phage pAh6.2TG (approx. 8.5×10^{10} PFU/mL) added to reach a final concentration of approx. 2×10^6 and 2×10^7 PFU/mL, respectively. Group 2 tank had 20 mL of SM buffer without phage added. The mixtures in groups 2, 5 and 6 were maintained at 29 ± 1.0 °C with aeration for 3 h. In culture medium exposure groups (1, 3 and 4), 1 L of TSB was added to 50 L water. Groups 3 and 4 tanks had 2 and 20 mL of phage pAh6.2TG (approx. 8.5×10^{10} PFU/mL) added, respectively. After 3 h, the fish were transferred to all groups, maintained at 29 ± 1°C with aeration for 14 days. In order to investigate the effect of phage on the concentration of *A. hydrophila* in rearing water, a volume of 25 mL water from groups 2, 5 and 6 were sampled at 3, 24 and 48 h after exposure with phage. A volume of 1 mL water was centrifuged at 4 °C, 10.000 x *g* , for 5 min. The supernatant were collected and diluted in SM buffer to measure concentration of phage by Plaque Assay method (Jun et al., 2013). The pellet was washed 1 time and re-suspended in 1 mL of PBS buffer. Bacterial concentration was then enumerated by conventional plate count method using RS supplemented with Novobiocin (Harrigan & McCance, 2014). In order to investigate the effect of phage on the concentration of *A. hydrophila* in liver, two fish from groups 2, 5 and 6 were sampled at 24, 48 and 72 h after exposure with phage. The fish were necropsied, and 0.1 g of live tissue was collected and homogenized in a microtube containing 900 µL of SM buffer. The samples were then centrifuged at 10.000 x *g* , for 5 min. The supernatant and pellet were used for respective phage and bacterial enumeration same as above.

The remaining fish were observed daily for 14 days, and mortality was recorded. Representative moribund or freshly dead fish were collected for bacterial re-isolation using RS supplemented with Novobiocin as described above. The RPS was calculated according to the formula described by Ellis (1988): RPS = (1 - % mortality in challenge / % mortality in control) * 100.

Determination of serum antibody by the enzyme-linked immunosorbent assay (ELISA)

For the comparison of specific antibody (IgM) levels against *A. hydrophila* between experimental groups, blood samples of 5 surviving fish in each tank (10 fish/group) were collected at the end of the experiment (day 14). Sera were collected after centrifugation at 8,000 x g for 15 min, stored at -20 °C until used. ELISA assay was carried out following the protocol described by Dien et al. (2021).

Statistical Analysis

Percent survival data from the challenge experiments was analyzed by the Kaplan-Meier method and differences among groups were tested using a log-rank test, p -values of 0.05 or less were considered to be statistically significant. Enumeration of *A. hydrophila*concentration and phage titer in rearing water and fish liver samples was analyzed by ANOVA. Dunnett post-hoc test was used to measure specific differences between pairs of mean. The OD_{450nm}readings from the indirect ELISA assay were analyzed using a Kruskal-Wallis test. Multiple comparison analyses were performed by Bonferroni test. All statistical analyses were performed using SPSS Software ver22.0 (IBM Corp., USA).

RESULTS

Prophage induction, phage isolation, and morphology

Although three doses of Mitomycin C (250, 500, and 1,000 ng/mL) were used for prophage induction, no plaque was detected, indicating that*A. hydrophila* BT09 did not contain prophage and was suitable as a bacterial host for lytic phage isolation. Subsequently, a phage, designated pAh6.2TG, was isolated from a freshwater sample. Phage pAh6.2TG produced medium, clear, and round plaques with diameter of 1.3 1.8 mm (Figure 1A-B) after 16 h of incubation. TEM morphology examination showed that the phage had an icosahedral head with 59.6 ± 2.5 nm diameter (n = 3) and a contractile tail which was 137 ± 10.2 nm in length and 20.2 ± 2.7 nm in diameter (n = 3) (Figure 1C-D). Based on the morphological features, phage pAh6.2TG was initially classified to the *Myoviridae* family.

Host range and specificity of phage pAh6.2TG

Among all bacterial isolates tested, pAh6.2TG showed lytic activity against 10/17 *A. hydrophila* isolates (Table 1) of which 8 isolates were MDR (Table S1). In contrast, no lytic activity was observed against other fish bacterial pathogens including *A. veronii* , *A. schubertii, E. ictaluri* , *S. agalactiae* as well as two probiotic bacteria *L. fermentum* , and *L. plantarum* (Table 1).

Stability of phage pAh6.2TG at different environmental conditions

Stability of pAh6.2TG at different temperatures (4 to 40°C) is shown in Figure 2A. Similar percentages of viable phage were detected after 1 h (96 ± 0.55 – 99.6 ± 0.08%) and 24 h (93 ± 0.23 – 98.6 ± 0.17%) of incubation, indicating that pAh6.2TG is a relatively thermostable phage.

Phage pAh6.2TG was stable (93.5 ± 1.69 – 97 ± 0.87%) at pH 7, 9 and 11 (Figure 2B). However, the phage pAh6.2TG was not stable at low pH. At pH 5, 93 ± 0.24% phage remained viable after 1h, and decreased sharply to 32.7 ± 0.44% (from 7.88 to 2.58 ± 0.06 logPFU/mL) after 24 h. At pH 3, only 15.2 ± 1.47% (1.19 ± 0.2 logPFU/mL) of phage was still viable after 1 h and reduced to undetectable level at 24 h (Figure 2B).

Phage pAh6.2TG was relatively stable at a wide range of salinity (0 – 40 viable after 1 and 24 h, respectively (Figure 2C).

In fish-rearing water (30 +- 1 °C, pH 6.9, 0% NaCl) spiked with phages, percentage of stability at 1 and 3 h were 99.5 +- 0.15% and 98.6 +- 0.11%, respectively. After 24 and 48 h, phage titer decreased slightly to 91.9 +- 0.85% and 91.3 +- 0.5%, equivalent to 6.52 +- 0.07 and 6.47 +- 0.03 logPFU/mL, respectively.

Genome characterization of pAh6.2TG phage

Based on the assembly graph generated by Unicycler software, pAh6.2TG was predicted to contain a circular genome with a length of 51,780 bp, a GC content of 52.48%, encoding 65 putative open reading frames (ORFs) (Table S2) without tRNA genes (Figure 3). According to bioinformatics prediction, pAh6.2TG genome consists of three main functional modules: i) phage structure and DNA packaging (major capsid protein, baseplate protein, tail fiber protein, and terminase subunit), ii) DNA metabolism and replication (RNA polymerase, DNA polymerase, DNA helicase, 5'-3' exonuclease, DNA ligase, and Ribonucleoside-diphosphate reductase large subunit), and iii) host lysis (cell wall hydrolase).

The closest phage taxonomic classification of pAh6.2TG toward other 91 *Myoviridae* phages in the public database revealed that *Aeromonas* phage pAh6.2TG and PVN02 (accession no. LR813619) were classified as the identical species with 99.33% identity. The result also showed total 64/65 ORFs were homologous between pAh6.2TG and PVN02 (97.3 - 100 % nt. identity), except for ORF03 that showed the highest homology (70%) to another *Aeromonas* phage pAh6-C (Table S2). Phylogenetic analysis based on whole genome (Figure 4A-B), major capsid protein sequence (Figure 4C), and terminase large subunit sequence (Figure 4D) confirmed high homology of phage pAh6.2TG and phage PVN02. In addition, pAh6.2TG was closely related to the *Aeromonas* phage pAh6-C (accession no. KJ858521), *Shewanella* phage Spp001 (accession no. NC023594), and *Shewanella* phage SppYZU05 (accession no. NC047824) (Figure 4).

In vivo **challenge results**

Phage pAh6.2TG improved survivability of Nile tilapia challenged with the MDR A. hydrophila

In vivo experiment showed that 100% fish in negative control group (no Ah + no phage) survived after 14 days, while only 25% survival was recorded in positive control group (Ah + no phage) (Figure 5). Interestingly, there was 62.5% and 80% survival in groups treated with pAh6.2TG with MOI = 0.1 (Ah + phage 0.1) and MOI = 1.0 (Ah + phage 1.0), respectively. These differences in percentage of survival of 2 phage treated groups were not statistically significant ($p = 0.154$) but statistically significant with positive control group ($p = 0.000$). The remaining two groups treated with phage without bacteria had 95 – 100% survival. The relative percent survival (RPS) of two treatments groups were 50% (MOI = 0.1) and 73.3% (MOI = 1), respectively. The moribund fish in challenge groups showed behavioral abnormalities (lethargy, loss of appetite, and surface swimming) and pale liver. Using selective medium, pure colonies with morphological characteristics of *A. hydrophila* were successfully isolated from representative dead fish (n=3).

Phage pAh6.2TG suppressed bacterial concentration in water and fish tissue

Fluctuation of bacterial concentration and phage titer in water and fish liver are shown in Figure 6 and Table S3. In rearing water, after 3 h of bacteria and phages exposure, bacterial concentration reduced 6.7 +- 0.5 folds in group treated with phage MOI = 0.1, and 18.1 +- 6.98 folds in group treated with phage MOI = 1.0 (Figure 6A). The calculation of fold changes is displayed in Table S3. In contrast, after 3 h, bacterial concentration increased 10.2 +- 3.15 folds in Ah + no phage group. Simultaneously, phage titer in groups treated with phage MOI = 0.1 and 1.0 after 3 h increased 51.04 +- 5.16 folds, and 20.98 +- 1.03 folds, respectively (Figure 6B). Phage was absent in Ah + no phage control group. At 24 h post-challenge, bacterial concentration in three groups was increased, while phage concentration in water slightly decreased. Besides, slight reduction of bacterial and phage concentration was observed in all groups at 48 h post-treatment (Figure 6A – B).

Moreover, in fish liver, bacterial concentrations of 5.8 +- 0.14, 5.52 +- 0.06, 5.51 +- 0.24 logCFU/g were recorded in Ah + no phage, Ah + phage 0.1, and Ah + phage 1.0 groups, respectively (Figure 6C). In Ah + phage 0.1 and Ah + phage1.0 groups, phage titers were 4.55 +- 0.2 and 4.75 +- 0.12 logPFU/g, respectively (Figure 6D). Similar pattern of phage concentration in rearing water was observed at 48 h post-challenge, while bacterial load decreased in all groups. In fish liver, compared to 24 h post-treatment, bacterial concentration in Ah + no phage groups increased 10.69 +- 3.85 folds, while in Ah + phage 0.1 and Ah + phage 1.0 groups, bacteria decreased 2.7 +- 0.24 and 34.08 +- 26.4 folds, respectively (Table S3).

The bacterial load in fish liver of Ah + no phage group decreased 3.8 +- 0.64 folds, from 6.58 x 10^6 +- 3.18 x 10^5 at 24 h post-challenge to 1.75 x 10^6 +- 2.12 x 10^5 CFU/g at 72 h post-challenge (Table S3). The same pattern was recorded in Ah+ phage 0.1 and Ah + phage 1.0 groups with 4.03 +- 0.83 and 2.18 +- 0.96 fold-reduction, respectively (Table S3). At 72 h post-challenge, phage titer in fish liver decreased 15.13 +- 3.35 and 13.96 +- 3.95 folds in groups treated with phage 0.1 and 1.0 at 24 h post-challenge, respectively (Table S3).

Surviving fish developed specific IgM against **MDR*A. hydrophila***

All surviving fish in three groups challenged with MDR *A. hydrophila* had significantly higher levels of

specific antibody (IgM) compared to the three unchallenged groups ($p < 0.05$) as measured by indirect ELISA, Kruskal - Wallis test: H (5) = 35.218,$p = 0.000$ (Figure 7). The serum from fish in the Ah + no phage, Ah + phage 0.1, and Ah + phage 1.0 groups had OD readings of 0.18 +- 0.09, 0.22 +- 0.17, and 0.22 +- 0.12, respectively. The IgM level was slightly higher in 2 phage treated groups but not statistically significant difference. In contrast, the low level of OD_{450} readings were recorded in the remaining groups (0.06 +- 0.003 to 0.08 +- 0.03) (Figure 7).

DISCUSSION

The *Myoviridae* phages specific to *A. hydrophila* are highly diverse in nature (Chandrarathna et al., 2020; Cheng et al., 2021; Jun et al., 2013). The lytic pAh6.2TG isolated in this study had genome characteristics most closely related to phage PVN02 (99.33% nt. identity) in the GenBank database, previously isolated from Vietnam (Tu et al., 2020). The origins of two phages from the closed geographical area of Mekong basin, although from different rivers, may explain the high genomic similarity of pAh6.2TG and PVN02. Compared to previously reported *A. hydrophila* -specific phages, pAh6.2TG (51,780 bp) had similar genome size with the phage PVN02 (51,668 bp) from Vietnam (Tu et al., 2020), and pAh6-C (53,744 bp) from Korea (Jun et al., 2015), but is larger than phage AhyVDH1 (39,175 bp) from China (Cheng et al., 2021), and smaller than phage LAh10 (260,310 bp) from Australia. The latter is the largest known phage infecting *A. hydrophila* (Kabwe et al., 2020). Genome analysis indicated that pAh6.2TG does not contain potential virulent genes or antimicrobial resistant genes, suggesting it is highly relevant as a biocontrol agent in aquaculture systems without concern of antimicrobial-resistant gene transmission.

Climate change has affected aquaculture environments by perturbing chemical and physical properties of water, particularly in the increase of water temperature and salinization (Maulu et al., 2021; Seggel & De Young, 2016). The stability of pAh6.2TG under a wide range of temperatures (4 – 40 ^{deg}C) and salinity (0 – 40 ppt) might be important characteristics for its wider application in diverse aquaculture environments. Relatively high stability of pAh6.2TG in fish-rearing water suggests that immersion route is practical. However, low viability of pAh6.2TG at pH 3 – 5 suggests that oral administration might not be applicable due to the low pH in gastrointestinal tract of aquatic animals, e.g. pH in Nile tilapia stomach range from 1.4 – 2.0 (Moriarty, 1973).

One of the major limitations of phage application is its narrow host range and geographical specificity (Culot et al., 2019; Perez-Sanchez et al., 2018; Ross et al., 2016). Although the newly isolated phage pAh6.2TG could lyse multiple isolates of MDR *A. hydrophila* from Vietnam, however, it does not lyse the isolates from Thailand and other bacterial species from the same or different genera. Therefore, to expand wider application of phage in aquaculture, a cocktail of multiple phage strains from different geographical locations might be the better approach to tackle not only AMR *A. hydrophila* but also other important bacterial pathogens in freshwater fishes. In addition, the specific infection of pAh6.2TG to *A. hydrophila* and not probiotic bacteria suggest the potential combination of phage therapy and probiotics to combat MDR *A. hydrophila* infection in aquaculture.

Carps, tilapias, and catfishes are crucial inland freshwater fish that play a vital role for food system transformation to tackle micronutrient deficiencies in LMICs (FAO, 2020; Hicks et al., 2019). *A. hydrophila* infection is one of the most important bacterial diseases responsible for the loss of millions of dollars in the global freshwater aquaculture industry (Hossain et al., 2014; Peterman & Posadas, 2019; Pridgeon & Klesius, 2012). Increasing prevalence of pathogenic MDR*A. hydrophila* in aquaculture poses the high risk for serious uncontrollable disease outbreaks and public health concern due to spread of AMR. Non-antibiotic approach using lytic phages, therefore, was explored to control disease caused by MDR *A. hydrophila* in aquaculture systems. In this study, we provided *in vivo* evidences for the efficacy of phage application in rearing water which is effective at suppressing bacterial concentration in water as well as reducing the bacterial load in fish liver. The presence of phages in the fish liver also suggests that immersion administration could deliver considerably large number of phages into the fish tissue. These factors may contribute to improvement of survivability (RPS = 50 – 75%) of tilapia. Importantly, not only was there higher survival in phage treated groups, but all surviving fish also developed specific IgM against *A. hydrophila* . This suggests that phages

possibly weakened the bacteria which allowed the fish immune system to respond more effectively and saved the fish from death. Similarly, there were several studies using phages as therapeutic agent to control *A. hydrophila* infection. Le et al. (2018) used phage cocktails (F2 and F5) with MOI = 0.01, 1.0, and 100 to control *A. hydrophila* infection in striped catfish (*Pangasianodon hypophthalmus*) by injection administration and obtained RPS of 16.33%, 44.9%, and 100%, respectively. Immersion treatment of 1 x 10^8 PFU/mL phage Akh-2 improved survivability of Nile tilapia with RPS of 41.1% (Akmal et al., 2020). Cao et al. (2020) applied phage MJG by injection, immersion, and oral administration to control a pathogenic *A. hydrophila* in rainbow trout and the fish gained RPS of 100%, 66.7%, and 50%, respectively. Dang et al. (2021) showed protective efficacy of phage PVN02-sprayed feed against *A. hydrophila* 4.4T in striped catfish with RPS from 75.6 – 87.8%.

The findings in this study suggest a potential approach using phage as prophylactic agent that was effective in protecting Nile tilapia from a MDR *A. hydrophila* . This approach provided comparable RPS to other promising alternatives to antibiotics, such as probiotic-based or plant-based products (Dawood et al., 2020; Kuebutornye et al., 2020; Naliato et al., 2021; Neamat-Allah et al., 2021). Apart from tilapia, pAh6.2TG has great potential to be applied in catfish aquaculture industry due to the lytic activity of pAh6.2TG against multiple MDR*A. hydrophila* strains isolated from diseased striped catfish.

In summary, this study reported a newly isolated lytic phage pAh6.2TG that infects several isolates of MDR *A. hydrophila* . The phage was classified as a member of *Myoviridae* based on a combination of morphology and genomic characterization. *In vitro* tests showed that pAh6.2TG was relatively stable at different environmental conditions. Using this phage as prophylactic agent was successful at reducing mortality in Nile tilapia. Phage pAh6.2TG application in rearing water not only suppressed MDR *A. hydrophila* loads in the rearing water and colonization of the bacteria in fish liver, but also improved fish survivability. These findings supported that pAh6.2TG could be used in rearing water for biocontrol of MDR *A. hydrophila* infection towards sustainable aquaculture.

NUCLEOTIDE SEQUENCE DATA

Phage pAh6.2TG sequence data has been submitted to the GeneBank databases under accession number MZ336020.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTION STATEMENT

Le Thanh Dien : Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft. **Le Buu Ky** : Investigation. **Bui The Huy** : Investigation. **Pattanapon Kayansamruaj:** Methodology, Data analysis. **Mohammad Fadhlullah Mursalim:** Investigation. **Saengchan Senapin** : Data curation, Review & Editing. **Channarong Rodkhum** : Supervision, Validation, Review & Editing. **Ha Thanh Dong** : Conceptualization, Data curation, Writing Review & Editing, Supervision, and Validation.

ETHICAL APPROVAL

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines

page, have been adhered to and the appropriate ethical review committee approval has been received. This project has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) in accordance with university regulations and policies governing the care and use of laboratory animals. The review has followed guidelines documented in Ethical Principles and Guidelines for Use of Animals for Scientific Purposes edited by the National Research Council of Thailand.

DATA AVAIBILITY STATEMENT

The data that support the findings of this study are available on request.

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Tables and Figures

Table 1. Bacterial strains used for determination of pAh6.2TG host range and specificity

Bacterial species	**Strain**	**Location**	**Source**	**Year**	**pAh6.2TG specific**	**References**
A. hydrophila	BT01	Ben Tre, Vietnam	Striped catfish	2018	-	Laboratory strain
	BT02	Ben Tre, Vietnam	Striped catfish	2018	-	Laboratory strain
	BT03	Ben Tre, Vietnam	Striped catfish	2018	+	Laboratory strain
	BT04	Ben Tre, Vietnam	Striped catfish	2018	+	Laboratory strain
	BT05	Ben Tre, Vietnam	Striped catfish	2018	+	Laboratory strain
	BT12	Ben Tre, Vietnam	Striped catfish	2018	+	Laboratory strain
	BT09^{+}	Ben Tre, Vietnam	Striped catfish	2018	+	Laboratory strain
	BT13	Ben Tre, Vietnam	Striped catfish	2018	+	Laboratory strain
	BT14$^{\#}$	Ben Tre, Vietnam	Striped catfish	2018	+	Dien et al. (2021)
	BT22	Ben Tre, Vietnam	Striped catfish	2018	+	Laboratory strain
	TG26	Tien Giang, Vietnam	Striped catfish	2018	+	Laboratory strain
	TG35	Tien Giang, Vietnam	Striped catfish	2018	+	Laboratory strain
	CUVET02	Chonburi, Thailand	Asian seabass	2020	-	Laboratory strain
	CUVET21	Chonburi, Thailand	Walking catfish	2020	-	Laboratory strain
	CUVET46	Kanchanaburi, Thailand	Nile tilapia	2020	-	Laboratory strain
	CUVET52	Uttaradit, Thailand	Nile tilapia	2020	-	Laboratory strain
	CUVET92	Kanchanaburi, Thailand	Nile tilapia	2020	-	Laboratory strain
A. veronii	NK01	Nongkhai, Thailand	Nile tilapia	2014	-	Dong et al. (2015a)
	NK02	Nongkhai, Thailand	Nile tilapia	2014	-	Dong et al. (2015a)
	NT03	Pathum Thani, Thailand	Nile tilapia	2016		Dong et al. (2017)
A. schubertii	N1	Tra Vinh, Vietnam	Snakehead fish	2016	-	Laboratory strain
	N3	An Giang, Vietnam	Snakehead fish	2016	-	Laboratory strain
	N7	Dong Thap, Vietnam	Snakehead fish	2016	-	Laboratory strain

E. ictaluri	T1-1	Ratchaburi, Thailand	Striped catfish	2014	-	Dong et al. (2015b)
S. agalactiae	2809	Thailand	Nile tilapia	2018	-	Jhunkeaw et al. (2021)
L. fermentum	VTCC 11051	Vietnam	Pickles	2009	-	Vietnam Type Culture Collection
L. plantarum	VTCC 10890	Vietnam	Pickles	2009	-	Vietnam Type Culture Collection

+MDR strain used in *in vitro* assays, #MDR strain used in challenge test.

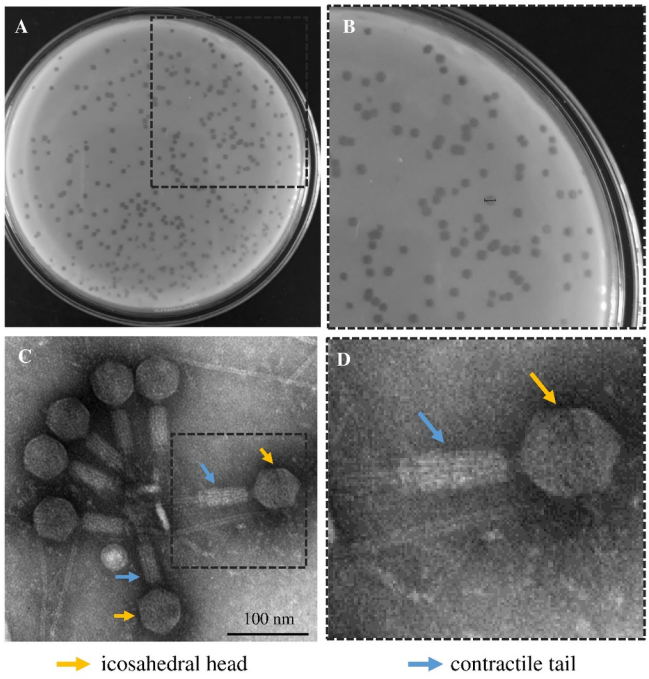


Figure 1. (A & B) Plaques of pAh6.2TG on double layer TSA. (C & D) Transmission electron micrographs of pAh6.2TG.

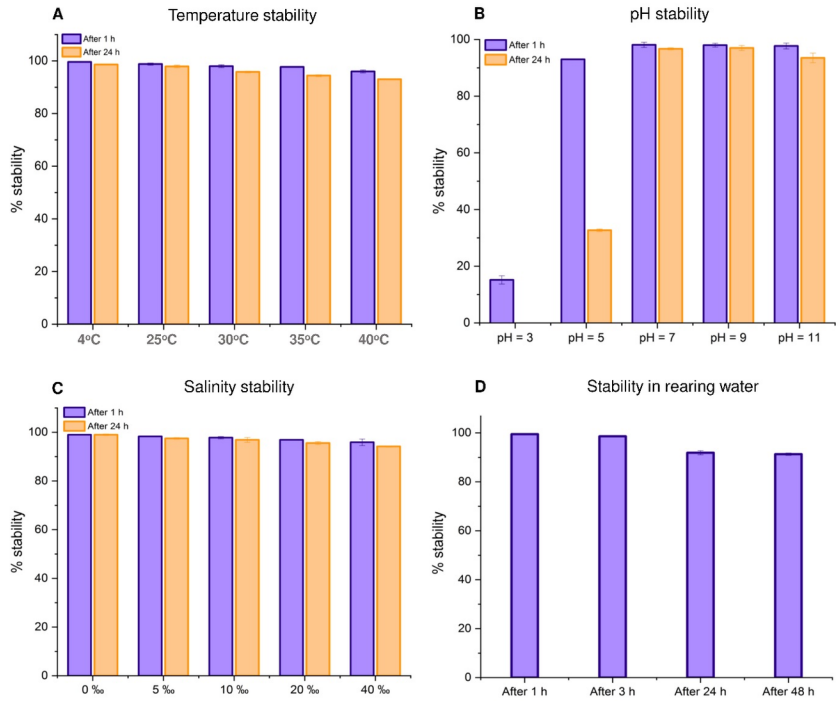


Figure 2. Stability of phage pAh6.2TG. (A) Temperature stability. (B) pH stability. (C) Salinity stability. (D) Stability in rearing water. Value of % stability are mean ± a standard error of the mean (SEM) bar (n = 3 in Figure A, B, C and n =2 in Figure D).

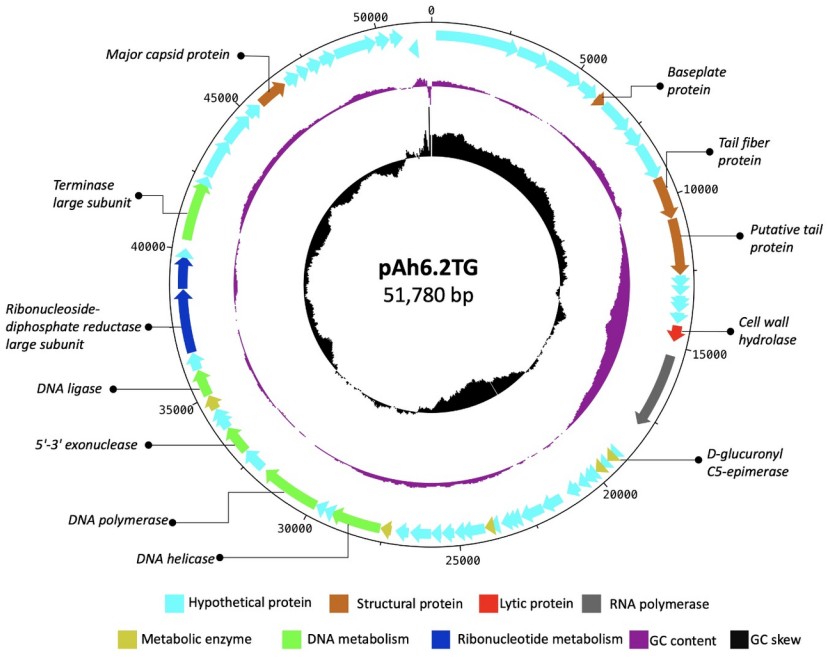


Figure 3. Genome map of phage pAh6.2TG. Arrows represent the annotated ORFs which are shown in various colors indicating their predicted protein function. Two inner rings represent the GC skew (in black) and GC content (in violet). Some genes of interest are marked.

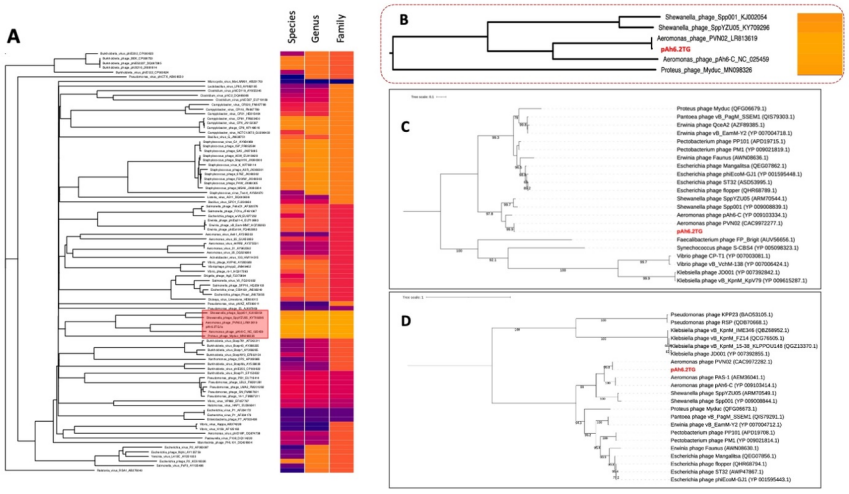


Figure 4. Phylogenetic analyses based on (A and B) whole genome, (C) major capsid protein sequence, and (D) terminase large subunit. (A) Comparative genomics ($n = 91$) was performed using VICTOR web server with settings recommended for prokaryotic viruses. Clustering of viruses at species, genus, and family level was determined automatically and allocated by color strips adjacent to the phylogenomic tree (same color representing identical taxonomic unit). Red box indicates phage pAh6.2TG and its monophyletic taxa which is magnified in panel B. Trees based on (C) major capsid protein and (D) terminase large subunit were constructed by the maximum-likelihood method with 5,000 ultrafast bootstrapping. Bootstrap value (in percentage) is shown at the node, whereas scale bar indicates amino acid substitution per site. Red taxon represents the phage of this study (pAh6.2TG).

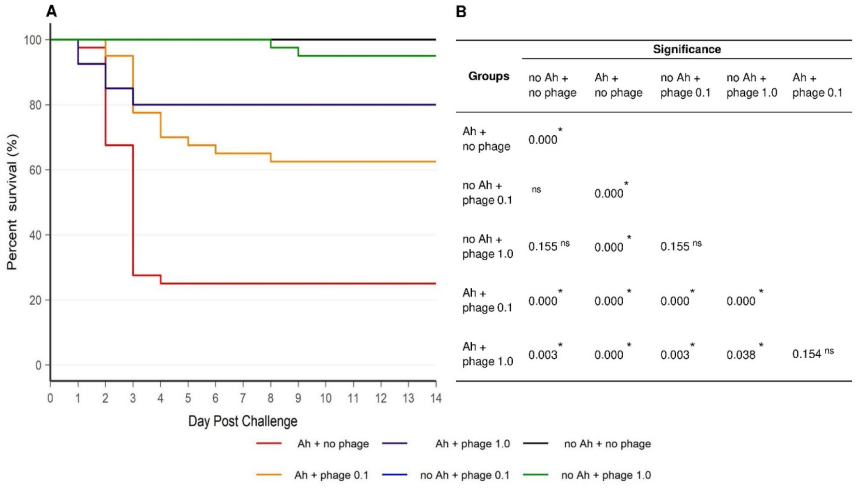


Figure 5. Kaplan - Meier analysis of percentage survival of Nile tilapia (n = 40) challenged with MDR *A. hydrophila* BT14 (A). Differences between groups were tested using log-rank test (B). "*" denotes significant difference ($p < 0.05$), and "ns" means not significant.

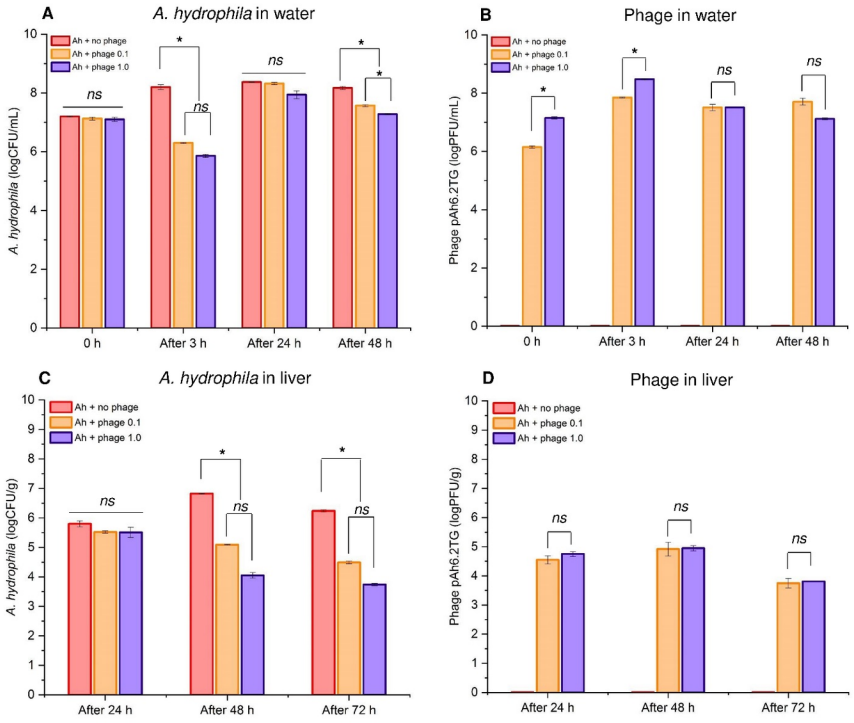
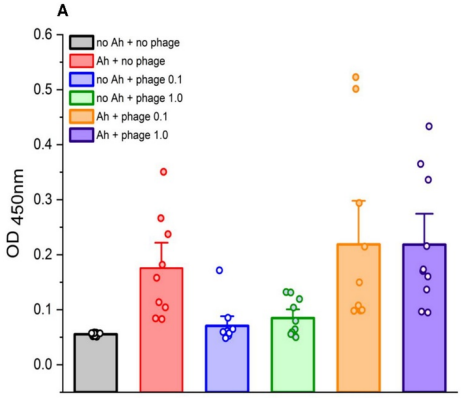


Figure 6. Enumeration of *A. hydrophila* concentration and phage titer in rearing water and fish liver samples. (A) *A. hydrophila* concentration in rearing water (logCFU/mL). (B) Phage pAh6.2TG titer in rearing water (logPFU/mL). (C) *A. hydrophila*concentration in fish liver (logCFU/g). (D) Phage pAh6.2TG titer in fish liver (logPFU/g). Value of *A. hydrophila* concentration and phage titer are mean ± a standard error of the mean (SEM) bar (n = 2) and "*" above the bar indicates significant difference between groups ($p < 0.05$), "*ns*" means not significant.



B

Groups	Significance				
	no Ah + no phage	Ah + no phage	no Ah + phage 0.1	no Ah + phage 1.0	Ah + phage 0.1
Ah + no phage	0.002 *				
no Ah + phage 0.1	1.000 ns	0.037 *			
no Ah + phage 1.0	1.000 ns	0.352 ns	1.000 ns		
Ah + phage 0.1	0.001 *	1.000 ns	0.018 *	0.212 ns	
Ah + phage 1.0	0.000 *	1.000 ns	0.005 *	0.069 ns	1.000 ns

Figure 7. Indirect ELISA analysis of *A. hydrophila*specific IgM antibody. Fish sera were collected on day 14 and dilutions with 1:256 were used to test for antigen specific IgM. Data were expressed as mean absorbance at OD_{450nm} with a SEM bar (A). One dot represents one biological replicate (n = 9 in group Ah + no phage, n = 10 in other groups). Differences between groups were tested using log-rank test (B). "*" denotes significant difference ($p< 0.05$), and "ns" means not significant.