Isolation and characterization of bacteriophages against virulent *Aeromonas hydrophila*

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Abstract

Background: *Aeromonas hydrophila* is an important water-borne pathogen that leads to a great economic loss in aquaculture. Along with the abuse of antibiotics, drug-resistant strains rise rapidly. In addition, the biofilms formed by this bacterium limited the antibacterial effect of antibiotics. Bacteriophages have been attracted increasing attention as a potential alternative to antibiotics against bacterial infections.

Results: Five phages against pathogenic *A. hydrophila*, named N21, W3, G65, Y71 and Y81, were isolated. Morphological analysis by transmission electron microscopy revealed that phages N21, W3 and G65 belong to the family *Myoviridae*, while Y71 and Y81 belong to the *Podoviridae*. These phages were found to have broad host spectra, although their burst sizes were varied per infected host cell at their optimal multiplicity of infection. They were sensitive to high temperature but had a wide adaptability to the pH. In addition, the phages G65 and Y81 showed considerable effect on growth and biofilm inhibition of *A. hydrophila*; and the phages G65, W3 and N21 were able to scavenge mature biofilm effectively. Phage treatments applied to the pathogenic *A. hydrophila* in mice model resulted in a significantly decreased bacterial loads in tissues.

Conclusions: Five *A. hydrophila* phages were isolated with broad host ranges, low latent periods, and wide pH and thermal tolerance. And the phages exhibited varying abilities in controlling *A. hydrophila* infection. *This work* presents promising data supporting the future use of phage therapy.

Background

Aeromonas hydrophila is a Gram-negative, rod-shaped bacterium that is ubiquitous found in natural aquatic environments. The bacterium is predominantly pathogenic to poikilothermy animals, including fish, frogs, turtles, snakes and amphibians [1]. It is responsible for the large-scale outbreak of fish hemorrhagic septicemia, leading to severe economic losses to aquaculture industry worldwide [2]. The bacterium is also an important pathogen that infects humans and other mammals, causing gastroenteritis and various systemic infections. *A. hydrophila* is a very typical pathogen of human-animal-fish comorbidity [1].

In general, the prevention and treatment of diseases depends mainly on extensive application of antimicrobial agents [3, 4]. Antibiotics not only kill the target bacteria, but might also disrupt the host's normal flora and the ecological balance of the water environment [5]. With the frequent use of antibacterial drugs in aquaculture, multidrug resistance (MDR) of *A. hydrophila* strains are emerging; and drug residues in aquaculture products and the environment are getting worse [6-8]. Vivekanandhan et al. [9] indicated that 99% of *A. hydrophila* strains isolated from fish and prawns were resistant to methicillin, rifampicin, bacitracin, and novobiocin. Recently, De Silva et al. [10] tested antibiotic resistance of 32 strains of *Aeromonas*, and found that all the isolates were multidrug resistant and 100% resistant to ampicillin, colistin, vancomycin and cephalothin. In addition, each of the 43 *Aeromonas* strains isolated by Hossain et al. [11] from 46 zebrafish was resistant to at least four antibiotics. MDR strains can

reproduce and pass its resistance on, creating many more antibiotic resistant bacteria [12, 13]. Additionally, the biofilm formation is also an important factor that obstructs bacterial killness with antibiotics and induces the generation of MDR strains, since biofilms can form a protective barrier for bacteria to avoid drug penetration[14, 15]. Thus, new antimicrobial therapies are urgent to be developed.

Bacteriophages, naturally-occurring bacterial viruses that can kill specific bacteria with no chemical residues and does not affect other flora, are one of the potential alternatives. A small dose of phage can achieve a good therapeutic effect as phage proliferation via auto “dosing” results in great bacterial killing [16]. One previous study from Nishikawa et al. [17] reported that phages KEP10 intraperitoneally injected into mice could immediately spread to all organs examined and maintained stability and a high titer; and treatment of the phages into the peritoneal cavity significantly decreased the mortality of mice inoculated transurethrally with a multidrug-resistant strain of uropathogenic *Escherichia coli* (UPEC). Another study indicated that infection of methicillin-resistant *Staphylococcus aureus* with subsequent administration of purified phage phi MR11 effectively suppressed *S. aureus*-induced bacteremia and lethality in mice [18]. Phage therapy has also shown its efficacy in several cases of *Aeromonas* diseases. Jun et al. [19] reported that phages pAh1-C and pAh6-C could provide protective effects of the against mass mortality of the cyprinid loach caused by *A. hydrophila*. Additionally, therapeutic treatments of *A. hydrophila*-phage 2 and *A. hydrophila*-phage 5 applied to the catfish during bacterial infection resulted in a significantly enhanced survival of the tested fishes [20]. However, not all phages make for good therapeutics. In general, the narrowness of phage host ranges will limit putative treatment and the poor of phage stability will reduce treatment efficiency. Thus, it is necessary to isolate stable phages with relatively wide host ranges for phage therapy.

In this study, we isolated and characterized five phages specific to *A. hydrophila*. Furthermore, we investigated the efficiency of the phages for biofilm inhibition and removal, and performed a phage therapy experiment in *A. hydrophila*-infected mice.

Results

Isolation of *A. hydrophila* phages

Three phages designated as N21, W3, G65 with *A. hydrophila* NJ-35 as an indicator host, and two phages Y71 and Y81 with *A. hydrophila* XY-16 as an indicator host, were isolated from fish ponds and polluted rivers in Nanjing. Clear plaques appeared after 12 h incubation at 28 °C. As shown in Fig. 1A, plaques of all the five phages were morphologically similar with diameter of 1 mm to 3 mm, and transparent in the middle. The plaque edges of phages W3, Y71 and Y81 were clear with no halo, while those of phages N21 and G65 were blurred with haloes.

Phage morphology

Purified phages were examined by TEM and classified based on the criteria proposed by Ackermann [21]. TEM observation revealed that all the phages (Fig. 1B) had tails and thus belonged to the order

Caudovirales. Phages N21, W3 and G65 possessed a morphology typical of the *Myoviridae* family, displaying an icosahedral head with the diameter of (62.6 ± 1.9) nm, (64.9 ± 3.2) nm and (58.8 ± 4.1) nm, respectively, a contractile tail with the length of (153.1 ± 6.2) nm, (154.1 ± 1.4) nm and (152.3 ± 9.8) nm, respectively, and collar or whisker structures. Phages Y71 and Y81 morphologically belong to the *Podoviridae* family, possessing an icosahedral head of (62.8 ± 1.1) nm and (54.8 ± 2.9) nm in diameter, respectively, and a short tail of (22.0 ± 0.2) nm, (20.0 ± 2.2) nm in length, respectively.

Host ranges

The host range was determined against a few *Aeromonas* species, representative of major pathogens of aquatic organisms. As shown in Table 1, of a total of 205 isolates tested, it was found that 22.67% (n=17), 21.33% (n=16), 21.33% (n=16), 20% (n=15) and 22.67% (n=17) of 75 *A. hydrophila* isolates were susceptible to phages N21, W3, G65, Y71 and Y81. Additionally, all of the phages could infect one strain of *A. caviae*; phages N21, W3, G65 also showed infectivity to *A. veronii* of 1, 3, and 3 strains, respectively; and phage W3 was able to infect the only *A. bestiarum* isolate tested in this study, showing broad infectivity against phylogenetically distant species in *Aeromonadaceae*.

Determination of the multiplicity of infection (MOI)

A. hydrophila cultures of exponential growth phase were infected with different amount of phages as designed. The phage titers were measured after incubation for 2 h. The results indicated that the optimal MOIs of phage isolates N21, W3, G65, Y71 and Y81 were 0.01, 1, 0.001, 0.1 and 0.001, respectively, which gave the highest production of phage progeny (Table 2).

Latent times and phage burst sizes

Single step growth experiment was performed to determine the latent time and phage burst size. As shown in Fig. 2, the latent periods of all five phages were found to be about 10 min, and the burst sizes of phages N21, W3, G65, Y71 and Y81 were 703 PFU, 99 PFU, 2031 PFU, 262 PFU and 5203 PFU per infected host cell at their optimal MOIs, respectively.

pH and thermal stability

The pH and thermal stabilities of phages were estimated by determining the changes in survival based on the number of plaque-forming units (PFU). As shown in Fig. 3, growth of phage N21 showed no obvious change after 2 h incubation at pH5.0-11.0, but 75.26% recovery at pH4.0. The survival of phage W3 could maintain relatively stable at pH4.0-10.0; very few phages could recovery at pH3.0 or pH11.0. Phage Y81 displayed similar pH stability to phage W3. More than 75% phage G65 could survive at pH4.0-11.0. Phage Y71 showed relatively stable between pH5.0-10.0. The data suggested that the phages can remain active under a wide range of pH conditions but sensitive to strong acid or alkali.

Thermal stability of the isolated phages was assayed at pH7.0. All of the five phages maintained almost 100% infectivity after culture at 4 °C or 30 °C for one day (data not shown). Fig. 4 showed that all the

phages remained relatively stable at 30°C and 40°C, but sensitive to higher temperatures. No more than 50% phages remained alive after a 40-minute incubation at 50°C. At 60°C, no more than 1% of phages W3, G65, and Y81 survived for 20 minutes, and phages N21 and Y71 for 40 minutes.

Inhibition and clearance of biofilms

As shown in Fig. 5A, 70.07% of the growth of *A. hydrophila* NJ-35 was significantly inhibited by phage G65 even at a small MOI of 0.01, and the inhibition rate could reach 72.01% by phage W3 at a MOI of 100; phage N21 showed limited growth inhibition to *A. hydrophila* NJ-35. Additionally, phage Y81 other than Y71 showed considerable growth inhibition to *A. hydrophila* XY-16. Similar to the growth inhibition, biofilm formation of *A. hydrophila* NJ-35 could be significantly repressed when cocultured with phage G65 at the MOI of 0.01 (63.11%) and of 100 (88.59%); similar but better biofilm inhibition could be observed when *A. hydrophila* XY-16 was cocultured with phage Y81. Phage W3 inhibited 74.41% biofilm formation at the MOI of 100. Phage N21 and Y71 exhibited poor inhibitory effects on biofilms (Fig. 5B).

The biofilms of *A. hydrophila* strain NJ-35 were formed for 24 h and then treated with phages N21, W3 and G65 with the titer of 1.0×10^8 PFU/mL, respectively; and the biofilms of *A. hydrophila* strain XY-16 were treated with phages Y71 and Y81, respectively. As shown in Fig. 6, total biofilm biomass showed an obvious reduction in a time-dependent manner after 6 h of phage treatment. For the biofilm formed by *A. hydrophila* NJ-35, phage G65 and W3 showed a strong clearance efficiency, and the clearance rates reached 65.24% and 62.68% after treating for 6 h and up to 75.12% and 73.35% after treating for 24 h, respectively. Phage N21 also showed well biofilm elimination efficiency after treating for 12 h (63.67%). However, the ability of phage Y81 and Y71 to remove biofilm formed by *A. hydrophila* XY-16 was limited.

Therapy assay

To corroborate whether the phages can prevent the proliferation of *A. hydrophila in vivo*, we performed an infection and therapy assay in mice. Mice infected with *A. hydrophila* were sacrificed at 6 h and 24 h post-treatment with phages, and hearts, livers, spleens, lungs and kidneys were collected for determination of bacterial loads. As shown in Fig. 7, phage N21 and Y81 treatment lead to significant decrease of the bacterial loads in all tested tissues at 6 h compared to the non-treated controls. After treating for 24 h, the bacterial loads in all tissues of phage-treated groups showed significant decrease compared to the non-treated controls. The results indicated that the phages can act as scavengers to eliminate pathogens *in vivo*.

Discussion

Generally, virion morphology is the most important criterion for the bacteriophage classification except the type of nucleic acid [22]. According to the order of Caudovirales, bacteriophages that are characterized as tailed phages have been classified into three families, including *Myoviridae*, *Podoviridae*, and *Siphoviridae*. Most bacteriophages reported against *Aeromonas* spp. have been characterized as tailed phages, and the family *Myoviridae* were found to be the majority [20, 21, 23, 24]. For instance,

Ackermann [21] indicated that 33 of 43 *Aeromonas* phages investigated belonged to the *Myoviridae* family. Also, several *Aeromonas* phages belonging to the family *Podoviridae* have been isolated and characterized [25-27]. In this study, phage N21, W3, and G65 had an icosahedral head, and a relatively short yet wide contractile tail with a complex baseplate structure at the remote end of the tail. The structures conformed to the typical structural features of *Myoviridae* phage and were similar to a novel virulent phage AHP-1 recently identified [28]. Phage Y71 and Y81, possessing an icosahedral head with a short tail, were tentatively classified as members of *Podoviridae* family.

Broad host range usually exhibits great advantages in combating multiple pathogen infections. In general, phage showed narrow host specificity that only infect its indicator host [29, 30], although there are also some *Aeromonas* bacteriophages that show wide host spectrum [31]. Our study revealed that the phages isolated here had relatively broad spectrum of infectivity against *A. hydrophila* and showed the potential to infect *A. caviae*, *A. veronii* and *A. bestiarum*. Short latent period and high burst size are essential features for highly effective phages serving as therapeutic agents [32]. Previous study demonstrated that a T4-like bacteriophage BPA6 infecting *A. hydrophila* had a burst size of 244 phage particles per cell with approximately 10 min of latent period [33]. Similar latent periods were observed in our five phages, and phages N21, G65 and Y81 showed higher burst sizes. Moreover, the phages isolated in our study, being more resistant to alkali than acid condition, showed a broad pH tolerance compared to most isolated phages [30, 34-36]. Additionally, the phages were found to be relatively stable up to 40 °C. The high pH and thermal resistance made the phages be potential for possible treatment of *A. hydrophila* infection or contamination in the diverse physicochemical environments. Expectively, phage therapy in mice model resulted in a decreased bacterial loads in tissues compared to the control groups, suggesting a potential role for phages in controlling *A. hydrophila* infection. However, the limitation is that mice might not be the best model to evaluate the treatment effect of phages on *A. hydrophila* infection, which mainly leads to the large-scale outbreaks in freshwater fish. Additionally, different bacterial strains of the same species or different bacterial species are often present in an infection [37]. Thus, the effectiveness of the phages against multiple bacterial strains might need to be tested and phage cocktails be prepared. In addition, previous studies have revealed that phage therapy may be most effective when combined with antibiotics since phages is likely to enhance the absorb of antibiotics into bacterial cells [38-41]. Further study is required to evaluate the effectiveness of the combining phages with antibiotics in the treatment of *A. hydrophila* infection.

In aquatic environments, biofilm formation plays important roles in the survival and pathogenicity of pathogens [42]. *A. hydrophila* is capable of forming biofilms on host tissues, as well as multiple biotic and abiotic aquaculture substrates[43]. The extracellular biofilm matrix serves as a scaffold that protects the bacteria encased in the matrix from various harmful environment including antimicrobial agents and cellular host defenses[44]. Biofilm formation is a major barrier to the current treatment method relying on antibiotics. Although there have been several studies on controlling *Aeromonas* spp. using bacteriophages [20, 29, 45], no reports are available regarding phages that can control biofilm formation of *A. hydrophila*. In this study, phages G65 and Y81 were found to inhibit biofilm formation by more than 50% when the MOI was greater than 0.001, although relatively poor performance was observed for the

other three phages. Similarly, phages G65 and Y81 were very efficient in reducing bacterial biomass even at a low MOI (0.001), while phages Y71 and Y81 barely achieve equivalent level of inhibition at a much higher MOI (100). The diverse roles of the five phages played in biofilm inhibition were likely to be related to the inhibition effect of phages on the bacterial growth. It was suspected that the bacteria became resistant to the bacteriophages after long bacteriophage exposure so that the bacterial growth causes biofilm formation. Bacterial hosts that are available to phages might evolve to phage-resistant mutants that avoid infection when facing antiphage pressure because of the loss or alteration of phage receptors [46]. Phage W3 showed increasing inhibition to bacterial growth and biofilm formation with the enhancement of the MOI. The different MOI needed for inhibition ability is probably owing to that MOI is another major factor determining phage's ability to adsorb host cells in addition to receptors[47].

The phages G65 and W3 reduced mature biofilm for more than 60% after treating for 6 h with the phage titer of 1×10^8 PFU/ml. Although phage Y81 showed considerable effect on growth and biofilm inhibition, its effect on mature biofilm was very low. Contrary to Y81, N21 was more effective in the reduction of biofilm and almost 60% biofilm was removed after treated with N21 for 12 h. As phage receptors vary with bacterial cells in planktonic and attached states [48], it is not surprising that phages exhibiting poor inhibition for bacterial growth and biofilm formation can have high lytic activity to attached bacteria. Moreover, production of structured extracellular polymers of biofilm, in some cases, provides a physical barrier between phages and their receptors. However, some phages can specifically recognize these polymers and even produce polysaccharide depolymerases to degrade them, allowing phages to come in contact with the encased bacteria [46]. For instance, *Pseudomonas* spp. phage F116 produces an alginate lyase that can reduce the viscosity of the alginate exopolysaccharide and promote phages to penetrate the alginate matrix and reach the bacterial surface [49]. Thus, diverse antibiofilm efficacy of the five phages investigated in this study is likely due to the different activities of enzymes that can degrade exopolysaccharides. In addition, phages N21 and G65 displayed semitransparent halos around phage plaques on the lawns of host bacteria, and halo formation has been supposed to play important roles in exopolysaccharide depolymerization and biofilm degradation [50, 51]. Whether the halo formation of phage G65 and N21 is associated with their efficient biofilm removing ability needs further to be confirmed.

Conclusions

In this study, five *A. hydrophila* phages were isolated, with three belonging to family *Myoviridae* and two belonging to family *Podoviridae* morphologically. All of the five phages have broad host ranges, low latent periods, wide pH and thermal tolerance, and effective treatment of *A. hydrophila* infection. More importantly, several phages exhibited varying abilities to repress and remove *A. hydrophila* biofilm. This study provides a basis for therapeutic applications of phages to control *A. hydrophila* infection.

Methods

Bacterial strains and growth conditions

Two epidemic strains of high virulence isolated from diseased crucian carp, *A. hydrophila* NJ-35 (Genbank: CP006870.1) from Nanjing, Jiangsu in 2010 and XY-16 (GenBank of *gyrB* gene: JX025797.1) from Xinyi, Jiangsu in 2009 [52, 53], were used for phage isolation. An additional 203 *Aeromonas* strains, including 73 *A. hydrophila*, 85 *A. veronii*, 12 *A. caviae*, 1 *A. bestiarum*, 12 *A. sobria*, 10 *A. media*, 3 *A. salmonicida*, 3 *A. jandaei* and 4 *A. aquariorum* were used for host range analysis. All bacterial strains were routinely cultured in Luria Bertani broth (LB) (Difco/Becton Dickinson) at 28 °C with shaking at 180 rpm. All *Aeromonas* strains used in this study are listed in Table 3.

Phage isolation, purification and propagation

A. hydrophila strains NJ-35 and XY-16 were used as indicator host for phages. Ten water samples were collected from ponds, sewage and rivers in different areas of Nanjing. The samples were centrifuged at 4000 *g* for 30 min and the supernatants were filtered through 0.22-μm membrane filters. A 10 mL of the supernatant was added into 20 mL of 3 × LB liquid media, and 1 mL cultures of NJ-35 or XY-16 (late logarithmic phase) were used to inoculate the mixture. After adding 5 M CaCl₂ to a final concentration of 0.1 mM, the phages were enriched by culturing for 14 h at 28 °C. A 3 mL of the culture was added to a 5 mL LB media, followed by the addition of chloroform with a final concentration of 3%, vigorously shaken for 2 min. After static layering, 1 mL of the supernatant was filtered through a 0.22 μm filter. To confirm the presence of the lytic phage in the filtrate, the double-layer agar method [54] was performed using the filtrate. After incubating at 28 °C for 8 h, a single plaque was picked up with a sterile pipette tip into LB media with the addition of host bacteria. After proliferation, the phages were purified several times using the double-layer agar method.

Host range

The host range of the harvested phages was determined using a spot assay. *Aeromonas* strains were spread evenly on the LB solid plate. Five microliters of the phage cultures of 10⁸ PFU/mL were dropped onto the overlaid top agar. After cultured for 12 h at 28 °C, the presence or absence of a lysis zone was observed.

TEM analysis

The phages were cultured at 28 °C for 8-10 h using the double-layer plates. A plate with plenty of plaques whose edges were faintly visible was added with 2 mL of sterilized ddH₂O. The plate was shaken horizontally for 5 minutes to fully wash off the phages. Suspensions in the plate were centrifuged at 6000 *g* for 5 min. For TEM analysis, 15 μL of the phage supernatant was spotted on a copper grid covered with a Formva film. After 3 min, the suspension was removed by filter paper and baked it under incandescent light for 5-10 s. The phages were negatively stained with 2% uranyl acetate for 1 min. Excess dye was removed by the filter paper and the grid was dried under an incandescent lamp. The morphology of the phage was imaged by transmission electron microscopy (H-7650 Hitachi, Japan) operated at 80 kV. Phage dimensions were calculated by measuring the dimensions of five independent phages.

Determination of optimal MOI

The optimal MOI is the ratio of the number of phages to that of host bacteria present in a defined space that is best for phage proliferation to obtain maximum titers. The host *A. hydrophila* strains grown to the log phase were washed three times with PBS and adjusted to corresponding densities of 10^5 , 10^6 , 10^7 and 10^8 CFU/mL, respectively. The phages and bacteria were mixed with MOIs of 100, 10, 1, 0.1, 0.01, 0.001 and 0.0001, respectively. After incubation for 2 h at 28 °C, the phage titers were measured by the double-layer agar method.

One-step growth curve

According to the optimal MOI, phage solutions were inoculated into 10 mL host bacteria in logarithmic phase (1×10^8 CFU/mL). The phages were allowed to absorb for 15 min and then centrifuged at 13,000 *g* for 5 min. After discarding the supernatants, the phage-infected bacterial pellets were resuspended in 10 mL of preheated LB media and incubated at 28 °C with shaking at 180 rpm. At a interval of 5 min, 100- μ L aliquots were taken until 115 min and phage titers were immediately determined by the double-layer agar method.

pH and thermal stability assays

For pH stability tests, 100 μ L phage suspension (1.0×10^7 PFU/mL) was used to inoculate 900 μ L physiological saline adjusted to pH values of 3~12 with NaOH or HCl. The mixtures were incubated at 28°C for 2 h and aliquots were taken to measure the titers of phages at different pH values. For thermal stability tests, 2 mL phage suspension (1.0×10^7 PFU/mL) was incubated at 30 °C, 40 °C, 50 °C, and 60 °C. At a interval of 20 min, 100- μ L aliquots were collected until 100 min. Survived phages were counted and the survival rates were calculated by the PFU at each time point divided by that at the primary PFU. All tests were performed in triplicate.

Inhibition assay of growth and biofilm formation

A. hydrophila strains were cultured in LB medium to logarithmic period and then normalized to 1×10^6 CFU/mL. The suspensions were mixed with equal volume of phages with the MOI of 0.01, 0.1, 0, 1, 10, and 100, respectively. Two hundred microliters of the mixtures were added into each well of the 96-well plates. Each treatment was performed in eight replicates. Fresh LB medium served as a blank control. Then the plates were incubated at 28 °C for 24h without shaking. The biomass of bacteria was evaluated by measuring OD₄₅₀ using a micro-plate reader (Tecan, Switzerland). Next, the culture supernatants were discarded and the plates were washed three times with sterile PBS to remove all planktonic cells. Biofilms formed in each well were fixed with 200 μ L of 99% (vol/vol) methanol for 15 min. After drying, biofilms were stained with 1% crystal violet for 10 min. Then the wells were washed with distilled water to remove unbound dye. Crystal violet was dissolved in 200 μ L of 95% ethanol for 10 min and the absorbance was measured at 595 nm (OD₅₉₅).

Biofilm clearance assay

A. hydrophila strains were cultured in LB medium to logarithmic period and then normalized to OD₆₀₀ of 1.0. The suspensions were inoculated to LB media (200 µL per well) in 96-well plates at a ratio of 1:1000. The plates were incubated at 28 °C for 24 h without shaking. After removing all planktonic cells, each well were treated with 200 µL phage dilution of 1×10^8 PFU/mL with sodium chloride-magnesium sulfate (SM) buffer (100 mM NaCl, 50 mM Tris pH 7.5, and 10 mM MgSO₄) for 6 h, 12 h, and 24 h. Biofilms treated with SM buffer served as the control. The microplates were then washed twice with PBS and the biofilms left were stained by crystal violet method described above.

Phage therapy assay in mice model

The animal experiment was performed in accordance with the animal welfare standards, complied with the guidelines of the Experimental Animal Welfare Ethics Committee, Chinese Association for Laboratory Animal Sciences, and was approved by the Ethical Committee for Animal Experiments of Nanjing Agricultural University, China (approval number: SYXK(Su) 2017-0007). Six-week-old female ICR mice (body weight, 18 ± 2 g) were purchased from the Experimental Animal Center of Yangzhou University and raised in the Experimental Animal Center of College of Veterinary Medicine, Nanjin Agricultural University under specific-pathogen-free (SPF) conditions. The dose of *A. hydrophila* used in this study was chosen on the basis of a preliminary study that the mice were expected to show obvious clinical signs but no death. *A. hydrophila* NJ-35 and XY-16 grown to log phase were washed three times with PBS and adjusted to CFU/mL, respectively. For phage therapy assay, a total of 120 mice were randomly divided into three groups and housed in cages (5 mice per cage). Feed and water were allowed *ad libitum*. To evaluate treatment efficiency of phages in *A. hydrophila* NJ-35 infection, 40 mice were intraperitoneally inoculated with 100 µL of bacterial suspension of *A. hydrophila* NJ-35; after 30 min, the infection group of 10 mice were then intraperitoneally injected with 100 µL of sterile PBS, while each 10 out of the other 30 mice in the treatment group were injected with 100 µL of phages N21, W3 or G65 at the MOI of 1. The blank control group of 30 mice were intraperitoneally injected with 100 µL of sterile PBS, followed by injecting 100 µL of each of the phage suspensions. Similarly, 50 mice were used to determine the therapy effect of phages Y71 and Y81 on *A. hydrophila* XY-16 infection. After 6 h and 24 h, five mice in each treatment were euthanized by CO₂, respectively. Hearts, livers, spleens, lungs and kidneys were aseptically removed from the mice, weighed, added to 1 mL of sterilized PBS, homogenized using a tissue homogenizer (Bioprep-24, Allsheng, Hangzhou, China), and then 10-fold serially diluted in PBS. Bacterial loads in tissues were determined by plating the dilutions on LB plates. The results are expressed as the numbers of CFU/g tissue.

Statistical analyses

Data were collected and analyzed using GraphPad Prism version 5 software. The bacterial loads in the phage-treatment and the control groups were analyzed using *t*-test. Error bars presented in the figures

represent standard deviations of the means of three replicate experiments. A *P*-value < 0.05 was considered as a significant difference.

Abbreviations

MDR: Multidrug resistance; UPEC: Uropathogenic *Escherichia coli*; LB: Luria Bertani broth; MOI: Multiplicity of infection; TEM: Transmission electron microscopy; CFU: Colony-forming units; PFU: Plaque forming units; PBS: Phosphate buffer saline; SM: Sodium chloride-magnesium sulfate; SPF: Specific-pathogen-free; ICR: Institute of Cancer Research

Declarations

Ethics approval and consent to participate

The animal experiment was performed in accordance with the animal welfare standards, complied with the guidelines of the Experimental Animal Welfare Ethics Committee, Chinese Association for Laboratory Animal Sciences, and was approved by the Ethical Committee for Animal Experiments of Nanjing Agricultural University, China (approval number: SYXK(Su) 2017-0007).

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JL analyzed the data and wrote the manuscript; SG and JL carried out most of the experiments; YD and CL participated in the design of the study and performed the statistical analysis. YL provided expertise and conceived the study. All authors read and approved the final manuscript.

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Tables

Table 1 The information of the phages isolated in this study

Phages	Sources	Indicator bacteria	Host ranges			
			<i>A. hydrophila</i>	<i>A. caviae</i>	<i>A. veronii</i>	<i>A. bestiarum</i>
N21	Pond water polluted by diseased fish	<i>A. hydrophila</i> NJ-35	NJ-35, XY-16, J-1, GY-23, NJ-34, XH-3, XH-4, XH-5, XH-6, CH-3, ML-4, ML-5, ML-11, ML-12, ML-23, CH-8, SQ-11	ML-27	CH-7	
W3	Pond water polluted by diseased fish	<i>A. hydrophila</i> NJ-35	NJ-35, XY-16, GY-23, NJ-34, XH-3, XH-4, XH-5, XH-6, CH-3, ML-4, ML-5, ML-11, ML-12, ML-23, CH-8, SQ-11	ML-27	GY-40, SQ-7, CH-7	NJ-24
G65	Polluted river	<i>A. hydrophila</i> NJ-35	NJ-35, XY-16, GY-23, NJ-34, XH-3, XH-4, XH-5, XH-6, CH-3, ML-4, ML-5, ML-11, ML-12, ML-23, CH-8, SQ-11	ML-27	GY-40, SQ-7, CH-7	
Y71	Pond water polluted by diseased fish	<i>A. hydrophila</i> XY-16	NJ-35, XY-16, J-1, GY-23, NJ-34, XH-3, XH-4, XH-5, XH-6, ML-4, ML-5, ML-12, ML-23, CH-3, SQ-11		CH-7	
Y81	Pond water polluted by diseased fish	<i>A. hydrophila</i> XY-16	NJ-35, XY-16, J-1, GY-23, NJ-34, XH-3, XH-4, XH-5, XH-6, CH-3, ML-4, ML-5, ML-11, ML-12, ML-23, CH-8, SQ-11		CH-7	

Table 2 The optimal MOI of phage N21, W3, G65, Y71 and Y81

Bacteria (CFU/mL)	Phages (PFU/mL)	MOI	Titers of phages after 2 h co-culture (PFU/mL)				
			N21	W3	G65	Y71	Y81
10 ⁶	10 ⁸	100	1.28×10 ⁸	2.00×10 ⁸	1.72×10 ⁷	4.00×10 ⁷	7.30×10 ⁷
10 ⁷	10 ⁸	10	2.50×10 ⁸	2.50×10 ⁸	5.50×10 ⁷	2.54×10 ⁸	3.55×10 ⁸
10 ⁸	10 ⁸	1	3.10×10 ⁸	6.03×10 ⁸	5.70×10 ⁷	2.03×10 ⁹	3.58×10 ⁸
10 ⁸	10 ⁷	0.1	4.10×10 ⁸	8.00×10⁸	1.17×10 ⁸	2.50×10⁹	3.60×10 ⁸
10 ⁸	10 ⁶	0.01	8.93×10⁸	1.00×10 ⁸	2.16×10 ⁸	1.15×10 ⁹	3.80×10 ⁸
10 ⁸	10 ⁵	0.001	2.06×10 ⁸	1.00×10 ⁸	2.31×10⁸	1.00×10 ⁹	4.46×10⁸
10 ⁸	10 ⁴	0.0001	2.0×10 ⁸	8.00×10 ⁷	2.00×10 ⁸	8.60×10 ⁸	3.76×10 ⁸

Table 3 *Aeromonas* strains used in this study

<i>Aeromonas</i> species	Sources			
	Fish	Shrimp	Crab	Water
<i>A. hydrophila</i>	NJ-35, XY-16, NJ-34, NJ-28, ML-2, ML-11, ML-15, XH-3, XS-1, XS-6, XS-7, XH-3 NJ-1, XX-11, XX-12, XX-13, XX-14, NJ-1, JH-19, CS-60, J-1, DW-4, ML-9, ML-13, ML-21, XH-5, CH-2, XS-5, XS-10, CH-8, XS-12, XH-5 XX-49, XX-52, XX-58, JH-17, DW-2, ML-4, ML-16, ML-22, CH-1, CH-6, XS-3, XS-9, XS-11, JD-3, GY-23, DW-3, ML-12, ML-19, ML-23, XH-1, XH-6, CH-3, CH-10, XS-4, JD-4, XH-1, XH-6, XX-62, DW-1, ML-5, ML-14, ML-17, ML-26, CH-4, CH-11, CH-9, XS-2, JD-1, JD-2			ML-30, ZG-22, SQ-11, NJ-3
<i>A. veronii</i>	XH-4, XH-5, CH-5, DS-4, DS-6, ML-10, LK-1, ZG-15, ZG-5, ZG-2, GY-37, GY-28, GY-13, ML-10 LK-1, ML-25, CH-7, CH-12, DS-1, ML-3, ZG-12, ZG-11, ZG-10, ZG-6, ZG-4, GY-41, GY-40, DS-7, ML-3, ML-25, GY-36, DS-2, DS-5, ML-24, ZG-17, ZG-13, DS-9, XH-4, ML-24, DS-3, ML-6, ZG-18, ZG-14, GY-36, DS-8, DS-10, LK-16, LK-15, ML-6, LK-3, SQ-4, SQ-2, ZG-16, ZG-9, ZG-7, ZG-3, LK-3	SQ-5, SQ-6, SQ-7, ML-8, WX-1, WX-2, WX-3, WX-4, LK-17	LK-19, LK-21, LK-22	GY-11, GY-32, GY-53, GY-54, GY-58, ZG-19, ZG-21, ZG-23, SQ-8, SQ-9, ZX-4, ML-29, LK-26, XS-13, LK-27, DS-11
<i>A. caviae</i>	ML-1, SQ-1, SQ-3, LK-6, LK-10, LK-4, LK-5, XS-8	ZX-2, LK-18	LK-20	ML-27
<i>A. bestiarum</i>				NJ-24
<i>A. sobria</i>	LK-2, LK-12, CS-40, GY-45, JH-1, ZG-8, GY-4, LK-14	ZX-1		LK-24, ZG-20, SQ-10
<i>A. media</i>	NJ-4, NJ-30, NJ-5, NJ-6, NJ-32			NJ-7, NJ-8, NJ-21, NJ-25, NJ-29
<i>A. salmonicida</i>				XX-27, XX-28, CS-2
<i>A. jandaei</i>	XH-2, ZG-1			LK-23
<i>A. aquariorum</i>	ML-18, LK-25, ML-20			ML-28

Figures

Figure 1

Morphology of phages. (A) Plaque morphology of phages N21, W3, G65, Y71 and Y81 that were plated on 0.7% LB agar overlays with suspensions of *A. hydrophila* and incubated at 28 °C for 16 h. (B) Transmission electron micrographs of phage virions negatively stained with 2% uranyl acetate.

Figure 2

One step growth curves of phages. Phages N21, W3 and G65 mixed with *A. hydrophila* NJ-35, Y71 and Y81 mixed with *A. hydrophila* XY-16 were cocultured at the optimal MOI for 2 h. The phage titers were measured by the double-layer agar method. Data are presented as the mean $\pm$ standard deviation (SD), and latent periods and burst sizes were inferred from three independent experiments, with each experiment being comprised of three individual measurements.

Figure 3

pH stability of phages N21, W3, G65, Y71 and Y81. Phages were incubated for 2 h under different pH values, and the survival rate of phages were shown as mean $\pm$ SD from the triplicate experiments.

Figure 4

Thermal stability of phages N21, W3, G65, Y71 and Y81. Phages were incubated for 100 min under different temperatures. At an interval of 20 min, the survival rates were calculated by the PFU of viable phages at each time point divided by that at the primary PFU. The test was performed in triplicate.

Figure 5

Growth and biofilm inhibition. The inhibition abilities to growth and biofilm formation of *A. hydrophila* NJ-35 by phages N21, W3, G65, and that of *A. hydrophila* XY-16 by phages Y71 and Y81 were detected at an

MOI of 0.01, 0.1, 1, 10 and 100. (A) Growth inhibition of phages on *A. hydrophila* NJ-35 or XY-16. (B) Inhibition ability of phages on biofilm formation of *A. hydrophila* NJ-35 or XY-16. Data are shown as the mean $\pm$ SD from the triplicate experiments.

Figure 6

Biofilm clearance ability of phages N21, W3, G65, Y71 and Y81. Biofilms of *A. hydrophila* NJ-35 formed in 96-well plates for 24 h were treated with N21, W3, G65 at concentrations of 108 PFU/mL, and biofilms of *A. hydrophila* XY-16 formed were treated with phages Y71 and Y81. Biofilms treated with SM buffer served as control. Total biofilm biomass was stained with 1% crystal violet and measured at OD595. Data are shown as the mean $\pm$ SD from the triplicate experiments.

Figure 7

Therapy treatment of phages on *A. hydrophila* infection in mice model. Bacterial loads in the tissues of mice infected with *A. hydrophila* NJ-35 after treating with phages N21, W3 or G65 at the MOI of 1 for 6 h (A) and 24 h (B). Bacterial loads in the tissues of mice infected with *A. hydrophila* XY-16 after treating with phages Y71 or Y81 for 6 h (C) and 24 h (D). Mice treated with PBS after infecting with *A. hydrophila* served as the control. Data are shown as the mean $\pm$ SD from five mice in three independent experiments.