Original

Selection of Phages to Control *Aeromonas hydrophila* – An Infectious Agent in Striped Catfish

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Striped catfish (*Pangasianodon hypophthalmus*) farming in the Mekong Delta Vietnam (MKDVN) importantly contributes to national aquaculture export. Currently, however, diseases occur more frequently across the entire MKDVN region. One of the most common types is hemorrhagic septicemia caused by *Aeromonas hydrophila*. In this study, isolation and selection of the phages for control *in vitro* *Aeromonas hydrophila* were conducted. 24 phages were isolated from 100 striped catfish pond water samples. Next, lytic activity of these phages was clarified. Four phages with short latent period (about 25 to 40 min) and/or high burst size (about 67 to 94 PFU/cell) were selected to evaluate their infection activity to different phage-resistant *A. hydrophila* strains. Two phages termed as TG25P and CT45P were subjected to the phage cocktail to inactivate *A. hydrophila*. Re-growth of the host bacteria appeared about eight hours after treatment. Usage of the phage cocktail that attach different host bacterial receptors is not always much effective than usage of single phage. This is the first report about phage therapy to control *A. hydrophila* isolated from striped catfish. Some challenges in the phage cocktail were shown to achieve strategies in prospective studies in the context of high antibiotic resistance of *A. hydrophila*.

Key words : Antibiotic resistance / Phage therapy / Striped catfish / *Aeromonas hydrophila* / Mekong Delta.

INTRODUCTION

Striped catfish (*Pangasianodon hypophthalmus*) is the native freshwater catfish species in the Mekong river (traversing Vietnam and Cambodia), Chao Phraya and Mae Klong rivers in Thailand. According to the United Nations' Food and Agriculture Organisation (FAO), with natural conditions and suitable environments, Vietnam has become a leading producer on farmed striped catfish, accounting for 90% of the total of 1.4 million tons

of total global striped catfish production in 2010 (De-Silva & Phuong, 2011). In addition, frozen striped catfish fillet is among the predominant aqua-produce exports from Vietnam. According to the report from Vietnam's Office of Statistics, in 2017, aqua-product export generated US\$8.3Billion dollars, in which striped catfish was the second highest contribution accounting for 22% (GSO Vietnam, 2017).

One major factor affecting the sustainable development of the MKDVN aqua-industry has been reported so far as the annual cycle of bacterial pathogen infections. In 2014, an area of over 730ha, or 12% of the total farming surface devoted to striped catfish, were bacteri-

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ally infected, in which one of the most common types being hemorrhagic septicemia caused by *A. hydrophila*. Usage of antibiotics as a measure of prevention and treatment of hemorrhagic septicemia disease has been commonly used in the region. However, it recently shows an inadequate control due to antibiotics resistance of *A. hydrophila* in striped catfish farms. In research of Quach et al. (2014), antibiotic resistance ratio of *A. hydrophila* isolates in ill striped catfish in the MKDVN was 100% counted for certain kinds of antibiotics, i.e., ampicillin, amoxicillin, cefalexin, trimethoprim/sulfamethoxazole and was 93% for tetracycline. Moreover, increase in antibiotic residuals has also been detected in exported stock. From 2014 to 2016, many consignments from leading Vietnamese producers have been rejected due to higher-than-approved-limit of antibiotic residuals by importing markets such as Russia, Japan, South Korea, Canada and the USA. Consumption of beyond-acceptable-limit of antibiotic residuals can affect people's health and well-being in the long term. In addition, improper usage of antibiotics can greatly affect the long term sustainability of the aqua industry in general, and striped catfish sector in particular, due to the negative biological impact within the farming environment and over time the eco system at large. Due to these adverse impacts of *A. hydrophila* in aqua-culture, there is an urgent need to come up with an alternative solution, effectively and eco-friendly such as phage therapy.

Principles of phage therapy using lytic phage as a treatment for pathogen bacterial infections became available a long time ago (Twort, 1915), but the method has only gained serious attention in the aqua industry in the last thirty years, especially in the face of wide spreading of antibiotic resistances in bacteria. Phage therapy has shown its efficacy in treatment of bacterial diseases in fish and shellfish in several cases, such as *Edwardsiella tarda* causing edwardsiellosis in Loach (Wu et al., 1981; Wu & Chao, 1982), *Lactococcus spp.* causing lactococcosis in Yellowtail (Nakai et al., 1999), *Aeromonas salmonicida* causing furunculosis in brook trout, rainbow trout, Atlantic salmon (Imbeault et al., 2006), *A. hydrophila* causing tail and fin rot in Loach (Wu et al., 1981; Jun et al., 2013). Phage therapy against various kinds of fish and shellfish pathogens have been reviewed by Richards (2014). However, there has been no approach on phage therapy to treat *A. hydrophila* causing hemorrhagic septicemia in striped catfish.

In this study, phages specific to *A. hydrophila* – a causative agent of striped catfish – were isolated and characterized. Furthermore, strategies and challenges in selection of phages were also investigated against *A. hydrophila in vitro*, a primarily step for further development of the phage therapy in treatment of hemorrhagic

septicemia in striped catfish.

MATERIALS AND METHODS

Isolation of phage

100 water samples were collected from striped catfish ponds in Can Tho and Tien Giang provinces, MKDVN. The samples were stored in cool condition and transferred to laboratory within 24 hours to isolate the phages infecting *A. hydrophila*. *A. hydrophila* strain A1 used in the current study was previously isolated and characterized from ill striped catfish samples by the colleagues in Can Tho University (data not shown). Preliminary centrifugation at 5,000 rpm ($2,432 \times g$) in 5 min was carried out to remove solid particles in the samples. Culture of A1 strain was inoculated into water sample with an addition of Brain Heart Infusion medium (Lab M, Heywood, UK) (Crothers-Stomps et al., 2010). The mixture was incubated overnight at 30°C, 150 rpm. It was passed through 0.22- μ m filter to obtain filtrate that was then used to the plaque assay (Hoang et al., 2014) with some modifications. Briefly, a mixture of 100 μ L of filtrate and 100 μ L of *A. hydrophila* A1 was added to 3 mL of molten 0.5% Tryptic Soy Broth (TSB) agar (maintained at 47°C). Mixture was then poured over 1.5% Luria-Bertani (LB) agar plate. After incubation for 6-8 hrs at 30°C, a single clear plaque was picked out, suspended in SM buffer [100 mM NaCl, 10 mM $MgSO_4$, 0.01% gelatin, and 50 mM Tris-HCl (pH 7.5)], incubated overnight at 4°C and passed through 0.22- μ m filter to obtain filtrate. The filtrate was applied to the above protocol in triplicate to purify the phage. The purified phage stock was properly prepared. After plaque assay, molten agar lawn was mixed with 2 mL of SM buffer and 200 μ L of Chloroform. The mixture was incubated at room temperature for 2 hours. The supernatant obtained after centrifugation at 10,000 rpm ($9,727 \times g$), 5 min was passed through 0.22- μ m filter to obtain phage stock. Concentration of phage stock was examined by the plaque assay as described above.

One-step growth curve

One-step growth curves of the isolated phages were determined according to the method of Verma et al. (2009) with some modifications. Culture of *A. hydrophila* A1 was incubated at 30°C, 120 rpm, in TSB medium until its optical density at 600 nm (OD_{600}) of 0.1 (approximately 10^7 CFU mL^{-1}) was attained. Phage was added into the culture at multiplicity of infection (MOI) of 0.01 (phage : host). The mixed culture was incubated as same condition above for 5 min and was then centrifuged at 10,000 rpm ($9,727 \times g$), 5 min, 4°C. The resulting pellet was re-suspended in the same volume of TSB medium and incubated following the above

condition. Samples were drawn every 5 min and diluted 100-fold in SM buffer on ice. The diluted solutions were centrifuged at 12,000 rpm (14,000 × g), 4°C, 5 min, to obtain the supernatant that was subjected to phage titration. Latent period and burst size of the phage were determined according to the previous research (Verma et al., 2009). The experiment was conducted in triplicate.

Isolation of phage-resistant bacteria

The host bacterial culture was incubated at 30°C, 120 rpm in TSB medium until its OD₆₀₀ of 0.1 was attained. The culture was divided into two aliquots, of which one aliquot was mixed with phage lysate at MOI of 2.0, one aliquot was left blank without phage addition. The mixtures were incubated as shown above. Samples were drawn every 30, 45 or 60 min and subjected to measure their OD₆₀₀. After observation of re-increased OD₆₀₀ of the host and phage mixture, the cultivation was maintained overnight to obtain the full growth of bacteria. The fully grown culture was then 10-fold serially diluted, spread on Tryptone Soya Agar (TSA) plate and incubated overnight at 30°C. A phage-resistant bacterium termed as R-XX (with “XX” is number on name of each phage) was isolated by examining its susceptibility to phage via drop plaque assay (Namura et al., 2008). The experiment was conducted in triplicate.

Cocktail of phages

Each newly isolated R-XX bacterial strain was examined its susceptibility to other phages. R-XX was cultivated overnight in TSB medium at 30°C, 150 rpm. Next day, 100 µL of the culture was added to 3 mL of molten 0.5% TSB agar (maintained at 47°C). Mixture was then poured over 1.5% LB agar plate and incubated at room temperature for 2 min. Then, 2 µL of each phage stock (~10⁹ PFU mL⁻¹) was dropped onto the plate. Plates were incubated overnight at 30°C and were then checked for clear zones on the bacterial lawn.

The most suitable phages were chosen for phage cocktail experiment. The host bacterial culture was incubated at 30°C, 120 rpm in TSB medium until its OD₆₀₀ of 0.1 was attained. The culture was divided into two aliquots. First aliquot was mixed with phage lysates at MOI of each phage of 1.0 (a total MOI of two phages of 2.0). The mixtures were incubated as shown above. The second aliquot was left as a blank without phage addition. Each sample was withdrawn at 30, 45 or 60 min intervals and subjected to measure the OD₆₀₀. The experiment was conducted in triplicate.

RESULTS AND DISCUSSION

TABLE 1. Lytic activity of selected phages

Name of phage	Latent period (min)	Burst size (PFU/cell)
TG22P	25	70±10.1
TG23P	25	94±15.9
TG25P	40	79±11.9
CT45P	25	67±1.4

Isolation of phages

Among 100 striped catfish pond water samples, 24 samples showed existence of phages specific to *A. hydrophila*. Possibility of finding phages in the samples was quite high. However, from each sample, only single transparent and large plaque (2-3 mm diameter) was selected for purification. Stocks of 24 phages were then prepared with concentration of 10¹⁰–10¹¹ PFU mL⁻¹.

Latent period and burst size of phages

Selection of appropriate phages is one of key factors in the success of phage therapy. Two criteria mainly involve in the selection of phages, i.e., latent period and burst size. A phage is considered as highly lytic phage when it owns short latent period and/or high burst size. In this study, latent period and burst size of 24 phages were determined, of which four phages with short latent period and/or high burst size were selected for further evaluation (Table 1). There were three phages with latent period of about 25 min. In terms of burst size, all phages had burst size greater than 60 (PFU/cell). Phages TG25P was chosen because of its high burst sizes of 79±11.9 (PFU/cell), although its latent periods of 40 min was longer than that of other phages. Lytic activity of these phages is considered as high as that of previous phages infecting *A. hydrophila* which causes other types of diseases in fish and shellfish (Wu et al., 1981; Chow and Rouf, 1983; Jun et al., 2013).

Inactivation of *A. hydrophila* by single phage

The four selected phages were further evaluated their capacity to inactivate growth of *A. hydrophila* in TSB medium. As shown in the figure 1, after incubation for about 30-45 min, increase of OD₆₀₀ of the four mixtures (*A. hydrophila* with each phage) was quite similar to that of the control (*A. hydrophila* without phage). The increase of OD₆₀₀ corresponded to latent period of phages when most of bacterial cells had not been lysed yet. However, further incubation for about 45-60 min, OD₆₀₀ of the four bacterium-phage solutions decreased sharply, while that of the control increased. It was expected that most of bacterial cells were infected and lysed by phages since MOI value applied in the four bacterium-phage solutions was 2.0. Further incubation

FIG. 1. Change of optical density at 600 nm during inactivation of *A. hydrophila* by single phage. Each of four phages, i.e. TG22P, TG23P, TG25P or CT45P was inoculated at a MOI of 2.0. Negative control was without phage addition. Error bars indicating 95% confidence intervals for the averaged values ($n = 3$) are not graphically detectable as the intervals were too narrow.

for 5-6 hours, all of bacterium-phage solutions became transparent, while the control constantly increased its turbidity, indicating that phages were efficient in inactivation of *A. hydrophila*. However, after about seven hours of incubation, OD_{600} of the four solutions started to increase due to regrowth of phage-resistant bacterial strains.

Candidates for the phage cocktail

After overnight incubation of the four bacterium – phage solutions, four respective phage-resistant bacterial strains were isolated. Name of these strains is shown in the first column in Table 2. Four phage-resistant *A. hydrophila* strains termed as R-22, R-23, R-25 and R-45 were resistant to phages of TG22P, TG23P, TG25P and CT45P, respectively. Suitable phage candidates for the phage cocktail were selected by susceptibility of each phage-resistant bacterial strain to other phages. For instance, R-22 was examined its susceptibility to other phages, i.e. TG23P, TG25P, and CT45P. It showed that R-22 and R-23 were not susceptible to other phages. Thus, TG22P and TG23P were not suitable to be involved in phage cocktail. In contrast, R-25 was susceptible to other phages, i.e. TG22P, TG23P, and CT45P. In addition, R-45 was susceptible to TG25P and TG23P. The susceptibility of bacteria to phages was the same among triplicated experiments. Therefore, TG25P and CT45P were suitable candidates to be involved in phage cocktail.

The major challenge of phage therapy is the regular emergence of phage-resistant bacteria (Labrie et al.,

TABLE 2. Susceptibility of phage-resistant cells to lysis

		Phage			
		TG22P	TG23P	TG25P	CT45P
Bacteria	R-22	-	-	-	-
	R-23	-	-	-	-
	R-25	+	+	-	+
	R-45	-	+	+	-

“+” susceptible; “-” unsuspensible

2010; Oliveira et al., 2012; Mateus et al., 2014). An effective way to tackle the problem is to apply phage cocktail to inactivate phage-resistant bacteria. A main factor for success of phage cocktail approach is a collection of appropriate phages that infect host bacterium via attaching to different receptors on the bacterial cell wall. Filippov et al. (2011) demonstrated that the phage cocktail can overcome the problem of the bacterial resistance only when the phages exploit different receptors. Similar observation was shown in research of Tanji et al. (2004) when cocktail of two phages can markedly delay the appearance of phage-resistant cells. Most phage-resistant cells exhibit changes in their outer membrane components such as OmpA, OmpC, and OmpF or lipopolysaccharide (LPS) (Tanji et al., 2004). In research of Mateus et al. (2014), however, the efficiency of cocktail of three phages did not increase significantly compared to the use of single phage. It could be explained by the fact that all of the three phages might use the same bacterial receptor. In addition, too many phages in the cocktail result in a higher development and manufacturing costs (Mateus et al., 2014). It is also time consuming and costly to identify kind of host bacterial receptor that is attached by a phage. Here, we present a susceptibility matrix among different phage-resistant bacteria and different phages. The matrix could be useful to clarify whether different phages use the same or different types of bacterial receptors. A similar matrix was successful to clarify this (Tanji et al., 2004). The matrix supports us to select suitable cocktail phages to control the host cells. Only common and simple microbiological works have been required to build the matrix. It seems that phage cocktail in the many published works was simple mixtures of different phages without a clear considering about difference of bacterial receptors that phages attached.

Cocktail of phages

A phage cocktail involved TG25P and CT45P was examined its capacity to inactivate *A. hydrophila*. The Figure 2 shows OD_{600} of the phage cocktail experiment. After incubation for about 45-60 min, OD_{600} of the bacterium-phage-cocktail solution decreased sharply,

FIG. 2. Change of optical density at 600 nm during phage cocktail treatment to *A. hydrophila*. Two phages, i.e. TG25P and CT45P, were inoculated at a MOI of 2.0. Negative control was without phage addition. Error bars indicating 95% confidence intervals for the averaged values ($n = 3$) are not graphically detectable as the intervals were too narrow.

while that of the control (host cells alone) maintained the increasing trend. After next 7 hours of incubation, bacterium-phage-cocktail solution became transparent, while the control constantly enhanced its turbidity, indicating the efficacy of phage cocktail for inactivation of *A. hydrophila*. However, after about eight hours of incubation, it was unexpected that OD₆₀₀ of the bacterium-phage-cocktail solution re-increased due to regrowth of phage-cocktail-resistant bacterial strains. Therefore, the usage of phage cocktail could prolong about one hour in inactivation time compared to the usage of single phage (Figure 1).

To clarify it, 20 strains of either R-25 or R-45 were isolated and subjected to examine their susceptibility to the other phage via drop plaque assay. The result were shown in the Table 3. It was revealed that, not all of R-25 or R-45 strains were susceptible to phage CT45P or TG25P, respectively. There were 4/20 R-25 strains (20%) unsusceptible to phage CT45P. Similar, there were 12/20 R-45 strains (60%) unsusceptible to phages TG25P. Re-growth of these unsusceptible strains was responsible to the re-increase of OD₆₀₀ in the phage cocktail experiment. In the current study, efficiency of the phage cocktail to delay the development of resistance by the host was not as expected although these phages might attach different types of host bacterial receptors (Tanji et al., 2004). The cocktail of the two phages TG25P and CT45P were not much effective than a single phage in inactivation of host cells. Table 3 explained reasons for the current ineffective phage cocktail when these phages could not infect all types of phage-resistant bacterial strains. It was proposed that

TABLE 3. Susceptibility of phage-resistant bacterial strains to cocktail phages

Bacterial type	No. of tested strains	No. of susceptible strains to each phage	
		TG25P	CT45P
R-25	20	(-)	16
R-45	20	8	(-)

(-): Not identified

the host bacteria might utilize different phage-resistant mechanisms other than alternation of their receptors such as the development of adaptive immunity via interfering CRISPR sequences (Hyman & Abedon, 2010; Labrie et al., 2010; Örmälä & Jalasvuori, 2013). The host bacteria in the current study might evolve different phage-resistant mechanisms after exposed to phages. Therefore, usage of cocktail of phages that attach different host bacterial receptors is not always more effective than usage of single phage. Table 3 also indicates that selection of cocktail phages based on the susceptibility matrix proposed by Tanji et al. (2004) might not always be reliable for *A. hydrophila* and their phages. In the future studies, the susceptibility matrix should be clarified with bigger number of the phage-resistant *A. hydrophila* strains and phages.

However, previous studies interestingly indicate that when the host bacteria are resistant to phage infection, the bacterial receptors change resulting in less toxic properties (Fillippov et al., 2011; León & Bastías, 2015). This may have meanings in control of bacterial diseases on fish farms using phage therapy. Jun et al. (2013) demonstrated high efficiency of phage therapy using two *Aeromonas* phages to reduce mortality of the ill cyprinid loach (*Misgurnus anguillicaudatus*) although their lytic activity (latent period and burst size) and inactivation of host bacterium *in vitro* were similar to that of the current study. Similar efficiency was demonstrated in phage cocktail to control *Vibrio parahaemolyticus* in aquaculture (Mateus et al., 2014). In the future studies, whole genomic information of the phages will be analyzed. Influence of different factors such as pH, organic solvents, temperature, etc., on stability of the phages will be also examined prior to applying the phage therapy for treatment of *A. hydrophila* infection in striped catfish.

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declared

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