Phage Application for the Protection from Acute Hepatopancreatic Necrosis Disease (AHPND) in *Penaeus vannamei*

Jin Woo Jun¹ · Jee Eun Han² · Sib Sankar Giri¹ · Kathy F. J. Tang² · Xiaohui Zhou³ · Luis Fernando Aranguren² · Hyoun Joong Kim¹ · Saekil Yun¹ · Cheng Chi¹ · Sang Guen Kim¹ · Se Chang Park¹

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Abstract Acute hepatopancreatic necrosis disease (AHPND) caused by *Vibrio parahaemolyticus* has been one of the most problematic diseases in marine shrimp aquaculture throughout Southeast Asia and Latin America. To evaluate the effectiveness of a bacteriophage (phage) treatment for AHPND, a series of bioassays were carried out in a marine shrimp (*Penaeus vannamei*) model using an AHPND-*V. parahaemolyticus* strain that is highly pathogenic to shrimp. We monitored the mortality and histopathological changes during phage treatment. Shrimps treated with phage prophylaxis and phage therapy displayed significant protection from AHPND and survived a lethal bacterial challenge.

Keywords Acute hepatopancreatic necrosis disease (AHPND) · *Vibrio parahaemolyticus* · Phage prophylaxis · Phage therapy

Introduction

Vibriosis is a major shrimp disease caused by *Vibrio* species and some *Vibrio parahaemolyticus* strains cause acute hepatopancreatic necrosis disease (AHPND), resulting in up to 100% of mortality in marine shrimp aquaculture [1–4]. It has become a serious issue in shrimp aquaculture around the world, especially in China, Thailand, Vietnam, Malaysia, and Mexico [5–7]. Although antibiotics have been commonly used for prophylaxis or therapy for AHPND, the increasing incidence of antibiotic resistance has limited the effectiveness of antibiotics [8, 9].

Phages have been proposed as a control for infectious diseases in humans and animals [10]. The use of a phage as a therapeutic agent (phage therapy) is advantageous as it is natural and relatively inexpensive, without serious or irreversible side effects reported to date [10, 11]. There have been only few attempts to use phages to control bacterial infections in shrimps [12]. There is no feasible remedy reported for AHPND, and we previously noted that the development of effective treatment methods is needed [13].

In our previous study, phage pVp-1 induced effective bacteriolysis of AHPND-*V. parahaemolyticus* strains from diverse regions [13]. Our aim was to determine whether this phage could be suitable for prophylactic or/and therapeutic use against AHPND-*V. parahaemolyticus* in the *Penaeus vannamei* model.

Materials and Methods

Experiment Preparations

Virulent *Siphoviridae* phage pVp-1, infecting AHPND-*V. parahaemolyticus* strains, was used [13–15]. In our

Jin Woo Jun and Jee Eun Han contributed equally to this work.

✉ Se Chang Park
parksec@snu.ac.kr

¹ Laboratory of Aquatic Biomedicine, College of Veterinary Medicine and Research Institute for Veterinary Science, Seoul National University, Seoul 151-742, Republic of Korea

² School of Animal and Comparative Biomedical Sciences, University of Arizona, Tucson, AZ, USA

³ Department of Pathobiology and Veterinary Science, University of Connecticut, Storrs, CT, USA

previous study [13], pVp-1 infected 90.9% (20 of 22 strains) of the AHPND-*V. parahaemolyticus* strains tested. *Vibrio parahaemolyticus* 13-028/A3 is known to be highly pathogenic, causing 100% mortality of shrimps within 24 h post-infection, and was used for the AHPND challenge [13].

SPF (Specific Pathogen Free) juvenile marine shrimps (*P. vannamei*, $n = 96$, average weight = 1.02 g) were obtained from the West Campus SPF facility, University of Arizona, Tucson, USA. Shrimps were divided into 24 groups and kept in 3 l glass tanks at appropriate conditions (water temperature 25 °C; salinity 25‰) for at least 72 h before experiments.

Phage Treatment of Infected Shrimps

In the first bioassay, all shrimps were challenged by bath immersion with *V. parahaemolyticus* 13-028/A3 (5.0×10^5 CFU/ml) for 24 h, except Tanks 1 and 2. Tank 1 was designated as a negative control without bacterial challenge or phage treatment; Tank 2 was designated as a phage control with phage treatment by bath immersion (1.5×10^6 PFU/ml) and feeding (1.5×10^8 PFU/shrimp)

using pellets (5% of body weight) that had been impregnated with the phage suspension, but not bacterially challenged. Tank 3 was designated as a positive control with a bacterial challenge but not phage treated. Two treatment groups (Tanks 4 and 5) were fed with pellets containing the phage 1 h after the bacterial challenge. The other two treatment groups (Tanks 6 and 7) were treated with bath immersion using the phage suspension 1 h after the bacterial challenge. In the second bioassay, all conditions were the same as the first bioassay, except that the phage treatment was applied at different time points (24, 6, and 1 h prior to the bacterial challenge). In the third bioassay, the experiment was performed as for the first and second bioassays, except that the treatment groups were fed with pellets that had been impregnated with the phage at various time points (24, 6, and 1 h prior to the bacterial challenge, and 1 h after the bacterial challenge).

In all bioassays, each group was monitored for symptoms of infection and cumulative mortality was recorded daily for 5 days after the bacterial challenge. Each experiment was performed twice, on separate occasions. All animal experiments were performed in accordance with

Table 1 Phage treatment of shrimps infected by AHPND-*V. parahaemolyticus*

Bioassay	Experimental group (type*/method [†])	Phage treatment (h [‡])	No. of dead shrimp/no. tested	Mortality (%)
I	Tank 1 (Negative control)	No	0/4	0
	Tank 2 (Phage control)	Yes	0/4	0
	Tank 3 (Positive control)	No	4/4	100
	Tank 4 (Therapy/feeding)	Yes (+ 1)	4/4	100
	Tank 5	Yes (+ 1)	4/4	100
	Tank 6 (Therapy/immersion)	Yes (+ 1)	4/4	100
	Tank 7	Yes (+ 1)	4/4	100
II	Tank 1 (Negative control)	No	0/4	0
	Tank 2 (Phage control)	Yes	0/4	0
	Tank 3 (Positive control)	No	4/4	100
	Tank 4 (Prophylaxis/feeding)	Yes (– 24, – 6, – 1 h)	2/4	50
	Tank 5	Yes (– 24, – 6, – 1 h)	2/4	50
	Tank 6 (Prophylaxis/immersion)	Yes (– 24, – 6, – 1 h)	2/4	50
	Tank 7	Yes (– 24, – 6, – 1 h)	1/4	25
III	Tank 1 (Negative control)	No	0/4	0
	Tank 2 (Phage control)	Yes	0/4	0
	Tank 3 (Positive control)	No	4/4	100
	Tank 4 (Prophylaxis + therapy/feeding)	Yes (– 24, – 6, – 1, + 1 h)	0/4	0
	Tank 5	Yes (– 24, – 6, – 1, + 1 h)	0/4	0

*Experimental group, type: negative control, neither bacterial challenged nor phage-treated; phage control, not challenged but treated; positive control, challenged but not treated; therapy, challenged and treated by therapeutic application; prophylaxis, treated by prophylactic application and challenged

[†]Experimental group, method: feeding, oral administration of phage-impregnated feed; immersion, bath immersion administration using phage suspension

[‡]Phage treatment, h: +, phage administration after bacterial challenge (therapeutic application); –, phage administration prior to bacterial challenge (prophylactic application)

Fig. 1 Histopathological features of the hepatopancreas of shrimps at 48 h of phage treatment, the shrimp was challenged by AHPND-*V. parahaemolyticus* 13-028/A3 strain and treated with the phage pVp-1. Negative control (a) and phage control (b) showed the normal

appearance of the hepatopancreas. Positive control (c), challenged but not treated, showed the acute sloughing of hepatopancreatic tubular epithelial cells. The phage-treated shrimp d demonstrated the protected morphology of the hepatopancreas. Scale bars 30 μ m

guidelines of the Animal Ethical Committee of University of Arizona.

Histopathology

A separate experiment was performed as the third bioassay for histopathology by a standardized method [16]. Histopathology was examined for the severity of infection.

Results and Discussion

In our previous in vitro study, the phage pVp-1 demonstrated substantial bacteriolytic activity against three representative AHPND-*V. parahaemolyticus* strains (13-028/A3, 13-511/A1, and 13-306D/4) causing 100% mortality within 24 h post-infection [13]. We hypothesized that phage therapy can be useful against AHPND in shrimp. We used a marine shrimp (*P. vannamei*) model to evaluate the therapeutic effect of pVp-1 against AHPND-*V. parahaemolyticus*. Among three highly pathogenic AHPND-*V.*

parahaemolyticus strains, 13-028/A3 was selected as a bacterial challenge strain since it induced the highest efficiency of plating value [13].

The protective effects of phage administration against experimental AHPND-*V. parahaemolyticus* infection are shown in Table 1. In the first bioassay, no promising result was achieved: all treatment groups showed 100% mortality without retardation of disease progression compared to the positive control group that was bacterially challenged but not treated with the phage (Table 1). We suggest that this was due to the extremely rapid progression of AHPND, so that the time delay of the phage treatment (attachment of phage to bacteria) resulted in decreased efficacy of protection.

The second bioassay was modified to evaluate the prophylactic effect of the phage. In this experiment, shrimps treated with the phage showed lower mortality rates than those in the positive control group. Cumulative mortality rates were 50% following phage treatment by phage-impregnated feeding, and 25 and 50% following phage treatment by bath immersion (Table 1).

Although the two bioassays indicated that prophylactic application of pVp-1 is more effective than the therapeutic application, we added another bioassay to examine if the combination of these procedures can reach a higher level of protection. In the third bioassay, the protective effect of the phage was evaluated after prophylactic and therapeutic administration. In the treatment groups, the phage was administered by only one method, feeding with phage-impregnated pellets. In most aquaculture environments, the oral method is considered a cost-effective and realistic method of large scale administration, which is suitable for shrimp farms.

Shrimps in the positive control groups showed 100% cumulative mortalities within 48 h post-bacterial challenge. In addition, administration of the phage by either feeding or bath immersion (phage control) did not affect the physical condition or survival of experimental shrimps during the week of observation.

Histological analysis of the hepatopancreas from shrimps was carried out to diagnose AHPND. In the positive control group, histopathological features of shrimps showed typical AHPND signs; the acute sloughing of hepatopancreatic tubular epithelial cells is evident (Fig. 1c). However, histopathological examination of the hepatopancreas of phage-treated shrimps revealed a significant recovery of AHPND lesions following prophylactic and therapeutic treatments (Fig. 1d). Also, histopathological features of shrimps in negative control (Fig. 1a) and phage control (Fig. 1b) showed the normal appearance of the hepatopancreas.

As Republic of Korea is officially AHPND-free country, AHPND-related study is not permitted by law; the current study was performed in OIE reference laboratory for crustacean diseases in United States. Although the number of animals used in vivo experiment was limited, significant result of phage treatment was observed from AHPND infection. In our previous studies, the administration of pVp-1 did not cause any harm to mice or oysters during the observation period [14, 15]. In the present study, a considerable degree of mortality was observed in the phage-treated shrimps, as the progression of AHPND is extremely rapid. The timing of phage addition in relation to pathogen development is a crucial factor in the case of vibriosis [15], which may be resolved by more frequent administration of the phage.

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References

- Burge CA, Mark Eakin C, Friedman CS, Froelich B, Hershberger PK, Hofmann EE, Petes LE, Prager KC, Weil E, Willis BL, Ford SE, Harvell CD (2014) Climate change influences on marine infectious diseases: implications for management and society. *Ann Rev Mar Sci* 6:249–277
- Lightner DV, Redman RM, Pantoja CR, Noble BL, Tran LH (2012) Early mortality syndrome affects shrimp in Asia. *Glob Aquac Advocate* 15:40
- Flegel TW (2012) Historic emergence, impact and current status of shrimp pathogens in Asia. *J Invertebr Pathol* 110:166–173
- Leaño EM, Mohan CV (2012) Early mortality syndrome threatens Asia's shrimp farms. *Glob Aquac Advocate* 2012:38–39
- NACA (2012) Asia Pacific emergency regional consultation on the emerging shrimp disease: early mortality syndrome (EMS)/ acute hepatopancreatic necrosis syndrome (AHPNS). Network of Aquaculture Centres in Asia-Pacific Bangkok, Bangkok
- Soto-Rodriguez SA, Gomez-Gill B, Lozano-Olvera R, Betancourt-Lozano M, Morales-Covarrubias MS (2015) Field and experimental evidence of *Vibrio parahaemolyticus* as the causative agent of acute hepatopancreatic necrosis disease of cultured shrimp (*Litopenaeus vannamei*) in Northwestern Mexico. *Appl Environ Microbiol* 81:1689–1699
- Zhang BC, Liu F, Bian HH, Liu J, Pan LQ, Huang J (2012) Isolation, identification, and pathogenicity analysis of a *Vibrio parahaemolyticus* strain from *Litopenaeus vannamei*. *Prog Fish Sci* 33:56–62
- Han JE, Mohny LL, Tang KFJ, Pantoja CR, Lightner DV (2015) Plasmid mediated tetracycline resistance of *Vibrio parahaemolyticus* associated with acute hepatopancreatic necrosis disease (AHPND) in shrimps. *Aquac Rep* 2:17–21
- Neela FA, Nonaka L, Suzuki S (2007) The diversity of multi-drug resistance profiles in tetracycline-resistant *Vibrio* species isolated from coastal sediments and seawater. *J Microbiol* 45:64–68
- Sulakvelidze A, Kutter E (2005) Bacteriophage therapy in humans. In: Kutter E, Sulakvelidze A (eds) *Bacteriophages: biology and application*. CRC Press, Boca Raton, pp 381–436
- Gutiérrez D, Martínez B, Rodríguez A, García P (2010) Isolation and characterization of bacteriophages infecting *Staphylococcus epidermidis*. *Curr Microbiol* 61:601–608
- Alagappan K, Karupiah V, Deivasigamani B (2016) Protective effect of phages on experimental *V. parahaemolyticus* infection and immune response in shrimp (Fabricius, 1798). *Aquaculture* 453:86–92
- Jun JW, Han JE, Tang KFJ, Lightner DV, Kim J, Seo SW, Park SC (2016) Potential application of bacteriophage pVp-1: agent combating *Vibrio parahaemolyticus* strains associated with acute hepatopancreatic necrosis disease (AHPND) in shrimp. *Aquaculture* 457:100–103
- Jun JW, Kim HJ, Yun SK, Chai JY, Park SC (2014) Eating oysters without risk of vibriosis: application of a bacteriophage against *Vibrio parahaemolyticus* in oysters. *Int J Food Microbiol* 188:31–35
- Jun JW, Shin TH, Kim JH, Shin SP, Han JE, Heo GJ, De Zoysa M, Shin GW, Chai JY, Park SC (2014) Bacteriophage therapy of a *Vibrio parahaemolyticus* infection caused by a multiple antibiotic resistant O3:K6 pandemic clinical strain. *J Infect Dis* 210:72–78
- Lightner DV (1996) A handbook of shrimp pathology and diagnostic procedures for diseases of cultured penaeid shrimp. World Aquaculture Society, Baton Rouge