



Short communication

Potential application of bacteriophage pVp-1: Agent combating *Vibrio parahaemolyticus* strains associated with acute hepatopancreatic necrosis disease (AHPND) in shrimp



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ABSTRACT

Acute hepatopancreatic necrosis disease (AHPND, also known as early mortality syndrome, EMS) caused by *Vibrio parahaemolyticus* has resulted in severe marine shrimp mortality and significant economic losses in related aquaculture throughout Southeast Asia and Central America. As no viable remedy has yet been reported, the main objective of this study was to develop an effective bacteriophage (phage)-based method of controlling AHPND/EMS. To determine the bacteriolytic activity of phage pVp-1 against *V. parahaemolyticus* strains causing AHPND/EMS, its infectivity was tested on 22 strains isolated from geographically diverse regions (5 of the Asian type and 17 of the Mexican type). This phage was able to infect 90.9% (20 strains among 22 strains) of the AHPND/EMS-related *V. parahaemolyticus* strains used in this study, and demonstrated substantial bacteriolytic activity against three strains known to be highly pathogenic. To the best of our knowledge, this is the first report of a virulent phage infecting *V. parahaemolyticus* strains responsible for AHPND/EMS, and indicates the potential utility of pVp-1 in phage therapy.

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1. Introduction

Vibrio species are among the most abundant bacteria in marine environments (Thompson et al., 2004). Of these, *V. anguillarum*, *V. harveyi*, and *V. parahaemolyticus* are known to be pathogenic to shrimp (Lightner and Mc Vey, 1993). Certain strains of the latter cause acute hepatopancreatic necrosis disease (AHPND, also known as early mortality syndrome, EMS), which have resulted in severe mortality (up to 100%) in aquacultures of the marine shrimp *Penaeus vannamei* and *P. monodon* (Flegel, 2012; Leño and Mohan, 2012; Lightner et al., 2012). This disease has caused significant economic losses in China since 2009, Southeast Asia (including Thailand, Vietnam, and Malaysia) since 2011, and Central American countries such as Mexico (since 2013), Costa Rica (from 2015), and Honduras (also from 2015) (NACA, 2012; Soto-Rodriguez et al., 2015; Zhang et al., 2012). As antibiotics are considered the standard treatment for this disease, they have frequently been used as prophylaxis or therapy for AHPND/EMS (Han et al., 2015a; Neela et al., 2007). The most commonly used of these is tetracycline (Neela et al., 2007). However, the imprudent use of antibiotics

has led to increasing numbers of antibiotic-resistant bacterial strains (Han et al., 2015a; Neela et al., 2007). For instance, according to a previous report (Han et al., 2015a), *V. parahaemolyticus* strains associated with AHPND/EMS in Mexico were found to be resistant to oxytetracycline and exhibited a high level of resistance to tetracycline.

Phages, viruses that infect bacteria, can be used to control infectious diseases in humans, animals, and plants (Biswas et al., 2002; Bruttin and Brüssow, 2005; Chhibber et al., 2008; Kumari et al., 2009; Nagy et al., 2012; Sulakvelidze and Kutter, 2005; Vinodkumar et al., 2005). Phages have been proposed as an alternative method since they show an effective bacteriolytic activity and possess advantages over conventional antibiotics: phages are natural and relatively inexpensive (Gutiérrez et al., 2010; Sulakvelidze and Kutter, 2005). More importantly, no serious side effects of phage therapy have been reported to date (Gutiérrez et al., 2010; Sulakvelidze and Kutter, 2005). Recently, an acceleration of phage therapy research has become desirable due to the increase of antibiotic-resistant bacteria, the limited choice of effective treatments, and a delay in the development of novel antibiotics (Hagens and Loessner, 2010). Considering the lack of feasible remedies reported for AHPND/EMS, the development of effective treatment methods is necessary.

In our previous study, we described a virulent *Siphoviridae* phage, pVp-1, capable of infecting *V. parahaemolyticus* (Jun et al., 2014b).

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pVp-1 showed effective bacteriolytic activity against 20 (74%) of 27 multiple-antibiotic-resistant *V. parahaemolyticus* strains (Jun et al., 2014b). The aim of the present study was to determine whether phage pVp-1 induces effective bacteriolysis of AHPND/EMS-associated pathogenic strains isolated from geographically diverse regions.

2. Materials and methods

2.1. AHPND/EMS-related bacterial isolates and DNA extraction

The *V. parahaemolyticus* strains associated with AHPND/EMS analyzed in this study are shown in Table 1. These bacterial isolates were obtained from pond water, sediment samples, and the stomachs of shrimp affected by AHPND/EMS in Southeast Asia and Central America. Pure cultures were obtained by streaking on 2% NaCl tryptic soy agar (TSA) plates.

For DNA extraction from pure bacterial cultures, each isolate was grown in 2% NaCl tryptic soy broth (TSB) at 28–29 °C with gentle shaking (100 rpm). The culture was then centrifuged at 5000 × g for 5 min, and the resulting pellet was resuspended in 1 ml water before being boiled for 10 min.

2.2. AHPND/EMS identification and typing

Conventional PCR assays were conducted to amplify *pirA*- and *pirB*-like genes for AHPND/EMS-associated *V. parahaemolyticus* identification, as previously described (Han et al., 2015c). PCR targeting a Tn3-like transposon region was performed for plasmid typing (Han et al., 2015b).

2.3. Phage host range and efficiency of plating (EOP) analysis

The phage's host range was determined by spot test. Ten microliter of concentrated phage lysate ($>10^9$ PFU/ml) was added dropwise to a TSA plate overlaid with AHPND/EMS-associated *V. parahaemolyticus* cells (10^9 CFU/ml). The plate was allowed to dry and then incubated overnight. The spots were classified into three categories according to their clarity: clear, turbid, and no reaction. These phage activity tests were repeated three times to obtain reliable results. Plaque-forming units (PFU) assays were performed using a conventional double-layer agar method (Adams, 1959). EOP values were determined by calculating the ratio of the PFU value obtained with each phage-

susceptible strain to that observed with the indicator strain (*V. parahaemolyticus* 13-028/A3).

3. Results and discussion

3.1. AHPND/EMS-associated *V. parahaemolyticus* identification and typing

A total of 22 *V. parahaemolyticus* strains responsible for AHPND/EMS were isolated from shrimp aquaculture environments and shrimp themselves in various geographical locations, and identified using PCR amplification with specific primers (Table 1). The typing method used revealed that five strains (isolated from Vietnam) belonged to the Asian type, while 17 strains (Mexico, three; Costa Rica, four; Honduras, two; Central American country 1, one; Central American country 2, six; Central American country 3, one) belonged to the Mexican type. In this study, the isolate typing results were consistent with the locations from which samples were sourced, as previously established using plasmid-based PCR (Han et al., 2015b).

3.2. Bacteriolytic activity of phage pVp-1

To evaluate the host range of pVp-1, its infectivity was tested on 22 strains of AHPND/EMS-associated *V. parahaemolyticus*. This phage infected 90.9% (20 strains among 22 strains) of AHPND/EMS *V. parahaemolyticus* strains isolated from geographically diverse regions, demonstrating that pVp-1 has a broad host range, as previously reported (Jun et al., 2014b). pVp-1 inhibited all (5/5) of the Asian type strains, and 88.23% (15/17) of the Mexican type strains, infecting all of those tested but for two strains isolated from Honduras. In our previous study, pVp-1 presented its broad host range toward multiple-antibiotic-resistant *V. parahaemolyticus* strains (Jun et al., 2014b). Considering that *Siphoviridae* phages are generally believed to have restricted host ranges (Wichels et al., 1998), these results highlight the unique characteristics of pVp-1. This phage formed clear plaques on plates of 15 (75%) of the 20 strains infected by pVp-1, including those from Vietnam (2/5), Mexico (3/3), Costa Rica (4/4), and Central America Countries 1 (1/1) and 2 (5/6).

The EOP varied among strains, with three (14-188/1, 15-142-32, and 15-234-18) exhibiting higher values than the indicator host strain (Table 1). Furthermore, pVp-1 was able to infect three representative AHPND/EMS-associated *V. parahaemolyticus* strains, resulting in clear plaques (Fig. 1) and high EOPs (≥ 0.91). These strains: 13-028/A3

Table 1
Bacterial strains used in this study and infectivity of *Vibrio* phage pVp-1.

Bacterial species	Strain	Source	Host range ^a (EOPs)	EMS type	Reference
<i>V. parahaemolyticus</i>	13-028/A3	Vietnam	++ (1.00)	Asia	Tran et al. (2013)
	14-188/1	Vietnam	+ (3.80)	Asia	Han et al. (2015b)
	14-188/2	Vietnam	++ (0.76)	Asia	Han et al. (2015b)
	VpK	Vietnam	+ (0.35)	Asia	
	12-194/g	Vietnam	+ (0.15)	Asia	Han et al. (2015b)
	13-511/A1	Mexico	++ (0.95)	Mexico	Nunan et al. (2014)
	13-306D/4	Mexico	++ (0.91)	Mexico	Nunan et al. (2014)
	14-231/D74	Mexico	++ (0.21)	Mexico	Han et al. (2015c)
	15-341-3	Costa Rica	++ (0.35)	Mexico	
	15-341-7	Costa Rica	++ (0.86)	Mexico	
	15-341-12	Costa Rica	++ (0.65)	Mexico	
	15-342-3	Costa Rica	++ (0.38)	Mexico	
	15-289-2	Honduras	-	Mexico	
	15-289-20	Honduras	-	Mexico	
	15-118-30	Central America 1	++ (0.45)	Mexico	
	15-142-32	Central America 2	+ (3.55)	Mexico	
	15-142-33	Central America 2	++ (0.93)	Mexico	
	15-234-17	Central America 2	++ (0.17)	Mexico	
	15-234-18	Central America 2	++ (6.20)	Mexico	
	15-234-22	Central America 2	++ (0.64)	Mexico	
15-234-27	Central America 2	++ (0.21)	Mexico		
15-268/A3	Central America 3	+ (0.12)	Mexico		

^a ++, clear plaque; +, turbid plaque; -, no plaque.

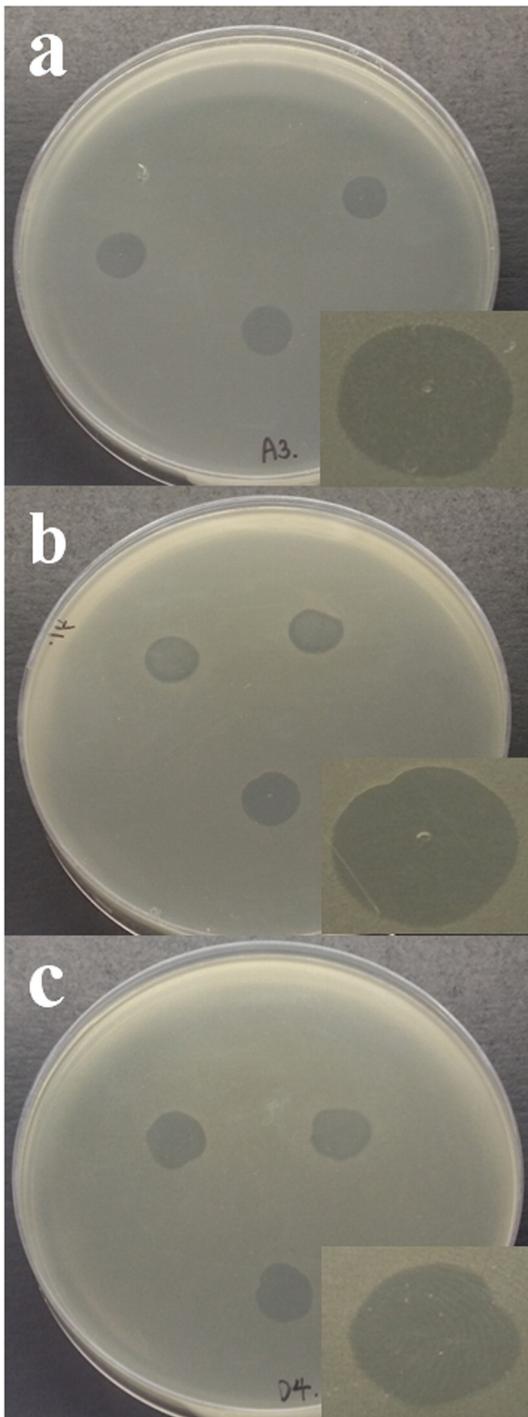


Fig. 1. Bacteriolytic activity of pVp-1 and its enlarged morphology against three representative AHPND/EMS *V. parahaemolyticus* strains: 13-028/A3 (a), Vietnam isolate; 13-511/A1 (b) and 13-306D/4 (c), Mexico isolates.

(from Vietnam), 13-511/A1, and 13-306D/4 (both from Mexico) were defined as representative isolates as they are known to be highly pathogenic to shrimp, causing 100% mortality within 24 hours post-infection (data not shown).

In our previous study, the therapeutic efficacy of pVp-1 was emphasized via a series of phage treatment trials in a mouse model of infection with a multiple-antibiotic-resistant *V. parahaemolyticus* pandemic strain (Jun et al., 2014b). In that investigation, the administration of pVp-1 at high doses did not negatively affect the physical condition or survival of the mice during the whole observation period (Jun et al., 2014b). In a separate study conducted using two models of oyster

infection, we demonstrated that pVp-1 is effective in controlling *V. parahaemolyticus* infection by bath immersion, and inhibits bacterial growth when applied to the oyster-surface (Jun et al., 2014a). As this phage did not cause any harm to oysters (Jun et al., 2014a), we expect no serious side effects to result from its use on shrimp.

The results of the current study imply that further research regarding the application of pVp-1 in shrimp aquaculture is justified. Despite the potential utility of pVp-1 in treating AHPND/EMS-related *V. parahaemolyticus* infection, further studies involving its use in a bona fide shrimp aquaculture environment are required. Moreover, in vivo experiments are now in progress to evaluate the protective effects conferred on shrimp by this phage.

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