Vibrio changes in *Penaeus vannamei* shrimp and water after infection of acute hepatopancreatic necrosis disease

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Introduction

Acute hepatopancreatic necrosis disease (AHPND), formerly known as early mortality syndrome (EMS), causes high mortality in major farmed shrimp species, including *Penaeus monodon* and *Penaeus vannamei*. Infected shrimp exhibit typical gross signs of AHPND, including a pale to whitish and atrophied hepatopancreas (HP) and an empty stomach and midgut, leading to rapid mortality within days of onset. Since its first report in China in 2009, AHPND has rapidly spread across major shrimp-producing countries, causing severe economic losses in the global aquaculture industry. The pathogenicity of AHPND is associated with the presence of *pir*AB toxin genes, which can be carried by multiple *Vibrio* species, including *Vibrio parahaemolyticus* and *Vibrio campbellii*. In shrimp farms, AHPND transmission occurs through multiple routes, including infected shrimp, feces, and contaminated water. However, most previous studies have relied on PCR-based detection methods, which do not distinguish between viable and non-viable bacteria, leaving a critical gap in understanding how infectious bacteria persist and spread in shrimp and their environment. Therefore, quantitative analysis of viable bacterial loads in both shrimp tissues and environmental samples is essential for understanding the dynamics of infection and identifying potential transmission pathways. In particular, investigating bacterial loads in hepatopancreas, feces, and tank water may provide key insights into how AHPND-causing *Vibrio* are maintained and transmitted within aquaculture systems.

Material and Methods

Juvenile *Penaeus vannamei* (0.5–2.0 g) were acclimated under controlled conditions (28°C, 25 ppt) and confirmed to be AHPND-negative by PCR prior to the experiment. Two AHPND-causing strains, *Vibrio parahaemolyticus* (VP) and *Vibrio campbellii* (VC), were cultured in tryptic soy broth supplemented with 2% NaCl (TSB+; Difco) and used for immersion challenge at 10^6 CFU/mL. Experimental shrimp were divided into three groups (VP, VC, and NC). Each group included one tanks for mortality monitoring, two tanks for sampling of the HP and tank water, and one tank for feces collection. Each 20-L tank contained 17 shrimp in 14-L of aerated artificial seawater under controlled conditions (28°C, 25 ppt).For feces sampling, shrimp were transferred to clean tanks after disinfection using formalin (300 ppm) and povidone-iodine (100 ppm) to minimize contamination from tank water and external surfaces. Samples including HP, feces, and tank water were collected at multiple time points (0–168 hpi). Samples from HP, feces, and tank water were serially diluted and plated on thiosulfate–citrate–bile salts–sucrose agar (TCBS; Difco), followed by incubation at 28°C for 24 h. Bacterial loads were calculated as CFU/g or CFU/mL, and representative green colonies were selected and confirmed by colony PCR for AHPND.

Figure 1. Overview of experimental procedures.

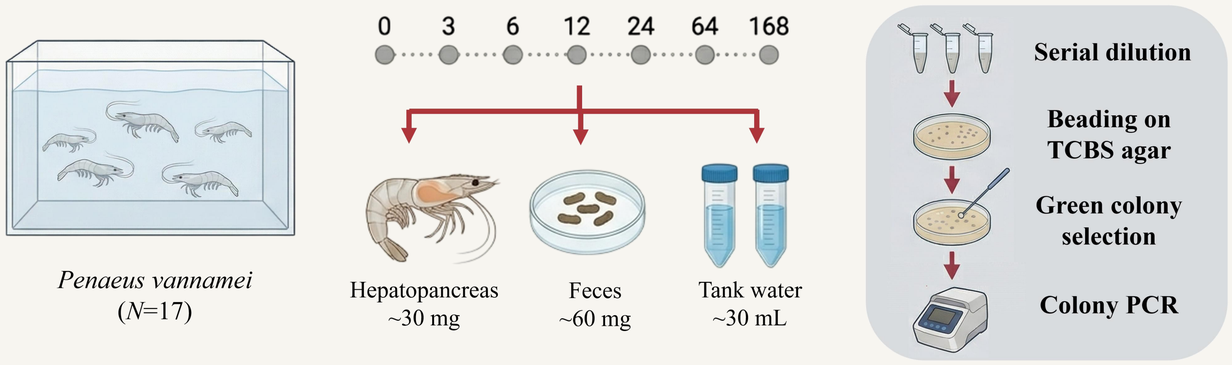


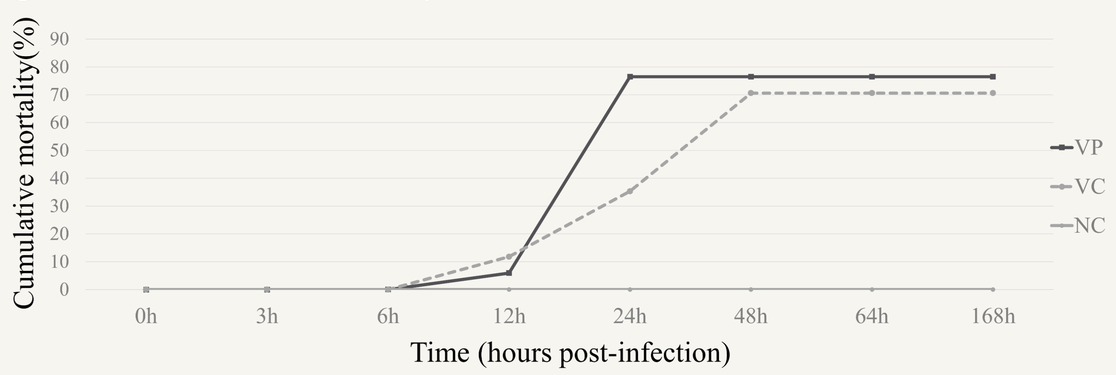
Table 1. The primers used in this study

Name	Primer sequence (5′-3′)	Size (bp)	Description	Reference
VpPirA-284F	TGACTATTCTCACGATTGGACTG	284	AHPND detection	Han et al. (2015)
VpPirA-284R	CACGACTAGCGCCATTGTTA			
VpPirB-392F	TGATGAAGTGATGGGTGCTC	392		
VpPirB-392R	TGTAAGCGCCGTTTAACTCA			

Results and Discussions

In the laboratory shrimp assay, shrimp exhibited typical symptoms of AHPND, and mortality was first observed at 12 hpi in both groups. The cumulative mortality reached 76.5% at 24 hpi in the VP group and 70.6% at 48 hpi in the VC group.

Figure 2. Cumulative mortality after AHPND infection



Vibrio loads increased rapidly in all sample types, including HP, feces, and tank water, during the early phase of infection. In HP samples, bacterial loads increased to 10^6 CFU/g at 3 hpi and reached approximately 10^8 CFU/g at 12–24 hpi. In feces samples, *Vibrio* counts increased after infection and reached 10^8–10^9 CFU/g at 24 hpi in both groups. Similarly, in tank water samples, bacterial loads increased to 10^6 CFU/mL at 6 hpi and reached 10^7 CFU/mL at 12 hpi. These results indicate rapid bacterial proliferation and severe environmental contamination during the acute phase of infection, coinciding with peak shrimp mortality.

Table 2. *Vibrio* counts from samples at various time points post-infection

Sample	Group	Time post-infection						
		0hpi	3hpi	6hpi	12hpi	24hpi	64hpi	168hpi
HP (CFU/g)	VP	1.48×10^4	1.25×10^6	1.25×10^6	TNTC(>10^8)	TNTC(>10^8)	4.73×10^7	1.43×10^5
	VC	5.15×10^5	7.90×10^6	8.64×10^7	2.90×10^7	TNTC(>10^8)	2.03×10^7	2.00×10^4
	NC	3.28×10^5	2.75×10^5	1.32×10^7	3.28×10^7	8.25×10^5	ND	4.75×10^3
Feces (CFU/g)	VP	1.80×10^5	7.58×10^7	2.27×10^8	8.10×10^7	2.10×10^8	3.28×10^7	2.47×10^7
	VC	5.00×10^6	1.03×10^8	2.33×10^9	TNTC(>10^9)	TNTC(>10^9)	5.25×10^7	8.78×10^7
	NC	3.01×10^7	2.50×10^8	4.50×10^7	2.95×10^8	6.15×10^7	1.27×10^7	1.41×10^7
Tank water (CFU/mL)	VP	<10	3.90×10^6	3.28×10^6	TNTC(>10^7)	TNTC(>10^7)	2.85×10^3	1.55×10^3
	VC	9.50×10^1	TNTC(>10^7)	4.48×10^6	TNTC(>10^7)	2.85×10^4	4.00×10^2	1.63×10^3
	NC	4.00×10^1	3.15×10^4	4.29×10^5	3.55×10^4	1.65×10^4	1.45×10^4	1.19×10^4

TNTC: too numerous to count not detected; ND: Not detected.

At 168 hpi, *Vibrio* counts decreased in all sample types, however, AHPND-associated *Vibrio* were still detected in surviving shrimp. Although *Vibrio* counts decreased over time, AHPND-associated *Vibrio* were still detected at the final time point. In HP samples, 100% (VP) and 60% (VC) of the colonies were positive at the final time point, whereas approximately 5% of colonies from feces samples remained positive in both groups. In contrast, no positive colonies were detected in tank water samples at 168 hpi. These results demonstrate that viable AHPND-causing bacteria persist in surviving shrimp even after the acute infection phase. Notably, feces contained the highest bacterial loads during infection and remained positive at later time points, suggesting continuous shedding of viable bacteria and highlighting its potential role as a major transmission route in shrimp culture systems. This persistence of viable bacteria in surviving shrimp may contribute to the maintenance of the pathogen within aquaculture environments, thereby increasing the risk of subsequent infection cycles.

Table 3. AHPND colony PCR results in the HP, feces, and tank water samples

Time	Sample	No. of positive colonies / No. of colonies tested (%)		
		VP group	VC group	NC group
Starting day (3hpi)	HP	9/17(53%)	17/17 (100%)	0/5 (0%)
	Feces	10/10 (100%)	10/10 (100%)	0/5 (0%)
	Tank water	10/10 (100%)	10/10 (100%)	0/5 (0%)
Terminal day (168hpi)	HP	15/15 (100%)	9/15 (60%)	0/15 (0%)
	Feces	1/20 (5%)	1/20 (5%)	0/15 (0%)
	Tank water	0/15 (0%)	0/15 (0%)	0/5 (0%)

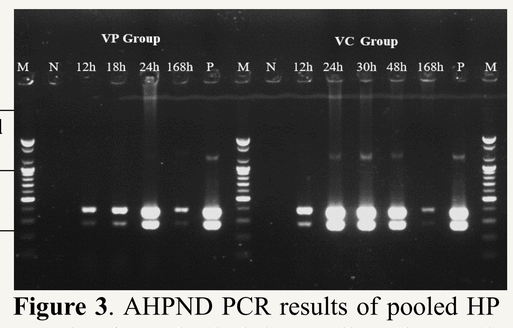


Figure 3. AHPND PCR results of pooled HP samples from dead shrimp collected at each mortality time point and surviving shrimp at 168 hpi. Lane M: 100-bp ladder; Lane N: negative control; Lanes 1–4: VP group pooled HP samples at 12, 18, and 24 hpi and surviving shrimp at 168 hpi; Lanes 5–9: VC group pooled HP samples at 12, 24, 30, and 48 hpi and surviving shrimp at 168 hpi; Lane P: positive control

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