Evaluation of temperature dependent pathogenicity of acute hepatopancreatic necrosis diseases

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Introduction

Acute hepatopancreatic necrosis disease (AHPND) is an economically devastating shrimp disease, which is caused by *Vibrio* species harboring the pVA1 plasmid that encodes binary Pir toxins: PirA and PirB. The World Organisation for Animal Healh (WOAH) states that AHPND outbreaks peak during hot and dry seasons, but field data indicate that AHPND occurrence is not limited to hot seasons. In addition, there are few studies controversy to WOAH description. Therefore, to comprehensively elucidate the temperature-dependent pathogenicity of AHPND-related *Vibrio* strains, this study performed *in vivo* (bioassay) and *in vitro* (bacterial growth kinetics and toxin gene expression) analyses using AHPND-causing *V. parahaemolyticus* ($AHPND_{VP}$) and *V. campbellii* ($AHPND_{VC}$) under three different temperature conditions.

Material and Methods

❖ Bacterial and shrimp preparation

In this study, two AHPND-causing strains, *V. parahaemolyticus* ($AHPND_{VP}$) and *V. campbellii* ($AHPND_{VC}$), were used. Both strains were cultured in tryptic soy broth supplemented with 2% NaCl (TSB^{+}) to reach approximately 10^9 CFU/mL for further analysis.

For the bioassay, juvenile *Penaeus vannamei*, weighing 0.5—2.0 g, were obtained from a local Korean shrimp farm, and confirmed to be AHPND-free by PCR using primers described in Table 1.

Table 1. Primers and probe used for this study

Type	Primers	Sequence of the primer (5'-3')	Size (bp)	Reference
PCR	AP4-F1	ATGAGTAACAATATAAAACATGAAAC	1,269	Dangtip et al. (2015)
	AP4-R1	ACGATTTCGACGTTCCCCAA		
	AP4-F2	TTGAGAATACGGGACGTGGG	230	
	AP4-R2	GTTAGTCATGTGAGCACCTTC		
qPCR	VpPirA-F	TTGGACTGTCGAACCAAACG	135	Han et al. (2015b)
	VpPirA-R	GCACCCCATTGGTATTGAATG		
	AHPND-P1	[FAM]AGACAGCAAACATACACCTATCATC CCGGA[TAMRA]		

❖ Temperature-dependent bacterial growth kinetics

Each bacterial inoculum was diluted 1:1,000,000 in fresh TSB^{+} and incubated at three different temperatures (32, 27, and 22 °C) at 100 rpm for 30 hours. Every 2 hours, viable bacteria were enumerated on tryptic soy agar supplemented with 2% NaCl. In addition, the generation time was calculated following the formulas.

$$n \text{ (Number of generations)} = (Log\ b - LogB)/Log2$$

$$g \text{ (Generation time)} = t/n$$

where B and b are the CFU counts at the beginning and end of the interval, respectively, and t represents the time interval.

❖ Bioassay

A bioassay was conducted using 20-L tanks filled with 14-L of seawater (25 ppt). Shrimp (N=306) were distributed across 18 tanks, with 17 shrimp per tanks. The tanks were assigned to three groups: negative control (NC), $AHPND_{VP}$, and $AHPND_{VC}$ challenge groups. Subsequently each group was divided into three temperatures (32, 27, and 22 °C) in duplicate. For artificial infection, each bacterial inoculum was added to the corresponding tanks to achieve a final bacterial concentration of 10^6 CFU/mL. For NC group, the same volume of TSB+ was added.

For molecular and histopathological analyses, two shrimp were collected from each tank at 12 hours post-infection (hpi) and 24 hpi. Of the collected shrimp, one was used for DNA extraction followed by PCR and qPCR using primer and probe sets (Table 1). The other was immediately fixed in Davidson's AFA, and transferred to 70% EtOH for tissue embedding and H&E staining. The degeneration grade was evaluated from G1 to G4 using light microscopy.

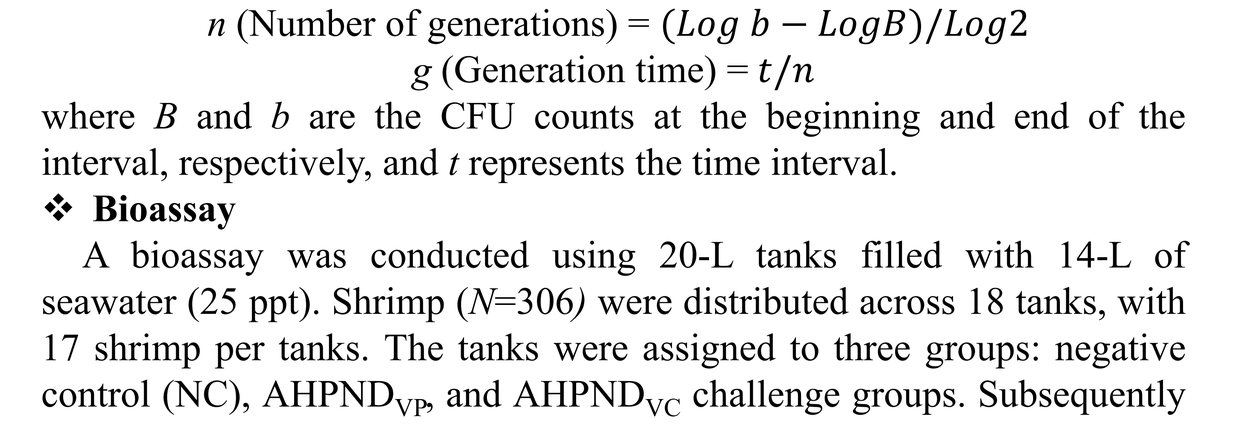


Figure 1. Temperature-dependent bacterial growth kinetics

Results

❖ Temperature-dependent bacterial growth kinetics

Based on CFU enumeration, $AHPND_{VP}$ and $AHPND_{VC}$ displayed the shortest lag phase and entered exponential phase most rapidly, compared to 22 and 27 °C. However, by 30h, both strains ultimately reached high bacterial counts (approximately 10^8 CFU/ml) regardless of the culture temperatures. $AHPND_{VP}$ and $AHPND_{VC}$ exhibited the shortest generation times at 32 °C (16.9 and 17.1 min), compared to 27 °C (28.4 and 18.5 min) and 22 °C (29.9 and 21.0 min).

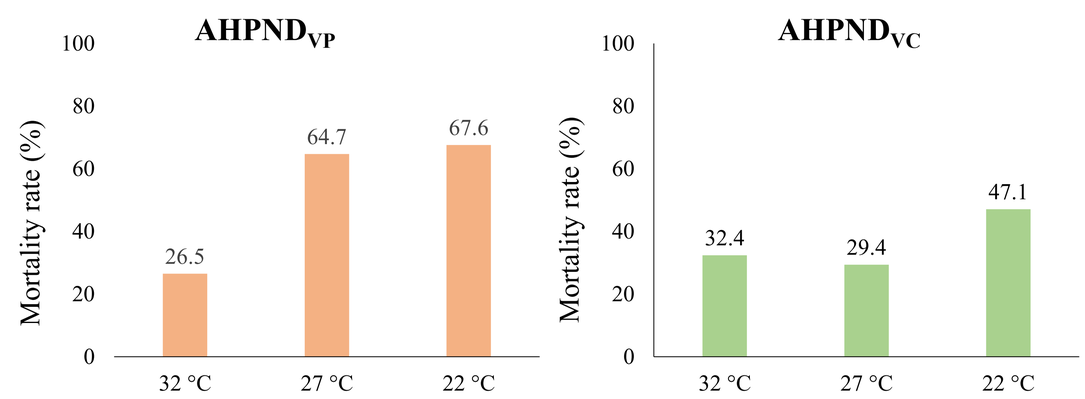


Figure 2. Cumulative mortality of the bioassay.

❖ Bioassay

In shrimp exposed to AHPND strains, mortalities were first observed at 12 hpi. On the other hand, no mortality was observed in the NC group. Interestingly, in both challenge groups, the highest mortality rates were observed at the lowest temperature groups (22 °C) (Fig 2).

All sampled shrimp were confirmed to be AHPND positives by PCR or qPCR (Table 2). In histopathological examination, shrimp exposed to $AHPND_{VP}$ exhibited a severe hepatopancreatic necrosis (G4) including a massive structural collapse across all temperatures. Notably, in $AHPND_{VC}$ group, the most pronounced necrosis was observed in 22 °C (G4), compared to 27 and 32 °C (G3) (Fig 3).

Table 2. Molecular analysis of shrimp collected at 12 and 24 hpi

Group	Temp	12 hpi		24 hpi	
		PCR	Ct ± SD (Copies/μL ± SD)	PCR	Ct ± SD (Copies/μL ± SD)
$AHPND_{VP}$	32	-	33.60±1.85 ($2.19\times10^3\pm1.30\times10^3$)	+	33.59±0.4 ($2.20\times10^3\pm3.05\times10^3$)
	27	+	29.69±0.12 ($2.72\times10^4\pm2.12\times10^3$)	+	30.36±0.16 ($1.76\times10^4\pm9.06\times10^2$)
	22	+	31.06 ± 0.39 ($1.14\times10^4\pm2.81\times10^3$)	+	34.84±0.75 ($9.86\times10^2\pm2.38\times10^2$)
$AHPND_{VC}$	32	+	34.99±0.34($8.96\times10^2\pm9.80\times10$)	+	25.65±0.39 ($3.65\times10^5\pm4.57\times10^4$)
	27	+	Undetermined	+	27.12±0.02 ($1.42\times10^5\pm9.10\times10^2$)
	22	+	31.91±0.93 ($7.11\times10^3\pm4.00\times10^3$)	+	34.57±0.48 ($1.17\times10^3\pm1.81\times10^2$)

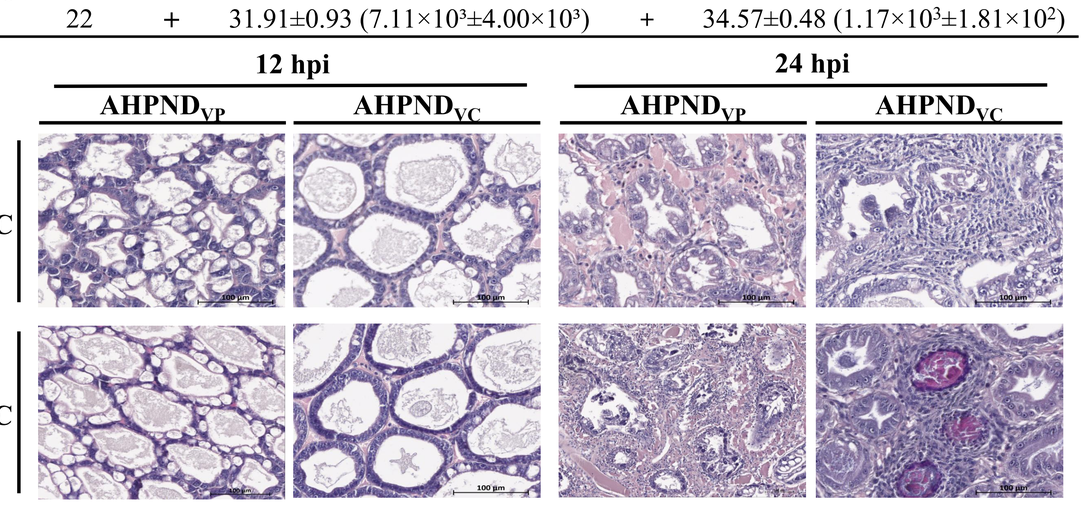


Figure 3. Histopathological analysis of shrimp collected at 12 and 24 hpi

Discussion & Conclusion

In the era of climate, extremely hot or cold water temperatures have been frequently observed. it is remarkable that AHPND-causing *Vibrio* strains are highly sensitive to temperature fluctuations, actively changing their genomes and pathogenicity. To mitigate the impact of AHPND, understanding the correlation between temperature and AHPND is necessary. However, research on the temperature-dependent interplay between AHPND and shrimp is still limited. This study revealed that low water temperature exacerbates AHPND pathogenicity *in vivo*, while bacterial growth was enhanced at high water temperatures. Therefore, implementing season-dependent management strategies is essential for AHPND prevention. For instance, environmental improvements to suppress bacterial proliferation are necessary in summer, whereas maintaining optimal temperatures is crucial during winter and transitional seasons. Ultimately, a comprehensive understanding of these temperature-dependent disease dynamics is fundamental for the sustainable future of the shrimp farming industry.