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Temporal Dynamics of *pirA* Expression During *Vibrio campbellii* Infection in *Litopenaeus vannamei*

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Abstract

Acute hepatopancreatic necrosis disease (AHPND) is one of the most devastating bacterial diseases affecting Pacific white shrimp (*Litopenaeus vannamei*) aquaculture worldwide. Although *Vibrio parahaemolyticus* has been recognized as the principal causative agent of AHPND, increasing evidence indicates that *Vibrio campbellii* also plays an important role in disease outbreaks. The present study investigated the pathogenicity and temporal dynamics of *pirA* transcription in *V. campbellii* isolated from cultured *L. vannamei*. The isolate was identified by 16S rRNA sequencing, and *pirA* expression was quantified during bacterial growth under *in vitro* conditions and throughout experimental infection *in vivo* using quantitative real-time PCR. *In vitro*, *pirA* transcription increased rapidly during the exponential growth phase and reached its highest level at 6 h after incubation, followed by a gradual decline despite continued bacterial growth. Immersion challenge experiments using juvenile shrimp (1×10^6 CFU/mL) resulted in cumulative mortality exceeding 90% within 48 h post-infection. *In vivo*, *pirA* transcription remained relatively low during the early stage of infection but increased markedly at 36 h post-infection, coinciding with rapid mortality and severe hepatopancreatic damage. Histopathological examination revealed progressive epithelial cell sloughing, degeneration of B- and F-cells, tubular collapse, and extensive tissue necrosis, which are characteristic lesions of AHPND. These findings demonstrate that *V. campbellii* possesses strong pathogenic potential. Temporal induction of *pirA* transcription was closely associated with severe hepatopancreatic lesions and mortality during experimental infection. Collectively, these findings provide new insights into the temporal dynamics of *pirA* transcription during AHPND progression and its association with tissue pathology and mortality, supporting the development of early molecular diagnostics, disease surveillance, and biosecurity strategies for sustainable shrimp aquaculture.

Keywords: acute hepatopancreatic necrosis disease (AHPND); *Vibrio campbellii*; *pirA*; pathogenicity; virulence; hepatopancreas; *Litopenaeus vannamei*

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1. Introduction

Pacific white shrimp, *Litopenaeus vannamei*, is one of the most economically important aquaculture species worldwide because of its rapid growth, high market demand, and

adaptability to intensive farming systems [1]. Global shrimp production has expanded continuously over the past two decades, driven by advances in hatchery technology and intensive culture practices. However, the intensification of shrimp farming has also accelerated the prevalence of infectious diseases, resulting in substantial economic losses and posing a major challenge to the sustainability of the shrimp aquaculture industry [2,3]. Among bacterial diseases affecting cultured shrimp, acute hepatopancreatic necrosis disease (AHPND), formerly known as early mortality syndrome (EMS), is considered one of the most devastating emerging diseases in shrimp aquaculture [4]. Since its first report in China in 2009, AHPND has rapidly spread throughout Asia and Latin America, causing severe economic losses due to high mortality during the early culture period [5]. Histopathologically, AHPND is characterized by massive sloughing of hepatopancreatic epithelial cells, degeneration of B-, F-, and R-cells, tubular atrophy, and extensive tissue necrosis, frequently resulting in cumulative mortalities exceeding 70–100% [2]. The disease is caused by plasmid-encoded binary toxins, PirA and PirB, which are secreted by pathogenic *Vibrio* species and play essential roles in hepatopancreatic tissue destruction [6,7]. Recent reviews have emphasized that AHPND pathogenesis involves complex interactions among Pir-AB-producing pathogens, environmental conditions, host susceptibility, and shrimp immune responses [8]. Although *V. parahaemolyticus* has long been recognized as the principal etiological agent of AHPND [4], increasing evidence indicates that several other *Vibrio* species carrying the *pirAB* toxin genes can also induce AHPND in cultured shrimp. Among these, *V. campbellii*, a member of the harveyi clade, has recently emerged as an important AHPND-associated pathogen responsible for severe disease outbreaks in shrimp aquaculture [9–11]. Several field investigations have reported the isolation of pathogenic *V. campbellii* from diseased shrimp exhibiting typical AHPND lesions, suggesting that this species is becoming an increasingly important pathogen in intensive shrimp farming systems [12]. Despite its increasing importance, relatively little information is available regarding the pathogenic characteristics and virulence-associated gene expression of *V. campbellii* during infection. Recent studies have further highlighted that AHPND is no longer exclusively associated with *V*. *parahaemolyticus*, but rather represents an emerging plasmid-mediated disease involving multiple members of the *V*. *harveyi* clade. In particular, *V*. *campbellii* has recently been identified as an important AHPND-associated pathogen in several countries, including South Korea, demonstrating pathogenicity comparable to that of classical AHPND-causing *V. parahaemolyticus*. Comparative genomic studies have suggested that horizontal transfer of the pVA1 virulence plasmid carrying the *pirA* and *pirB* toxin genes enables rapid dissemination of pathogenicity among closely related *Vibrio* species, thereby increasing the epidemiological complexity of AHPND [12]. In addition, recent investigations have emphasized that bacterial virulence is governed not only by the presence of toxin genes but also by their temporal regulation, which is influenced by bacterial growth phase, quorum sensing systems, environmental conditions, and host-associated signals. Consequently, characterization of the temporal dynamics of *pirA* expression during both bacterial growth and host infection has become increasingly important for understanding disease progression, improving early diagnosis, and developing more effective surveillance strategies for shrimp aquaculture [11,13].

Previous studies have primarily focused on detecting the presence of the *pirA* and *pirB* toxin genes or confirming pathogenicity through challenge experiments [11,14]. However, disease progression is a dynamic process, and the temporal expression patterns of virulence genes may differ substantially between bacterial growth under laboratory conditions and infection within the host. Understanding these transcriptional dynamics is important for elucidating AHPND pathogenesis and may provide useful biomarkers for early disease diagnosis and outbreak prediction. Furthermore, few studies have simultane-

ously investigated bacterial growth, temporal *pirA* transcription, cumulative mortality, and histopathological progression during experimental infection with pathogenic *V. campbellii*. Although both PirA and PirB function together as binary toxins responsible for AHPND pathogenesis, previous studies have widely used temporal changes in *pirA* transcription as an indicator of virulence activation [7,11]. Characterizing the temporal dynamics of *pirA* transcription may therefore improve our understanding of bacterial virulence regulation during infection and contribute to the development of early molecular diagnostics, disease surveillance, and biosecurity strategies for sustainable shrimp aquaculture. Furthermore, variation in *pirA* expression has been suggested to reflect changes in toxin production more sensitively than simple detection of the *pirAB* genes, which remain constitutively present on the pVA1 virulence plasmid. To address this knowledge gap, the present study investigated the pathogenicity and temporal dynamics of *pirA* transcription in *V. campbellii* isolated from cultured Pacific white shrimp. Specifically, bacterial growth, temporal *pirA* transcription under both *in vitro* and *in vivo* conditions, cumulative mortality, and histopathological alterations were evaluated to determine whether temporal changes in *pirA* transcription are associated with disease progression during experimental AHPND infection.

2. Materials and Methods

2.1. Bacterial Isolation and Identification

Pathogenic bacterial strains were isolated from diseased Pacific white shrimp (*L. vannamei*) collected from commercial shrimp farms in Korea during AHPND outbreaks in 2023. Shrimp showing typical clinical signs of acute hepatopancreatic necrosis disease (AHPND), including pale and atrophied hepatopancreas, empty gut, and lethargic swimming behavior, were selected for analysis.

The hepatopancreas was aseptically removed and homogenized using sterile phosphate-buffered saline (PBS). Homogenized samples were streaked onto thiosulfate citrate bile sucrose (TCBS; Difco, Detriot, MI, USA) agar and tryptic soy agar supplemented with 1.5% NaCl (TSA). Plates were incubated at 25 °C for 24 h, and morphologically distinct colonies were selected and purified by repeated streaking.

Genomic DNA from isolated bacteria was extracted using a Pathogen-spin DNA/RNA extraction kit (Intron Biotechnology, Seongnam, Republic of Korea). Identification of bacterial isolates was performed by PCR amplification of species-specific genes and 16S rRNA sequencing. PCR amplification targeting the *pirA* and *pirB* toxin genes was conducted using primer sets described by WOAH [15]. The amplified products were electrophoresed on 2.0% agarose gels and visualized under UV illumination.

The nucleotide sequences obtained from 16S rRNA PCR products were analyzed using BLAST (National Center for Biotechnology Information, Bethesda, MD, USA), and phylogenetic trees were constructed using the neighbor-joining method in MEGA 12.0 software. Among the *pirAB*-positive isolates, the *V. campbellii* isolate used in the subsequent growth kinetics and immersion challenge experiments was recovered from Farm G during a confirmed AHPND outbreak in July 2023. This isolate was selected because it exhibited the highest pathogenicity during preliminary screening and was therefore considered representative for investigating temporal *pirA* expression during experimental infection. The *V. alginolyticus* isolate was not examined further because the primary objective of this study was to characterize the temporal dynamics of *pirA* transcription in AHPND-associated *V. campbellii*.

2.2. Experimental Shrimp and Rearing Conditions

Juveniles Pacific white shrimp (*L. vannamei*) with a mean body weight of 0.6–1.0 g were obtained from a commercial hatchery and acclimated for 7 days before experimental

infection. Shrimp were maintained in aerated seawater tanks at 25–27 °C with a salinity of 28–30 psu under continuous aeration.

Prior to the experiment, shrimp were confirmed negative for AHPND, white spot disease, and hepatopancreatic microsporidiosis (HPM) by PCR analysis. During acclimation and challenge experiments, shrimp were fed a commercial shrimp diet twice daily.

Water quality parameters were maintained as follows: dissolved oxygen > 5.0 mg L^{-1}, pH 7.8–8.2, and ammonia nitrogen < 0.5 mg L^{-1}.

2.3. In Vitro Bacterial Growth and pirA Expression Kinetics

Pure cultures of *V. campbellii* were inoculated into tryptic soy broth supplemented with 1.5% NaCl (TSB) and incubated at 30 °C with shaking at 180 rpm. Samples were collected at 2, 6, 12, 24, 36, and 48 h after incubation. Bacterial growth was quantified by serial dilution and colony counting on TCBS agar plates, and results were expressed as CFU mL^{-1}. For toxin gene expression analysis, total RNA was extracted from bacterial cultures using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentration and purity were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA (cDNA) was synthesized using a reverse transcription kit (Takara, Shiga, Japan). Quantitative real-time PCR (RT-qPCR) targeting the *pirA* gene was performed using a QuantStudio™ 3 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) according to the protocol described by Cruz-Flores et al. [16] with minor modifications. Primer sequences, reaction conditions, and amplification parameters followed the published protocol. Relative expression levels were normalized to the bacterial 16S rRNA gene and calculated using the $2^{-\Delta\Delta C}{}_{T}$ method [14].

2.4. Immersion Challenge Test

The pathogenicity of the selected *V. campbellii* isolate was evaluated by immersion challenge according to previously described methods with slight modifications. Healthy shrimp (mean body weight 1.54 ± 0.25 g) were randomly assigned to the experimental tanks and acclimated for 7 days before bacterial challenge. Each treatment consisted of three independent tanks, with ten shrimp per tank. Each tank was considered an independent experimental unit for all statistical analyses. The selected *V. campbellii* isolate was cultured in TSB at 30 °C for 24 h and adjusted to a final concentration of 1×10^6 CFU mL^{-1} using sterile seawater. The bacterial suspension concentration was confirmed by serial dilution and plate counting immediately before challenge. Shrimp were randomly distributed into experimental tanks (10 shrimp per tank, triplicate groups) and immersed in seawater containing bacterial suspensions for 120 h. A mock-infected control group was maintained under identical conditions using sterile TSB without bacteria. During the challenge period, no water exchange was performed, and shrimp mortality was recorded every 12 h. Dead shrimp were immediately removed and subjected to PCR analysis for confirmation of *pirA* and *pirB* toxin genes. A bacterial concentration of 1×10^6 CFU mL^{-1} was selected because previous studies have demonstrated that this inoculum consistently induces typical AHPND lesions and reproducible mortality in immersion challenge models while minimizing excessive variability among replicate tanks [4,13,17].

2.5. Quantification of pirA Expression in Shrimp Tissues

To investigate *in vivo pirA* transcription dynamics, hepatopancreas tissues were sampled at 0, 6, 12, 24, 36, and 48 h post-infection. Total RNA extraction and cDNA synthesis were performed as described above. Quantitative RT-qPCR targeting *pirA* was conducted using gene-specific primers. Expression levels were expressed as log10-transformed rela-

tive expression values. Detection of *pirA* and *pirB* genes was performed according to the multiplex real-time PCR assay described by Cruz-Flores et al. [16].

2.6. Histopathological Analysis

Hepatopancreas tissues collected from experimental shrimp were fixed in Davidson's fixative for 24 h and subsequently transferred to 70% ethanol. Fixed tissues were dehydrated through a graded ethanol series, embedded in paraffin, sectioned at 4–5 μm, and stained with hematoxylin and eosin (H&E). Histological sections were examined using a BX53 light microscope (Olympus, Tokyo, Japan) at 100× and 400 × magnification. Digital images were captured using an Olympus DP74 digital camera and processed using cellSens Standard software (version 2.3; Olympus, Tokyo, Japan). Histopathological lesions, including epithelial cell sloughing, degeneration of B-, F-, and R-cells, tubular collapse, hemocytic infiltration, and tissue necrosis, were evaluated qualitatively. Histopathological changes were evaluated qualitatively rather than quantitatively, and lesion severity was described based on representative microscopic observations.

2.7. Statistical Analysis

All experimental data are presented as the mean ± standard deviation (SD). Prior to statistical analysis, data were tested for normality using the Shapiro–Wilk test and for homogeneity of variances using Levene's test. Differences among groups for bacterial growth, relative *pirA* expression, and other quantitative variables were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) post hoc test. Cumulative survival was analyzed using the Kaplan–Meier method, and differences between survival curves were evaluated using the log-rank (Mantel–Cox) test. Statistical analyses were performed using IBM SPSS Statistics version 29.0 (IBM Corp., Armonk, NY, USA), with replicate tanks considered as the experimental units. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Identification of Pathogenic Vibrio Isolates

Bacterial isolates obtained from diseased Pacific white shrimp (*L. vannamei*) showing typical clinical signs of acute hepatopancreatic necrosis disease (AHPND) were successfully cultured on TCBS and TSA agar plates. Sampling information, average *Vibrio* counts, and bacterial isolates obtained from shrimp farms are summarized in Table 1. The isolates produced green colonies on TCBS agar and smooth circular colonies on TSA agar, which are characteristic morphological features of pathogenic *Vibrio* species. PCR analysis targeting *pirA* and *pirB* toxin genes confirmed that both *V. campbellii* and *V. alginolyticus* isolates carried AHPND-associated toxin genes. Species-specific PCR amplification further identified the isolates as *V. campbellii* and *V. alginolyticus*, respectively. Phylogenetic analysis based on 16S rRNA gene sequences demonstrated that the isolates clustered closely with reference strains of *V. campbellii* and *V. alginolyticus* retrieved from the GenBank database. The *V. campbellii* isolate formed a distinct clade within the harveyi group with high bootstrap support values (>95%, Figure 1), confirming species-level identification. The results indicated that both isolates belonged to pathogenic *Vibrio* species associated with AHPND outbreaks in cultured shrimp.

Table 1. Sampling information, *Vibrio* abundance, and bacterial isolates were obtained from AHPND-affected shrimp farms in 2023.

Type	Farm	Average *vibrio* Number (CFU/mL)	Bacteria (16s rRNA)	*pirA* PCR Result	Occurrence of AHPND **
BFT *	A	$8.7 \times 10^3 \pm 7.9 \times 10^3$	*V. campbellii* *V. alginolyticus* *Vibrio* sp.	Positive	July: AHPND (3/3)
	B	$5.1 \times 10^3 \pm 2.1 \times 10^3$	*V. alginolyticus* *V. harveyi* *Vibrio* sp.	Negative	Negative
	C	$9.6 \times 10^3 \pm 7.7 \times 10^3$	*V. alginolyticus* *V. owensii* *Vibrio* sp.	Positive	July, September: AHPND
	D	$2.5 \times 10^3 \pm 2.3 \times 10^3$	*V. alginolyticus* *V. harveyi* *Vibrio* sp.	Negative	Negative
	E	$2.5 \times 10^3 \pm 1.5 \times 10^3$	*V. harveyi* *V. alginolyticus* *Vibrio* sp.	Negative	Negative
	F	$3.4 \times 10^3 \pm 3.2 \times 10^3$	*V. owensii* *Vibrio* sp.	Negative	Negative
Pond	G	$6.1 \times 10^2 \pm 5.4 \times 10^2$ (July: 2.1×10^5)	*V. campbellii* *V. alginolyticus* *Vibrio* sp.	Positive	July: AHPND
	H	$4.6 \times 10^3 \pm 3.0 \times 10^3$	*P. damselae* *V. alginolyticus* *Vibrio* sp.	Negative	Negative
	I	$1.0 \times 10^4 \pm 1.0 \times 10^4$	*A. enteropelogenes* *V. owensii* *V. alginolyticus* *P. damselae* *Vibrio* sp.	Negative	Negative
	J	$1.2 \times 10^3 \pm 1.1 \times 10^3$	*V. alginolyticus* *V. owensii* *V. anguillarum* *Vibrio* sp.	Negative	Negative
	K	$1.1 \times 10^3 \pm 1.0 \times 10^3$	*V. owensii* *V. alginolyticus* *Vibrio* sp.	Negative	Negative
	L	$1.0 \times 10^4 \pm 1.0 \times 10^4$	*V. alginolyticus* *V. owensii* *Vibrio* sp.	Negative	Negative

* BFT = Biofloc Technology. ** Occurrence of AHPND was confirmed by farm disease records and PCR diagnosis.

3.2. In Vitro Bacterial Growth and pirA Gene Expression Kinetics

The relationship between bacterial growth and relative *pirA* expression in *Vibrio campbellii* cultured in TSB medium is shown in Figure 2. The isolate exhibited rapid exponential growth during the early incubation period. Bacterial density increased continuously from 2 to 36 h, reaching its maximum at 36 h before declining at 48 h. In contrast, relative *pirA* expression increased rapidly during the early exponential phase and reached its highest level at 6 h post-incubation, followed by a gradual decline despite continued bacterial growth. During the later stages of incubation (24–48 h), relative *pirA* expression remained detectable but at substantially lower levels than those observed at 6 h. Overall, relative pirA expression reached its peak before maximal bacterial density, indicating that pirA

transcription was transiently induced during the early exponential growth phase rather than being directly proportional to bacterial cell density.

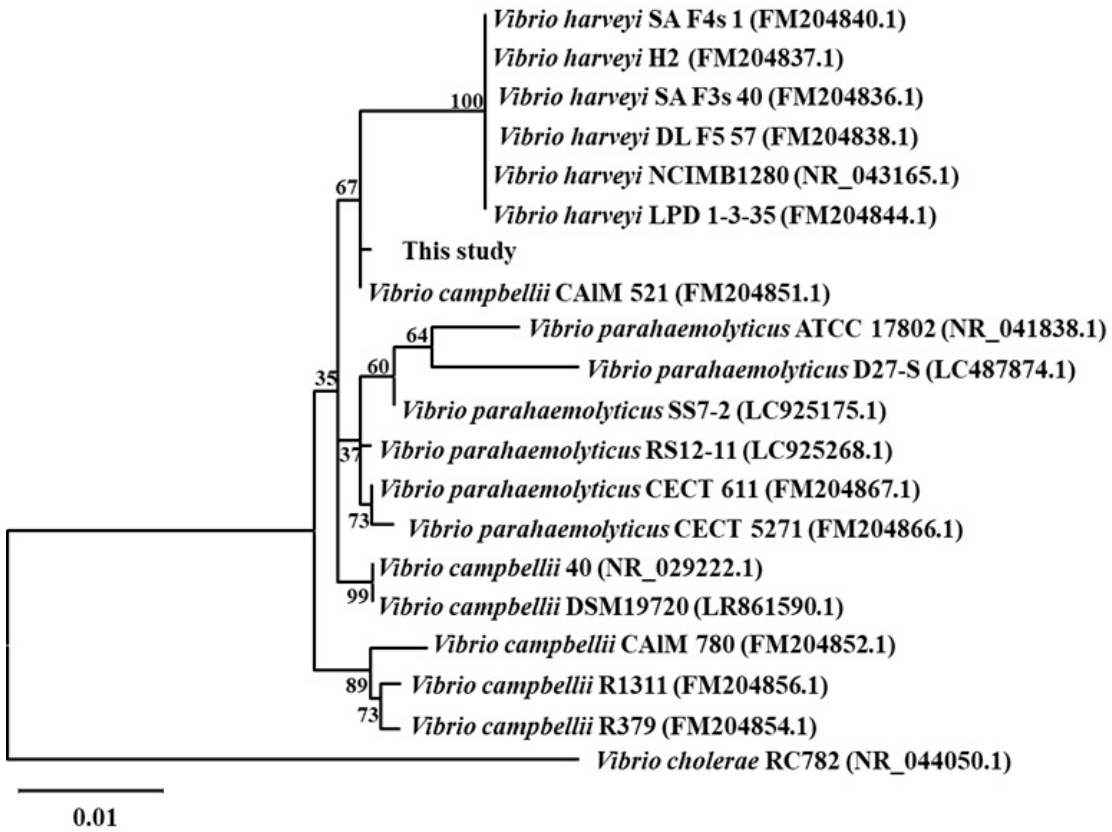

Figure 1. Phylogenetic analysis of pathogenic *Vibrio* isolates obtained from diseased *Litopenaeus vannamei*. The phylogenetic tree was constructed based on 16S rRNA gene sequences using the neighbor-joining method with 1000 bootstrap replicates. Bootstrap values greater than 50% are indicated at branch nodes. Evolutionary distances were calculated using the Kimura two-parameter model. Reference sequences were obtained from GenBank.

3.3. Temporal Induction of In Vivo pirA Gene Expression During Infection

Temporal changes in *pirA* transcription in the hepatopancreas of shrimp experimentally challenged with *V. campbellii* are presented in Figure 3. *pirA* expression remained relatively low during the early stage of infection (0–12 h). However, transcription increased markedly after 24 h post-infection and reached its highest level at 36 h, with a maximal expression of approximately 9.03 ± 0.27 $\log_{10}$ expression units. Following this peak, *pirA* expression gradually declined at 48 h post-infection, coinciding with extensive hepatopancreatic tissue damage and high cumulative mortality. The temporal pattern of *pirA* induction corresponded closely with disease progression, suggesting that expression of this virulence gene is highly associated with active host–pathogen interactions during AHPND development. The delayed peak in *pirA* transcription observed *in vivo*, compared with the rapid induction observed under *in vitro* culture conditions, indicates that virulence gene expression may be influenced by host-derived environmental signals during infection.

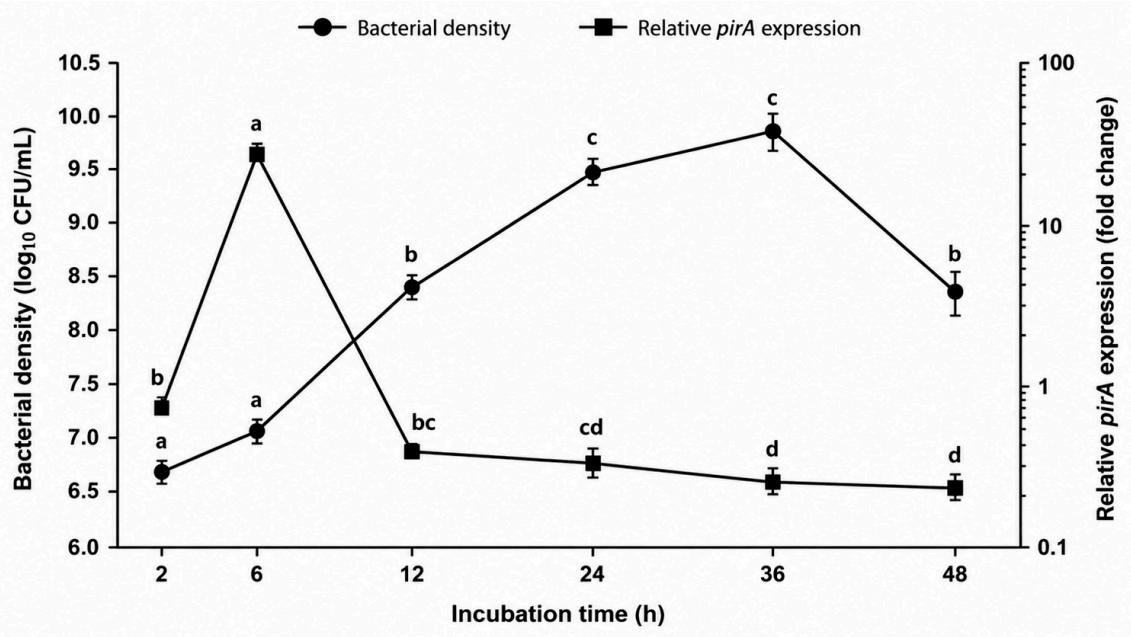

Figure 2. Temporal relationship between bacterial growth and relative pirA gene expression in Vibrio campbellii during *in vitro* culture. Bacterial growth and relative pirA gene expression were determined by plate counting and RT-qPCR, respectively. Data are presented as mean ± SD (n = 3). Different lowercase letters indicate significant differences among sampling times (one-way ANOVA followed by Tukey's HSD test, $p < 0.05$).

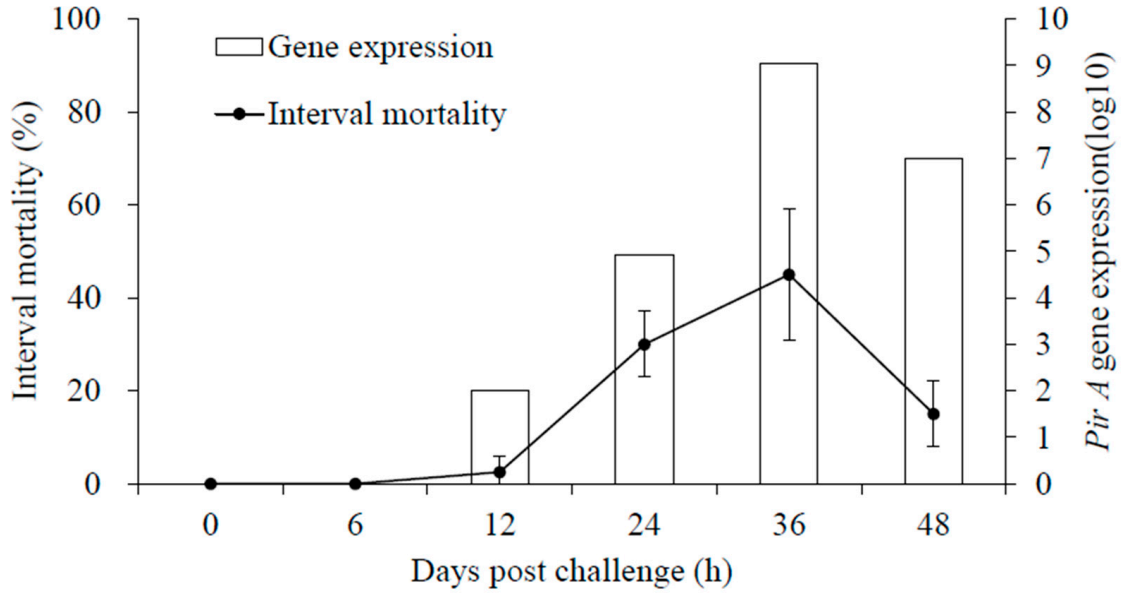

Figure 3. Temporal relationship between relative pirA expression and interval mortality in *Litopenaeus vannamei* experimentally challenged with *Vibrio campbellii*. Interval mortality represents the percentage of shrimp that died during each observation interval and is not cumulative mortality. Relative pirA expression was quantified by RT-qPCR. Data are presented as mean ± SD (n = 3).

3.4. Mortality Progression Following Immersion Challenge

Immersion challenge experiments demonstrated that *V. campbellii* caused severe mortality in juvenile Pacific white shrimp (Figure 4). No mortality was observed in the mock-infected control group throughout the 120 h experimental period. Mortality in the challenged group began to increase at approximately 24 h post-infection and progressed rapidly thereafter, with cumulative mortality reaching approximately 55% at 36 h and exceeding

90% by 48 h post-infection. Kaplan–Meier survival analysis showed that shrimp challenged with *V. campbellii* had significantly lower survival than the control group (log-rank test, $p < 0.001$). Moribund shrimp exhibited characteristic clinical signs of AHPND, including pale hepatopancreas, reduced feeding activity, lethargic swimming behavior, and empty gastrointestinal tracts. PCR analysis of dead shrimp confirmed the presence of the *pirA* and *pirB* toxin genes, whereas no toxin genes were detected in the control group.

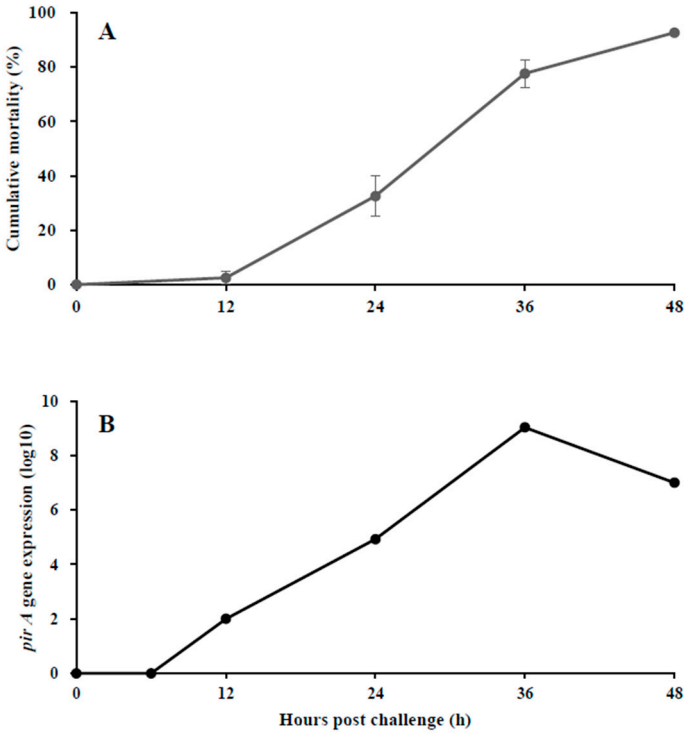

Figure 4. Temporal relationship between *in vivo pirA* transcription and cumulative mortality in *Litopenaeus vannamei* experimentally challenged with *Vibrio campbellii*. (**A**) Relative *pirA* expression in hepatopancreatic tissues quantified by RT-qPCR at different time points following immersion challenge. (**B**) Cumulative mortality of shrimp following experimental infection with *V. campbellii*. Data are presented as the mean ± SD ($n = 3$).

3.5. Histopathological Changes in the Hepatopancreas Following Experimental Infection

Histopathological examination revealed progressive hepatopancreatic lesions characteristic of acute hepatopancreatic necrosis disease (AHPND) in shrimp experimentally infected with *V. campbellii* (Figure 5). In the control group, hepatopancreatic tubules exhibited normal architecture with intact epithelial cells and well-defined tubular structures. Distinct B-, F-, and R-cells were clearly observed along the tubular epithelium. At 24 h post-infection, initial pathological alterations were evident, including degeneration of epithelial cells and partial sloughing of B- and F-cells into the tubular lumen. Although the overall tubular structure remained relatively intact, cellular debris had begun to accumulate within the lumen. At 36 h post-infection, hepatopancreatic lesions became markedly more severe. Extensive detachment of epithelial cells from the basement membrane and collapse of hepatopancreatic tubules were frequently observed. Large amounts of cellular debris and pronounced hemocytic infiltration were also present within the affected tissues. By 48 h post-infection, severe necrosis and extensive sloughing of hepatopancreatic epithelial cells were evident throughout the tissue. Tubular boundaries became indistinct owing to extensive tissue destruction, resulting in complete collapse of the hepatopancreatic architecture. These pathological changes were accompanied by widespread hemocytic

infiltration and advanced tissue necrosis, representing the typical histopathological features of AHPND.

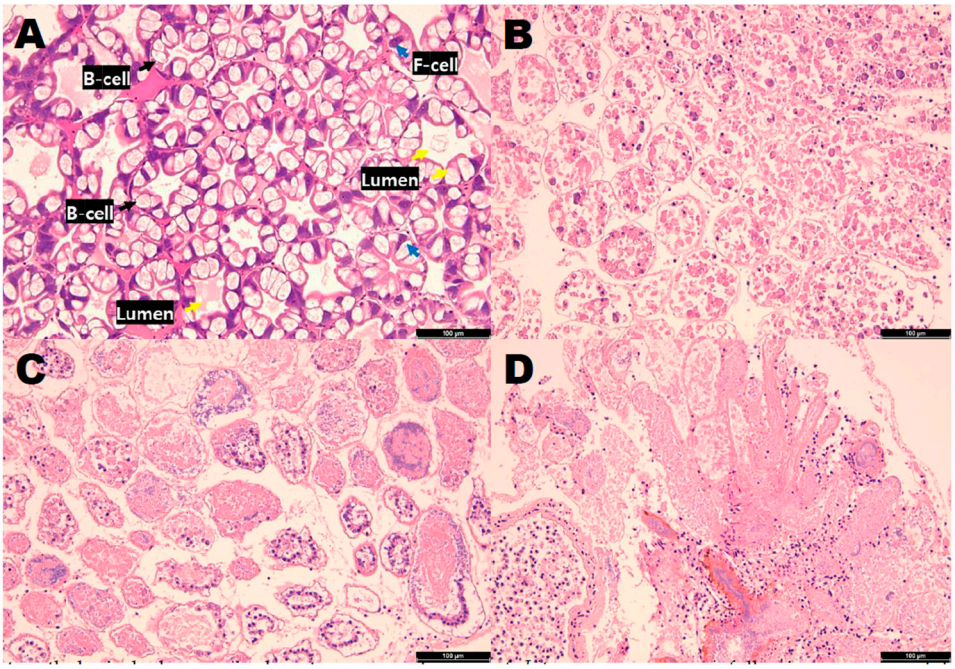

Figure 5. Histopathological changes in hepatopancreas tissues of *Litopenaeus vannamei* following experimental infection with pathogenic *Vibrio campbellii* (H&E staining). (**A**) Normal hepatopancreas of control shrimp showing intact tubular structures and normal epithelial cells. (**B**) Degeneration and sloughing of hepatopancreatic epithelial cells at 24 h post-infection. (**C**) Severe tubular collapse and hemocytic infiltration at 36 h post-infection. (**D**) Massive tissue necrosis and extensive epithelial sloughing at 48 h post-infection. Scale bar = 100 μm.

4. Discussion

The present study demonstrated that *Vibrio campbellii* isolated from diseased Pacific white shrimp possessed strong pathogenicity and was capable of inducing severe mortality and characteristic AHPND lesions in experimentally infected shrimp. The challenged shrimp exhibited cumulative mortality exceeding 90% within 48 h post-infection, indicating rapid disease progression and high virulence of the isolate. Similar pathogenic characteristics of *pirAB*-positive *V. campbellii* have previously been reported in shrimp affected by AHPND [9,18]. Traditionally, AHPND has mainly been associated with *V. parahaemolyticus* carrying *pirA* and *pirB* toxin genes [4,6]. However, increasing evidence indicates that other *Vibrio* species belonging to the harveyi clade can also acquire *pirAB*-containing plasmids and induce AHPND-like pathology in shrimp [19]. The present results further support the hypothesis that *V. campbellii* is an important AHPND-associated pathogen in shrimp aquaculture systems. The rapid induction of mortality observed in this study may be associated with efficient bacterial colonization and early *pirA* gene expression during infection. Previous studies demonstrated that PirAB toxins cause extensive sloughing of hepatopancreatic epithelial cells, leading to impaired digestive function and rapid systemic deterioration in infected shrimp [20]. In the present study, severe mortality coincided with increased *pirA* expression and progressive hepatopancreatic destruction, suggesting a close relationship between *pirA* transcription, tissue pathology, and disease severity.

A major finding of the present study was the distinct temporal regulation of *pirA* transcription under both *in vitro* and *in vivo* conditions. Under *in vitro* conditions, *pirA* expression increased rapidly during the exponential growth phase and reached its maximum at 6 h post-incubation before declining during the stationary phase. Similar growth

phase-dependent expression has been reported for several virulence-associated genes in pathogenic *Vibrio* species, in which toxin production is coordinated with bacterial population density through quorum sensing systems [11,21–23]. These observations suggest that *pirA* transcription is temporally regulated rather than being constitutively expressed throughout bacterial growth. In contrast, *pirA* expression during experimental infection remained relatively low during the early stage of infection but increased markedly at 36 h post-infection, coinciding with rapid mortality and severe hepatopancreatic degeneration. This delayed induction suggests that, in addition to bacterial growth, host-derived environmental and physiological factors may contribute to the temporal regulation of *pirA* transcription during infection. Such factors may include oxidative stress, osmotic fluctuations, antimicrobial peptides, digestive metabolites (e.g., bile acids and taurocholate), nutrient limitation, and interactions with host immune cells, all of which have been reported to influence bacterial virulence gene expression. In particular, bile acids and taurocholate may modulate biofilm formation, *pirA*/*pirB* expression, and PirAB toxin release in AHPND-causing *Vibrio parahaemolyticus* [24]. However, because these host-derived factors were not directly investigated in the present study, their contribution to the delayed *pirA* expression observed *in vivo* remains hypothetical and warrants further investigation. In addition, differences in incubation temperature between the *in vitro* (30 °C) and *in vivo* (25–27 °C) experiments may also have influenced bacterial growth and the temporal pattern of *pirA* transcription. Temperature has been reported to influence bacterial metabolism, growth kinetics, quorum sensing, and virulence gene expression in several *Vibrio* species and therefore may have contributed to the delayed *pirA* peak observed during experimental infection [22,25]. One possible explanation for the delayed induction of *pirA in vivo* is that *V. campbellii* may first require successful colonization of the shrimp hepatopancreas before activating quorum sensing-dependent virulence pathways. Unlike nutrient-rich laboratory media, the hepatopancreatic environment is characterized by dynamic host-derived factors, including osmotic fluctuations, nutrient limitation, digestive metabolites, and host immune responses, all of which may influence bacterial transcriptional responses. Furthermore, host immune defenses, including oxidative stress and antimicrobial peptides produced by shrimp hemocytes, may further modulate bacterial gene expression during infection. As bacterial colonization progresses, local bacterial populations may gradually increase to levels that favor quorum sensing-mediated regulation of virulence genes. Such density-dependent regulation has been described in several pathogenic *Vibrio* species and has been proposed as one possible mechanism coordinating toxin production after bacterial populations become established within host tissues [13,21,23]. Recent functional evidence has shown that the quorum-sensing-associated transcriptional regulator AphB directly interacts with the *pirAB* promoter and is required for efficient *PirA* and *PirB* expression in AHPND-causing *V. parahaemolyticus*. Moreover, acidic conditions enhanced AphB-dependent toxin expression, indicating that environmental cues may modulate *pirAB* transcription [13]. Furthermore, because the *pirAB* toxin genes are located on the pVA1 virulence plasmid, their expression is likely to be influenced by complex interactions between plasmid-borne regulatory elements and chromosomal regulatory networks rather than by constitutive transcription alone. Horizontal transfer of the pVA1 plasmid among different *Vibrio* species has been recognized as a major mechanism underlying the emergence of new AHPND-causing pathogens [6,9,19]. Collectively, these observations suggest that environmental stress, quorum sensing, and plasmid-associated regulation may act together to coordinate *pirA* expression during infection. Although the precise molecular mechanisms regulating *pirA* expression remain to be elucidated, the present findings support the hypothesis that activation of this virulence gene results from a combination of bacterial growth phase, local cell density, host-derived environmental signals, and plasmid-mediated regulatory

mechanisms. Future studies integrating transcriptomic analysis, quorum sensing pathways, and host immune responses will be necessary to clarify the regulatory network controlling *pirA* expression during AHPND progression. Collectively, these findings suggest that temporal monitoring of *pirA* expression may serve as a promising molecular biomarker for assessing disease progression, virulence activation, and the early diagnosis of AHPND outbreaks in shrimp aquaculture. These findings may also facilitate the development of transcription-based monitoring tools for assessing the infection status and virulence activation of AHPND-causing Vibrio under field conditions.

Histopathological examination revealed progressive hepatopancreatic lesions that were consistent with classical AHPND pathology previously described in shrimp infected with *pirAB*-positive *Vibrio* species [20,26]. During the early stage of infection (24 h post-infection), degeneration and sloughing of hepatopancreatic epithelial cells were observed while the overall tubular architecture remained relatively intact. At 36 h post-infection, severe tubular collapse, epithelial detachment, and accumulation of cellular debris became evident, indicating extensive tissue destruction. By 48 h post-infection, massive sloughing and necrosis resulted in almost complete collapse of hepatopancreatic tubules. The hepatopancreas plays a critical role in digestion, nutrient absorption, energy storage, and immune defense in shrimp [20]. Therefore, severe degeneration of hepatopancreatic tissues likely contributes directly to physiological dysfunction, reduced feeding activity, and mortality in infected shrimp. The progressive histopathological changes observed in the present study closely paralleled the temporal increase in *pirA* expression, supporting the hypothesis that Pir toxins are directly involved in hepatopancreatic tissue destruction during AHPND progression.

The present findings have several important implications for shrimp disease management and aquaculture biosecurity. First, the rapid increase in *pirA* expression and mortality observed during the early infection stage highlights the importance of early diagnosis and continuous disease monitoring in shrimp farms. Since clinical signs and mortality progressed rapidly after toxin induction, early molecular detection of *pirAB*-positive *Vibrio* strains may improve disease control and reduce production losses. Second, the present results suggest that monitoring toxin gene expression may provide more accurate information regarding disease progression than simple bacterial detection alone. Previous field studies demonstrated that pathogenic *Vibrio* populations can persist in shrimp farming environments without necessarily causing disease outbreaks [18]. Therefore, monitoring systems integrating bacterial abundance, *pirA* expression, and environmental conditions may provide improved predictive capacity for AHPND outbreaks. Environmental stressors such as salinity fluctuation, high temperature, and organic matter accumulation are known to influence *Vibrio* proliferation and shrimp susceptibility [11]. Therefore, maintaining stable water quality and minimizing environmental stress may play critical roles in reducing disease outbreaks in intensive shrimp farming systems. Finally, the emergence of *pirAB*-positive *V. campbellii* further emphasizes the importance of strengthened biosecurity measures, including SPF seed usage, water disinfection, and routine pathogen surveillance in shrimp hatcheries and grow-out farms.

Although the present study demonstrated clear relationships among *pirA* expression, pathogenicity, and histopathological progression, several limitations should be considered. First, the molecular mechanisms regulating *pirA* expression during host infection remain unclear. Comprehensive transcriptomic analyses may provide valuable information regarding virulence regulation, host immune responses, and metabolic pathways associated with AHPND progression. Second, quorum sensing systems are known to play important roles in virulence regulation in many pathogenic *Vibrio* species. However, quorum sensing-related regulatory pathways were not investigated in the present study. Further studies

examining quorum sensing gene expression and cell-density-dependent toxin regulation may improve understanding of AHPND pathogenesis. Third, environmental factors such as salinity, temperature, and organic loading may strongly influence bacterial virulence and host susceptibility under field conditions. Another limitation of the present study is that only a single bacterial concentration (1×10^6 CFU mL^{-1}) was evaluated. This inoculum was selected because it has been widely used in experimental AHPND immersion challenge models to produce reproducible infection, characteristic histopathological lesions, and consistent mortality, thereby enabling the temporal regulation of *pirA* expression to be investigated under standardized experimental conditions. Since the primary objective of this study was to characterize the temporal dynamics of *pirA* expression rather than dose-dependent pathogenicity, a single challenge dose was considered appropriate. Nevertheless, future studies should compare multiple bacterial concentrations to further elucidate dose-dependent *pirA* expression patterns and disease progression. Furthermore, our evaluation was restricted solely to *pirA* transcription. Although PirA and PirB function together as binary toxins responsible for AHPND, the present study focused on *pirA* because temporal changes in *pirA* transcription have been widely used as a molecular indicator of virulence activation in AHPND-associated *Vibrio* species [7,11]. Previous studies have suggested that *pirA* expression exhibits greater temporal variation during infection, whereas *pirB* expression tends to remain relatively stable after toxin gene activation. Therefore, monitoring *pirA* transcription provides a sensitive approach for evaluating temporal changes in virulence regulation during disease progression. Nevertheless, simultaneous analysis of *pirA*, *pirB*, and Pir toxin production will be necessary to fully elucidate the molecular mechanisms underlying AHPND pathogenesis. Despite these limitations, the present study provides novel insights into the pathogenicity and temporal regulation of *pirA* expression in *V. campbellii*, thereby improving our understanding of AHPND pathogenesis. These findings establish a scientific foundation for future investigations into the molecular mechanisms governing virulence regulation and support the development of more effective diagnostic and disease management strategies for shrimp aquaculture.

5. Conclusions

The present study demonstrated that *pirAB*-positive *V. campbellii* isolated from diseased Pacific white shrimp possesses strong pathogenicity and is capable of inducing severe mortality and characteristic AHPND lesions under experimental conditions. Temporal analysis revealed distinct *pirA* transcription patterns, with rapid induction during the exponential growth phase *in vitro* and delayed peak expression during host infection *in vivo*. Notably, maximal *pirA* expression coincided with severe hepatopancreatic degeneration and rapid mortality, indicating a temporal association among virulence gene expression, tissue destruction, and disease progression. These findings provide new insights into the pathogenic mechanisms of *V. campbellii* and highlight the potential value of temporal *pirA* monitoring as an indicator of AHPND progression. The present study contributes to a better understanding of AHPND pathogenesis and provides a scientific basis for the development of early diagnostic tools, disease surveillance, and biosecurity strategies in shrimp aquaculture.

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Institutional Review Board Statement: Ethical review and approval were waived because shrimp are invertebrates and are not classified as laboratory animals under the Laboratory Animal Act of the Republic of Korea (Act No. 19918). Article 2(2) of the Act defines laboratory animals as vertebrate animals used for animal experiments. Therefore, Litopenaeus vannamei is not subject to mandatory Institutional Animal Care and Use Committee (IACUC) review under the applicable Korean legislation.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available from the corresponding author upon reasonable request.

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