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Article

First Report and Pathogenicity of *Vibrio campbellii* (VCAHPND) Isolated in South Korea

Hee-Jae Choi [1], Da-Yeon Choi [1], Ji-Hoon Lee [2], Jun-Hwan Kim [3] and Yue Jai Kang [1,*]

[1] Department of Aquatic Life Medicine, Kunsan National University, Gunsan City, Jeonbuk-do, South Korea
[2] Aquatic Animal Disease Control Center, National Institute of Fisheries Science (NIFS), Busan City, South Korea
[3] Department of Aquatic Life Medicine, College of Ocean Sciences, Jeju National University, Jeju City, South Korea
* Correspondence: email: kyj5088@hanmail.net; Tel.: +82-(0)63-469-1888

Abstract: The global shrimp farming industry is impacted by various infectious diseases, leading to marked production losses. Virulent *Vibrio parahaemolyticus* strains (VPAHPND) and other *Vibrio* species cause acute hepatopancreatic necrosis disease (AHPND) in shrimp. We isolated a binary toxin PirA/B-bearing *V. campbellii* strain (HJ-2023) from a Korean shrimp farm. The biochemical characteristics and antibiotic susceptibility of HJ-2023 were analyzed and compared to those of PirA/B-bearing VPAHPND. Non-AHPND-causing *V. campbellii* was the control. The pathogenicity of each strain was analyzed in white-leg shrimp. Phylogenetic analysis classified the isolated HJ-2023 strain as *V. campbellii*. Biochemical tests confirmed distinct properties of HJ-2023, compared to the genetically similar *V. harveyi* group. The AHPND-causing toxin gene of HJ-2023 showed 100% similarity to the *rpoD* gene of VCAHPND. An antibiotic disc susceptibility test indicated that HJ-2023 showed resistance to ampicillin, clindamycin, lincomycin, and erythromycin, which is effective against gram-positive bacteria, and showed weak sensitivity to gentamycin. Furthermore, HJ-2023 was 25% less toxic than VPAHPND but could be lethal during aquaculture-related outbreaks. These results highlight the complexity and virulence of AHPND-causing strains through multiple pathways and confirm the pathogenicity of *V. campbellii*, which increases the risk posed by AHPND to shrimp aquaculture. Our findings could help prevent AHPND.

Keywords: acute hepatopancreatic necrosis disease; *Vibrio parahaemolyticus*; *Vibrio campbellii*; *Litopenaeus vannamei*

Key Contribution: *V. campbellii* (VCAHPND; HJ-2023) can cause acute hepatopancreatic necrosis disease (AHPND). First report the presence of HJ-2023 on a shrimp farm in South Korea and evaluated its pathogenicity

1. Introduction

Of the aquatic invertebrate species that are farmed worldwide, shrimp comprise a fast-growing species with a wide global market, with approximately 4 million tons being produced annually [1]. However, infectious diseases can impede productivity in shrimp farms; additionally, novel pathogens arising from mutations can emerge in farm environments. Acute hepatopancreatic necrosis disease (AHPND), a representative bacterial disease, has occurred frequently in shrimp farms over the past 12 years, causing enormous economic losses of more than $1 billion annually throughout Asia, where approximately 70% of global shrimp is produced [2–4]. Before being named AHPND, it was known as early mortality syndrome (EMS) [5]. AHPND was first discovered in China in 2009 [6] and has since spread to various countries in Southeast Asia and North America [7–9].

In 2013, the main causative agent of AHPND was identified to be *Vibrio parahaemolyticus*, a gram-negative, rod-shaped bacterium [10]. *V. parahaemolyticus* contains the pVA1 plasmid that expresses the fatal binary toxin PirA/B, which causes sudden death in shrimp [11]. This plasmid contains the toxin PirA/B genes of *Photorhabdus* and *Xenorhabdus* and is homologous to the insect toxin expression

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plasmid. When *V. parahaemolyticus* acquires a plasmid expressing the toxin genes PirA and PirB (15.75 and 56.12 kDa, respectively), it causes AHPND in shrimp [12–15].

V. parahaemolyticus AHPND is called EMS because it causes high mortality rates of 40–100% for approximately 8–30 d in giant tiger prawns (*Penaeus monodon*) and white-leg shrimp (*Penaeus vannamei*) in the post-larval stage after stocking [13,16]. However, AHPND has been shown to affect not only post-larval stages but also various growth stages, including the adult stage [17]. Additionally, shrimp affected by AHPND exhibit extensive necrosis and the detachment of tubular epithelial cells, empty digestive tracts, pale-to-white hepatopancreases, lethargy, anorexia, and slow growth [18,19].

Outbreaks of *V. parahaemolyticus* AHPND first occurred in China in 2009, followed by Vietnam (2010), Malaysia (2011), Thailand (2012), Mexico (2013), the Philippines (2015), and South America (2016) [11,14,19,20]. Initially, *V. parahaemolyticus* was identified as the only causative agent of AHPND; however, other *Vibrio* strains carrying a similar pVA1 plasmid containing PirA/B toxin genes have recently been reported in different parts of the world, including in *Vibrio campbellii* from Latin America [21], *V. owensii* from China [16], *V. harveyi* from Malaysia [22], and *V. punensis* from South America [23].

In South Korea, AHPND outbreaks caused by *V. parahaemolyticus* strains were reported in a shrimp farm on the west coast in 2016 and have continued to occur [24], but there have been no reports of AHPND outbreaks caused by other *Vibrio spp*. strains. In this study, we identified a novel *Vibrio* strain encoding an AHPND-causing toxin gene. The strain was isolated from a domestic vannamei shrimp farm, identified, and evaluated for its characteristics and toxicity.

2. Results

2.1. Bacterial identification via PCR

To identify the collected bacteria, PCR was performed on the HJ-2023 strain isolated from the hepatopancreases of white-leg shrimp and on the control bacteria (VP_{AHPND} and $VC_{non\text{-}AHPND}$). PCR analysis was performed using primers targeting the *16S* rRNA and *rpoD* genes that can detect the *Vibrio* strains HJ-2023, VP_{AHPND}, and $VC_{non\text{-}AHPND}$. The *toxR* gene that detects *V. parahaemolyticus* was detected only in VP_{AHPND}, and the *vch* gene sequence that detects *V. campbellii* was detected in the isolated HJ-2023 strain and $VC_{non\text{-}AHPND}$. Similarly, only HJ-2023 and VP_{AHPND} were positive for gene sequences targeting the virulence genes *PirA* (*AP3*) and *PirB*, which were present in the pVA1 plasmid (Figure 1).

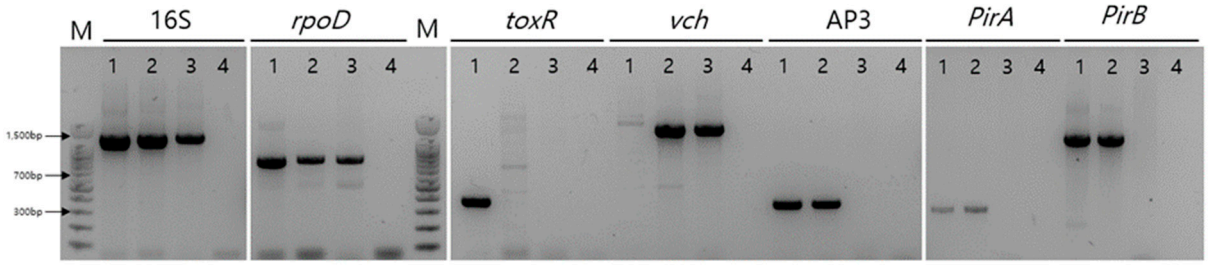

Figure 1. PCR detection of the toxR, vch, AP3, rpoD, 16S, PirA, and PirB genes. Line M, 100-bp DNA ladder; Line 1, VPAHPND; Line 2, HJ-2023; Line 3, VCnon-AHPND; Line 4, Negative control.

2.2. Phylogenetic tree based on pathogenic rpoD

Phylogenetic analysis of the *rpoD* gene grouped the isolated HJ-2023 strain with *V. campbellii* strains registered in GenBank and showed a close relationship between *V. campbellii* and *V. harveyi* (Figure 2).

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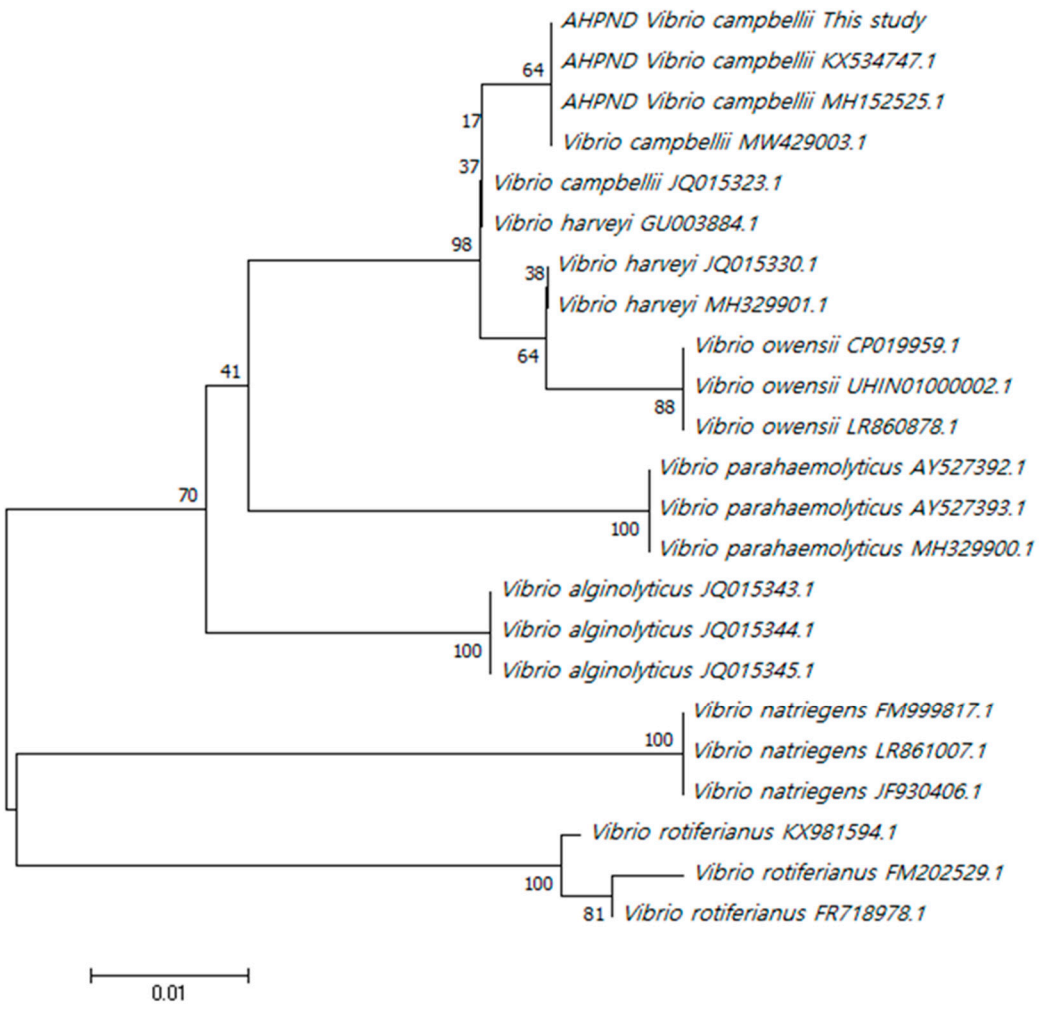

Figure 2. Phylogenetic tree based on concatenated rpoD gene sequences. The analysis was performed using the neighbor-joining method. Bar = 0.01 expected nucleotide substitutions per site. The neighbor-joining tree reflects the percentage of trees based on 1,000 bootstrap sets possessing the branch (MEGA 7 software). Percentages are displayed for the results that were ≥ 10% at the internal nodes.

2.3. Biochemical characteristics

The biochemical properties of the isolated HJ-2023 strain were determined using API Tests 20E and 20NE. Biochemical tests revealed differences in ODC, GEL, ARA, PNPG, MNE, and GNT between the AHPND and non-AHPND strains (Table 1). Furthermore, differences were observed in GLU, NAG, ADI, and CIT between the VP_{AHPND} and HJ-2023 strains. The biochemical reactions of the isolated HJ-2023 strain were different from those of the existing strains; the HJ-2023 strain showed positive reactions for ONPG and ESC (Tables 1 and 2).

Table 1. API test 20E.

Isolates		VP_{AHPND}	HJ-2023	$VC_{Non\text{-}AHPND}$
Biochemical test	Abbreviation			
β-galactosidase	ONPG	-	+	-
Arginine dihydrolase	ADH	-	-	-

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Lysine décarboxylase	LDC	+	+	+
Ornithine décarboxylase	ODC	-	+	+
Citrate utilization	CIT	-	-	-
H_2S production	H_2S	-	-	-
Urease production	URE	-	-	-
Tryptophane désaminase	TDA	-	-	-
Indole production	IND	+	+	+
Acétoïne production (Vogues–Proskawer reaction)	VP	-	-	-
Gelatinase production	GEL	-	+	+
Glucose production	GLU	+	+	+
Manitol acidification	MAN	+	+	+
Inositol acidification	INO	-	-	-
Sorbitol acidification	SOR	-	-	-
Rhamose acidification	RHA	-	-	-
Saccharose acidification	SAC	-	-	-
Melobiose acidification	MEL	-	-	-
Amygdalin acidification	AMY	+	+	-
Arabinose acidification	ARA	-	-	+
Nitrite metabolism	NO_2	+	+	+
Oxidase test	OXY	+	+	+

Table 2. API test 20NE.

Isolates		VP_{AHPND}	HJ-2023	$VC_{Non\text{-}AHPND}$
Biochemical test	Abbreviation			
Nitrate reduction	NO3	+	+	+
Indole production	TRP	+	+	+
Glucose fermentation	GLU	+	+	+
Arginine Dihydrolase	ADH	-	-	-
Urease production	URE	-	-	-
Hydrolysis (β-glucosidase) (ESCulin)	ESC	-	+	-
Hydrolysis (protease) (GELatin)	GEL	-	+	+
β-galactosidase (Para-Nitrophenyl-βD-Galactopyanosidase)	PNPG	-	+	+
Glucose assimilation	GLU	-	-	+
Arabinose assimilation	ARA	-	-	+
Mannose assimilation	MNE	+	+	+
Mannitol assimilation	MAN	-	-	+
N-acetyl-glucosamine assimilation	NAG	-	-	+
Maltose assimilation	MAL	+	+	+
Potassium gluconate assimilation	GNT	-	+	+
Capric acid assimilation	CAP	-	-	-

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Adipic acid assimilation	ADI	-	-	+
Malate assimilation	MLT	+	+	+
Trisodium citrate assimilation	CIT	-	-	+
Phenylacetic acid assimilation	PAC	-	-	-
Oxidase test	OXY	+	+	+

2.4. Antibiotic susceptibility test

An antibiotic susceptibility test of the isolated HJ-2023 strain and the control bacteria VP_{AHPND} and $VC_{non\text{-}AHPND}$ revealed that the strains were resistant to streptomycin, erythromycin, cephalothin, kanamycin, vancomycin, ampicillin, clindamycin, and lincomycin. Furthermore, the strains exhibited sensitivity to norfloxacin, nalidixic acid, chloramphenicol, oxytetracycline, and tetracycline. The isolated HJ-2023 strain and VP_{AHPND} showed an intermediate level of resistance to ciprofloxacin, whereas $VC_{non\text{-}AHPND}$ showed sensitivity. The isolated HJ-2023 strain also showed an intermediate level of resistance to gentamicin, whereas the VP_{AHPND} and $VC_{non\text{-}AHPND}$ strains were gentamicin-resistant (Table 3).

Table 3. Mean and standard deviations of the diameters of the antibiotic inhibition zones (mm).

Antibiotics (Conc.)	HJ-2023	VP_{AHPND}	$VC_{non\text{-}AHPND}$
CIP (5 μg)	18.8 ± 0.2 (I)	16.5 ± 0.1 (I)	23.0 ± 0.1 (S)
NoR (10 μg)	20.3 ± 0.3	20.0 ± 0.0	17.5 ± 0.1
GM (10 μg)	12.3 ± 0.1 (I)	11.2 ± 0.2 (R)	11.5 ± 0.1 (R)
NA (30 μg)	21.0 ± 0.2 (S)	23.2 ± 0.2 (S)	28.2 ± 0.2 (S)
C (30 μg)	23.3 ± 0.2 (S)	19.7 ± 0.1 (S)	32.5 ± 0.2 (S)
T (30 μg)	22.3 ± 0.3 (S)	20.5 ± 0.3 (S)	31.2 ± 0.1 (S)
S (10 μg)	8.3 ± 0.1 (R)	9.5 ± 0.1 (R)	0.0 ± 0.0 (R)
TE (30 μg)	23.3 ± 0.1 (S)	19.5 ± 0.3 (S)	21.0 ± 0.1 (S)
E (15 μg)	0.0 ± 0.0 (R)	9.5 ± 0.1 (R)	11.0 ± 0.1 (R)
CF (30 μg)	9.8 ± 0.1 (R)	0.0 ± 0.0 (R)	0.0 ± 0.0 (R)
K (30 μg)	12.3 ± 0.1 (R)	12.0 ± 0.2 (R)	10.5 ± 0.1 (R)
VA (30 μg)	11.0 ± 0.0 (R)	0.0 ± 0.0 (R)	0.0 ± 0.0 (R)
AM (10 μg)	0.0 ± 0.0 (R)	0.0 ± 0.0 (R)	0.0 ± 0.0 (R)
CC (2 μg)	0.0 ± 0.0 (R)	0.0 ± 0.0 (R)	0.0 ± 0.0 (R)
L (2 μg)	0.0 ± 0.0 (R)	0.0 ± 0.0 (R)	0.0 ± 0.0 (R)

The non-observation of the inhibition zone around the disc was considered a 0-mm inhibition diameter. Resistant (R), sensitive (S), and intermediate (I). Ciprofloxacin (CIP; 5 μg), norfloxacin (NoR; 10 μg), gentamicin (GM; 10 μg), nalidixic acid (NA; 30 μg), chloramphenicol (C; 30 μg), oxytetracycline (T; 30 μg), streptomycin (S; 10 μg), tetracycline (TE; 30 μg), erythromycin (E; 15 μg), cephalothin (CF; 30 μg), kanamycin (K; 30 μg), vancomycin (VA; 30 μg), ampicillin (AM; 10 μg), clindamycin (CC; 2 μg), and lincomycin (L; 2 μg).

2.5. Comparison of the virulence of the isolated HJ-2023 strain and that of the controls

After inoculating white-leg shrimp with the isolated HJ-2023 strain and the control strains, the cumulative mortality rate was observed for 5 d. In the high challenge concentration (2×10^6 CFU/50 μL) experiments, the average cumulative mortality rates were 90%, 95%, and 10% in the isolated HJ-2023 strain, VP_{AHPND}, and $VC_{non\text{-}AHPND}$ groups, respectively, compared to 0% mortality in the control group (no bacteria inoculated) at 12 h after infection. Comparing the virulence of the isolated HJ-2023 strain and $VC_{non\text{-}AHPND}$ groups with that of the PBS control group further showed average cumulative

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mortality rates of 95% and 85%, respectively, in the high concentration challenge experiment and 30% and 0%, respectively, in the low concentration challenge experiment at 72 h. In the low challenge concentration (2×10^5 CFU/50 μL) experiments, the average cumulative mortality rates of the shrimp in the HJ-2023, VP_{AHPND}, and $VC_{non\text{-}AHPND}$ groups were 40%, 55%, and 0%, respectively. The cumulative mortality rate was maintained for 120 h (Figure 3).

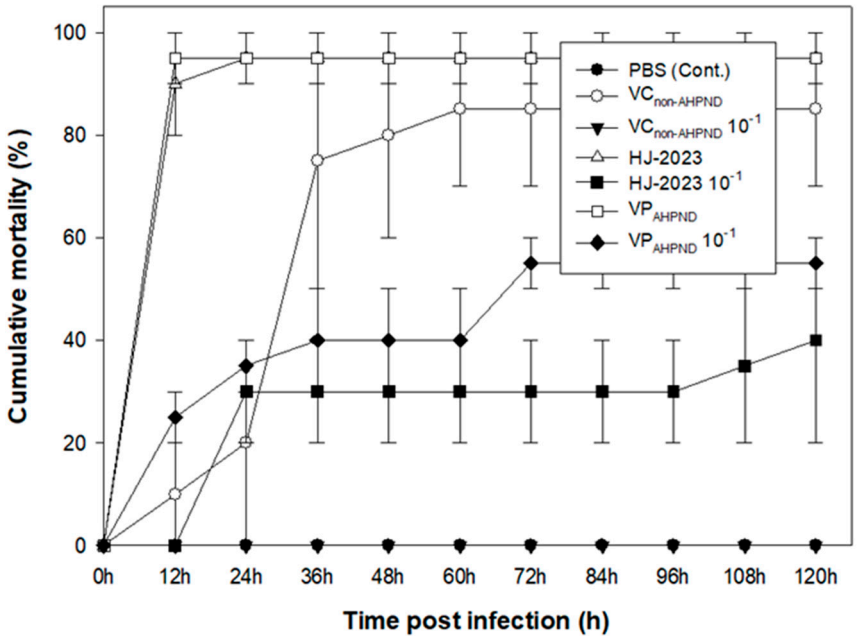

Figure 3. Cumulative mortality rates of white-leg shrimp injected with HJ-2023, VP_{AHPND}, or $VC_{non\text{-}AHPND}$ (high concentration, 2×10^6 CFU/50 μL; and low concentration, 2×10^5 CFU/50 μL). The control groups were injected with phosphate-buffered saline. The groups were observed every 12 h for 5 d after the injection.

3. Discussion

AHPND can cause serious economic losses by affecting major aquaculture species, such as *Litopenaeus vannamei*, *Penaeus monodon*, and *M. rosenbergii* [18], and has been listed by the World Organization for Animal Health in 2017 [10]. In South Korea, AHPND was designated as a statutory infectious disease for aquatic life in species subject to slaughter in 2021 [1]. White-leg shrimp, which is the most important species in the global shrimp farming industry and accounts for the majority of shrimp in South Korea, with high production and consumption rates, is one of the main species affected by AHPND [24].

We found that the bacteria from the white-leg shrimp farm were positive for the *PirA/B* toxin genes; however, the presence of *V. parahaemolyticus* could not be detected using PCR. Therefore, we assumed that it was the AHPND of another species and identified and characterized the isolated strain. In the case of *Vibrio spp*., it is difficult to distinguish between species because of their high genetic similarity. In particular, the *16S* rRNA gene sequence of the *Harveyi* clade, which causes AHPND, shares more than 99% sequence homology with other species [25]. Therefore, we performed a PCR analysis using the *rpoD* and *toxR* gene sequences, which showed the minimum intraspecific similarity between *V. parahaemolyticus* and other *Vibrio spp.* [26]. It was confirmed that the isolated strain was not *V. parahaemolyticus*. The isolated strain was suspected to be *V. campbellii* among various *Vibrio spp*. Therefore, PCR was performed targeting the *Vch* gene, a *V. campbellii* detection gene, and the bacterium was identified as *V. campbellii*. The presence of the pVA1 plasmid containing the AHPND-inducing toxin genes (*PirA/B*) was confirmed using PCR followed by gene sequencing analysis. Most of the bacterial strains harboring pVA1 had 99.92–100% nucleotide sequence homology with each other [27]; the gene sequence from the isolated strain was 100% identical to the

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PirA/*B* toxin gene sequences of VP_{AHPND}, the control strain used in this study (data not shown). The isolated strain was named *V. campbellii* AHPND (HJ-2023), and its characteristics and virulence were studied.

The PirA/B binary toxin is essential for the development of pathogenic lesions in AHPND.

According to reports on the *PirA*/*B* toxin genes present in pVA1, *V. parahaemolyticus* strains lacking *PirA*/*B* fail to cause AHPND; however, the strains transformed with plasmids containing *PirA*/*B* have been found to show clinical signs of AHPND and cause 100% mortality in shrimp larvae [28,29]. Additionally, pVA1 can be transferred among similar clusters and is capable of self-transfer using the *pndA PSK* gene system, which is a mobilizing gene associated with conjugative transfer [14]. Therefore, the pVA1 plasmid has the potential to be transferred among different *Vibrio spp.* or bacterial species through horizontal transfer [14,15,30]. Dong et al. [27] showed that the pVA1 plasmid can be transferred from AHPND-causing *V. campbellii* to non-AHPND-causing *V. owensii* in a controlled laboratory environment. The authors further provided direct evidence of horizontal plasmid transfer among *Vibrio* species. Accordingly, it is highly likely that the pVA1 plasmid from the *V. parahaemolyticus* was horizontally transferred to the *V. campbellii* strain.

Based on the phylogenetic analysis, the isolated HJ-2023 strain was classified into the *V. campbellii* group and confirmed to be biochemically different from the genetically similar *V. harveyi* group using biochemical tests [31]. The AHPND-causing gene from the isolated HJ-2023 strain also showed 100% similarity to the *rpoD* gene of VC_{AHPND}, which was previously reported in China and Thailand [20,32]. To date, the *Vibrio spp*. that have been reported to cause AHPND are *V. campbellii*, *V. harveyi*, and *V. owensii*, all of which belong to the *Harveyi* clade of the *Vibrionaceae* family and are considered as the pathogenic clade of AHPND [33]. However, a recent study reported that *V. punensis*, a member of the Orientalis clade, was introduced with a toxin plasmid that causes AHPND [23]. Therefore, AHPND is not limited to one bacterial clade, and there is a possibility that the toxin plasmid can be transferred to various bacterial strains. This is likely to increase the virulence and spread of the disease via transfer of the toxin plasmid to potential bacterial pathogens via various routes.

Based on the biochemical characteristics determined using API Test 20E and 20NE and the percentage of identification (% ID), the isolated HJ-2023 strain showed 99.3% and 84.8% similarity to *V. vulnificus* and *V. alginolyticus*, respectively. $VC_{non\text{-}AHPND}$ was identified as *V. parahaemolyticus* (81.8% similarity) and *V. alginolyticus* (97.9% similarity). Additionally, VP_{AHPND} was found to be similar to *V. parahaemolyticus* (99.9%). A previous study reported that the classification of *Vibrio* species based on the biochemical characteristics of the API test is somewhat inappropriate [34]. As a result of the reading, however, the control VP_{AHPND} was clearly classified as *V. parahaemolyticus*, and the isolated HJ-2023 and $VC_{non\text{-}AHPND}$ strains were classified as *V. alginolyticus* and *V. vulnificus*. The isolated HJ-2023 strain was confirmed to be *V. campbellii* via gene sequence identification, but the same result was not obtained in the bacterial identification based on the biochemical characteristics determined using the API tests, as the information on *V. campbellii* was not registered in the bacterial identification reading software; therefore, it was likely confirmed as a similar *Vibrio* species. Because of this diversity in biochemical characteristics, when bacteria are isolated from diseased white-leg shrimp using TCBS medium, approximately 84.3% of them form green colonies [22]. *V. campbellii* also forms green colonies because it lacks a sucrose hydrolase gene, making it difficult to distinguish it from *V. parahaemolyticus* [20].

In the antibiotic disc susceptibility test, both the isolated HJ-2023 strain and the control bacteria VP_{AHPND} and $VC_{non\text{-}AHPND}$ showed strong resistance to ampicillin, clindamycin, and lincomycin, with a small inhibition area of 0 mm. Most *Vibrio* strains are resistant to ampicillin and lincomycin; therefore, they showed similar results [35]. The isolated HJ-2023 strain showed strong resistance against erythromycin, which is effective against gram-positive bacteria, in an area of 0 mm, whereas VP_{AHPND} and $VC_{non\text{-}AHPND}$ showed weak resistance. The isolated HJ-2023 strain further showed weak sensitivity to gentamycin, but VP_{AHPND} and $VC_{non\text{-}AHPND}$ showed resistance. Taken together, these results indicate that even within the same *Vibrio* genus, susceptibility and resistance to antibiotics differ depending on the species [36].

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To compare the pathogenicity of the isolated HJ-2023 strain and that of the control bacteria VP_{AHPND} and $VC_{non\text{-}AHPND}$, we observed the cumulative mortality rate after infection in white-leg shrimp. When the shrimp were artificially challenged with high concentrations of the isolated HJ-2023 strain and VP_{AHPND} strains, more than 90% mortality was observed within 12 h. These results were similar to those of multiple studies that showed 100% mortality within 12–36 h in VP_{AHPND} and VC_{AHPND} artificial infection experiments [27,30]. In the low concentration experiments, when the concentration was 10-times lower than the high concentration, the cumulative mortality rates of the isolated HJ-2023 strain and VP_{AHPND} were 30% and 55% within 72 h, respectively. Accordingly, the isolated HJ-2023 strain showed approximately 25% less toxicity than VP_{AHPND}; however, it is undoubtedly a lethal strain when outbreaks occur in aquaculture fields.

V. campbellii is widely distributed in marine environments and is an important pathogen in marine shrimp and fish [37]. For a long time, *V. campbellii* was often misidentified because of its high similarity to *V. harveyi* and was considered non-pathogenic to shrimp. However, according to the results of the present study, although $VC_{non\text{-}AHPND}$ itself causes mortality in shrimp, *V. campbellii* is conferred this ability by the pVA1 plasmid and appears to potentially cause stronger pathogenicity in shrimp. AHPND-causing *V. harveyi* is more virulent than the *V. harveyi* involved in non-AHPND [22]. The present study indicates that when devising measures to prevent the spread of AHPND, quarantine focusing on the *V. parahaemolyticus* strain, which is believed to be a unique strain of AHPND, is not a clear solution. In the present study, even the same *Vibrio spp.* showed various differences in their antibiotic susceptibility and biochemical characteristics. These results highlight the complexity of AHPND-causing strains and their virulence through multiple pathways, which may increase the risk of AHPND in the shrimp aquaculture industry.

Additional studies are needed to determine how the biochemical properties of $VC_{non\text{-}AHPND}$ change as toxin plasmids are acquired. The present study may serve as a basis for attaining a better understanding of and preventing AHPND caused by various bacteria in shrimp aquaculture. Based on the results of this study, effective biosecurity measures should be considered to prevent the spread of AHPND caused by various bacterial strains.

4. Materials and methods

4.1. Sampling and polymerase chain reaction (PCR)

Penaeus vannamei was sampled from a shrimp farm in Chungcheongnam-do, Taean-gun, South Korea. The hepatopancreases of five randomly selected shrimp were aseptically obtained and plated on thiosulfate citrate bile salt sucrose (TCBS) (Difco, Franklin Lakes, NJ, USA). The plate was then incubated at 27 °C for 48 h, and a single colony was selected and subjected to PCR. The control strains used included *V. parahaemolyticus* [VP_{AHPND} (ATCC 17802)], which causes AHPND, and *V. campbellii* [$VC_{non\text{-}AHPND}$ (KCTC 2716)], which does not cause AHPND. Primer sets used to identify the *Vibrio* species are provided in Table 4. For the PCR, a 20-μL reaction was set up with 1 μL of each colony dilution as a template, 1 μL of each primer, and 10 μL of ExPrime Taq DNA Premix (Genetbio, Daejeon, South Korea). The thermal cycling conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 30 cycles of 5 min denaturation at 95 °C, 30 s annealing at 42 °C (for *PirA*), 45 °C (for *PirB*), 50 °C (for *rpoD* and AP3), 52 °C (for *vch*), and 54 °C (for *16S* rRNA and *toxR*), and extension at 72 °C for 1 min, with a final extension step at 72 °C for 7 min. The PCR products were electrophoresed on 1% agarose gels and analyzed using a Gel-Doc UV transilluminator (Bio-Rad, Hercules, CA, USA). We named the identified *Vibrio campaberii* AHPND strain as HJ-2023.

Table 4. Primers used for bacterial identification.

Target	Primer	Sequences (5′-3′)	Product size (bp)	Reference
16S rRNA	V16S-F	GGG GAT AAC CAT TGG AAA CGA T	1,313	This study

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	V16S-R	AAA CTA CCT ACT TCT TTT GCA GC		
rpoD	VRpoD-F	ATT GAA GAA GGT ATT AAC CAA G	755	This study
	VRpoD-R	GAC ATR CGA CGG CTG ATG TCT TT		
toxR	toxR-F	AAC CCG CTT TCT TCA GAC TC	399	[38]
	toxR-R	AAC GAG TCT TCT GCA TGG TG		
Vch	VCH-F	ATT AGA TAT TTG ACT GAA TGG CG	1,359	[31]
	VCH-R	CTT AGA ATG GAT GAT TCG AAA GT		
AP3	AP3-F	ATG AGT AAC AAT ATA AAA CAT GAA AC	333	[39]
	AP3-R	GTG GTA ATA GAT TGT ACA GAA		
PirA	VpPirA-F	ATG AGT AAC AAT ATA AAA C	336	[40]
	VpPirA-R	TTA GTG GTA ATA GAT TG		
PirB	VpPirB-F	ATG ACT AAC GAA TAC GT	1,317	
	VpPirB-R	CTA CTT TTC TGT ACC AA		

16S rRNA, *16S ribosomal* RNA; AP3, AHPND primer 3; *PirA* and *PirB*, Photorhabdus insect-related (Pir) toxins A and B; *rpoD*, RNA polymerase sigma factor; *toxR*, Cholera toxin regulator; *Vch*, *V. campbellii* hemolysin.

4.2. Phylogenetic analysis based on pathogenic rpoD

The *rpoD* gene amplified via PCR from the isolated HJ-2023 strain was cloned using pGEM®-T Easy Vector Systems (Promega, Madison, WI, USA). Sequencing was performed by COSMOgenetech (Seoul, Korea). The sequences were further compared with those of other available *Vibrio* core group member (*V. campbellii*, *V. harveyi*, *V. rotiferianus*, *V. parahaemolyticus*, and *V. alginolyticus*) *rpoD* gene sequences using a BLAST search in the National Center for Biotechnology Information GenBank database (www.ncbi.nlm.nih.gov/BLAST). The *rpoD* gene sequences were then aligned, and a phylogenetic tree was constructed by bootstrapping 1,000 replicates using the neighbor-joining method in Molecular Evolutionary Genetic Analysis 11 software (http://www.megasoftware.net/).

4.3. Biochemical tests

The biochemical identification of the isolated HJ-2023 strain and control bacteria, VP_{AHPND} and $VC_{non\text{-}AHPND}$, was performed using commercially available API 20E and API 20NE TEST Kits (BioMérieux, Marcy-l'Étoile, France). Each bacterial suspension was prepared in 1.5% NaCl solution and analyzed at 37 ± 1 °C for 24 h according to the manufacturer's instructions. The results were interpreted on the APIWEB™ (BioMerieux) website.

4.4. Antimicrobial susceptibility test

For the antibiotic susceptibility test, the isolated HJ-2023 strain and the control bacteria VP_{AHPND} and $VC_{non\text{-}AHPND}$, at 0.5 McFarland turbidity and in total volumes of 50 µL, were spread on Luria broth (LB) agar plates containing 1.5% NaCl. Fifteen types of antibiotic discs were then prepared and

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incubated at 27 °C for 24 h. After the incubation period, the diameters of the transparent area surrounding the antibiotic discs were measured in millimeters to calculate the inhibition area. The strains were classified into three grades—Resistant (R), Intermediate (I), and Sensitive (S)—based on the standards of the Clinical and Laboratory Standards Institute. The types and concentrations of the 15 antibiotic discs used for the strains are as follows: ciprofloxacin (CIP; 5 μg), norfloxacin (NoR; 10 μg), gentamicin (GM; 10 μg), nalidixic acid (NA; 30 μg), chloramphenicol (C; 30 μg), oxytetracycline (T; 30 μg), streptomycin (S; 10 μg), tetracycline (TE; 30 μg), erythromycin (E; 15 μg), cephalothin (CF; 30 μg), kanamycin (K; 30 μg), vancomycin (VA; 30 μg), ampicillin (AM; 10 μg), clindamycin (CC; 2 μg), and lincomycin (L; 2 μg).

4.5. In vivo experiments to assess pathogenicity

To compare the virulence of the isolated HJ-2023 strain and the control bacteria VP_{AHPND} and $VC_{non\text{-}AHPND}$, *in vivo* tests were performed on white-leg shrimp. White-leg shrimp (average weight: 25 ± 5 g) were collected from a prawn farm located in Gunsan-si, Jeollabuk-do, placed in a breeding tank maintained at 25 °C for 1 week, and used for *in vivo* infection experiments. The water used for breeding the shrimp was maintained with 30% salinity using sea salt (KENT Marine Reef Salt, Franklin, WI, USA). To confirm the presence or absence of an AHPND infection, the hepatopancreases of five white-leg shrimp were isolated, and non-infection was confirmed via PCR. The strains (isolated HJ-2023 and the control bacteria VP_{AHPND} and $VC_{non\text{-}AHPND}$) were cultured in LB supplemented with 1.5% NaCl and incubated at 27 °C with shaking until the absorbance at 600 nm (OD_{600}) reached 1.1. The shrimp were then divided into two replicates, with seven groups in total (a control group and six experimental groups). Each group comprised 10 randomly assigned white-leg shrimp. Each group was inoculated via intramuscular injection of phosphate-buffered saline (control group) or 2×10^6 CFU/50 μL (high challenge concentration) and 2×10^5 CFU/50 μL (low challenge concentration) of bacteria. After inducing the infection, the cumulative mortality rate was observed for 5 d.

5. Patents

Author Contributions: Yue Jai Kang, Hee-Jae Choi, and Ji-Hoon Lee: Conceptualization and methodology. Hee-Jae Choi and Ji-Hoon Lee: Investigation. Da-Yeon Choi and Jun-Hwan Kim: Formal analysis. Ji-Hoon Lee and Yue Jai Kang: Writing—original draft preparation and reviewing. Yue Jai Kang: editing. All authors have read and agreed to the published version of the manuscript.

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