



Isolation and Antibiotic Sensitivity of *Vibrio* spp. Bacteria Causing Acute Hepatopancreatic Necrosis Disease (AHPND) in Whiteleg Shrimp (*Litopenaeus vannamei*) in Gia Lai, Vietnam

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ABSTRACT

Background: Acute hepatopancreatic necrosis disease (AHPND) is a serious bacterial infection that causes high mortality in whiteleg shrimp (*Litopenaeus vannamei*), leading to significant losses in shrimp farming. In recent years, the disease has severely impacted the aquaculture industry in Gia Lai province and across Vietnam. The primary causative agents of AHPND are strains of *Vibrio* spp., particularly *V. parahaemolyticus*, which produce PirAB toxins that damage the hepatopancreas of infected shrimp.

Methods: To investigate the bacterial pathogens associated with AHPND, six bacterial strains were isolated: five from live shrimp exhibiting typical AHPND symptoms and one from a shrimp that had died from the disease. Shrimp samples were collected from May to August 2024 in Hoai Nhon district, Gia Lai province, Vietnam. Morphological observations of colonies after isolation were first used to select strains with characteristics consistent with *Vibrio* spp. These strains were then subjected to 16S rRNA gene sequencing for molecular identification. In addition, antibiotic susceptibility testing was performed using the standard disk diffusion method to evaluate resistance to commonly used antibiotics in aquaculture.

Result: Two bacterial isolates, designated VhnT5 and VhnT6, were identified as *V. parahaemolyticus* based on 16S rRNA gene analysis. Antibiotic susceptibility tests revealed that both strains were completely resistant (100%) to Ampicillin (10 µg) and Erythromycin (15 µg), but remained sensitive to Chloramphenicol (30 µg) and Levofloxacin (5 µg). These findings suggest a concerning pattern of antibiotic resistance potentially linked to overuse or misuse of antibiotics in shrimp farming practices. The results emphasize the need for better antibiotic management and informed strategies to handle bacterial infections in aquaculture.

Key words: AHPND, Antibacterial Activity, Antibiotic resistance, *Vibrio parahaemolyticus*.

INTRODUCTION

Vietnam is considered to have great potential for aquaculture development. The coastline is more than 3,260 km long with 112 estuaries and streams flowing into the sea, which has potential for brackish and saltwater aquaculture. In addition, thousands of large and small islands scattered along the coastline are areas that can be used for aquaculture all year-round. According to the Ministry of Agriculture and Rural Development, the total aquatic product output in September 2023 is estimated at 857,7 thousand tons, up 2.8% compared with previous year; and in the first 9 months of 2023, the aquatic product output is estimated at 6 796,7 thousand tons, up 2.1% compared with previous year. More specifically, the contribution of white-leg shrimp farming in these numbers is important due to the rapid increase of its intensive farming, as well as the surface area of its farming in Vietnam in general and in the Central region in particular. In Gia Lai, white-leg shrimp is the main farming species for aquaculture. The province aims to have 4,700 ha of aquaculture by 2025: 2,400 ha of freshwater surface and 2,300 ha of saltwater and brackish water surface. High-tech aquaculture alone strives to account for 30% of the total intensive and semi-intensive shrimp farming area.

Acute hepatopancreatic necrosis disease (AHPND), formerly known as early mortality syndrome (EMS), is a

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disease that causes significant economic losses in the aquaculture industry. AHPND can cause sudden and massive mortalities in shrimp, which can be observed within 30-35 days of stocking (de la Pena *et al.*, 2015). AHPND is highly susceptible in whiteleg shrimp (Zorriehzahra and Banaederakhshan, 2015). The main causative agent of AHPND was found to be *V. parahaemolyticus*

(Tran *et al.*, 2013; To *et al.*, 2022), a gram-negative, rod-shaped bacterium with a plasmid encoding a lethal dual toxin Pir A/Pir B that causes rapid death of infected shrimp (Lin *et al.*, 2017; Lin *et al.*, 2019). The clinical signs of AHPND are severe atrophy of the shrimp hepatopancreas and histopathology such as sloughing, accumulation of surrounding blood cells and damaged intertubular spaces of the hepatopancreas and severe secondary bacterial infection during the acute infection phase (Tran *et al.*, 2013).

In Vietnam, AHPND appeared in shrimp farming areas in the Mekong Delta since 2010 such as Ninh Thuan (16 ha), Soc Trang (1,719 ha), Bac Lieu (346 ha) and Ca Mau (3,493 ha) (Nguyen *et al.*, 2016). According to the Department of Animal Health, 2015, AHPND spread widely, affecting 19 provinces in 2012, 22 provinces in 2015 and 25 provinces in 2017. However, the total affected area decreased significantly, from 28,005 ha in 2012 to 9,284 ha in 2015 and further down to 6,793 ha in 2017 (Dang *et al.*, 2018). Research from 2014 and 2018 in four provinces of Ben Tre, Long An, Bac Lieu and Kien Giang showed that the area damaged by AHPND fluctuated between 2% and 57.2% of the total shrimp farming area (Mai *et al.*, 2021). In Gia Lai, in 2023, the total area of shrimp ponds affected by disease in the province was 1.9 ha. Of which, AHPND was recorded on 0.7 ha in Hoai Nhon district. This study was conducted to assess the share of genetic factors and the share of antibiotic resistance in microorganisms responsible for AHPND in Gia Lai province and thus contribute to the sustainable management and development of whiteleg shrimp farming in Gia Lai province and the Central region in general.

MATERIALS AND METHODS

Collection of diseased shrimp samples

We collected two types of whiteleg shrimp samples from ponds in Hoai Nhon district, Gia Lai province from May to August 2024: 1) live shrimps with clinical signs of AHPND such as empty digestive tract, opaque white abdomen, atrophied white hepatopancreas, lethargy, anorexia, soft shell (de la Pena *et al.*, 2015; Zorriehzahra and Banaederakhshan, 2015); 2) shrimps dead from disease. The diseased shrimp samples were placed in sterile bags and stored in ice before being brought to the Microbiology Laboratory of Quy Nhon University, Gia Lai province, Vietnam, for analysis.

Microbial isolation method

For live shrimps with clinical signs of AHPND samples, intestines, hepatopancreas and tail bulge were collected and then finely ground and diluted to appropriate concentration with 0.9% NaCl solution. For both concentrations of 10^{-6} or 10^{-4} we used, 100 μ l of supernatant was aspirated and spread on Alkaline Peptone medium plates according to the plating method (WHO and FAO, 2016). And then, these plates were incubated at 37°C for 24 hours. After incubation, different

single colonies were selected and continued to be subcultured on Tryptic soy agar (TSA, Merck) medium (supplemented with 1.5% NaCl) plates.

For samples of shrimps dead from disease, intestines and hepatopancreas were collected and then finely ground and diluted to appropriate concentration with 0.9% NaCl solution. For both concentrations of 10^{-6} or 10^{-4} we used, 100 μ l of supernatant was aspirated and spread on Thiosulfate-Citrate Bile-Sucrose agar (TCBS, Merck) plates specifically used for *Vibrio* spp. according to the plating method. After plating, the plate was incubated at 37°C for 24 hours. After incubation, different single colonies were selected and continued to be subcultured on TCBS agar plates.

All single isolates were stocked in Tryptone Soy Broth (TSB, Merck) pre-added with 20% glycerol and stored at -40°C for other usages.

Total DNA extraction of bacterial strains

Bacterial strains were grown on Alkaline Peptone Water (APW, Merck) medium, shaken at 200 rpm for 24 hours. The culture was centrifuged at 13,000 rpm at 4°C to collect the cells. Total DNA was extracted by the cetyl trimethylammonium bromide (CTAB, BioBasic-Canada) method: The cells were disrupted by adding 500 μ L CTAB, 30 μ L Sodium dodecyl sulfate 10%, 25 μ L lysozyme to the eppendorf tube containing the cells, vortexed and then incubated at 70°C for 30 minutes. The DNA was precipitated by adding two volumes of ice-cold ethanol and 10% 3M sodium acetate. The precipitate was collected by centrifugation at 13,000 rpm for 30 minutes, washing the DNA twice with 70% ethanol and drying. The pellet was resuspended in 1X TE. DNA concentration was determined spectrophotometrically at 260 nm absorbance (A260) using a Nano Drop-1000 (Thermo Scientific).

DNA amplification and sequence analysis

The bacterial 16S rRNA gene sequence was amplified by PCR using primer pair 27F (5'-TAACACAT GCAAGT CGAACG-3') and 1492R (5'-GGTGTGACGG GCGGTG TGTA-3') with the thermal cycle for PCR reaction: predenaturation at 94°C for 3 min, denaturation at 94°C for 30s, annealing at 52°C for 30s, extension at 72°C for 45s with 35 cycles and extension at 72°C for another 5 min. PCR reaction products were checked by electrophoresis on 1% agarose gel. The size of total DNA obtained after PCR reaction was compared with standard DNA ladder (Thermo Scientific, USA) and sent for sequencing to DNA SEQUENCING Company, Vietnam.

Sequencing results of the research samples were checked, processed and edited using BioEdit 7.2. The raw sequences were then compared with reference sequences in the GenBank database using the BLAST tool (<https://blast.ncbi.nlm.nih.gov/>) to identify the *Vibrio* strains in the study samples at the species level. The phylogenetic tree was constructed based on the similarity of nucleotide sequences between species, which was statistically calculated using MEGA 11 software (Kumar *et al.*, 2016;

Tamura and Nei, 1993). The phylogenetic tree was established based on genetic distances according to Tamura-Nei, using the Neighbor-joining method. The Bootstrap value of the phylogenetic tree was analyzed on 1000 samples.

Determination of antimicrobial activity

We tested the sensitivity of the bacteria to several antibiotics using the disk diffusion method (Fiebelkorn *et al.*, 2003). One colony of identified bacteria was spread on each Petri dish with Müller-Hinton agar medium. Antibiotic discs were from HiMedia (India). We then placed four regular paper antibiotic disks on each of the aforementioned inoculated media and incubated them at 37°C for 24 hours (Rodloff *et al.*, 2008). Lastly, we measured the zones of inhibition to the nearest millimetre as recommended by the National Committee for Clinical Laboratory Standards (National Committee for Clinical Laboratory Standards, 2012). The sensitivity of the bacteria to the antibiotics was then assessed using the Clinical and Laboratory Standards Institute (CLSI) specifications (National Committee for Clinical Laboratory Standards, 2012), listed in Table 1.

Table 1: Acceptable susceptible zone of inhibition values for antibiotics used based on CLSI* (2012).

Antibiotics	Inhibitory zone diameter to nearest millimeter (mm)		
	Sensitive (S)	Intermediate (I)	Resistant (R)
Ampicillin 10 µg	≥17	14-16	≤13
Chloramphenicol 30 µg	≥18	13-17	≤12
Levofloxacin 5 µg	≥17	14-16	≤13
Erythromycin 15 µg	≥23	14-22	≤11

Data analysis

Data on antibiotic resistance was analyzed using Microsoft Excel program.

RESULTS AND DISCUSSION

Results of isolation of vibrio bacteria on typical media

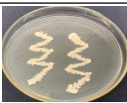
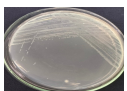
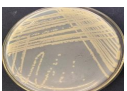
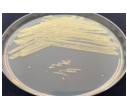
Vibrio spp. belongs to the group of gram-negative, rod-shaped, non-spore-forming bacteria, of the Vibrionaceae family (Farmer, 2021) and is a common pathogen for aquatic animals (Laith *et al.*, 2020; Sowmiya *et al.*, 2025), seriously affecting the aquaculture industry in Vietnam as well as in the rest of the world. Based on the isolation of bacteria from shrimps with signs of AHPND disease presented in Table 2, we selected the VhnT5 bacteria strain, which is rod-shaped, non-spore-forming, to transfer to Tryptic soy agar (TSA agar) medium (supplemented with 1.5% NaCl). Our results showed that when strain VhnT5 was transferred to TSA medium, the colonies developed were round and smooth, cream or opaque white (Fig 1 a, c).

In addition, we also took the hepatopancreas of dead whiteleg shrimps to isolate directly on TCBS agar (Thiosulfate-Citrate Bile-Sucrose Agar), a typical medium for selecting *Vibrio* spp. The results showed the appearance of round, green colonies and were designated as VhnT6 (Fig 1 b, d).

Identification of *Vibrio* spp. using 16S rRNA molecular markers

Total DNA of single colony strains VhnT5 and VhnT6 was used for species identification based on 16S rRNA gene similarity. The length of the 16S rRNA gene sequence from sample 1 (VhnT5) was 976 bp and sample 2 (VhnT6) was 837 bp (Fig 2, 3). The phylogenetic tree was constructed using the Maximum Likelihood algorithm based on the

Table 2: Results of isolation of bacteria from shrimps with clinical signs of AHPND disease.

Isolate designation	Description of colony and cell morphology	Colony morphology
VhnT1	Colonies are irregular, circular, white in color, the surface is dry, serrated edges; rod-shaped cells, with spores.	
VhnT2	Colonies are irregular, circular, white in color, the surface is dry; rod-shaped cells, with spores.	
VhnT3	Colonies are regular, circular, opaque; the surface is mucoid; spherical cells.	
VhnT4	Colonies are regular, circular, yellow in color; the surface is glistening, mucoid; spherical cells.	
VhnT5	Colonies are circular, pale yellow; the surface is glistening and mucoid; rod-shaped cells, without spores.	

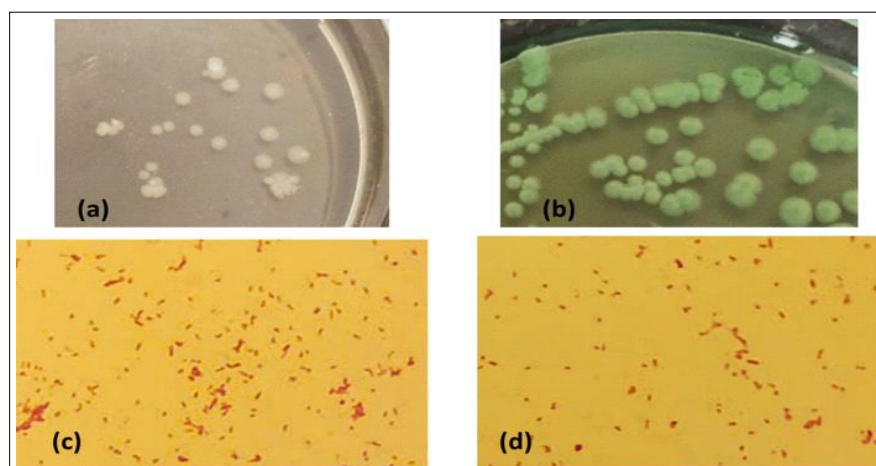


Fig 1: Colony morphology of *Vibrio* spp. on TSA agar (a) and TCBS agar (b); Gram-stained image of *Vibrio* spp. cells (c) (d).

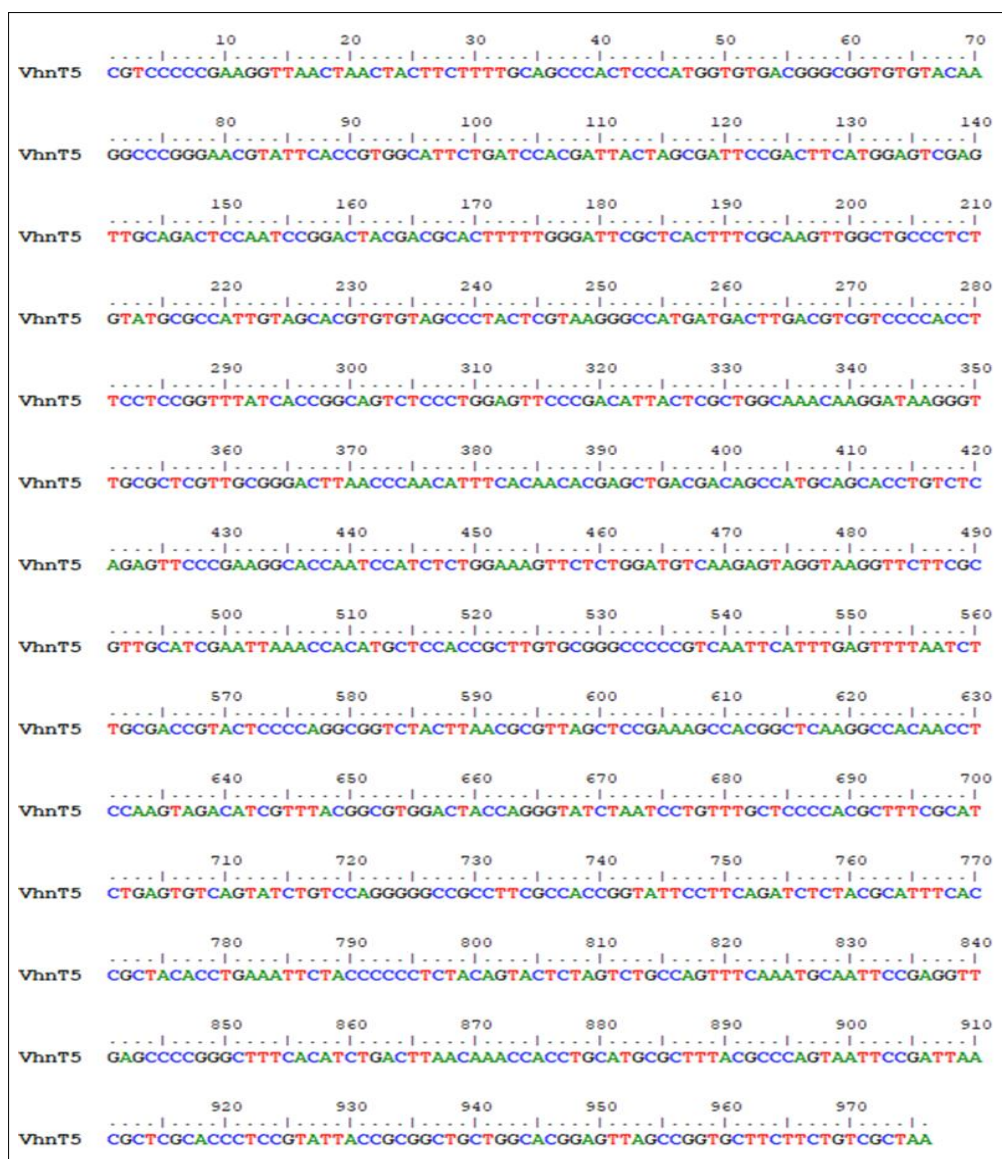


Fig 2: Nucleotide sequence of 16S rRNA gene isolated from single colony strain VhnT5.

Tamura-Nei model (Kumar *et al.*, 2016; Tamura and Nei, 1993) with some *Vibrio* spp. species published on Genbank used as reference. The classification tree constructed based on MEGA11 software is shown in Fig 4.

The BLAST analysis results on GenBank showed that the 16S rRNA gene sequence of strain VhnT5 had

99.90% similarity with the 16S rRNA gene sequence of *V. parahaemolyticus* (Table 3).

Genetic relationship analysis based on 16S rRNA sequences also showed that strain VhnT5 showed high similarity with reference strains *V. parahaemolyticus* G551 (MG970576.1) and *V. parahaemolyticus* NSTH33

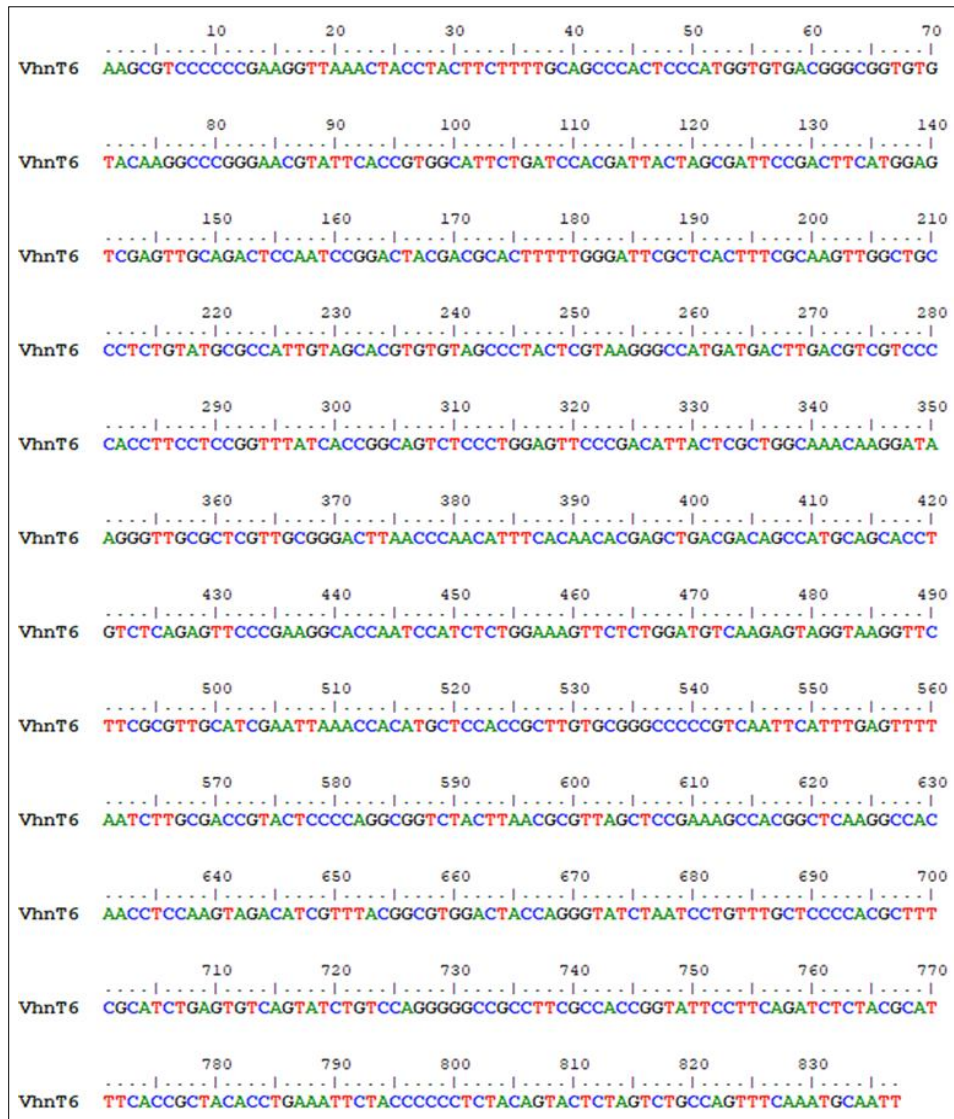


Fig 3: Nucleotide sequence of 16S rRNA gene isolated from single colony strain VhnT6.

Table 3: Highest similarity of the 16S rRNA region sequence of VhnT5 when comparing the sequence with the reference sequence database on GenBank.

GenBank number	Species	Similarity rate	Location	Source
MG593210.1	<i>V. parahaemolyticus</i> GM1314	99.90%	India	Paria <i>et al.</i> (2017)
MG593225.1	<i>V. parahaemolyticus</i> GH351	99.90%	India	Paria <i>et al.</i> (2017)
MG970576.1	<i>V. parahaemolyticus</i> G551	99.90%	India	Paria <i>et al.</i> (2018)
KF886644.1	<i>V. parahaemolyticus</i> NSTH33	99.90%	Thailand	Changchai and Suanjit (2014)
MG970577.1	<i>V. parahaemolyticus</i> G558	99.80%	India	Paria <i>et al.</i> (2018)

(KF886644.1) with a Bootstrap value of 100 (Fig 4a). VhnT5 formed a separate group with strains of the genus *V. parahaemolyticus* on the taxonomic tree. Thus, combined with morphological characteristics and 16S rRNA gene sequence analysis, it was shown that strain VhnT5 belongs to the species *V. parahaemolyticus*. Similarly, comparing the 16S rRNA gene sequence of strain VhnT6 with reference genes on the GenBank database showed that strain VhnT6 showed 100% similarity with *V. parahaemolyticus* reference strains (Table 4). Furthermore, it formed a separate branch with strains belonging to the species *V. parahaemolyticus* 2012V-1116 (CP051114.1) on the phylogenetic tree with a Bootstrap value of 100 (Fig 4b). This result also showed that strain VhnT6 belongs to the species *V. parahaemolyticus*.

Thus, our analysis results show that the *Vibrio* strain isolated from shrimp collected in Hoai Nhon district, Gia Lai province belongs to the species *V. parahaemolyticus*. AHPND caused by bacteria in seafood, especially shrimp, is seriously threatening the aquaculture industry in Asia, including many countries such as China, Malaysia, Philippines, Thailand, or Vietnam and even some other countries in the world. This disease leads to mortality rates of up to 100% in whiteleg shrimp and tiger shrimp populations and has caused significant economic losses

to the shrimp farming industry (de la Pena *et al.*, 2015). In our country, AHPND has been announced as a disease that seriously affects shrimp ponds in many localities such as Hai Phong, Quang Ninh, Nghe An, Quang Tri, Thua Thien Hue and the Mekong Delta. Regarding the occurrence of this disease, there have been many published studies stating that the main agent causing hepatopancreatic necrosis in shrimp is caused by bacteria *V. parahaemolyticus*, *V. vulnificus*, *V. fluvialis*, *V. cholerae* and *V. alginolyticus* (Nguyen and Pham, 2016; Dang *et al.*, 2016). Our study also showed similar results. The results of in-depth research on the causative agent of AHPND showed that the disease is caused by virulence genes of the plasmid carried by the bacteria (Lightner, 2014).

Identification of *Vibrio* spp. using 16S rRNA molecular markers

The results of the antibiotic susceptibility test showed that both *V. parahaemolyticus* VhnT5 and VhnT6 strains isolates exhibited sensitivity (S) to Chloramphenicol 30 µl and Levofloxacin 5 µg, while they were resistant (R) to Ampicillin 10 µg and Erythromycin 15 µg (Table 5).

Erythromycin is a macrolide antibiotic, which prevents bacterial protein synthesis by binding to the 23S rRNA of bacterial 50S ribosomal subunits and inhibiting the

Table 4: Highest similarity of the 16S rRNA region sequence of VhnT6 when comparing the sequence with the reference sequence database on GenBank.

GenBank number	Species	Similarity rate	Location	Source
CP051114.1	<i>V. parahaemolyticus</i> 2012V-1116	100%	Virginia (USA)	Rigney <i>et al.</i> (2020)
CP047985.1	<i>V. parahaemolyticus</i> HZ-52	100%	China	Fu (2023)
PP404004.1	<i>V. parahaemolyticus</i> N9	100%	Vietnam	Ngo <i>et al.</i> (2024)
CP068627.1	<i>V. parahaemolyticus</i> LH80	100%	China	Fu (2022)
MZ328009.1	<i>V. parahaemolyticus</i> vp-pir-201806	100%	China	Yang <i>et al.</i> (2021)

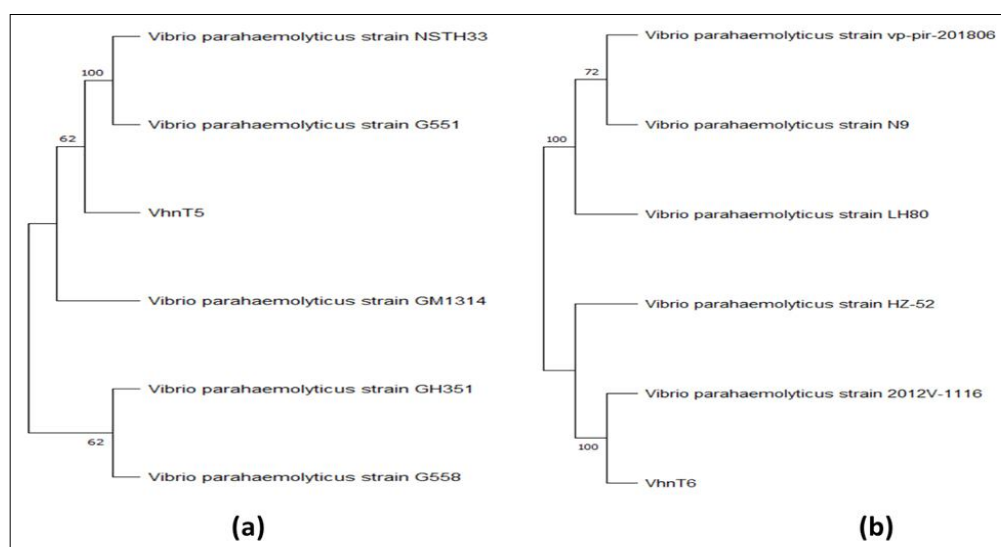


Fig 4: Genetic relationship based on 16S rRNA gene sequences of bacterial isolates VhnT5 and VhnT6 with reference strains on GenBank.

Table 5: The minimum inhibitory concentrations (MICs) against *V. parahaemolyticus* using agar disk diffusion assay.

Antibiotic	Inhibition zone diameter (mm)		According to CLSI 2012
	VhnT5	VhnT6	
Ampicillin 10 µg	0	0	R
Erythromycin 15 µg	0	0	R
Chloramphenicol 30 µg	20.4±0.5	21.0±1.3	S
Levofloxacin 5 µg	22.7±2.1	20.6±1.6	S

transpeptidation or translocation process of protein synthesis (Parnham *et al.*, 2014). Chloramphenicol is a phenicol antibiotic, which binds reversibly to the L16 protein of the 50S ribosomal subunit of bacteria, inhibiting peptide bond formation and preventing amino acid transfer to expand peptide chains (Dinos *et al.*, 2016). Levofloxacin is a fluoroquinolone antibiotic that works by inhibiting the bacterial DNA-gyrase enzyme, preventing DNA replication and repair, thereby killing bacteria (Sartini *et al.*, 2021). Penicillin is an antibiotics, it can kill bacteria by inhibiting the activity of the enzyme transpeptidase that catalyzes the final step in cell wall biosynthesis, the cross-linking peptidoglycan (Yocum *et al.*, 1980).

A previous study by Ngo *et al.* (2022), identified four species of *Vibrio* spp. from aquaculture water in five areas in the northern provinces of Vietnam, in which the most common were *V. parahaemolyticus* and *V. alginolyticus* with very high resistance rates to other antibiotics such as ampicillin (100%); amoxicillin (98.84%); streptomycin (84.88%) and oxytetracycline (69.77%) (Ngo *et al.*, 2022). Nine strains of *V. parahaemolyticus* isolated from shrimps in Nghe An were resistant to ampicillin 30 µg (100%) and tetracycline 30 µg (55.6%) (Truong *et al.*, 2016). Evaluation of 133 samples of *Vibrio* spp. isolated from shrimps in Ho Chi Minh City showed a resistance rate of 82.7% to ampicillin (Huynh *et al.*, 2019). The study results showed that most *V. parahaemolyticus* bacteria were highly resistant to β-lactam antibiotics such as amoxicillin and ampicillin. According to Le (2011), β-lactam antibiotics used through food treat intestinal bacteria and are quickly eliminated, this drug is not used for vibriosis. Streptomycin, gentamycin and neomycin are 3 antibiotics in the aminoglycosides group (Le, 2011). Aminoglycoside antibiotics are not absorbed through the intestinal tract and can only be used by injection in medicine and veterinary medicine, so they are rarely used in aquaculture. Antimicrobial resistance is a complex mechanism; many researchers have recommended that we reduce and use better antimicrobials to control infections.

CONCLUSION

We identified two strains of *Vibrio* spp.: VhnT5 and VhnT6, isolated from whiteleg shrimp samples cultured in Hoai Nhon, Gia Lai, belonging to the species *V. parahaemolyticus* causing AHPND. The isolated bacteria were assessed to have a high resistance rate to Erythromycin. The results of

this study show that antibiotic resistance has appeared in the aquaculture environment in Gia Lai province, causing difficulties for shrimp farmers in treating shrimp diseases. Therefore, the provincial agricultural management agencies need to propagate and advise people on how to use antibiotics appropriately, especially not to use banned antibiotics in disease prevention and treatment for aquatic animals. At the same time, other research directions to find solutions to treat shrimp diseases when infected with *Vibrio* spp. bacteria without using antibiotics are also explored.

Author contributions

Conceived and designed the experiments: Thi Mong Diep Nguyen. Performed the experiments: Thi Tho Nguyen, Thi Lam Phuong Ngo, Nguyen Thanh Binh Le. Analyzed the data: Thi Mong Diep Nguyen, Thi Lam Phuong Ngo. Contributed reagents/materials/analysis tools: Thi Mong Diep Nguyen. Wrote the paper: Thi Mong Diep Nguyen.

Conflict of interest statement

The authors declare no conflicts of interest.

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