Hue University Journal of Science: Natural Science
Vol. 134, No. 1D, 57–72, 2025

pISSN 1859-1388
eISSN 2615-9678

Characterisation of *Vibrio* spp. isolated from shrimp pond water in Hue City, Vietnam

Nguyen Thi Kim Co[1,2,3]*, Le Thi Thuy Trang[3], Bui Thi Chinh[3], Hoang Xuan Thao[3], Nguyen Ty[3], Nguyen Duc Hoang[1,2]

[1] Center for Bioscience and Biotechnology, University of Science, Ho Chi Minh City, Vietnam
[2] Vietnam National University, Ho Chi Minh City, Vietnam
[3] Faculty of Biology, University of Education, Hue University, Hue City, Vietnam

* Correspondence to Nguyen Thi Kim Co <ntkimco@hueuni.edu.vn>

(*Received: 23 December 2024; Revised: 26 April 2025; Accepted: 18 July 2025*)

Abstract. This study investigates the characteristics of *Vibrio* strains isolated from shrimp pond water in Hue City, focusing on their biochemical properties, antibiotic resistance, extracellular enzyme production, hemolytic activity, and toxin-encoding genes. Five Gram-negative, oxidase- and catalase-positive, motile bacterial strains forming colonies on TCBS and CHROMagar™ *Vibrio* were selected and designated as V06, V16, V17, V25, and V29. The analysis of their 16S rRNA sequences revealed 98.78 to 99.99% similarity with four *Vibrio* species: *V. parahaemolyticus*, *V. alginolyticus*, *V. azureus*, and *V. sinaloensis*. All isolates exhibited multidrug resistance to at least four antibiotics. Notably, all strains exhibited 100% resistance to vancomycin, penicillin, and ampicillin. Hemolytic activity varied among the strains, in which V06 displayed beta-hemolysis; V17 and V29 showed alpha-hemolysis, while V16 and V25 exhibited indeterminate hemolytic activity on blood agar. Additionally, all strains demonstrated the ability to produce extracellular proteases and lipases, which have been formerly demonstrated to be related to virulence in *Vibrio* species. The molecular analysis revealed the presence of *toxR* and *tlh* genes in all strains; however, no *trh*, *tdh*, *pirA*, or *pirB* genes were detected. These findings suggest that the isolated *Vibrio* strains possess numerous virulence-associated traits, including enzyme production, the presence of *toxR* and *tlh* genes, hemolytic activity, and significant antibiotic resistance, which may enhance their pathogenic potential in aquaculture environments.

Keywords: *Vibrio* spp., 16S rRNA sequence, antibiotic resistance, extracellular enzyme, hemolytic activity, toxin-encoding gene

1 Introduction

The genus *Vibrio* includes a diverse group of more than 100 Gram-negative, motile, curved-shaped recognised species that are primarily found in marine and estuarine environments [1]. *Vibrio* comprises both non-pathogenic and pathogenic species that present considerable risks to human health, aquaculture industries, and marine ecosystems [2]. In aquaculture, numerous *Vibrio* species, including *V. parahaemolyticus*, *V. alginolyticus*, *V. harveyi*, and *V. vulnificus*, are considered opportunistic bacteria that pose a significant threat because of their pathogenicity in fish and shellfish [3]. *Vibrio*-related diseases cause significant economic losses globally, with vibriosis outbreaks, leading to high mortality rates in cultured species [4].

The economic impact of *Vibrio*-related infections is exacerbated by the increasing prevalence of these diseases as water temperatures rise because of climate change [3]. Rising sea surface temperatures create favourable conditions for *Vibrio* species, expanding their

geographic range and seasonal prevalence. This has led to a remarkable increase in cases of *Vibrio* infections over the world [5]. The prevalence of pathogenic *Vibrio* bacteria in aquaculture has been increasing in Vietnam, particularly in the Mekong Delta, a key region for seafood production [6]. Notably, *V. parahaemolyticus*, which causes acute hepatopancreatic necrosis disease (AHPND) in shrimp, accounts for 78.1% of the bacterial isolates obtained from water samples in the region, with some strains exhibiting multidrug resistance and virulence factors, including *tdh* and *trh* genes [6, 7]. Nowadays, multidrug-resistant *Vibrio* strains are increasingly prevalent, with resistance observed against commonly used antibiotics, such as ampicillin, amoxicillin, and erythromycin, among others [8–10]. The emergence of such resistant pathogens not only threatens aquaculture productivity but also poses risks to public health through the contamination of seafood [11]. The pathogenicity of *Vibrio* is influenced by various virulence factors, including hemolytic activity, production of extracellular enzymes, and the presence of specific toxin-encoding genes such as *toxR*, *tlh*, *tdh*, *trh*, *pirA*, and *pirB*, which encode various toxins and regulatory proteins critical for the expression of pathogenic characteristics in *Vibrio* strains [12–14]. Hemolytic activity, a hallmark of virulence in *Vibrio*, involves the lysis of red blood cells [15]. For example, *in V. parahaemolyticus, hemolysin contributes to cytotoxicity, and it is regarded as* a *primary* virulence factor in infections [16]. Additionally, extracellular enzymes from *Vibrio*, such as proteases, lipases, and DNases, enhance pathogenicity by degrading host tissues and facilitating bacterial invasion [17]. Therefore, understanding these characteristics is essential for developing effective management strategies to mitigate the impact of *Vibrio* infections in aquaculture.

2 Materials and methods

2.1 Strain isolation and 16S rRNA identification

Sample collection and strain isolation

Water samples for *Vibrio* isolation were collected from shrimp ponds along the coastal region and the Tam Giang-Cau Hai lagoon system in Thua Thien Hue Province. The samples were enriched in Alkaline Peptone Water (Oxoid) and incubated at 30 °C for 12 hours. Following enrichment, 100 μL of aliquots of diluted samples were inoculated at 30 °C for next 24 hours on TCBS (Thiosulfate-Citrate-Bile Salts-sucrose) agar (Himedia, India). Colony morphology and pigmentation were also assessed on CHROMagar™ *Vibrio* (Microbiology, France) [11].

Biochemical characterisation

Colonies with distinct morphologies were selected and subjected to preliminary identification by using Gram staining, an oxidase test with oxidase paper discs containing a 1% tetramethyl-p-phenylenediamine dihydrochloride solution (Nam Khoa Biotek CO., Ltd), and a catalase test with 3% H_2O_2. Additionally, these strains were evaluated for various physiological characteristics. Endospore-forming ability was assessed with the Schaeffer-Fulton method using malachite green [18]. Motility capacity was recorded by using a soft agar medium composed of Luria-Bertani (LB) broth (Himedia, India) supplemented with 0.3% agar, while biofilm formation was determined by using a Congo red medium containing Brain heart infusion (BHI) broth (Himedia, India), supplemented with sucrose (50 g/L) and a Congo red dye (0.8 g/L) [19, 20].

pISSN 1859-1388
eISSN 2615-9678

Molecular identification

Colonies tentatively identified as belonging to the genus *Vibrio* were cultured in the LB medium supplemented with 1% NaCl at pH 8.0 and incubated at 30 °C for 14–16 hours to extract genomic DNA. DNA extraction was conducted with the HTOne™ Bacterial Genomic DNA Extraction Kit (HT Biotechnology Company Limited, Vietnam), following the protocol for Gram-negative bacteria. The 16S rDNA region was amplified with the primer pairs 16S rRNA_27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 16S rRNA_1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3') [21]. Amplified products were electrophoresed on a 1% agarose gel in the 1× TAE buffer for 40 minutes, stained with ethidium bromide, and visualised by using a UV transilluminator (DyNa Light). PCR products were purified and sequenced with the Sanger method at Macrogen (Korea). The resulting sequences were edited and aligned by means of Clone Manager 9.0 and ChromasPro software. Sequence comparisons were conducted against the NCBI gene database with the BLAST tool. Phylogenetic analysis was performed by means of IQ-TREE software with the Neighbor-Joining method to infer evolutionary relationships among species [22].

Extracellular enzyme activity

The isolated *Vibrio* strains were cultured overnight in the LB medium at 30 °C with shaking at 200 rpm for 72 hours. The medium was supplemented with 1% specific substrates to test enzyme production: carboxymethyl cellulose (CMC) for cellulase, starch for amylase, gelatin for protease, and olive oil (emulsified in PBS, pH 9.0) for lipase. After incubation, the cultures were centrifuged at 6,000 × g for 15 minutes at 4 °C to collect the supernatant. For assessing enzyme activity, LB agar plates containing 1% of the corresponding substrates were prepared. Wells with a diameter of 6 mm were punched into the agar plates, and 100 µL of the collected supernatant was added to each well. The plates were initially incubated at 4 °C for 60 minutes to allow diffusion of the supernatant into the agar and then transferred to an incubator at 30 °C and maintained there for 24 hours. Substrate degradation zones were visualised by adding a 1% Lugol's iodine solution for cellulase, amylase, and lipase activity, and a 10% mercuric chloride ($HgCl_2$) solution for protease activity [23, 24].

Antibiotic sensitivity testing

The antibiotic resistance of the isolate was evaluated with the Kirby-Bauer disk diffusion method, following the Clinical and Laboratory Standards Institute (CLSI) guidelines 63 [25]. The strains were cultured overnight in the Muller-Hinton medium (Oxoid) at 30 °C and standardised to 1.5 × 10^8 CFU/mL with sterile saline. 100 µL of the bacterial culture was spread onto the entire surface of a Muller-Hinton Agar (MHA) plate (Oxoid). The antibiotic disks containing doxycycline 30 µg, florfenicol 30 µg, chloramphenicol 30 µg, levofloxacin 5 µg, ofloxacin 5 µg, gentamicin 10 µg, erythromycin 15 µg, clindamycin 2 µg, tetracycline 30 µg, ampicillin 10 µg, azithromycin 15 µg, penicillin 10UI, streptomycin 10 µg, kanamycin 30 µg, and vancomycin 30 µg (Nam Khoa Biotek CO., Ltd) were aseptically placed on the agar surface with sterile forceps. Then the MHA plates were incubated at 30 °C for 24 hours. After incubation, the diameter of the inhibition zones around each antibiotic disk was determined. Bacterial sensitivity to antibiotics was categorised as sensitive (S), intermediate (I), or resistant (R), following CLSI63 guidelines [26].

Hemolytic activity assay

The isolated *Vibrio* strains were cultured on the LB agar plates supplemented with 5% sterilised

sheep blood and incubated at 30 °C for 24–48 hours. Hemolytic activity was assessed through the appearance of transparent zones surrounding the bacterial colonies (indicative of β-hemolysis) or green discolouration of the colonies (indicative of α-hemolysis). The positive control included in the assay was *Staphylococcus aureus* FS8300 [27].

Detection of toxin-encoding genes

To determine the presence of toxin genes, namely *toxR* (toxin Regulator), *tdh* (thermostable direct hemolysin), *trh* (thermostable-related hemolysin), *tlh* (thermolabile hemolysin), *pirA* (Primosome Assembly Protein A), and *pirB* (Primosome Assembly Protein B) in the isolated *Vibrio* strains, we performed the PCR amplification with specific primers designed to detect *Vibrio* toxin-encoding genes (Table 1). Initially, bacterial cell suspensions were prepared in a lysis buffer. These suspensions underwent heating at 95 °C to facilitate cell lysis. Following this step, the samples were centrifuged at 16,000 × g for 5 minutes to separate the lysate from cellular debris. The lysate was used as a template for PCR amplification, which began with an initial denaturation step at 95 °C for 5 minutes, followed by 35 cycles consisting of denaturation at 95 °C for 30 seconds, an annealing step at 55 °C for 20 seconds, and an extension step at 72 °C for 1 minute.

Subsequently, the PCR products were analysed by means of electrophoresis on a 1.5% agarose gel at a voltage of 120 V and a current of 300 mA to visualise the presence of the target toxin genes [28].

Table 1. Sequence of primers used for detecting *Vibrio* toxin-encoding genes

Target Genes	Primer names	Nucleotide sequences 5′ → 3′	Annealing temp. (°C)	Amplicon size (bp)	References
toxR	*toxR*-F	GTCTTCTGACG CAATCGTTG	63	367	[29, 30]
	toxR-R	ATACGAGTGG TTGCTGTCATG			
tdh	*tdh*-F	GTAAAGGTCT CTGACTTTTGG AC	55	500	[31, 32]
	tdh-R	TGGAATAGAA CCTTCATCTTC ACC			
trh	*trh*-F	TTGGCTTCGAT ATTTTCAGTAT CT	55	269	[31, 32]
	trh-R	CATAACAAAC ATATGCCCAT TTCC			
tlh	*tlh*-F	AAAGCGGATT ATGCAGAAGC ACTG	60	450	[30]
	tlh-R	GCTACTTTCTA GCATTTTCTCT GC			

pISSN 1859-1388
eISSN 2615-9678

Target Genes	Primer names	Nucleotide sequences 5′ → 3′	Annealing temp. (°C)	Amplicon size (bp)	References
pirA	*pirA*-F	TGACTATTCTCACGATTGGACTG	55	284	[31]
	pirA-R	CACGACTAGCGCCATTGTTA			
pirB	*pirB*-F	TGACTAACGAATACGTTGTAAC	55	1316	[32]
	pirB-R	CTACTTTTCTGTACCAAATTCA			

2.2 Data analysis

The data were measured in triplicate and analysed by using R version 4.2.1 (2022-06-23).

3 Results and discussion

3.1 Strain isolation and 16S rRNA identification

The *Vibrio* species were isolated from brackish water samples collected from shrimp ponds. Five bacterial strains were isolated by using selective media-Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) agar and CHROMagar™ Vibrio. The selected isolates formed green colonies (V16, V25, and V29 strains) and yellow colonies (V06 and V17 strains) on TCBS agar. For CHROMagar™ Vibrio, strains V06 and V17 developed purple colonies, V16 and V29 strains showed white colonies, while V25 colonies were light green. The analysis of cellular morphology and key biochemical characteristics resulted in five gram-negative strains with short rod-shaped or vibrio-like cells, motile, catalase-positive, oxidase-positive, biofilm-forming, and non-spore-forming. These characteristics indicated that the isolates belonged to the genus *Vibrio*. These strains were designated as V06, V16, V17, V25, and V29 and selected for further evaluation. The colonies and cellular features of the isolated strains are illustrated in Fig. 1.

The BLAST results for the 16S rRNA amplification products of the isolates are presented in Table 2. All isolates showed an E-value of 0.0, highlighting significant matches with their respective top-hit strains. The percent identity (Pct) values are all above 98%, confirming similarity to the identified genetic species. Notably, the query cover is either 99 or 100%, which means that nearly the entire sequence of the isolates aligns with the reference strains in the database [33]. In this study, five isolated strains showed alignment with different *Vibrio* species. Specifically, V06, V16, and V17 strains were matched to *V. parahaemolyticus*, *V. sinaloensis*, and *V. azureus* with 99.32, 98.78, and 99.16% identity, respectively. Notably, the remaining two isolates, V25 and V29, were matched to the same species (*V. alginolyticus*), suggesting these might be closely related or the same strain.

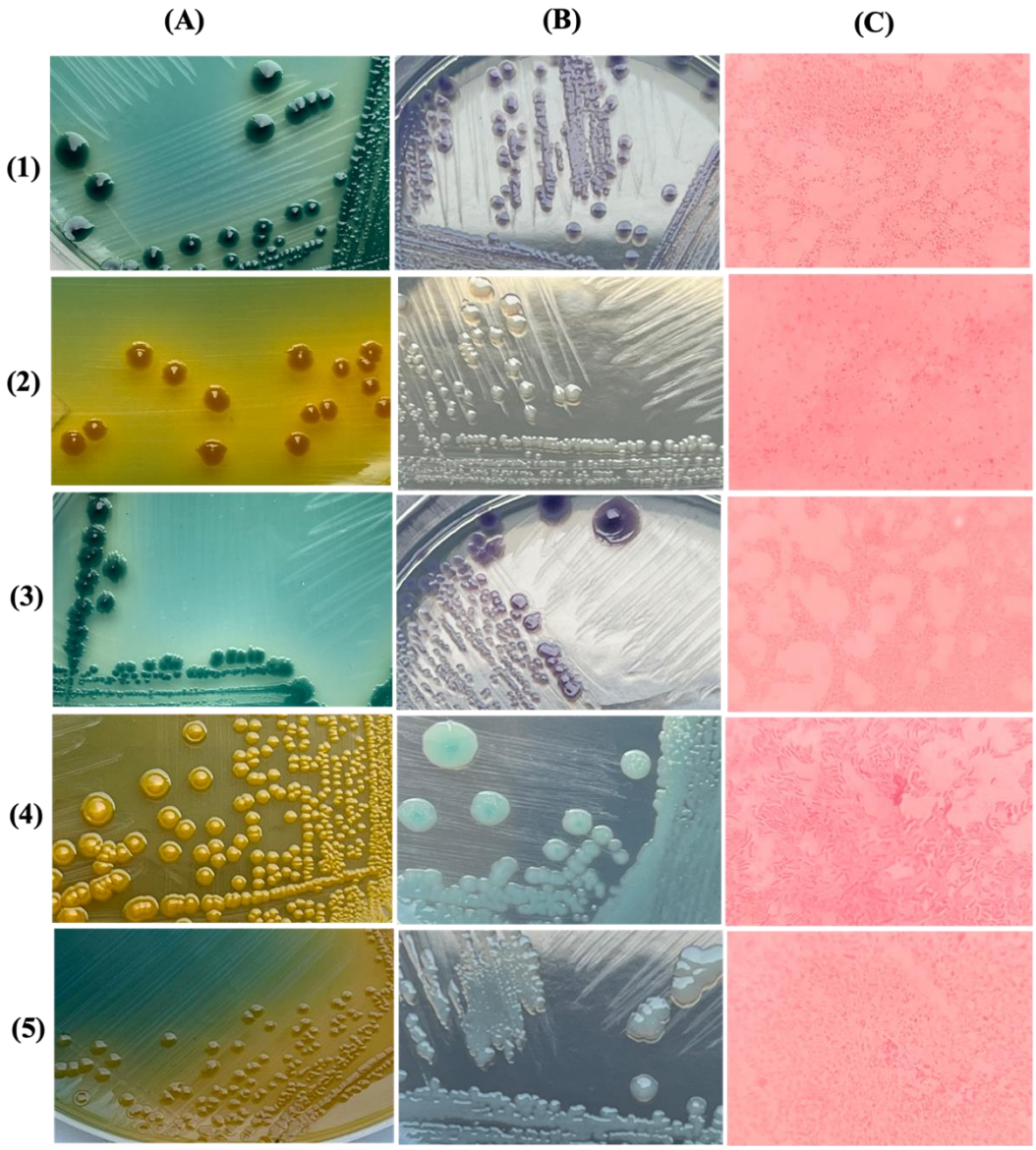

Fig. 1. Colony morphology on TCBS agar (A), CHROMagar™ Vibrio (B), and cell morphology under Light microscop of isolated strains (C) (Strain V06 (1); Strain V16 (2); Strain V17 (3); Strain V25 (4); Strain V29 (5))

Table 2. BLAST results for isolated strains

Strain ID	BLAST results					Registered Genbank Accession Number
	Top-hit taxon	Top-hit strain	E-value	Pct (%)	Query cover	
V06	*Vibrio parahaemolyticus*	ATCC 17802	0.0	99.32	100	PQ422922
V16	*Vibrio sinaloensis*	CAIM 797	0.0	98.78	99	PQ422917
V17	*Vibrio azureus*	NBRC 104587	0.0	99.16	100	PQ422918
V25	*Vibrio alginolyticus*	NBRC 15630	0.0	99.30	100	PQ422919
V29	*Vibrio alginolyticus*	NBRC 15630	0.0	99.66	99	PQ422920

pISSN 1859-1388
eISSN 2615-9678

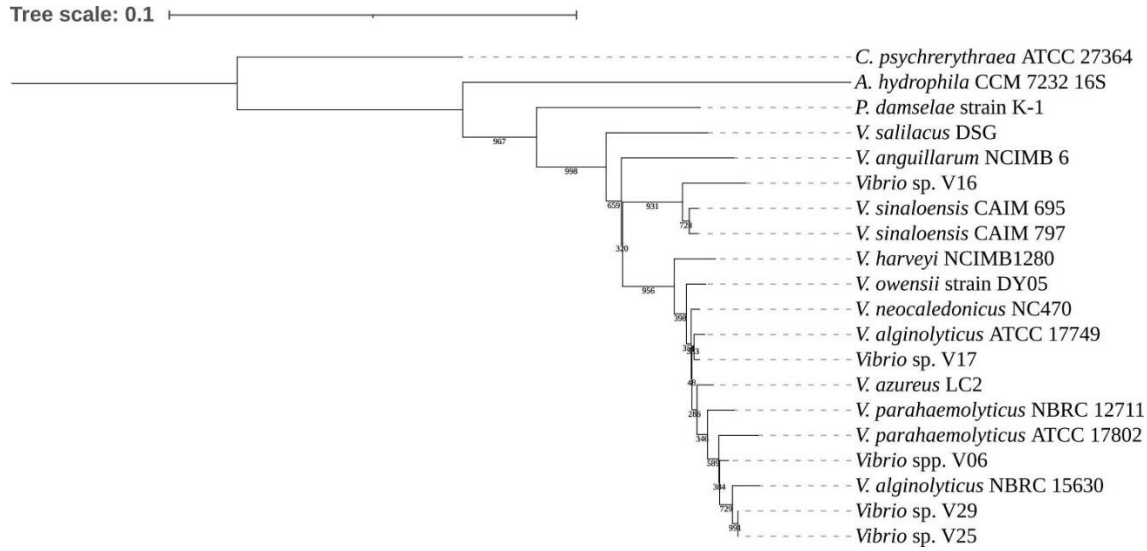

Fig. 2. Phylogenetic tree of five *Vibrio* isolated strains

Additionally, the phylogenetic positions and evolutionary relationships of five *Vibrio* strains (V06, V16, V17, V25, and V29) were analysed and depicted in Fig. 2. Strain V16 was positioned near *V. anguillarum* NCIMB 6 and *V. sinaloensis* CAIM 695, indicating a close evolutionary relationship with these species. Similarly, strain V17 was closely related to *V. azureus* LC2, which demonstrated that this strain was closely related evolutionarily to *V. azureus*. Strain V06 was located adjacent to two *V. parahaemolyticus* strains (NBRC 12711 and ATCC 17802), strongly suggesting that it belongs to the *V. parahaemolyticus* species. Strains V25 and V29 were closely positioned on the phylogenetic tree, highlighting a close evolutionary relationship. Additionally, both strains exhibited a clear phylogenetic association with *V. alginolyticus*. Despite their similarities, the colony colours on CHROMagar™ *Vibrio* differed. Therefore, further analysis is required to identify strain V25 accurately.

3.2 Extracellular enzyme activity

The amylase-producing ability of *Vibrio*, especially *V. parahaemolyticus*, has also been previously reported, with the maximum activity observed at the end of the exponential growth phase [34, 35]. Recent studies reported cellulolytic activity in *V. xiamenensis* and *V. alginolyticus* isolated from mangrove soil samples, suggesting potential for cellulase production in this genus [36]. However, in this study, none of the strains exhibited amylase or cellulase activity. Conversely, the results of the extracellular enzyme production of the isolated strains indicated that all of them were capable of producing protease and lipase. The activities of extracellular protease and lipase are illustrated in Fig. 3.

Strain V25 exhibited limited protease production, as indicated by its minimal gelatin degradation activity. In contrast, strains V06 and V29 demonstrated protease activity by producing substrate degradation zones with a diameter of 18 mm. Notably, strains V16 and V17 exhibited significantly higher protease production, with degradation zone diameters of 19 mm and 22 mm, respectively.

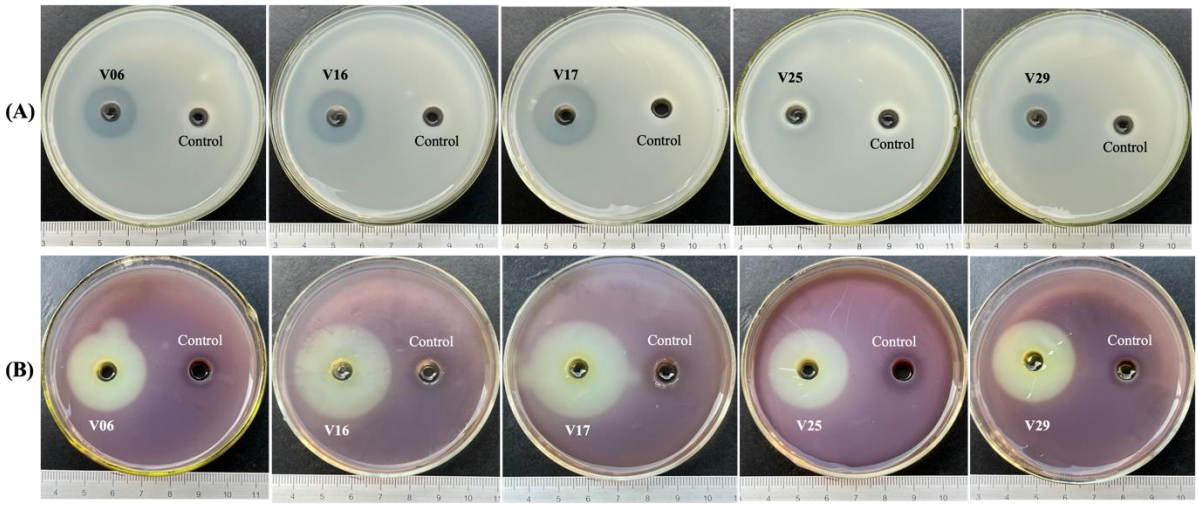

Fig. 3. Extracellular enzyme activity of isolated *Vibrio* strains: Protease activity (A); Lipase activity (B)

The production of protease by *Vibrio* species is a critical component of their pathogenic mechanisms [37–39]. The protease from *V. cholerae* exhibited cytotoxic effects on human intestinal cells, resulting in cell detachment and death [40]. In contrast, proteases secreted by *V. parahaemolyticus* and *V. alginolyticus* facilitated bacterial spread by degrading structural proteins like collagen, thereby contributing to infections, including wound infections and gastroenteritis in humans [41]. Previous studies reported that proteases produced by *V. parahaemolyticus*, including Vibrio High Virulent Protein-2 (VHVP-2), played a critical role in the pathogenicity of shrimp diseases. These enzymes degraded host tissues, leading to hepatopancreatic atrophy and high mortality rates in shrimp. In cases of Translucent Post-Larvae Disease (TPD), protein fragments with molecular weights exceeding 100 kDa have been recognised as the key virulence factors. These exhibited fragments of lethality comparable with live bacterial infections, leading to histopathological changes in the hepatopancreas and midgut of shrimp post-larvae [42].

The proteases produced by *Vibrio* species contribute to their pathogenicity through several mechanisms. These include the degradation of host proteins, which disrupt cellular structures and lead to tissue damage or cell death [39]. Additionally, proteases facilitate immune evasion by degrading immune-related proteins, thereby impairing host defense mechanisms [43]. Furthermore, proteolysis provides essential nutrients for bacterial growth, supporting the survival and proliferation of *Vibrio* pathogens within the host environment [44].

Similarly, all examined *Vibrio* strains exhibited high lipase activity. The diameters of the lipase substrate degradation zones produced by the cell-free supernatants of strains V06, V29, and V25 were recorded as 23, 24, and 28 mm, respectively. Notably, the remaining two strains, V16 and V17, demonstrated exceptionally high lipase capacity, with degradation zone diameters of 34 and 33 mm, respectively.

Specifically, lipase production by *V. parahaemolyticus* has been implicated in the pathogenicity of shrimp diseases, particularly Acute Hepatopancreatic Necrosis Disease and Translucent Post-Larvae Disease. *V. parahaemolyticus* has been reported to secrete extracellular lipase through the Type 2 Secretion System (T2SS), which plays a critical role in its pathogenicity by degrading host tissues [42]. This enzymatic activity contributes to hepatopancreatic

pISSN 1859-1388
eISSN 2615-9678

atrophy and tissue damage in shrimp, hallmarks of Acute Hepatopancreatic Necrosis Disease. Similar to proteases, lipases from *Vibrio* species facilitate nutrient acquisition and immune evasion, enhancing bacterial survival and dissemination within the host [45]. In the present study, all isolated strains demonstrated the ability to produce extracellular proteases and lipases. While these enzymes are hypothesised to contribute to the pathogenicity of *Vibrio* species, further research is required to elucidate the precise relationship between their enzymatic activity and virulence.

3.3 Antibiotic sensitivity patterns

The antibiotic susceptibility profiles of the tested *Vibrio* strains revealed significant variability, with most strains exhibiting resistance to antibiotics such as vancomycin, penicillin, streptomycin, ampicillin, and chloramphenicol. In contrast, intermediate sensitivity was frequently noted for gentamicin and azithromycin. However, broad-spectrum antibiotics, including florfenicol and tetracyclines (doxycycline and tetracycline), maintained efficacy against the majority of these *Vibrio* strains. Notably, five *Vibrio* strains demonstrated multidrug resistance to at least four antibiotics (Fig. 4).

All strains consistently showed resistant profiles to vancomycin, penicillin, and ampicillin. The observed vancomycin resistance in this study aligned with that reported in previous studies, which delineated the intrinsic resistance of *Vibrio* species, caused by their cell wall composition and the absence of target binding sites for this glycopeptide antibiotic [46]. Additionally, resistance to beta-lactam antibiotics, such as penicillin and ampicillin, has been recorded as being related to the production of beta-lactamases in these bacteria [47].

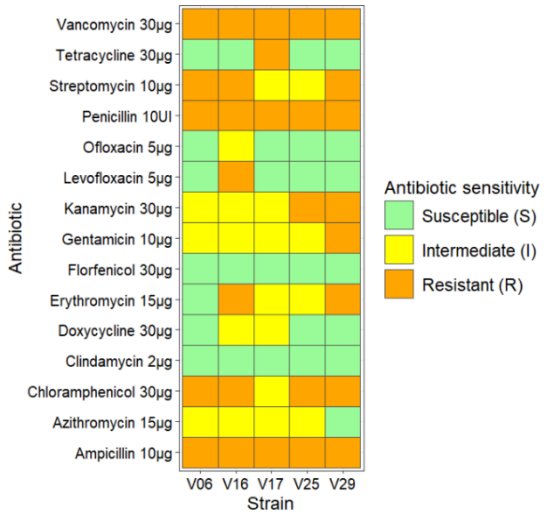

Fig. 4. Heat map showing the antibiotic sensitivity patterns of isolated *Vibrio* strains

Most strains indicated resistance to streptomycin and chloramphenicol, except for intermediate strain V25 and strain V17. Streptomycin resistance is widespread among aquatic bacteria, due to aminoglycoside-modifying enzymes, while chloramphenicol resistance in *Vibrio* is often attributed to cat genes encoding chloramphenicol acetyltransferase [48, 49]. Notably, all the *Vibrio* strains in this study were susceptible to florfenicol, which is an effective antibiotic against many aquatic pathogens and is widely used in aquaculture [4]. Numerous studies have documented the antibiotic resistance of *Vibrio*. Among the 315 *Vibrio* strains isolated in Southwest Nigeria, the resistance rates to erythromycin, sulfamethoxazole, and tetracycline were 95, 94, and 75%, respectively [50]. *V. parahaemolyticus* and *V. aglinolyticus* isolated from fish and shrimp farming in Northern Vietnam exhibited a high rate, exceeding 69.77%, of resistance to oxytetracycline, streptomycin, amoxicillin, and ampicillin [8]. Another example is *V. parahaemolyticus* isolated from the Mekong Delta, Vietnam, which is resistant to amoxicillin, cephalexin, sulfadiazine sodium, and erythromycin [51]. Most recently, *V. vulnificus* and *V. parahaemolyticus* isolated from the

Chesapeake Bay showed resistance patterns to third-generation cephalosporins, tetracyclines, sulfamethoxazole-trimethoprim, and aminoglycosides [52]. Globally, there is an increase in antibiotic resistance, which is facilitated by mobile genetic elements and horizontal gene transfer, exacerbated by environmental factors such as pollution and climate change [53].

In this study, strain V29, which showed 99.66% similarity to *V. alginolyticus*, exhibited the highest multi-antibiotic resistance among the five *Vibrio* species. It is resistant to eight of the fifteen antibiotics tested, namely penicillin, ampicillin, streptomycin, clindamycin, gentamicin, kanamycin, erythromycin, and vancomycin (Fig. 5). These findings highlight the increasing concern of multidrug-resistant *Vibrio* strains in aquaculture and emphasise the importance of monitoring antibiotic use and exploring alternative biocontrol strategies in this field study.

3.4 Hemolytic activity

The hemolytic activity of hemolysin proteins refers to their ability to lyse red blood cells. Hemolysins are considered virulence factors for many *Vibrio* species, facilitating tissue invasion, immune evasion, and the manifestation of disease symptoms through their pore-forming capabilities [16, 54]. In this study, we evaluated the hemolytic activity of the isolated *Vibrio* strains, and the results are presented in Fig. 6. Among the five strains analysed, strain V06 exhibited beta-hemolytic activity, indicating a higher potential for pathogenicity. In contrast, strains V17 and V29 demonstrated alpha-hemolytic activity, suggesting moderate virulence. The remaining two strains showed minimal hemolytic activity. *Vibrio* species are known to exhibit both alpha and beta hemolysis, notably *V. parahaemolyticus* and *V. alginolyticus.* Interestingly, *V. parahaemolyticus* isolated from tissue infections predominantly displayed alpha hemolytic reactions, whereas strains from sea fish typically exhibited beta hemolysis, often associated with the presence of the thermostable direct hemolysin (*tdh*) gene [15, 55].

In contrast to our findings for strain V29, *V. alginolyticus* isolated from Alaskan oysters possessed an alpha-hemolysin linked to a thermostable direct hemolysin-related hemolysin (*trh*) gene [56]. Recent studies have shown that *V. alginolyticus* can exhibit both alpha and beta hemolysis [57]. Strains V16 and V25 exhibited non-specific hemolytic activity, which may be attributed to the production of other virulence factors contributing to their hemolytic activity. Therefore, further investigation is necessary to elucidate the factors involved in the hemolytic activity and virulence of these strains.

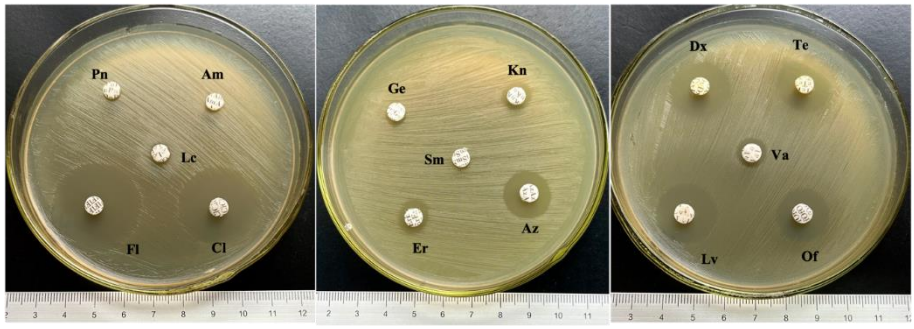

Fig. 5. Antibiotic susceptibility of isolated *V. alginolyticus* strain V29
Ge: Gentamicin 10 μg; Kn: Kanamycin 30 μg; Sm: Streptomycin 10 μg; Er: Erythromycin 15 μg; Az: Azithromycin 15 μg; Dx: Doxycycline 30 μg; Te: Tetracycline 30 μg; Va: Vancomycin 30 μg; Lv: Levofloxacin 5 μg; Of: Ofloxacin 5 μg; Pn: Penicillin 10 UI; Am: Ampicillin 10 μg; Lc: Clindamycin 2 μg; Fl: Florfenicol 30 μg; Cl: Chloramphenicol 30 μg

pISSN 1859-1388
eISSN 2615-9678

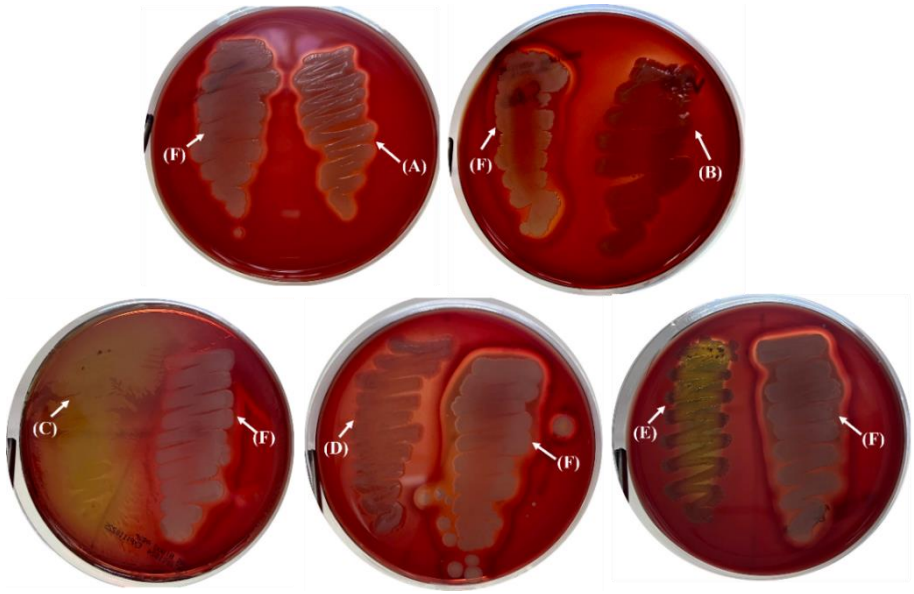

Fig. 6. Hemolytic phenotype of isolated *Vibrio* strains
Strain V06 (A); Strain V16 (B); Strain V17 (C); Strain V25 (D); Strain V19 (E); *S. aureus* SF8300 (F)

3.5 Presence of toxin-encoding genes

The results of the assessment of the presence of *toxR*, *tdh*, *trh*, *tlh*, *pirA*, and *pirB* genes in the investigated *Vibrio* strains (Table 3 and Fig. 7) revealed a consistent genetic profile across all isolates (V06, V16, V17, V25, and V29). Specifically, the *toxR* gene (367 bp) was detected in all strains, indicating the presence of the toxin regulator gene, which plays a critical role in regulating toxin production and other virulence-associated functions in *Vibrio* [58]. *ToxR* is a transmembrane DNA-binding protein that regulates the transcription of virulence genes in *V. cholerae*, such as those encoding cholera toxin and toxin-coregulated pilus, by controlling the expression of *toxT*, which is a direct activator of these virulence factors [59].

In the case of *V. parahaemolyticus*, *toxR* regulates multiple virulence phenotypes, including enterotoxicity and cytotoxicity, by acting on genes like T3SS1 and Vp-PAI. *ToxR*-carrying *Vibrio* species can cause disease in shrimp. The most recent study on *Penaeus monodon* in Peninsular Malaysia demonstrated that *V. parahaemolyticus* isolates carrying the *toxR* genes were pathogenic. In an immersion challenge test, *V. parahaemolyticus* caused significant mortality in shrimp exhibiting symptoms such as a pale hepatopancreas, empty guts, and histopathological damage like the loss of hepatopancreatic tubule structure and hemocytic infiltration [60]. This highlights the role of the *toxR* gene in controlling pathogenic mechanisms across *Vibrio* species [27, 61].

Similarly, the *tlh* gene (450 bp) encoding thermolabile hemolysin was also present in all isolates. This gene is commonly associated with *Vibrio* pathogenicity [62]. The *tlh* gene is recognised as a molecular marker for *V. parahaemolyticus* and is expressed in both clinical and environmental strains [12]. This gene also encodes a hemolysin capable of lysing red blood cells and demonstrating lecithin-dependent phospholipase activity, which is integral to its negative impacts on human infections and cytotoxicity [63]. Furthermore, its expression is markedly upregulated under conditions that mimic intestinal infection, reinforcing its association with pathogenicity [12].

Table 3. Presence of toxin-encoding genes in isolated *Vibrio* strains, indicated as positive (+) or negative (–)

Gene	Amplicon size (bp)	V06	V16	V17	V25	V29
toxR	367	+	+	+	+	+
tdh	500	–	–	–	–	–
trh	269	–	–	–	–	–
tlh	450	+	+	+	+	+
pirA	284	–	–	–	–	–
pirB	1316	–	–	–	–	–
16S rDNA	~1500	+	+	+	+	+

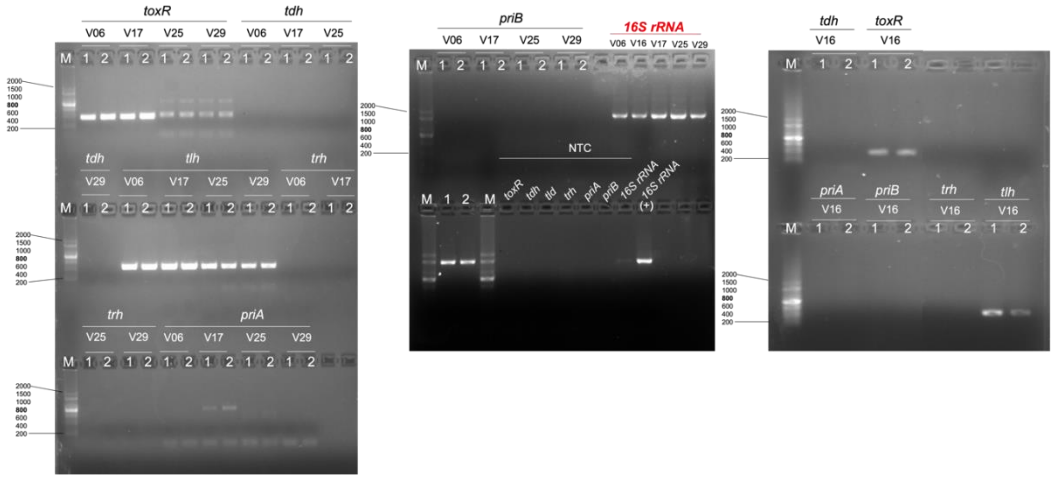

Fig. 7. Gel electrophoresis showing the presence of toxin-encoding genes of *Vibrio* strains (16S rRNA was used as a control)

In contrast, the *tdh* (500 bp) and *trh* (269 bp) genes, which are related to thermostable direct hemolysin and thermostable-related hemolysin, respectively, were absent in all the investigated strains. Additionally, the replication-associated genes *pirA* (284 bp) and *pirB* (1316 bp) were not observed in any of the isolates. These findings suggest that in this study, while the *Vibrio* strains possessed potential pathogenicity because of the presence of *toxR* and *tlh*, they lacked virulence factors associated with thermostable hemolysins (*tdh* and *trh*). These results indicated that their pathogenic potential may be mediated through mechanisms independent of these specific toxins.

In a previous study conducted in Hue City, Vietnam, the presence of toxin genes in *Vibrio* spp., which were isolated from AHPND shrimp and hemorrhagic fishes, was evaluated. The findings revealed that the key toxin genes identified included $pirA^{vp}$, $pirB^{vp}$, and *tlh*. Specifically, 14 out of 54 isolates from shrimp were found to carry the $pirA^{vp}$ and $pirB^{vp}$ genes, while 18 out of 66 isolates from fish harbored the *tlh* gene. Notably, none of the strains contained the *tdh* or *trh* genes [13, 64].

Another study analysing the existence of toxin genes in *Vibrio* isolates associated with hemorrhagic disease in red drum (*Sciaenops ocellatus*) indicated that the majority of the isolates were found to carry the *tlh* gene, while the *tdh* and *trh* genes were not detected [65]. These results

pISSN 1859-1388
eISSN 2615-9678

underscore the predominance of the *tlh* gene in these strains and its potential role in pathogenesis.

4 Conclusion

This study characterises *Vibrio* strains from Vietnamese shrimp aquaculture through biochemical and molecular analyses. The five bacterial strains isolated in this study exhibited genetic similarity exceeding 98.78% to four *Vibrio* species, namely *V. parahaemolyticus*, *V. alginolyticus*, *V. azureus*, and *V. sinaloensis*, with the latter two representing novel regional findings requiring genomic confirmation. All strains exhibited multidrug resistance, including universal resistance to vancomycin, penicillin, and ampicillin, highlighting antimicrobial misuse concerns. Phenotypic analyses revealed hemolytic activity and extracellular protease and lipase production in most isolates, indicating pathogenic potential, though amylase and cellulase activity was absent. Molecular profiling identified conserved *toxR* and *tlh* genes across all strains, but the absence of *tdh*, *trh*, *pirA*, and *pirB* defined a unique virulence signature. Collectively, these findings provide important insights into the pathogenicity, resistance patterns, and molecular characteristics of *Vibrio* strains in shrimp farming, underscoring the necessity for continuous surveillance and improved disease management strategies.

Acknowledgment

This work was supported by Hue University Science and Technology Project [Project code: DHH2024-03-193]. We also appreciate the technical support provided by HT Biotechnology (HTBiotech) Company Limited, Vietnam.

References

1. Marques PH, Prado LC da S, Felice AG, Rodrigues TCV, Pereira U de P, Jaiswal AK, et al. Insights into the *Vibrio* Genus: A One Health Perspective from Host Adaptability and Antibiotic Resistance to In Silico Identification of Drug Targets. Antibiotics (Basel). 2022;11(10):1399.
2. Sampaio A, Silva V, Poeta P, Aonofriesei F. *Vibrio* spp.: Life Strategies, Ecology, and Risks in a Changing Environment. Diversity. 2022;14(2):97.
3. Sanches-Fernandes GMM, Sá-Correia I, Costa R. Vibriosis Outbreaks in Aquaculture: Addressing Environmental and Public Health Concerns and Preventive Therapies Using Gilthead Seabream Farming as a Model System. Front Microbiol. 2022;Volume 13 - 2022.
4. Baker-Austin C, Oliver JD, Alam M, Ali A, Waldor MK, Qadri F, et al. *Vibrio* spp. infections. Nat Rev Dis Primers. 2018;4(1):8.
5. Sheahan M, Gould CA, Neumann JE, Kinney PL, Hoffmann S, Fant C, et al. Examining the Relationship between Climate Change and Vibriosis in the United States: Projected Health and Economic Impacts for the 21st Century. Environ Health Perspect. 2022;130(8):87007.
6. Tran THT, Yanagawa H, Nguyen KT, Hara-Kudo Y, Taniguchi T, Hayashidani H. Prevalence of *Vibrio parahaemolyticus* in seafood and water environment in the Mekong Delta, Vietnam. J Vet Med Sci. 2018 Nov; 80(11):1737–42.
7. Nguyen KCT, Truong PH, Thi HT, Ho XT, Nguyen PV. Prevalence, multidrug resistance, and biofilm formation of *Vibrio parahaemolyticus* isolated from fish mariculture environments in Cat Ba Island, Vietnam. Osong Public Health Res Perspect. 2024;15(1):56-67.
8. Ngo TH, Vu XN, Le DLO, Dang LQ, Le TTM, Pham TT, et al. Antibiotics resistance of *Vibrio* spp. isolated from aquaculture water in some areas of Northern Vietnam. Hue University Journal of Science: Natural Science. 2022;131(1C):83-93.
9. Ha PTH, Thi QVC, Thuy NP, Luan NT. Multi-antibiotics resistance phenotype of pathogenic *Vibrio parahaemolyticus* isolated from acute hepatopancreatic necrosis disease in *Litopenaeus vannamei* farmed in the Mekong Delta. Journal of the World Aquaculture Society. 2023;54(4):1070-87.

10. Deng Y, Xu L, Chen H, Liu S, Guo Z, Cheng C, et al. Prevalence, virulence genes, and antimicrobial resistance of *Vibrio* species isolated from diseased marine fish in South China. Sci Rep. 2020;10(1):14329.

11. Zhang F, Zhang J, Lin G, Chen X, Huang H, Xu C, et al. Antibiotic Resistance and Genetic Profiles of *Vibrio parahaemolyticus* Isolated from Farmed Pacific White Shrimp (*Litopenaeus vannamei*) in Ningde Regions. Microorganisms. 2024;12(1):152.

12. Wang R, Zhong Y, Gu X, Yuan J, Saeed AF, Wang S. The pathogenesis, detection, and prevention of *Vibrio parahaemolyticus*. Front Microbiol. 2015;6:144.

13. Quang HT, Lan TT, Thi PTD, Nhan LTT, Ngoc LMT, Tram NDQ, et al. Screening and analysing toxic genes from *Vibrio* isolates causing acute hepatopancreatic neorosis disease in white-leg shrimp at Thua Thien Hue. Hue University Journal of Science: Natural Science. 2020;129(1A):115-23.

14. Pérez-Reytor D, Jaña V, Pavez L, Navarrete P, García K. Accessory Toxins of *Vibrio* Pathogens and Their Role in Epithelial Disruption During Infection. Front Microbiol. 2018;Volume 9 - 2018.

15. Twedt RM, Novelli RE, Spaulding PL, Hall HE. Comparative Hemolytic Activity of *Vibrio parahaemolyticus* and Related Vibrios. Infect Immun. 1970;1(4):394-9.

16. Letchumanan V, Chan KG, Lee LH. *Vibrio parahaemolyticus*: a review on the pathogenesis, prevalence, and advance molecular identification techniques. Front Microbiol. 2014;5:705.

17. Oliver JD, Wear JE, Thomas MB, Warner M, Linder K. Production of extracellular enzymes and cytotoxicity by *Vibrio vulnificus*. Diagn Microbiol Infect Dis. 1986;5(2):99-111.

18. Aryal S. Endospore Staining- Principle, Reagents, Procedure and Result [Internet]. Microbiology Info.com. 2015 [cited 2024 Dec 11]. Available from: https://microbiologyinfo.com/endospore-staining-principle-reagents-procedure-and-result/

19. Palma V, Gutiérrez MS, Vargas O, Parthasarathy R, Navarrete P. Methods to Evaluate Bacterial Motility and Its Role in Bacterial–Host Interactions. Microorganisms. 2022;10(3):563.

20. Golnari M, Bahrami N, Milanian Z, Rabbani Khorasgani M, Asadollahi MA, Shafiei R, et al. Isolation and characterization of novel *Bacillus* strains with superior probiotic potential: comparative analysis and safety evaluation. Sci Rep. 2024;14(1):1457.

21. King WL, Siboni N, Kahlke T, Green TJ, Labbate M, Seymour JR. A New High Throughput Sequencing Assay for Characterizing the Diversity of Natural *Vibrio* Communities and Its Application to a Pacific Oyster Mortality Event. Front Microbiol. 2019;10:2907.

22. Tamura K, Stecher G, Kumar S. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. Mol Biol Evol. 2021;38(7):3022-7.

23. Costa RA, Conde Amorim LMM, Araújo RL, dos Fernandes Vieira RHS. Multiple enzymatic profiles of *Vibrio parahaemolyticus* strains isolated from oysters. Rev Argent Microbiol. 2013;45(4):267-70.

24. Denkin SM, Nelson DR. Induction of Protease Activity in *Vibrio anguillarum* by Gastrointestinal Mucus. Appl Environ Microbiol. 1999;65(8):3555-60.

25. Jorgensen JH, National Committee for Clinical Laboratory Standards ib. Performance standards for antimicrobial disk susceptibility tests: approved standard. Sixth edition ed. Wayne, PA: NCCLS; 1997.

26. Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol. 1966;45(4):493-6.

27. Kim YB, Okuda J, Matsumoto C, Takahashi N, Hashimoto S, Nishibuchi M. Identification of *Vibrio parahaemolyticus* strains at the species level by PCR targeted to the toxR gene. J Clin Microbiol. 1999;37(4):1173-7.

28. Chen S, Ge B. Development of a toxR-based loop-mediated isothermal amplification assay for detecting *Vibrio parahaemolyticus*. BMC Microbiology. 2010;10(1):41.

29. Bej AK, Patterson DP, Brasher CW, Vickery MC, Jones DD, Kaysner CA. Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tl*, *tdh* and *trh*. J Microbiol Methods. 1999;36(3):215-25.

30. Rizvi AV, Bej AK. Multiplexed real-time PCR amplification of tlh, tdh and trh genes in *Vibrio parahaemolyticus* and its rapid detection in shellfish and Gulf of Mexico water. Antonie Van Leeuwenhoek. 2010;98(3):279-90.

31. Jeon HJ, Song JW, Lee C, Kim B, Park SY, Kim JH, et al. Antibacterial Activity of *Bacillus* Strains against Acute Hepatopancreatic Necrosis Disease-

pISSN 1859-1388
eISSN 2615-9678

Causing Vibrio campbellii in Pacific White Leg Shrimp. Fishes. 2022;7(5):287.

32. Zheng Z, Li R, Aweya JJ, Yao D, Wang F, Li S, et al. The PirB toxin protein from *Vibrio parahaemolyticus* induces apoptosis in hemocytes of Penaeus vannamei. Virulence. 2021;12(1):481-92.

33. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215(3):403-10.

34. Iuchi S, Tanaka S. Catabolite-like repression of extracellular enzyme production in *Vibrio parahaemolyticus*. Microbiol Immunol. 1980;24(9):803-14.

35. Najafi MF, Kembhavi A. One step purification and characterization of an extracellular α-amylase from marine *Vibrio* sp. Enzyme and Microbial Technology. 2005;36(4):535-9.

36. Gao ZM, Xiao J, Wang XN, Ruan LW, Chen XL, Zhang YZ. *Vibrio xiamenensis* sp. nov., a cellulase-producing bacterium isolated from mangrove soil. Int J Syst Evol Microbiol. 2012;62(Pt 8):1958-62.

37. Shinoda S, Miyoshi SI. Proteases Produced by Vibrios. Biocontrol Science. 2011;16(1):1-11.

38. Osei-Adjei G, Huang X, Zhang Y. The extracellular proteases produced by *Vibrio parahaemolyticus*. World J Microbiol Biotechnol. 2018;34(5):68.

39. Mondal A, Tapader R, Chatterjee NS, Ghosh A, Sinha R, Koley H, et al. Cytotoxic and Inflammatory Responses Induced by Outer Membrane Vesicle-Associated Biologically Active Proteases from *Vibrio cholerae*. Infect Immun. 2016;84(5):1478-90.

40. Vaitkevicius K, Rompikuntal PK, Lindmark B, Vaitkevicius R, Song T, Wai SN. The metalloprotease PrtV from *Vibrio cholerae* Purification and properties. FEBS J. 2008;275(12):3167-77.

41. Miyoshi SI. Extracellular proteolytic enzymes produced by human pathogenic *Vibrio* species. Front Microbiol. 2013; 4:339.

42. Liu S, Wang W, Jia T, Xin L, Xu T ting, Wang C, et al. *Vibrio parahaemolyticus* becomes lethal to post-larvae shrimp via acquiring novel virulence factors. Microbiol Spectr. 11(6):e00492-23.

43. Vandeputte M, Kashem MdA, Bossier P, Vanrompay D. *Vibrio* pathogens and their toxins in aquaculture: A comprehensive review. Reviews in Aquaculture. 2024;16(4):1858-78.

44. Onohuean H, Okoh AI, Nwodo UU. Epidemiologic potentials and correlational analysis of *Vibrio* species and virulence toxins from water sources in greater Bushenyi districts, Uganda. Sci Rep. 2021;11(1):22429.

45. Truong NTT, Nguyen TH, Bui CC, Nguyen TTH. Effects of different shrimp extracts on *Vibrio parahaemolyticus* growth and virulence. Vietnam Journal of Science, Technology and Engineering. 2021;63(4):62-5.

46. Zhang XH, Austin B. Haemolysins in *Vibrio* species. J Appl Microbiol. 2005;98(5):1011-9.

47. Baker-Austin C, Oliver JD, Alam M, Ali A, Waldor MK, Qadri F, et al. *Vibrio* spp. infections. Nat Rev Dis Primers. 2018;4(1):8.

48. Zhang G, Sun K, Ai G, Li J, Tang N, Song Y, et al. A novel family of intrinsic chloramphenicol acetyltransferase CATC in *Vibrio parahaemolyticus*: Naturally occurring variants reveal diverse resistance levels against chloramphenicol. Int J Antimicrob Agents. 2019;54(1):75-9.

49. Elmahdi S, DaSilva LV, Parveen S. Antibiotic resistance of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in various countries: A review. Food Microbiol. 2016;57:128-34.

50. Adesiyan IM, Bisi-Johnson MA, Okoh AI. Incidence of antibiotic resistance genotypes of *Vibrio* species recovered from selected freshwaters in Southwest Nigeria. Sci Rep. 2022;12(1):18912.

51. Dang THO, Truong QP, Nguyen TP. Antibacterial resistance of *Vibrio parahaemolyticus* isolated from shrimp farms located in east coastal region of the Mekong Delta, Vietnam. CTU J of Inn & Sus Dev. 2022;14(2):1-7.

52. Huang Z, Anokyewaa MA, Wang J, Jian J, Lu Y. Pathogenicity and antibiotic resistance analysis of *Vibrio* species found in coastal water at mainly beach of Shenzhen, China. Front Mar Sci. 2022;Volume 9 - 2022

53. Fang GY, Liu XQ, Mu XJ, Huang BW, Jiang YJ. Distinct increase in antimicrobial resistance genes among *Vibrio parahaemolyticus* in recent decades worldwide. Chemosphere. 2023;340:139905.

54. Chiu YC, Yeh MC, Wang CH, Chen YA, Chang H, Lin HY, et al. Structural basis for calcium-stimulating pore formation of *Vibrio* α-hemolysin. Nat Commun. 2023;14(1):5946.

55. Chen AJ, Hasan NA, Haley BJ, Taviani E, Tarnowski M, Brohawn K, et al. Characterization of

Pathogenic *Vibrio parahaemolyticus* from the Chesapeake Bay, Maryland. Front Microbiol. 2017;Volume 8 - 2017

56. González-Escalona N, Blackstone GM, DePaola A. Characterization of a *Vibrio alginolyticus* Strain, Isolated from Alaskan Oysters, Carrying a Hemolysin Gene Similar to the Thermostable Direct Hemolysin-Related Hemolysin Gene (trh) of Vibrio parahaemolyticus. Appl Environ Microbiol. 2006;72(12):7925-9.
57. Mohamad N, Amal MNA, Yasin ISM, Zamri Saad M, Nasruddin NS, Al-saari N, et al. Vibriosis in cultured marine fishes: a review. Aquaculture. 2019;512:734289.
58. Parsot C, Mekalanos JJ. Expression of ToxR, the transcriptional activator of the virulence factors in *Vibrio cholerae*, is modulated by the heat shock response. Proc Natl Acad Sci U S A. 1990;87(24):9898-902.
59. Mey AR, Butz HA, Payne SM. *Vibrio cholerae* CsrA Regulates ToxR Levels in Response to Amino Acids and Is Essential for Virulence. mBio. 2015;6(4):e01064-15.
60. Haifa-Haryani WO, Azzam-Sayuti M, Amatul-Samahah MdA, Chin YK, Zamri-Saad M, Annas S, et al. Characterization of the Virulency of Vibrio spp. in Giant Tiger Shrimp (*Penaeus monodon*) from Peninsular Malaysia. Aquaculture Research. 2023;2023(1):7259748.
61. Zhang Y, Hu L, Osei-Adjei G, Zhang Y, Yang W, Yin Z, et al. Autoregulation of ToxR and Its Regulatory Actions on Major Virulence Gene Loci in *Vibrio parahaemolyticus*. Front Cell Infect Microbiol. 2018;Volume 8 - 2018
62. Gutierrez West CK, Klein SL, Lovell CR. High Frequency of Virulence Factor Genes tdh, trh, and tlh in *Vibrio parahaemolyticus* Strains Isolated from a Pristine Estuary. Appl Environ Microbiol. 2013;79(7):2247-52.
63. Raghunath P. Roles of thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) in *Vibrio parahaemolyticus*. Front Microbiol. 2015;Volume 5 – 2014.
64. Quang HT, Lan TT, Hai TTH, Yen PTH, Van TQK, Tung HT, et al. Genetic diversity and toxic genes analysis of *Vibrio* spp. isolated from white leg shrimp and marine fishes cultured in Tam G iang lagoon in Thua Thien Hue province, Vietnam. INDJST. 2020;13(13):1412-22.
65. Yen PTH, Linh NQ, Tram NDQ. The identification and determination of toxin genes of *Vibrio* strains causing hemorrhagic disease on red drum (Sciaenops ocellatus) using PCR. AMB Express. 2021;11(1):4.