Isolation and Activity Assessment of *Vibrio parahaemolyticus* Causing Translucent Post-Larvae Disease from Shrimp Samples

[1,2]Diem Hong Tran, [1,2]Vu Nguyen Ngo, [1,2]Huong Thi Thu Phung
[1]Center for Hi-Tech Development, Nguyen Tat Thanh University, Saigon Hi-Tech Park, Ho Chi Minh City, Vietnam
[2]NTT Hi-Tech Institute, Nguyen Tat Thanh University, Ho Chi Minh City, Vietnam
nnvu@ntt.edu.vn, thdiem@ntt.edu.vn, ptthuong@ntt.edu.vn

Abstract

Translucent Post-Larvae Disease (TPD) is an emerging threat in shrimp aquaculture, primarily caused by a hypervirulent strain of *Vibrio parahaemolyticus*. This study aimed to isolate and characterize *V. parahaemolyticus* strains from shrimp farming environments and assess their pathogenic potential. Samples were collected from various shrimp farms in Mekong Delta, Vietnam. A total of 5 *V. parahaemolyticus* strains were isolated and screened for the presence of the *vhvp-2* gene encoding the Vibrio High Virulent Protein-2 (vhvp-2), a key virulence factor associated with TPD. The isolates were also evaluated for antibiotic resistance, salt tolerance, and extracellular enzyme activities. The results revealed a high prevalence of antibiotic-resistant *V. parahaemolyticus* strains, with one isolate carrying the *vhvp-2* gene. These findings provide valuable insights into the distribution of TPD-causing *V. parahaemolyticus* strains in Vietnamese shrimp farms and highlight the need for improved disease management strategies in shrimp aquaculture.

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TPD, *vhvp-2*, *V. parahaemolyticus*, shrimp

1. Introduction

Shrimp aquaculture plays a key role in global seafood production, but it is increasingly threatened by emerging new diseases. One such disease is Translucent Post-Larvae Disease (TPD), recently reported in several Asian countries, including China and Vietnam[1]. TPD typically affects shrimp at the post-larval stage, causing translucent body appearance, weakness, and high mortality shortly after stocking, leading to significant economic losses[2]. Recent findings have linked TPD to a hypervirulent strain of *Vibrio parahaemolyticus* carrying the vhvp-2 gene, which encodes a novel virulence factor (Vibrio High Virulent Protein-2)[3, 4]. This distinguishes TPD-causing strains from other non-virulent or opportunistic *V. parahaemolyticus* commonly present in marine environments[3, 5].

Due to the novelty of this pathogen and limited data in Vietnam, especially in the Mekong Delta where shrimp farming is intensive, preliminary isolation and identification of *V. parahaemolyticus* strains associated with TPD are essential. Detection of the *vhvp-2* gene provides a valuable marker for identifying potential pathogenic strains and understanding their distribution[4]. The primary aim of this study is to isolate and preliminarily characterize *V. parahaemolyticus* strains from TPD-suspected shrimp samples. In addition, basic assessments of antibiotic resistance, salt tolerance, and extracellular enzyme activity were conducted to provide initial insights into their physiological traits. These findings lay the groundwork for future research on pathogenicity and control of TPD in Vietnamese shrimp aquaculture.

2. Materials and methods

2.1. Sample collection

Shrimp samples at day 20–25 of culture exhibiting clinical signs of TPD—including anorexia, lethargic swimming, empty gut, poor reflexes, and hepatopancreas discoloration (from dark brown to pale/light brown)[6]—were collected from five shrimp farms in the Mekong Delta, Vietnam, during routine monitoring in 2024. Samples were transported on ice and processed within 12 hours of collection.

Figure 1. Hepatopancreas of shrimp post-larvae exhibiting typical clinical signs of Translucent Post-

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Larvae Disease (TPD), including pale coloration, tissue degeneration, and fragile structure.

2.2. Bacterial isolation and culture

Hepatopancreas tissues were aseptically dissected from shrimp samples exhibiting clinical signs of TPD. The tissues were homogenized in sterile phosphate-buffered saline (PBS, pH 7.2) using a glass homogenizer under sterile conditions. The homogenates were serially diluted (10^{-1} to 10^{-6}) in PBS, and 100 µL aliquots from each dilution were spread evenly onto ChromAgar plates, a selective medium for *Vibrio* species [7–9]. Plates were incubated at 30°C for 18–24 hours. Colonies displaying mauve to purple centers—morphologically consistent with *V. parahaemolyticus*—were presumptively identified and selected for further analysis. These colonies were sub-cultured onto Tryptic Soy Agar (TSA) plates supplemented with 1% NaCl and incubated under the same conditions to obtain pure cultures for downstream testing.

2.3. Biochemical identification

Presumptive isolates obtained from ChromAgar were subjected to a series of standard biochemical tests to confirm their identity as *V. parahaemolyticus*. These tests included oxidase and catalase reactions, as well as assessments for gelatinase, caseinase, and chitinase activities using agar-based enzymatic assays. Salt tolerance was evaluated by culturing isolates in TSB supplemented with NaCl concentrations ranging from 1% to 10%. Carbohydrate utilization was assessed using fermentation tests with glucose, sucrose, and mannitol as carbon sources. Final identification was confirmed by 16S rRNA gene sequencing. Genomic DNA was extracted from pure bacterial cultures, and the 16S rRNA gene was amplified by PCR using universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). PCR products were purified and sequenced. The obtained sequences were compared with reference sequences in the NCBI GenBank database using the BLAST algorithm to determine species-level identity.

2.4. PCR detection of vhvp-2 gene

Genomic DNA was extracted from pure bacterial cultures using a commercial bacterial DNA extraction kit (NEB, UK). PCR amplification targeted the *vhvp-2* gene using specific primers: forward 5′-GGAGTATTGGTGGGCTGAAA-3′ and reverse 5′-GGTAGGCATGGACCGTAAAG-3′. The PCR protocol consisted of an initial denaturation at 94°C for 3 minutes; 35 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 1 minute; followed by a final extension at 72°C for 5 minutes. PCR products were separated on a 1.5% agarose gel and visualized under blue-light LED transilluminator.

2.5. Antibiotic susceptibility testing

Isolates were tested against a panel of antibiotics commonly used in aquaculture, including ampicillin, tetracycline, streptomycin, chloramphenicol, and ciprofloxacin, using the disk diffusion method following CLSI guidelines. Results were interpreted as sensitive, intermediate, or resistant based on the diameter of the inhibition zones.

2.6. Salt tolerance and enzyme activity

Salt tolerance was assessed by culturing isolates in TSB containing different NaCl concentrations (0%, 3%, 6%, 8%, and 10%). Growth was measured by OD_{600} after 24 hours of incubation. Extracellular enzyme activities, including gelatinase and caseinase, were evaluated using plate assays with gelatin or skim milk agar. The presence of clear zones around colonies indicated positive enzyme production.

3. Results

3.1. Isolation and identification of *V. parahaemolyticus*

3.2. From shrimp samples collected at five different farms, five distinct bacterial isolates exhibiting characteristic purple colonies on ChromAgar were successfully obtained. These isolates tested positive for oxidase and showed halophilic properties, aligning with the typical biochemical traits of *V. parahaemolyticus*. Subsequent 16S rRNA gene sequencing confirmed the identity of all five isolates, with each sequence sharing more than 99% similarity to reference *V. parahaemolyticus* strains listed in the NCBI GenBank database.

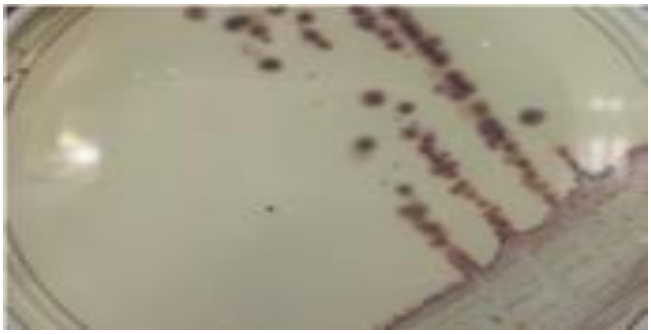

Figure 2. Morphology of *V. parahaemolyticus* colonies on ChromAgar Vibrio. Typical mauve to purple colonies were observed after 24 hours of incubation at 30°C.

3.3. Antibiotic Susceptibility Profiles

All five isolates exhibited resistance to at least two antibiotics, indicating emerging antimicrobial tolerance patterns.. High resistance rates were observed for ampicillin (5/5) and tetracycline (4/5), while lower resistance frequencies were recorded for streptomycin (2/5) and ciprofloxacin (1/5). All isolates remained sensitive to chloramphenicol (5/5). There was no clear distinction in resistance patterns between *vhvp-2*-positive and *vhvp-2*-negative strains.

Table 1. Antibiotic susceptibility profiles of *V. parahaemolyticus* isolates recovered from shrimp samples. R: Resistant, S: Sensitive.

Strain code	Ampicillin	Tetracycline	Streptomycin	Chloramphenicol	Ciprofloxacin
VP1	R	R	R	S	S
VP2	R	R	S	S	S
VP3	R	S	S	S	R
VP4	R	R	R	S	S
VP5	R	R	S	S	S

3.4. Salt Tolerance

All five *V. parahaemolyticus* isolates exhibited clear halophilic characteristics, as evidenced by their growth responses across varying NaCl concentrations. Optimal growth was recorded in TSB medium containing 3% NaCl, where all isolates showed high turbidity and rapid proliferation. Moderate growth was sustained at 6% NaCl, though a noticeable reduction in cell density was observed. At 8% NaCl, bacterial growth was significantly impaired, and only weak turbidity was detected. When exposed to 10% NaCl, growth was minimal and often delayed, suggesting that this concentration approaches the upper limit of salt tolerance for these strains. No visible growth was observed in NaCl-free medium, confirming that all isolates are obligate halophiles and require a saline environment for survival. These observations are consistent with the ecological niche of *V. parahaemolyticus* as a marine bacterium and support its adaptation to saline conditions typically found in shrimp aquaculture systems.

3.5. Extracellular Enzyme Activity

Gelatinase activity was observed in three out of five isolates, as indicated by the presence of clear zones surrounding the colonies on gelatin agar plates. Two isolates exhibited the ability to produce both caseinase and chitinase. Notably, both *vhvp-2*-positive strains tested positive for these enzymatic activities, suggesting a potential role for these enzymes in virulence, although further functional validation is required. All isolates were positive for oxidase and catalase, consistent with the general biochemical characteristics of *V. parahaemolyticus*. These findings reflect the enzymatic potential of the isolates and may contribute to their ability to degrade host tissues during infection.

Table 2. Extracellular enzyme activities of *V. parahaemolyticus* isolates. Positive (+) indicates clear hydrolysis zones; negative (–) indicates absence of enzyme activity.

Strain code	Gelatinase	Caseinase	Oxidase	Catalase	Chitinase
VP1	+	+	+	+	+
VP2	-	+	+	+	-
VP3	+	+	+	+	+
VP4	+	+	-	+	-
VP5	-	+	+	+	-

3.6. Detection of the *vhvp-2* Gene

PCR screening revealed that 1 out of the 5 isolates tested positive for the *vhvp-2* gene, producing a distinct amplicon of the expected size (~351 bp). The remaining four isolates were negative for this gene. Notably, *vhvp-2*-positive strains were recovered from farms reporting high levels of post-larval mortality, suggesting a possible association with TPD outbreaks.

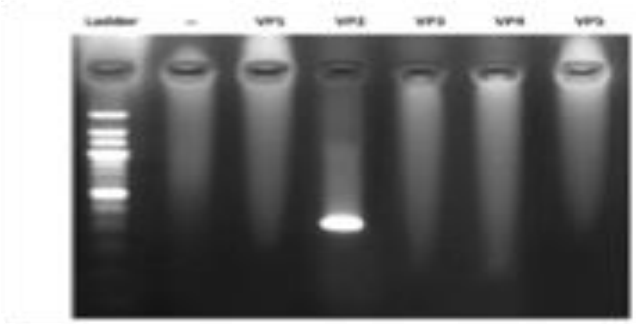

Figure 3. PCR detection of the *vhvp-2* gene in *V. parahaemolyticus* isolates. Ladder: 100 bp ladder; Lane 1: negative control. (–) Lane 2: VP1; Lane 3: VP2 (*vhvp-2* positive, ~351 bp); Lanes 4–6: VP3–VP5.

4. Discussion

V. parahaemolyticus is recognized as a key pathogen in shrimp aquaculture, with virulence typically associated

with factors such as extracellular enzymes, salt tolerance, and antibiotic resistance. However, in this study, VP2—the only strain carrying the *vhvp-2* gene—showed no remarkable differences in colony morphology, salt tolerance, or antibiotic susceptibility compared to *vhvp-2*-negative strains. All isolates grew optimally at 3% NaCl and displayed similar resistance patterns, particularly against ampicillin and tetracycline. In terms of enzyme production, VP2 was positive for caseinase but negative for gelatinase and chitinase, while several *vhvp-2*-negative strains demonstrated stronger gelatinase and chitinase activity. This suggests that the acquisition of *vhvp-2* does not impact core physiological or biochemical characteristics. Instead, *vhvp-2* likely acts as a key genetic determinant directly associated with TPD pathogenicity, independent of common phenotypic traits.

This observation supports the hypothesis that TPD-causing strains may originate from environmental *V. parahaemolyticus* populations through horizontal gene transfer of *vhvp-2*. Consequently, routine monitoring based solely on phenotypic characteristics may be insufficient for early detection of TPD-associated strains. Molecular surveillance targeting *vhvp-2* is essential for accurate identification and risk assessment. These findings highlight the importance of incorporating molecular diagnostics into disease management strategies in shrimp aquaculture.

Acknowledgments

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Phân lập và đánh giá độc lực của *Vibrio parahaemolyticus* gây bệnh mờ đục ấu trùng (TPD) trên tôm

[1,2]Trần Hồng Diễm,[1,2] Ngô Nguyên Vũ, [1,2]Phùng Thị Thu Hường
[1]Viện Kỹ thuật Công nghệ cao NTT, Trường Đại học Nguyễn Tất Thành, Thành phố Hồ Chí Minh
[1]Trung tâm Phát triển Công nghệ cao Đại học Nguyễn tất Thành, Khu Công nghệ cao Thành phố Hồ Chí Minh
nnvu@ntt.edu.vn, thdiem@ntt.edu.vn, ptthuong@ntt.edu.vn

Tóm tắt Bệnh ấu trùng trong suốt (TPD) là một thách thức mới nổi trong nuôi tôm, chủ yếu gây ra bởi một chủng *Vibrio parahaemolyticus* có độc lực cao. Nghiên cứu này nhằm phân lập và nhận diện các chủng *V. parahaemolyticus* từ môi trường nuôi tôm, đồng thời đánh giá khả năng gây bệnh của chúng. Mẫu được thu thập từ nhiều trại nuôi tôm tại khu vực Đồng bằng sông Cửu Long, Việt Nam. Tổng cộng, 5 chủng *V. parahaemolyticus* đã được phân lập và sàng lọc gen *vhvp-2* – gen mã hóa cho Vibrio High Virulent Protein-2, một yếu tố độc lực chủ chốt liên quan đến bệnh TPD. Các chủng này cũng được kiểm tra về khả năng kháng kháng sinh, chịu mặn và hoạt tính enzyme ngoại bào. Kết quả cho thấy sự phổ biến cao của các chủng *V. parahaemolyticus* kháng kháng sinh, trong đó có một chủng mang gen *vhvp-2*. Những phát hiện này cung cấp hiểu biết quan trọng về sự phân bố của các chủng gây TPD tại các trại nuôi tôm ở Việt Nam, đồng thời nhấn mạnh nhu cầu cấp thiết về các chiến lược quản lý dịch bệnh hiệu quả hơn trong ngành nuôi tôm.

Từ khóa TPD, *vhvp-2*, *V. parahaemolyticus*, tôm