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DETECTION OF *Vibrio parahaemolyticus* AND *Aeromonas veronii* CAUSING DISEASE IN SHRIMP (*Litopenaeus vannamei*) BY MULTIPLEX PCR

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Abstract

This study presents the development of a multiplex PCR assay for the simultaneous detection of two major shrimp pathogens. In Vietnam, the co-culture of shrimp and fish in the same pond increases the risk of shrimp infection due to cross-contamination from fish waste, feed, and environmental sources, contributing to disease outbreaks and shrimp mortality rates exceeding 80%. This study aimed to detect Vibrio parahaemolyticus and Aeromonas veronii, two major etiological agents associated with Acute Hepatopancreatic Necrosis Disease (AHPND)/Early Mortality Syndrome (EMS) in Litopenaeus vannamei by using a multiplex polymerase chain reaction (PCR) technique. Two gene fragments, GSTT2 and pepO, were selected as species-specific molecular markers for V. parahaemolyticus and A. veronii, respectively. Multiplex PCR successfully amplified both target genes with clear and specific bands (236 bp for GSTT2, 520 bp for pepO) without cross-reactivity with other bacterial species. These results confirm the reliability and specificity of the method, offering a rapid and accurate diagnostic tool for bacterial diseases in shrimp farming, thereby contributing to improved disease management and sustainable aquaculture development in Vietnam.

Keywords: *Aeromonas veronii, Vibrio parahaemolyticus,* Multiplex PCR, *Litopenaeus vannamei,* and AHPND/EMS.

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PHÁT HIỆN VI KHUẨN *Vibrio parahaemolyticus* VÀ *Aeromonas veronii* GÂY BỆNH TRÊN TÔM THẺ CHÂN TRẮNG (*Litopenaeus vannamei*) BẰNG PHƯƠNG PHÁP MULTIPLEX PCR

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Tóm tắt

Nghiên cứu này trình bày việc phát triển một kỹ thuật PCR đa mồi nhằm phát hiện đồng thời hai tác nhân vi khuẩn gây bệnh chính trên tôm thẻ chân trắng (Litopenaeus vannamei). Tại Việt Nam, mô hình nuôi ghép tôm và cá trong cùng ao nuôi làm gia tăng nguy cơ lây nhiễm chéo qua chất thải, thức ăn dư thừa và các yếu tố môi trường, dẫn đến các đợt bùng phát dịch bệnh với tỷ lệ tử vong vượt quá 80%. Nghiên cứu được thiết kế nhằm phát hiện Vibrio parahaemolyticus và Aeromonas veronii, hai tác nhân liên quan mật thiết đến Hội chứng hoại tử gan tụy cấp tính (AHPND)/Hội chứng chết sớm (EMS), đồng thời cũng là hai loài vi khuẩn được phát hiện phổ biến nhất trong các ao nuôi ghép. Hai đoạn gen đặc hiệu loài được sử dụng trong bài là GSTT2 (đặc hiệu cho V. parahaemolyticus) và pepO (đặc hiệu cho A. veronii). Phản ứng PCR đa mồi khuếch đại thành công cả hai gen mục tiêu với các dải DNA rõ ràng, đặc hiệu (236 bp cho GSTT2 và 520 bp cho pepO), đồng thời không ghi nhận phản ứng chéo với các loài vi khuẩn khác. Những kết quả này cho thấy độ đặc hiệu và độ tin cậy cao của phương pháp, cung cấp một công cụ chẩn đoán nhanh chóng và chính xác các bệnh do vi khuẩn trong nuôi tôm, từ đó hỗ trợ nâng cao hiệu quả quản lý dịch bệnh và hướng đến phát triển bền vững ngành thủy sản tại Việt Nam.

Từ khóa: *Aeromonas veronii, Vibrio parahaemolyticus,* Multiplex PCR, *Litopenaeus vannamei,* and AHPND/EMS.

1. Introduction

Whiteleg shrimp (*L. vannamei*) is one of the most widely farmed aquaculture species, with production spanning Asia, the Americas, the Caribbean, and the Pacific Islands. Its global economic importance has driven the rapid expansion of shrimp farming, particularly in countries such as Vietnam, China, Ecuador, and India (Effendi & Elizal, 2020). However, the intensification of farming practices has been accompanied by increasing disease outbreaks, most notably Acute Hepatopancreatic Necrosis Disease (AHPND), also known as Early Mortality Syndrome (EMS), which can cause shrimp mortality rates exceeding 80%.

Two major bacterial pathogens associated with AHPND/EMS are *V. parahaemolyticus* and *A. veronii. V. parahaemolyticus* is a Gram-negative, rod-shaped, motile bacterium that produces several hemolysins, including thermostable direct hemolysin (TDH) and TDH – related hemolysin (TRH), which are key virulence factors. Infections can occur through the consumption of contaminated seafood or exposure to seawater, and severe cases may lead to systemic infections (Li et al., 2019,Khanh et al., 2019,Hoa et al., 2023). *A. veronii*, a Gram-negative facultative anaerobe, secretes multiple toxins such as Act, Alt, Ast, and hemolysins like AerA and Asa1. These toxins damage intestinal epithelial cells by disrupting membrane integrity and suppressing essential RNAs, leading to significant impairment of cellular function (Koutsoumanis et al., 2014; Samanta & Bandyopadhyay, 2020, Lee et al., 2023).

Despite the severe impact of these pathogens, existing research has yet to address co-infections or the simultaneous detection of multiple pathogens, a condition frequently observed in co-culture systems. Current detection methods rely solely on separate assays for each bacterium, significantly increasing diagnostic time, cost, and complexity. Therefore, a molecular approach such as the multiplex Polymerase Chain Reaction (PCR) proposed in this study represents a high-value solution. This technique facilitates the rapid, specific, and simultaneous identification of multiple pathogens in a single reaction, thereby improving diagnostic efficiency and optimizing resource utilization, although the *pepO* gene was selected instead of other virulence genes such as 16s rRNA or *toxR*, the *pepO* gene is present in most *A. veronii* strains, the primer designed on this gene has no conflict with the *gstt2* gene, consistent with the study's aim of detecting the presence of bacteria. This study contributes significantly to improving disease management and sustainable shrimp farming.

2. Materials and Methods

2.1. Searching sequences and designing primer pairs

To identify novel gene segments for the characterization of *V. parahaemolyticus* and *A. veronii*, genomic data were retrieved from the National Center for Biotechnology Information (NCBI) database, specifically the complete genomes of *V. parahaemolyticus* ATCC 17802 and *A. veronii* ANYA 19083. Sequence homology analyses were conducted using the Nucleotide BLAST tool to assess gene specificity. Genes were selected based on high identity and query coverage percentages when aligned to sequences from the same species, and low similarity metrics when compared to sequences from other bacterial species. For each selected gene, details including genomic position, accession number, and predicted encoded protein were systematically recorded for downstream analyses.

2.2. Primer set design

Table 1. presents the target gene sequences used for primer design, specifically *gstt2* and *pepO*. These sequences were submitted to Primer3Plus (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) to generate specific

primer pairs for multiplex PCR. The specificity of each primer set was verified using the Primer – BLAST tool to ensure accurate amplification of the target regions.

Table 1. Target sequences for amplification

Sequence	Genbank ID	Product
gstt2	CP014046.2	Glutathione S – transferase
pepO	CP121822.1	Metallopeptidase

2.3. Culturing and enrichment of *V. parahaemolyticus*, *A. veronii* and control bacteria

V. parahaemolyticus, *A. veronii*, and the control bacterial strains (*P. shigelloides*, *V. vulnificus*, and *S. agalactiae*) were cultured to obtain sufficient biomass for genomic DNA extraction and purification, following the procedures described above.

For *V. parahaemolyticus* and *A. veronii*, strains stored at – 80 °C were inoculated onto Tryptic Soy Agar (TSA; HiMedia Laboratories, India) supplemented with 2% NaCl and incubated at 37 °C for 18 hours. For primary enrichment, a single colony was transferred into 5 mL of sterilized Tryptic Soy Broth (TSB; HiMedia Laboratories, India) supplemented with 2% NaCl in a 15 mL Falcon tube and incubated at 37 °C with shaking at 180 rpm for 18 hours. After 24 hours, 1 mL of the culture was transferred into a flask containing 100 mL of sterilized TSB supplemented with 2% NaCl for secondary enrichment (Cheng, 2016; Pham et al., 2020).

Before co-culturing *V. parahaemolyticus* and *A. veronii*, the bacterial density of each strain is first determined individually by measuring optical density at 600 nm (OD600) using a UV-Vis spectrophotometer (Thermo Scientific Evolution 201, USA). According to standard correlations, an OD_{600} of 1 corresponds to approximately 10^8 CFU/mL for *V. parahaemolyticus* and 10^9 CFU/mL for *A. veronii* (Yen et al., 2019; Thi et al., 2023). To equalize the bacterial densities to 10^8 CFU/mL for both strains, the appropriate volume ratio of each bacterial suspension from the secondary culture is calculated. The adjusted volumes are then mixed and transferred into an erlenmeyer flask containing 50 mL of TSB medium for primary enrichment. The culture is incubated at 37°C with shaking at 180 rpm for 24 hours. After primary enrichment, 1 mL of the culture is transferred into another erlenmeyer flask containing 100 mL of TSB medium for secondary enrichment.

2.4. Purification of bacterial genome from the culturing medium

Genomic DNA from *V. parahaemolyticus*, *A. veronii*, and the control bacterial strains (*P. shigelloides*, *V. vulnificus*, and *S. agalactiae*) were extracted and purified using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Briefly, 1 mL of the gently shaken secondary culture was transferred into labeled 1.5 mL eppendorf tubes and centrifuged at 13,000 rpm for 5 min. The supernatant was discarded, and the pellet was resuspended in 200 µL of Lysis Buffer ATL. Subsequently, 25 µL of Proteinase K and 200 µL of Lysis Buffer AL were added, and the mixture was incubated at 56 °C for 10 min and inverted every 2 minutes. Next, add 210 µL of ethanol, and the mixture was thoroughly mixed. The entire solution was transferred to a silica spin column placed in a 1.5 mL Eppendorf tube and centrifuged at 13,000 rpm at 4 °C for 1 min; the flow-through was discarded. The column was then washed with 500 µL of Wash Buffer 1 AW1, followed by centrifugation under the same conditions, and the washing step was repeated using Wash Buffer 2 AW2. The column was dried by centrifugation without any

added solution. Finally, 50 µL of Elution Buffer AE was applied, incubated at room temperature for 1 min, and centrifuged at 13,000 rpm at 4 °C for 1 min. The purified genomic DNA was stored at – 20 °C until further use.

2.5. *in vitro* Singleplex PCR

DNA templates isolated from the genomes of *V. parahaemolyticus* and *A. veronii* were used in this procedure. Additionally, two custom-designed primer pairs targeting the *gstt2* and *pepO* genes were employed to amplify species-specific genetic regions. This PCR reaction was performed using an exogenous internal control to monitor the amplification process and simultaneously assess the specificity of the primer pairs listed in Table 2. The optimized thermal cycling conditions detailed in Table 3 were uniformly applied to all subsequent PCR experiments in this study.

Table 2. Primer pairs characteristics

Primers	Sequence 5' – 3'	Product length	Tm (°C)	References
VP – GSTT2 – F	TGGGTAAGTTGGTCGAAGG	236 bp	58	This study
VP – GSTT2 – R	CGTCACATCAATGTGTGGC		58	
AV – pepO – F	CATCTGCTACGCACTTATGC	520 bp	60	This study
AV – pepO – R	CGATCTCGTTGTTGTTCGG		58	
IC – F	ACATACTACAGGTCCAAGTG	102 bp	58	This study
IC – R	AGGGAGAACTGGTTCTTGG		58	
IC – LV – F	ATCCTGGTCTTGTCGGTAG	98 bp	58	This study
IC – LV – R	CATGTAGAATCCTCACTCTG		58	

Table 3. Thermo cycle for PCR reaction

Temperature °C	Time	Cycle
94	5 minutes	
94	30 seconds	30
53	30 seconds	
72	30 seconds	
72	7 minutes	1
4		storage

Singleplex PCR reactions were prepared using a primer mix consisting of 80 µL of ultrapure water, 10 µL of forward primer (100 µM), and 10 µL of reverse primer (100 µM). The final volume of the primer mix was 100 µL. For PCR reaction of *V. parahaemolyticus*, a 10 µL reaction mixture was prepared containing 3.05 µL of ultrapure water, 5 µL of MyTaq™ Mix (Meridian Bioscience, Germany), 1 µL of GSTT2 - primer mix, and 0.95 µL of extracted genomic DNA (at a concentration of 105 µg/mL). For PCR reaction of *A. veronii*, a 10 µL reaction mixture was prepared containing 3.55 µL of ultrapure water, 5 µL of MyTaq™ Mix (Meridian Bioscience, Germany), 1 µL of pepO - primer mix, and 0.45 µL of extracted genomic DNA (at a concentration of 222 µg/mL).

2.6. *in vitro* Multiplex PCR

For the multiplex PCR protocol, the primer mixture containing three primer pairs (GSTT2, pepO, and exogenous IC) was first prepared by using a primer mix consisting of 40 µL of ultrapure water, 10 µL of forward primer (100 µM) of each primer, and 10 µL of reverse primer (100 µM) of each primer. Next, for the multiplex PCR reaction, a 10 µL reaction mixture was prepared containing 2.2 µL of ultrapure water, 5 µL of MyTaq™ Mix (Meridian Bioscience, Germany), 1 µL of primer mix, 1 µL of DNA IC, and 0.8 µL of extracted genomic DNA (at a concentration of 229.5 µg/mL).

2.7. *in vitro* Multiplex PCR with control bacteria

Three mixtures of primer including GSTT2 with exogenous IC, pepO with exogenous IC, respectively, were prepared with 60 µL of ultrapure water with 10 µL of each forward and reverse primer listed in Table 2 (at a concentration of 100 µM). The final volume of the primer mix was 100 µL. The PCR samples were prepared into a 0.2 mL PCR tube with a DNA template of genome extract from control bacteria: *P. shigelloides*, *V. vulnificus*, and *S. agalactiae.*

2.8. Shrimp Cultivation and Sample Collection

Healthy and disease-free whiteleg shrimp (*L. vannamei*) with an average weight of 5.0–6.5 g was purchased from a hatchery in Binh Chanh District, Ho Chi Minh City. Shrimp were reared in 10–20 L plastic tanks filled with water up to 2/3 of the tank volume. The water temperature was maintained at 30 ± 2 °C with continuous aeration. Shrimp were fed pelleted feed twice a day at a rate of 10% of their body weight. The optimal salinity for shrimp rearing was maintained within the range of 28 – 34, and the pH was controlled between 7.7 and 8.5. Water quality parameters were monitored every two days to ensure suitable environmental conditions. Shrimp were fed pelleted feed twice daily at a rate of 10% of their body weight.

Shrimp were acclimatized for 3 days before being subjected to infection procedures according to the treatments outlined in Table 4. Each treatment consisted of 3 replicates with 4 shrimp per tank. After acclimation, shrimp were transferred to 10 L tanks and infected with 2 mL of bacterial suspension ($OD_{600} = 1$, equivalent to 10^8 CFU/mL for *V. parahaemolyticus*, 10^9 CFU/mL for *A. veronii*). The negative control group received 1 mL of sterile TSB medium, autoclaved at 121 °C, 1 atm for 15 minutes. Shrimp behavior was observed for 60 minutes and monitored continuously for 5 – 7 days before tissue sampling.

2.9. Purification and extraction of genomic DNA from infected shrimp tissues

Following an incubation period of 6 – 8 days, dead shrimp were collected from each treatment group, with leg muscle tissue selected as the primary sampling site. Genomic DNA from shrimp in 4 treatments was extracted and purified using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Table 4. Infectious treatment on *Litopenaeus vannamei*

No.	Treatments	Quantity (shrimp)	Experiment layout
1	TM1	4	2 mL NaCl 2%
2	TM2	4	2 mL TSB medium with 2% NaCl
3	TM3	4	2 mL *S. agalactiae* cell suspension
4	TM4	4	2 mL *A. veronii* and *V. parahaemolyticus* cell suspension

2.10. *in vivo* Multiplex PCR experiment

For the multiplex PCR *in vivo* protocol, the primer mixture containing three primer pairs (GSTT2, pepO, and endogenous IC) was first prepared by using a primer mix consisting of 40 µL of ultrapure water, 10 µL of forward primer (100 µM), and 10 µL of reverse primer (100 µM) of each primer.

Multiplex PCR *in vivo* reactions were performed using genomic DNA from TM1, TM2, TM3, and TM4 treatment groups. Each 10 µL reaction mixture consisted of 5.0 µL of MyTaq™ Mix (Meridian Bioscience, Germany), 1.0 µL of primer mix, 1.0 µL of DNA extracted from shrimp (endogenous IC), and variable volumes of genomic DNA depending on concentration: 1.9 µL for TM1 (52.5 µg/mL), 1.5 µL for TM2 (63.0 µg/mL), 0.8 µL for TM3 (124.5 µg/mL), and 1.0 µL for TM4 (91.5 µg/mL). Ultrapure water was added to adjust the total reaction volume to 10 µL.

2.11. Agarose gel electrophoresis reusults analysis

To prepare samples for agarose gel electrophoresis, 5 µL of each extracted genomic DNA sample was mixed with 1 µL of 6X GelRed Loading Buffer with Tricolor (ABT, Vietnam). For PCR products, the reaction mixture was prepared by combining 2 µL of PCR products, 3 µL of ultrapure water, and 1 µL of the same loading buffer. All samples were subsequently subjected to electrophoresis on a 1% agarose gel at 100 V for 15 – 20 minutes. A 1 kb DNA ladder was used as a molecular size marker for genomic DNA, while a 100 bp DNA ladder was used for PCR products. DNA bands were visualized under ultraviolet (UV) illumination.

3. Results

3.1. Identification of *V. parahaemolyticus*- and *A. veronii*-specific genes

The *gstt2* gene (Genbank ID: CP014046.2) encodes the enzyme Glutathione S-transferase (GST), which plays a key role in phase II of the cellular detoxification process. According to Valenzuela-Chavira et al. (2021), this gene produces a structurally unique GST enzyme that detoxifies xenobiotics and heavy metals via a novel mechanism. By bolstering bacterial resilience in toxic aquaculture environments, it enhances the pathogen's ability to infect shrimp, making VpGSTT2 a significant auxiliary virulence factor (Valenzuela-Chavira et al., 2021). The *pepO* gene (Genbank ID: CP121822.1) encodes a neprilysin-type metallopeptidase that is secreted extracellularly via the type II secretion system (T2SS). This enzyme functions as a membrane-disrupting and immune-evasive factor by degrading host defense proteins and cleaving antimicrobial peptides. Through these mechanisms, *pepO*

facilitates bacterial invasion and persistence within the host, contributing significantly to pathogenesis under aquaculture conditions (Bianchetti et al., 2002; Abolghait et al., 2010).

3.2. Designing primers

Two novel primer pairs were designed to target the genes encoding Glutathione S-transferase (GST) and neprilysin-type metallopeptidase, yielding expected amplicon sizes of 236 bp and 520 bp, respectively, with melting temperatures (Tm) ranging from 58 °C to 60 °C. The amplicon sizes were selected to ensure sufficient separation (≥100 bp) for clear visualization during electrophoresis. In addition, an internal control (IC) primer set was incorporated to validate the efficiency of DNA extraction and monitor PCR performance.

3.3. Culturing and proliferating of *V. parahaemolyticus* and *A. veronii*

After *A. veronii* was cultured on Tryptic Soy Agar (TSA) medium and incubated at 37 °C for 24 hours, colonies appeared cream-colored, round, and convex. Similarly, *V. parahaemolyticus* was streaked onto TSA and incubated under the same conditions, resulting in colonies that were opaque white, round, convex, with smooth surfaces and regular edges, measuring 1 – 2 mm in diameter.

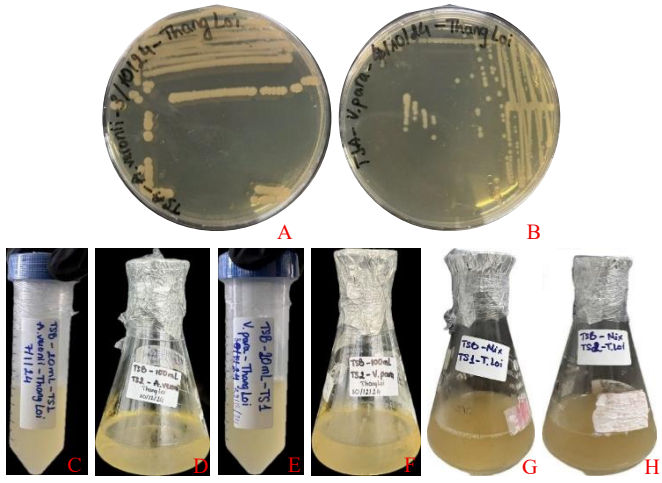

Figure 1. Culturing and proliferating of *V. parahaemolyticus* and *A. veronii*. A – *A. veronii* cultured on TSA medium; B – *V. parahaemolyticus* cultured on TSA medium; C, D – *A. veronii* was enriched in TSB broth medium; E, F – *V. parahaemolyticus* was enriched in TSB broth medium; G, H – The mixture of two bacteria was cultured and enriched in TSB broth

After primary enrichment, the bacterial densities of *A. veronii* and *V. parahaemolyticus* were measured using a spectrophotometer at 600 nm. *A. veronii* showed an OD_{600} of 0.970, corresponding to approximately 9.7×10^8 CFU/mL (OD = 1 ≈ 10^9 CFU/mL), while *V. parahaemolyticus* showed an OD_{600} of 1.072, equivalent to 1.072×10^8 CFU/mL (OD = 1 ≈ 10^8 CFU/mL). Based on the OD_{600} measurements, the inoculation volumes for the secondary enrichment step were calculated to ensure equivalent bacterial densities. Specifically, 970 µL of *A. veronii* and 1072 µL of *V. parahaemolyticus* were added to erlenmeyer flasks containing TSB medium supplemented with 2% NaCl. The secondary enrichment was carried out under the same conditions as the primary enrichment

3.4. Extracting and purifying of *V. parahaemolyticus, A. veronii* and mixture of 2 bacteria genome

As illustrated in **Figure 2**, clear and bright bands were seen in Lanes 1, 2, and 3, around 10 kb up the DNA ladder.

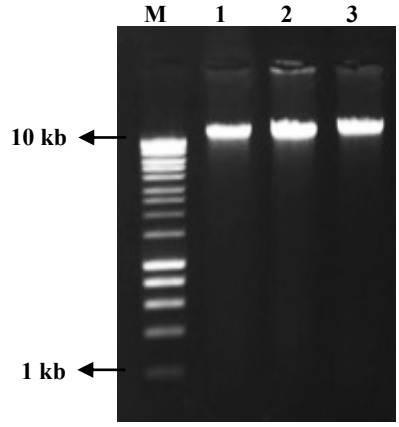

Figure 2. The electrophoresis result of *V. parahaemolyticus*, *A. veronii* and mixture of 2 bacteria genome. Lane M: DNA HyperLadder 1 kb; Lane 1: *A. veronii*; Lane 2: *V. parahaemolyticus*; Lane 3: Mixture of 2 bacteria genome

The intensity and integrity of the DNA band indicated successful genomic extraction, with a high yield of intact bacterial DNA. The observed band size was consistent with that of a typical bacterial genome, confirming the efficiency and reliability of the extraction protocol.

3.5. Results of Singleplex and Multiplex PCR *in vitro*

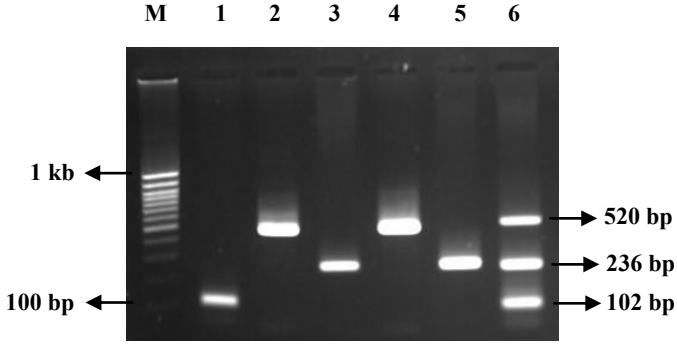

Figure 3. The electrophoresis result of Singleplex PCR and Multiplex PCR *in vitro*. Lane M: DNA HyperLadder 100 bp; Lane 1: Exogenous IC – 102 bp; Lane 2: *A. veronii* – pepO – 520 bp; Lane 3: *V. parahaemolyticus* – GSTT2 – 236 bp; Lane 4: Mixture of 2 bacteria – pepO – 520 bp; Lane 5: Mixture of 2 bacteria – GSTT2 – 236 bp; Lane 6: V. *parahaemolyticus – A. veronii* (mPCR)

An exogenous internal control (IC, 102 bp) appeared in all lanes (1–10), confirming the absence of nonspecific amplification. Notably, lane 8 displayed a distinct band at 236 bp (GSTT2), and lane 9 showed a band at 520 bp (pepO). These results validate that the primers specifically amplified the *GSTT2* and *pepO* genes, demonstrating high specificity for *V. parahaemolyticus* and *A. veronii*, respectively. This outstanding specificity serves as an ideal foundation for optimizing the PCR protocol into a rapid, reliable diagnostic kit that can be utilized directly at aquaculture facilities, thus enabling farmers to make timely and effective intervention decisions.

3.6. Multiplex PCR with control bacteria

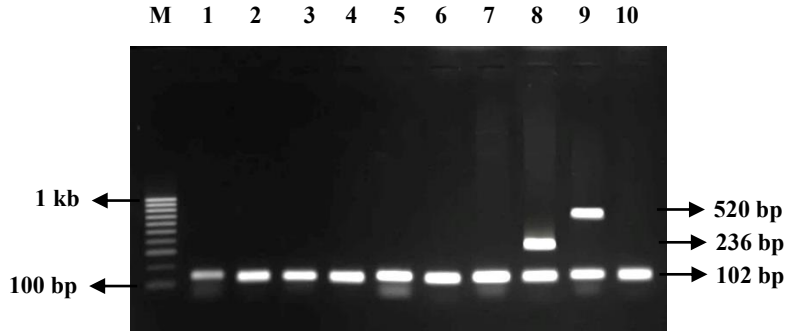

Figure 4. The electrophoresis result of Multiplex PCR with control bacteria. Lane M: DNA HyperLadder 100 bp; Lane 1: *P. shigelloides* (pepO – 520 bp); Lane 2: *P. shigelloides* (GSTT2 – 236 bp); Lane 3: *V. vulnificus* (pepO – 520 bp); Lane 4: *V. vulnificus* (GSTT2 – 236 bp); Lane 5: *S. agalactiae* (pepO – 520 bp); Lane 6: *S. agalactiae* (GSTT2 – 236 bp); Lane 7: *V. parahaemolyticus* (pepO – 520 bp); Lane 8: *V. parahaemolyticus* (GSTT2 – 236 bp); Lane 9: *A. veronii* (pepO – 520 bp); Lane 10: *A. veronii* (GSTT2 – 236 bp)

The exogenous internal control (IC) at 102 bp appeared in all lanes (1 – 10), confirming no non-specific amplification. Notably, lane 8 showed a clear band at 236 bp (GSTT2), and lane 9 showed a band at 520 bp (pepO). These results confirm that the primers specifically amplified the *GSTT2* and *pepO* genes, indicating high specificity for *V. parahaemolyticus* and *A. veronii*, respectively.

3.7. *L. vannamei* cultivation, infection and morphology observation

Clinical symptoms, including lethargy, anorexia, and abnormal swimming behavior, were observed within 1 – 3 days post-infection in Treatments 2, 3, and 4. *V. parahaemolyticus* was identified as the primary pathogen, while *A. veronii* acted as an opportunistic co-infectant. By day 7, complete mortality was recorded in Treatments 3 and 4.

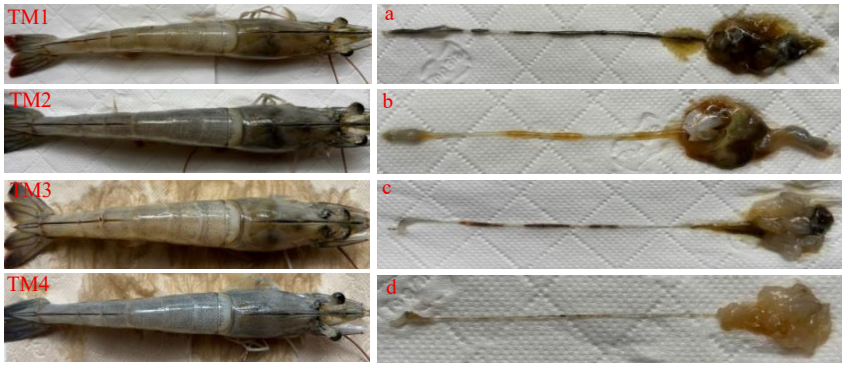

Figure 5. External morphological observations and shrimp hepatopancreas collected from each of the four infective treatments (TM). TM1/a: Uninfected whiteleg shrimp; TM2/b: Infected whiteleg shrimp with TSB medium; TM3/c: Shrimp infected with *S. agalactiae*; TM4/d: Shrimp infected with *V. parahaemolyticus* and *A. veronii*.

Shrimp in Treatment 1 (uninfected; TM1/a) and Treatment 2 (TSB-supplemented; TM2/b) exhibited no signs of health deterioration. The hepatopancreas and liver appeared normal, and the intestines were full, indicating good feeding response and overall health. In contrast, shrimp in Treatment 3 (infected with *S. agalactiae*; TM3/c) showed gradual

hepatopancreatic atrophy and uneven intestinal contents, reflecting a poor feeding response. Severe hepatopancreatic degeneration and empty intestines were evident in Treatment 4 (co – infected with *V. parahaemolyticus* and *A. veronii*; TM4/d), suggesting strong bacterial invasion and serious physiological decline.

3.8. Extraction and Purification of Genomic DNA from Infected Shrimp

Genomic DNA was successfully extracted from *L. vannamei* across all four treatment groups. Agarose gel electrophoresis revealed bright, high-molecular-weight DNA bands exceeding 10 kb in size, as compared to the 1 kb DNA ladder, indicating high integrity and absence of degradation.

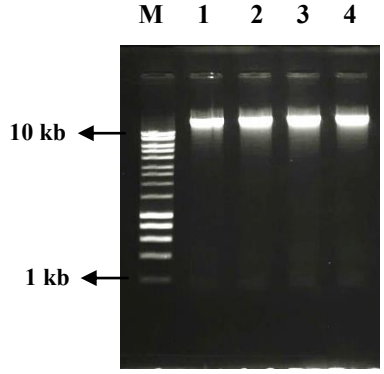

Figure 6. The electrophoresis result of genomic DNA extracted from shrimp samples across four treatments. Lane M: DNA HyperLadder 1 kb; Lane 1: Shrimp non infected with bacteria; Lane 2: Shrimp infected with TSB broth; Lane 3: Shrimp infected with *S. agalactiae*; Lane 4: Shrimp infected with *V. parahaemolyticus* and *A. veronii*.

3.9. Results of Multiplex PCR *in vivo*

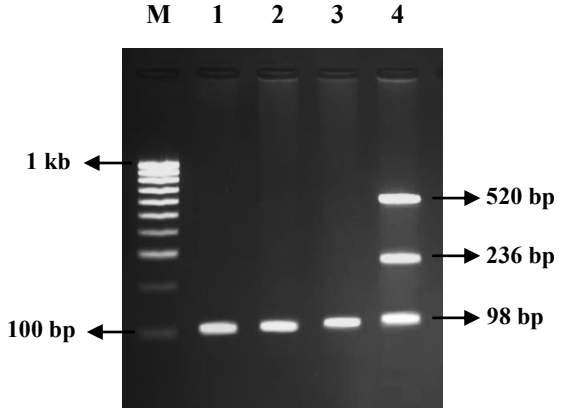

Figure 7. The electrophoresis result of multiplex PCR products from shrimp samples across four treatments. Lane M: DNA HyperLadder 100 bp; Lane 1: Shrimp non infected with bacteria; Lane 2: Shrimp infected with TSB broth; Lane 3: Shrimp infected with *S. agalactiae*; Lane 4: Shrimp infected with *V. parahaemolyticus* and *A. veronii*.

Endogenous internal control (IC, 98 bp) was detected in all lanes from lane 1 to lane 4, confirming the presence of shrimp genomic DNA. No non – specific amplification was observed in lanes 1 – 3, indicating that the IC primers were specific to *L. vannamei*, and that the bacterial primers (GSTT2 and pepO) did not cross – react with either shrimp DNA or *Streptococcus agalactiae* (lane 3). In lane 4, three distinct bands at 98 bp, 236 bp, and 520 bp

corresponded to the IC, GSTT2 (*V. parahaemolyticus*), and pepO (*A. veronii*), respectively. These results confirmed the high specificity and effectiveness of the multiplex PCR system. The successful amplification of *gstt2* and *pepO* genes from infected shrimp demonstrates the effectiveness of the assay in detecting target pathogens in actual biological samples.

4. Discussion

The multiplex PCR method developed in this study demonstrates clear practical advantages. Compared to singleplex PCR, this method allows for the simultaneous detection of two target genes in a single reaction. This approach not only has comparable analysis time (approximately 2 hours) but also leads to significant savings in reagents and the required sample volume. Although multiplex qPCR is also an option, that technique requires specialized equipment and expensive probes, making the procedure complex and costly. Given that the study's objective was focused on detecting two major bacterial pathogens in shrimp, this multiplex PCR assay represents a suitable and cost-effective choice. Its simplicity, speed and ability to be run on standard PCR machines make it a practical and accessible tool, effectively supporting pathogen surveillance directly at shrimp farms.

This study selected the *pepO* gene as a species-specific marker for *A. veronii* rather than classical virulence genes. BLAST analysis (accession number CP121822.1, locus 3,117,412 – 3,117,931) revealed 100% identity and more than 96% coverage among multiple *A. veronii* strains, indicating that this sequence is highly conserved within the species. Moreover, no significant similarity was observed with other bacterial species, further supporting the specificity of the *pepO* gene. This gene encodes a member of the neprilysin (M13) family of endopeptidases, a well characterized group of enzymes found across various organisms (Bland et al., 2008;Abolghait et al., 2010). The majority of M13 peptidases described so far are endopeptidases with a strong preference for cleaving the amino-terminal bond of hydrophobic residues (Turner et al., 2001). Therefore, selecting *pepO* as the detection target ensures high accuracy in identifying *A. veronii* strains present in samples, consistent with the purpose of the study which is to use multiplex PCR technique to detect *V. parahaemolyticus* and *A. veronii* bacteria in the samples.

4. Conclusion

The successful development of this Multiplex PCR system establishes a robust foundation for future applications in aquaculture health management. The assay demonstrated unwavering specificity, accurately detecting *V. parahaemolyticus* and *A. veronii* while showing no cross-reactivity with non-target bacteria such as *S. agalactiae, V. vulnificus*, and *P. shigelloides*. The operational parameters (including bacterial culture conditions and the Multiplex PCR thermal cycling profile) were carefully optimized to significantly minimize both the run-time and the operational costs, yet the practical results maintained high accuracy and reliability. The superior specificity and simultaneous detection capability of this multiplex PCR method make it an ideal candidate for immediate development into a field-based rapid diagnostic kit (particularly suited for the increasingly popular integrated farming systems), enabling timely disease surveillance and on-farm intervention decisions. Furthermore, the current multiplex PCR platform holds strong potential for expansion to incorporate additional primer sets targeting other common pathogens, such as WSSV, IMNV, and *Vibrio* spp. causing Acute Hepatopancreatic Necrosis Disease (AHPND), as well as those causing septicemia, encephalitis, or ulcerative diseases in both fish and shrimp, such as *Aeromonas* spp. and *Streptococcus* spp. This expansion will pave the way for the development of a more comprehensive and efficient disease surveillance system in aquaculture in general and in fish and shrimp farming in particular.

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