DOI: https://doi.org/10.62985/j.huit_ojs.vol25.noS2_ICA.216

A NEW NESTED PCR METHOD FOR EARLY DETECTION OF PATHOGENIC *Vibrio* spp. CAUSING ACUTE HEPATOPANCREATIC NECROSIS DISEASE IN SHRIMP

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Received: 2 May 2025; Revised: 19 May 2025; Accepted: 31 May 2025

ABSTRACT

Acute hepatopancreatic necrosis disease (AHPND) is a disease in shrimp caused by infection with toxic *Vibrio* strains, predominantly *Vibrio parahaemolyticus,* that harbor a plasmid containing the binary toxin genes *pir*A and *pir*B. This disease not only causes significant damage to the aquaculture industry but can also lead to antibiotic misuse, which poses environmental risks. Because the essential genes responsible for *Vibrio's* pathogenicity (*pir*A/B) are located on the plasmid, detection of AHPND based solely on the presence of *Vibrio parahaemolyticus* alone is not accurate, as it does not assess whether the bacteria carry a plasmid containing the *pir*A/B genes. Currently, the PCR method using the AP3 primer, the standard method for detecting AHPND-causing *Vibrio* in Vietnam (TCVN 8710-19:2019), is a single-step PCR method with limited sensitivity. Thus, a new high-sensitivity nested PCR method was developed in this study for early AHPND detection by targeting the toxin genes. The AP5 primer set was specifically designed for amplification of the *pir*A-*pir*B region in the pVPA3-1 plasmid (GB: KM067908.1), with product size of 1,970 bp for the 1st round and 433 bp for the 2nd round of amplification, respectively. The adjusted concentration of outer primers was 0.3 pmol/µL. The optimized annealing temperatures for the outer primers and inner primers were 69 ºC and 70 ºC, respectively. Optimized reactions can detect as few as 10^3 plasmid copies per reaction. The sensitivity of this method was 100 times higher compared to PCR with AP3 primers and 1,000 times higher compared to AP4 method. Such a significant improvement in sensitivity opens up the possibility of developing these optimized reactions into a commercial kit that would enable the direct detection of AHPND using samples with low bacterial loads, such as larval shrimp and farming pond water.

Keywords: AHPND, nested PCR, *pir*A, *pir*B, plasmid, *Vibrio.*

1. INTRODUCTION

Acute Hepatopancreatic Necrosis Disease (AHPND) is a devastating disease in shrimp caused by bacterial strains of *Vibrio,* most commonly *Vibrio parahaemolyticus*, that carry toxic genes [1]. The disease was first discovered in China in 2009 and subsequently detected in other countries: Vietnam and Malaysia (2011), Thailand (2012), Mexico (2013), the Philippines (2015), and South America (2016)—coinciding with the start of intensive shrimp farming. Shrimps infected with AHPND often lose their appetite, crowd at the pond edges, and develop a pale hepatopancreas. Histopathological examination reveals cell detachment in the hepatopancreas, gastrointestinal tract, especially concentrating in the major blood vessels

that supply nutrients to these organs. The cellular detachment in the hepatopancreas and gut leads to the formation of acute infectious areas, progressing to severe stages in the final phase, ultimately causing shrimp death due to an inability to digest food [2]. This disease causes significant losses in the shrimp industry, leading to a sharp decline in global and regional shrimp production. The economic damage is estimated at around 7 billion USD annually [3]. In Vietnam, the affected area accounts for 2% to 57.2% of the total shrimp farming area [4]. Besides economic losses, the disease also has negative environmental consequences due to the large quantities of antibiotics and drugs used in pond water. Detecting the causative agent is essential for selective breeding, pond treatment, and preventing disease spread.

Vibrio parahaemolyticus bacteria are common in water environments; however, not all strains cause disease. Only those carrying plasmids with toxic genes can cause AHPND in shrimp. Moreover, some other species within the *Vibrio* genus, when harboring toxic plasmids, can also cause this disease [1, 5, 6]. Therefore, culture-based methods on selective media merely detect the presence of *Vibrio parahaemolyticus* populations but do not precisely identify the pathogenic strains. Currently, most methods for detecting AHPND are molecular biology techniques based on the presence of pathogenic gene sequences. Several PCR-based methods have been developed and used. The AP1 and AP2 PCR methods were the first designed to detect *Vibrio parahaemolyticus*, but because the primers AP1 and AP2 target regions at the plasmid boundary, their results are not entirely accurate [7]. The PCR method with primers for AP3 [8] has high specificity; however, this pair amplifies only the *pir*A region and cannot distinguish strains with mutations or deletions in the *pir*B gene that cause loss of toxicity. The nested PCR method with primers AP4 [9] enables detection of *pir*A and *pir*B genes with high sensitivity but produces many nonspecific products. Realtime PCR offers high sensitivity and specificity but requires expensive equipment and complex protocols. This study develops a new nested PCR assay (AP5), which amplifies both toxic genes *pir*A and *pir*B, offering high sensitivity and conditions suitable for most laboratories in Vietnam.

2. MATERIALS AND METHODS

2.1. Materials

Chemicals used for plasmid DNA extraction and the nested PCR reaction include 2X My Taq Mix (Bioline, UK), 6X GelRed Buffer (ABT, Vietnam), 100 bp ladder (Bioline, UK), 1 kb ladder (Bioline, UK), deionized water (ABT, Vietnam) and other basic common chenicals in molecular biology. The *Vibrio parahaemolyticus* strains carrying plasmids with toxic genes were supplied by the Aquatic Biotechnology Department, Ho Chi Minh City Center for Biotechnology.

2.2. Methods

2.2.1. Primer design

Primers were designed using Primer-BLAST software from NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast) based on the plasmid pVPA3-1 sequence (GenBank accession number KM067908.1). The pVPA3-1 plasmid, measuring 69,168 bp, has been proven to carry the toxic genes responsible for AHPND in shrimp [5]. The outer primers were designed within the region spanning nucleotides 15,046 to 20,580, covering *pir*A, *pir*B, and a portion of the transposon, with annealing temperatures ranging from 55 °C to 65 °C—optimized at 60 °C—and product sizes between 500 and 2,000 bp. The inner primers targeted nucleotides 16,834 to 18,466, with annealing temperatures from 58 °C to 65 °C—optimized at 61 °C—and product sizes between 200 and 1,000 bp. Both primer pairs were validated for

specificity against pathogenic plasmid sequences of *Vibrio* in GenBank using BLASTn. Their potential for primer dimer formation was assessed using IDT OligoAnalyzer (https://www.idtdna.com).

2.2.2. Optimizing PCR reactions

The first-round PCR reaction has a total volume of 12 µL, consisting of 1 µL of forward primer (AF5-F1) and reverse primer (AF5-R1) (final concentrations tested from 0.05 to 0.5 pmol/µL), 1 µL of plasmid DNA (at a concentration of 10^6 copies/µL), 6 µL of 2X MyTaq Mix (Bioline), and 4 µL of nuclease-free deionized water. Thermal cycle for the first-round PCR starts with an initial denaturation step at 94 °C for 2 minutes, followed by 25 cycles of denaturation at 94 °C for 30 seconds, annealing for 30 seconds (with temperature tested from 50 °C to 70 °C), and extension at 72 °C for 90 seconds.

The second-round reaction has a total volume of 12 µL, comprising 1 µL of forward primer (AF5-F2) and reverse primer (AF5-R2) (at the final concentration of 0.417 pmol/µL), 1 µL of the first PCR product as template, 6 µL of 2X MyTaq Mix (Bioline), and 4 µL of nuclease-free deionized water. Thermal cycling conditions for the nested PCR include an initial denaturation at 94 °C for 2 minutes, followed by 20 cycles of denaturation at 94 °C for 20 seconds, annealing step for 20 seconds (with temperature tested from 50 °C to 70 °C), and extension at 72 °C for 20 seconds.

2.2.3. Evaluation of nested PCR sensitivity on plasmid samples

Plasmid DNA was extracted from toxic *Vibrio parahaemolyticus* strain using alkaline lysis method [10]. DNA concentration was determined by spectrophotometry at 260 nm using a UV-VIS SP3000-nano spectrophotometer (OPTIMA). The number of plasmid DNA copies was calculated from the concentration based on plasmid size. The sensitivity of the AP5 nested PCR method was evaluated over a range from 10^9 to 10^1 plasmid copies per reaction and compared with the sensitivity of the PCR method using primer AP3 [8] - the standard PCR method for *Vibrio parahaemolyticus* detection (according to TCVN 8710-19:2019) - and with the high-sensitivity nested PCR using primer AP4 [9].

The PCR with AP3 primers and nested PCR with AP4 primers were performed using previously designed primers and the optimal conditions published by the authors. Specifically, AP3 primer sequences include **AP3-F:** 5′-ATG AGT AAC AAT ATA AAA CAT GAA AC-3′ and **AP3-R:** 5′-GTG GTA ATA GAT TGT ACA GAA-3′. PCR conditions for the AP3 primer pair involved an initial denaturation at 94 °C for 5 minutes, followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 53 °C for 30 seconds, extension at 72 °C for 40 seconds, and finish with a final extension at 72 °C for 7 minutes [7]. The primer set for AP4 includes **AP4-F1:** 5′-ATG AGT AAC AAT ATA AAA CAT GAA AC-3′, **AP4-R1:** 5′-ACG ATT TCG ACG TTC CCC AA-3′, **AP4-F2:** 5′-TTG AGA ATA CGG GAC GTG GG-3′ and **AP4-R2:** 5′-GTT AGT CAT GTG AGC ACC TTC-3′ [8]. All sensitivity testing reactions contained 1 µL of primers (at final concentration of 5 pmol/µL), 1 µL of plasmid DNA, 6 µL of My Taq Mix 2X (Bioline), 4 µL of nuclease-free deionized water. All reactions were performed in triplicate.

3. RESULTS AND DISCUSSION

3.1. Primer design

The outer primer pair AP5-F1/R1 was designed to target the DNA region containing *pir*A and *pir*B from the pVPA3-1 plasmid (Figure 1), producing a product of 1,970 bp (Table 1).

The inner primer pair AP5-F2/R2 specifically amplifies the DNA segment containing *pir*A and *pir*B, yielding a product of 433 bp (Figure 1 and Table 1).

Table 1. Designed AP5 primer pairs for nested PCR detection of AHPND-related plasmid

Primer	Sequence (5' – 3')	Primer length (base)	%GC	Tm (°C)	Product length (bp)
AP5 - F1	CGGATGCCCAAGTCTTTCCT	20	55	60,04	1970
AP5 - R1	TATTGGAGGCGGTGATACGC	20	55	59,97	
AP5 - F2	AACAATGGCGCTAGTCGTGG	20	55	61,02	433
AP5 - R2	TTGCCGCAGCATAACCGAAG	20	55	61,64	

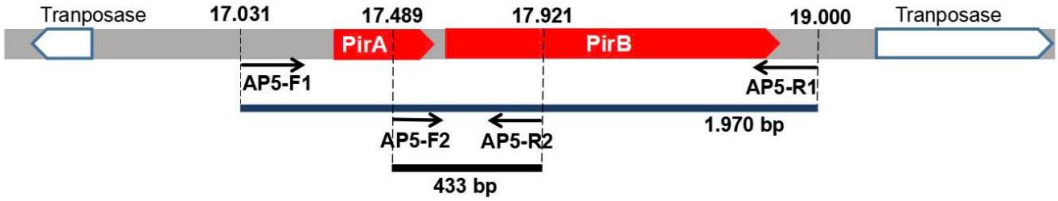

Figure 1. Diagram of the AP5 primer positions on the plasmid pVPA3-1

The primers were tested for specificity against pathogenic plasmids from *Vibrio parahaemolyticus* using NCBI BLAST (https://www.ncbi.nlm.nih.gov), yielding 100% similarity with all known *pir*A/B gene sequences deposited in the GenBank database. Primer dimer formation capacity was assessed using IDT (https://www.idtdna.com), indicating only a few nucleotides in the primers pairing with each other at very low energy levels. The first-round PCR produces a larger product (1970 bp) compared to the AP4 method (1269 bp) [9]. The amplified region covers the entire *pir*A and *pir*B gene sequences, allowing the exclusion of false positives from strains with deletions or mutations—such as deletions of *pir*A, *pir*B, or both— which result in loss of toxicity [5].

3.2. Optimized conditions for PCR

The annealing temperatures for both steps were tested across a range from 50 °C to 70 °C, approximately 10°C around the primers' theoretical melting temperatures. Both the outer and inner primers annealed effectively within this temperature range, producing clear, specific bands at the expected sizes, with no primer dimer formation observed, as evidenced by the absence of signals below 100 bp on the gel (Figure 2). However, regarding specificity, both the outer and nested PCR reactions exhibited nonspecific bands at annealing temperatures from 50 °C up to 67.1 °C. These nonspecific bands decreased as the primer annealing temperature increased, as shown in Figure 2. The results indicated that an annealing temperature of 69.4 °C for the outer primers and 69.9 °C for the inner primers produced sharp bands with minimal nonspecific amplification. Based on the observed trend that annealing temperatures affect nonspecific amplification and primer dimer formation, the annealing temperature was set at 69 °C for the outer primers and 70 °C for the inner primers. Higher primer temperatures help decrease mismatched pairing and nonspecific amplification [11].

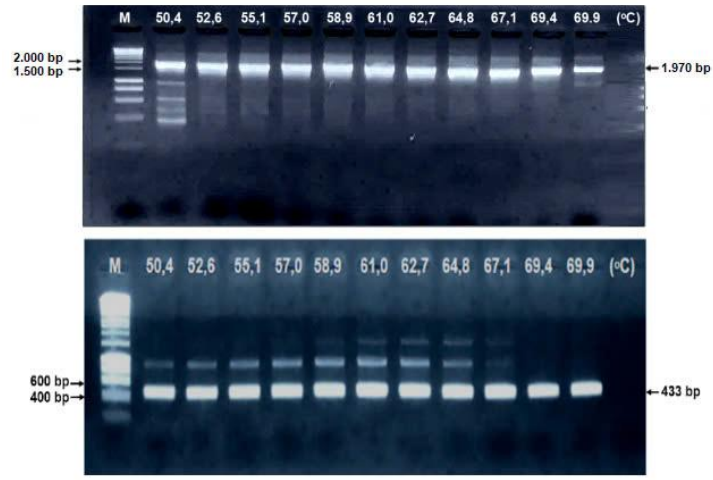

Figure 2. Results of primer annealing temperature analysis for the outer primer AP5F1/R1(A - M: 1 kb Ladder, product size 1970 bp) and the inner primer AP5F2/R2 (B - M: 100 bp Ladder, product size 433 bp)

Typically, primer concentrations in PCR mixtures are kept in excess to ensure efficiency during later cycles; however, in nested PCR, excessive outer primer amounts can lead to nonspecific bands and hinder amplification of the target region when combined with inner primers in the second round. Therefore, outer primer concentrations were optimized to achieve the best reaction efficiency while minimizing nonspecific amplification. The results showed that primer concentrations of 0.4 pmol/µL or higher produced additional nonspecific bands, while at 0.2 pmol/µL, the target band was faint. An outer primer concentration of 0.3 pmol/µL resulted in minimal nonspecific bands in nested PCR, with clear and strong amplification of the target sequence (Figure 3).

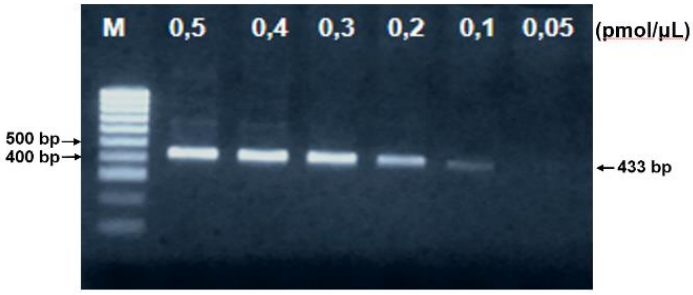

Figure 3. The effect of outer primer (AP5F1/R1) concentration on nested PCR products (M: 100 bp Ladder)

3.3. Sensitivity of AP5 nested PCR reaction

The plasmid DNA extracted from the pathogenic bacterial strain had a initial concentration of 330 ng/µL, corresponding to approximately 4.42×10^9 copies/µL for a plasmid size of 69,168 bp. This DNA solution was diluted to a concentration of 10^9 copies/µL, then serially diluted from 10^9 down to 10^1 copies/µL to serve as samples for sensitivity testing of the AP5 nested PCR. The sensitivity of the optimized AP5 method was subsequently compared with two previously established conventional PCR methods: AP3 [8] and AP4 [9].

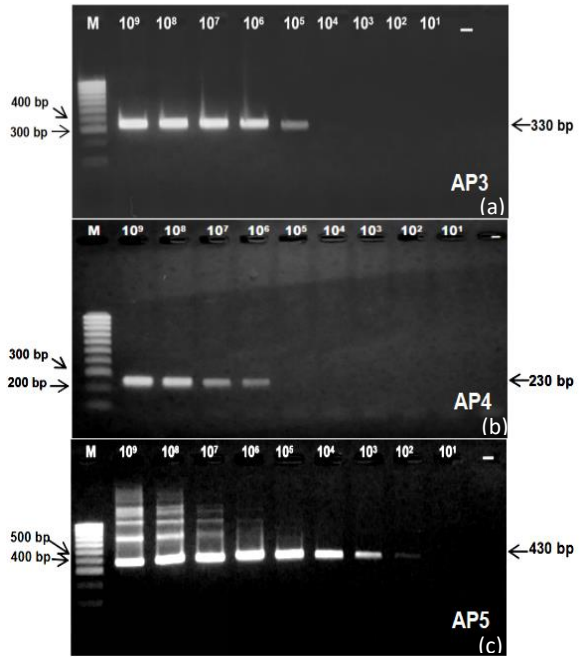

Figure 4. Comparison of the sensitivity of the PCR methods (AP3, AP4 and AP5) in detection of AHPND plasmid (M: 100 bp Ladder)

The PCR method using primer AP3 (the current standard PCR method for detecting *Vibrio parahaemolyticus*) detected a minimum of 10^5 copies of plasmid DNA (Figure 4-a). The AP4 nested PCR, reported by Sirintip et al. (2015), was claimed to have higher sensitivity—100 times greater than the AP3 PCR. However, in this parallel study, the AP4 method could only detect down to 10^6 copies (Figure 4-b). In contrast, the nested PCR method with AP5 primers could detect DNA plasmid at the lowest concentration of 10^3 copies (Figure 4-c). Thus, the AP5 nested PCR is approximately 1,000 times more sensitive than AP4 and 100 times more sensitive than AP3—the standard PCR for AHPND detection in Vietnam. As discussed by Ratchanok et al. (2015), the conventional AP3 PCR, with just one step, has limited sensitivity and therefore is mainly suitable for enriched *Vibrio* samples. In AP4 method, the melting temperatures of primers differ by a considerable margin [9]. This can lead to a reduction in primer annealing efficiency and thus lower reaction sensitivity. The AP5 primer set was designed with melting temperatures of the forward and reverse primers within 1 °C of each other [12], ensuring optimal primer annealing at the same temperature and improving the overall PCR sensitivity.

4. CONCLUSION

The study successfully developed a nested PCR method with primers specifically designed to target the *pir*A/B gene region. The optimal outer primer concentration was 0.3 pmol/µL. The ideal annealing temperatures were 69 °C for the first-round PCR and 70 °C for the second-round PCR. This method could detect as few as 10^3 plasmid DNA copies, making it over 100 times more sensitive than the AP3 primer-based PCR and 1,000 times more sensitive than the AP4 method. After further validation of its specificity and efficiency on field samples, this AP5 nested PCR method can be used for early detection of AHPND pathogens, identifying *pir*A/B genes in isolated *Vibrio* spp. bacterial samples as well as samples with low bacterial loads such as larval shrimp and farming pond water.

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TÓM TẮT

PHƯƠNG PHÁP NESTED PCR MỚI NHẰM PHÁT HIỆN VI KHUẨN *Vibrio* spp. GÂY BỆNH HOẠI TỬ GAN TỤY CẤP TRÊN TÔM

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Bệnh hoại tử gan tụy cấp (AHPND) trên tôm nuôi do một số vi khuẩn thuộc chi *Vibrio,* chủ yếu là *Vibrio paraheamolyticus*, mang plasmid có 2 gen độc *pir*A và *pir*B gây ra. Bệnh không chỉ gây thiệt hại lớn cho ngành nuôi trồng thủy sản mà còn có thể dẫn đến việc sử dụng kháng sinh tùy tiện, gây ảnh hưởng tới môi trường. Do các gen thiết yếu đối với khả năng gây bệnh của *Vibrio* (*pir*A/B) nằm trên plasmid, các phương pháp phát hiện bệnh thông qua sự có mặt của vi khuẩn *Vibrio paraheamolyticus* không hoàn toàn chính xác do không đánh giá được vi khuẩn có mang plasmid chứa gen *pir*A/B hay không. Hiện nay, phương pháp PCR dùng mồi AP3, phương pháp tiêu chuẩn để phát hiện *Vibrio* gây bệnh AHPND tại Việt Nam (TCVN 8710-19:2019) có độ nhạy hạn chế của PCR một bước. Nghiên cứu này xây dựng phương pháp nested PCR mới (AP5) có độ nhạy cao nhằm phát hiện sớm tác nhân gây bệnh thông qua sự có mặt của các gen gây độc. Bộ mồi nested PCR AP5 được thiết kế đặc hiệu để khuếch đại vùng *pir*A-*pir*B trên plasmid pVPA3-1 (GB: KM067908.1), cho sản phẩm vòng ngoài có kích thước 1.970 bp và sản phẩm vòng trong có kích thước 433 bp. Nồng độ mồi ngoài thích hợp cho phản ứng là 0,3 pmol/µL. Nhiệt độ lai mồi tối ưu cho phản ứng vòng ngoài là 69 ºC và cho phản ứng vòng trong là 70 ºC. Sau khi được tối ưu, phản ứng nested PCR phát hiện tối thiểu 10^3 bản sao plasmid/phản ứng, nhạy hơn 100 lần so với phương pháp dùng mồi AP3 và 1.000 lần so với phương pháp AP4. Phản ứng này có triển vọng phát triển thành bộ kit thương mại, cho phép phát hiện trực tiếp vi khuẩn gây bệnh AHPND trên các mẫu có tải lượng vi khuẩn thấp như tôm giống và nước ao nuôi.

Từ khóa: AHPND, nested PCR, *pir*A, *pir*B, plasmid, *Vibrio.*