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QUY TRÌNH DUPLEX PCR PHÁT HIỆN ĐỒNG THỜI *Vibrio parahaemolyticus* VÀ WHITE SPOT SYNDROME VIRUS (WSSV) GÂY BỆNH TRÊN TÔM BIỂN

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TÓM TẮT

Với việc tăng cường diện tích nuôi cùng với biến đổi khí hậu, ngành nuôi tôm hiện đang gặp nhiều trở ngại, đặc biệt là thiệt hại do mầm bệnh. Sự cần thiết phải phát hiện và sàng lọc các mầm bệnh là một trong những nhiệm vụ quan trọng nhất vì rất khó để kiểm soát khi nào dịch bùng phát. Nghiên cứu này được thực hiện nhằm tối ưu hóa quy trình duplex PCR (dPCR) phát hiện đồng thời vi-rút gây bệnh đốm trắng (WSSV) và *Vibrio parahaemolyticus*, hai mầm bệnh gây thiệt hại lớn về mặt kinh tế cho ngành nuôi tôm biển. Mục tiêu chính của nghiên cứu này là chuẩn hóa giảm nồng độ mồi, dNTPs và $MgCl_2$ nhằm phát triển quy trình có độ nhạy và độ đặc hiệu cao và khả năng phát hiện thời gian ngắn. Kết quả cho thấy: (i) Phương pháp PCR được tối ưu hóa phát hiện đồng thời vi-rút gây bệnh đốm trắng (WSSV) và *Vibrio parahaemolyticus* (Vp_{AHPND}); (ii) Cụ thể của PCR tối ưu hóa là 100 pg/µL đối với WSSV và 10 ng/µl đối với *V. parahaemolyticus*; (iii) Xác định tính đặc hiệu và ứng dụng của PCR duplex để phát hiện đồng thời vi-rút gây bệnh đốm trắng (WSSV) và *V. parahaemolyticus* (Vp_{AHPND}) trên tôm biển.

Từ khóa: *PCR duplex, tôm biển, Vibrio parahaemolyticus, vi-rút gây bệnh đốm trắng*

DUPLEX PCR PROTOCOL FOR THE DETECTION OF *Vibrio parahaemolyticus* (Vp_{AHPND}) AND WHITE SPOT SYNDROME VIRUS (WSSV) CAUSING DISEASES IN MARINE SHRIMP

ABSTRACT

The intensification of farming areas and climate change have made the shrimp farming industry suffer from various obstacles, especially the loss of productivity due to pathogens. White Spot Syndrome Virus (WSSV) and *Vibrio parahaemolyticus* (Vp_{AHPND}) were reported to cause huge economic losses to marine shrimp farmers . When causing diseases or outbreaks, they are extremely hard to be controlled. Therefore, detecting and screening out the pathogens are crucial. This study was conducted to optimize a duplex PCR (dPCR) protocol, which is able to detect WSSV and Vp_{AHPND} simultaneously. Benefits of the protocol are noticeable, such as resulting fast, and reducing the concentration of primer, dNTPs, and $MgCl_2$ while providing substantial sensitivity and specificity. The results showed that (i) The PCR protocol was successfully optimized to detect WSSV and Vp_{AHPND} simultaneously and its specificity was determined. (ii) For the DNA template of WSSV and *V. parahaemolyticus*, the protocol required 100 pg/µL and 10 ng/µl, respectively. (iii) The duplex PCR was successfully applied to detect WSSV and *V. parahaemolyticus* in marine shrimp samples.

Keywords: *duplex PCR, marine shrimp, Vibrio parahaemolyticus (Vp_{AHPND}), White Spot Syndrome Virus*

1. INTRODUCTION

White Spot Syndrome Virus (WSSV) and *V. parahaemolyticus* are fatal pathogens that cause mass mortality because they are able to spread widely and are difficult to be controlled. WSSV first appeared in Taiwan in 1992 (Chou *et al*., 1995), then spread to many countries around the world (Escobedo-Bonilla *et al.*, 2008). This disease inflicts a high mortality rate of up to 100% within 3-10 days (Lightner, 1996). The virus enters the stomach, gills, epidermis, and connective tissues of the hepatopancreas. Subsequently, WSSV attacks the lymph organs, the antenna glands, muscle tissue, hematopoietic tissue, heart, posterior intestine, and part of the middle intestine. The nervous system is only infected in the last stage (Marks *et al.*, 2005). Transmission of WSSV occurs vertically from infected bloodstocks to larvae, horizontally through water or infected carrier, and by cannibalism of infected organisms. WSSV can survive in the laboratory condition for 30 days at 30^{o}C, in ponds for 3-4 days, it can be inactivated at 50^{o}C for 120 minutes and grows best at 30^{o}C. It could also survive in soil and sediment for an extended period. Specifically, it could survive up to 180 days in sandy soil (Yeager and O'Brien,1979), and its DNA could persist in soil for up to 10 months (Natividad *et al*., 2008). WSSV can be chronically infected for a lifetime if the host is healthy and the number of pathogens is negligible (OIE, 2009).

V. parahaemolyticus is a short, rod-shaped and gram-negative bacterium living in a moist environment that utilizes inorganic compounds as nutrition. It was identified as the causative agent of acute hepatopancreatic necrosis disease (AHPND) when it contains the virulence gene called pVA1 (Tran *et al.*, 2013) in the plasmid, causing necrosis in cultured shrimp (Kongrueng *et al*., 2015). The plasmid pVA1 can be transmitted to the other species in the *Vibrio* family; therefore, it was recorded that some other *Vibrio* species infected shrimp showed clinical symptoms of AHPND (OIE, 2019). AHPND caused devastating loss to the farms in South East Asia, and infected to hepatopancreas of cultured shrimp, especially *Penaeus monodon* and *Penaeus vannamei*. AHPND has been found to cause up to a 100% mortality rate in shrimp on 20-30 days after stocking (NACA, 2012).

Polymerase chain reaction (PCR) is a method used to make millions to billions of copies of DNA. In recent years, it is widely used in aquaculture to check for seed quality and diagnose some diseases in fish and shrimp because the method is able to detect pathogens quickly and accurately. PCR assays to detect *Vp*$_{AHPND}$ in shrimp have been studied and applied with different primer sets such as AP3, AP4 primers (Tinwongger *et al*., 2014; Sirikharin *et al.*, 2015; Han *et al*., 2015), where the AP4 primer set is thought to be specific for *Vp*$_{AHPND}$ because of AP4 primer is believed to be 100 times more sensitive than those using AP3 primer (Sritunyalucksana *et al*., 2015). In addition, the PCR assay using AP4 primer set can be useful in detecting isolated *Vp*$_{AHPND}$ in samples where the target material is restricted. Besides, the duplex PCR method is based on the principle of PCR and employs different pairs of primers to amplify different target fragments on the same DNA molecule or on different DNA subdivisions (Chamberlain *et al*., 1988). The duplex PCR method has been widely applied because it is able to simultaneously detect more than one type of disease consisting of *Vp*$_{AHPND}$ and WSSV. The detection of four shrimp diseases by amplifying the WSSV, IHHN (infectious hypodermal and hematopoietic necrosis virus), AHPND and EHP (enterocytozoon hepatopenaei) by multiplex PCR (Koiwai *et al.*, 2018). Duplex PCR method approaches a detection limit of 2.3 copies/µl and 4.6 copies/µl of EHP and *Vp*$_{AHPND}$, respectively (Zhang *et al*., 2022).

The invention of Korean (No. KR101999070B1) provides a multiplex PCR primer set for simultaneous diagnosis of acute hepatopancreatic necrosis disease and white spot syndrome virus of shrimp breeds.

This research was conducted to optimize the chemical ingredients and cycling conditions of dPCR for the detection of WSSV and AHPND. The research aims to apply a specific and sensitive protocol for the detection of WSSV and AHPND from shrimp. The results indicated that the dPCR method shows superiority for detection in samples with low pathogen loads, which will facilitate monitoring of the source and transmission of WSSV and AHPND and will help control shrimp epidemic disease.

2. MATERIALS AND METHODS

2.1. Materials

WSSV and *V. parahaemolyticus* (Vp_{AHPND}) DNA were extracted from infected marine shrimp and stored at -20°C. Besides, other sources of DNA from *Vibrio harveyi*, tissues samples from affected shrimp previously confirmed to be infected with MBV, IHHNV from the collection of Aquatic Pathology Department, College of Aquaculture and Fisheries, Can Tho University were used in this study.

Shrimp samples (10 samples) were used for application of the optimized duplex PCR.

2.2. Methods

2.2.1. DNA extraction

(i) *Vibrio parahaemolyticus* DNA Extraction:

The bacteria were cultured in 5 mL Nutrition Broth (NB) containing NaCl 1.5% for 16 - 18 hours. The sample of 1.5 mL bacteria suspension was centrifuged at 5000 rpm for 5 minutes at 4°C. The pellet was washed with 500 µL NaCl solution 0.9% and then centrifuged at 5000 rpm for 5 minutes at 4°C. The supernatant was discharged. The remained pellet was added with 100 µL pure water. The sample was boiled at 100°C for 10 minutes, then centrifuged 12,000 rpm for 10 minutes. The supernatant was collected and stored for the PCR reaction.

(ii) WSSV DNA Extraction:

Shrimp samples (50 - 100µg) were added to the 1.5 mL eppendorf containing 500 µL lysis buffer (50 mM Tris, 1 mM EDTA, 500 mM NaCl, 1% SDS, 10 µg/mL proteinase K). The samples were boiled at 100°C for 10 minutes. Subsequently, They were centrifuged at 13,000 rpm for 10 minutes. The supernatant (100 µL) was transferred to a new eppendorf containing 200 µL ethanol 100%. After that, it was centrifuged at 13,000 rpm for 10 minutes. The pellet was dried at room temperature and then diluted in 500 µL TE buffer. Finally, the samples are stored at -20°C for further analysis.

2.2.2. Polymerase chain reaction

(i) PCR method to detect WSSV:

To detect WSSV, four primers P1, P2, P3, and P4 were employed (Kimura *et al*., 1996). Their sequences and size of DNA targets are shown in Table 1. The method was conducted under two steps. The total volume of step 1 was 25 µL, containing 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs mix, 20 pmol P1, 20 pmol P2, 1.5 U/µl Taq DNA Polymerase and 1 µl DNA extract; The total volume of step 2 was also 25 µL, containing 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs mix, 20 pmol P3, 20 pmol P4, 1.5 U/µL Taq DNA Polymerase, and 1 µL product from step 1 reaction. The thermal cycles of two steps were the same. Initial denaturation was at 94°C for 5 minutes. To amplify the DNA target, 30 cycles were run with denaturation at 94°C for 30 seconds, annealing at 56°C in 30 seconds, extension at 72°C for 1 minute. Final extension was conducted at 72°C for 5 minutes. The relative sizes of DNA products of step 1 and step 2 were 982 bp and 570 bp, respectively. Electrophoresis was conducted to check the relative size of amplified products.

(ii) PCR method to detect *V. parahaemolyticus*:

To detect *V. parahaemolyticus,* PCR reactions were conducted under two steps with two sets of primers AP4-F1 and AP4-R1, AP4-F2 and AP4-R2 (Table 1). DNA targets of step 1 and step 2 had 1269 bp and 230 bp, respectively. The PCRs were performed in a 25 µL total reaction mixture. The mixture of step 1 contained 1X PCR buffer, 3 mM $MgCl_2$, 200 µM dNTPs mix, 5 mM AP4-F1, 5 mM AP4-R1, 1.5 U/µl Taq DNA Polymerase and 1 µl DNA extract. The thermal cycle of step 1 started with an initial denaturation at 94ºC in 2 minutes, followed by 30 cycles: denaturation at 94ºC for 30 seconds, annealing at 55ºC for 30 seconds, extension at 72ºC for 1 minute. The final extension was at 72ºC in 2 minutes. For the step 2, the mixture included 1X PCR buffer, 3 mM $MgCl_2$, 200 µM dNTPs mix, 3.75 mM AP4-F2, 3.75 mM AP4-R2, 1.5 U/µl Taq DNA Polymerase and 1 µl product of step 1. The thermal profile of step 2 began at 94ºC for 2 minutes. Sequently, 25 cycles were conducted: denaturation at 94ºC for 20 seconds, annealing at 55ºC for 20 seconds, extension at 72ºC for 20 seconds. Finally, the temperature was kept at 72ºC for 2 minutes. Electrophoresis was conducted to check relative size of DNA products.

2.2.3. Duplex PCR optimization

To detect WSSV and *V. parahaemolyticus* simultaneously, a duplex PCR was optimized. The duplex PCR comprised 2 steps. The reaction mixture of step 1 contained 1X PCR buffer, 3 mM $MgCl_2$, 200 µM dNTPs, 20 pmol P1, 20 pmol P2, 5 mM AP4-F1, 5 mM AP4-R1, 2 U/µl Taq DNA Polymerase and 1 µl extract of WSSV DNA and 1 µl extract of *V. parahaemolyticus* DNA. The amplification of step 1 consisted of 30 cycles: denaturation at 94ºC for 30 seconds, annealing at 55ºC for 30 seconds, extension at 72ºC for 1 minute. Initial denaturation and final extension were at 94ºC for 2 minutes and at 72ºC for 2 minutes, respectively. For step 2, PCR components included 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 20 pmol P1, 20 pmol P2, 3.75 mM AP4-F2, 3.75 mM AP4-R2, 2 U/µl Taq DNA Polymerase and 1 µl product of step 1. The amplification of step 2 also consisted of 30 cycles: denaturation at 94ºC for 30 seconds, annealing at 55ºC for 30 seconds, extension at 72ºC for 30 seconds. Initial denaturation and final extension were at 94ºC for 2 minutes and at 72ºC for 2 minutes, respectively.

Table 1. The sequence of primers used in the optimized method

Primers	Size of amplicon	Resources
WSSV		
P1: 5'-ATCATGGCTGCTTCACAGAC-3'	982 bp	Kimura *et al*., 1996
P2: 5'-CGCTGGAGAGGACAAGACAT-3'		
P3: 5'-TCTTCATCAGATGCTACTGC-3'	570 bp	
P4: 5'-TAACGCTATCCAGTATCACG-3'		
Vibrio parahaemolyticus		
AP4-F1: 5'-ATGAGTAACAATATAAAACATGAAAC-3'		
AP4-R1: 5'-ACGATTTCGACGTTCCCCAA-3'	1269 bp	Dangtip *et al*., 2015
AP4-F2: 5'-TTGAGAATACGGGACGTGGG-3'		
AP4-R2: 5'-GTTAGTCATGTGAGCACCTTC-3'	230 bp	

2.2.4. Sensitivity and specificity testing

The sensitivity is known as the lowest DNA concentration, at which the PCR reaction can amplify and detect the disease. For the sensitivity test, the duplex PCRs were conducted at the optimized condition (reaction components, thermal cycle), in which the concentrations of DNA template were diluted to have an arrangement of 10-time reduction. To test the specificity of the duplex PCR, DNA samples of *V. harveyi*, IHHNV, and MBV were used. These pathogens were isolated from farmed shrimps. The duplex PCRs were operated under the optimized condition.

2.2.5. Application of the optimized PCR for the detection of WSSV and V. parahaemolyticus in shrimp

To apply the optimized duplex PCR, extracted DNA from 3 shrimp samples were used. Before being tested, shrimps at PL15 stage and after 30-45 days of culture were challenged with WSSV and *V. parahaemolyticus*. The shrimp were immersed in bacterial and viral solutions. After immersion, the shrimp were placed in a net, rinsed with flowing seawater for 3 min, and then returned to the rearing tanks (Satoh *et al.*, 2008; Tran *et al*., 2013). Moreover, 7 samples of PL12-15 were also taken from hatcheries for this application.

3. RESULTS AND DISCUSSION

3.1. Optimization of PCR for detection of WSSV and *V. parahaemolyticus*

3.1.1. PCR method for detection of WSSV and V. parahaemolyticus

Gel electrophoresis was used to separate DNA molecules based on their size and electrical charge. Electrophoresis results of single detections of WSSV, V. *parahaemolyticus* and the duplex method are shown in Figure 1. Relative sizes at 230 bp and 570 bp were specific to *V. parahaemolyticus* and WSSV, respectively (Figure 1A, 1B). This result insisted with previous studies (Kimura *et al*., 1996; Dangtip *et al*., 2015). This result of single detections of WSSV, V. *parahaemolyticus* was used as positive control for the duplex PCR procedure to detect *V. parahaemolyticus* (Vp_{AHPND}) and WSSV simultaneously.

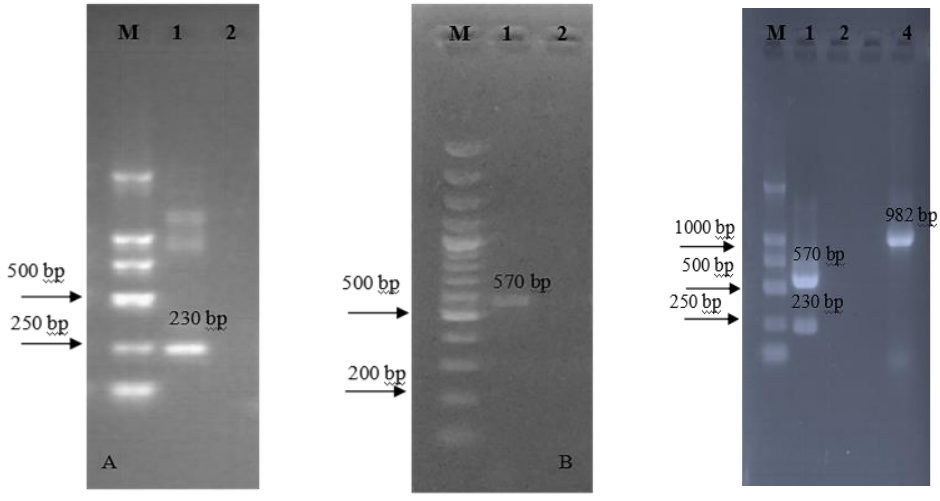

Figure 1. (A) PCR result of detecting *V. parahaemolyticus*; (B) PCR result of detecting WSSV; (C) Duplex PCR result of detecting *V. parahaemolyticus* and WSSV simultaneously (*Lane M: 1kb ladder; lane 1: DNA product of step 2 of dPCR V. parahaemolyticus and WSSV simultaneously; lane 2: no DNA template* (*negative control); lane 4: DNA product of step 1 of dPCR V. parahaemolyticus and WSSV simultaneously*).

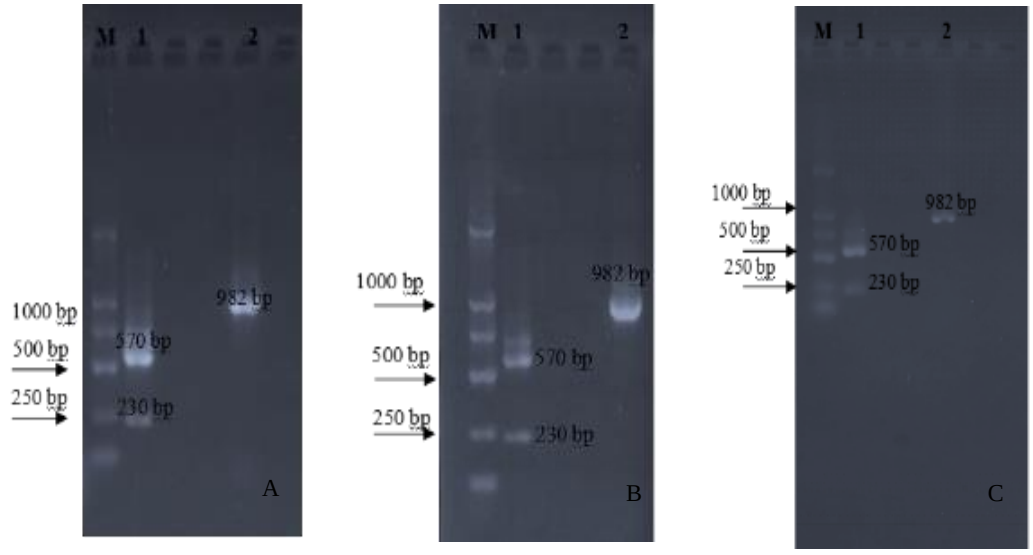

Figure 2. (A) Duplex PCR result of Taq DNA polymerase concentration optimization. *Lane M: 1kb ladder; lane 1: DNA product of step 2 of dPCR with 1U Taq DNA polymerase concentration; lane 2: DNA product of step 1 of dPCR with 1U Taq DNA polymerase concentration.* **(B) Duplex PCR result of primers P1/P2 and P3/P4 concentration optimization.** *Lane M: 1kb ladder; lane 1: DNA product of step 2 of dPCR with 10 pmol/reaction of P3/P4 concentration; lane 2: DNA product of step 1 of dPCR with 10 pmol/reaction of P1/P2 concentration*. **(C) Duplex PCR result of primers AP4-F1/AP4-R1 and AP4-F2/AP4-R2 concentration optimization.** *Lane M: 1kb ladder; lane 1: DNA product of step 2 of dPCR with 3 mM/reaction of AP4-F2/AP4-R2 concentration; lane 2: DNA product of step 1 of dPCR with 3 mM/reaction of AP4-F1/AP4-R1 concentration*

3.1.2. Duplex PCR method for detection of WSSV and V. parahaemolyticus (VpAHPND) simultaneously

DNA products of both two steps of the duplex PCR were separated under electrophoresis. PCR products in step 2 (lane 1) contained two DNA bands at 230 bp and at 570 bp, which correspond to *V. parahaemolyticus* and WSSV, respectively (Figure 1C). The results indicated that the duplex PCR method was able to detect WSSV and *Vp*$_{AHPND}$ simultaneously. To reduce process costs, it is essential to minimize the ingredients of the reaction but the stability and sensitivity of the method remain.

3.1.3. Optimization of the Taq DNA polymerase concentration

Taq DNA polymerase concentration was decreased from 2U/reaction to 1.5U and 1U/reaction. Two bands at 570 bp (WSSV) and at 230 bp (*V. parahaemolyticus*) were clearly visible although the concentration was reduced to 1U per reaction (Figure 2A). Therefore, the concentration of Taq DNA polymerase at 1U/ reaction was chosen for further experiments.

3.1.4. Optimization of primers concentration

The concentration of both primer sets P1/P2 and P3/P4 for WSSV reduced from 20 pmol to 10 pmol per reaction. As can be seen from the electrophoresis gel, a band at 570 bp was clear and bright (Figure 2B). Therefore, the concentrations of primers P1, P2, P3, and P4 at 10 pmol were suitable for the PCR reaction. In terms of primer for *V. parahaemolyticus*, the concentrations of set AP4-F1/AP4-R1 and set AP4-F2/AP4-R2 were reduced from 5 mM to 3 mM and from 3.75 mM to 3 mM, respectively. Light bands were visible (Figure 2C).

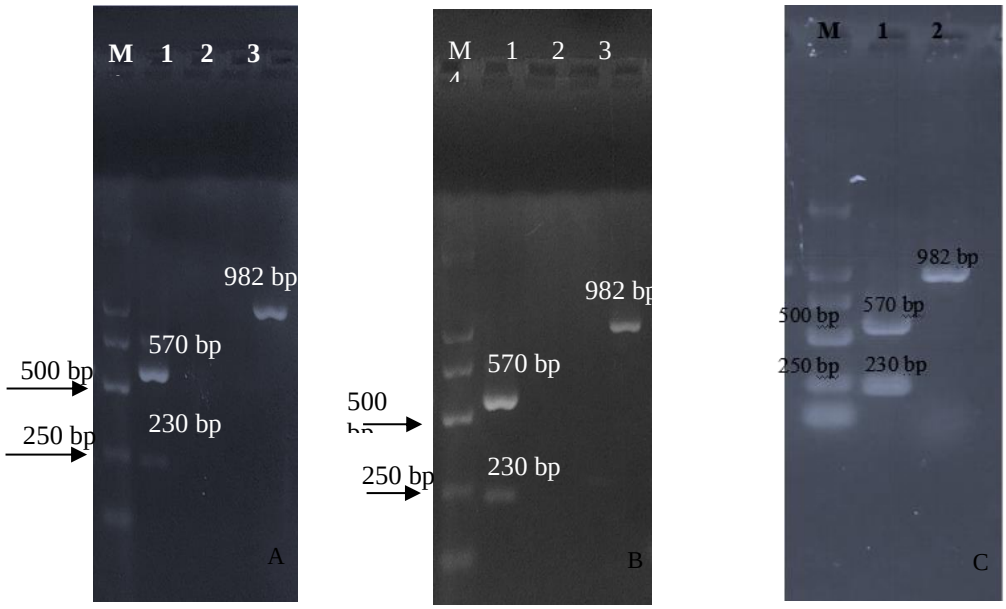

Figure 3. Duplex PCR result of dNTPs concentration optimization. *Lane M: 1kb ladder; lane 1: DNA product of step 2 of dPCR with 120 µM dNTPs; lane 2: DNA product of step 1 of dPCR with 120 µM dNTPs.* **(B) Duplex PCR result of $MgCl_2$ concentration optimization.** *Lane M: 1kb ladder; lane 1: DNA product of step 2 of dPCR with 120 µM dNTPs; lane 2: DNA product of step 1 of dPCR with 120 µM dNTPs*. **(C) Duplex PCR result of temperature optimization**. *Lane M: 1kb ladder; lane 1: DNA product of step 2 of dPCR with 25 temperature cycles; lane 2: DNA product of step 1 of dPCR with 25 temperature cycles.*

3.1.5. Optimization of dNTPs concentration

dNTPs concentration was declined from 200 µM to 120 µM to reduce the appearance of non-specific products. However, this was not much effective to reduce the non-specific bands (Figure 3A). The appropriate concentration of dNTPs for PCR reactions is about 200-400 µM (Markoulatos *et al*, 2002). Concentrations of dNTPs can be used up to 1.5 mM, because dNTPs catalyze with Mg^{2+}, excess dNTPs can increase erroneous detection and potently inhibit Taq DNA polymerase of PCR that reducing dNTPs can reduce reaction failure rates (Quyen Dinh Thi and Nong Van Hai, 2008).

3.1.6. Optimization of MgCl2 concentration

When the concentration of $MgCl_2$ was reduced from 3 mM to 1.5 mM, the electrophoresis results showed clear and bright bands at 230 bp and 570 bp (Figure 3B). There were no nonspecific bands. $MgCl_2$ concentration is reported to affect the specificity and the association between primers and DNA strands (Quyen Dinh Thi and Nong Van Hai, 2008). Thus, if there was too high or too low the $MgCl_2$ concentration, it would increase the nonspecific coupling and the unwanted PCR products $MgCl_2$ (Markoulatos *et al*, 2002).

3.1.7. Optimization of the number of thermal cycles

The thermal cycling is proportional to the number of DNA copies produced and increases the clarity of the amplified product lines. In this study, when the number of thermal cycles was reduced from 30 cycles to 25 cycles, there were no nonspecific products; meanwhile, specific bands were clearly visible (Figure 3C) affecting the process outcome, thereby reducing the reaction time Therefore, the results were found to be suitable for duplex PCR process.

3.2. Optimization of sensitivity of the duplex PCR method

The concentration of the DNA template is one of the main factors influencing the efficiency of the PCR process. DNA samples were diluted to have 10 time reduction of concentrations, such as 10 ng /µL, 1 ng /µL, 100 pg /µL, etc. The electrophoresis result showed that the bands specific to WSSV appeared lane 3, 4 and 5 (10 ng /µL, 1 ng /µL, 100 pg /µL, respectively) but not in the remaining lanes. This indicated that the minimum concentration of DNA template is not lower than 100 pg/µL. For *V. parahaemolyitcus* DNA, the minimum concentration was 10 ng/µL (Lane 3-Figure 4). It was considered to have higher sensitivity than the method of the previous study (Dangtip *et al*, 2015) with the DNA template concentration at 50 ng/µL.

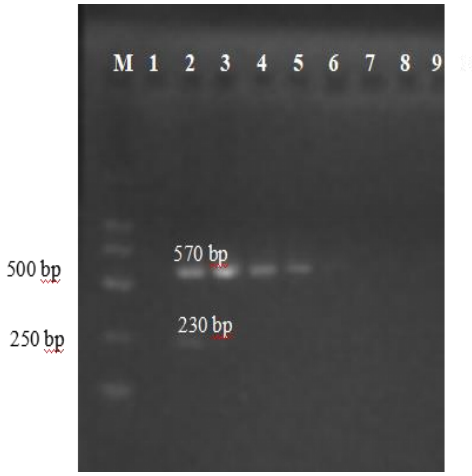

Figure 4. Duplex PCR result of sensitivity test with different DNA template concentrations. *Lane M: 1kb ladder;lane 1: negative control; lane 2: [WSSV + V.p DNA] = 10 ng/µL; lane 3: [WSSV + V.p DNA] = 1 ng/µL; lane 4: [WSSV + V.p DNA] = 100 pg/µL; lane 5: [WSSV + V.p DNA] = 10 pg/µL; lane 6: [WSSV + V.p DNA] = 1 pg/µL; lane 7: [WSSV + V.p DNA] = 100 fg/µL; lane 8: [WSSV + V.p DNA] = 10 fg/µL; lane 9: [WSSV + V.p DNA] = 1 fg/µL*

3.3. Optimization of specificity of the duplex PCR method

DNA samples of WSSV and *V. parahaemolyticus, V. harveyi*, IHHNV and MBV were used as templates for the duplex PCRs. DNA in PCR products was separated under gel electrophoresis. The result of the duplex PCR to detect WSSV and *V. parahaemolyticus* showed that the electrophoresis line appeared at 570 bp and 230 bp, which were specific for WSSV and *V. parahaemolyticus* (Lane 1 of Figure 5). The remaining lanes were DNA samples of *V. harveyi*, IHHNV and MBV respectively, which did not appear in any bands. This demonstrated that the optimized duplex PCR is specific for detecting WSSV and *V. parahaemolyticus* simultaneously.

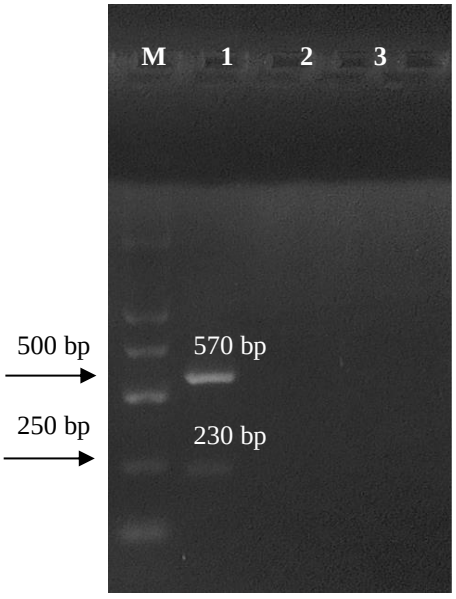

Figure 5. Duplex PCR result of specificity test. *Lane M: 1kb ladder; lane 1: WSSV and V. parahaemolyticus DNA; lane 2: V. harveyi DNA; lane 3: IHHNV DNA; lane 4: MBV DNA.*

3.4. Application of the improved PCR in infected white leg shrimp

10 shrimp samples at the PL15 stage, after 30-45 days of culture, and shrimp seed samples (PL12-15) taken from hatcheries,

were challenged with WSSV and *V. parahaemolyticus*. DNA extracts from shrimp which were challenged with WSSV and *V. parahaemolyticus and taken f*rom hatcheries were used for the experiment. The electrophoresis results are shown in Figure 6. Lane 1 was negative control with not band. Lane 2 was positive control, which containing two bands of WSSV and *V. parahaemolyticus* DNA. Lanes 3, 4, and 5 were sample of DNA extracts from the challenged shrimps. These three lanes had clearly visible bands at 570 bp and 230 bp specific for WSSV and *V. parahaemolyticus.* Lanes 6 to 12 were loaded with DNA samples of the PLs taken from the hatcheries, in which lanes 6, 10, 11, and 12 showed a slight band at 570 bp, which indicated that WSSV DNA existed in the sample. Lanes 7, 8, and 9 did not appear any bands, indicating that the samples were not infected with diseases.

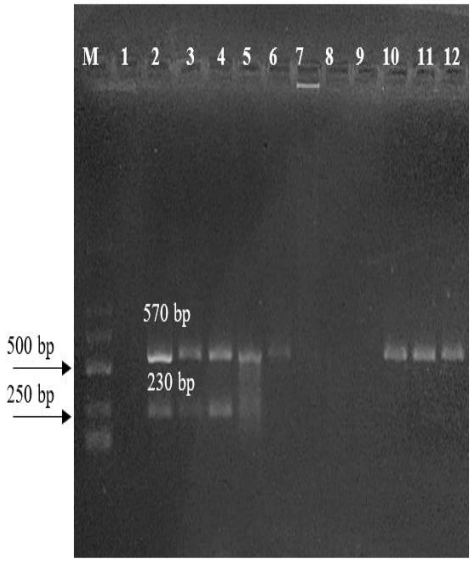

Figure 6. Application of duplex PCR detecting WSSV and *V. parahaemolyticus*.
Lane M: 1kb ladder; lane 1: no DNA template (negative control); lane 2: DNA template (positive control); lane 3-5: DNA of shrimp samples challenged with both WSSV and V. parahaemolyticus; lane 6-12: DNA of shrimp samples taken from hatcheries

The duplex PCR protocol that is able to simultaneously detect WSSV and *V. parahaemolyticus* is an excellent diagnostic method for single or double shrimp infection. The method is not only fast but also reliable to detect pathogens in shrimps. Therefore, it can save time, prevent and control the pathogen spreading, as well as promptly evaluate the quality of the bloodstocks before stocking.

Due to higher sensitivity limits than most classical diagnostic methods, PCR has become the preferred method for the diagnosis of WSSV and Vp_{AHPND} in shrimp. Molecular-based detection methods for shrimp include singelex PCR, duplex PCR, and multiplex PCR. Koiwai *et al*., (2018) developed multiplex PCR and STH chromatographic PAS, named PCR-DNA chromatography have been widely used for the detection of four shrimp diseases by amplifying the WSSD, IHHN, AHPND, and EHP infections and 16S rRNA/tRNAVal/12S rRNA mitochondrial region (an internal control). In addition, the duplex droplet digital PCR (ddPCR) method can simultaneously detect and quantify the two pathogens EHP and V_{pAHPND} specifically. The sensitivity levels of ddPCR for EHP and Vp_{AHPND} were 2.3 copies/µl and 4.6 copies/µl, respectively, which were 10-fold higher than the sensitivity of the qPCR assay and showed good reproducibility (Zhang *et al*., 2022).

4. CONCLUSIONS

In conclusion, the duplex PCR of the product amplification procedure was based on WSSV and Vp_{AHPND} specific genomic regions at 570 bp and 230 bp, respectively. The chemical composition of the reaction including 1X PCR buffer, 1.5 mM $MgCl_2$, 120 µM dNTPs, 10 pm P3 and P4; 3 mM AP4-F2 and AP4-R2, 1U Taq DNA polymerase, total reaction volume of 25 µl. The thermal profile began at 94ºC for 2 minutes. Sequently, 25 cycles were conducted denaturation at 94ºC for 20

seconds, annealing at 55ºC for 20 seconds, and extension at 72ºC for 20 seconds. Finally, the temperature was kept at 72ºC for 2 minutes. The total amplification time is approximately 30 minutes. This duplex PCR was high specificity for some diseases caused by bacteria, and viruses causing diseases in shrimp. The concentration for demand sensitivity was 100 pg/µL specific for WSSV and 10 ng/µL specific for *V. parahaemolyticus*. This PCR assay can be a rapid, sensitive, and specific method for simultaneous and unambiguous detection of WSSV and AHPND from pure cultures and shrimp clinical samples. Therefore, it can be a good PCR method candidate applied for the detection of WSSV and AHPND in cultured shrimp in Vietnam and in the cultural shrimp industry in the world.

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