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Research Article

Establishment of pre-enrichment step and DNA extraction method for the novel PCR procedure of acute hepatopancreatic necrosis disease (AHPND) and mutant-AHPND fast detection in shrimp

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Abstract

After the first outbreak of Acute Hepatopancreatic Necrosis Disease (AHPND) in China in 2009, this disease has been still considered a globally dangerous disease in shrimp aquaculture industry. Currently, there is no effective method to prevent and treat AHPND. Therefore, rapid detection methods which could avert and control this disease in shrimp were considered the most effective strategy. In 2021, a new PCR reaction was established that can simultaneously detect AHPND and mutant-AHPND. Intending to develop a PCR kit, the PCR procedure including a pre-enrichment step and DNA-extracted method was established to conduct the PCR reaction. The new PCR procedure was verified with a detection limit of 5.10^3 CFU/mL. This detection limit was two times higher than the conventional PCR methods, which are currently used to detect AHPND. *Vibrio parahaemolyticus* showed the best growth at 37°C in the broth with hepatopancreas of shrimp. A simple boiling method to extract DNA in bacterial broth with shrimp tissues was also modified. The PCR procedure has successfully been validated on 42 AHPND-suspecting samples. These results would be developed into a novel standard procedure using a PCR kit for rapid detection of AHPND and related mutant-AHPND for rapid disease diagnosis in shrimp farms.

Keywords: AHPND, mutant-AHPND, DNA extraction, PCR, *Vibrio parahaemolyticus*

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Introduction

Amidst the COVID-19 challenge, in most of the farmed shrimp-producing countries, annual production figures for 2020 are yet to be released. However, preliminary news and the industry analysis show that production in Ecuador, Indonesia, and Vietnam increased moderately, but the harvest in India, Thailand, Malaysia, and Bangladesh has decreased compared to 2019. The production drop was possibly much higher in China than in other countries in Asia. As of November 2020, shrimp farming in Asia entered a period of low production including the north-eastern region of India, Vietnam, Thailand, Myanmar, and Bangladesh (FAO, 2021a). Despite these restrictions, the global shrimp trade has remained relatively stable with reduced supplies, particularly from Asia during April – August of 2020. However, imports increased in the two largest markets in China and the United States, where retail sales were at record highs in April – September of 2020 (FAO, 2021b).. This proved that the reduced supply could not meet the increased demand for shrimp in major import markets. Besides the objective cause of the COVID-19 epidemic, shrimp diseases were a great concern for the major shrimp-producing countries in the world.

After the first outbreak of acute hepatopancreatic necrosis disease (AHPND) in China in 2009, this infectious disease has been still considered a globally dangerous disease in shrimp aquaculture industry. Currently, there is no effective method to prevent and treat AHPND. The treatment of antibiotics in shrimp farming can affect the health of consumers, and major shrimp export markets such as the EU and the US, where antibiotic usage is banned for shrimp farming. Therefore, rapid detection methods which could prevent and control hazardous diseases in farmed shrimp were considered the most effective strategy. In addition to the rapid spread and mortality rate (up to 100%) of AHPND, the presence of different types of mutations also affected the diagnosis of the disease. Recently, toxA and toxB deletion mutations on the pVA1 plasmid in some strains of *Vibrio parahaemolyticus* (V.p) have been reported. Two mutant types were collected and identified from AHPND-affected farms in Vietnam, Thailand, Mexico, and Latin America. In type 1, both toxA and toxB genes were mutated, indicating toxABvp (-). Type 2 was a mutation that lost the toxA gene and a part of the toxB gene, indicating toxAvp (-). Studies showed that the mentioned mutant strains did not cause AHPND (Han *et al.,* 2017).

However, in 2017, Phiwsaiya found another case of early mortality syndrome (it was the primary name of the disease and then was changed to AHPND) in Vietnam. Bacterial strain *V. parahaemolyticus* XN87 contains plasmid with mutant toxA and normal toxB genes. The study indicated that in this mutant form, shrimp did not show hepatopancreatic necrosis symptoms, but the early mortality rate in shrimp was

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still up to 50% (Phiwsaiya *et al.*, 2017). This mutation was called mutant-AHPND. In our previous study, a new PCR reaction was established that can simultaneously detect AHPND and mutant-AHPND, while the latest detected methods were unable to identify the mutant. This PCR reaction has been evaluated to be effective and has a sensitivity of four times higher than that of AP3, which is commonly used in the diagnosis of AHPND (Mai-Hoang *et al.*, 2021). Intending to develop a PCR kit, this study focused on establishing the PCR procedure including a pre-enrichment step and DNA extraction method to conduct a faster PCR reaction.

A single-pair-of-primers PCR procedure for the detection of *V. parahaemolyticus* causing AHPND/mutant-AHPND requires time to enrich the bacteria in the initially suspecting shrimp sample. The growth, as well as the viability of *V. parahaemolyticus*, depends heavily on environmental factors such as temperature, salinity, and dissolved oxygen. As halophilic bacteria, their salt tolerance ranges from 0.5% to 7.5%. V.p bacteria are reported to have a heat tolerance range of 11 – 37°C, with an optimal temperature of 30°C (Kim *et al.*, 2012). However, in a study when V.p proliferated in the environment with the composition of the host organism such as salmon meat, oyster juice, and halibut meat, the maximum temperature in the test was 36°C, which resulted in the best growth rate index and gradually decreases at a lower temperature. *V. parahaemolyticus* and *Vibrio* species in general grow strongly in conditions of abundant oxygen. When proliferating V.p with different shaking conditions (6 ×g), they grew strongly under conditions for higher dissolved oxygen content (Hanh *et al.*, 2020). It is necessary to shorten the time of pre-enrichment, thereby shortening the time to perform the diagnostic PCR procedure. In addition, the PCR process should be a convenient and feasible method so it has to have parameters that are compatible with the facilities and equipment of most local laboratories, especially in developing countries. This study chose to investigate two parameters of temperature and shaking conditions to reduce the time of the V.p pre-enrichment step. Also, these two parameters come with incubation and shaking devices, investigation of different temperatures and shaking conditions would provide possible parameters to perform PCR procedures for unequipped laboratories. Moreover, to shorten the time to perform the PCR procedure, a DNA collection step by high temperature was also conducted. Therefore, the parameters of speed and centrifugation time would be investigated to choose the most optimal values. Afterwards, a novel standard procedure would be developed and used as a PCR kit for rapid detection of AHPND and related mutant-AHPND for rapid disease diagnosis in dominant shrimp farming areas.

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Materials and methods

Bacterial strains and suspected shrimp samples

Bacterial strains used and recovered during this study were shown in the study of Mai-Hoang *et al.* (2021). Positive control VP_{AHPND} XN89 strain, and mutant-VP_{AHPND} XN87 strain were mainly used in most experiments. All *Vibrio* spp. were inoculated and cultured in tryptic soy broth agar plus (TSB with 1.5% NaCl), and HiCrome™ Candida Differential Agar. Plate and broth cultures were incubated at 37°C for 24 hours. Other bacterial species were cultured using the recommended methods for the respective species. The bacterial isolates were preserved in cryovial to store at -80 °C and revived as needed (Mai-Hoang *et al.,* 2021).

P. vannamei samples were purchased in Ho Chi Minh City, Vietnam, and tested negative for AHPND for infection tests. *V. parahaemolyticus* can be isolated on standard media used for the isolation of bacteria from shrimp (Lee *et al.,* 2015; Soto-Rodriguez *et al.,* 2015). Negative samples identification were carried out using AP3 PCR (Sirikharin *et al.,* 2015). Samples suspecting AHPND were provided by personnel from the Sub-Departments of Livestock and Animal Health of Tien Giang and Kien Giang provinces, Vietnam for validation after the establishment of the PCR procedure.

The detection limit of the PCR technique with GMIF1-2 primers for bacterial broth

To determine the bacterial density at OD600 0.697–0.700, colonies of bacteria were inoculated from the HiCrome Vibrio Agar plates to TSB with 1.5% NaCl and incubated for 4–6 h at 37°C. The optical density (OD) at 600 nm of the resulting bacterial cultures was measured using a spectrophotometer (Thermo Scientific, United States). Bacterial solution at OD_{600} 0.697–0.700 was 10^{th} serial diluted to concentrations 10^{-5}, 10^{-6}, and 10^{-7}. The CFU/50 µl of all of these solutions were then plated onto TSA agar, and subsequently counted the number of colonies on the plate (Pollack *et al.,* 2018). Therefore, the density of XN89 and XN87 strains was determined at OD_{600} 0.697–0.700.

Briefly, one single colony of the specific isolate from the recommended agar plate was suspended in 5 mL TSB. Bacterial growth broth XN89 (5.10^8 CFU/mL at OD=0.697) and XN87 (8.10^8 CFU/mL at OD=0.700) were diluted in 10-fold order into smaller concentration range from 5.10^7 to 5.10^2 CFU/mL. Tubes of 1 mL of the bacterial broth in these concentration ranges were centrifuged at 18,928 ×g for 10 min, the cell pellet was re-dissolved with 500 µl MiliQ, then boiled at 100 °C for 10 min to release DNA in the cells. Cell lysis was centrifuged at 18,928 ×g for 10 min. Finally, 1 µl of the supernatant was conducted PCR to determine the detection limit of the technique. Reaction components, primers, and

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thermocycler parameters are shown in Table 1 (Mai-Hoang *et al.*, 2021).

Table 1: Primers for new PCR procedure (Mai-Hoang *et al.*, 2021).

Primer (5' – 3' sequence)	Amplicon (bp)
GMIF1-F: TTCTCACGATTGGACTGTCG	366 (normal)
GMIF2-R: GGGTTAAATTCCGTCAAAGATG	1429 (mutant)

Optimization of the pre-enrichment time and shaking conditions combination

After determining the lowest cell density for positive PCR results, the bacterial broth with the density was inoculated into the negative shrimp samples to simulate the AHPND detection by PCR procedure, then cultured under shaking or no shaking conditions. Cell broth was collected at each 2, 4, 6, 8, and 24 h; subjected to heat treatment and conducting PCR to determine the minimum pre-enrichment time for positive PCR results. The procedure was carried out as in Table 2.

Optimization of the growth temperature

The bacterial broth was inoculated into negative shrimp samples and allowed V.p to grow under set pre-enrichment time and shaking conditions at 25°C (room temperature), 30°C, and 37°C. The procedure was carried out as in Table 2.

Table 2: The PCR procedure in this study.

Steps	Initial procedure	Optimized procedure
Pre-enrichment Crush 2–3 shrimp heads in falcon 50 ml, submerge the shrimp heads into TSB+ to reach a volume of 20 ml and culture under specific shaking conditions and temperature	Shaking at 37°C overnight	Shaking at 37°C for 4 h
DNA extraction - Centrifuge 200 µl bacterial broth at a specific speed and time, and discard the supernatant. - Re-suspense with 500 µL of MiliQ in a tube. - Boil at 100°C for 10 min. - Cool the tube, centrifuge at a specific speed and time, and collect the supernatant.	Centrifuge at 18,928 ×g for 10 min	Centrifuge at 11,200 ×g for 5 min

PCR: 1 µL of the supernatant was conducted for the PCR reaction, which was established in the previous study (Mai-Hoang *et al.*, 2021).

Optimization of the centrifugation speed and time for the boiling method

The bacterial broth was boiled at 100°C for 10 min with the centrifugation speed of 4,032 ×g; 11,200 ×g; and 18,828 ×g (maximum speed of quick-spin centrifuge devices), for 5, 10, 15, and 20 min. The procedure was carried out as in Table 2. After analyzing the PCR product bands with ImageJ and

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Graphpad, the criteria for parameter selection was the highest brightness. If there was no significant difference, the variable with the lowest time was selected for time-saving achievement (Mai-Hoang *et al.*, 2021).

Validation of the PCR procedure and specificity tests

Forty-two samples (10-20 shrimps for each sample) from ponds with the unknown status of AHPND infection were collected from the Mekong Delta region of Vietnam (Tien Giang and Kien Giang provinces). The PCR procedure was shown in Table 2 with optimum parameters for evaluation of the monoplex PCR process. The negative control was added with broth without bacterial infection as a template. The procedure with the AP3 method was performed at the same time to verify the results shown by the new PCR process. The specificity of the PCR procedure was tested using all strains in Table 2 that originated from our previous study (Mai-Hoang *et al.*, 2021).

Results

The detection limit of the PCR technique with GMIF1-2 primers for bacterial broth

From the results of Figure 1, it showed that the minimum bacterial density that could be detected by PCR technique was 5.10^3 CFU/mL (equivalent to 10 CFU/reaction) for strain XN89 and 5.10^5 CFU/mL (equivalent to 10^3 CFU/reaction) for strain XN87. Therefore, the above concentrations were chosen as the inoculating concentration of shrimp samples to survey the pre-enrichment time and shaking conditions matrix for the PCR procedure to detect AHPND/mutant-AHPND pathogenic strains in shrimp.

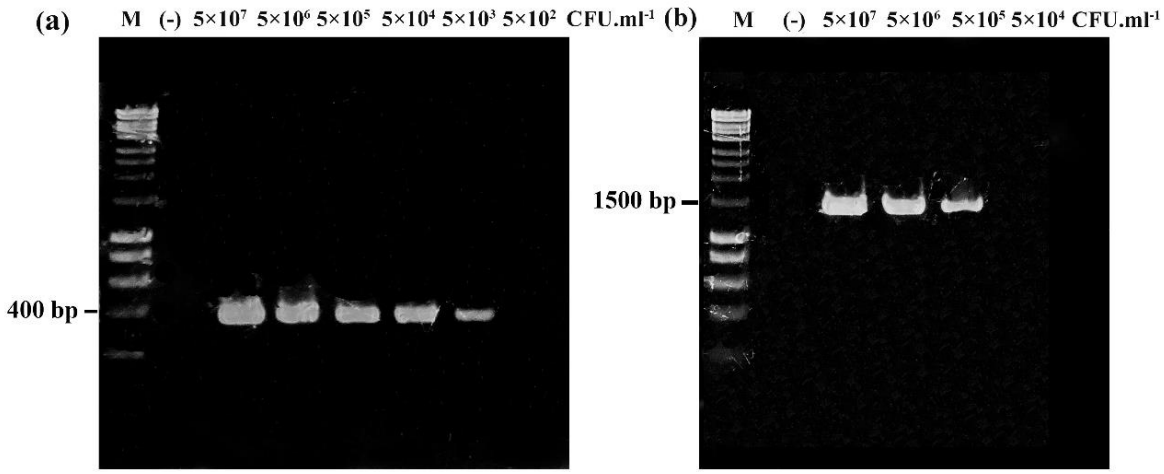

Figure 1: The detection limit of PCR technique with XN87 and XN89 strains. (M): 1kb ladder; (-) negative control; A: lanes 1 to 6 is the diluted concentration of bacterial broth of *V. parahaemolyticus* XN89 strain. B: lane 1 to 4 is the diluted concentration bacterial broth from *V. parahaemolyticus* XN87 strain. Data represent three repetitions.

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Optimal combination of pre-enrichment time and shaking condition

The survey aimed to determine the optimal combination of shaking conditions and the shortest growth time that still gave positive PCR results. One milliliter of the bacterial broth was inoculated with a concentration of 5.10^5 CFU/mL for the XN87 strain and 5.10^3 CFU/mL for the XN89 strain into shrimp samples independently. Bacterium in samples was grown under shaking or no shaking at 37°C and 200 µL of the enrichment broth was collected every 2, 4, 6, 8, and 24 h. Shrimp samples inoculated with XN87 strain when shaking gave a positive result as early as 4 h and gave a positive result as early as 8 h when not shaking (Fig. 2). As for shrimp samples inoculated with the XN89 strain, when shaking or not shaking, the earliest positive result was at 4 h (Fig. 3). The results appeared in all three replicates. Therefore, we selected XN87 strain and shaking growth for 4 h of the pre-enrichment step was the optimal condition for further investigations.

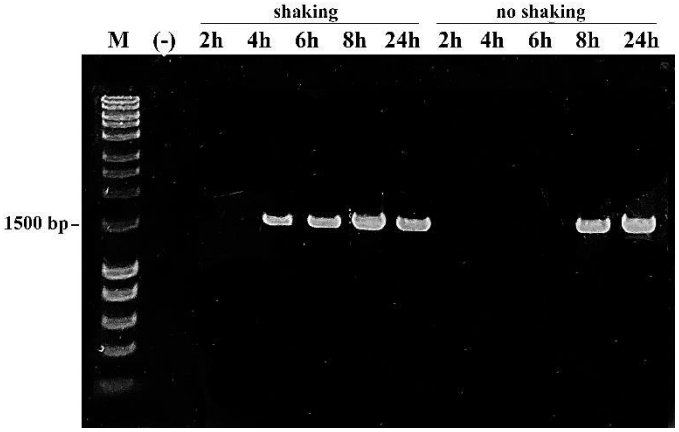

Figure 2: Results of optimization with different combinations of pre-enrichment time (h) and shaking conditions on PCR procedure with XN87 strain. (M): 1 kb ladder; (-) negative control; lanes 1 to 10: Different combinations of pre-enrichment time and shaking conditions respectively. Data represent three repetitions.

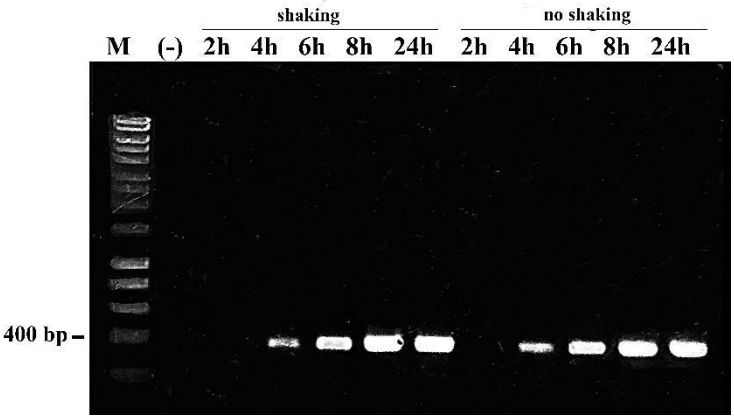

Figure 3: Results of optimization with different combinations of pre-enrichment time (h) and shaking conditions on PCR procedure with XN89 strain. (M): 1 kb ladder; (-) negative control; lanes 1 to 10: Different combinations of pre-enrichment time and shaking conditions respectively. Data represent three repetitions.

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Optimal growth temperature of V. parahaemolyticus in spiked shrimp tissues

The survey aimed to determine the optimal growth temperature for *V.parahaemolyticus* in spiked shrimp tissues. One milliliter of the bacterial broth was inoculated with a concentration of 5.10^5 CFU/mL for strain XN87 into shrimp samples, which were cultured under shaking conditions for 4 h at temperatures of 25, 30, and 37°C. The result showed that when pre-enriched for 4 h, the shrimp samples only gave positive results at 37°C, this result appeared in all three replicates (Fig. 4). So, 37°C was selected as the optimal temperature for the next surveys.

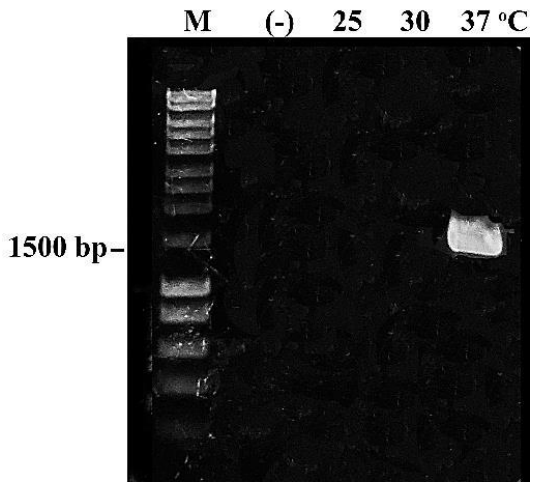

Figure 4: Results of optimization with different growth temperatures in broth with shrimp tissues. (M): DNA ladder 1 kb; (-) negative control; lanes 1 to 3: different growth temperature. Data represent three repetitions.

Optimal centrifugation speed and time combination for the boiling method

The survey aimed to choose the optimal centrifugation speed with the shortest time. Similarly, One milliliter of the bacterial broth was inoculated with a concentration of 5.10^5 CFU/mL for strain XN87 into the shrimp samples, which were cultured under shaking conditions for 4 h at 37 °C. The boiling method was conducted as shown in Table 2 with centrifugation speeds of 4,032; 11,200; and 18,928 ×g, and centrifugation times of 5, 10, 15, and 20 min, respectively. The results show that with the centrifugation time from 10 to 15 min at 4,032 ×g and 5 to 20 min at the remaining speed parameters, there was no significant difference (p= 0.2253>0.05) but there was a significant difference between the combination of 4,032 ×g and 5 min for the other combinations (p=0.0235<0.05) (Fig. 5). Therefore, the combination of 11,200 and 5 min was chosen as the optimal rate for the extracted DNA boiling method.

Validation of the PCR procedure and specificity tests

This survey aimed to evaluate the possibility of using a new PCR procedure to replace the AP3 method, which is the current standard method in AHPND diagnosis, by comparing technical parameters such as accuracy, sensitivity, specificity, negative deviation, and positive deviation. Accuracy (%) is the ratio of the co-positive (PA) and co-negative (NA) results by the two methods to the total number of samples tested (N).

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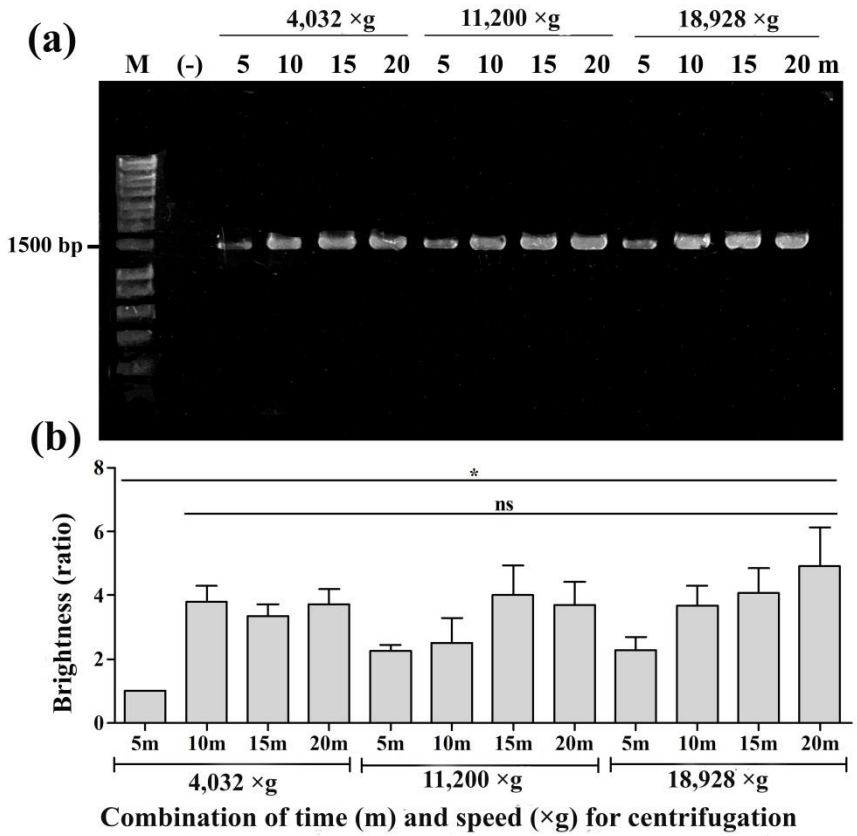

Figure 5: Results of optimization of different combinations of time (m) and speed (×g) for centrifugation of the boiling method. A: Electrophoresis image (M): DNA ladder 1 kb; (-) negative control; lanes 1 to 12: the different combination of time (m) and speed (×g) for centrifugation. B: Results of brightness analysis. Data represent three repetitions.

Sensitivity (%) is the ratio of the number of positive results (TP) obtained from the alternative method to the number of positive results (N+) obtained from the reference method (AP3 method). Specificity (%) is the ratio of the number of negative results (TN) obtained from the substitution method to the number of negative results (N-) obtained from the reference method. Positive deviation (%) is the ratio of false-positive results (PD) obtained from the alternative method to the number of results proven negative by the reference method. Negative deviation (%) is the ratio of the number of false negative results (ND) obtained with the alternative method to the number of results that have been proven positive (ISO 1589:2012). The experiment was conducted on 42 samples suspecting of AHPND provided by personnel from the Sub-Departments of Livestock and Animal Health of Tien Giang and Kien Giang provinces, Vietnam (10 samples from Tien Giang and 32 samples from Kien Giang). The PCR procedure set up with optimal parameters was presented in Table 2. The comparative method between AP3 and the new PCR procedure was based on the establishment of the AP4 method by Dangtip *et al.*, 2015. The results of the relative technical parameters between the new PCR procedure and the AP3 method were 100% accuracy, 100% sensitivity, and 100% specificity, and

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both negative deviation, and positive deviation were 0 (calculated from results shown in Table 3):

$$\text{Accuracy (\%)} = = \frac{6 + 36}{42} \text{ x } 100\ \% = 100\ \%$$

$$\text{Sensitivity (\%)} = \frac{TP}{N+} = \frac{6}{6} \text{ x } 100\ \% = 100\ \%$$

$$\text{Specificity (\%)} = \frac{TN}{N-} = \frac{36}{36} \text{ x } 100\ \% = 100\ \%$$

$$\text{Positive deviation (\%)} = \frac{PD}{PD+NA} = \frac{0}{0 + 36} \text{ x } 100\ \% = 0\ \%$$

$$\text{Negative deviation (\%)} = \frac{ND}{ND+PA} = \frac{0}{0 + 6} \text{ x } 100\ \% = 0\ \%$$

PA: the number of co-positive results
NA: the number of co-negative results
N: total number of samples tested
TP: the number of positive results obtained from the alternative method
N+: the number of positive results obtained from the reference method
TN: the number of negative results obtained from the substitution method
N-: the number of negative results obtained from the reference method
PD: the number of false-positive results
ND: the number of false negative results

Table 3: Correlation between results of AHPND diagnosis by new PCR procedure and AP3 method

AP3 analysis results	PCR procedure analysis results	
	Positive	**Negative**
Positive	PA = 6	PD = 0
Negative	ND = 0	NA = 36

The specificity tests of the PCR procedure gave positive results for all VP_{AHPND} strains and negative results for the other non-AHPND bacterial species and WSSV (Fig. 6).

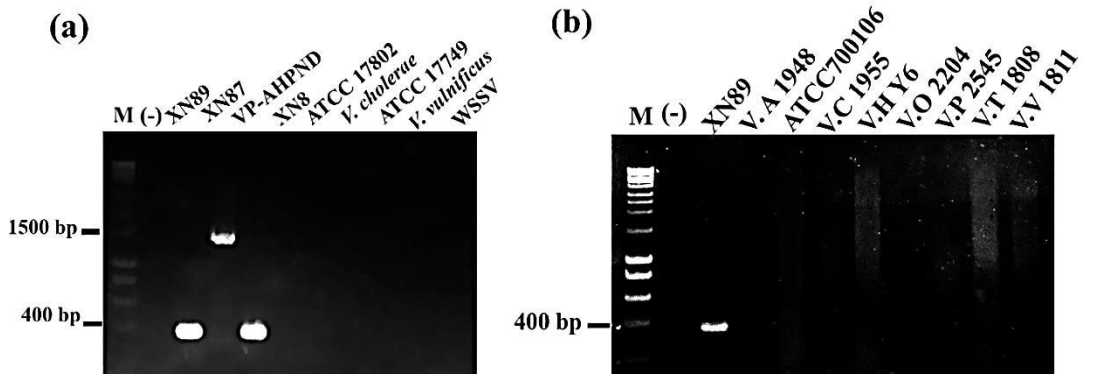

Figure 6: Results of the specificity of the PCR procedure. (M): DNA ladder 1 kb; (-) negative control; (a): lanes 1 to 9 are strains XN89, XN87, Vp-AHPND, *Vibrio* non-AHPND and WSSV, respectively; (b): lanes 1 to 9 are strains XN89 and non-AHPND *Vibrio*, respectively. The results represent three replicates.

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Discussion

The lowest cell density which gave positive PCR results were determined. The detection limit of most conventional PCR methods was ~10^4 CFU/mL of VpAHPND pure culture (Tang *et al.*, 2020). Therefore, with a detection limit of 5.10^3 CFU/mL VpAHPND (10 CFU per reaction), our PCR procedure was two times more sensitive than the conventional PCR methods. With the same reaction composition and optimal thermocycler conditions, the XN87 strain has a larger target product size, indicating that XN89 strain has a lower detection limit than strain XN87. Therefore, both XN87 and XN89 strains were selected for the follow-up investigation.

V. parahaemolyticus is a rod-shaped, Gram-negative (-), aerobic, the halophilic bacterium of the Gamma Proteobacteria class, whose natural habitat is coastal waters worldwide. Therefore, *V. parahaemolyticus* has a better proliferative ability when providing higher dissolved oxygen content. Because the XN89 strain gave similar results to the XN87 strain in shaking conditions and was detected even earlier in non-shaking conditions, the XN87 strain was selected as the representative inoculate strain for subsequent investigations. The AP3 method, as recommended, required a pre-enrichment time of 4 – 6 hours (Sirikharin *et al.*, 2015). However, according to FAO, the pre-enrichment time when using PCR to diagnose AHPND was 6 hours (Tang *et al.*, 2020). Therefore, the mentioned PCR procedure required less pre-enrichment time than the standard PCR process. Also, this 4-hour step could even detect the lowest bacterial density limit of the PCR technique in the suspecting shrimp samples.

Also, further investigation showed that at 6 h, shrimp samples revealed positive results at all three temperatures (Supplementary material, Fig. 1S). The result indicated that from 6 h of shaking, all three temperatures gave good amplified signals. The above results also showed that for laboratories without thermostatic shaking devices but with an incubator, the optimal pre-enrichment time and temperature to detect AHPND/mutant-AHPND was 8 h at 37°C. In addition, in cases where thermostatic shaking devices and incubators were not available, the pre-enrichment step could be conducted at room temperature (25°C) overnight.

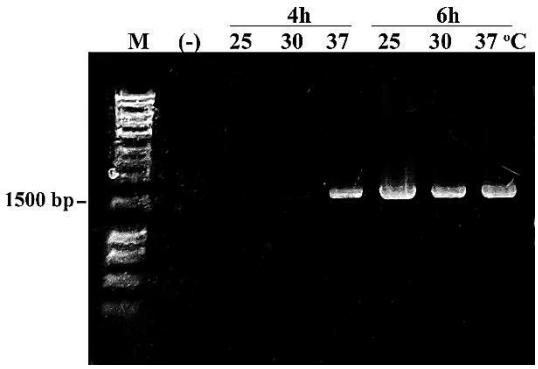

Figure 1S: Results of optimization with different growth temperatures in broth with shrimp tissues at 4 h and 6 h. (M): DNA ladder 1 kb; (-) negative control; lane 1 to 6: different growth temperature at 4 h and 6 h. Data represent three repetitions.

In the PCR process to detect AHPND, after the pre-enrichment step, the

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template should be prepared for the PCR reaction. According to research by many authors, the DNA used as a template for PCR reactions did not necessarily have to be extracted and purified from cells (Simmon *et al.,* 2004; Zou *et al.,* 2017). Thus, a simple DNA-extracted method is to break the bacterial cell, releasing the DNA template, and then the cell lysis is directly used for PCR reaction (Simmon *et al.,* 2004). Among the methods of cell disruption to release DNA, the boiling method was the easiest, fastest, and least expensive one. The parameters of time and speed for centrifugation in steps of biomass and DNA collection would be investigated to shorten the method implementation time as well. DNA-extracted kits were almost used in current PCR procedures (Dangtip *et al.,* 2015; Lai *et al.,* 2015; Hossain *et al.,* 2020). Therefore, the preparation of DNA in the current AHPND-diagnostic PCR procedures is expensive and quite time-consuming. The boiling method should be tested as an alternative. The boiling method also required a sub-culturing step to extract DNA effectively from colonies (Dashti *et al.,* 2009; Barbosa *et al.,* 2016). In this study, the boiling method was employed to extract total DNA directly from the cultured broth. Proliferation in broth including shrimp tissues requires VpAHPND to compete against microorganisms in shrimp hepatopancreas tissue. The boiling method also needs to be optimized to obtain a sufficient amount of DNA for a direct PCR reaction consequently. In addition, the results from Figure 5 also showed that for laboratories without high-speed centrifuges but with quick-spin centrifuge machines (4,032 ×g) required the shortest centrifugation time was 10 min.

The diagnostic results on 42 samples suspecting AHPND showed the same result between AHPND detection by the new PCR procedure and the AP3 method. It was also found that the microbiota and chemical composition in shrimp tissues did not significantly affect the detection of AHPND by the new PCR procedure.

The new PCR procedure was verified with a detection limit of 5.10^3 CFU/mL for bacterial broth. This detection limit was two times higher than that of the conventional PCR methods which are currently used to detect AHPND in shrimp. The shortest time for pre-enrichment with this minimum density in AHPND-suspected samples was 4 h. The published AHPND-detected procedures enriched V.p at 30°C for 6 h, but our study reported that 37°C was the best growth temperature for V.p in a competitive environment. These experimental parameters help avoid missing samples infected with V.p at low density but still at high risk of causing AHPND/mutant-AHPND. A simple boiling method to extract DNA directly from the cultured broth was also modified this new PCR procedure was the basis for the development of a standard kit for the rapid detection of AHPND/mutant-AHPND.

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