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# Development of a diagnostic scar marker for *Vibrio shilonii* caused acute hepatopancreatic necrosis disease in whiteleg shrimp

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## Abstract

Background: In a previous report, we showed that *Vibrio shilonii* was found on whiteleg shrimp (*Litopenaeus vannamei*) with acute hepatopancreatic necrosis disease in Thua Thien Hue province, Vietnam. This study was performed to develop a diagnostic molecular marker generated by random amplified polymorphic DNA (RAPD) for *V. shilonii* rapid detection.

Methods: Pathogen *Vibrio* spp. were isolated from shrimps and fishes, and were identified by 16S rRNA sequencing. Genetic diversity of *Vibrio* strains was analysis by RAPD technique. Specific PCR product for *V. shilonii* was cloned and sequenced. SCAR marker was developed from specific PCR product.

Result: Twenty random primers were evaluated for RAPD to identify DNA polymorphisms between *Vibrio* species. The random primer OPN-06 generated a 468-bp DNA fragment specific for *V. shilonii*. This was then converted into a sequence-characterized amplified region (SCAR) marker designated N6-441.

Conclusion: Specific primers (Vshi-441F/Vshi-441R) amplified a unique DNA fragment in all *V. shilonii* isolates but not in the other *Vibrio* spp. This PCR assay showed significantly sensitive to the target DNA and reliably for the amplification the *V. shilonii* genome.

## Introduction

Whiteleg (*Litopenaeus vannamei)* is the most popular shrimp species for commercial cultivation due to their survival advantages, fast growth rate and strong adaptability to environment [1]. The shrimp is currently cultivated in various areas in the world including Southeast Asia, China, India, United States, Mexico, and Latin America countries. Shrimp industry produced more than 4 million tons in 2016 [2]. Whiteleg and black tiger (*Penaeus monodon*) shrimps are the most common farm-based shrimp species cultured in Vietnam [3]. However, the diseases are highly occurred and directly affected to shrimp farming industry [4].

Thua Thien Hue is a province that is located in Central of Vietnam. It has a biggest lagoon in the Southeast Asia (Tam Giang lagoon) which plays significantly importance role in the aquaculture farming industry. Aquaculture has been developing in Thua Thien Hue province for 50 years ago and became the most important livelihood activity. However, aquaculture productivity decreased continuously since 2009 until now. The main reason is because of the disease outbreak, natural disasters, climate change and the environment issues, especially water pollution, impacting on the health of aquatic animals. Many farmers lost their production due to the bloom of pathogenic *Vibrio* sp. and viral shrimp diseases related to increasing water temperature [5].

In 2009, acute hepatopancreatic necrosis disease (AHPND) has caused remarkable mortality (up to 100%) in populations of shrimp cultured in South East Asia and Latin America countries. This disease is referred to as early mortality syndrome (EMS). AHPND is caused by several *Vibrio* species, which carry out the genes encode for toxic proteins similar to *Photorhabdus* insect-related (Pir) toxins [6,7]. The marine bacterium *Vibrio shilonii* was firstly identified in the coastal areas of the Mediterranean Sea and has been proposed as the agent factor causing the bleaching disease for *Oculina patagonica* [8]. It was recently reported that *V. shilonii* caused the acute hepatopancreatic necrosis disease (AHPND) on shrimp in Ecuador and Vietnam [5,7].

Random amplified polymorphic DNA (RAPD) is rapid and powerful tool for generating molecular markers to differentiate organism population. However, RAPD is limited by inherent low reliability and reproducibility of the PCR reaction. Thus, a RAPD-derived codominant and reproducible marker was developed and called as the sequence-characterized amplified region (SCAR) [9]. Using SCAR markers for identification of pathogens such as *Pseudomonas syringae* [10], *Pseudofabraea citricarpa* [11], and *Bipolaris sorokiniana* [12]. However, SCAR markers to identify *Vibrio* species have not been previously reported.

In Thua Thien Hue, five strains of *V. shilonii* were found on whiteleg shrimp with AHPND [5]. However, no more information of these five strains has been published. This study aimed to develop a molecular marker for the rapid *V. shilonii* identification and to provide scientific data as the basis for the study, and to contribute to the production of rapid diagnostic kits for pathogens *Vibrio* in the future.

## Methods

### Vibrio strains

*Vibrio* strains were provided by the Laboratory of Gene Technology, Institute of Biotechnology, Hue University, Vietnam (Table 1). Characteristics of each strain were previously described [5].

### DNA isolation

*Vibrio* colonies were cultured in alkaline saline peptone water medium with 2% peptone and 2% NaCl, pH 8.6, with shaking at 180 rpm for 18 h at 30°C. Cells were collected using centrifugation with 13,000 rpm at 4°C for 1 min. Total genomic DNA was extracted by the AquaPure Genomic DNA Isolaton Kit (Cat. 732-6340, Bio-rad) according to the manufacturer's recommendations, and stored at 4°C. Total DNA concentrations were determined using a photo spectrometer at 260/280 nm. Genomic DNA was adjusted to final concentration of 50 ng/µL for PCR amplification.

### RAPD analysis

Genomic DNA pools of each species were used as templates for RAPD analysis (Table 1). DNA pool samples were prepared by mixing genomic DNA of all strains from each species into one unique sample with an equal ratio. A total of 20 RAPD primers (Operon Technologies, USA) were assayed for detecting *Vibrio shilonii* species (Table 2), these primers were used in our previous study [5] of randomly selected. Specific bands (absent in the other *Vibrio* species) were replicated twice for confirmation used for further studies. PCR-RAPD reactions were carried out according to Quang *et al*. [5].

| DNA pool codes | Species | No. of strains | Sample codes |
|---|---|---|---|
| N1 | *Vibrio parahaemolyticus* | 13 | VT19, VT34, VT41, VT44, VT59, VT62, VX01, K5, VT56, VC85, VC59, VC39, VC41 |
| N2 | *Vibrio shilonii* | 5 | VT23, VT26, VT27, VT29, VT33 |
| N3 | *Vibrio vulnificus* | 6 | VC15, VC17, VC21, VC28, VC37, VC67 |
| N4 | *Vibrio harveyi* | 3 | VC24, VC25, VC45 |
| N5 | *Vibrio cholera* | 3 | VC43, VC53, VC61 |
| N6 | *Vibrio communis* | 1 | VT47 |
| N7 | *Vibrio furnissii* | 1 | VT65 |
| N8 | *Vibrio brasiliensis* | 1 | VC52 |

**Table 1:** DNA pool of *Vibrio* samples [5].

### DNA fragment cloning and sequencing

The specific amplicon of *V. shilonii* was extracted, then purified with GeneJET Gel Extraction Kit (Thermo Scientific, Lithuania). The purified PCR product was cloned into pGEM-T Easy vector (Promega, USA) and transferred into *Escherichia coli* strain TOP10 competent cells heat shock method [13]. Positive clones were confirmed by PCR with M13 primers and sequenced at Firstbase company (Malaysia).

### SCAR marker development

Specific SCAR primers Vshi-441F and Vshi-441R were designed based on the sequenced RAPD amplicon (Table 3). Genomic DNA samples, including those from five *V. shilonii* strains and other pathogen *Vibrio* species, were amplified using the Vshi-441F and Vshi-441R primers to verify the SCAR marker specificity. A 12 µL PCR reaction system was developed containing 50 ng of total DNA, 10 pmol of each primer, 6 µL 2× GoTaq® Green Master Mix (Promega, USA), and sterile distilled water. PCR amplification was performed as follows: 95°C for 5 min; 35 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min; the final cycle at 72°C for 10 min.

To test detection sensitivity, 0.01–10 ng/µL of diluted genomic DNA of *V. shilonii* were evaluated as DNA templates for PCR amplification.

| Primer Code | Nucleotide sequence (5'-3') | Number of amplified bands for *V. shilonii* | Number of specific bands | Name of specific band |
|---|---|---|---|---|
| OPA-01 | CAGGCCCTTC | 1 | 0 | - |
| OPA-02 | TGCCGAGCTG | 3 | 1 | A02-350 |
| OPA-03 | AGTCAGCCAC | 3 | 0 | - |
| OPA-09 | GGGTAACGCC | 5 | 1 | A09-2000 |
| OPA-11 | CAATCGCCGT | 2 | 2 | A11-850 and A11-500 |
| OPB-01 | GTTTCGCTCC | 2 | 1 | B01-1200 |
| OPB-04 | GGACTGGAGT | 5 | 1 | B04-1600 |
| OPB-18 | CCACAGCAGT | 2 | 1 | B18-250 |
| OPC-13 | AAGCCTCGTC | 0 | 0 | - |
| OPD-02 | GGACCCAACC | 6 | 1 | D02-1200 |
| OPD-07 | TTGGCACGGG | 1 | 0 | - |
| OPD-11 | AGCGCCATTG | 3 | 1 | D11-1400 |
| OPF-04 | GGTGATCAGG | 4 | 0 | - |
| OPF-08 | GGGATATCGG | 0 | 0 | - |
| OPG-06 | GTGCCTAACC | 0 | 0 | - |
| OPG-10 | AGGGCCGTCT | 1 | 1 | G10-750 |
| OPG-17 | ACGACCGACA | 3 | 0 | - |
| OPK-13 | GGTTGTACCC | 2 | 0 | - |
| OPN-03 | GGTACTCCCC | 2 | 1 | N03-900 |
| OPN-06 | GAGACGCACA | 1 | 1 | N06-450 |
| | Total | 46 | 12 | |

**Table 2:** RAPD primer selection for *Vibrio shilonii.*

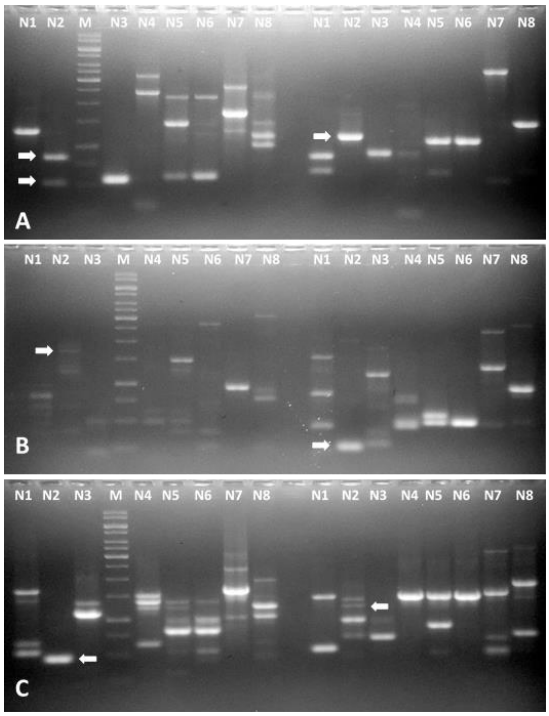


**Figure 1. Representative specific DNA bands from PCR-RAPD with a DNA pool template.** M. DNA molecular size marker (GeneRulerTM DNA 1 kb ladder, Thermo Fisher Scientific). White arrows indicate specific bands. (A) OPA-11 and OPB-01 primers. (B) OPB-04 and OPB-18 primers. (C) OPN-06 and OPD-02 primers.

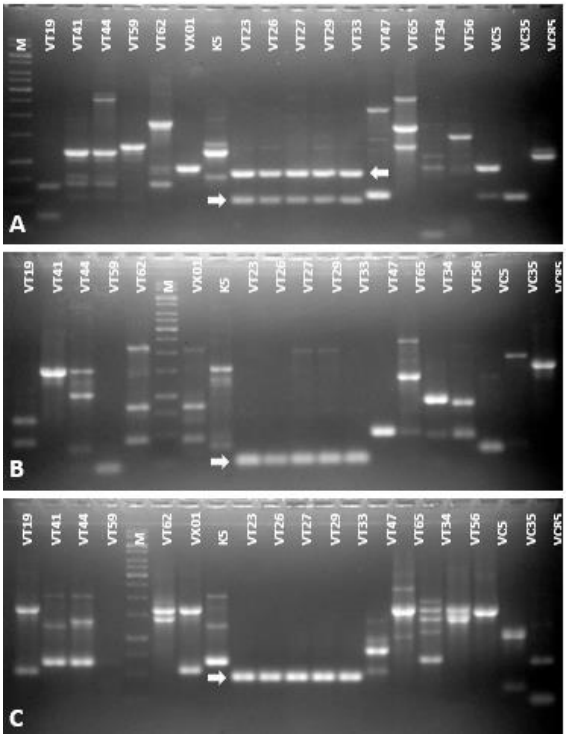


**Figure 2: RAPD profile of *Vibrio* spp. with selected primer.** M. DNA molecular size marker (GeneRulerTM DNA 1 kb ladder, Thermo Fisher Scientific). White arrows indicate the specific bands. (A) Primer OPA-11. (B) Primer OB-18. (C) Primer OPN-06.

## Results

### RAPD analysis

To reduce the number of test samples, we used a DNA pool instead of individual genomic DNA for specific RAPD amplicons. The DNA pool in this study was a mixture of genomic DNA of strains of each *Vibrio* species at an equal ratio: eight DNA pool samples for *V. parahaemolyticus*, *V. vulnificus*, *V. shilonii*, *V. cholera*, *V. communis*, *V. harveyi*, *V. furnissii*, and *V. brasiliensis* (Table 1).

From 20 tested random primers, we obtained 46 DNA fragments from *Vibrio shilonii* (0–6 DNA bands/primer); most of these were polymorphic bands (Figure 1). Twelve specific bands for *V. shilonii* were generated from 11 primers (Table 2), and these primers were used for

```
N06_468         GAGACGCACATAAGGTAAGTGCAGATTGTTACGCGGAAAATGTCGGTTCTTACAAAACTC
CP009355        TTAAGGCGCATAAAGTCAGTGCAGATTGCTACTCATCGAATGTGGGTTCATACAAAACGT
CP025797        TAAACGCCCATAAAGTCAGTGCCGATTGCTACGCTTCTAATGTTGGTTCTTACAAGACGT
CP025793        TAAACGCCCATAAAGTCAGTGCCGATTGCTACGCTTCTAATGCTGGCTCTTACAAGACGT
CP013485        TAAACGCCCATAAAGTCAGTGCGGATTGCTACGCTTCTAATGCTGGTTCTTACAAAACGT
                   * ** ***** ** ***** ***** *** *    ****  ** ** ***** **

N06_468         TTGAGAAGTGTGGCTTCGTTCAGGAAGGACGTCAGAAAGACTACTATAAGCTTGAGCACG
CP009355        TAGAGAAATGCGGCTTTAGCCAAGAAGGACGTCAGACAGACTATTACAAGCTAGAGGAAG
CP025797        TAGAGAAAAATGGTTTTACTCAAGAAGGCCGTCAGAAAGACTACTACAAACTAGAGCAAG
CP025793        TAGAGAAAAATGGTTTTACTCAGGAAGGCCTTCAGAAAGACTACTATAAACGAGAGCAAG
CP013485        TAGAGAAAAATGGTTTTACTCAAGAAGGCCGTCAGAAAGACTACTACAAACGAGAGCAAG
                * *****    ** **    ** ***** * ***** ****** ** ** *  *** * *

N06_468         GTTTTGATGACGAGCTCTATTATGGCATGACAGCCGAGCAGTTCAGTGCATTAAACCGCT
CP009355        GCTTGGAAGACAAGCTTTACTACGGTATGACAGCAGAGCAATTCGCCTCTTTCAATCGTT
CP025797        GCTTTGACGACAAGCTTTACTACGGTATGACCGCAGAACAATTCGCGTCTTTAAATCGCT
CP025793        GCTTTGACGATAAACTTTACTACGGTATGACCGCAGAGCAATTCGCGTCTTTAAATCGCT
CP013485        GCTTTGACGACAAGCTTTACTACGGCATGACCGCAGAACAATTCGCGTCTTTAAATCGCT
                * ** ** **  * ** ** ** ** ***** ** ** ** ***    * ** ** ** *

N06_468         -----------GATAAAGC--------------------------AAAAGGCCGTTAAA
CP009355        TAG-------TGATCAAGCGACGCGTTTAAACAGC------AAACGAAAAGGCCGCTA--
CP025797        AATAATTAGCTGACAAAAAAGTAGCTAACAACAACGTAGCCAACAAAAAAGGCCGTTAA-
CP025793        GATTATTAGCCGACAAAAAAGTAGCTAAAAACAAAGTAGTGAACAAAAAAGGCCGTTAA-
CP013485        AATAATTAGCTAACAAA----------------------------AAAGGCCGTTAA-
                           *  **                              ******** **

N06_468         CACCTTAGTCGGATTTAACGGCCTTTT-TGTATCAAGTG--------------CTTAGTT
CP009355        TGCCCAAGATCGGGTTAGCGGCCTTTTCTATATTAATAGACC-----CTAGGTCTTAGTT
CP025797        CCCACATGCTCGGGTTAACGGCCTTTTCTATATTCAGCGCTAAATGGCTAAGACTTAGTT
CP025793        CCCACATGCTCGGGTTAACGGCCTTTTCTATATTCAGCGCTAAATGGCTAAGACTTAGTT
CP013485        CCCAAATGCTCGGGTTAACGGCCTTTTCTATATTCAGCGCTAAATGGCAAAGGCTTAGTT
                  *    *   *  *** ********* * ***  *  *             *******

N06_468         AGCCTGGCTCTGGCTCTGAAGCTTCTCTTTCGCTTCTTCAACTTTTGAGAAGTCCAATCC
CP009355        GCCTTGATTTTGGCTTTGAAGCTTCTCTTTTGCTTCTTCAACTTTTGAGAAATCTAAACC
CP025797        GCCTTGGTTTTGGCTTTGAAGCTTCTCTTTTGCTTCTTCAACCTTTGAGAAATCCAATCC
CP025793        GCCTTGGTTTTGGCTTTGAAGCTTCTCTTTTGCTTCTTCAACCTTTGAGAAATCCAATCC
CP013485        GCCTTGGTTTTGGCTTTGAAGCTTCTCTTTTGCTTCTTCAACCTTTGAGAAATCCAATCC
                  * **  * ***** ************** *********** ******** ** ** **

N06_468         CAACTCTTCAACCGCTTCTTTGATTAGCGCAGGGTTTTGCATGACTTGCGCCATCAGCAT
CP009355        CAACTCATCGACGGCTTCTTTGATTAGCGCAGGGTTTTGCATCACTTGCGCCATCAGCAT
CP025797        CAACTCGTCTACGGCTTCTTTGATTAGCGCAGGGTTTTGCATCACTTGCGCCATCAGCAT
CP025793        CAACTCATCTACGGCTTCTTTGATTAGCGCAGGGTTTTGCATCACTTGCGCCATCAGCAT
CP013485        CAATTCGTCTACCGCTTCTTTGATTAGCGCAGGGTTTTGCATCACTTGCGCCATCAGCAT
                *** ** ** ** ***************************** *****************

N06_468         TTGAAGTTTGTCTTGAGGTAAGCCCAATTGACTGATAGTTGCCATTGCAGCAAAAGGGTT
CP009355        TTGTAGCTTGTCTTGTGGCAGACCTAGTTGGCTGATCGTTGCCATCGCTGCAAGAGGGTT
CP025797        TTGTAGCTTGTCTTGTGGTAAACCTAGTTGGCTGATTGTTGCCATTGCCGCTAGAGGGTT
CP025793        TTGTAGCTTGTCTTGTGGCAAACCTAGTTGGCTGATTGTTGCCATTGCCGCTAGAGGGTT
CP013485        TTGTAGCTTGTCTTGTGGCAAGCCTAGTTGGCTGATTGTTGCCATTGCCGCTAGAGGGTT
                *** ** ******** ** *  ** * *** ***** ******** ** ** * ******

N06_468         TTGTGTCAGAGTCTGAAAAATCTCGTTGATTTGTGCGTCTC
CP009355        TTGCGTCAGAGTTTGGAAGATTTCGTTGATCTGTTCATCGC
CP025797        TTGCGTCAGAGTCTGAAAAATCTCGTTGATTTGCTCATCGC
CP025793        TTGCGTCAGAGTCTGAAAAATCTCGTTGATTTGCTCATCGC
CP013485        TTGCGTCAGAGTCTGAAAAATCTCGTTGATTTGTTCGTCGC
                *** ******** ** ** ** ******** ** * *** *
```

**Figure 3: Multi-alignment of N06-450 marker with other *Vibrio* sequences.** CP009355, *V. tubiashii* ATCC 19109; CP025797, *V. owensii* 051011B; CP025793, *V. jasicida* 090810c; CP013485, *V. alginolyticus* ATCC 33787. Underlined sequences indicate SCAR primers.

amplification of all samples. Specific PCR products generated from these 11 primers were used to screen specific markers for *V. shilonii*. Results showed that the 12 specific bands were present in five strains of *V. shilonii* and absent in other *Vibrio* species (Figure 2). Of 12 PCR products, N06-450 fragment (designated as the primer name and expected size of the band) was chosen to develop the SCAR marker because it produced a clear band of the suitable size (~450 bp) and was sufficiently different from RAPD patterns of other pathogens. However, the OPN-06 primer amplified only one PCR product, so further research was easily performed with this band.

### SCAR marker development

The N06-450 band was chosen to develop the SCAR marker. Thus, the PCR band was cloned into pGEM-T Easy vector and performed DNA sequencing. The results indicated that the length of the amplicon was 468 bp with 44.4% GC content (A = 119, T = 141, G = 111, and C = 97) (Figure 3).

BLAST results showed no significant similar sequence on Genbank database. The similarity coefficient of the N06-450 sequence with other *Vibrio* species ranged from 74%–78%: *V. alginolyticus* ATCC 33787 (78.19%), *V. hyugaensis* 090810a (74.13%), *V. tubiashii* ATCC 19109 (73.52%), *V. owensii* 051011B (73.53%), or *V. jasicida* 090810c (72.75%) (Figure 3). No similar sequences were found from popular *Vibrio* pathogens such as *V. parahaemolyticus*, *V. brasiliensis*, *V. communis*, *V. furnissii*, and *V. harveyi.*

The primer pair Vshi-441F/Vshi-441R (Table 3) was designed based on the comparison of N06-450 sequences and others from Genbank (Figure 3 and Table 3). The Vshi-441F/Vshi-441R primers were selected to amplify on genomic DNA of pathogenic *Vibrio* isolates. A unique band with the size approximately of 441 bp was observed in *V. shilonii* isolates only (Figure 4), indicating that the designed SCAR marker was specific for this species, (this SCAR marker was named N6-441).

| Primer name | Sequence | Product length |
|---|---|---|
| Vshi-441F | 5'-GTTACGCGGAAAATGTCGGT-3' | 441 bp |
| Vshi-441R | 5'-GAGACGCACAAATCAACGAG-3' | |

**Table 3:** Sequences of SCAR primers.

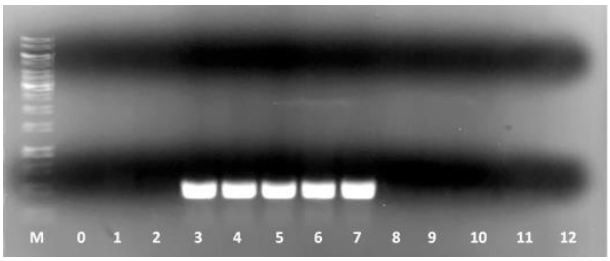


**Figure 4: Specific SCAR marker test.** M. DNA molecular size marker (GeneRulerTM DNA 1 kb ladder, Thermo Fisher Scientific). Lane 0, negative control; lane 1, *V. parahaemoliticus*; lane 2, *V. vulnificus*; lane 3-7, *V. shilonii*; lane 8, *V. cholera*; lane 9, *V. harveyi*; lane 10, *V. communis*; lane 11, *V. furnissii*; lane 12, *V. brasiliensis.*

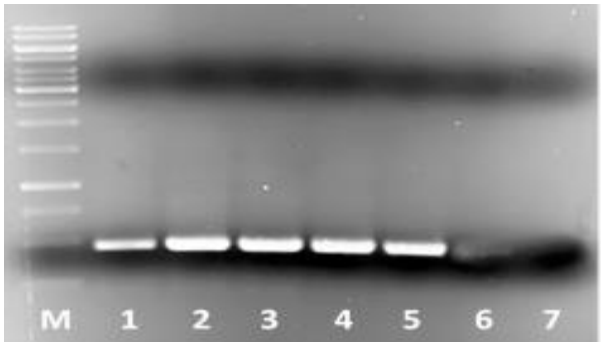


**Figure 5: Sensitivity test of SCAR primers**. M. DNA molecular size marker (GeneRulerTM DNA 1 kb ladder, Thermo Fisher Scientific). lane 1: 10 ng/µL; lane 2: 5 ng/µL; lane 3: 1 ng/µL; lane 4: 0.5 ng/µL; lane 5: 0.1 ng/µL; lane 6: 0.01 ng/µL; lane 7: negative control.

To evaluate the SCAR marker sensitivity for *V. shilonii* identification, serial diluted *V. shilonii* genomic DNA were used as templates for the PCR assay using Vshi-441F/Vshi-441R primers. The results revealed that this SCAR marker detected *V. shilonii* DNA at concentration of 100 pg/µL or higher (Figure 5).

## Discussion

*Vibrio* spp. have been traditionally identified by culture the isolate on selective blood agar medium in integration with biochemical and serological testing. These methods are simple but require a lot of time and technicians. Meanwhile, PCR method has high advantages for rapid identification of *Vibrio* spp. in laboratory targeting the defined DNA sequences on genomic DNA. Furthermore, this method is often selected to amplify the specific targeted DNA sequence of the heterogeneous collection [14].

RAPD is a PCR-based technique to amplify the target or random DNA fragment using random primers. RAPDs are simple assay with fast results. This technique does not require sequence data as well as randomly genome distribution, and nature dominant. RAPD marker disadvantage is that this method is not able to identify the organism species due to their low reproducibility and nature dominant. Thus, RAPD marker recently is being developed into a codominant and reproducible marker (SCAR), which is able for species identification [15]. Using SCAR markers for identification of animal pathogens has been reported for such pathogens as *Eimeria* species [9] and *Actinobacillus pleuropneumoniae* [16].

Molecular diagnostic methods for *Vibrio* spp. detection based on PCR have been reported including loop-mediated isothermal amplification assay for *V. parahaemolyticus* [17], multiplex PCR for *Vibrio* spp. [18], or *V. vulnificus* [19]. Conventional PCR for specific *Vibrio* spp. genes also have been reported [20]. However, to our knowledge, no SCAR marker methods for diagnosing *Vibrio* spp. have been published heretofore.

In this study, we developed a rapid and highly specific SCAR marker for *V. shilonii* identification and detection.

The method was developed based on specific primers designed for conventional PCR which is able to replace the traditional microbiology method. Our newly developed method is applicable for epidemiological studies as well as contribution to the protection programs for aquaculture.

Using the RAPD technique, we found a high diversity of *Vibrio* species except for *V. shilonii*, which is crucial for specific DNA fragments selection of the species. The designed primers for *V. shilonii* was evaluated the specificity by sequencing the amplified regions, followed by BLAST analysis because these sequences did not show significant similarities within the NCBI database (Figure 3).

The results in this study therefore show the SCAR primers can be applied to directly detect *V. shilonii* strains. The specificity of these primers was evaluated based on genomic DNA from eight *Vibrio* spp. demonstrated the positive amplification only observed on DNA of the targeted species.

The results of this study show a high level of polymorphism in the RAPD pattern of the different *Vibrio* species, and a SCAR marker (N6-441) was developed based on the 468-bp sequence specific to *V. shilonii*. Specific primers (Vshi-441F/Vshi-441R) amplified an unique DNA fragment for all *V. shilonii* isolates but not in the other *Vibrio* spp. This PCR method was highly sensitive to the *V. shilonii* genome and thus may be useful in rapid detection of this pathogenic bacterium.

## Authors' Contribution

Hoang Tan Quang: experimental design, data analysis, paper preparation
Pham Thi Diem Thi: experimental performant
Tran Thuy Lan: experimental performant
Nguyen Duc Huy: paper preparation
Nguyen Duy Quynh Tram: experimental performant
Nguyen Thi Thu Lien: project leader, experimental design, paper preparation

## Competing Interest

The authors declare that they have no competing interests.

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