INDIAN JOURNAL OF SCIENCE AND TECHNOLOGY

ORIGINAL ARTICLE

Genetic diversity and toxic genes analysis of *vibrio* spp. isolated from white leg shrimp and marine fishes cultured in Tam G iang lagoon in Thua Thien Hue province, Vietnam

Hoang Tan Quang[1], Tran Thuy Lan[1], Truong Thi Hong Hai[1], Pham Thi Hai Yen[2], Tran Quang Khanh Van[2], Ho Thi Tung[2], Mac Nhu Binh[2], Nguyen Khoa Huy Son[2], Nguyen Quang Linh[3], Nguyen Duy Quynh Tram[2]*

1 Institute of Biotechnology, Hue University, Hue, 530000, Vietnam
2 University of Agriculture and Forestry, Hue University, Hue, 530000, Vietnam
3 Hue University, Hue, 530000, Vietnam

OPEN ACCESS

Received: 16-04-2020
Accepted: 23-04-2020
Published: 23-05-2020

Editor: Dr. Natarajan Gajendran

Citation: Quang HT, Lan TT, Hai TTH, Yen PTH, Van TQK, Tung HT, Binh MN, Son NKH, Linh NQ, Tram NDQ (2020) Genetic diversity and toxic genes analysis of vibrio spp. isolated from white leg shrimp and marine fishes cultured in Tam Giang lagoon in Thua Thien Hue province, Vietnam. Indian Journal of Science and Technology 13(13): 1412-1422. https://doi.org/10.17485/IJST/v13i13.161

***Corresponding author**.
Nguyen Duy Quynh Tram

University of Agriculture and Forestry, Hue University, Hue, 530000, Vietnam
ndqtram@hueuni.edu.vn

Funding: This study was financial supported by the grant from Vietnam Ministry of Education and Training (Project No. CT-2018-DHH-01)

Competing Interests: None

Published By Indian Society for Education and Environment (iSee)

Abstract

Objective: This study was done to report the results of genetic diversity and toxic genes analysis of *Vibrio* pathogen isolated from white leg shrimp and marine fishes cultured in Thua Thien Hue province, Vietnam. **Methods/statistical analysis:** Pathogen *Vibrio* spp. were isolated from shrimps and fishes, and were identified by 16S rRNA sequencing. The presence of toxin genes in *Vibrio* spp. strains were determined through the presence of genes encoding toxic proteins (*pirAvp, pirBvp, tlh, tdh and trh*) based on specific primers for these genes. Genetic diversity of *Vibrio* strains was analysed by RAPD technique. **Findings:** A total of 120 *Vibrio* colonies from shrimps (with Acute Hepatopancreatic Necrosis Disease) and fishes (with hemorrhagic disease) cultured in Tam Giang lagoon in Thua Thien Hue, Vietnam were isolated. Of which, 14/54 strains from shrimps had *pirAvp* and *pirBvp* genes and 18/66 strains from fishes had *tlh* gene, and none of *Vibrio* strains had *tdh* and *trh* genes. Randomly amplified polymorphic DNA (RAPD) analysis of 36 *Vibrio* samples showed the 148 polymorphic DNA fragments from ten random primers. The genetic diversity is high within studied species. In there, *V. parahaemolyticus* has the highest diversity level (h=0.1645 and I=0.2695) while *V. shilonii* is lowest (h=0.0136) and I=0.0207). The degree of genetic differentiation among species is also high (Gst=0.4827). Genetic identity between *V. parahaemolyticus* and *V. vulnificus* (0.9545) is highest while between *V. shilonii* and *V. harveyi* (0.7416) is lowest. The dendrogram also showed that *V. parahaemolyticus* is closely related to *V. vulnificus* whereas *V. shilonii* and *V. harveyi* have large distance. **Application/improvements:** This study is aimed to provide scientific data as the basis for the study and production of rapid diagnostic kits in the future.

Keywords: AHPND; Genetic diversity; RAPD; toxic genes; Vibrio; Vietnam

1 Introduction

Vibrio spp. are found in all over the world in marine and estuarine environments. In fish, this genus is present as a part of the normal intestinal microflora. Some *Vibrio* spp. (such as *V. anguilarum, V. harveyi,* and *V. parahaemolyticus*) can cause dangerous diseases in shrimp [(1)].

In shrimp, Acute Hepatopancreatic Necrosis Disease (AHPND) has caused remarkable mortality (up to 100%) in populations of shrimp cultured in South East Asian and Latin American countries. This disease is referred to as early mortality syndrome (EMS). AHPND is caused by several *Vibrio* species, which secretes proteins similar to Photorhabdus insect-related (Pir) toxins (PirAvp and PirBvp) [(1,2)]. *Vibrio* spp. are also mainly pathogenic to brackish water and marine fish. The distribution of vibriosis is worldwide and causes economic loss to the aquaculture industry [(3)].

The Tam Giang lagoon in Thua Thien Hue province of Vietnam is the biggest lagoon in the Southeast Asia. Aquaculture has developed in the Tam Giang Lagoon since the late 1970s, and has become the most important livelihood activity since the early 1990s. However, aquaculture production 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 has serious impacts on the health of the aquatic animals. In addition, the increased intensity of the flood seriously affects aquaculture production through changing of water quality and salinity. Many farmers lost their production due to serious Vibrio bacterial and viral shrimp diseases related to increasing water temperature [(4,5)].

Randomly amplified polymorphic DNA (RAPD) is a PCR-based genotyping technique, using random primers to detect changes in the DNA sequence. According to Behura et al (2015), RAPD was a reliable and fast technique for discriminating between the species of Vibrio, therefore, this is a powerful tool for these prawn pathogens study [(6)].

In Thua Thien Hue, to our knowledge there is limited published data available on the genetic diversity and toxic genes analysis of *Vibrio* isolated from white leg shrimp and marine fishes cultured. This study is therefore aimed to provide scientific data as the basis for the study and production of rapid diagnostic kits.

2 Materials and Methods

2.1 Sample collection

Seventy samples of shrimp (body weight of 0,64 g $\pm$ 0,22 and length of 4,37 cm $\pm$0.29 Hepatopancreatic Necrosis Disease syndromes were collected from white-leg shrimp (*Litopenaeus vannamei*) at farms in Dien Hai (n=8), Dien Mon (n=12) and Dien Huong (n=15) communes (Phong Dien district), Phu Thuan town (n=15) (Phu Vang district) and Loc Binh commune (n=20) (Phu Loc district) (Figures 1 and 2). Sampling was done with live shrimps which showed signs of pathology: stopped eating; swim slowly; pale, atrophy, and toughness in the liver and empty intestine. The outer surface of the shrimp body was disinfected with 70° alcohol before bacterial isolation. *Vibrio* sp. was isolated from their liver and pancreas, then samples were cultured on TCBS medium (Thiosulfate Citrate Bile Sucrose Agar) at 30°C for 24 hours.

Thirty-two samples of fishes including seabass (*Lates calcarifer*, n=12), red drum (*Sciaenops ocellatus*, n=15) and grouper (*Epinephelus fuscoguttatus*, n=5) with haemorrhage were collected from cages (in Hai Duong communes and Thuan An town, Phu Vang district) had typical signs: stopped eating, and swimming slowly (Figure 1 and 2). The external signs of three fish species were hemorrhage on body, abdomen, fin erosion, tailless condition and protruding eyes. The fish was disinfected with 70° alcohol and cleaned, using the sterile implants for sampling bacteria from the hemorrhage areas on the body and bacterial samples in the heart, liver, kidneys, intestines, spleen and brain for culturing on TCBS medium, incubated at 30°C for 24 hours.

After 24 hours, the development of colonies was checked. The types of colonies on plates were distinguished based on color, shape and size of colonies. The dominant colonies were transferred to TCBS medium to select pure colonies for further studies.

A total of 120 Vibrio spp. colonies were isolated on TBSC medium from shrimps (54 colonies) and fishes (66 colonies) in Thua Thien Hue province, Vietnam for toxic genes determination.

2.2 DNA isolation

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

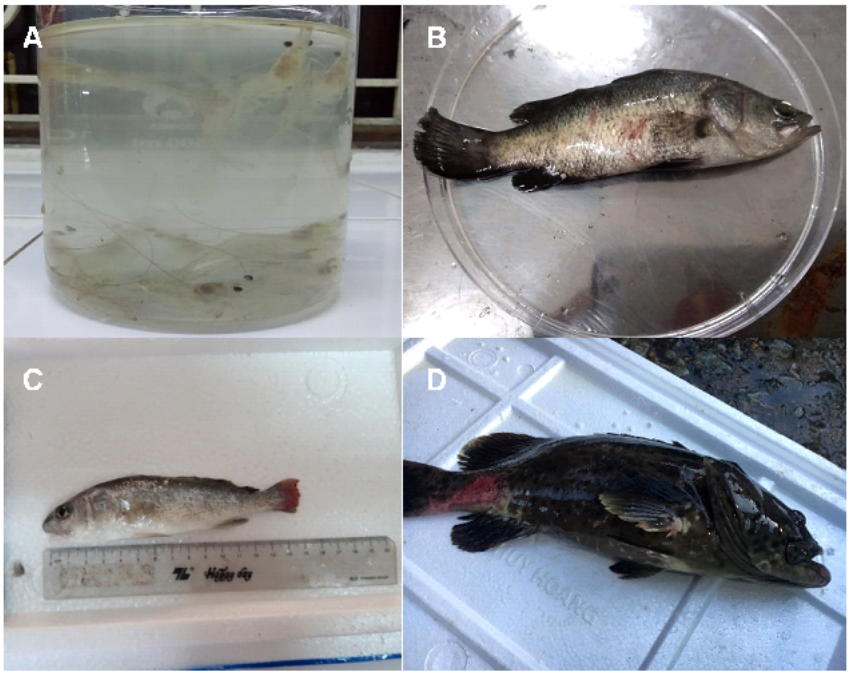

Fig 1. Samples for *Vibrio* isolation. A. Litopenaeus vannamei, B. Lates calcarifer, C. Sciaenops ocellatus, D. Epinephelus fuscoguttatus.

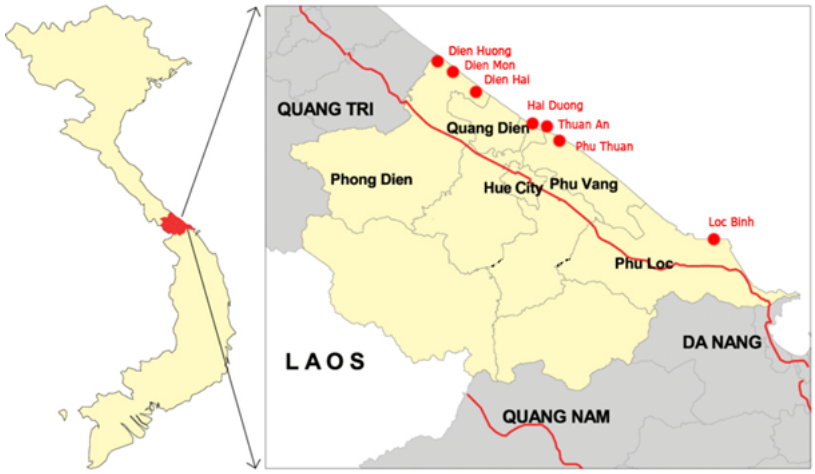

Fig 2. Map of samples collection (red spots)

2.3 Determination of toxin gene

The presence of toxin genes in *Vibrio* spp. strains were determined through the presence of genes encoding toxic proteins (*pirAvp, pirBvp, tlh, tdh* and *trh*) based on specific primers for these genes (Table 1).

PCR procedure: 50 ng of total DNA, 10 pmol of each primer, 6 μL 2× Go Taq® Green Master Mix (M7502, Promega, USA), and sterile distilled water (total volume of 12 μL). PCR amplification was performed in MJ Mini ™ Thermal Cycler (Bio-Rad, USA) as follow: 95°C for 10 minutes; followed by 30 cycles at 95°C for 30 seconds, 53°C for 30 seconds, and 72°C for 1 minute; last cycle was 72°C for 10 minutes. PCR products were used for electrophoresis on 0.8% agarose gel, stained by SafeView™ Classic Nucleic Acid Stain (Applied Biological Materials Inc., Canada) and determined by Ultra Slim LED Illuminator system.

Table 1. Sequence of primers

Genes	Primer names	Nucleotide sequences		Size (bp)	References
pirAvp	VpPirA-284	5'-TGACTATTCTCACGATTGGACTG-3' CACGACTAGCGCCATTGTTA-3'	5'-	284	(1)
pirBvp	VpPirB-392	5'-TGATGAAGTGATGGGTGCTC-3' TGTAAGCGCCGTTTAACTCA-3'	5'-	392	(1)
tlh	tl	5'-AAAGCGGATTATGCAGAAGCACTG-3' GCTACTTTCTAGCATTTTCTCTGC-3'	5'-	450	(7)
trh	L-trh R-trh	5'-TTGGCTTCGATATTTTCAGTATCT-3' CATAACAAACATATGCCCATTTCCG-3'	5'-	410	(5)
tdh	L-tdh R-tdh	5'-GTAAAGGTCTCTGACTTTTGGAC-3' TGGAATAGAACCTTCATCTTCACC-3'	5'-	245	(5)

2.4 Vibrio identification

Vibrio strains with *pirAvp* and *pirBvp* genes (from shrimps) or *tlh, tdh* and *trh* genes (from fishes) were identification based on the sequencing of 16S rDNA genes (Table 2 and 3). The total DNA was used as a template for the 16S rDNA amplification using the primer pair of 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR products are purified and sequenced at Firstbase company (Malaysia). The nucleotide sequences of 16S rDNA were used for species identification with BLAST tool on Genbank. Phylogenetic tree was built with MEGA X software.

2.5 Genetic diversity

Genomic DNA were used as template for RAPD analysis, including 14 strains with *pirABvp* genes, 18 strains with *tlh* gene and 4 non-toxic genes strains (Table 2 and 3). PCR reactions were carried out according to Quang et al (2016). Each reaction contained 10 μl 2× PCR master mix (GoTaq Green Master Mix 2X, Promega, USA), 20 pmol each primers and 50 ng genomic DNA in a 20 μL final volume. Amplification was performed in a thermocycler (SimpliAmp, ThermoFisher Scientific, USA) under the following conditions: 3 min at 95°C; followed by 42 cycles for 1 min of denaturing at 92°C; 1 min of annealing at 36°C and a 2 min extension at 72°C; a final extension for 10 min at 72°C. PCR products were separated on a 1.4% agarose gel for 5 hr at 40 V[8]. Samples with lack of bands in agarose gel were replicated twice to get confirmation. Ten random decamer oligonucleotide primers (Operon Technologies, USA) were selected for evaluation (Table 4).

PCR-RAPD amplicons were scored as presence (1) or absence (0) of a band in each sample. The sizes of the RAPD markers were estimated by using the DNA size standard (GeneRuler 1kb DNA Ladder, Thermo Scientific). The genetic identity and genetic distance between populations were expressed using Nei's (1972) genetic distance[9]. Genetic parameters were calculated as the total genotype diversity in populations (Ht), total genotype diversity within populations (Hs), mean coefficient of gene differentiation (Gst), and estimate of gene flow (Nm); number of polymorphic bands, observed number of alleles (na), effective number of alleles (ne), Nei's (1973) gene diversity (h), Shannon's information Index (I) for RAPD data using the POPGENE software ver. 1.31[10].

3 Results

3.1. Isolation of pathogenic *Vibrio*

From 120 *Vibrio* samples, we found that 14 strains (from shrimps) and 18 strains (from fishes) had *pirAvp* and *pirBvp* genes or tlh gene, respectively (Tables 2 and 3). *V. parahaemolyticus* is the most popular species that cause diseases in shrimp and fish, and has both *pirABvp* and tlh genes. *V. shilonii* displayed only in shrimp while *V. vulnificus, V. harveyi* and *V. cholerae* appeared only in fish.

In shrimp, the pVPA3-1 plasmid causes AHPND was identified in *V. parahaemolyticus* strain 13-028/A3, it consists of genes that encode mobilization proteins, replication enzymes, virulence-associated proteins, and proteins similar to Pir toxins. These Pir toxin-like proteins are encoded by 2 genes (*pirA*- and *pirB*-like), also known as *pirAvp* and *pirBvp* genes[1]. In Thua Thien Hue province, 14/54 samples from shrimp had *pirABvp* genes (25.93%), including *V. parahaemolyticus, V. shilonii*, *V. communis* and *V. furnissii* species. The bacterium *V. shilonii* was found in the first time in Vietnam. *V. shilonii* had been found to be a cause of bleaching in the coral *Oculina patagonica*[11], in recent paper, *V. shilonii* caused AHPND was found in Ecuador[1]

In fish, the majority of *Vibrio* strains isolated were not pathogenic. The pathogenic strains are those that produce thermostable

Table 2. Isolation of pathogenic Vibrio isolates from shrimp

	Sample codes	Strain	Colonies morphology	Location	Genes	
					pirA	*pirB*
1	VT19	*V. parahaemolyticus* TX07-3/3	Green, small center, serrated edges	Phu Thuan	+	+
2	VT23	*V. shilonii* TX05-3/3	Green, big center, non-round edges	Phu Thuan	+	+
3	VT26	*V. shilonii* TX03-3/3	Green, non-center, round edges	Phu Thuan	+	+
4	VT27	*V. shilonii* TX02-3/3	Green, big center, round, medium from green to yellow	Phu Thuan	+	+
5	VT29	*V. shilonii* TX01-3/3	Green, dark center, round	Phu Thuan	+	+
6	VT33	*V. shilonii* TV02-3/3	Yellow, big center, round, medium from green to yellow	Phu Thuan	+	+
7	VT34	*V. parahaemolyticus* 29X2 - 13/5	Green, round, smooth, 2 mm diameter	Dien Huong	-	-
8	VT41	*V. parahaemolyticus* K31-X-13/6/2019	Green to yellow, round, smooth, 2 mm diameter, medium from green to yellow	Dien Huong	+	+
9	VT44	*V. parahaemolyticus* K39-X-13/6	Green to yellow, round, smooth, 2 mm diameter	Dien Huong	+	+
10	VT47	*V. communis* R-PD-K4-V-13/6	Yellow, round, smooth, 2 mm diameter, medium from green to yellow	Dien Mon	+	+
11	VT56	*V. parahaemolyticus* PD-K1-10/6	Green, round, smooth, 2 mm diameter	Dien Mon	-	-
12	VT59	*V. parahaemolyticus* R-PD-K3-10/6	Yellow, round, 1.5 mm diameter, medium from green to yellow	Dien Mon	+	+
13	VT62	*V. parahaemolyticus* R-PD-K3-10/6	Green, round, smooth, 2 mm diameter	Dien Mon	+	+
14	VT65	*V. furnissii* R-K39-13/6	Yellow, round, 2 mm diameter	Dien Huong	+	+
15	VX01	*V. parahaemolyticus* VX01	Green, viscous, round, smooth, 2 mm diameter	Dien Huong	+	+
16	K5	*V. parahaemolyticus* K5	Green, round, convex	Dien Huong	+	+

direct haemolysin (TDH) toxin. TDH is an enzyme that lyses human red blood cells on Wagatsuma blood agar plates, which is referred to as the Kanagawa phenomenon positive. Another toxin produced by Kanagawa phenomenon negative *Vibrio* strains is the TDH-related hemolysin (TRH) toxin encoded by *trh* gene[12]. Thermolabile hemolysin (TLH) is an another *Vibrio* enterotoxin that cause blood cell lysis in infected fish, TLH is encoded by *tlh* gene[13]. In this study, 18/66 strains had *tlh* gene (27.27%), including *V. vulnificus*, *V. harveyi*, *V. parahaemolyticus*, *V. cholerae*, *V. brasiliensis, V. fluvialis* and *V. natriegens.* None of *Vibrio* strains had *tdh* and *trh* genes, so maybe hemorrhagic disease in fish at Thua Thien Hue province was caused by TLH toxin. Our study is in accordance to report by Chosin et al. (2015), none of the eight AP-positive AHPND *V. parahaemolyticus* strains possesses the genes for the conventional virulence factors affecting humans, such as *tdh*, *trh* and type III secretion system 2[14]. In Bangladesh, Ahmmed et al. (2019) found *tlh*, *pirA* and *pirB* genes from two AHPND positive *V. parahaemolyticus* strains isolated from shrimp (*Penaeus monodon*)[15].

The previous studies in Malaysia showed that the most virulent of the non-cholera vibrios (*V. vulnificus*) have various virulence factors that facilitate the development of clinical disease[16]. *V. vulnificus* also have been implicated in fish diseases such as septicemia[3]. In our study, *V. vulnificus* (6/18) was the most popular patogen species in fish.

Vibrio spp. that caused disease in shrimp and marine fish are widely distributed in brackish and saline waters worldwide. They exist in the aquatic environment and can be harmful to shrimp and fish in favorable conditions.

In shrimp, the presence of *Vibrio* spp. such as *V. parahaemolyticus, V. alginolyticus, V. vulnificus*, etc. are likely to cause AHPND[17]. The disease has caused huge losses, high mortality, and a very high risk for shrimp farming from 2010 to the

Table 3. Isolation of pathogenic *Vibrio* isolates from fishes with hemorrhagic disease

	Sample codes	Strains	Colonies morphology	Location	Organs	Host	Genes		
							tlh	***tdh***	***trh***
1	VC15	*V. vulnificus* HM-TA-D2-L2-V2	Yellow, medium from green to yellow	Thuan An	Skin	*Sciaenops ocellatus*	+	-	-
2	VC17	*V. vulnificus* HM-TA-G2-V1-Đ2	Yellow, medium from green to yellow	Thuan An	Liver	*Sciaenops ocellatus*	+	-	-
3	VC21	*V. vulnificus* CTX1-3/3	Green, non-center, round	Hai Duong	Kidney	*Lates calcarifer*	+	-	-
4	VC24	*V. harveyi* CDV1-3/3	Yellow, non-center, serrated edges, medium from green to yellow	Hai Duong	Skin	*Lates calcarifer*	+	-	-
5	VC25	*V. harveyi* CTV1-3/3	Yellow	Hai Duong	Kidney	*Lates calcarifer*	+	-	-
6	VC28	*V. vulnificus* CTX3-3/3	Green, small center, non-round edges	Hai Duong	Kidney	*Lates calcarifer*	+	-	-
7	VC35	*V. vulnificus* LTA.CC.X.01-24/3	Green, non-center, non-round edges, smooth	Thuan An	Kidney	*Lates calcarifer*	-	-	-
8	VC37	*V. vulnificus* LTA.CC.X.02	Green, non-center, non-round edges, smooth, medium from green to yellow	Thuan An	Kidney	*Lates calcarifer*	+	-	-
9	VC39	*V. parahaemolyticus* LTA.CC.X.03	Green, big-center, round, smooth, medium from green to yellow	Thuan An	Kidney	*Lates calcarifer*	+	-	-
10	VC41	*V. parahaemolyticus* LTA.CC.X.04	Green, big-center, non-round edges, smooth	Thuan An	Kidney	*Lates calcarifer*	+	-	-
11	VC43	*V. cholerae* LTA.CC.V.01	Yellow, small-center, round, smooth, medium from green to yellow	Thuan An	Kidney	*Lates calcarifer*	+	-	-
12	VC45	*V. harveyi* LTA.CC.V.02	Yellow, dark center, non-round edges, rough, medium from green to yellow	Thuan An	Kidney	*Lates calcarifer*	+	-	-
13	VC52	*V. brasiliensis* HM-X-13/6	Green, round, 2 mm	Hai Duong	Kidney	*Sciaenops ocellatus*	+	-	-
14	VC53	*V. cholerae* V-13/6	Yellow, round, smooth, 3 mm diameter	Hai Duong	Spleen	*Sciaenops ocellatus*	+	-	-
15	VC59	*V. parahaemolyticus* HM-17/6	Green, round, 2 mm diameter	Hai Duong	Gut	*Sciaenops ocellatus*	+	-	-
16	VC61	*V. cholerae* HM-V-13/6	Green, round, 1 mm diameter, medium from green to yellow	Hai Duong	Skin	*Sciaenops ocellatus*	+	-	-
17	VC67	*V. vulnificus* HM-X-13/6	Green, round, 4 mm diameter	Hai Duong	Skin	*Sciaenops ocellatus*	+	-	-
18	VC72	*V. fluvialis* ML2-13/8	Yellow, non-center, 4 mm diameter, medium from green to yellow	Thuan An	Spleen	*Epinephelus fuscoguttatus*	+	-	-
19	VC81	*V. natriegens* MT6-13/8	Yellow, non-round edges, 4 mm diameter, medium from green to yellow	Thuan An	Kidney	*Epinephelus fuscoguttatus*	+	-	-
20	VC85	*V. parahaemolyticus* MD8-13/8	Yellow, round, 2 mm diameter, medium from green to yellow	Thuan An	Skin	*Epinephelus fuscoguttatus*	-	-	-

present. Clinical signs of the disease include an empty gastrointestinal tract, an opaque stomach, a white atrophic pancreas, lethargic shrimps, anorexia and soft shells[18]. Diseases caused by *Vibrio* bacteria appear all year round but often out break when high water temperature and in high salinity waters, somehow the disease can still occur in the estuaries or for a number of freshwater fish species.

Although bacteria can be infected in different parts, they often attack the heart and muscles of the fish. Because they have a strong ability to attack the muscular system, hence it is very serious and difficult to treat, bacteria can cause chronic diseases in adults. If the disease occurs acute, the consequences of damage are very heavy.

In fish larvae, fry or fingerlings, when seriously infected they can be killed up to 50%. In adult fish, the rate of damage is lower but the fish will develop anorexia or stop eating or ceases in growth and at harvest it is possible to observe necrotic wounds on the skin and muscles of the fish. When the fish were infected with Vibrio, fish eats less or stops eating, they swim on the surface layer and around the cage edge. On the fish body, often appear various small reddish ulcers with the skin bulging surrounding it and a lot of viscous. The internal organs, liver, kidney, spleen with hemorrhage would have occurred and empty gastrointestinal tract[5].

Restrepo et al. (2018) reported that the gene transfer capacity of *Vibrio* species goes beyond the clade classification, creating new *Vibrio* pathogenesis and has major implications for the spread of emerging diseases[19]. In our study, ten *Vibrio* species contain toxic genes (*pirA, pirB*, and *tlh*), they seem to be the results of gene transfer of among *Vibrio* species in Vietnam sea.

3.2 RAPD analysis

A total of 20 RAPD primers were assayed for their specificity in detecting *Vibrio* species, ten primers were highly reproducible and found suitable for use in RAPD-PCR. These selected primers generated total of 148 amplified bands, ranging in size from approximately 200 (OPB-18) to 4000 bp (OPB-01 and OPN-03), and the number of PCR products of each primer varied from 11 to 17 (Table 4). All of bands were polymorphic (with a mean of 14.80 bands per primer). OPG-17 is the best primer for *Vibrio* with 100% genotypes were amplified (15 bands) (Figures 3 and 4).

Table 4. Sequence of RAPD primer, band sizes, and number of amplified bands

Primercode	Nucleotide sequence (5'-3')	Number of amplification genotypes	Size range of amplified bands (bp)	Number of amplified bands
OPA-03	AGTCAGCCAC	32	250-3500	15
OPA-11	CAATCGCCGT	35	350-3100	16
OPB-01	GTTTCGCTCC	33	500-4000	16
OPB-18	CCACAGCAGT	34	200-2500	15
OPC-13	AAGCCTCGTC	31	320-3500	13
OPD-02	GGACCCAACC	34	300-3300	17
OPD-07	TTGGCACGGG	35	320-2700	11
OPG-17	ACGACCGACA	36	320-3000	15
OPN-03	GGTACTCCCC	30	350-4000	13
OPN-06	GAGACGCACA	35	250-3000	17
Overall			200-4000	148
Mean				14.80

In this study, the percentage of polymorphic bands (PPB) of each population was ranged from 25.80% (*V. shilonii*) to 100% (*V. parahaemolyticus* and *V. vulnificus*) (Table 5). The PPB of all samples was 79.08%. The total amplicon among all *Vibrio* species varied from 45 (*V. cholerae*) to 105 (*V. parahaemolyticus*). *V. shilonii* had the different RAPD pattern from other *Vibrio* species in number of PCR amplicons (31), number of polymorphic loci (8), percentage of polymorphic bands (25.80%) and number of specific loci (1).

3.3 Genetic diversity

Data for observed number of alleles (na), effective number of alleles (ne), Nei's (1973) genetic diversity (h), Shannon's information index (I), for all the *Vibrio* species were analyzed using ten RAPD primers and their respective values were found as 2.0000, 1.2885, 0.2046, and 0.3435, respectively (Table 6). *V. shilonii* displayed the lowest value of Nei's (1973) gene diversity (h=0.0136) and Shannon's information index (I=0.0207), whereas the highest population was *V. parahaemolyticus* (h=0.1645

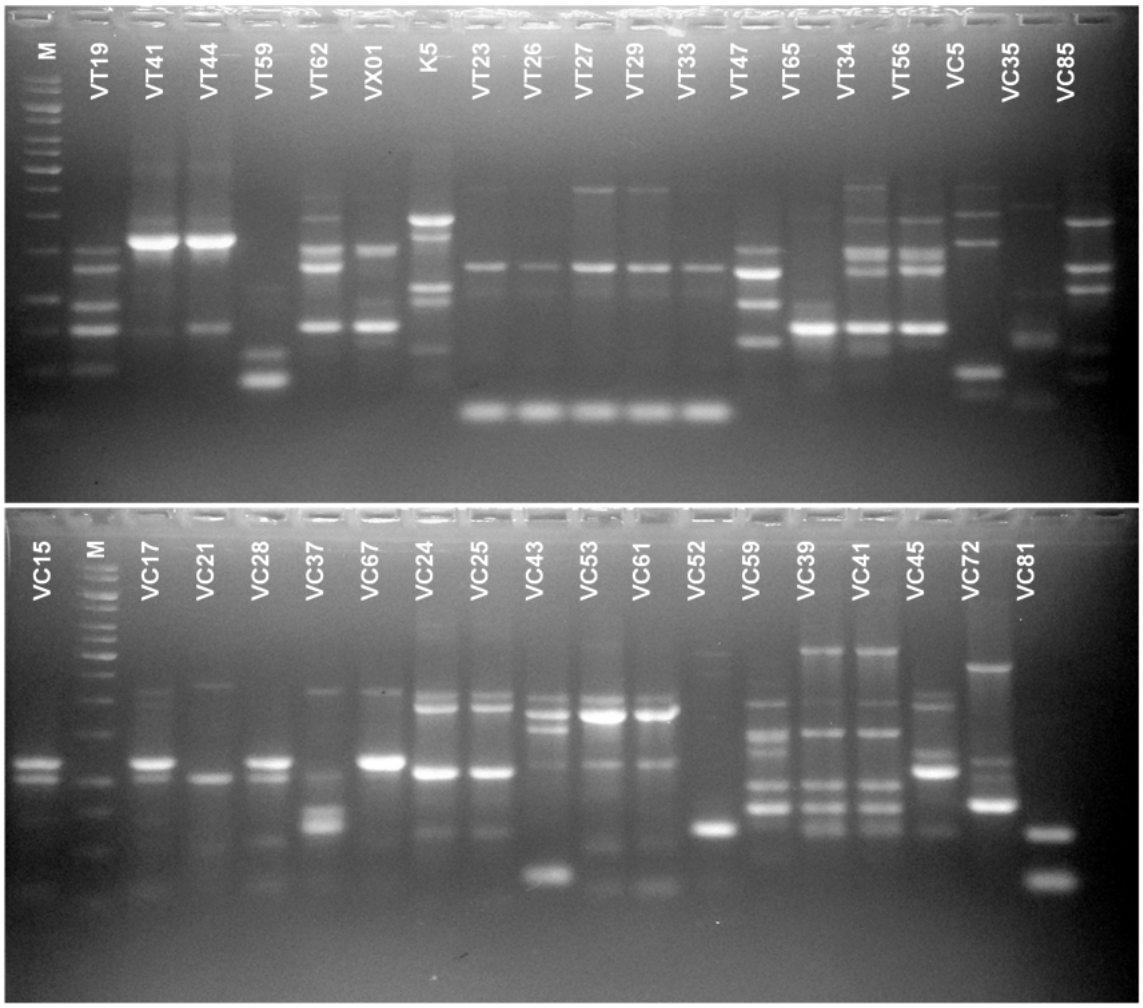

Fig 3. PCR products of OPG-17 primer of 36 samples. M. DNA molecular size marker (GeneRuler^{TM} DNA 1 kb ladder, Thermo Fisher Scientific). Sample codes were located at the top of lane.

Table 5. Summary of PCR amplicons of *Vibrio* in Thua Thien Hue using RAPD markers

Species	Number of PCR amplicons	Number of polymorphic loci	Percentage of polymorphic bands	Number of specific loci
V. parahaemolyticus	105	105	100	0
V. vulnificus	61	61	100	0
V. shilonii	31	8	25.80	1
V. harveyi	55	42	76.36	3
V. cholerae	45	33	73.33	0
Others*	97	96	98.97	0
Mean	65.67	57.5	79.08	0.67
Overall				4

*Others: samples including *V. communis, V. furnissii, V. brasiliensis, V. fluvialis* and *V. natriegens* species.

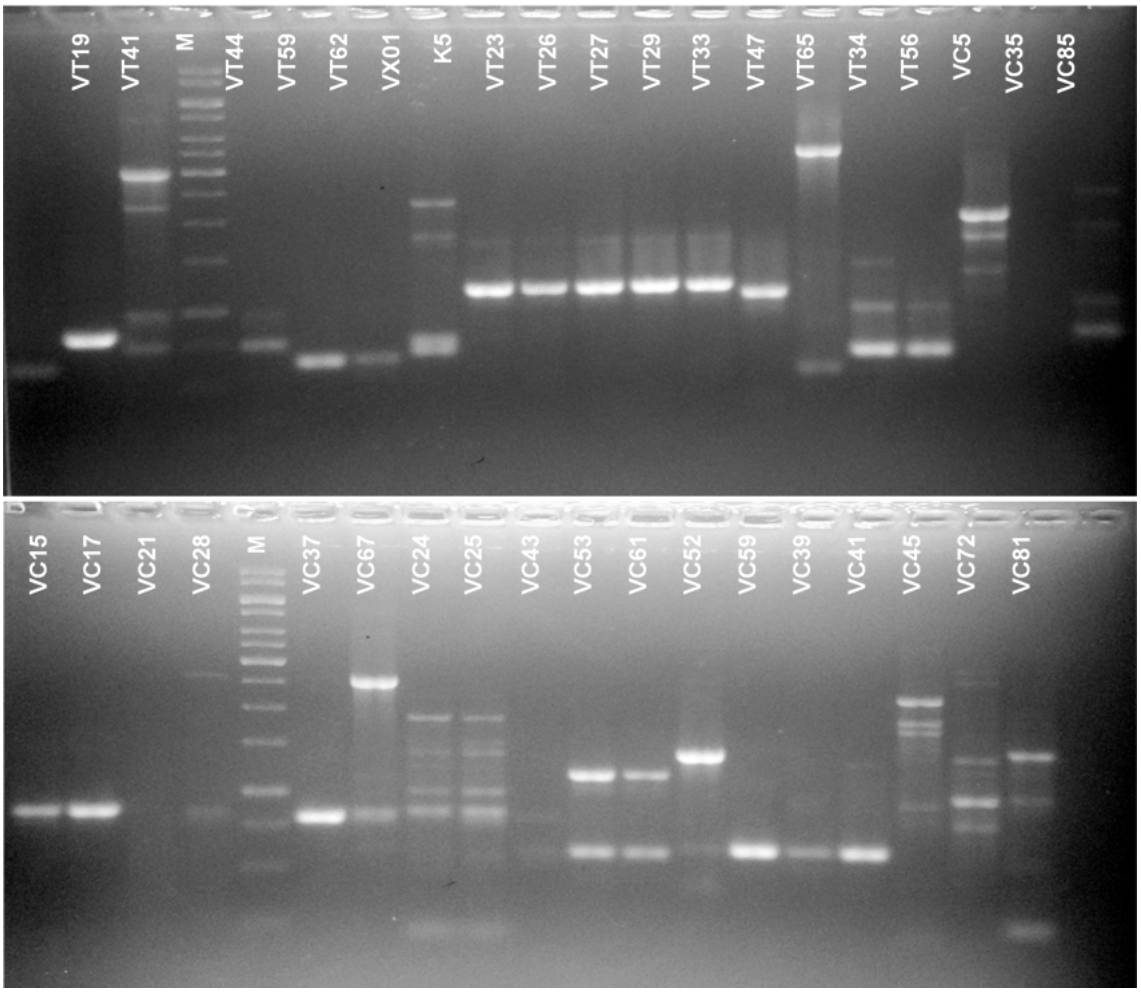

Fig 4. PCR products of OPB-01 primer of 36 samples

and I=0.2695) (Table 6).

Table 6. Summary of genetic parameters estimate for 5 species of *Vibrio* in Thua Thien Hue, Vietnam

Populations	na	ne	h	I
V. parahaemolyticus	1.7095	1.2440	0.1645	0.2695
V. vulnificus	1.4122	1.2047	0.1247	0.1925
V. shilonii	1.0405	1.0221	0.0136	0.0207
V. harveyi	1.2838	1.1782	0.1054	0.1574
V. cholerae	1.2230	1.1558	0.0885	0.1298
Others	1.6419	1.2560	0.1719	0.2768
Overall	2.0000	1.2885	0.2046	0.3435
Standard deviation (SD)	0.0000	0.2139	0.1195	0.1612

The average diversity within populations (Hs) was 0.1114, which accounted for 51.72% of the total diversity found in the populations (Ht = 0.2154). The mean coefficient of gene differentiation (Gst) value of 0.4827 indicated a very high degree of genetic differentiation among species. The gene flow (Nm) was 0.5359, which indicated that it was small among the species (Table 7).

The values of genetic identity and genetic distance between *Vibrio* species were given in Table 8. The analyzed data indicated

Table 7. Summary analysis of genetic variability across all species ofVibrio in Thua Thien Hue, Vietnam

Parameters	Ht	Hs	Gst	Nm
Across all populations	0.2154	0.1114	0.4827	0.5359
SD			0.0176	0.0048

that the values of genetic identity between populations were low, ranging from 0.7416 to 0.9545. *V. parahaemolyticus* and *V. vulnificus* had the highest genetic identity (0.9545) while *V. shilonii* and *V. harveyi* had the lowest genetic identity (0.7416). The values of genetic distance between populations were high, varying from 0.0465 to 0.2989 and the two species of *V. shilonii* and *V. harveyi* have the largest genetic distance (0.2989).

Table 8. Nei's (1978) genetic identity (above diagonal) and genetic distance (below diagonal) between 5 species of *Vibrio* in Thua Thien Hue, Vietnam

Populations	*V. parahaemolyticus*	*V. vulnificus*	*V. shilonii*	*V. harveyi*	*V. cholerae*	Others
V. parahaemolyticus	****	0.9545	0.8196	0.8840	0.9024	0.9582
V. vulnificus	0.0465	****	0.8375	0.8838	0.8791	0.9474
V. shilonii	0.1989	0.1774	****	0.7416	0.7579	0.8381
V. harveyi	0.1233	0.1235	0.2989	****	0.7897	0.8670
V. cholerae	0.1027	0.1289	0.2772	0.2361	****	0.8794
Others	0.0427	0.0541	0.1766	0.1427	0.1285	****

3.4 Cluster analysis

In the genetic similarity dendrogram constructed on the basis of comparative analysis of the total loci obtained with the ten RAPD primers, the *Vibrio* species gets distributed based on their genetic distance (Figure 5). The dendrogram showed that *V. parahaemolyticus* was closely related to *V. vulnificus* whereas *V. shilonii* and *V. harveyi* had large distance.

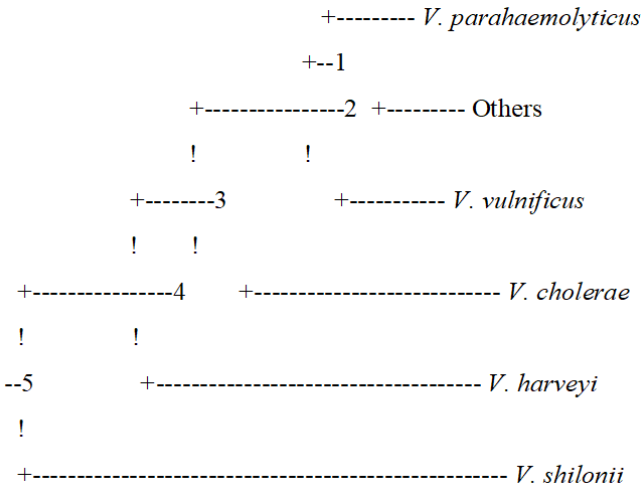

Fig 5. Dendrogram obtained with UPGMA method based on Nei's (1972) distance for all species of *Vibrio* in Thua Thien Hue, Vietnam

4 Conclusion

In our study, 120 *Vibrio* samples were isolated from white leg shrimp and fishes cultured in Tam Giang lagoon in Thua Thien Hue province (Vietnam). In shrimp samples, 14/54 strains had *pirAvp* and pirBvp genes and in fish samples, 18/66 strains had tlh gene, and none of Vibrio strains had *tdh* and *trh* genes. RAPD analysis of 36 *Vibrio* samples showed genetic identity between *V. parahaemolyticus* and *V. vulnificus* is highest while between *V. shilonii* and *V. harveyi* is lowest. *V. shilonii* had the most different RAPD pattern than other species.

Acknowledgments

This study was financial supported by the grant from Vietnam Ministry of Education and Training (Project No. CT-2018-DHH-01).

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