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Identification of pathogenicity, investigation of virulent gene distribution and development of a virulent strain-specific detection PCR method for *Vibrio harveyi* isolated from Hainan Province and Guangdong Province, China

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ABSTRACT

A collection of 46 *Vibrio harveyi* strains were isolated from *Epinephelus* spp., *Lutjanus erythopterus*, and other maricultured fish in coastal areas of Hainan Province and Guangdong Province, China, between 2011 and 2013. Eighteen strains were determined to be pathogenic via artificial infection of healthy *Epinephelus coioides* at 10^7 colony-forming units (CFU) mL^{-1}. Mortality occurred within 2 to 6 h after injection. Genotypic assays of the 46 strains by enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) revealed a similar genotype profile, referred to as the ERIC-1 profile, for all 18 pathogenic strains. This finding indicates that pathogenic *V. harveyi* strains in south China have similar genetic backgrounds and might be representative pathogenic strains of this region. All 46 strains were screened for the presence of virulence genes typical of *V. harveyi*, of zoonotic *Vibrio* species such as *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* and of the aquatic pathogen *V. anguillarum*. Virulence genes were amplified by PCR using specific primers, and five typical virulence genes of the Harveyi clade, *luxR*, *toxRvh*, *chiA*, *serine protease* and *vhh*, were detected in all pathogenic isolates. Non-pathogenic strains carried only 1 to 4 of these genes, indicating that these five genes might be the main virulence genes of ERIC-1 strains. Strain-specific PCR primers were designed based on the sequences of distinct ERIC-PCR bands for the 18 pathogenic strains. Species-specific primers exhibited high specificity and sensitivity. This study demonstrates that bacteria that are highly important to mariculture could be specifically detected using ERIC-PCR fingerprint-based amplification.

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1. Introduction

The southern coastal regions (108°–117°E, 18°–23°N), including Hainan Province, Guangdong Province and the Guangxi Zhuang Autonomous Region, are the major maricultural areas in China. The annual average temperature in these regions ranges from 22 °C to 26 °C, which is suitable for aquaculture of grouper and other tropical marine animals. However, with the development of aquaculture, vibriosis, which is caused by *Vibrio* spp., has become a serious problem, and more than 10 species of *Vibrio*, including *V. harveyi*, *V. alginolyticus*, *V. parahaemolyticus*, *V. anguillarum*, *V. vulnificus*, and *V. ponticus*, contribute to vibriosis in this region (Xie et al., 2005, 2007). Among them, *V. harveyi* was verified to be the dominant pathogen in recent years (Austin and Zhang, 2006; Haldar et al., 2010; Mei et al., 2010; Rattanama et al., 2012; Xu et al., 2012; Zhou et al., 2012; Cui et al., 2014).

V. harveyi strains are known to differ in their ability to cause aquatic animal diseases (Austin and Zhang, 2006). Several virulence genes are thought to be associated with pathogenicity of vibrios (Ruwandeepika et al., 2010). Previous studies have identified major virulence genes contributing to *V. pathogenicity* and *V. cholera* (Waldor and Mekalanos, 1996; Iida et al., 1998; McCarthy et al., 1999; Zhang et al., 2003). In addition, horizontal gene transfer, a driving force in *Vibrio* evolution and speciation, also appears to be an efficient mechanism for introducing new phenotypes into the bacterial genome (Ochman et al., 2000; Gogarten et al., 2002; Hacker et al., 2003). Although *V. harveyi* has caused severe losses in the aquaculture industry, little is known about its key virulent genes and its pathogenic mechanisms.

Because the pathogenic mechanism of *V. harveyi* is still unknown and no effective method is currently available to control its spread, developing a rapid diagnostic to support a prophylactic approach is particularly important. Although several rapid detection methods have recently been developed for *V. harveyi* (Conejero and Hedreyda, 2003, 2004; Oakey et al., 2003; Pang et al., 2006; Sun et al., 2009), methods

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that detect a specific pathogenic genotype have not been reported. Thus, the design of molecular methods for rapid detection of pathogenic *V. harveyi* is essential for improving industrial culture production. The key step in developing such a method is identification of a target gene or molecular biomarker. Due to the tremendous diversity of bacterial genomic DNA, the sequences of ERIC-PCR bands are often unique to the genome of the strain used for amplification. Therefore, these sequences have been used as biomarkers for designing primers used to discriminate closely related bacterial strains or to detect specific bacteria in various samples, especially with regard to understanding the epidemiology of pathogens and food-contaminating bacteria (Ventura et al., 2003; Alippi et al., 2004; Yan et al., 2007; Ye et al., 2008). To our knowledge, however, this method has not been used in maricultural studies. In this paper, strain-specific PCR primers were designed based on the sequences of specific bands amplified from pathogenic strains of *V. harveyi* by ERIC-PCR fingerprinting. We demonstrate that the pathogenic strains of *V. harveyi* could be specifically detected using ERIC-PCR fingerprint-based amplification. Our technique will help in developing measures to control *V. harveyi* infection.

To explore the pathogenic characteristics of *V. harveyi* isolated from maricultural areas in southern China, the pathogenicity, genotyping and virulent gene distribution of *V. harveyi* strains obtained from these areas were investigated, and a rapid detection method for such pathogenic *V. harveyi* strains was developed in this paper.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A total of 46 *V. harveyi* strains originally isolated from diseased or moribund fish between 2011 and 2013 from mariculture systems of Hainan Province and Guangdong Province, southern China were used in this study. All strains were identified as *V. harveyi* using standard biochemical testing described in Bergey's Manual of Systematic Bacteriology and multilocus sequence analysis (Cano-Gomez et al., 2009). The strains were stored in broth medium containing 25% glycerol (v/v) at −80 °C. The sampling sites are illustrated in Fig. 1, and the sources and origins of these *V. harveyi* isolates are listed in Table 1.

2.2. Artificial infection

To confirm the pathogenicity of *V. harveyi*, experimental challenges were conducted using healthy *Epinephelus coioides* (mean weight 15.0 ± 0.5 g) obtained from culture ponds in Wenchang City, Hainan Province, China. Artificial infection of 46 *V. harveyi* strains was performed by intraperitoneal injection. The artificial infection method and the criterion for classifying a strain as pathogenic was referred to Xie et al. (2005) and Bai et al. (2008), with slight modifications. Six fish were maintained in a 50 L tank containing 40 L filtrated seawater (30 g L^{-1} salinity). The fish were maintained at 30 ± 1 °C and were fed commercial pellets twice a day. Compressed air was pumped continuously into water, and 50% of the water was exchanged daily. Fish were kept for 7 days before artificial infection. All bacterial strains were cultured in Marine Broth 2216E for 24 h with constant shaking at 30 °C. The cultures were diluted with sterile phosphate-buffered saline (PBS, pH 7) to 1×10^7 CFU mL^{-1}, as determined by plate counting. Fish was infected intraperitoneally with 100 μL of the diluted bacterial suspension, and the same volume of PBS was injected as the control. Two parallel experiments were performed per treatment. After artificial injection, the symptoms caused by virulent bacteria and the mortality of the fish were recorded for 7 days. The injected isolate was considered the cause of death only if it could be re-isolated as single colonies in pure culture from the liver, spleen and brain of a moribund or dead fish. Histopathological examination of liver, spleen and brain was also performed to diagnose the results of infection. The strains which could cause all the infected fish to death in 7 days were defined as pathogenic strains and the other strains were non-pathogenic.

To determine the toxicity of the *V. harveyi* culture supernatant, experimental challenges with the supernatant of strain GDH11385 were conducted in *E. coioides.* Briefly, strain GDH11385 cells were grown in Zobell 2216E broth to the logarithmic phase and then serially diluted 10-fold to obtain 1×10^7 CFU mL^{-1}. The cultures were centrifuged to collect the supernatants, which were then filtered through a 0.2-μm syringe filter to remove all remaining cells. Experimental challenge of six fish with 100 μL of the supernatant was performed by intraperitoneal injection. As a positive control, the same volume of an untreated 10^7 CFU mL^{-1} GDH11385 culture was injected; as a negative control, the same volume of the 2216E broth filtered through a 0.2-μm syringe filter was injected. Two parallel experiments were performed per treatment.

2.3. LD_{50} assays

To determine the 50% lethal dose (LD_{50}) of the pathogenic *V. harveyi* strain GDH11385, bacterial doses ranging from 10^3 to 10^8 CFU mL^{-1} were used for intraperitoneal inoculation. Three tanks (6 fish per tank) were used for each treatment. The LD_{50} value was calculated by the classical Bliss method (Bliss, 1935).

2.4. Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR)

All isolated strains of *V. harveyi* (n = 46) were grown overnight at 28 °C on Zobell 2216E agar plates (Sigma). One colony of each strain was suspended in 5 mL Zobell 2216E broth (Sigma) and grown for 24 h at 28 °C. Chromosomal DNA was extracted using a TaKaRa

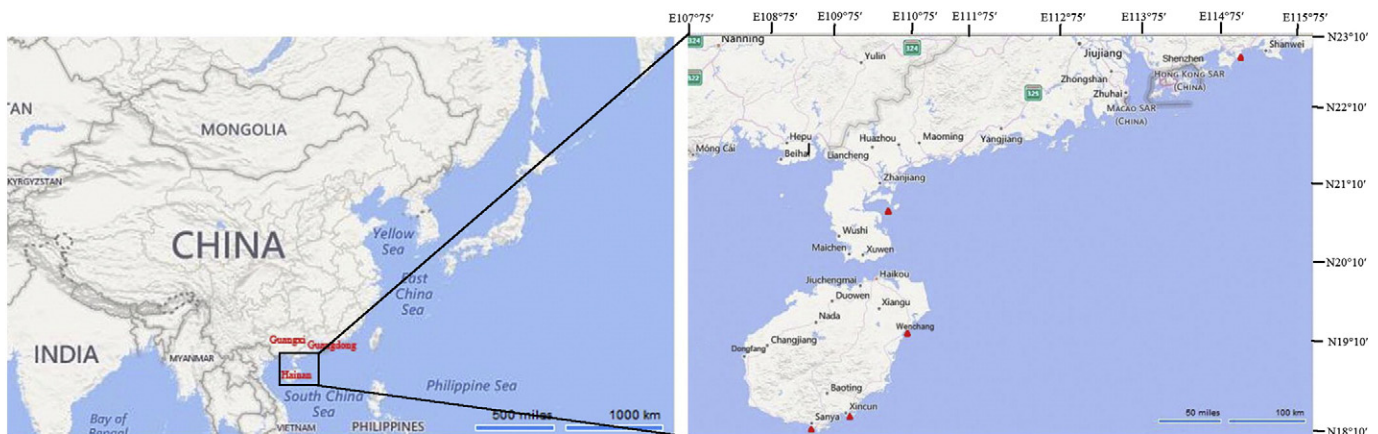

Fig. 1. Sampling area and the locations of sampling sites (red triangles). (http://www.worldmapfinder.com/BingMaps/Cn.html).

Table 1
Characteristics of 46 *Vibrio harveyi* isolates from mariculture systems.

Name of isolate	Source	City	Year of isolation	ERIC profile	Virulence	LD_{50} value (CFU g^{-1} fish body weight)
GDH11385	*Epinephelus coioides*	Shenzhen	2011	ERIC-1	Pathogenic	8.7×10^3
GDH11388	*E. coioides*	Shenzhen	2011	ERIC-1	Pathogenic	
GDH11392	*E. coioides*	Shenzhen	2011	ERIC-1	Pathogenic	
GDH11399	*E. coioides*	Shenzhen	2011	ERIC-1	Pathogenic	
NS131241	*E. coioides*	Sanya	2013	ERIC-1	Pathogenic	
NS131651	*E. coioides*	Sanya	2013	ERIC-1	Pathogenic	
NS131652	*E. coioides*	Sanya	2013	ERIC-1	Pathogenic	
NS131841	*E. coioides*	Sanya	2013	ERIC-1	Pathogenic	
WC13D121	*Lutjanus erythopterus*	Wenchang	2013	ERIC-1	Pathogenic	
WC13D151	*L. erythopterus*	Wenchang	2013	ERIC-1	Pathogenic	
WC13D231	*L. erythopterus*	Wenchang	2013	ERIC-1	pathogenic	
WC13D251	*L. erythopterus*	Wenchang	2013	ERIC-1	Pathogenic	
WC13D262	*L. erythopterus*	Wenchang	2013	ERIC-1	Pathogenic	
WC13DH11	*L. griseus*	Wenchang	2013	ERIC-1	Pathogenic	
WC13DH21	*L. griseus*	Wenchang	2013	ERIC-1	Pathogenic	
WC13DH31	*L. griseus*	Wenchang	2013	ERIC-1	Pathogenic	
WC13H252	*L. griseus*	Wenchang	2013	ERIC-1	Pathogenic	
XC130452	*E. coioides*	Lingshui	2013	ERIC-1	Pathogenic	
GDH11387	*E. coioides*	Shenzhen	2011	ERIC-7	Non-pathogenic	
GDH11389	*E. coioides*	Shenzhen	2011	ERIC-14	Non-pathogenic	
HNH1101	*L.erythopterus*	Zhanjiang	2010	ERIC-7	Non-pathogenic	
HNH1102	*Fugu* sp.	Zhanjiang	2012	ERIC-7	Non-pathogenic	
HNH1103	*L. erythopterus*	Zhanjiang	2010	ERIC-3	Non-pathogenic	
HNH1104	*L. erythopterus*	Zhanjiang	2010	ERIC-7	Non-pathogenic	
HNH1105	*Sciaenops ocellatus*	Zhanjiang	2012	ERIC-7	Non-pathogenic	
HNH1106	*Siganus* sp.	Zhanjiang	2011	ERIC-7	Non-pathogenic	
NS131341	*E. coioides*	Sanya	2013	ERIC-12	Non-pathogenic	
HNH1108	*Trachinotus ovatus*	Zhanjiang	2012	ERIC-6	Non-pathogenic	
HNH1109	*L. erythopterus*	Zhanjiang	2010	ERIC-7	Non-pathogenic	
HNH1111	*E. coioides*	Zhanjiang	2011	ERIC-7	Non-pathogenic	
HNH1112	*S. ocellatus.*	Zhanjiang	2012	ERIC-7	Non-pathogenic	
HNH1113	*S. ocellatus*	Zhanjiang	2012	ERIC-10	Non-pathogenic	
NS131051	*E. fuscoguttatus*	Sanya	2013	ERIC-11	Non-pathogenic	
NS131251	*E. fuscoguttatus*	Sanya	2013	ERIC-13	Non-pathogenic	
HNH1107	*E. coioides*	Sanya	2013	ERIC-10	Non-pathogenic	
NS131451	*E. coioides*	Sanya	2013	ERIC-13	Non-pathogenic	
NS131531	*E. coioides*	Sanya	2013	ERIC-12	Non-pathogenic	
NS131631	*E. coioides*	Sanya	2013	ERIC-8	Non-pathogenic	
NS131751	*E. coioides*	Sanya	2013	ERIC-15	Non-pathogenic	
NS131851	*E. coioides*	Sanya	2013	ERIC-4	Non-pathogenic	
WC13D222	*L. erythopterus*	Wenchang	2013	ERIC-16	Non-pathogenic	
NS131632	*E. coioides*	Sanya	2013	ERIC-9	Non-pathogenic	
WC13DH51	*L. griseus*	Wenchang	2013	ERIC-2	Non-pathogenic	
WC13DH52	*L. griseus*	Wenchang	2013	ERIC-12	Non-Pathogenic	
XC130211	*T. ovatus*	Lingshui	2013	ERIC-5	Non-pathogenic	
XC130352	*E. lanceolatus*	Lingshui	2013	ERIC-4	Non-pathogenic	

MiniBEST Bacteria Genomic DNA Extraction Kit, Ver.3.0 (TaKaRa, Japan). The concentration of the purified DNA was determined using an Ultraspec 2100 pro (Amersham Biosciences, USA) and adjusted to 10 ng/mL. ERIC-PCR was performed in a final volume of 25 μL containing 50 ng extracted DNA, 2.5 μL Ex Taq buffer (10×), 200 mM each deoxynucleoside triphosphate (dNTP), 2.5 mM $MgCl_2$, 10 pmol each ERIC 1R primer (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC2 primer (5′-AAGTAAGTGACTGGGGTGAGCG-3′), and 1 U Ex Taq DNA polymerase (TaKaRa, Japan). Amplification was performed using a Biometra T-Gradient thermoblock (Biometra, Germany) under following conditions: 7 min initial denaturation at 95 °C followed by 35 cycles of 30 s denaturation at 90 °C, 1 min annealing at 52 °C, and 8 min elongation at 65 °C, with a final elongation step at 68 °C for 16 min. The PCR products (5 μL) were analysed on a 1.5% agarose gel stained with Goldview I nucleic acid stain (Solarbio, China), which was then visualized under UV transillumination and photographed using a Gel Doc XR apparatus (Bio-Rad, USA).

2.5. Investigation of the distribution of 17 known virulence genes

Seventeen virulence genes associated with the virulence of *V. harveyi*, *V. cholera*, *V. parahaemolyticus*, *V. anguillarum* or *V. vulnificus* (8 for *V. harveyi*, 5 for *V. cholerae*, 2 for *V. parahaemolyticus*, 1 for *V. anguillarum* and 1 for *V. vulnificus*) were examined by PCR in the 46 *V. harveyi* isolates. Specific primers for the *V. harveyi* genes *luxR*, *toxRVh* (*V. harveyi* toxR), *vhpA* (metalloprotease), *chiA* (chitinase), and *vhml* (*V. harveyi* myovirus-like), the gene encoding serine protease, and *vhs* (*V. harveyi* siphovirus-like) were designed by Ruwandeepika et al. (2010). Primers specific for *vhh* (*V. harveyi* haemolysin) were designed by Zhang et al. (2001). Primers for other *Vibrio* genes include *zot* (zonula occludens toxin; Rivera et al., 2001), *toxRVc* (a virulence regulator; Miller et al., 1987), *tcpA* (toxin-coregulated pilus; Keasler and Hall, 1993), *ctxA* (cholera toxin; Fields et al., 1992) and *hlyA* (haemolysin; Saravanan et al., 2007) from *V. cholera*; *flaC* (flagella C subunit; Bai et al., 2008) from *V. anguillarum*; *tdh* (thermostable direct haemolysin) and *trh* (TDH-related haemolysin; Tada et al., 1992) from *V. parahaemolyticus*; and *vvh* (haemolysin; Lee et al., 1999) from *V. vulnificus*. The sequences of primers used for amplifying virulence genes from *V. harveyi* and other *Vibrio* spp. are provided in Table 2.

PCR was performed in a 25 μL volume containing 25 ng extracted DNA, 2.5 μL Ex Taq buffer (10×), 200 mM each deoxynucleoside triphosphate (dNTP), 2.5 mM $MgCl_2$, 10 pmol each primer, and 1 U Ex Taq DNA polymerase (TaKaRa, Japan). The reaction was performed using a Biometra T-Gradient thermoblock (Biometra, Germany). The cycling conditions were as follows: 30 cycles of 95 °C for 1 min; annealing at 50 °C for 1 min for *luxR*, *toxRVh*, *serine protease*, *chiA* and *vhpA*, 55 °C

Table 2
Nucleotide sequences of primers used for PCR amplification.

Gene	Primer sequence (5′–3′)	Reference	Product size (bp)
luxR	F: ATGGACTCAATTGCAAAGAG R: TTAGTGATGTTCACGGTTGT	Ruwandeepika et al., 2010	618
toxRVh	F: CGACAACCAAAATACGGAA R: AGAGCAATTTGCTGAAGCTA	Ruwandeepika et al., 2010	131
chiA	F: GGAAGATGGCGTGATTGACT R: GGCATCAATTTCCCAAGAGA	Ruwandeepika et al., 2010	232
vhpA	F: CTGAACGACGCCCATTATTT R:CGCTGACACATCAAGGCTAA	Ruwandeepika et al., 2010	201
vhml	F: TGATCATGCCGATGGTCTTA R: GGTCAAAATCCCACACATCC	Ruwandeepika et al., 2010	180
Serine protease	F: TGCACGACCAGTTGCTTTAG R: AAGTGGTCGTCAGCAAATCC	Ruwandeepika et al., 2010	232
vhs	F: CCGGAAGGTTACAGCATTGT R: GCGTCGGTCTTCTCAAGTTC	Ruwandeepika et al., 2010	185
vhh	F: GAGGACGTTTGGTGAGATAA R: ACGACGAATACAATCTCTGG	Zhang et al., 2001	1404
ctxA	F: CGGGCAGATTCTAGACCTCCTG R: CGATGATCTTGGAGCATTCCCAC	Fields et al., 1992	564
toxRVc	F: ATGTTCGGATTAGGACAC R: TACTCACACACTTTGATGGC	Miller et al., 1987	883
tcpA	F: CACGATAGGAAAACCGGTCAAGAG R: ACCAAATGCAACGCCGAATGGAG	Keasler and Hall, 1993	617
hlyA	F: GGCAAACAGCGAAACAAATACC R: CTCAGCGGGCTAATACGGTTTA	Saravanan et al., 2007	738
zot	F: TCGCTTAACGATGGCGCGTTTT R: AACCCCGTTTCACTTCTACCCA	Rivera et al., 2001	947
vvh	F: GCTATTTCACCGCCGCTCAC R: CCGCAGAGCCGTAAACCGAA	Lee et al., 1999	222
flaC	F: AAATCATTCCAAATCGGTGC R: TCTTTGATTCGGCTCTTA	Bai et al., 2008	580
trh	F: CCCTCAAAATGGTTAAGCG R: CATTTCCGCTCTCATATGC	Tada et al., 1992	235
tdh	F: CCACTACCACTCTCATATGC R: ATACGAGTGGTTGCTGTCATG	Tada et al., 1992	250

for *vhh*, *vhm*, *vvh*, *trh*, *tdh* and *flaC*, and 60 °C for *vhs*, *ctxA*, *toxRVc*, *tcpA*, *hlyA* and *zot*; and an extension step of 72 °C for 1 min. All cycles included an initial denaturation of 95 °C for 5 min and a final elongation of 72 °C for 10 min. The PCR products (5 μL) were analysed on a 1.5% agarose gel stained with Goldview I nucleic acid stain (Solarbio, China), and the results were visualized using a UV transilluminator (Bio-Rad, USA). Each PCR was performed in triplicate to confirm reproducibility.

2.6. Rapid-detection PCR for virulent strains

Specific ERIC-PCR bands of approximately 2000 bp (indicated by the black arrow in Fig. 2) from pathogenic strains of *V. harveyi* were excised from the agarose gel and purified using an Agarose Gel DNA Purification Kit (TaKaRa, Japan). The bands were cloned as described by Sambrook et al. (2001). The sequences of the fragments were determined by DNA sequencing, and the sequences have been deposited in the GenBank database. A BLAST search against the GenBank nucleotide sequence database was performed. The sequence with high specificity was used to design specific primers with the software Primer Premier 5.0 (http://www.premierbiosoft.com/primerdesign/).

The specificity of the primers was determined by PCR amplification of the DNA extracted from the pathogenic and non-pathogenic *V. harveyi* isolates used in this study. Reference cultures of the Harveyi clade, including 33 *V. alginolyticus* strains stored in our lab and 5 ATCC strains (*V. alginolyticus* ATCC 33787, *V. parahaemolyticus* ATCC17802, *V. mimicus* ATCC 33653, *V. furnissii* ATCC 33813, and *V. natriegens* ATCC 33788) purchased from American Type Culture Collection (ATCC, Mannasas, VA, USA), were used in this study.

To determine the sensitivity of the ERIC-PCR method, the GDH11385 strain was grown in Zobell 2216E medium to the logarithmic phase and serially diluted 10-fold from 2×10^2 to 2×10^6 CFU mL^{-1}. One microlitre of each of the diluted cultures was used in PCR amplification with the designed primers.

2.7. Nucleotide sequence accession numbers

Sequence data obtained from this work were submitted to NCBI GenBank and assigned the following accession numbers: KR003746 through KR003758.

3. Results

3.1. Artificial infection

A total of 46 *V. harveyi* strains were isolated from Shenzhen and Zhanjinag in Guangdong Province and Wenchang, Lingshui and Sanya in Hainan Province. After artificial infection, 18 isolates of *V. harveyi* were verified to be pathogenic to *Epinephelus coioides* (Table 1 and Fig. 2). Among them, 4 strains were isolated from Shenzhen, 9 from Wenchang, 4 from Sanya and 1 from Lingshui (Table 1 and Fig. 2). The infected fish became lethargic, disorientated, loss of appetite and developed eye lesions (Supplementary Fig. 1), and also exhibited unique behaviours, such as shortness of breath, continuously open operculum, and repeated an "up-down" movement wherein they occasionally sprinted to the surface and then free-fell to the bottom of the enclosure. Moribund fish rampaged, repeatedly leaping out of the water and falling to the bottom, laid on the bottom of the enclosure and died (Supplementary video).

The results of artificial infection using suspensions of different strains showed that some resulted in mortality after 2 h and generally resulted in 100% mortality after 4 to 6 h. Approximately 90% of the dead fish opened their operculum (Supplementary Fig. 1). Based on necropsy and histopathological examination, some fish had developed ascites, liver splenomegaly, and/or cells disintegration in liver and spleen (Supplementary Fig. 1 and Supplementary Fig. 2). LD_{50} assays revealed the highest virulence for isolate GDH11385 (8.7×10^3 CFU g^{-1}

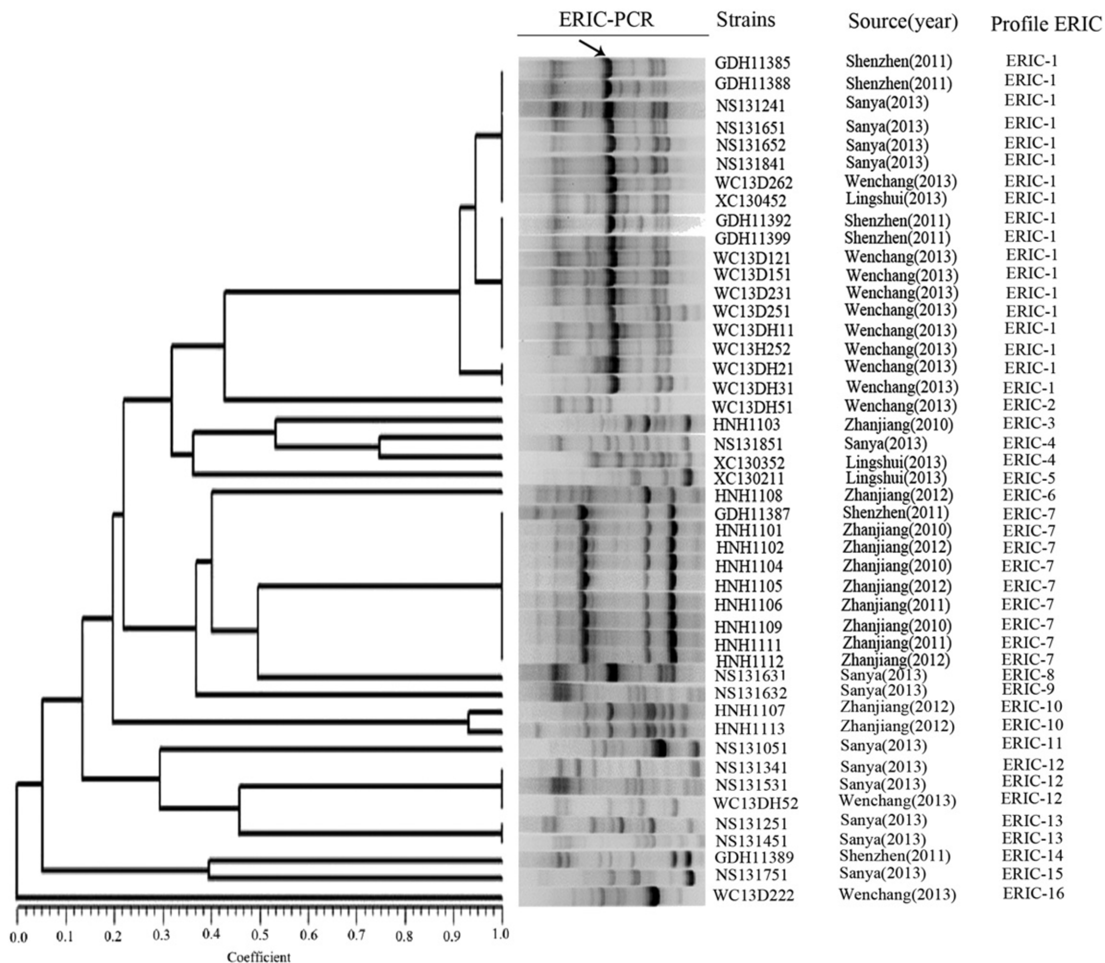

Fig. 2. Dendrogram showing the ERIC profiles of the *V. harveyi* isolates used in this study. Note: The black arrow indicates the 2000-bp band that was sequenced.

fish body weight). The same strain of *V. harveyi* was re-isolated in the form of single colonies from the brain, liver and spleen of moribund or dead fish. No mortality occurred after injection with sterile PBS or the culture supernatant of isolate GDH11385. The results show that some strains of *V. harveyi* are pathogenic to *E. coioides* but their culture supernatants are not toxic to it.

3.2. ERIC-PCR fingerprint analysis

ERIC primers produced between 3 and 13 bands per reaction, with sizes ranging from 0.1 to 8.0 kb (Fig. 2). The *V. harveyi* isolates from mariculture areas of south China produced 44 ERIC patterns, and all isolates yielded products with the primers used. Gel Compare Software 4.1 was used to construct a phylogenetic dendrogram to estimate relationships among isolates. The *V. harveyi* strains comprised sixteen genotypes (ERIC-1 through ERIC-16), and the ERIC-1 profile was the most common genotype among the 18 pathogenic strains, all of which shared a unique 2000 bp band (black arrow in Fig. 2).

3.3. PCR amplification of virulence genes

Of the genes known to be virulence determinants in *V. harveyi*, all 18 pathogenic isolates were positive for *chiA*, *serine protease*, *vhh*, *luxR* and $toxR_{vh}$. Fourteen of the 18 isolates were positive for *vhml,* whereas none of the isolates was found to carry *vhpA* or *vhs.* The presence of virulence genes of other *Vibrio* spp., such as *flaC*, *zot*, *vvh*, $toxR_{Vc}$, *trh* and *tdh*, in the Harveyi clade was observed in 17 (94%), 13 (72%), 6 (33%), 4 (22%), 8 (44%) and 3 (17%) of the 18 isolates, respectively. The remaining three virulence genes from other *Vibrio* spp. (i.e., *hlyA, tcpA* and *ctxA*) were not detected in any of the 18 pathogenic isolates. The isolate in this study possessing the fewest virulence-related genes was NS131841, with only 5 genes (i.e., *chiA*, *serine protease*, *vhh*, *luxR* and $toxR_{vh}$). Isolate GDH11399 was found to possess the most virulence-related genes (12), with only 6 to 10 virulence-related genes in other pathogenic isolates. In total, 155 positive amplicons (104 *V. harveyi* genes and 51 genes from other *Vibrio* spp.) were found among the 18 pathogenic isolates.

Genes known to be virulence determinants in *V. harveyi* and other *Vibrio* spp. were also detected in 28 non-pathogenic isolates used in this study. However, the total number of virulence genes was fewer than the pathogenic isolates. Only 87 (60 *V. harveyi* genes and 27 genes from other *Vibrio* spp.) positive amplicons were noted among all 28 non-pathogenic isolates. For genes of virulence determinants in the 28 non-pathogenic *V. harveyi* isolates, 20 isolates were found to be positive for *luxR* and *chiA*, 10 for *vhh*, 6 for *serine protease* and 3 for *toxRvh. vhml*, *vhpA* and *vhs* was not found in any of the non-pathogenic isolates. The presence of virulence genes from other *Vibrio* spp., such as *flaC*, *zot*, *vvh*, $toxR_{Vc}$, *trh* and *tdh*, was observed in 14 (50%), 3 (11%), 5 (18%), 1 (4%), 2 (7%) and 1 (4%) of the 28 non-pathogenic isolates, respectively. No positive amplicons were detected in non-pathogenic isolates NS131341 and WC13D222 (Table 3).

3.4. Development of rapid detection technology for pathogenic V. harveyi strains

Three replications were generated by ERIC-PCR amplification using genomic DNA samples from 18 pathogenic isolates as templates. All strains displayed similar fingerprints (see the ERIC-1 profile in Fig. 2). Fragments of genomic DNA from GDH11385, serving as candidates for

Table 3
The presence of virulence genes in pathogenic and non-pathogenic strains.

Isolates	*luxR*	*toxRvh*	*vhpA*	*chiA*	*Serine protease*	*vhh*	*vhml*	*vhs*	*toxRvc*	*hlyA*	*flaC*	*vvh*	*trh*	*tdh*	*tcpA*	*zot*	*ctxA*
Pathogenic strains	There were 155 positive amplicons (including faint amplicons).																
GDH11385	+	+	−	+	+	+	+	−	−	−	+	−	−	−	−	+	−
GDH11388	+	+	−	+	+	+	+	−	−	−	+	−	−	−	−	+	−
GDH11392	+	+	−	+	+	+	−	−	−	−	+	−	−	−	−	−	−
GDH11399	+	+	−	+	+	+	+	−	+	−	+	+	*	*	−	+	−
NS131241	+	+	−	+	+	+	+	−	−	−	+	−	−	−	−	+	−
NS131651	+	+	−	+	+	+	+	−	−	−	+	+	−	−	−	+	−
NS131652	+	+	−	+	+	+	+	−	−	−	+	−	−	−	−	−	−
NS131841	+	+	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−
WC13D121	+	+	−	+	+	+	+	−	−	−	+	−	−	−	−	+	−
WC13D151	+	+	−	+	+	+	+	−	−	−	+	−	−	−	−	+	−
WC13D231	+	+	−	+	+	+	+	−	+	−	+	−	−	−	−	+	−
WC13D251	+	+	−	+	+	+	−	−	−	−	+	+	*	−	−	−	−
WC13D262	+	+	−	+	+	+	+	−	−	−	+	−	*	−	−	+	−
WC13DH11	+	+	−	+	+	+	+	−	+	−	+	+	*	−	−	+	−
WC13DH21	+	+	−	+	+	+	+	−	+	−	+	+	*	*	−	−	−
WC13DH31	+	+	−	+	+	+	−	−	−	−	+	+	*	*	−	+	−
WC13H252	+	+	−	+	+	+	+	−	−	−	+	−	*	−	−	+	−
XC130452	+	+	−	+	+	+	+	−	−	−	+	−	*	−	−	+	−
Non-pathogenic strains	There were 87 positive amplicons (including faint amplicons).																
WC13DH51	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
HNH1103	+	−	−	+	+	−	−	−	*	−	−	−	−	−	−	−	−
NS131851	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
XC130352	+	+	−	+	+	−	−	−	−	−	+	−	−	−	−	−	−
XC130211	+	+	−	+	+	−	−	−	−	−	+	+	−	*	−	+	−
HNH1108	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
GDH11387	+	−	−	+	−	−	−	−	−	−	+	+	*	−	−	+	−
HNH1101	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
HNH1102	+	−	−	+	−	+	−	−	−	−	+	+	*	−	−	+	−
HNH1104	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
HNH1105	+	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−
HNH1106	+	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−
HNH1109	+	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−
HNH1111	+	−	−	+	−	+	−	−	−	−	+	−	−	−	−	−	−
HNH1112	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
HNH1107	+	−	−	+	+	−	−	−	−	−	+	−	−	−	−	−	−
NS131451	+	−	−	+	−	+	−	−	−	−	−	+	−	−	−	−	−
NS131631	+	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−
HNH1113	+	−	−	+	+	−	−	−	−	−	+	−	−	−	−	−	−
NS131051	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−
NS131341	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
NS131531	+	−	−	+	−	+	−	−	−	−	+	−	−	−	−	−	−
WC13DH52	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
NS131251	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
NS131632	+	−	+	+	−	+	−	−	−	−	+	+	−	*	−	−	−
GDH11389	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
NS131751	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
WC13D222	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Note: "*" indicates a faint amplicon.

the design of strain-specific primers, were obtained by ERIC-PCR amplification. One ERIC-PCR band of 2000 bp was excised and used to construct clone libraries, generating 106 clones. Fifty-two clones were randomly selected for sequencing, and 13 different sequences (GenBank accession numbers KR003746 through KR003758) were obtained. Sequence alignments revealed that the sequence KR003746, with high abundance (27 in the 52 clones) and specificity, displayed similarity to a membrane protein of *V. harveyi* ATCC33843 (DNA identity of 98%, see supplementary Doc. 1). Based on this sequence, we designed a pair of specific primers named 3858P1F/R (Table 4) using the software Primer Premier 5.0. Amplification consisted of denaturation at 94 °C for 5 min, followed by 20 cycles at 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 1 min, with final extension at 72 °C for 10 min. Using these primers, a PCR product of 656 bp was expected (Fig. 3) from each of the 18 pathogenic isolates. The reference cultures and non-pathogenic isolates failed to produce amplicons (Fig. 3). The sensitivity assay revealed positive PCR products from cultures of 2×10^4 to 2×10^6 CFU mL^{-1}. A weakly positive result (i.e., a faint band) was observed using a culture of 2×10^3 CFU mL^{-1}, but this result was not always reproducible (Fig. 4).

4. Discussion

4.1. Pathogenicity of *V. harveyi*

V. harveyi is a serious pathogen in mariculture that can infect both vertebrates and invertebrates, including fish, shrimp and molluscs. It can cause vasculitis, gastro-enteritis and eye lesions in fish; and it is also associated with luminous vibriosis and *Bolitas negricans* in shrimp. Infected animals become lethargic and disorientated, exhibit a loss of appetite and develop necrotic subdermal cysts (Austin and Zhang, 2006; Austin, 2010). In the present study, *Epinephelus coioides* infected with *V. harveyi* exhibited the symptoms common to other fish diseases caused by *V. harveyi*, such as eye lesions, lethargy and disorientation. However, some other symptoms not previously reported were also found in *E. coioides*, including shortness of breath with the operculum opening continuously, frequent swims to the surface, and collapse. Perhaps different symptoms were caused by different strains of *V. harveyi* or different hosts. The results of artificial infection showed that the strains fitting the ERIC-1 profile were acutely lethal to *E. coioides* (all infected animals died after 4–6 h) and the same strain was re-isolated

Table 4
Characteristics of the primer pairs designed for specific amplification of *V. harveyi*.

Primers	Sequence (5′–3′)	Tm (°C)	Specificity
3858P1F	TACATCTACCATTTCCGTAAACATG	60	Strains with the ERIC-1 profile
3858P1R	ATTGAGGTAAAAGTTTCAGCACATC	60	

from the liver, spleen, and brain of dead or moribund fish. Histopathologic examination of the tissue also confirmed the death of the fish was indeed due to *V. harveyi* infection. However, the culture supernatants of the pathogenic strains and the bacterial supernatants filtered through a 0.2-μm syringe filter were not toxic to *E. coioides*. According to the results of this study, we infer that *V. harveyi* is the pathogen of *E. coioides.* It invades the brain of fish and cause the host to swim erratically, to experience difficulty breathing, and to suffocate subsequently.

4.2. Genotype of V. harveyi

ERIC-PCR is a DNA-based typing method. Compared with similar methods such as amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD) and pulsed-field gel electrophoresis (PFGE), it is more rapid and economical, technically simpler and has a higher discriminatory power for determining the distribution of a single strain among individuals (Yuan et al., 2010; Guimarães Ade et al., 2011). This technique has been used successfully to discriminate strains of *V. alginolyticus* (Kahla-Nakbi et al., 2006), *V. tapetis* (Rodríguez et al., 2006) and *V. parahaemolyticus* (Bhowmick et al., 2008), indicating that it is effective for typing and epidemiological studies of *Vibrio* species. The approach has also been used to establish DNA fingerprints for *V. harveyi* with the aim of evaluating the applicability of these techniques in epidemiological studies. For example, Ruwandeepika et al. (2010) used ERIC-PCR to type 45 *V. harveyi* and 1 *V. campbellii* strain isolated from different areas and different hosts. In this study, ERIC-PCR is used for first time to type *V. harveyi* isolated from aquaculture systems in a specific geographic region. The results showed that 46 *V. harveyi* strains isolated from mariculture areas in south China had high genetic diversity and reproducibility, and 16 different ERIC profiles were obtained. The predominant ERIC profile was ERIC-1, which was observed in 18 strains (39%) isolated between 2011 and 2013. Among all 46 *V. harveyi* strains, 18 isolated from different sites exhibited high pathogenicity (LD_{50} of ~10^3 CFU g^{-1} fish body weight) to *E. coioides*. The result demonstrates that *V. harveyi* strains with similar ERIC profiles share similar characteristics (i.e., pathogenicity) and that the ERIC-PCR technique is useful for investigating epidemic diseases as well as for basic molecular characterization of pathogenic *V. harveyi*.

4.3. Virulent gene distribution of V. harveyi

In this study, 17 virulent-related genes, including 8 genes known to be virulence determinants in *V. harveyi* (typical virulence genes) and 9 virulent-related genes from other *Vibrio* species (atypical virulence genes), were examined by PCR. No obvious correlations were established between virulent strains and the number of virulence genes harboured in *V. harveyi*. Compared with strains carrying 7 or 8 virulence genes (e.g., XC130211 and GDH11387), the strain with the fewest pathogenic genes (NS131841) exhibited high pathogenicity, indicating that pathogenicity of a strain has no direct correlation to the number of virulence genes that it possesses. However, the total number of virulence genes, both typical and atypical, of the 18 pathogenic isolates was higher than that of the 28 non-pathogenic isolates. The result suggests that the cluster of pathogenic *V. harveyi* strains serves as an abundant reservoir of virulence genes. Nakamura et al. (2004) reported that virulence genes have high transferability due to their benefit to survival in a variety of environments and host organisms. Therefore, the virulence genes of pathogenic *V. harveyi* maybe also have the potential to be transferred. Furthermore, based on the number of other *Vibrio* genes obtained by horizontal gene transfer (Ochman et al., 2000), we inferred that the pathogenic strains of *V. harveyi* may have a higher capacity to acquire extrinsic DNA compared with non-pathogenic strains. Therefore, this cluster of *V. harveyi* strains could act as either donors or recipients. As donors, the strains disseminate their own virulence genes to other *Vibrio* spp. In contrast, as recipients, they acquire

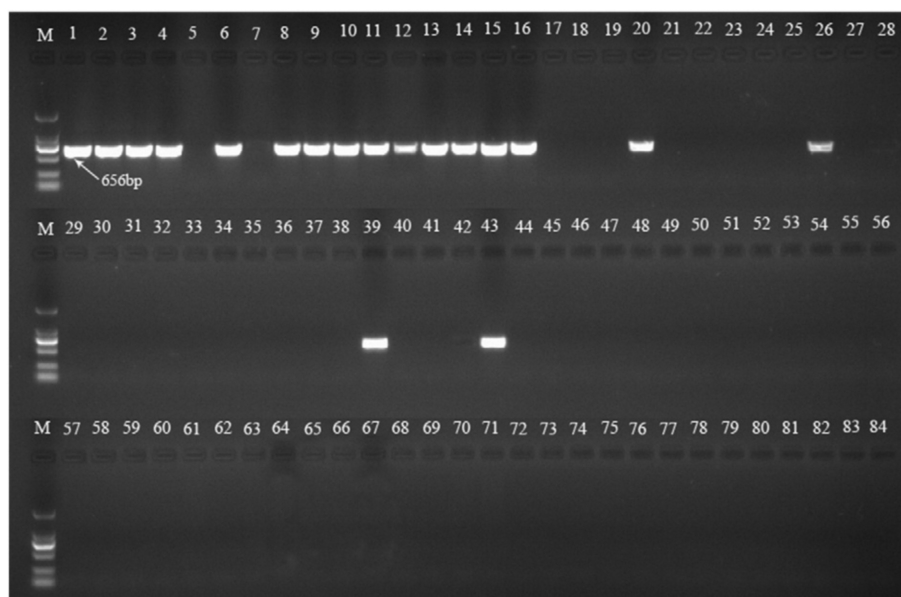

Fig. 3. PCR profiles obtained using the 3858P1F and 3858P1R primers and DNA templates from 18 pathogenic *V. harveyi* isolates, 28 non-pathogenic isolates, 5 ATCC strains and 33 *V. alginolyticus* isolates. M: DL2000 marker (Takara, Japan). The eighteen lanes (Lane 1–4, Lane 6, Lane 8–16, Lane 20, Lane 26, Lane 39, Lane 43) with a 656-bp band were obtained from pathogenic *V. harveyi*. The other lanes without any band were obtained from the non-pathogenic *V. harveyi* isolates and the reference strains.

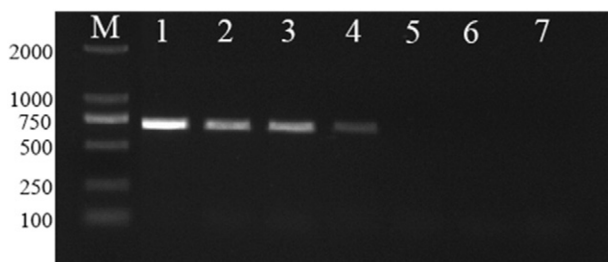

Fig. 4. PCR profiles obtained using the 3858P1F and 3858P1R primers and serially diluted cultures of the isolate GDH11385. M: the DL2000 marker (Takara, Japan). Lane 1: 2×10^6 CFU mL^{-1}. Lane 2: 2×10^5 CFU mL^{-1}. Lane 3: 2×10^4 CFU mL^{-1}. Lane 4: 2×10^3 CFU mL^{-1}. Lane 5: 2×10^2 CFU mL^{-1}. Lane 6: 2×10^1 CFU mL^{-1}. Lane 7: negative control.

virulence genes from other *Vibrio* spp. via horizontal gene transfer and increase their ability to infect aquatic organisms by increasing their virulence towards a specific host and/or by broadening their host range (Ruwandeepika et al., 2010). Therefore, the ability to effectively detect these strains may have a positive impact on aquaculture and marine environment. To date, no obvious correlation has been established between the pathogenicity of a strain and the presence of virulent genes (Bai et al., 2008; Ruwandeepika et al., 2010). Accordingly, the results of this study provide clues to the relationship between *V. harveyi* virulent strains and their virulence gene genotypes. All the tested pathogenic strains possess 5 virulence-related genes (i.e., *luxR*, *toxRvh*, *vhh*, *chiA* and *serine protease*), whereas the non-pathogenic strains only carry 1 to 4 of these genes, suggesting that co-existence of these 5 genes may contribute to strain pathogenicity. This finding is consistent with previous reports that the pathogenicity of *V. harveyi* may be due to the presence of multiple virulent factors (Ruwandeepika et al., 2010). Of course, it would be premature to conclude that the virulence of *V. harveyi* is exclusively caused by these 5 genes, and we cannot exclude the possibility that other virulence genes (even critical virulence genes from other species) may also contribute to the pathogenicity of this species.

The atypical virulence genes of four other *Vibrio* species were used in this study to explore cases of horizontal gene transfer involving *V. harveyi* in the mariculture system of south China. The atypical virulence genes belonging to each *Vibrio* species used in this study can be detected by PCR amplification. Our results are partially consistent with previous reports, in which *toxRvc and flaC* were previously detected in *V. harveyi* (Ruwandeepika et al., 2010). However, the results of our study also showed *V. harveyi* isolates to be positive for *zot*, *vvh*, *trh* and *tdh*, which were not detected in *V. harveyi* in previous reports (Kaper et al., 1995; Bai et al., 2008; Ruwandeepika et al., 2010; Klein et al., 2014). It indicates that *V. harveyi* in the mariculture system of south China might acquire an extensive set of foreign genes, causing it to exhibit novel functions to promote fitness in a particular niche (Ochman et al., 2000).

4.4. Detection of pathogenic V. harveyi

PCR is a rapid and simple technique for the detection and identification of bacteria. Numerous methods use a single gene, such as *16S rDNA*, *ToxR*, *vhhp2*, or *vhh*, for *V. harveyi* detection and identification based on PCR amplification (Conejero and Hedreyda, 2003, 2004; Oakey et al., 2003; Pang et al., 2006; Sun et al., 2009). However, no reports for detecting strain-specific genotypes of *V. harveyi* have been published. As ERIC-PCR band sequences are often unique to the genome of a particular strain, these sequences can be used to design primers for discriminating among closely related bacterial strains or for specific detection of bacteria in various samples, especially epidemiology investigation of pathogens (Rivera et al., 2001; Alippi et al., 2004; Ye et al., 2008; Wang et al., 2009). Indeed, ERIC-PCR generates highly specific genomic DNA fingerprints (Di Giovanni et al., 1999) with unique sequences. Therefore, ERIC-PCR is useful for generating species- or strain-specific fragments, and as demonstrated by Alippi et al. (2004), species-specific primers can be developed from ERIC-PCR fragments to detect certain bacteria. These results suggest that screening suitable fragments from an ERIC-PCR amplicon, which exhibits low similarity to other bacterial DNA fragments, might allow for the design of high-specificity primers. In this study, 84 strains were used for extensive validation of primers, and only 18 pathogenic strains with the ERIC-1 profile produced amplicons. The other strains, including non-pathogenic *V. harveyi* strains and the reference Harveyi clade strains *V. alginolyticus*, *V. parahaemolyticus*, *V. mimicus*, *V. furnissii*, and *V. natriegens*, failed to produce amplicons. These results show that the specific primers are highly sensitive and can directly detect the ERIC-1 profile of *V. harveyi* from samples of both organisms and seawater. However, in consideration of the diversity of pathogenic strains, the use of the developed ERIC primers should be validated extensively before they are used for distinguishing between pathogenic and non-pathogenic strains.

5. Conclusion

In this study, we determined that pathogenic *Vibrio harveyi* strains in south China have similar genetic backgrounds and might be its representative pathogenic strains, and a rapid PCR detection technique for such strains was established to facilitate rapid disease diagnosis for prevention and treatment efforts. In the future, we will explore the pathogenesis of these strains through genome sequencing, bioinformatics and comparative genomics, with a view to providing a theoretical basis for the development of vaccines against such strains for thorough and effective prevention and treatment of diseases caused by such bacteria.

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Competing interests

The authors declare that we have no competing interests.

Author contributions

Xu Xiandong, Xie Zhenyu and Zhou Yongcan participated in designing of the study, analyzing data and drafting the manuscript. Liu Kaifang collected the bacteria samples and performed the PCRs. Wang Shifeng and Guo Weiliang participated in the artificial infection experiment, helped to interpret the results and participated in finalizing the manuscript. All authors read and approved the final manuscript.

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