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COMMUNICATION

Development of a real time PCR assay for rapid detection of *Vibrio parahaemolyticus* from seafood

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ABSTRACT

A real time PCR assay for the detection of *Vibrio parahaemolyticus* in seafood samples was developed using a novel specific target and a competitive internal amplification control (IAC). The specificity of this assay was evaluated using 390 bacterial strains including *V. parahaemolyticus*, and other strains belonging to *Vibrio* and non-*Vibrio* species. The real time PCR assay unambiguously distinguished *V. parahaemolyticus* with a detection sensitivity of 4.8 fg per PCR with purified genomic DNA or 1 CFU per reaction by counting *V. parahaemolyticus* colonies. The assays of avoiding interference demonstrated that, even in the presence of 2.1 μg genomic DNA or 10^7 CFU background bacteria, *V. parahaemolyticus* could still be accurately detected. In addition, the IAC was used to indicate false-negative results, and lower than 94 copies of IAC per reaction had no influence on the detection limit. Ninety-six seafood samples were tested, of which 58 (60.4%) were positive, including 3 false negative results. Consequently, the real time PCR assay is effective for the rapid detection of *V. parahaemotyticus* contaminants in seafood.

KEYWORDS *Vibrio parahaemolyticus*, real time PCR, internal amplification control, seafood

INTRODUCTION

Vibrio parahaemolyticus is an important food-borne pathogen widely distributed in marine and estuarine environments, which can cause gastroenteritis through consumption of the raw, undercooked or mishandled seafood. This pathogen has been an increasing concern and become the largest percentage of bacterial food poisoning in littoral provinces of China (Wang et al., 2007). Its detection still depends on traditional culture methods, which are labor-intensive, complicated and time-consuming (Luan et al., 2007). Therefore, it is necessary to develop methods for rapid and accurate detection of *V. parahaemolyticus* from seafood.

Real time PCR is a widely accepted method for detection and quantification of several food-borne pathogens. Although this method is highly sensitive, the risk of false-negative results is still one limitation that cannot be overlooked (Maaroufi et al., 2006), which may be due to the presence of inhibitory factors, including food residues, organic solvents from nucleic acid extraction, or other unknown factors (Kreader, 1996; Abu Al-Soud and Rådström, 1998; Al-Soud and Rådström, 2001; Maaroufi et al., 2004). To address this challenge, another nucleic acid sequence was suggested to serve as an internal amplification control (IAC) and monitor amplification processes during PCR (Rosenstraus et al., 1998; Courtney et al., 1999; Niesters, 2004; Hartman et al., 2005; Raggam et al., 2005) and distinguish false-negative from true-negative results.

The non-competitive IACs were initially used to avoid false-negative results in previous PCR detection systems (Blackstone et al., 2007; Nordstrom et al., 2007). The disadvantage of non-competitive IACs is that amplification may not reflect the primary target accurately, because of differences in amplification efficiency and relative abundance of targets and IACs. Moreover, there is another disadvantage that fails to

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avoid the interferences between the target primers and IAC primers, which may often be less efficient for one or both reactions (Hoorfar et al., 2004). To overcome these disadvantages, some factors must be carefully considered, such as the nucleotide compositions of the target and IAC, the length of the IAC, and the amplification efficiency of primers.

In addition, detection targets of *V. parahaemolyticus* lack specificity, such as *gyrB*, *tlh, tdh*, *trh*, *groEL* and *toxR* genes (Miller et al., 1987; Shirai et al., 1990; Tada et al., 1992; Venkateswaran et al., 1998; Bej et al., 1999; Kim et al., 1999; Luan et al., 2007; Hossain et al., 2012). Consequently, it is necessary to identify other target sequences, which show no or low similarity to other bacterial species, but exhibit high specificity for *V. parahaemolyticus*.

In this study, a bioinformatics approach was applied to identify a more specific target sequence of *V. parahaemolyticus*. Real time PCR was then employed with a competitive IAC to detect this pathogen in seafood.

RESULTS

Real time PCR: specificity, sensitivity, and standard curve

Specificity of the real time PCR was evaluated among 390 strains. To consistently collect and analyze data, the baseline and threshold of PCR amplification profiles were set to the same for all the targets. As shown in Table 1, only *V. parahaemolyticus* strains produced a positive fluorescent signal, with C_t values between 17 and 20. The other bacteria tested were negative with only IAC signals.

The detection limit of the assay was 4.8 fg per reaction using the templates of purified *V. parahaemolyticus* ATCC33846 genomic DNA. As the template DNA concentration decreased, the target C_t value increased (Table S1 in supplementary data). For pure cultures of *V. parahaemolyticus*, PCR was capable of detecting as low as 1.61×10^2 CFU/mL (about 1 CFU from a pure culture per reaction) per reaction. The C_t value for the targeted gene increased as the concentration of target bacteria decreased (Table S2 in supplementary data).

Standard curves showed that there was a good linear correlation between the C_t values and the concentrations of added genomic DNA or bacteria, as the R^2 values for the purified DNA and bacterial cultures were 0.998 and 0.993, respectively.

Influence of background non-*V. parahaemolyticus* DNA or bacteria

When 2.4 ng–0.24 fg of purified *V. parahaemolyticus* genomic DNA was tested with a background of 2.1 μg non-*V. parahaemolyticus* bacterial DNA, the C_t values increased by approximately one cycle or less as compared to the C_t values obtained without background DNA (Table 2). However, the increase was not statistically significant ($p > 0.05$), implying that no significant inhibition to the target reaction occurred in the presence of 2.1 μg of background DNA. When background DNA was included at levels below 2.1 μg, no notable change in the C_t value was observed (data not shown).

The presence of non-*V. parahaemolyticus* (*V. vulnificus*, *V. alginolyticus*, and *V. harveyi*) background bacteria at a level of 10^7 CFU had no effect on the sensitivity of detection of *V. parahaemolyticus*, although the C_t values did increase slightly but not significantly ($p > 0.05$) compared to PCRs without background bacteria (Table 3).

Detection of *V. parahaemolyticus* in naturally contaminated seafood samples

Ninety-six seafood samples selected randomly from local markets were tested for the presence of *V. parahaemolyticus.* The results showed that 55 samples (57.3%) were identified as positive using the real time PCR assay, while only 39 positive samples were detected with traditional culture methods. Among the negative samples, five seafood samples were categorized as false-negative (i.e. absence of the amplification signal for both IAC and target DNAs), indicating the absence of PCR inhibitors (Table 4). The results were verified with 5-fold serially diluted template DNAs of the five false-negative samples. Three of the five samples were then positive for *V. parahaemolyticus*, while two samples remained negative. Therefore, 58 samples were positive.

DISCUSSION

Thus far, most of the target genes used in PCR detection of *V. parahaemolyticus* encode proteins involved in virulence. When these genes are aligned with genomic sequences of foodborne pathogens in the NCBI genome databank, some of them have similar sequences to other *Vibrio* species, such as *groEL*, *gyr*B and *tox*R genes, while others do not exist in some strain of this pathogen, such as *tlh, tdh* and *trh* gene. In this study, a new gene, VP1332, which encodes the binding protein component of the ABC transporter, was identified using comparative genomic methods. Specificity of this gene has been verified using the PCR method to detect 390 strains of *V. parahaemolyticus* and other genus/species from clinic, food and environment samples.

In previous reports, a non-competitive IAC was used to indicate PCR inhibition with two pairs of primers being complementary to both the target DNA and non-target DNA (Nordstrom et al., 2007). However, it is difficult to avoid the interference among the primer sets or to optimize reaction conditions for all the primer sets. Therefore, a competitive IAC used for real time PCR detection was developed in this study, which could more effectively indicate false-negative results. The competitive IAC contained the same primer rec-

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Table 1 Bacterial strains used and the detection results

Bacterial species	No. of strains studied	No. of positive results by real time PCR
Vibrio parahaemolyticus ATCC17802	1[a]	1
V. parahaemolyticus ATCC33846	1[a]	1
V. parahaemolyticus	307[b]	307
V. vulnificus ATCC 27562	1[a]	0
V. harveyi ATCC33842	1[a]	0
V. mimicus ATCC 33653	1[a]	0
V. fluvialis ATCC33810	1[a]	0
V. anguillarum	1[c]	0
V. damsel	2[c]	0
V. campbeffi ATCC33863	1[a]	0
V. alginolyticus	2[c]	0
V. cholerae ATCC25871	1[a]	0
*SGL**	1[c]	0
Staphylococcus aureaus	12[c]	0
Salmonella Typhimurium ATCC14028	1[a]	0
*S.*Typhimurium ATCC13311	1[a]	0
S. Arizonae ATCC13314	1[a]	0
S. Paratyphi A ATCC9150	1[a]	0
S. Paratyphi B CMCC50004	1[a]	0
S. Paratyphi C CMCC50017	1[a]	0
S. Enteritidis ATCC13076	1[a]	0
S. Vellore ATCC15611	1[a]	0
S. Tallahassee ATCC12002	1[a]	0
S. Abaetetuba CMCC51812	1[a]	0
S. Choleraesuis ATCC10708	1[a]	0
S. Infantis CMCC51741	1[a]	0
S. Typhi CMCC 50098	1[a]	0
S. Typhimurium AS1.1174	1[a]	0
S. Typhimurium CMCC50180	1[a]	0
Escherichia coli O157:H7 ATCC43889	1[a]	0
Escherichia coli	8[c]	0
Hemoclastic *Escherichia coli*	1[c]	0
Listeria monocytogenes ATCC7644	1[a]	0
L. monocytogenes ATCC27708	1[a]	0
L. monocytogenes ATCC54002	1[a]	0
L. monocytogenes ATCCBAA-751	1[a]	0
L. monocytogenes ATCC15313	1[a]	0
L. monocytogenes ATCC13932	1[a]	0
L. monocytogenes ATCC21-AB	1[a]	0
L. monocytogenes	6[c]	0
L. ivanovii AB 97016	1[a]	0
L. innocua AB 97022	1[a]	0
Enterobacter sakazakii ATCC29544	1[a]	0
E. cloacae ATCC700323	1[a]	0
E. cloacae ATCC13047	1[a]	0
Proteus mirabilis ATCC12453	1[a]	0
P. vulgaris ATCC33420	1[a]	0
Klebsiella peneumoniae ATCC27336	1[a]	0
Shigella flexneri CMCC51311	1[a]	0
S. dysenteriae CMCC51335	1[a]	0
Enterococcus avium ATCC14025	1[a]	0
E. faecium ATCC27270	1[a]	0
E. faecalis ATCC49452	1[a]	0
Citrobacter freundii ATCC8090	1[a]	0
Pseudomonas aeruginosa CDC B32116	1[a]	0
Serratia marcescens ATCC27592	1[a]	0
Bacillus subtilis ATCC6633	1[a]	0
Micrococcus luteus ATCC9341	1[a]	0
Yersinia enterocolitica	1[c]	0

[a] Standard strains were kindly provided by Shanghai Entry-Exit Inspection and Quarantine Bureau (Shanghai, China) or purchased from American Type Culture Collection and China Medical Culture Collection.

[b] Isolates were kindly provided by Shanghai Entry-Exit Inspection and Quarantine Bureau (Shanghai, China) and Ningbo Entry-Exit Inspection and Quarantine Bureau (Zhejiang, China).

[c] Other bacterial strains were acquired from our laboratory, Shanghai Jiao Tong University, Shanghai, China.

* *Vibrio* group I freshwater subgroup.

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Table 2 Effect of background bacterial DNA on the sensitivity of detection of *V. parahaemolyticus* DNA

Genomic DNA concentrations	With no background non-specific DNA Ct (dR)	With 2.1 μg of background non-specific DNA Ct (dR)*
2.4 ng/μL	18.93 ± 0.15	19.6 ± 0.11
240.0 pg/μL	22.19 ± 0.83	22.91 ± 0.23
24.0 pg/μL	25.96 ± 0.11	26.37 ± 0.82
2.4 pg/μL	29.26 ± 0.64	29.57 ± 0.13
240.0 fg/μL	32.63 ± 0.01	33.14 ± 0.22
24.0 fg/μL	36.28 ± 0.28	37.26 ± 0.32
2.4 fg/μL	37.78 ± 1.06	38.4 ± 0.66
0.24 fg/μL	No Ct	No Ct
Negative control	No Ct	No Ct

* *F* test analysis indicates there was no significant difference between with and without 2.1 μg of background non-*V. parahaemolyticus* DNA in C_t values ($F = 0.051$, $p = 0.825$, $p > 0.05$).

Table 3 Effect of background bacteria on the sensitivity of detection of *V. parahaemolyticus* in pure culture

V. parahaemolyticus concentrations (CFU/ mL)	With no background non-specific bacteria C_t (dR)	With a level of 10^7 CFU of background non-specific bacteria C_t (dR)*	With a level of 10^9 CFU of background non-specific bacteria C_t (dR)
1.61×10^7	21.28 ± 0.11	23.14 ± 0.23	23.76 ± 0.21
1.61×10^6	22.56 ± 0.13	26.01 ± 0.14	27.80 ± 0.18
1.61×10^5	26.42 ± 0.09	30.81 ± 0.11	32.01 ± 0.16
1.61×10^4	29.77 ± 0.15	33.86 ± 0.06	34.82 ± 0.11
1.61×10^3	32.81 ± 0.14	37.85 ± 0.02	38.75 ± 0.08
1.61×10^2	36.07 ± 0.26	39.95 ± 0.01	No C_t
1.61×10^1	No C_t	No C_t	No C_t
Negative control	No C_t	No C_t	No C_t

* *F* test indicates there was no significant difference between with and without a level of 10^7 CFU of background non-target bacteria in Ct values ($F = 0.379$, $p = 0.553$, $p > 0.05$).

Table 4 Real time PCR results of seafood samples

Food samples	Number	Traditional culture method (P*/N#)	Positive results (*n*)	Negative results (*n*)	False-negative results (*n*)
Shrimp	37	18/19	26	11	0
Fish	34	2/32	8	22	4
Crab	15	13/2	14	1	0
Mussels	10	6/4	7	2	1
Total	96	39/57	55	36	5

* P: positive results.
# N: negative results.

ognition sequences as the target DNA, but with a different probe hybridization site. Two probes labeled with distinct reporter dyes (FAM and HEX) were used to differentiate target DNA and IAC amplification, and the concentration of the IAC templates was optimized. As shown in Table 5, high concentrations of IAC inhibited the amplification signal of low concentration target DNA, whereas the two lowest IAC concentrations were not sufficient to produce desired IAC amplification signal. The results showed that less than 94 copies of IAC per reaction had no influence on the detection limit of the target reaction.

The detection limit of this real time PCR system was 4.8 fg/PCR with genomic DNA or 1 CFU/ PCR for pure culture of *V. parahaemolyticus*, which is more sensitive than previously reported (4 pg and 5 CFU/PCR, 200 pg and 30 CFU/PCR, 0.17 pg/PCR) (Venkateswaran et al., 1998; Wardet al., 2006; Yu et al., 2010). Moreover, the sensitivity of the reaction was still not influenced even at high concentrations of DNA (2.1 μg per reaction) or bacterial cells (10^7 CFU per reaction). Inhibition was not observed until the level of background bacteria was increased to 10^9 CFU per reaction (Table 3). The results indicate that this real time PCR is highly sensitive and can be

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Table 5 Effect of adding different concentrations of IAC on the sensitivity of detection of *V. parahaemolyticus* purified genomic DNA

Genomic DNA concentrations	Without IAC Ct (dR)	Adding 188 copies/PCR of IAC Ct (dR)	Adding 94 copies/PCR of IAC Ct (dR)*
2.4 ng/μL	18.93 ± 0.15	19.03 ± 0.16	18.83 ± 0.42
240.0 pg/μL	22.19 ± 0.83	22.41 ± 0.92	21.81 ± 0.98
24.0 pg/μL	25.96 ± 0.11	26.50 ± 0.56	25.68 ± 0.55
2.4 pg/μL	29.26 ± 0.64	29.71 ± 0.73	28.91 ± 0.78
240.0 fg/μL	32.63 ± 0.01	33.47 ± 0.55	32.47 ± 0.67
24.0fg/μL	36.28 ± 0.28	No Ct	36.15 ± 0.86
2.4 fg/μL	37.78 ± 1.06	No Ct	39.54 ± 0.64
0.24 fg/μL	No Ct	No C_t	No Ct
Negative control	No Ct	No Ct	No Ct

* *F* test analysis indicates there was no significant difference between with and without 94 copies/ PCR of IAC in C_t values ($F = 0.199$, $p = 0.664$, $p > 0.05$).

used for successful detection with a relatively high level of background noise.

The TaqMan real time PCR assay was developed and evaluated using a novel target gene with an IAC to indicate false-negative results for the detection of *V. parahaemolyticus* in seafood samples. Five of 96 seafood samples were considered false-negative due to the lack of the IAC signal. These false-negative samples were 5-fold serially diluted to 10^{-3}, and *V. parahaemolyticus* was detected in 3 samples in which inhibition was no longer present. Even though inhibition was infrequent, these results demonstrated that the reliability of the PCR assay can be raised by the inclusion of an internal control.

In conclusion, this real time PCR detection system using a novel target gene with a competitive IAC was a highly specific (only positive for *V. parahaemolyticus* strains), sensitive (4.8 fg of purified genomic DNA and about 1 CFU from a pure culture per reaction), rapid (approximately 12 h including time for sample enrichment), accurate (able to distinguish false-negative from true negative results), and efficient (an improvement by 19.79% compared to traditional cultural methods) method for the detection of *V. parahaemolyticus* in seafood. The multiplex assay developed herein appears to be a promising tool for high-throughput screening of food samples for *V. parahaemolyticus.*

MATERIALS AND METHODS

Bacterial culture and DNA extraction

A total of 390 bacterial strains were used in this study (Table 1), including 2 *V. parahaemolyticus* standard strains (ATCC33846 and ATCC17802), 307 *V. parahaemolyticus* isolates and 81 non-*V. parahaemolyticus* strains. All *Vibrio* species were cultured in Luria-Bertani (LB) broth with 2% sodium chloride, while the other bacterial strains were cultured in LB or Tryptone Soya Broth (TSB), or Brain Heart Infusion (BHI). The strains were incubated at 37°C for 8–12 h with shaking at 150 rpm, and then the total genomic DNAs of different strains were extracted according to methods described by Nichols et al. (2003). DNA concentrations were determined by a spectrophotometer (DU-800 Spectrophotometer, Beckman Coulter, Fullerton, CA, USA).

Primer and probe

To identify species specific sequences for *V. parahaemolyticus*, bioinformatic methods were employed to compare genomic sequences of *V. parahaemolyticus* with all other prokaryotic genomes (Zhu et al., 2009). After evaluating the specificity both by on-line BLAST (Basic Local Alignment Search Tool) and PCR amplification and the sensitivity by PCR amplification, a species specific CDS (VP1332), encoding a putative binding protein component of the ABC transporter (Makino et al., 2003), was selected as the target gene for *V. parahaemolyticus* detection. The primers were designed using the Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA), and the probe was designed by TaKaRa Biotechnology (Dalian, China), tagged with FAM. Both primers and the probe were synthesized by TaKaRa Biotechnology (Dalian, China).

IAC construction

The IAC was constructed using PCR as previously reported (Sachadyn and Kur, 1998; Abdulmawjood et al., 2002; Wieczorek and Osek, 2004; Rodríguez-Lázaro et al., 2005). Briefly, a region of the VPA0739 gene (Makino et al., 2003) of *V. parahaemolyticus* was selected as IAC and its DNA sequence was not homologous with the target gene (the homologous sequence was less than 20 bp). Firstly, the VPA0739 gene was amplified by a long primer set, of which the 3′ end sequences corresponded to the IAC primer set whereas the 5′ hanging end sequences corresponded to the VP1332 primer set (Table 6). Then, the 218 bp PCR product, including the flanking regions of the target primer set, was cloned into a plasmid vector pMD18-T (TaKaRa Biotechnology, Dalian, China) and sequenced for verification. Primer and probe sequences are listed in Table 6. The

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IAC probe was labeled with HEX (Fig. 1).

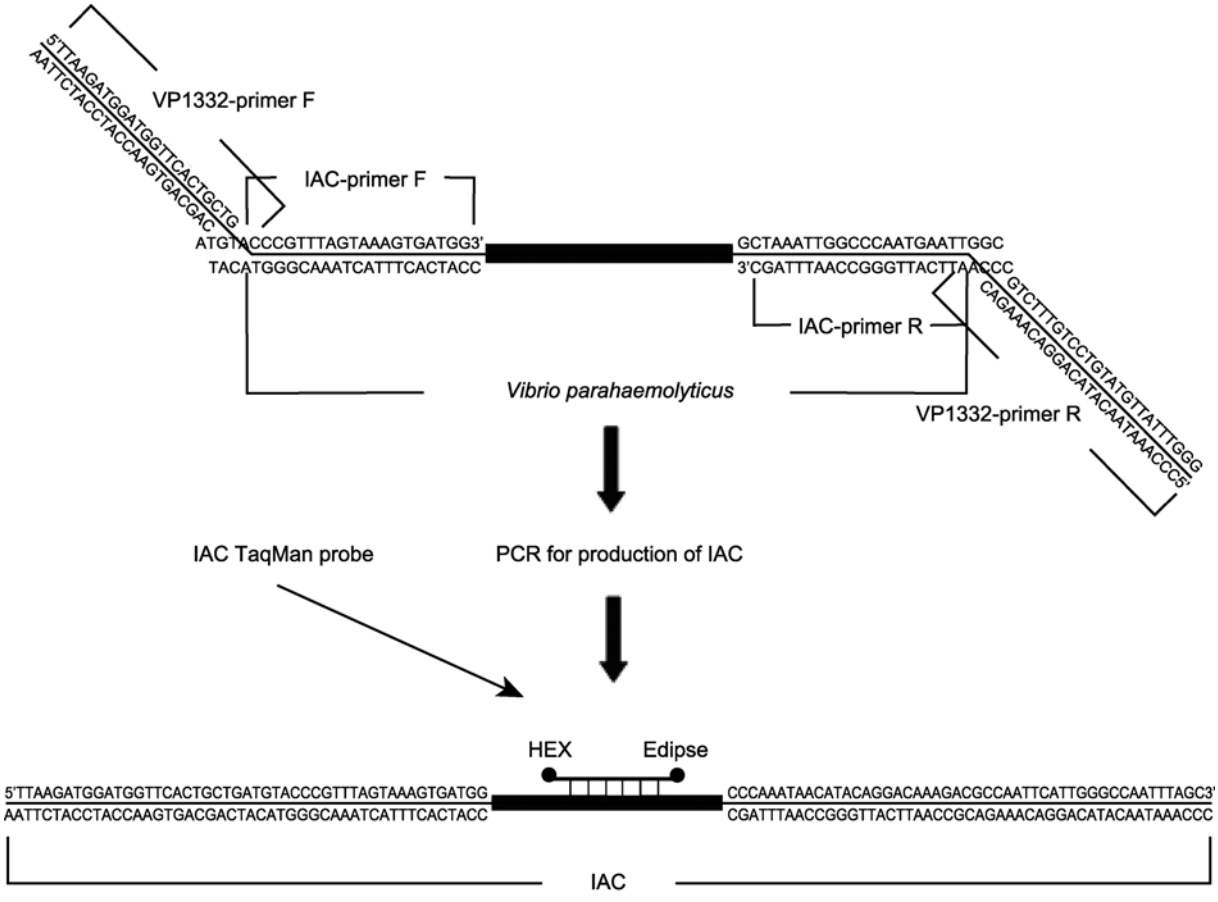

Figure 1. Scheme for construction of the IAC by compound primers technology.

Table 6 Sequences of primers and probes utilized

	Names	Sequences
	Primers	
	vp1332PF	5′-TTAAGATGGATGGTTCACTGCTG-3′
	vp1332PR	5′-GGGTTTATTGTATGTCCTGTTTCTG-3′
	IAC-primer F	5′- ATGTACCCGTTTAGTAAAGTGATGG-3′
	IAC-primer R	5′-CGGTTAAGTAACCCGGTTAAATCG-3′
	Long primer F*	5′-TTAAGATGGATGGTTCACTGCTGATGTACCCGTTTAGTAAAGTGATGG-3′
	Long primer R*	5′-GGGTTTATTGTATGTCCTGTTTCTGCGGTTAAGTAACCCGGTTAAATCG-3′
	Probes	
	vp1332-probe	5′-(FAM [a]) ACGCCAAACCGAACCTTTCTGCTGA (Eclipse)-3′
	IAC-probe	5′-(HEX [b]) ACATTCGTCAGTGCCGTCGCCAGA (Eclipse)-3′

* The underlined letters are derived from the diagnostic primers.
[a] 6-carboxyfluorescein.
[b] 5-hexachlorofluorescein.

Real time PCR amplification

The real time PCR assays were performed using a Stratagene Mx3005P™ Quantitative PCR System (Strategene, La Jolla, CA, USA). The final reaction volume (25 μL) consisted of 1×Taq buffer (Mg^{2+} plus, 20 mmol/L), 0.1 mmol/L dNTPs, 0.2 mmol/L of each primer, 1 U of Ex Taq HS DNA polymerase (TaKaRa Biotechnology, Dalian, China), 2 μL of template DNA, 300 nmol/L of vp1332-probe,

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and 200 nmol/L of IAC-probe. All real time PCR assays were performed in triplicate using the following program: 95°C for 2 min, followed by 40 cycles of 95°C for 10 s, and 60°C for 30 s.

PCR specificity and sensitivity

Specificity of the real time PCR assay was tested using genomic DNA from a panel of 390 bacterial strains (Table 1[A16]). For sensitivity testing, purified genomic DNA and pure culture of *V. parahaemolyticus* ATCC33846 was 10-fold serially diluted in sterile water to 10^{-9}. The DNA of pure culture was extracted using the boiling lysis method (Ward and Bej, 2006). The real time PCR was then carried out twice in triplicate.

Standard curve for real time PCR assay

PCR amplification efficiency, detection sensitivity and quantitative capabilities were determined using a series of 10-fold dilutions of genomic DNA and pure culture from *V. parahaemolyticus* standard strain (ATCC33846) with predetermined DNA and cell concentrations. The amplification efficiency was calculated from the formula $E = (10^{-1/slope}) - 1$ using the slopes based on standard linear curves plotted from cycle thresholds and the $\log_{10}$ values of genomic copies of each DNA per reaction. The linear regression was calculated using the MxPro™ QPCR Software (Strategene, La Jolla, CA, USA).

Detection of *V. parahaemolyticus* in the background of non-*V. parahaemolyticus*

The ability of the assay to detect known detectable amounts of *V. parahaemolyticus* DNA (2.4 ng–0.24 fg) in the presence of non-*V. parahaemolyticus* DNA was evaluated by adding serial dilutions of mixed genomic DNA (21.4 ng to 2.1 μg) from five bacterial strains (*V. vulnificus* ATCC27562, *V. harveyi* ATCC33842, *V. campbellii* ATCC33863, *V. fluvialis* ATCC33810 and *SGL*) to the PCR system. All the data were analyzed using SPSS 16.0 software (SPSS, Inc, Chicago, IL, USA).

The detection capability of *V. parahaemolyticus* in the background of other *Vibrio* species was also evaluated by mixing their exponential phase cultures. The *V. parahaemolyticus* cultures were 10-fold serially diluted to 10^{-9} in sterile water. A mixture containing equal amounts (approximately 10^5, 10^7 or 10^9 CFU per reaction) of *V. vulnificus* cultures, *V. alginolyticus* cultures, and *V. harveyi* cultures was added as background bacteria to each dilution (Fig. 2[A17]). DNA extraction was performed by boiling lysis method (Ward and Bej, 2006). All the data were analyzed using SPSS 16.0 software.

Examination of seafood samples

Sample collection

Ninety-six seafood samples were collected from a local market or supermarket, stored at 4ºC, and tested within 24 h after purchasing.

Sample preparation

All the samples were treated according to the ISO/PDTS 21872-1 draft dated November, 29th 2004 and the National Standard Method

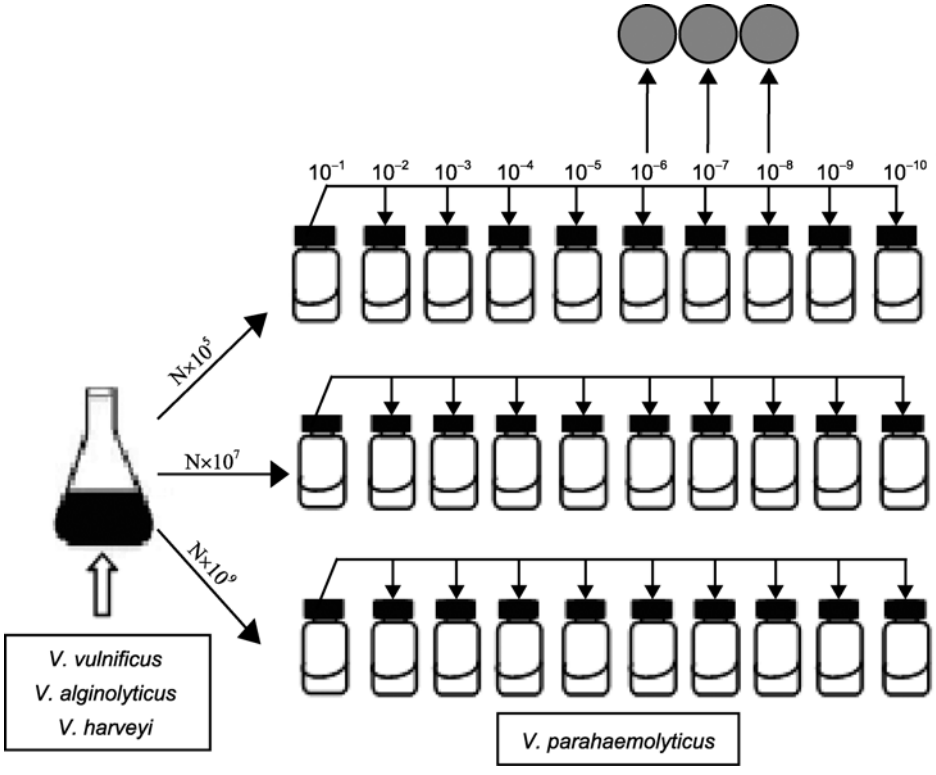

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Figure 2. Scheme for determining the presence of background bacteria on detection of *V. parahaemolyticus*.

of China (Microbiological examination of food hygiene-Examination of *V. parahaemolyticus* (GB/T 4789.7--2008)) with slight modifications. Oysters were scrubbed and shucked. The entire shell contents (animal and liquor) were shucked and homogenized at a high speed for 90 s with a sterile blender. Ten grams of homogenate were added to 90 mL of alkaline peptone water (APW; 1% peptone, 3.5% NaCl, pH 8.5 ± 0.2) in each of three separate flasks. All the samples were incubated for 10 h in a rotary shaker at 150 rpm and 30°C.

Culture isolation

After being incubated, the enrichments were streaked onto thiosulfate-citrate-bile salts-sucrose (TCBS; Difco, Sparks, MD, USA) plates and incubated overnight at (37 ± 1)°C. Suspicious colonies (typically blue–green) were streaked onto non-selective media (TSB with an additional 2.5% NaCl added) and were subjected to further biochemical identification, including oxidase, TSI (with an additional 2.5% NaCl added), and salt tolerance tests. Suspicious *V. parahaemolyticus* isolates were finally confirmed by using an API 20E assay (bioMérieux Inc., Durham, NC, USA).

DNA extraction and real time PCR detection

After incubation in APW (2.5% NaCl added), one milliliter portion from each sample was removed and put into a sterile 1.5 mL centrifuge tube and centrifuged for 10 min at 12,000 rpm, rinsed with sterile water and treated by the boiling lysis method for DNA extraction. Two microliters of the extracted DNA were used for real time PCR amplification.

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