Letters in Applied Microbiology

Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

A pentaplex PCR assay for detection and characterization of *Vibrio vulnificus* and *Vibrio parahaemolyticus* isolates

N. Bhattacharyya and A. Hou

Department of Environmental Sciences, School of the Coast and Environment, Louisiana State University, Baton Rouge, LA, USA

Significance and Impact of the Study: This study, to the best of our knowledge, has been the first effort to develop a fast, cost-effective multiplex PCR (a pentaplex PCR termed pPCR) assay for simultaneous detection of bacterial isolates for pathogenic and nonpathogenic strains of *Vibrio parahaemolyticus* and *Vibrio vulnificus*. The fact that the assay can be applied to screen and characterize large numbers of bacterial isolates for the two most concerned *Vibrio* spp. from clinical/environmental samples will help detect pathogens, prevent disease outbreaks and plan better risk management strategies to protect public health.

Keywords
bacterial isolates, pathogenic vibrios, pentaplex PCR, seafood, simultaneous detection.

Correspondence
Aixin Hou, Department of Environmental Sciences, School of the Coast and Environment, Louisiana State University, Baton Rouge LA70803, LA, USA.
E-mail: ahou@lsu.edu

2013/0511: received 15 March 2013, revised 25 April 2013 and accepted 15 May 2013

doi:10.1111/lam.12101

Abstract

Vibrio parahaemolyticus and *Vibrio vulnificus* are the leading causes of seafood-related illnesses and also can cause wound infections. These bacteria often co-exist in marine and estuarine environments. However, there have been no reported protocols that can detect and characterize (i.e. pathogenic or nonpathogenic) them in a single PCR. In this study, we developed a pPCR assay with a combination of two species-specific and three pathogenic-specific PCR primers to simultaneously detect virulent (*viuB* in *V. vulnificus* and *tdh*/*trh* in *V. parahaemolyticus*) and nonvirulent (*vvhA* in *V. vulnificus* and *tlh* in *V. parahaemolyticus*) markers of the two species in bacterial isolates. The assay was validated by three methods. First, the pPCR was used to characterize 300 bacterial isolates consisting of seven reference strains and 293 environmental strains isolated from the Gulf of Mexico water. Results were compared with characterizations based on single-gene PCR amplifications and previously published multiplex PCR protocols. Second, 51 isolates characterized with the pPCR were analysed by 16S rRNA sequencing to confirm any false-negative/positive reaction. Finally, the effectiveness of the assay for heterogeneous bacterial samples was validated. The pPCR correctly characterized isolates from the Gulf with an efficiency of 96·6–98·7%.

Introduction

Vibrio parahaemolyticus and *Vibrio vulnificus* are motile, gram-negative, halophilic bacteria that are commonly found in marine and estuarine environments. They were identified as the cause of human infections as early as 1951 (Fujino *et al.* 1951) and 1979 (Blake *et al.* 1979), respectively. In the United States (US), the two *Vibrio* spp. are the leading causes of seafood-related illnesses. According to the Centers for Disease Control and Prevention (CDC), the incidence of *V. parahaemolyticus* and *V. vulnificus* infections in the United States due to eating raw or undercooked oysters has shown a sustained increase since year 2000 (CDC, 2009). In 2011, the CDC estimates 45 000 cases of *V. parahaemolyticus* yearly in the United States and 207 cases of *V. vulnificus* (Scallan *et al.* 2011). These bacteria reside in seawater in association with phytoplankton. Oysters, which are filter feeders, trap bacteria in the mucus on their gills, and the bacteria bioaccumulate in oyster tissues (Potasman *et al.* 2002). There is thus an increased risk of food poisoning through consumption of raw seafood. *Vibrio vulnificus* and *V. parahaemolyticus* are also reported to cause 'necrotizing fasciitis' (Fujioka *et al.* 2003; Tena *et al.* 2010), which

gain entry into human soft tissues when skin lesions are exposed to contaminated waters during recreational activities. The detection and monitoring of these microorganisms in the environment is therefore of great importance from a public health standpoint.

Various conventional methods have been developed for the detection of *V. vulnificus* and *V. parahaemolyticus*, for example, use of selective, differential agar media, biochemical tests and examination of their colony morphology (Kaysner and DePaola 2004). These tests usually involve a lengthy laboratory procedure and provide very limited knowledge about bacterial pathogenicity.

In recent years, techniques based on the polymerase chain reaction (PCR) have been used to detect these species and their pathogenic strains thereof by targeting the amplification of specific gene sequences with appropriate primers. Species of *V. parahaemolyticus* and *V. vulnificus* are commonly differentiated by the presence of the thermolabile haemolysin *tlh* gene and the cytolysin *vvhA* gene, respectively (Bej *et al.* 1999; Panicker *et al.* 2004a). The pathogenic strains of *V. parahaemolyticus* contain a thermostable direct haemolysin (tdh)-encoding or tdh-related haemolysin (trh)-encoding gene, or sometimes both in one strain (Tada *et al.* 1992). The Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM) has accordingly given priority to the detection of pathogenic *V. parahaemolyticus* strains possessing the *tdh* and *trh* genes in the environment and seafood samples (Kaysner and DePaola 2004). In most cases, the strains with these genes have been isolated from clinical samples (Shirai *et al.* 1990). The proteins encoded by these genes have proved to cause gastroenteritis and can lyse red blood cells (Miyamoto *et al.* 1969; Honda and Iida 1993). A clinically important *viuB* gene found in *V. vulnificus* is a siderophore-encoding gene associated with iron acquisition and presents a risk to patients with elevated iron levels in their serum (Bogard and Oliver 2007). *Vibrio vulnificus* infections are greater threats to people with compromised immune systems, that is, patients with diabetes, liver diseases, AIDS, β-thalassaemia or haemochromatosis (Bogard and Oliver 2007).

The simultaneous detection of *V. vulnificus* and *V. parahaemolyticus* has been made possible by the development of several multiplex PCR assays in which relevant genes can be detected in one reaction (Neogi *et al.* 2010; Izumiya *et al.* 2011). The PCR protocols that have been developed make use of various combinations of unique primers for the detection of these five molecular biomarkers namely *tlh, tdh, trh, vvhA* and *viuB*. However, none of the previously reported protocols has combined the five pairs of primers into one PCR for the simultaneous detection of all five respective genes associated with each species. The purpose of this study was to develop a PCR assay for rapid detection of all the aforementioned genetic markers in a single PCR tube and to determine the efficiency of the assay by analysis of bacterial isolates collected from the surface waters of the northern Gulf of Mexico. We anticipate that the assay will be a cost-effective approach for screening large numbers of putative bacterial isolates for virulent and nonvirulent strains of these *Vibrio* species.

Results and discussion

The pPCR assay employed an optimal annealing temperature of 57°C and a higher concentration (see Materials and Methods) of primers than that previously reported. Five temperatures (55, 57, 59, 62 and 65°C) were tested in this study, and the 57°C was best suited to anneal all the primers needed to amplify the five target genes (Table 1; see gel image in Fig. 1). We were unable to amplify all five of the target genes simultaneously at the other annealing temperatures, perhaps because differences in the melting temperatures (T_m) of the primers caused unstable annealing to the target genes (White 1993). There were no interfering bands associated with the

Table 1 The five target genes, their primer sequences, previously reported annealing temperatures (T) and amplicon sizes of the genes

Target genes	Primer sequences*† (F: forward; R: reverse)	Multiplex PCR annealing *T* (°C)*†	FDA manual annealing T (°C)‡	Amplicon size (bp)*†
vvhA	F-5′- TTC CAA CTT CAA ACC GAA CTA TGA C -3′ R-5′-ATT CCA GTC GAT GCG AAT ACG TTG-3′	65*	62	205
viuB	F- 5′-GGT TGG GCA CTA AAG GCA GAT ATA-3′ R- 5′-CGG CAG TGG ACT AAT ACG CAG C-3′		NA	504
tlh	F- 5′-AAA GCG GAT TAT GCA GAA GCA CTG-3′ R- 5′-GCT ACT TTC TAG CAT TTT CTC TGC-3′	55†	60	450
tdh	F-5′-GTA AAG GTC TCT GAC TTT TGG AC-3′ R- 5′-TGG AAT AGA ACC TTC ATC TTC ACC-3′		60	269
trh	F- 5′-TTG GCT TCG ATA TTT TCA GTA TCT-3′ R- 5′-CAT AAC AAA CAT ATG CCC ATT TCC G-3′		58	500

*Panicker *et al.* (2004a); †Bej *et al.* (1999); ‡Kaysner and DePaola (2004); N/A: not available.

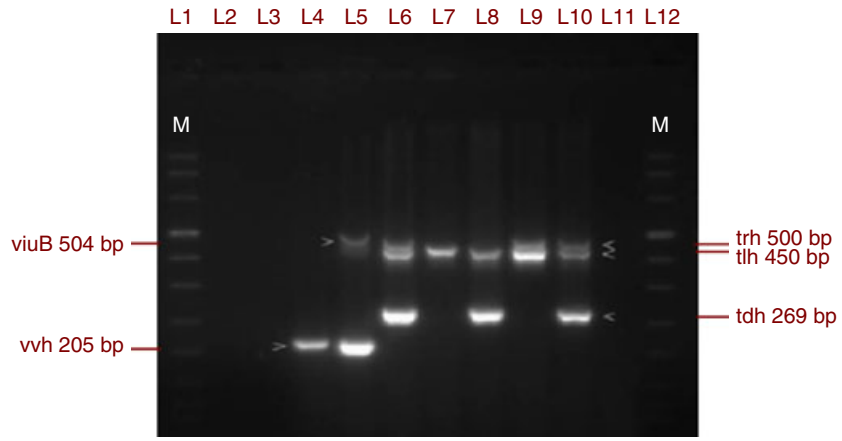

Figure 1 Gel image of the pPCR amplicons for the five target genes in the positive and negative controls, amplified with an annealing temperature of 57°C. L = Lane where, L1 & L12: 100 bp DNA marker; L2: Negative control - *V. cholera* ATCC 14035; L3: Negative control - Autoclaved LB glycerol stock; L4: *V. vulnificus* Vv3138-001 (*vvhA*$^+$); L5: *V. vulnificus* 1007 (*viuB* $^+$ *vvhA*$^+$); L6 & L10: *V. parahaemolyticus* F11-3A (*tlh*$^+$ *trh*$^+$ *tdh*$^+$); L7: *V. parahaemolyticus* FIHES98 (*tlh*$^+$); L8: *V. parahaemolyticus* Tx-2103 (*tlh*$^+$ *tdh*$^+$); L9: *V. parahaemolyticus* AQ 4037 (*tlh*$^+$ *trh*$^+$); L11: Negative control - *Nuclease free water*. The white arrow (>and<) signs represent the base pairs of the PCR amplicons shown on the left- & right-hand side of the gel image.

Table 2 Positive and negative controls used for the PCR analyses

Positive and negative controls	Genotype	Source/origin
Positive controls		
Vibrio parahaemolyticus F11-3A	*tlh*$^+$ *trh*$^+$ *tdh*$^+$	Washington clams (Bej *et al.* 1999)*
Vibrio parahaemolyticus AQ 4037	*tlh*$^+$ *trh*$^+$	A. DePaola, US FDA, Gulf Coast Seafood Lab (Nordstrom *et al.* 2007)*
Vibrio parahaemolyticus Tx-2103	*tlh*$^+$ *tdh*$^+$	A. DePaola, US FDA, Gulf Coast Seafood Lab (Nordstrom *et al.* 2007)*
Vibrio parahaemolyticus FIHES98	*tlh*$^+$	A. DePaola, US FDA, Gulf Coast Seafood Lab (Nordstrom *et al.* 2007)*
Vibrio vulnificus 1007	*vvhA*$^+$ *viuB*$^+$	Blood, Louisiana Department of Health and Hospitals (Ge and Han 2008)*
Vibrio vulnificus (Vv3138-001)	*vvhA*$^+$	Our lab (isolated from Breton Sound water sample)†
Negative controls		
Vibrio cholera ATCC 14035		Clinical isolate (ATCC)*
Nuclease-free water		Provided by Promega Corp.
Autoclaved LB glycerol stock		Prepared in our lab

*Isolates were provided by Dr. Beilei Ge, Dept. of Food Science, Louisiana State University, Baton Rouge, LA, USA.
†Isolated at our laboratory (Microbial Ecology Laboratory), Department of Environmental Science, Louisiana State University, Baton Rouge, LA, USA.

negative controls, including *V. cholera* ATCC 14035, autoclaved LB glycerol stock and nuclease-free water (Fig. 1). When individual primer pairs were tested separately, they worked perfectly at 57°C, without producing any spurious bands (gel image not shown).

The assay was validated using seven reference strains with known genotypes (Table 2) and 293 environmental strains isolated from Gulf of Mexico water samples on TCBS and CC agar. We compared the results obtained with the pPCR assay, individual primer pair tests and previously published multiplex PCR protocols (Bej *et al.* 1999; Panicker *et al.* 2004a). Among the 293 environmental isolates (Table 3), 117 isolates were determined to contain the *vvhA* gene and were identified as *Vibrio vulnificus* by the pPCR assay, a result consistent with the individual *vvhA* tests and the multiplex PCR protocol of Panicker *et al.* (2004a). The pPCR assay identified 30 environmental isolates as *Vibrio parahaemolyticus* based on the presence of the *tlh* gene, a result also in agreement with the individual *tlh* tests and the multiplex PCR protocol of Bej *et al.* (1999). The remainder of the environmental isolates contained none of the five genes targeted by the pPCR assay; likewise, the individual primer tests and the multiplex protocols detected none of the target genes in the unidentified bacteria. Several of these isolates, however, generated spurious bands, which appeared either near or distant from the location of the target gene on the agarose gel (Fig. S1). The spurious bands appeared in both the pPCR assay and the multiplex PCR assays (Table 3). Use of the pPCR assay enabled us to detect the five target

Table 3 Detection and characterization of bacterial isolates for *Vibrio parahaemolyticus* and *Vibrio vulnificus* with different PCR assays

	Characterization of isolates		Number of occurrences of target genes in tested isolates using different PCR assays			
Isolates tested	Species	Genotype	pPCR (this study)	Multiplex PCR* for *tlh*, *trh* and *tdh*	Multiplex PCR† for *vvhA* and *viuB*	single -gene PCR
Environmental Isolates (n = 293)	*Vibrio parahaemolyticus* (n = 30)	*tlh*$^+$	28	28	0	28
		tlh$^+$ *trh*$^+$ *tdh*$^+$	2	2	1§	2
	Vibrio vulnificus (n = 117)	*vvhA*$^+$	116	0	116	116
		vvhA$^+$ *viuB*$^+$	1	0	1	1
	Non- *Vibrio parahaemolyticus* / *Vibrio vulnificus* (n = 146)	*vvh*$^-$ *viuB*$^-$/*tlh* $^-$ *trh* $^-$ *tdh* $^-$	4§, 2¶	1§	3§	0
Reference strains (n = 7)‡	*Vibrio parahaemolyticus* (n = 4)	*tlh*$^+$	1	1	0	1
		tlh$^+$ *tdh*$^+$	1	1	1§	1
		tlh$^+$ *trh*$^+$	1	1	0	1
		tlh$^+$ *trh*$^+$ *tdh*$^+$	1	1	0	1
	Vibrio vulnificus (n = 2)	*vvhA*$^+$	1	0	1	1
		vvhA$^+$ *viuB*$^+$	1	1§	1	1
	Vibrio cholera (n = 1)	*vvhA*$^-$ *viuB*$^-$/*tlh*$^-$ *trh*$^-$ *tdh*$^-$	0	0	0	0

*Panicker *et al.* (2004a); Bej *et al.* (1999).

†Panicker *et al.* (2004a).

‡The seven reference strains are listed in Table 2.

§Refers to phantom bands, which appeared near the location of the target gene on the agarose gel.

¶Refers to phantom bands, which appeared distant from the location of the target gene on the agarose gel.

genes with 100% efficiency in the reference strains and with 96·6% efficiency in 293 environmental isolates. If the number of occurrences of the distant spurious bands that did not interfere with the detection of the target genes was excluded, the efficiency of the pPCR increased to 98·6%. The efficiencies of the multiplex PCR protocols by Panicker *et al.* (2004a) and Bej *et al.* (1999) were 98·3 and 99·3%, respectively, with the 300 tested strains.

To further confirm that there were no false-negative results, 41 environmental isolates that contained none of the five target genes and two environmental isolates showing spurious bands based on the pPCR assay were analysed by 16S rRNA sequencing. The sequence analyses showed that these isolates had higher similarities (>98%) to known strains belonging to eight other *Vibrio* spp. and seven non-*Vibrio* species. The other *Vibrio* spp. included *Vibrio orientalis* (n = 3), *Vibrio natriegens* (n = 2), *Vibrio rotiferianus* (n = 8), *Vibrio ponticus* (n = 9), *Vibrio aestuarianus* (n = 2), *V. cholera/Vibrio mimicus* (n = 6), *Vibrio mediterranei/Vibrio neptunius/ V. orientalis* (n = 1) and *Vibrio ordalii* (n = 3); the non-*Vibrio* species included *Bacillus gibsonii* (n = 2), *Bacillus aerophilus* (n = 1), *Providencia rettgeri* (n = 1), *Photobacterium rosenbergii* (n = 1), *Exiguobacterium indicum* (n = 1), *Aeromonas hydrophila* (n = 2) and *Bacillus safensis/Bacillus pumilus* (n = 1). The isolates that produced a spurious band in the 400- to 420-bp range showed a maximum similarity to *V. cholera/V. mimicus*. Eight environmental isolates that were positive for *V. vulnificus* (n = 4) or *V. parahaemolyticus* (n = 4) showed a 16S rRNA sequence similarity of 99·81 and 97·7% to these two species, respectively. In addition, the spurious band near the 510- to 550-bp range did not interfere with the observation of the bands of the target genes in the pPCR assay, and we were able to resolve this problem by decreasing the PCR cycle from 30 to 27 (data not shown). An increase in the number of PCR cycles has been reported to cause spurious bands because of non-specific amplification (Bell and DeMarini 1991; Roux 2009).

The pPCR assay specificity assessment with different combinations of pooled non-*V. vulnificus/V. parahaemolyticus* environmental isolates and reference strains showed that the pPCR assay was able to detect the target genes and did not produce any spurious bands (Table 4), revealing that the assay was able to successfully detect the target genes in a complex matrix.

In this study, the detection limit for strains of *V. vulnificus* and *V. parahaemolyticus* by the pPCR assay was 30 cells ml^{-1}, which lies within the ranges reported by Panicker *et al.* (2004a; 10^2–10^3 CFU ml^{-1}) and Bej *et al.* (1999; 10–100 CFU 10 g^{-1} APW enriched oyster tissue homogenates).

Several observations during this study are worth mentioning. First, use of the published protocols by Panicker *et al.* (2004a) in our laboratory produced spurious bands

Table 4 Specificity assessment for the pPCR assay using different combinations of type strains of *Vibrio parahaemolyticus* and *V. vulnificus* and pooled environmental isolates

Strains	pPCR analysis	Spurious bands
Pooled environmental isolates*	*vvhA*$^-$ *viuB*$^-$ *tlh*$^-$ *trh*$^-$ *tdh*$^-$	–
Pooled environmental isolates and *Vibrio vulnificus* 1007	*vvhA*$^+$ *viuB*$^+$	–
Pooled environmental isolates and *Vibrio parahaemolyticus* F II 3A	*tlh*$^+$ *trh*$^+$ *tdh*$^+$	–
Vibrio vulnificus 1007 – positive control	*vvhA*$^+$ *viuB*$^+$	–
Vibrio parahaemolyticus F II 3A– positive control	*tlh*$^+$ *trh*$^+$ *tdh*$^+$	–

*The pooled environmental isolates consist of species of *Vibrio cholerae/Vibrio mimicus* (*n* = 2), *Vibrio ordalii/Listonella anguillarum* (*n* = 2), *Vibrio ponticus* (*n* = 2) and *Providencia rettgeri* (*n* = 1), isolated from the Gulf water on TCBS agar and identified using 16S rRNA sequencing. The absence of the five target genes in these strains was also confirmed with single-gene PCR amplifications. Sign (–) represents absence and (+) presence.

in the negative controls. This might be because our primers did not include the base modification at the 5′ end of the primers used by Panicker *et al.* (2004a). When we changed the annealing temperature to 57°C, these spurious bands disappeared. Second, the Hot Start Green PCR mix (Promega Corporation, Madison, WI, USA) includes dyes incorporated by the manufacturer to facilitate loading of agarose gels, and those dyes may result in PCR inhibition in some cases (Hoppe *et al.* 1992). In our study, however, there was no inhibition, and we observed no false-negative reactions. Third, incorporation of glycerol (5–10%) has proved to be suitable for enhancing the specificity of the PCR (Pomp and Medrano 1991). The fact that we used a suspension of isolates stored in LB 20% glycerol stock for direct preparation of crude DNA templates may therefore explain why the efficiency of our assay was high (98·6%). Fourth, we used a premade PCR mixture from Promega Corporation and did not optimize factors such as the concentration of magnesium ions, pH, Taq polymerase dose or dNTP concentration. Optimization of one or more of these factors might help minimize the occurrence of nonspecific bands. Another observation made during this study was that the haemolysin genes *trh* and *tdh* appeared in a few isolates that were negative for the *tlh* gene. This result could reflect the fact that these pathogenic genes are mobile genetic elements. Horizontal gene transfer may account for the presence of *trh* and *tdh* haemolysin genes in strains of other *Vibrio* species (Bej *et al.* 1999; Dobrindt *et al.* 2004; Hurley *et al.* 2006).

To the best of our knowledge, this pPCR assay is the first protocol that is able to detect and characterize (i.e. pathogenic or nonpathogenic) a bacterial isolate of *V. vulnificus* or *V. parahaemolyticus* using five pairs of gene-specific primers (two species-specific and three specific for virulence) in a single reaction mixture. A multiplex PCR that involved ten primer pairs was once tried by Panicker *et al.* (2004a, b), but did not give reliable results. Our results demonstrated that the pPCR assay described here for the detection of the five target genes can be completed in a laboratory setting within 5–6 h after collection of isolates. The assay has the potential to largely reduce the time and cost of laboratory supplies required for sample processing compared with previously published PCR methods for the separate detection of each of the two *Vibrio* species. The study suggests that the assay has great advantages of potential utility to the food industry, to clinical laboratories and to environmental and water quality monitoring laboratories. In addition, this pPCR methodology could be incorporated into the current FDA BAM for rapid screening of bacterial isolates for *V. vulnificus* and *V. parahaemolyticus* and their virulent strains. Furthermore, the pPCR might provide additional time-saving solutions for the detection of both *V. vulnificus* and *V. parahaemolyticus* cells in a mixture such as MPN broths. In that case, however, the challenge of differentiating the two amplicon products of *viuB* (504 bp in *V. vulnificus*) and *trh* (500 bp in *V. parahaemolyticus*) in close proximity needs to be resolved.

Materials and methods

Reference strains and environmental isolates

Six reference strains of *V. parahaemolyticus* and *V. vulnificus* and one type strain of *V. cholera* were used for optimization of the pPCR assay and as positive and negative controls in the PCR analyses. Their genotypes and sources/origins are described in Table 2. The strains were checked and reconfirmed with the individual primers listed in Table 1. An additional 293 environmental isolates were obtained from water samples collected from Breton Sound, Barataria Bay and Bay Jimmy of the Gulf of Mexico in 2011 and 2012. These putative *V. parahaemolyticus* and *V. vulnificus* strains were isolated with 24-h incubations at 35 ± 2°C on BD™ Difco TCBS (Kobayashi *et al.* 1963) and Cellobiose-Colistin (CC) agar (BAM Media M189, 2004). They were used to evaluate the specificity and efficiency of the pPCR assay. The reference strains and environmental isolates were stored in 2× Luria–Ber-

tani broth with 20% glycerol at –80°C for further use in this study.

DNA template preparation

An aliquot of 20 μl of a cell suspension of a strain in 20% glycerol LB broth was used for the preparation of crude DNA templates. The DNA contents were released via lysis of cells by heating at 98°C for 10 min in a thermocycler (Bio-Rad iCycler™ MyiQ™ optical module, Bio-Rad Laboratories, Hercules, CA, USA).

Primer selection and concentration

The primers (Table 1) used for this experiment have been previously published (Bej *et al.* 1999; Panicker *et al.* 2004a) and reported in the FDA manual (Kaysner and DePaola 2004). The primer concentration used by Panicker *et al.* (2004a) did not produce clear bands in this study. We overcame this problem by increasing the primer concentration, the higher concentration then being used without further optimization throughout the experiment.

Optimization of pPCR annealing temperature

An optimization study was performed at annealing temperatures of 55, 57, 59, 62 and 65°C to determine the temperature best suited for specific binding of the 5 pairs of primers (Table 1) to enable simultaneous amplification of the specific target genes in one PCR. The optimization effort employed the positive and negative controls given in Table 2. To check the reproducibility of the assay, we used the PCR conditions described below to carry out the PCR assay five times for each of the five different annealing temperatures. The optimal annealing temperature was further tested with individual primer pairs for all the positive and negative controls given in Table 2.

PCR conditions for pentaplex assay

The PCR mixture contained 1 μl of the heat-treated crude DNA template, 12·5 μl of 1 × GoTaq® Hot Start Green Master Mix (Promega Corporation) and the primers (F-*vvhA* and R-*vvhA*, each at a concentration of 1·35 μmol l^{-1}; F-*tlh*, R-*tlh*, F-*trh* and R-*trh*, each at a concentration of 1·40 μmol l^{-1}; F-*tdh* and R-*tdh*, each at a concentration of 1·35 μmol l^{-1}; and F-*viuB* and R-*viuB*, each at a concentration of 3 μmol l^{-1}). Nuclease-free water (Promega Corporation) was used to bring the final reaction volume to 25 μl. The primers were purchased from Integrated DNA Technologies Inc., Iowa, USA. All PCR amplifications were performed on an iCycler Thermal Cycler (Bio-Rad Laboratories), with the following thermal cycle profile: an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min and extension at 72°C for 1 min, and a final extension step at 72°C for 6 min 50 s. Alternatively, the initial denaturation duration was increased to 10 min as pre-amplification heating for direct PCR of colonies (D'Aquila *et al.* 1991), and the assay produced good results (data not shown). This alternative can avoid the boiling step for the preparation of crude DNA template, that is to say isolates picked from TCBS and CC agar plates can be directly used for the PCR.

Validation of pPCR assay

The validation was conducted in three ways. First, seven reference strains (Table 2) and 293 environmental isolates from the Gulf of Mexico were assayed with the pPCR assay (this study) and previously published PCR protocols (Bej *et al.* 1999; Kaysner and DePaola 2004; Panicker *et al.* 2004a). The results were compared, and the efficiency of the pPCR was calculated based on the number of false-positive bands, false-negative bands and spurious bands, if any were recorded. Second, 51 of the 293 environmental isolates were selected for species identification with partial 16S rRNA sequencing (described below) to evaluate the specificity of the pPCR assay. Third, the specificity of the pPCR assay was further evaluated using different combinations of *V. parahaemolyticus* F11-3A and *V. vulnificus* 1007 reference strains and seven environmental isolates (16S rRNA sequenced) of *Vibrio* species other than *V. parahaemolyticus* and *V. vulnificus* (Table 4).

Analysis of PCR amplicons

Four microlitres of the amplified gene products was electrophoresed at 100 V for ~60 min in 2% agarose gel (ISC Bio Express, Gene Pure Sieve GQA Agarose) with 1·25 μl of ethidium bromide (Bio-Rad 10 mg ml^{-1} stock) using 0·25× TAE buffer. A 100- or 50-bp DNA ladder was used as DNA size marker. The fluorescent bands were visualized by a Bio-Rad Gel Doc System Universal Hood II S.N 76S/02980.

16S rRNA sequencing

The PCR cycle conditions for 16S rRNA amplification of the isolates were provided by Functional Biosciences Inc. (Madison, WI, USA). We used 16S forward 5′- AGA GTT TGA TCC TGG CTC-3′ and 16S reverse 5′-TAC CGC GGC TGC TGG CAC-3′ as primers (Functional Biosciences). The PCR cycle steps were as follows: 96°C for 5 min, followed by 35 cycles, with each cycle involving

94°C for 30 s, 60°C for 45 s and 72°C for 90 s. The PCR mixture contained 12·5 μl of Go-Taq Hot Start Green *Taq* polymerase (Promega Corp.), 0·4 μmol l^{-1} of each 16S primer and 2 μl of template DNA. The final reaction volume was made to 25 μl with nuclease-free water (Promega Corp.). The PCR products were further processed and sequenced at Functional Biosciences using the 16S-F primer having sequences similar to the 8F universal primers (Amann *et al.* 1995; Liu *et al.* 1997). Sequences ranging between 400 and 489 bp were blasted with the Standard Nucleotide NCBI BLAST tool (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PAGE=Nucleotides).

Acknowledgements

This work was supported by the U.S.EPA (Agreement No. MX95460010-0). Dr. Robert lane and Mrs James Manly Lee, Spence Colwell and Martin Maxwell at Louisiana State University helped collect environmental samples from the Gulf of Mexico. We thank Drs Vincent L. Wilson and Edward A. Laws for their critical review of this manuscript. We have no conflict of interest to declare.

References

Amann, R.I., Ludwig, W. and Schleifer, K.H. (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol Rev* **59**, 143–169.

BAM (2004) Media M189, Cellobiose-Colistin (CC) Agar. http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm064571.htm.

Bej, A.K., Patterson, D.P., Brasher, C.W., Vickery, M.C.L., Jones, D. and Kaysner, C.A. (1999) Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tl*, *tdh* and *trh*. *J Microbiol Methods* **36**, 215–225.

Bell, D.A. and DeMarini, D.M. (1991) Excessive cycling converts PCR products to random-length higher molecular weight fragments. *Nucleic Acids Res* **19**, 5079.

Blake, P.A., Merson, M.H., Weaver, R.E., Hollis, D.G. and Heublein, P.C. (1979) Disease caused by a marine Vibrio. *N Engl J Med* **300**, 1–5.

Bogard, R.W. and Oliver, J.D. (2007) Role of iron in human serum resistance of the clinical and environmental *Vibrio vulnificus* genotypes. *Appl Environ Microbiol* **73**, 7501–7505.

CDC (2009) *Cholera and Other Vibrio Illness (COVIS) Annual Summary*. Available at: http://www.cdc.gov/nationalsurveillance/PDFs/CSTEVibrio2009.pdf.

D'Aquila, R.T., Bechtel, L.J., Videler, J.A., Eron, J.J., Gorczyca, P. and Kaplan, J.C. (1991) Maximizing sensitivity and specificity of PCR by preamplification heating. *Nucleic Acids Res* **19**, 3749.

Dobrindt, U., Hochhut, B., Hentschel, U. and Hacker, J. (2004) Genomic islands in pathogenic and environmental microorganisms. [10.1038/nrmicro884]. *Nat Rev Microbiol* **2**, 414–424.

Fujino, T., Okuno, T., Nakada, D., Aoyama, A., Fukai, K., Mukai, T. and Ueho, T. (1951) On the bacteriological examination of Shirasu-food poisoning. *J Jpn Asso Infect Dis* **25**, 11–12.

Fujioka, M., Nishimura, G., Miyazato, O., Yamamoto, T., Okamoto, F. and Tsunenori, K. (2003) Necrotising fasciitis and myositis that originated from gastrointestinal bacterial infection: two fatal cases. *Scand J Plast Reconstr Surg Hand Surg* **37**, 239–242.

Ge, B. and Han, F. (2008) Evaluation of a loop-mediated isothermal amplification assay for detecting *Vibrio vulnificus* in raw oysters. *Foodborne Pathog Dis* **5**, 311–320.

Honda, T. and Iida, T. (1993) The pathogenicity of *Vibrio parahaemolyticus* and the role of the thermostable direct haemolysin and related haemolysins. *Rev Med Microbiol* **4**, 106–113.

Hoppe, B.L., Conti-Tronconi, B.M. and Horton, R.M. (1992) Gel-loading dyes compatible with PCR. *Biotechniques* **12**, 679–680.

Hurley, C., Quirke, A., Reen, F.J. and Boyd, E.F. (2006) Four genomic islands that mark post-1995 pandemic *Vibrio parahaemolyticus* isolates. *BMC Genomics* **7**, 104.

Izumiya, H., Matsumoto, K., Yahiro, S., Lee, J., Morita, M., Yamamoto, S., Arakawa, E. and Ohnishi, M. (2011) Multiplex PCR assay for identification of three major pathogenic *Vibrio* spp., *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*. *Mol Cell Probes* **25**, 174–176.

Kaysner, C.A. and DePaola, A. Jr. (2004) Bacteriological Analytical Manual, Chapter 9 *Vibrio*. http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070830.htm.

Kobayashi, T., Enomoto, S., Sakazaki, R. and Kuwahara, S. (1963) A new selective isolation medium for the Vibrio group; on a modified Nakanishi's medium (TCBS agar medium). *Nippon Saikingaku Zasshi* **18**, 387–392.

Liu, W.T., Marsh, T.L., Cheng, H. and Forney, L.J. (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Appl Environ Microbiol* **63**, 4516–4522.

Miyamoto, Y., Kato, T., Obara, Y., Akiyama, S., Takizawa, K. and Yamai, S. (1969) *In Vitro* hemolytic characteristic of *Vibrio parahaemolyticus*: its close correlation with human pathogenicity. *J Bacteriol* **100**, 1147–1149.

Neogi, S.B., Chowdhury, N., Asakura, M., Hinenoya, A., Haldar, S., Saidi, S.M. and Yamasaki, S. (2010) A highly sensitive and specific multiplex PCR assay for

simultaneous detection of *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*. *Lett Appl Microbiol* **51**, 293–300.

Nordstrom, J.L., Vickery, M.C.L., Blackstone, G.M., Murray, S.L. and DePaola, A. (2007) Development of a multiplex real-time PCR assay with an internal amplification control for the detection of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters. *Appl Environ Microbiol* **73**, 5840–5847.

Panicker, G., Vickery, M.C.L. and Bej, A.K. (2004a) Multiplex PCR detection of clinical and environmental strains of *Vibrio vulnificus* in shellfish. *Can J Microbiol* **50**, 911–922.

Panicker, G., Call, D.R., Krug, M.J. and Bej, A.K. (2004b) Detection of pathogenic Vibrio spp. in shellfish by using multiplex PCR and DNA microarrays. *Appl Environ Microbiol* **70**, 7436–7444.

Pomp, D. and Medrano, J.F. (1991) Organic solvents as facilitators of polymerase chain reaction. *Biotechniques* **10**, 58–59.

Potasman, I., Paz, A. and Odeh, M. (2002) Infectious outbreaks associated with bivalve shellfish consumption: a worldwide perspective. *Clin Infect Dis* **35**, 921–928.

Roux, K.H. (2009) Optimization and troubleshooting in PCR. *Cold Spring Harb Protoc* **2009**. doi:10.1101/pdb.ip66.

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.A., Roy, S.L., Jones, J.L. and Griffin, P.M. (2011) Foodborne illness acquired in the United States–major pathogens. *Emerg Infect Dis* **17**, 7–15.

Shirai, H., Ito, H., Hirayama, T., Nakamoto, Y., Nakabayashi, N., Kumagai, K., Takeda, Y. and Nishibuchi, M. (1990) Molecular epidemiologic evidence for association of thermostable direct hemolysin (TDH) and TDH-related hemolysin of *Vibrio parahaemolyticus* with gastroenteritis. *Infect Immun* **58**, 3568–3573.

Tada, J., Ohashi, T., Nishimura, N., Shirasaki, Y., Ozaki, H., Fukushima, S., Takano, J., Nishibuchi, M. *et al.* (1992) Detection of the thermostable direct hemolysin gene (*tdh*) and the thermostable direct hemolysin-related hemolysin gene (*trh*) of *Vibrio parahaemolyticus* by polymerase chain reaction. *Mol Cell Probes* **6**, 477–487.

Tena, D., Arias, M., Álvarez, B.T., Mauleón, C., Jiménez, M.P. and Bisquert, J. (2010) Fulminant necrotizing fasciitis due to *Vibrio parahaemolyticus*. *J Med Microbiol* **59**, 235–238.

White, B.A. (1993) *PCR Protocols: Current Methods and Applications, Volume 15 of Methods in Molecular Biology*. Totowa, NJ: Humana Press.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Gel images of spurious bands near (upper panel) or distant (lower panel) from the location of the target genes in the Pentaplex PCR analyses. L = Lane. In upper panel, L1: *V. vulnificus* 1007 & L2: *V. parahaemolyticus* F11-3A as positive controls; M: 50 bp DNA marker; L3: *V. cholera* ATCC 14035 as negative control; *L4*, L5 & L11: environmental isolates possessing vvh^+ gene in *V. vulnificus*; L7: environmental isolate possessing tlh^+ gene in *V. parahaemolyticus*; L6, L8, L9, L10 & L14: environmental isolates negative for all the target genes. L12 & L13: *V. parahaemolyticus* isolates with trh^+, tlh^+ *and* tdh^+ genes. The arrow sign in L12 represents a faint but detectable spurious band near the 206–205 bp location. In lower panel, L1: *V. vulnificus* 1007 & L2: *V. parahaemolyticus* F11-3A as positive controls; L3: 50 bp DNA marker; L4: *V. cholera* ATCC 14035 as negative control; *L5 & L6*: environmental isolates negative for all the target genes; L7, L8 & L9: the three arrow signs show spurious bands in the range 380–400 bp; L7: the arrow sign shows a faint, spurious band 510-520 bp; L10: the arrow indicates a false positive band at the *trh* location; L11 & L12 environmental isolates of *V. vulnificus* possessing vvh^+.