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Detection of Toxigenic *Vibrio cholerae, V. parahaemolyticus* and *V. vulnificus* in Oyster by Multiplex-PCR with Internal Amplification Control

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ABSTRACT

This study developed a multiplex-polymerase chain reaction (m-PCR) method that targets the toxin genes, *vvhA* and *vvp* for *Vibrio vulnificus*, *tdh* for *V. parahaemolyticus*, *ctx* for *V. cholera*, to detect these three species simultaneously. A chimeric DNA consisting of a fragment of the green fluorescent protein gene (*gfp*) flanked by sequences of the *vvhA* primers was used as the internal amplification control (IAC). In the presence of these three *Vibrio* species, amplicons of IAC (753 bp), *vvhA* (505 bp), *vvp* (383 bp), *tdh* (256 bp) and *ctx* (167 bp) could be simultaneously detected. The accuracy of the m-PCR approach was evaluated with a Kappa index of 0.96 using 28 strains of *V. vulnificus*, 18 *V. parahaemolyticus*, 14 *V. cholerae* and 40 other *Vibrio* species and non-*Vibrio* strains. As little as 10^1 CFU/mL of these *Vibrio* species in spiked oyster homogenate could be detected by this m-PCR method following enrichment at 37°C for 8 h. This method can be adopted for the rapid detection of the toxigenic strains of *Vibrio* species in seafood.

Key words: *Vibrio vulnificus*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, internal amplification control, multiplex PCR, virulence, oyster

INTRODUCTION

Vibrio parahaemolyticus and *V. vulnificus* are the most frequently encountered pathogenic vibrios in marine environments. As a marine and estuarine bacterium, *Vibrio vulnificus* is commonly associated with seawater, sediment, shellfish and the intestinal contents of fish[1,2]. It may cause septicemia and severe wound infection with high mortality in susceptible persons[3]. *V. parahaemolyticus* is one of the most frequently occurring food-borne enteric pathogen in many Asian countries, where seafood is often consumed[4]. Pandemic O3:K6 strains of *V. parahaemolyticus* have exhibited global dissemination since 1996[5,6]. *V. cholerae* is the etiological agent of cholera, an acute dehydrating diarrhea that occurs in epidemic form in many developing countries[7], while the non-O1 and non-O139 strains of *V. cholerae* are involved in sporadic infections. *V. cholerae* is a normal inhabitant of aquatic environments, survives in a wide range of salinity[8], and it often associated with *V. parahaemolyticus* and *V. vulnificus* in these environments[9-11].

Various molecular approaches have been developed to detect these *Vibrio* species. *V. vulnificus* can be detected by DNA probe hybridization or polymerase chain reaction (PCR) targeting a specific fragment of 16S rRNA[12], 23S rRNA[13] or specific target genes, such as *gyrB*[14] and hemolysin-cytolysin gene (*vvhA*)[15]. Different formats of PCR have also been developed to detect *V. parahaemolyticus* by targeting on the *tdh* and related genes[16], *gyrB*[17] or collagenase gene[18]. *V. cholerae* could also be detected by various PCR methods mostly based on the *ctx* gene[19] or the toxin coregulated pilus gene (*tcpA*)[20]. Toxin genes are often used as the targets in detecting toxigenic strains by molecular methods.

V. parahaemolyticus[21] and *V. cholerae*[22] are frequently isolated from environmental samples, nevertheless, high proportion of their environmental isolates are non-toxigenic strains. For accurate assessment of the risk of seafood or environment, detection of the toxigenic strains of these *Vibrio* species is necessary[23]. Only the toxigenic *V. parahaemolyticus* strains isolated from USA are usually positive in TDH, TRH and urease[4], and those from other countries are TDH positive with a small proportion of these strains being TRH positive[24,25]. Therefore, *tdh* has been the most frequently used target gene in molecular detection

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methods[26,27]. Cholera toxin (CTX) is the major enterotoxin produced by the toxigenic strain of *V. cholerae*[28]. Unlike *V. cholerae* and *V. parahaemolyticus*, a high percentage of environmental isolates of *V. vulnificus* exhibit animal virulence[29]. In another work, environmental *V. vulnificus* strains are phenotypically indistinguishable from clinical isolates and most of the environmental strains produce *in vitro* virulence factors[30]. Our previous study on virulence-associated genes in different strains of *V. vulnificus* revealed that *vvhA* was present in 91.4% of the mouse-virulent strains, while the extracellular protease gene (*vvp*) was identified in all virulent and non-virulent strains[31]. This extracellular protease of *V. vulnificus* exhibits elastolytic and collagenolytic activities[32]. To minimize false negative detection of toxigenic *V. vulnificus* strains, duo targets, *vvhA* and *vvp*, were detected in this study.

Internal amplification control (IAC) was incorporated into the multiplex real-time PCR detection of *V. parahaemolyticus*[33], but missed in most of these PCR methods. Two or three of these pathogenic vibrios are detected simultaneously by m-PCR targeting the collagenase gene[18] or *toxR* gene[34]. However, IAC is not included in these methods.

IAC is a non-target DNA sequence of known concentration and can be amplified concomitantly with the target genes using a single set of primers into amplicons of different sizes[35]. The presence of IAC ensures proper PCR procedures and the production of the amplified IAC fragment in the absence of the target gene further eliminates the false-negative result in the assay so that standard methods for interlaboratory use have thus been developed[36]. The European Standardization Committee, in collaboration with International Standard Organization, has offered a general guideline for PCR testing that requires the presence of IAC in the reaction mixture[35].

This report is the first one to develop an m-PCR by targeting on the common toxin genes of these three *Vibrio* species (*ctx* for *V. cholerae*, *tdh* for *V. parahaemolyticus*, *vvhA* and *vvp* for *V. vulnificus*) with IAC incorporated. Evaluation of this m-PCR method was also performed in spiked oyster homogenates.

MATERIALS AND METHODS

I. *Bacterial Cultures*

A total of 100 strains were used in this study, including 14 *V. cholerae*, 18 *V. parahaemolyticus*, 28 *V. vulnificus*, 13 other vibrios and 27 non-vibrios (Table 1). *V. vulnificus* VV45, *V. parahaemolyticus* ST550 and *V. cholerae* 294-94 (O139 strain) were used as reference strains for the development and optimization of method. *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* strains were identified by phenotypic traits and characterized by molecular methods[37-39]. These cultures were stored frozen at -85°C in bead-containing cryovials (Protect Bacterial Latex Preservers, Technical Service Consultants Ltd., England), and cultured at 37°C for 16 h in Luria-Bertani broth (LB, Difco, Becton-Dickinson Diagnostic Systems, Sparks, MD, USA) that was supplemented with 0.85 or 3% NaCl or in Bacto-Heart Infusion Broth (BHI, Difco) – 0.85% NaCl.

II. *DNA Purification*

Chromosomal DNA was extracted and purified by the conventional method[40]. Briefly, bacteria were cultured in 5 mL BHI– 0.85% NaCl at 37°C for 16 h, and cell mass out of 1 mL culture was harvested by centrifugation and subjected to DNA extraction. Aliquots (1 mL) of the oyster homogenates with/without spiked vibrios (10^1 - 10^6 CFU/mL) or the enriched spiked cultures were also harvested by centrifugation. The harvested cells were resuspended in 567 μL TE (10 mM Tris-Cl, 1.0 mM EDTA, pH 8) buffer that contained 3 μL proteinase K (20 mg/mL) and 30 μL sodium dodecyl sulfate (SDS) solution (10%), incubated at 37°C for about 1 h, and then mixed with 100 μL 5 M NaCl and 80 μL CTAB (N-cetyl-N,N,N-trimethyl ammonium bromide)-0.7M NaCl at 65°C. DNA was extracted using a mixture of chloroform/isoamylalcohol followed by phenol/chloroform/isoamyl alcohol, precipitated in 95% ethanol and resuspended in TE buffer. The DNA sample was quantified by absorbance at 260 nm on a GeneQuant II UV/VIS spectrophotometer (Pharmacia Biotech, Uppsala, Sweden).

III. *Design of PCR Primers*

The primers for amplifying the green fluorescent protein gene (*gfp*) and *ctx* genes were designed using Oligo-perfect Designer (Invitrogen) or Primer Design Assistant software[41]. The properties of these oligonucleotides were analyzed using the Oligonucleotide Properties Calculator (http://www.unc.edu/~cail/biotool/oligo/index.html). Other PCR primers were synthesized based on published sequences (Table 2). The fragments, generated by PCR using these primers, were further verified by DNA sequencing[42].

IV. *Construction of Internal Amplication Control*

The IAC was a chimeric DNA that consisted of a fragment of the coding region of *gfp*, flanked by the sequence of *V. vulnificus* primers. The internal fragment of the *gfp* gene of pPROBE-*gfp*[43] was amplified by chimeric oligonucleotide primers that included the *vvhA* primers at the 5' end (Table 1) to generate an amplicon of 753 bp. This amplicon was inserted into the vector pTZ57R/T, which was thus transformed to *Escherichia coli* XL1 Blue, using a TA cloning kit, by following the directions of the manufacturer (InsTAclone PCR Cloning Kit Fermentas International Inc., Burlington, Ontario, Canada). Ampicillin-resistant white colonies were screened and cultured to prepare the IAC-containing plasmid by conventional method[42]. The plasmids were digested by *Eco*RI and *Apa*I, separated by agarose gel electrophoresis, and eluted to obtain the fragment of 803 bp in length that contained the IAC (Figure 1).

Table 1. Bacterial strains examined in this study

Species/Strain	Serotype	Source	No of Strain	Target gene[a]	m-PCR
V. vulnificus					
	ND	Clinical	15	+	+
	ND	Environmental	13	+	+
V. parahaemolyticus					
	O3:K6	Clinical	9	+	+
ST550	04:K13	Clinical	1	+	+
1012	ND	Environmental	1	+	+
1015	ND	Environmental	1	±	+
999, 1003	ND	Environmental	2	±	-
	ND	Environmental	4	-	-
V. cholerae					
	O139	Clinical	5	+	+
	O139	Environmental	5	+	+
	Non-O1non-O139	Environmental	4	-	-
Other vibrios					
V. furnissii 159	ND	Environmental	1	-	-
V. hollisae 161	ND	Environmental	1	-	-
V. harveyi 164	ND	Environmental	1	-	-
V. campbellii 168	ND	Environmental	1	-	-
V. diazotrophicus 178	ND	Environmental	1	-	-
V. fluvialis 182	ND	Environmental	1	-	-
V. natriegens 185	ND	Environmental	1	-	-
V. splendidus 187	ND	Environmental	1	-	-
V. cincinnstriensis 190	ND	Environmental	1	-	-
V. orientalis 191	ND	Environmental	1	-	-
V. tubiashii 192	ND	Environmental	1	-	-
V. anguillarum 198	ND	Environmental	1	-	-
V. tubiashii 200	ND	Environmental	1	-	-
Non-vibrios					
Alcaligenes faecalis 31	ND	ND	1	-	-
Bacillus subtilis 4	ND	ND	1	-	-
B. cereus	ND	ND	2	-	-
E. coli JM109	ND	ND	6	-	-
E. aerogenes 36	ND	ND	1	-	-
Enterococcus faecalis 505	ND	ND	1	-	-
Klebsiella pneumoniae 40	ND	ND	1	-	-
L. monocytogenes	ND	ND	3	-	-
L. ivanovii SV5	ND	ND	1	-	-
Micrococcus luteus 11	ND	ND	1	-	-
Proteus vulgaris 42	ND	ND	1	-	-
M. varians 12	ND	ND	1	-	-
Pseudomnas aeruginosa 18	ND	ND	1	-	-
Serratia marcescens 21	ND	ND	1	-	-
Staphylococcus aureus	ND	ND	2	-	-
S.epidermidis 23	ND	ND	1	-	-
Streptococcus pyrogenes 28	ND	ND	1	-	-
Yersinia entercolitica 30	ND	ND	1	-	-

[a] Target genes; *vvhA* and *vvp* genes in *V. vulnificus*; *tdh* gene in *V. parahaemolyticus*, and *ctx* gene in *V. cholerae* strains.
+, positive response; -, negative response, ±, weak positive response; ND, not determined.

Table 2. Primers used in this study

Target	Primer	Sequence	Annealing temperature,°C	Amplicon, bp	Reference
IAC	FDA-GFP F	5'-CCGCGGTACAGGTTGGCGCAATGAGTAAAGGAGAAGAACTTTTCACTG	65.0	753	This study
	FDA-GFP R	5'-CGCCACCCACTTTCGGGCCTTATTTGTATAGTTCATCCATGCCATG			
vvhA	FDA F	5'-CCGCGGTACAGGTTGGCGCA	62.0	505	(54)
	FDA R	5'-CGCCACCCACTTTCGGGCC			
vvp	vvp F	5'-GCTCGTAGTCTTGCGCCAGTC	57.8	383	(55)
	vvp R	5'-AGGCTCTTCTTCCGCGACAAA			
tdh	tdh F	5'-GTAAAGGTCTCTGACTTTTGGAC	61.8	256	(16)
	tdh R	5'-TGGAATAGAACCTTCATCTTCACC			
ctx	ctx F2	5'-CAGTCAGGTGGTCTTATGCCAAGAGG	60	167	This study
	ctx R2	5'-CCCACTAAGTGGGCACTTCTCAAACT			

IAC, internal amplification control.

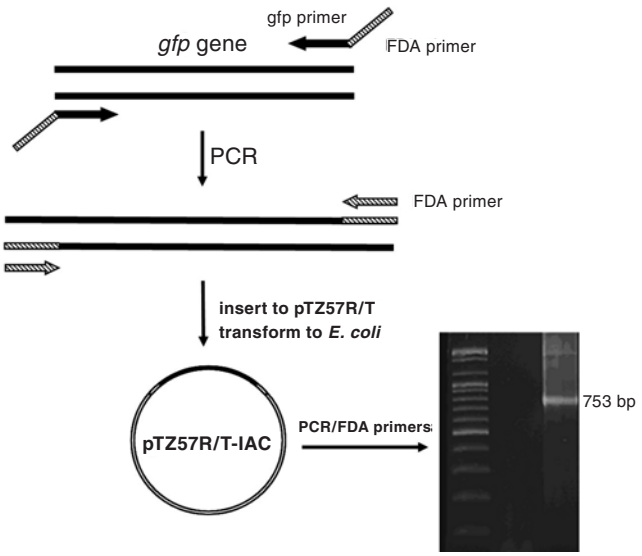

Figure 1. Schematic diagram to show the making of the Internal Amplification Control (IAC). The internal fragment of the *gfp* gene of pPROBE-*gfp*[56] was amplified by chimeric oligonucleotide primers that included the *vvhA* primers (FDA primer) at the 5'-end and inserted into the vector p TZ57R/T, which was transformed to *E. coli* XL1 Blue.

V. *PCR Conditions*

PCR was conducted on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) at 94°C for 3 min, followed by 30 cycles of denaturing at 94°C for 1 min, annealing at 62°C for 1 min, extension at 72°C for 1 min and finally 72°C for 10 min[44]. The reaction mixtures (50 μL) comprised 1 μL 10 mM dNTP (Finnzymes Oy, Espoo, Finland), 5 μL 10X PCR buffer (1.5 mM Mg^{2+}, QIAGEN GmbH, Hilden, Germany), 2 units DNA *Taq* polymerase (QIAGEN), 1 μL template DNA, 1 μL of each pair of primers and sterile distilled water. For optimal m-PCR conditions, 0.3 μM of FDA *vvhA* primers, 0.075 μM of *vvp* primers, 0.3 μM *tdh* primers, 0.075 μM *ctx* primers, and 5 pg IAC were added. PCR-amplified products were resolved by 2% agarose gel electrophoresis. 100 bp DNA ladder markers (New England BioLabs, Inc., Ipswich, MA, USA) were used.

VI. *Accuracy of m-PCR*

A total of 100 strains were used to evaluate the effectiveness of the present m-PCR procedure for simultaneous detection of the toxigenic *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* (Table 1). Presence of virulence-associated genes in these strains have been previously verified [37-39] and reconfirmed in this study by PCR using the specific primers (Table 2). The presence of TDH in *V. parahaemolyticus* was also confirmed on a specific Wagatsuma blood agar plate exhibiting β-hemolysis[45]. Chromosomal DNA was extracted from these strains and used as template DNA in this m-PCR; true positive, false negative, false positive and true negative results were identified. Inclusivity, exclusivity, positive predictivity, negative predictivity, analytical accuracy and Kappa index were calculated by the published method[46].

VII. *Evaluation of m-PCR in Oyster Homogenate*

The oysters purchased from the supermarket were shucked, and the tissue was homogenized by the standard method of the American Public Health Association in the presence of phosphate-buffered saline to produce 10-fold diluted homogenate[47]. The oyster homogenate was filtered through sterile cheesecloth, irradiated under UV for one hour and freeze/thaw-treated three times to eliminate viable vibrios[48]. The oyster homogenate was spiked with different levels (10^1 - 10^6 CFU/mL) of *V. vulnificus* VV45, *V. parahaemolyticus* ST550 or *V. cholerae* 294-94. The spiked oyster homogenate (1 mL) was enriched for 8 h at 37°C in 50 mL 0.1% peptone water that contained 1% NaCl. The

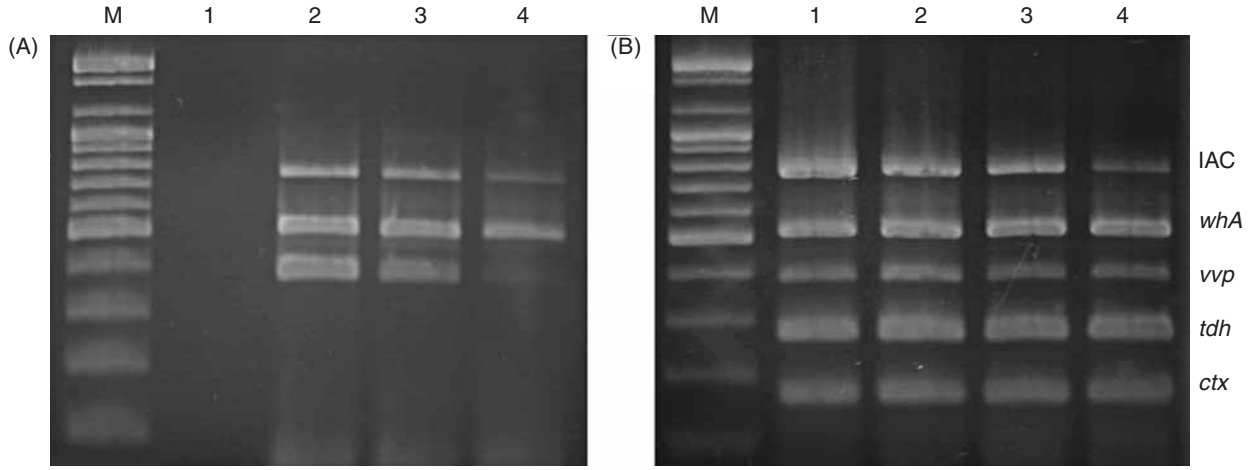

Figure 2. Detection of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* by m-PCR. Panel A, detection of *V. vulnificus* by m-PCR in presence of 3 ng IAC, 20 ng *V. vulnificus* VV45 DNA, FDA-F/R primers (each 0.3 μM) and various amounts of *vvp* primers. Lane 1, water; 2, 0.15 μM; 3, 0.075 μM; 4, 0.0325 μM of *vvp* primers. Panel B, different amounts of IAC were added to the PCR reaction mixtures which contained 20 ng *V. vulnificus* VV45 DNA, 0.5 ng *V. parahaemolyticus* ST550 DNA, 10 ng *V. cholerae* 294-94 DNA, 0.3 μM FDA *vvhA* primers, 0.075 μM *vvp* primers, 0.3 μM *tdh* primers and 0.075 μM *ctx* primers and different quantities of IAC. Lane 1, 75 pg; 2, 15 pg; 3, 5 pg; 4, 1 pg of IAC. M, 100 bp molecular ladder markers with sizes (bp) indicated.

chromosomal DNA was extracted from the spiked oyster homogenate with or without enrichment, and then subjected to m-PCR.

RESULTS

Since competition occurred during the amplification of IAC and *vvhA* due to the primers sharing in m-PCR, conditions for the detection of target genes in *V. vulnificus* were determined first. Primers for *V. cholerae* and *V. parahaemolyticus* were then added to this reaction mixture and the conditions were optimized. This m-PCR method was then evaluated with pure cultures and spiked oyster homogenates.

I. *Development of m-PCR for V. vulnificus with IAC*

The m-PCR conditions in this work were based on those recommended by FDA for the detection of *vvhA* (http://www.cfsan.fda.gov/~ebam/bam-9.html) and the optimal concentrations of the IAC, target templates and primers were determined by experiments with combinations of different concentrations of these factors (data not shown). The relative concentrations of the IAC and target gene are critical. When the amount of IAC was adjusted to 3 ng (about 3.5×10^9 copies) and VV45 DNA was adjusted to 20 ng (about 3.5×10^6 copies); in the presence of 0.3 μM FDA *vvhA* primers and 0.075 μM *vvp* primers, *vvhA*, *vvp* and IAC were successfully amplified (Figure 2A). The change of IAC and VV45 DNA did not substantially alter their relative levels in this m-PCR, and the relatively high level of IAC was further reduced.

II. *Detection of V. cholerae, V. parahaemolyticus and V. vulnificus by m-PCR*

The concentration effect of chromosomal DNA and primers for *V. cholerae* and *V. parahaemolyticus* was examined in order to assay these three toxigenic vibrios. In the presence of 20 ng *V. vulnificus* VV45 DNA (about 3.5×10^6 copies), 0.5 ng *V. parahaemolyticus* ST550 DNA (about 9.0×10^4 copies), 10 ng *V. cholerae* 294-94 DNA (about 1.8×10^6 copies), 0.3 μM FDA *vvhA* primers, 0.075 μM *vvp* primers, 0.3 μM *tdh* primers and 0.075 μM *ctx* primers, m-PCR successfully amplified IAC, *vvhA*, *vvp*, *tdh* and *ctx* concomitantly. The amount of IAC was lowered to 5 pg (about 5.7×10^6 copies) (Figure 2B). Under the above PCR conditions, the minimum detection levels for *V. vulnificus*, *V. parahaemolyticus* and *V. cholerae* DNAs were determined to be 20 ng (about 3.5×10^6 copies), 0.5 ng (about 9.0×10^4 copies) and 10 ng (about 1.8×10^6 copies), respectively (Figure 3). These amplified fragments were verified by nucleotide sequencing and comparing to the nucleotide sequences in the GenBank database (data not shown).

When various amounts of *E. coli* JM109 DNA (8 ng to 5.3 μg, about 1.9×10^6 to 1.2×10^{12} copies) were added to the reaction mixtures that contained the *Vibrio* template DNAs at these minimal detection levels, some nonspecific amplification products appeared in those mixtures containing a high level of background DNA (above 200 ng, about 4.6×10^7 copies), but the amplification of the target genes and IAC was unaffected (Figure 3E).

III. *Accuracy of m-PCR*

The accuracy of the present m-PCR method was evaluated using 28 strains of *V. vulnificus*, 18 strains of *V. parahaemolyticus*, 14 strains of *V. cholerae* and 40 other *Vibrio* species and non-*Vibrio* strains (Table 1). The chromosome DNA of each strain was isolated and assayed by the present

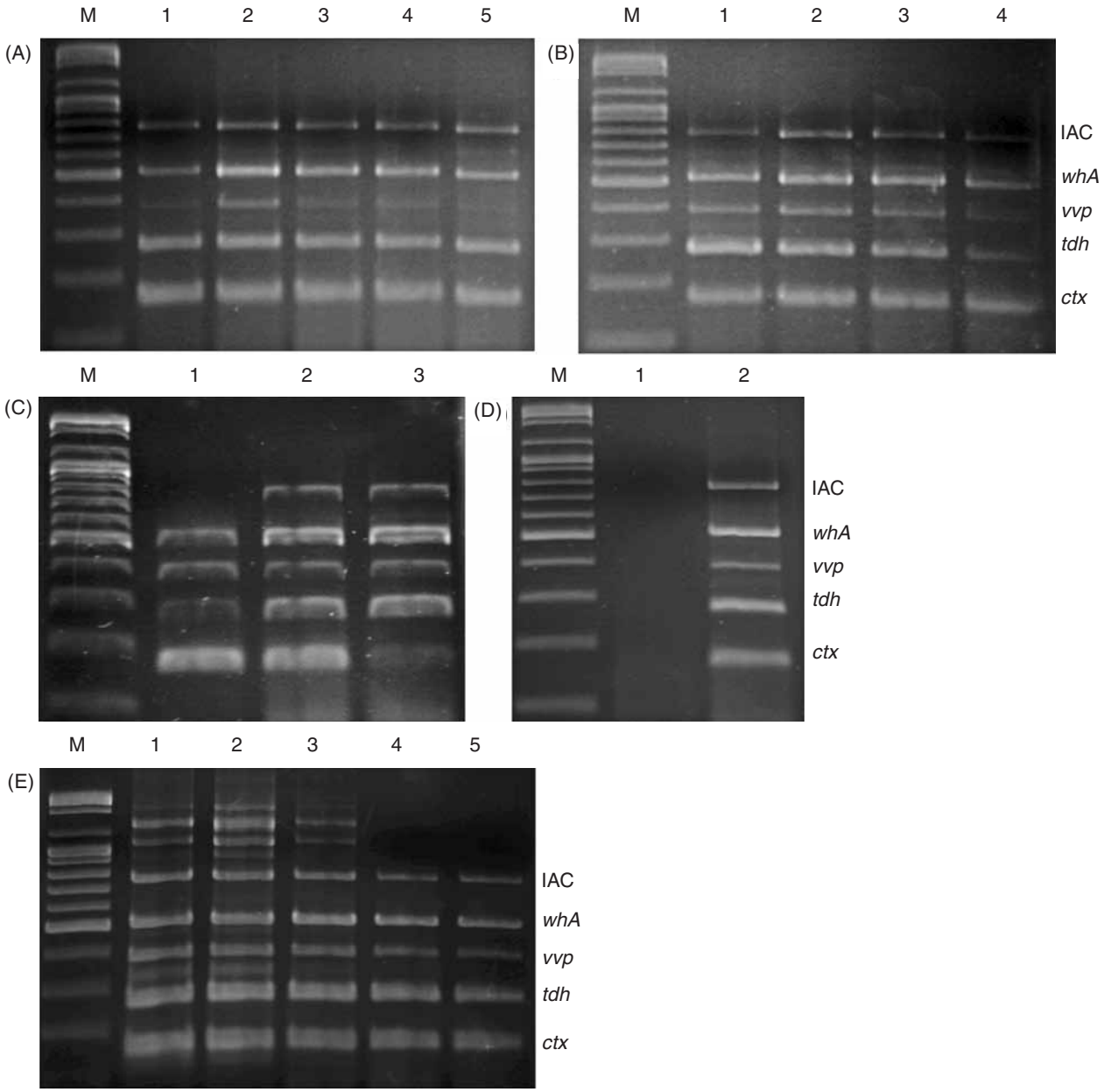

Figure 3. Minimum amounts of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* DNA detected by m-PCR. The PCR reaction mixtures contained 0.3 μM FDA *vvhA* primers, 0.075 μM *vvp* primers, 0.3 μM *tdh* primers, 0.075 μM *ctx* primers, 5 pg IAC and various amounts of template DNAs. Panel A, PCR performed with 0.5 ng *V. parahaemolyticus* ST550 DNA, 10 ng *V. cholerae* 294-94 DNA and various amounts of *V. vulnificus* VV45 DNA. Lane 1, 25 ng; 2, 20 ng; 3, 15 ng; 4, 10 ng; 5, 5 ng VV45 DNA. Panel B, PCR performed with 20 ng *V. vulnificus* VV45 DNA, 10 ng *V. cholerae* 294-94 DNA and various amounts of *V. parahaemolyticus* ST550 DNA. Lane 1, 10 ng; 2, 1 ng; 3, 0.5 ng; 4, 0.25 ng of ST550 DNA. Panel C, PCR performed with 20 ng *V. vulnificus* VV45 DNA, 0.5 ng *V. parahaemolyticus* ST550 DNA and various amounts of *V. cholerae* 294-94 DNA. Lane 1, 15 ng; 2, 10 ng; 3, 5 ng 294-94 DNA. Panel D, PCR performed with 20 ng *V. vulnificus* VV45 DNA, 0.5 ng *V. parahaemolyticus* ST550 DNA and 10 ng *V. cholerae* 294-94 DNA (lane 2). Lane 1, distilled water control. Panel E, PCR performed under the same condition for Panel D with various amounts of *E. coli* JM109 DNA added. Lane 1, 5.3 μg; 2, 1.06 μg; 3, 200 ng; 4, 40 ng; 5, 8 ng of JM109 DNA. Lane M in all panels, 100 bp molecular ladder markers, sizes as indicated in Figure 2.

m-PCR with or without the presence of the chromosomal DNAs of *V. vulnificus* VV45 (20 ng), *V. parahaemolyticus* ST550 (0.5 ng) and *V. cholerae* 294-94 (10 ng) to investigate whether the accuracy of this method would be influenced by other vibrios. In the presence or absence of *V. parahaemolyticus* and *V. cholerae* DNA, all of the *V. vulnificus* strains (40 ng) exhibited the typical *vvhA* and *vvp* amplicons (Table 1). In the presence or absence of *V. vulnificus* and *V. parahaemolyticus* DNA, only the *V. cholerae* O139 strains (10 ng) that originated in clinical and environmental sources exhibited the typical *ctx* amplicon, based on the present m-PCR, while the four environmental non-O1 non-O139 strains were negative (Table 1). In the presence or absence of *V. vulnificus* and *V. cholerae* DNA, only the *V. parahaemolyticus* (0.5 ng)

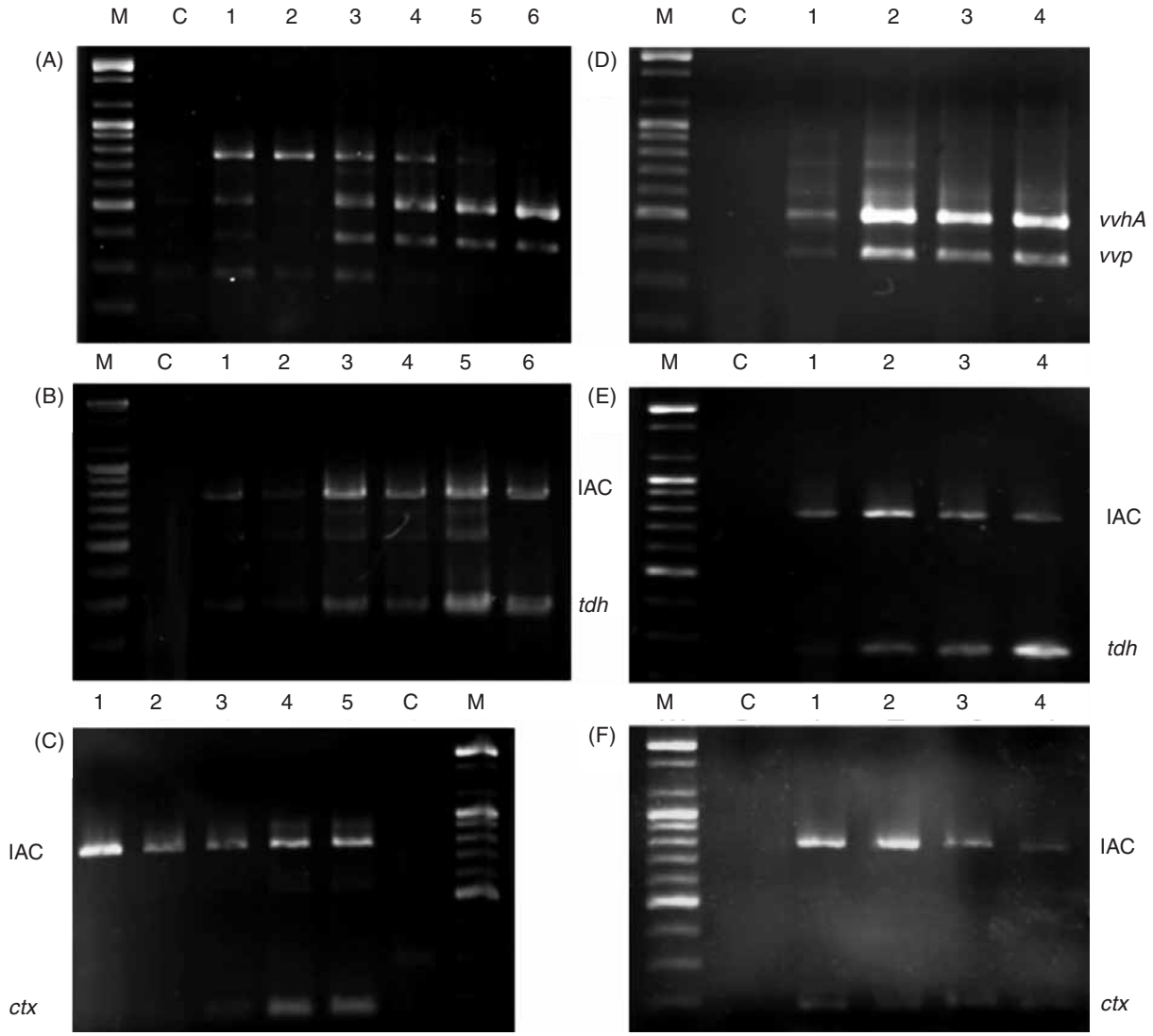

Figure 4. Detection of *V. vulnificus*, *V. parahaemolyticus* and *V. cholerae* in oyster homogenate by m-PCR. DNAs in oyster homogenate spiked with different levels of vibrios were extracted directly (Panel A, B and C) or after 8 h of enrichment at 37°C (Panel D, E and F) and m-PCR was performed in a mixture that contained 1 μL DNA extract, 0.3 μM FDA *vvhA* primers, 0.075 μM *vvp* primers, 0.3 μM *tdh* primers, 0.075 μM *ctx* primers and 5 pg IAC. Panels A and D, different levels of *V. vulnificus* VV45. Panels B and E, different levels of *V. parahaemolyticus* ST550. Panels C and F, different levels of *V. cholerae* 294-94. In these panels, lane 1, 10^1 CFU/mL; 2, 10^2 CFU/mL; 3, 10^3 CFU/mL; 4, 10^4 CFU/mL; 5, 10^5 CFU/mL; 6, 10^6 CFU/mL; C, control, only distilled water added; M, 100 bp molecular ladder markers, sizes as indicated in Figure 2.

strain ST550, O3:K6 strains, 1012 and 1015 exhibited the typical *tdh* amplicon, according to the present m-PCR. The ST550 and O3 : K6 strains were clinical strains that exhibited beta-hemolysis on Wagatsuma Agar (Kanagawa phenomenon positive, KP+) and the presence of TDH determined using a reverse passive latex agglutination kit (KAP-RPLA, Denka Seiken, Tokyo, Japan), while the environmental strains 1012 and 1015 did not exhibit beta-hemolysis, and should be regarded as false-positives. Strain 1012 reacted positively with *tdh* (Table 1). Also, strains 999, 1003 and 1015 reacted weakly with *tdh* (Table 1). The presence of non-functional *tdh* homologous sequences in these strains may cause false positive results in strains 1012 and 1015.

The chromosome DNAs of other *Vibrio* and non-*Vibrio* species (40 or 200 ng) were also assayed by the present m-PCR method, and all of these strains yielded negative results regardless of their levels (Table 1). In summary, the numbers of true positive, false negative, false positive and true negative results were 48, 0, 2 and 50, respectively[46]. Inclusivity, exclusivity, positive predictivity, negative predictivity, analytical accuracy and Kappa index were calculated to be 100%, 96%, 96%, 100%, 98% and 0.96, respectively[46]. These data supported the claim that the developed m-PCR was an accurate method.

IV. *Evaluation of m-PCR in Oyster Homogenate*

The oyster homogenates were spiked with various levels (10^1 - 10^6 CFU/mL) of *V. vulnificus*, *V. parahaemolyticus* or *V. cholerae* and assayed by the present m-PCR with

or without prior enrichment. Without enrichment, samples spiked with 10^3 CFU/mL of these pathogenic vibrios were successfully detected with concurrent IAC amplification (Figure 3A, B and C), and the detection sensitivity was close to those reported elsewhere[40]. In panel A of Figure 4, *vvhA* and *vvp* were successfully amplified at 10^1 CFU/mL and faint bands were also observed at 10^2 CFU/mL, whereas consistent results were observed at or above 10^3 CFU/mL. In panel A, amplified bands similar to the size of *tdh* amplicons were observed in the control and in lanes with low level of *V. vulnificus* cells. This result may indicate that the oyster medium treated by UV irradiation and freeze-thaw treatments[48] was not sterile and contained contaminated bacteria, nevertheless, the amplification of the target *V. vulnificus* was not influenced. When the samples were enriched by incubation at 37°C for 8 h, those spiked with as little as 10^1 CFU/mL of these toxigenic vibrios were successfully detected minimal non-specific amplification bands (Figure 4 D, E and F).

DISCUSSION

Although new technologies have been adopted for the detection of pathogenic bacteria, such as microarrays[40] and real-time PCR[33], m-PCR methods were also developed for the same purpose[34,40]. Other than regular temperature cycler, no expensive instrument is required in m-PCR and the detection process can be performed in diagnostic laboratories in a short time. m-PCR has been employed in simultaneous detection of several target genes in a single species, for example, the toxin gene (*rtxA*), extracellular secretory protein gene (*epsM*), mannose-sensitive pili gene (*mshA*) and the toxin coregulated pilus gene (*tcpA*) of *V. cholerae*[20], and the *tlh*, *tdh* and *trh* genes of *V. parahaemolyticus*[33]. It has also been developed for simultaneous detection of three *Vibrio* species based on the unique and specific sequence of a single gene, *toxR*[34]. For a reliable diagnostic PCR, IAC is mandatory to identify PCR inhibitors from seafood and environmental matrices[35], nevertheless, most of the assays developed do not include IAC. This study developed an m-PCR method targeting on four specific genes with IAC incorporated for detecting toxigenic *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* in oysters.

The experimental data here support the claim that the present m-PCR procedure was effective for detecting the toxigenic strains of these three *Vibrio* species. Competition for PCR reagents by individual targets is a concern in any multiplex PCR assay. Due to the high specificity of these primers used, amplification of these target genes in pure culture was not affected by the presence of high quantity of *E. coli* DNA or the competition of other *Vibrio* or non-*Vibrio* species (Table 1, Figure 3). Presence of the *tdh* elements in some strains of *V. alginolyticus*[49], *V. cholerae*[50], *V. hollisae*[50] and *V. mimicus*[51] may alter the accuracy of the detection of *tdh*-positive *V. parahaemolyticus* strains. Nevertheless, the presence of *tdh* elements is not a general phenomenon in these *Vibrio* species and the *tdh* elements found in these species are usually variants of the *tdh* of *V. parahaemolyticus*[51]. Also, the PCR primer tdhR exhibits no sequence identity to the *tdh* of *V. hollisae* and less sequence identity to the *tdh* sequences of *V. alginolyticus*, *V. cholerae* and *V. mimicus* than those of *V. parahaemolyticus*. Therefore, it is less likely to amplify these documented *tdh* variants by the present m-PCR. However, there are countless variants of most *Vibrio* species in nature, and relatively few genes from limited strains have been sequenced. In fact, in an early study using these *tdh* primers, positive amplifications were observed in two strains of *V. hollisae* and one strain of *V. damsela* whereas these strains were KP negative[16]. Modification of the *tdh* primers may be made to decrease the frequency of false positive detection which is regarded as a potential drawback of PCR methods[33]. Also, addition of the primers for *trh* in this method may avoid missing tdh^- and trh^+ strains of *V. parahaemolyticus*[16].

Under the present m-PCR condition, competition only affected the amplification of IAC, especially in enriched samples. The detection of bacteria in food by PCR is often hindered by the presence of inhibitors in food. Enrichment procedures can minimize the interference of these PCR inhibitors and also increase the concentration of the target microorganisms. Since the *V. vulnificus* DNA and IAC used the same set of PCR primers and a definite amount (5 pg) of IAC was used in the m-PCR procedure, the raised level of *V. vulnificus* DNA after enrichment reasonably weakened the amplification of the IAC. The results revealed a weak IAC amplicon in *V. vulnificus* after enrichment, while enrichment in *V. parahaemolyticus* and *V. cholerae* assays did not affect the amplification of IAC (Figure 4). This detection sensitivity is sufficient. The probability of illness is relatively low (< 0.001%) for consumption of 10,000 *V. parahaemolyticus* cells/serving, and consumption of about 100 million *V. parahaemolyticus* cells/serving (500 thousand cells/gram oysters) increases the probability of illness to about 50% (USFDA, http://www.fda.gov/).

The present m-PCR method aims to identify the toxigenic *V. cholerae*, *V. parahaemolyticus* or *V. vulnificus* in environmental samples. The target genes (*ctx* and *tdh*) used for the detection of *V. cholerae* and *V. parahaemolyticus* are well documented as the major virulence factors in these species and present in most of the clinical isolates and a few of the enivornmental isolates. The positive samples identified by this m-PCR method apparently raise high risk to human health. In *V. vulnificus*, the *vvhA* and *vvp* are common genes in the clinical and environmental strains of *V. vulnificus*[31], and these genes can be used as markers for species identification, but not suitable to distinguish clinical strains from environmental strains. Recently, the clinical and the environmental strains of *V. vulnificus* can be differentiated by the 16S rRNA genotype[52] or a randomly amplified polymorphic DNA fragment[53]. Nevertheless, no specific virulence gene associated with these indicators has been addressed.

In conclusion, this work developed an m-PCR procedure for the simultaneous detection of toxigenic *V. vulnificus*, *V. parahaemolyticus* and *V. cholerae* with an IAC incorporated

and a new pair of ctx primers designed. This method was proven accurate and could detect as little as 10^1 CFU of these vibrios in oyster homogenate following enrichment.

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