Journal of Food Safety

Journal of Food Safety ISSN 1745-4565

A RAPID MULTIPLEX PCR-DHPLC METHOD OF DETECTION AND IDENTIFICATION OF PATHOGENIC BACTERIA IN AQUATIC PRODUCTS

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Received for Publication February 26, 2014
Accepted for Publication September 7, 2014

doi: 10.1111/jfs.12156

Funding: The Public Welfare Industry Scientific Research Projects Foundation of General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China – "Research of detection and traceability of drug-resistance for foodborne pathogens by DHPLC and MALDI-TOF-MS" (No. 201210043)

ABSTRACT

In this study, we established a multiplex polymerase chain reaction-denaturing high-performance liquid chromatography (MPCR-DHPLC) method for rapid detection of the aquatic-associated pathogens *Vibrio cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. mimicus*, *V. alginolyticus* and *Listeria monocytogenes*. Specific primer sets targeting the *dnaJ* gene of the *Vibrio* species and the *hly* gene of *L. monocytogenes* were used. We also defined the optimal primer concentrations to detect each *Vibrio* species. We used the MPCR-DHPLC method to identify target bacteria in aquatic products. Performance characteristics of the MPCR-DHPLC system showed that it is specific for six common pathogenic bacterial species and can be used for the practical detection of these pathogens in aquatic products. Furthermore, two practical applications of two environmental water samples were tested according to this method by MPCR-DHPLC, then the results of detection were consistent with conventional tests but faster. It showed that this method proved to be a fast, sensitive and specific tool for *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. mimicus*, *V. alginolyticus* and *L. monocytogenes* detection in a routine microbiological laboratory.

PRACTICAL APPLICATIONS

Various bacterial species can be simultaneously detected with the same PCR reaction. A MPCR-DHPLC method of identification of various bacterial species was established. This method improves test efficiency and minimizes test time.

INTRODUCTION

Contamination of aquatic products has been a source of frequent food safety problems and economic losses. It is thus essential to develop rapid and reliable methods for the detection of pathogenic bacteria to reduce the frequency of outbreaks. The pathogens most commonly associated with aquatic products include *Listeria monocytogenes* and *Vibrio* spp. Vibrios are gram-negative bacteria and comprise the genus most frequently observed in seawater and marine food animals. Among them, *Vibrio cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. mimicus* and *V. alginolyticus* are the frequently occurring pathogens (Masini *et al.* 2007; Dumonteta *et al.* 2000; Soto-Rodriguez *et al.* 2003; Dalmasso *et al.* 2009; Huss 1997). *V. cholerae* is the causal agent of epidemic cholera. There have been seven large-scale outbreaks of *V. cholerae*-related epidemic cholera (Fykse *et al.* 2012; Spagnoletti *et al.* 2012). Common methods for *V. cholerae* detection include 01 group serotyping and 0139 group serotyping, in addition to enterotoxin gene (*ctxA*)-based detection (Ito *et al.* 1991; Varela *et al.* 1993; Blackstone *et al.* 2003). *V. parahaemolyticus* is a halophilic pathogen that causes acute proctitis. It is commonly detected by the Kanagawa test and thermostable direct hemolysin (Bej *et al.* 1999; Wang *et al.* 2011; Hossain *et al.* 2013). *V. vulnificus* is a halophilic pathogen found in coastal regions and estuaries; it is pathogenic in marine animals and thus creates a threat to the fish farming industry, whereas direct infection causes septicemia in humans. In addition to ecological and

biochemical methods, gene detection of 16sRNA and cytolysin are also used to detect *V. vulnificus* (Kim and Jeong 2001; Harwood *et al*. 2004; Lee *et al*. 2004; Li *et al*. 2006). *V. mimicus* can cause diarrhea and traumatic infection in humans. Its morphological features and DNA sequence are similar to those of *V. cholera*, and its thermolabile hemolysin gene is frequently targeted for detection and identification (Kim *et al*. 1997; Kang *et al*. 1998; Bi *et al*. 2000; Shinoda *et al*. 2004). *V. alginolyticus* is an opportunistic pathogen that can cause disease in humans and fish and is the most frequently detected marine *Vibrio* species (Buchanan and Gibbons 1974; Burrows and Freeman 1985; Lee *et al*. 1996).

L. monocytogenes is a widely distributed extremophilic gram-positive bacterium that can cause disease in humans and other animals. It grows slowly at 4C and can contaminate most types of foods; indeed, aquatic products can be easily contaminated during processing (Tully *et al*. 2006; Bej *et al*. 1999; Rantsiou *et al*. 2008; Vaz-Velho *et al*. 2001; Duffes *et al*. 1999; Johansson *et al*. 1999). Methods have been established for the molecular detection of gene-encoding virulence factors of *L. monocytogenes*, including *inl*, *hly*, *prf* and *iap* (Soni *et al*. 2013).

Polymerase chain reaction (PCR) is often used to detect pathogenic *Vibrio* spp. and *L. monocytogenes*, in addition to the traditional biochemical and serotype-based identification methods. However, only a few methods allow for the simultaneous detection of multiple pathogens. To address the need for a rapid and accurate method for the detection of multiple contaminants in aquatic products and processes, we established a multiplex PCR (MPCR) to detect *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. mimicus*, *V. alginolyticus* and *L. monocytogenes*. We used primers targeted at the *dnaJ* gene of *Vibrio* spp., encoding heat shock protein 40 (Nhung *et al*. 2007a) and the *L. monocytogenes hly* gene and combined this assay with a denaturing high-performance liquid chromatography (DHPLC) method. The procedure was optimized for multispecies detection in real environmental samples.

MATERIALS AND METHODS

Bacterial Strains

All reference bacterial strains were purchased from the American Type Culture Collection (ATCC) and China Microbiological Culture Collection Center (Table 1). Other strains (59 strains of *V. cholerae*, 8 strains of *V. parahaemolyticus*, 1 strain of *V. mimicus* and 1 strain of *Pseudomonas aeruginosa*) were isolated and identified from aquatic products, and they were stored by the Liaoning, Shandong and Guangdong Entry-Exit Inspection and Quarantine Bureaus.

MPCR primer sequences are presented in Table 2; they were manufactured by Takara Bioengineering (Dalian, China). The MPCR included a single upstream primer designed to bind a conserved sequence in all five targeted vibrios and species-specific downstream primers. The reaction also contained primers specific for the *hly* gene of *L. monocytogenes*.

TABLE 1. STRAIN SOURCE

	The number of reference strains	
Strains	ATCC	CMCC
Vibrio cholerae	14035, 14035, 35971, 55188, 51394, 25872	
V. parahaemolyticus	17802, 27519, 27968, 27969, 33845	
V. alginolyticus	17749, 33787, 33839, 19108, 51160	
V. vulnificus	27562, 14033, 33149, 29306, 43382	
V. mimicus	33654, 33655, 700326	
Aeromonas hydrophila	7966	
Listeria monocytogenes	7644	
L. ivanovii	19119	
L. innocua	33090	
L. welshimeri	35897	
L. seeligeri	35967	
Salmonella choleraesuis	10708	
Enterobacter sakazakii	51329	
Escherichia coli	25922	
Shigella flexneri	12022	
Staphylococcus aureus	29213	
Yersinia enterocolitica	9610	
Streptococcus hemolyticus		32121
Proteus vulgaris	49027	
Campylobacter jejuni	33560	

TABLE 2. MULTIPLEX PCR PRIMERS FOR FIVE *VIBRIO* SPECIES AND *LISTERIA MONOCYTOGENES*

Target	Primer sequences	Amplicon size	Reference
Common *Vibrio* primer	5′-CAGGTTTGYTGCACGGCGAAGA-3′		Nhung *et al*. (2007b)
Vibrio cholerae	5′-AGCAGCTTATGACCAATACGCC-3′	375 bp	Nhung *et al*. (2007b)
V. parahaemolyticus	5′-TGCGAAGAAAGGCTCATCAGAG-3′	96 bp	Nhung *et al*. (2007b)
V. vulnificus	5′-GTACGAAATTCTGACCGATCAA-3′	412 bp	Nhung *et al*. (2007b)
V. alginolyticus	5′-GATCGAAGTRCCRACACTMGGA-3′	144 bp	Nhung *et al*. (2007b)
V. mimicus	5′-YCTTGAAGAAGCGGTTCGTGCA-3′	177 bp	Nhung *et al*. (2007b)
Listeria monocytogenes	5′-CGCAACAAACTGAAGCAAAGG-3′ 5′-TTGGCGGCACATTTGTCAC-3′	210 bp	Park *et al*. (2006)

Equipment and Reagents

PCR amplifications were performed in thermal cyclers from Applied Biosystems (Foster City, CA). DHPLC was performed on a Transgenomic instrument (New Haven, CT). Taq DNA polymerase, 10 × PCR buffer, dNTPs and the bacterial DNA isolation kit were purchased from Takara. Triethylammonium acetate (TEAA, chromatography grade) was purchased from Transgenomic. Acetonitrile (chromatography grade) was obtained from Fisher Scientific (Jessup, MD). The DHPLC buffer comprised buffer A (5% TEAA and 0.025% acetonitrile) and buffer B (5% TEAA and 25% acetonitrile). Buffer D was 75% acetonitrile.

DNA Extraction and Sample Preparation

Sample preparation, enrichment culture and DNA extraction for *Vibrio* were performed as described by MNKL NO.156 2nd ed. 1997 and the corresponding ISO 11290-1:2004 standards, *L. monocytogenes* according to GB 4789.30-2010. Enriched bacterial solution (1 mL; for those bacterial strains that need a second culture, 1 mL of the secondary culture was used) was collected, and DNA was extracted with the Takara MiniBEST Bacterial Genomic DNA Extraction Kit (Takara). DNA yield was measured with a micro ultraviolet photospectrometer. DNA was diluted to a final stock concentration of 50 ng/mL in Tris-EDTA buffer solution and was stored at −20C.

Aquatic production samples (25 g) were obtained under sterile conditions. After the samples were cut into pieces, they were placed in sterile triangular flasks containing 225 mL double-buffered peptone water and 450 mL 3% NaCl peptone water, shaken gently for 3–5 min, and incubated at 36C for 4–6 h. The enriched solution (10 mL) was transferred to a test tube containing 10 mL double-buffered peptone water and 20 mL 3% NaCI peptone water, mixed gently and incubated at 36C for 18–24 h.

The (M) PCR-DHPLC Method

Standard strains of *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *V. mimicus*, *V. cholerae* and *L. monocytogenes* were used to establish the PCR-DHPLC detection method for individual *Vibrio* species.

Each 25μL PCR mixture contained 2.5 μL of 10 × PCR buffer, 2 μL of dNTP mixture, 1 U of Taq DNA polymerase, 10 pmol of each primer, 2 μL of DNA template (50 ng/μL) and dH_2O up to 25 μL. Reactions were performed with the following cycling conditions: 94C for 3 min; followed by 35 cycles of 94C for 60 s, 60C for 60 s and 72C for 60 s; with a final extension at 72C for 7 min. The reaction products were stored at 4C. For DHPLC (Transgenomic, Inc.), the column temperature was set to 50C. The mobile phase was composed of 50.2% buffer A (5% TEAA, 0.025% acetonitrile) and 49.8% buffer B (5% TEAA, 25% acetonitrile). The flow rate was set to 0.9 mL/min. The injected sample volume was 5 μL.

Multiplexed bacterial cultures were prepared and DNA extracted as described in *DNA Extraction and Sample Preparation* section. Because the target gene copy numbers varied, primer concentrations were adjusted to ensure similar yields and, thus, similar DHPLC peak heights. Based on the individual PCR-DHPLC detection method, the MPCR-DHPLC method was optimized by adding the primer sets individually to determine the optimal primer concentrations. The 25 μL of MPCR reaction system was performed, containing 2.5 μL of 10 × PCR buffer, 0.5 μL of each primer for *V. parahaemolyticus*, *V. alginolyticus* and *V. mimicus*, 1 μL of each primer for *V. cholerae* and *V. vulnificus*, 0.5 μL of the *L. monocytogenes* primer (all at 10 μmol/L), 2 μL of dNTPs (10 mmol/L), 0.2 μL of Taq DNA polymerase, DNA template (1 μL for single sample; 2 μL for mixed samples) and dH_2O up to 25 μL.

Method Validation

The specificity of the MPCR-DHPLC method was determined using DNA samples from the bacterial strains

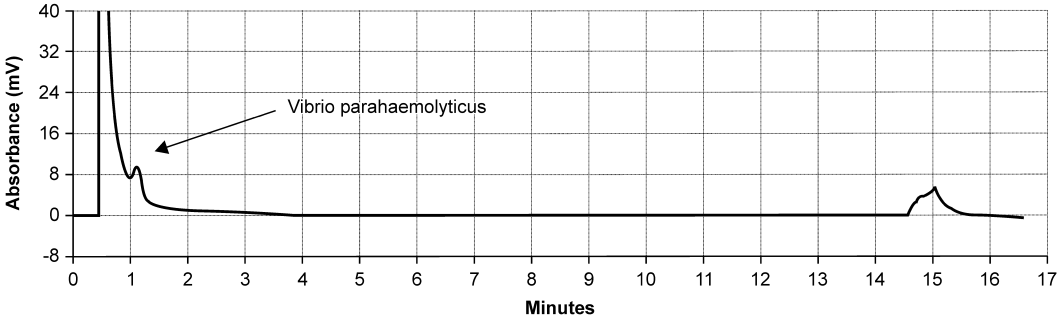

FIG. 1. THE RESULTS OF PCR-DHPLC FOR *VIBRIO PARAHAEMOLYTICUS*

described in *Bacterial Strains* section for the MPCR, and 5 μL of the PCR product was analyzed by DHPLC. The sensitivity of the method sensitivity was measured by using enriched bacterial solutions that were measured by turbidimetric assessment. The bacterial solutions were serially diluted to 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} dilutions and enriched as described in *DNA Extraction and Sample Preparation* section. Each dilution was replicated three times and the number of colonies was measured; the average value for the three replicates was then calculated. Bacterial DNA was extracted from each dilution and assessed by the MPCR-DHPLC method. The sensitivity was defined as the lowest concentration at which positive results were obtained. Accuracy was assessed by comparing methods in real environmental samples.

Practical Applications

Two environmental water samples, collected from Dandong, Liaoning province, China, by conventional microbiological detection method according to MNKL NO.156 2nd ed. 1997 and ISO 11290-1:2004, were tested by aforementioned MPCR-DHPLC. Those two samples were disposed according to MNKL NO.156 2nd ed. 1997, ISO 11290-1:2004 and GB 4789.30-2010 standards. Then DNA templates were extracted as description of *DNA Extraction and Sample Preparation* section. The PCR reagent, primers and DNA template were mixed and amplified in the PCR amplification, and the conditions were set as the optimized annealing temperature and cycle number in *The (M) PCR-DHPLC Method* section. Next, 5 μL of PCR amplification products were added and tested by aforementioned method in *Method Validation* section. Finally, the method developed in this test was verified by comparing the identification results with traditional bacterial identification method.

RESULTS

PCR-DHPLC Detection of Individual Species

The optimized PCR-DHPLC method amplified fragments from templates derived from six species of bacteria. PCR products were examined by DHPLC (Figs. 1–6). At 50C under nondenatured conditions, each targeted bacterium yielded a single absorption peak for the expected PCR product; each product was easily distinguishable.

MPCR-DHPLC Detection of Multiple Species

Each bacterial target yielded peaks with individually distinctive retention times and peak shapes that were consistent

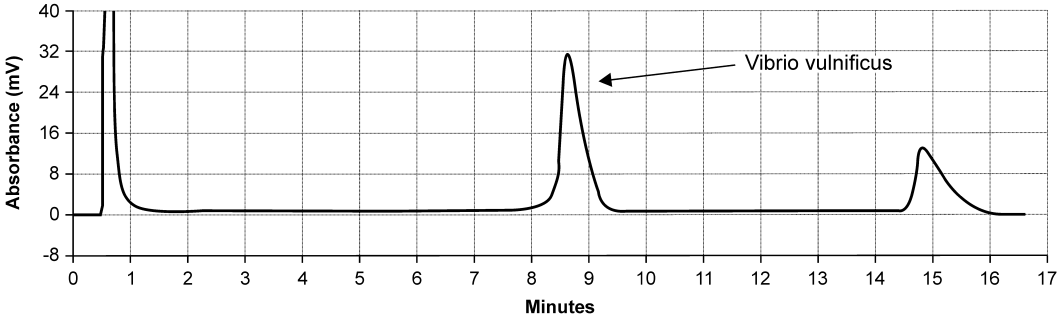

FIG. 2. THE RESULT OF PCR-DHPLC FOR *VIBRIO VULNIFICUS*

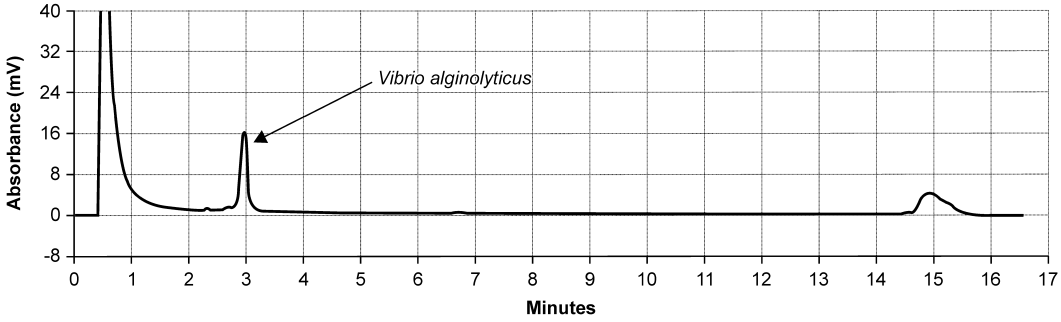

FIG. 3. THE RESULT OF PCR-DHPLC FOR *VIBRIO ALGINOLYTICUS*

between the PCR-DHPLC and MPCR-DHPLC methods. The DHPLC assay diagram is shown in Fig. 7.

Assay Performance

Assay positivity was obtained at dilutions of 10^{-8}; this is equivalent to a lower limit of detection of 120 cfu/mL for *L. monocytogenes*, 220 cfu/mL for *V. cholerae*, 60 /mL for *V. parahaemolyticus*, 9 /mL for *V. vulnificus*, 230 cfu/mL for *V. alginolyticus* and 370 cfu/mL for *V. mimicus*. Specificity was determined by using the MPCR method to test a combination of five species of *Vibrio* and *L. monocytogenes* with 15 strains of nontargeted species. The assay yielded positive results only for the targeted strains; none of the nontargeted strains yielded positive results and there were no false-positive strains (Fig. 8). It was indicated that the established MPCR method was highly specific for identification of *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *V. mimicus*, *V. cholerae* and *L. monocytogenes*.

Assay Results for Actual Samples

Among the environmental water samples collected from Dandong, Liaoning province, China, two different samples

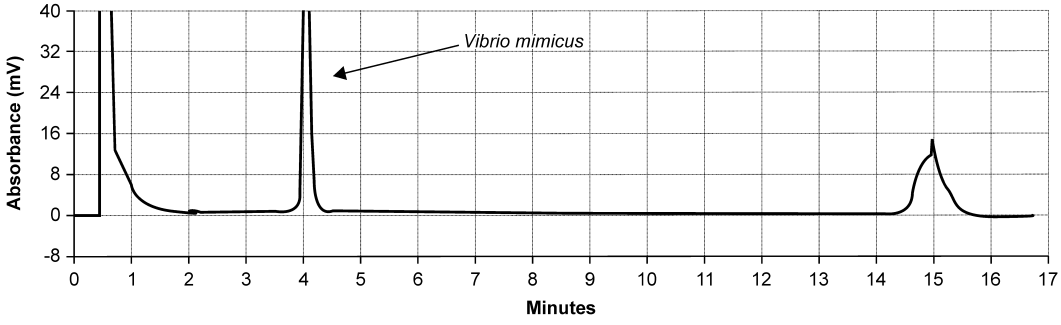

FIG. 4. THE RESULT OF PCR-DHPLC FOR *VIBRIO MIMICUS*

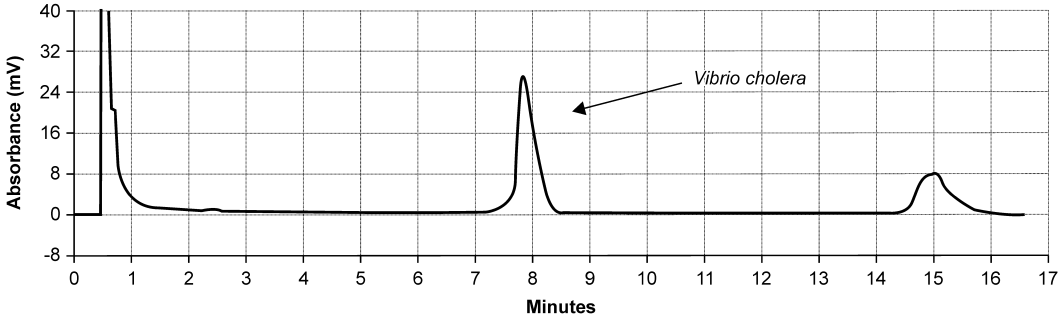

FIG. 5. THE RESULT OF PCR-DHPLC FOR *VIBRIO CHOLERA*

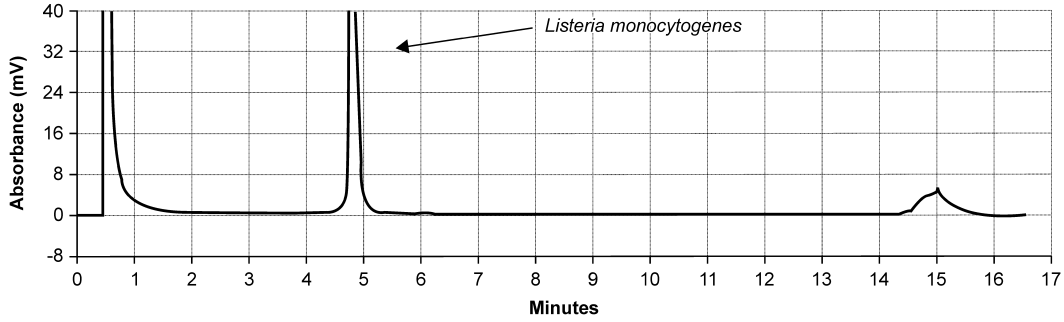

FIG. 6. THE RESULT OF PCR-DHPLC FOR *LISTERIA MONOCYTOGENES*

were found to contain both *V. cholerae* and *V. parahaemolyticus* by using the classic culture biochemical detection method. We used the MPCR-DHPLC methods established in this study to evaluate these two samples. Using the simultaneous enrichment method, both *V. cholerae* and *V. parahaemolyticus* were detected in these two samples by MPCR-DHPLC. Then the DNA templates of two samples were extracted according to *DNA Extraction and Sample Preparatio*n section. We applied the MPCR-DHPLC method to these two samples. PCR amplification products were analyzed by DHPLC, and obtained the testing results that both were detected two peaks. The two peaks respectively indicated *V. cholerae* and *V. parahaemolyticus* according to the amplification product fragment sizes. These identification results of the two samples were both positive of *V. cholerae* and *V. parahaemolyticus* (Fig. 9), and they were identical with the results of identification by the classic culture biochemical detection method. Therefore,

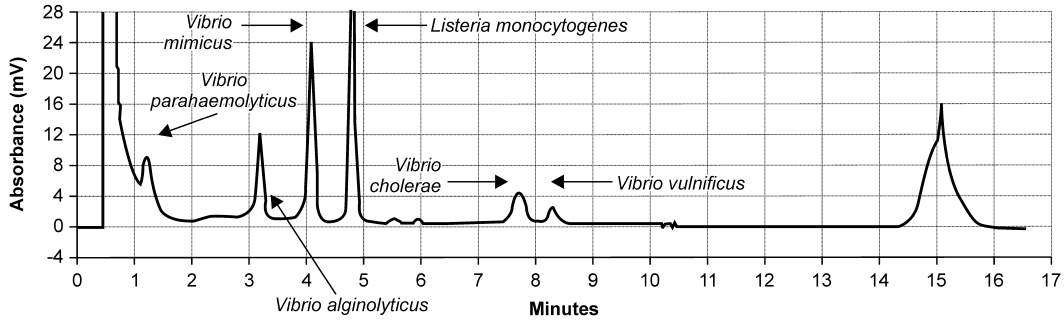

FIG. 7. THE RESULTS OF THE MULTIPLEX PCR-DHPLC AFTER OPTIMIZATION

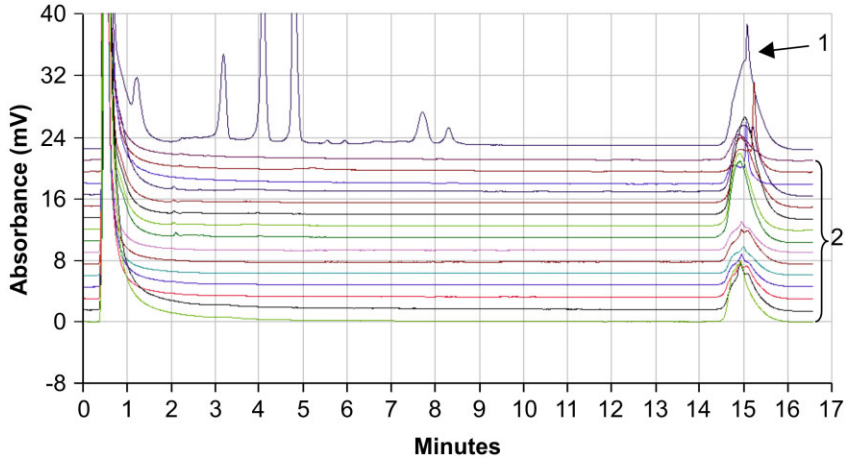

FIG. 8. THE MPCR-DHPLC DIAGRAMS FOR DETECTION OF *VIBRIO PARAHAEMOLYTICUS*, *V. VULNIFICUS*, *V. ALGINOLYTICUS*, *V. MIMICUS*, *V. CHOLERAE*, *L. MONOCYTOGENES* (1) AND 15 NONTARGETED BACTERIAL SPECIES (2)

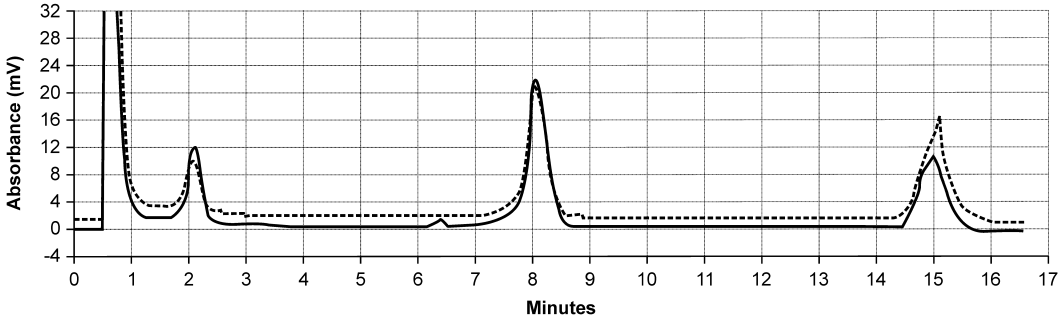

FIG. 9. THE RESULTS OF THE TWO ACTUAL SAMPLES DETECTED WITH MPCR-DHPLC

our method is accurate and can detect multiple species in a single sample.

DISCUSSION

In this study, we established a rapid MPCR-DHPLC method based on *dnaJ* gene targeting for the detection of *Vibrio* species using previously described primers (Nhung *et al.* 2007a). The method also detects the *hly* gene of *L. monocytogenes* (Park *et al.* 2006). Monitoring of food samples requires specificity for multiple pathogenic bacteria, which can be time consuming and tedious. Since the first MPCR technology was reported by Chamberlain *et al.* (1990), this method has been widely applied to the detection of pathogenic bacteria. The DHPLC methodology was developed by Oefner and Underhill (1995). DHPLC is a rapid, automatic and high-throughput technology for nucleic acid detection. Combining DHPLC with MPCR can increase the accuracy and efficiency of bacterial detection.

Most *Vibrio* detection methods target the pathogenesis genes for only a single species. To provide rapid and accurate identification of multiple *Vibrio* species, we used the *dnaJ* gene-specific primers reported by Nhung *et al.* (2007a). *DnaJ* is a housekeeping gene that is unaffected by phenotype, serotype and pathogenic factors; it is thus a stable target for identification. Our MPCR-DHPLC method provided the same results and degree of specificity as the PCR-agarose electrophoresis method for pathogen detection in fecal samples reported by Nhung *et al.* (2007a). However, our method provided greater sensitivity.

The *hly* gene encodes *L. monocytogenes* cytolysin and is relatively specific to the species; thus, we chose it as a target for identification. The DHPLC absorption peak was highest for the single enriched *L. monocytogenes*; thus, the efficiency of the reaction is sufficient to overcome the naturally low levels of *L. monocytogenes* in the environment and to compensate for the relatively poor DNA extraction efficiency from this gram-positive organism.

In this study, a rapid MPCR-DHPLC method was developed based on the individual PCR assay to identify different species in the single sample of aquatic product for *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *V. mimicus*, *V. cholerae* and *L. monocytogenes*. This method was applied to detect two actual samples, the simultaneous presence of *V. cholerae* and *V. parahaemolyticus*, in the aquatic environment from Dandong in China. The result of MPCR-DHPLC from this study demonstrated that it was consistent with the identification result by the classic culture biochemical detection method according to MNKL NO.156 2nd ed. 1997 and ISO 11290-1:2004. Thus far, PCR method aimed at *Vibrio* and *L. monocytogenes*, these pathogenic bacteria are found in water environment and host in various aquatic productions, and are used in the actual detection. However, one sample is likely to hide two or more kinds of pathogenic bacteria simultaneously. So it could be highly efficient and rapid for aquatic product test when six species of bacteria were assayed simultaneously in one reaction system. In conclusion, this MPCR-DHPLC method can become a suitable tool for preventing spread and infection of pathogenic bacteria via aquatic product foods and monitoring occurrence of *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *V. mimicus*, *V. cholerae and L. monocytogenes* in aquatic products.

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