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Simultaneous detection of *Vibrio cholerae*, *Vibrio alginolyticus*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* in seafood using dual priming oligonucleotide (DPO) system-based multiplex PCR assay

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ABSTRACT

In this study, a rapid and reliable multiplex PCR assay for simultaneous detection of *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio alginolyticus* in seafood was developed using the dual priming oligonucleotide (DPO) system. Species-specific DPO primers were designed targeting the *mdh*, *vvhA*, *colH* and *toxR* genes for the discrimination of *V. cholerae*, *V. vulnificus*, *V. alginolyticus* and *V. parahaemolyticus*, respectively. Compared to conventional PCR assay, the DPO system-based multiplex PCR assay allowed a wider annealing temperature at 48 °C–68 °C to effectively amplify target genes followed an analytical detection limit of $<1.5 \times 10^2$ CFU/mL (or g) for each *Vibrio* species in pure cultures or artificially contaminated food matrix. A total of 396 bacterial strains including 209 targets and 187 other bacterial strains were used to test the specificity of the DPO system-based multiplex PCR assay, and results showed that specific PCR product was only observed in target bacterial strains without nonspecific priming. Practical application of the assay indicated that target *Vibrio* species in seafood, clinical samples and foodborne outbreaks can be accurately detected. This DPO system-based multiplex PCR assay developed in this study would be a powerful tool for the rapid and reliable detection of the target *Vibrio* species.

1. Introduction

The genus *Vibrio* includes more than 30 species, and at least 12 *Vibrio* species are pathogenic to humans and/or have been associated with foodborne diseases due mainly to the larger consumption of raw or uncooked shellfish (Chakraborty, Nair, & Shinoda, 1997). Among these species, *Vibrio cholerae* is the most important *Vibrio* species that has been associated with epidemic and pandemic cholera outbreaks (Dalsgaard et al., 1995; López-Hernández et al., 2015; Ottaviani et al., 2009), in particular serogroups O1 and O139 (Dalsgaard, Forslund, Sandvang, Arntzen, & Keddy, 2001; Kaper, Morris, & Levine, 1995; Sack, Sack, Nair, & Siddique, 2004). Other *Vibrio* species capable of causing gastrointestinal diseases and acute septicaemia in humans have received more attention during the last decades, including *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio alginolyticus* (Altekruse et al., 2000; Dalsgaard, 1998; Dalsgaard, Möller, Brin, Hoei, & Larsen, 1996; Fuenzalida et al., 2007; Matsumoto et al., 2010; Scallan et al., 2011). In recent years, the prevalence of foodborne diseases caused by *V. cholerae*, *V. parahaemolyticus*, *V. alginolyticus* and *V. vulnificus* appears to has increased in China (Cai, Guo, Peng, & Pan, 2015; Huehn et al., 2014; Ji et al., 2011; Ou Yang et al., 2011; Wei et al., 2008; Wu, Wen, Ma, Ma, & Chen, 2014; Xu et al., 2014). Therefore, the development of a reliable method for simultaneous, accurate and specific detection of these *Vibrio* species is of great importance for seafood safety management and foodborne diseases active surveillance.

Currently, the identification of *Vibrio* species from clinical, environmental and seafood sources still relies on culture-based conventional diagnostics method. Although the conventional culture-based method is generally recognized as the classical method, it is laborious, time-consuming, and complex process involving the isolation of *Vibrio* species using commercial selective

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media followed by various biochemical and serological tests for identifying the presumptive colonies (Neogi et al., 2010; Yamazaki, Seto, Taguchi, Ishibashi, & Inoue, 2008), and the specificity of using selective media is still questionable (Pinto, Ciccarese, Tantillo, Catalano, & Forte, 2005). Polymerase chain reaction (PCR)-based methods have been suggested as an alternative (Chow, Yuen, & Yam, 2001; Imani, Iman, Hosseini, Karami, & Marashi, 2013; Nhung, Ohkusu, Miyasaka, Sun, & Ezaki, 2007; Xu et al., 2015a). Although PCR methods provide rapid detection, fundamental solution for eliminating false positive still remains a challenge, which requires very rigid primer parameters including primer specificity, primer length, melting temperature, GC content, less secondary structure and PCR annealing temperature to achieve a high specificity, in particular the development of a multiplex PCR assay, even when these requirements are satisfied, current conventional primers-based PCR assays still need to be more thoroughly validated (Hindiyeh et al., 2005; Välimaa, Tilsala-Timisjärvi, & Virtanen, 2015).

Dual priming oligonucleotide (DPO) system, a kind of PCR primer design method, can effectively eliminate non-specific priming without disrupting amplification of target sequences (Chun et al., 2007). The DPO system consists of three regions: a longer 5′-segment, a shorter 3′-segment and a polydeoxyinosines (poly I) linker bridging 5′ and 3′ segments. Compared to conventional PCR primer, the design of DPO system is greatly simplified. Firstly, the position of 3′ segment at a site containing 6–12 bases with 40–80% GC content is determined, then five deoxyinosines are designated for the poly I linker, and then the sequence upstream of 3′ segment are automatically extended 18–25 bases until $T_M > 65$ °C to generate the 5′ segment of DPO. Interestingly, the DPO system is particularly suitable for developing multiplex PCR assay due to a wide annealing temperature (Horii, Ohtsuka, Osaki, & Ohkuni, 2009; Lee et al., 2010b; Lee et al., 2008; Lee et al., 2010a; Woo et al., 2009; Xu, Liu, Guan, Cui, & Li, 2015b; Yeh et al., 2011). In the present study, a DPO system-based multiplex PCR (DPO system-based mPCR) assay for the simultaneous detection of *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and *V. alginolyticus* was developed.

2. Materials and methods

2.1. Bacterial strains

In this study, a total of 396 bacterial strains including 209 target *Vibrio* strains and 187 other bacterial strains were used for specificity testing. Details of these bacterial strains are described in Table 1. All *Vibrio* strains were cultured in tryptic soy broth, TSB (Becton Dickinson, USA) supplemented with 3% (w/v) NaCl (Xu et al., 2015a). *Campylobacter* strains were grown under microaerophilic conditions. Other bacterial strains were cultured in nutrient broth (BD, USA).

2.2. DPO system

Species-specific DPO systems used for the discrimination of *V. cholerae*, *V. parahaemolyticus*, *V. alginolyticus* and *V. vulnificus* were designed targeting the *mdh* gene of *V. cholerae*, the *toxR* gene of *V. parahaemolyticus*, the *colH* gene of *V. alginolyticus* and the *vvhA* gene of *V. vulnificus*, respectively. Moreover, a pair of DPO systems used for internal amplification control (IAC) was designed according to the genomic DNA of human adenovirus (AY601634). Details of the primers used in this study are listed in Table 2.

2.3. DPO-based mPCR assay

The optimized DPO system-based mPCR reaction system in a total volume of 50 μL was as follows: 10 × PCR buffer (500 mmol/L KCl, 15 mmol/L $MgCl_2$, 1% Triton X-100 and 100 mmol/L Tris-HCl, pH 8.8) 5 μL, dNTP (2.5 mmol/L for each) 8 μL, template DNA for each *Vibrio* species 1 μL, 5 U/μL of HiFi *Taq* DNA polymerase (TaKaRa, Japan) 1 μL, DPO primer pair (10 μmol/L) for each target 1 μL, and deionized water added up to 50 μL. PCR condition was that 95 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, 58 °C (48 °C–68 °C) for 45 s, 72 °C for 45 s, then with a final extension at 72 °C for 10 min. IAC was implemented to ensure the absence of false negative results in the assay development, sensitivity testing and the detection of clinical samples due to PCR reaction inhibition, in which a 508 bp of DNA fragment originating from the genomic DNA of human adenovirus (AY601634) harbored in plasmid pMD19-T was used as the control.

2.4. Determination of detection limit of the DPO-based mPCR assay

Detection limits of the DPO system-based mPCR assay for the simultaneous detection of the target *Vibrio* species from pure cultures, artificially contaminated seafood or spiked human feces were determined, respectively. *Vibrio* strains were cultured in TSB-3% NaCl broth at 37 °C for 8 h, respectively, and then were subjected to serial 10-fold dilutions in PBS (pH 7.4). The bacterial genomic DNA was extracted from the pure cultures using TaKaRa MiniBEST Bacterial Genomic DNA Extraction Kit (TaKaRa, Japan) according to the manufacturer's instructions. The template DNA of spiked food or human feces samples was prepared as the method previously described (Yamazaki et al., 2008), and with some modifications. Briefly, 100 μL of each dilution was spiked into 100 mg of target *Vibrio* species-negative homogenized shrimp meat and human feces, respectively, followed by the total DNA extraction from the homogenates using DNeasy Blood and Tissue Kit (Qiagen, Germany) according to manufacturer's instructions. Subsequently, the extracted template DNA was detected by the DPO system-based mPCR assay, and the detection limit of the assay was defined as the last positive dilutions. The bacterial amounts of each target *Vibrio* species determined by standard plate count method were that *V. cholerae* 1.23×10^8 CFU/mL, *V. parahaemolyticus* 1.27×10^8 CFU/mL, *V. alginolyticus* 1.11×10^8 CFU/mL and *V. vulnificus* 1.07×10^8 CFU/mL, respectively. All experiments were repeated at least three times.

2.5. Specificity testing of the DPO-based mPCR assay

In this study, a total of 396 bacterial strains including 209 target and 187 other bacterial strains were used to test the specificity of DPO system-based mPCR assay. The template DNA was prepared by the boiling method. In brief, 50 μL of mid-log phase bacterial cultures was added into 150 μL of TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0) and boiled for 10 min followed by quick cooling on ice for 5 min, then was centrifugated at 12, 000*g* for 5 min, the supernatants containing genomic DNA of bacteria were detected by the DPO system-based mPCR assay. All experiments were repeated in triplicate.

2.6. Evaluation of the assay using artificially contaminated food matrixes

We evaluated the DPO system-based mPCR assay for the detection of target *Vibrio* species using artificially contaminated food matrixes. Briefly, the artificially contaminated food matrixes were prepared using homogenized shrimp meat with a random