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Identification of *Vibrio cholerae* serotypes in high-risk marine products with non-gel sieving capillary electrophoresis

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ABSTRACT

Vibrio cholerae, a natural inhabitant of the marine environment, poses a threat to human health, and its new epidemic variants have been reported. A method of multiplex polymerase chain reaction–capillary electrophoresis–laser-induced fluorescence (PCR–CE–LIF) detection has been developed to detect and identify *V. cholerae* in marine products sensitively, rapidly, and reliably. Four sets of primers were selected to amplify genus-specific VCC gene, O139 serogroup-specific O139 gene, O1 serogroup-specific O1 gene, and ctxA gene associated with the CT toxin of enterotoxigenic *V. cholerae*. The PCR products were detected using CE–LIF with SYBR Gold serving as the DNA fluorescent dye. The parameters of PCR and the separation conditions of CE–LIF were optimized. Under the optimal conditions, *V. cholerae* was detected and four serotypes were identified simultaneously within 8 min. The alignment analysis showed that the PCR products had good agreement with the published sequences from GenBank, indicating that the primers selected in this study had high specificity and the PCR results were reliable. The proposed method could detect 5 to 20 cfu/ml *V. cholerae*. The intraday precisions of migration time and peak area of DNA marker and PCR products were in the ranges of 1.60–2.56% and 1.60–6.29%, respectively. The specificity results showed that only five standard bacteria used in this study showed the specific peaks when the target bacteria were mixed with seven other common intestinal pathogenic bacteria at the same concentration. The assay was applied to 71 high-risk marine products, and different serotypes of *V. cholerae* could be identified sensitively and reliably.

Cholera is an ancient and acute intestinal infection worldwide. Since 1817, seven worldwide cholera pandemics have occurred. Nowadays, it continues to be a global public health problem. In 2013, a total of 129,064 cholera cases were reported to the World Health Organization, with more than 47% of cases occurring in the Americas and 43.6% in Africa [1]. During recent years, several cholera outbreaks also occurred in developing countries and regions [2–6]. Cholera has the characteristics of sudden onset, rapid transmission, and wide range of spread. It often exhibits some typical symptoms such as vomiting and watery diarrhea, which may quickly lead to severe dehydration and even death without efficient and prompt treatment [7,8]. It is *Vibrio cholerae* that causes cholera. The bacteria are autochthonous inhabitants in brackish water and estuarine systems [9], and they can be divided into more than 200 serotypes. Among them, the O1 and O139 serotypes are recognized as the main etiological agents of epidemic cholera [10]. Other *V. cholerae* strains, such as non-O1/non-O139 *V. cholerae*s, can cause occasional outbreaks of diarrhea. In China, although cholera is under control, occasional outbreaks of diarrhea caused by *V. cholerae* may occur in some areas, especially through aquatic products. In addition, marine products contaminated by *V. cholerae* in coastal epidemic areas may be transmitted to the inland and result in cholera spread. Moreover, with global warming, cholera outbreaks may become increasingly common because the bacteria grow more rapidly in warmer environments [8]. The toxigenic ability of *V. cholerae* is closely associated with its serotype and biological properties. Very few invasive *V. cholerae* may represent

Abbreviations used: PCR, polymerase chain reaction; CE, capillary electrophoresis; LIF, laser-induced fluorescence; dNTP, deoxynucleoside triphosphate; MC, methylcellulose; EDTA, ethylenediaminetetraacetic acid; APW, alkaline peptone water; RSD, relative standard deviation; AGE, agarose gel electrophoresis.

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an alarm for public health. Therefore, it is imperative to develop a rapid, sensitive, and effective method to detect and identify *V. cholerae* in marine products.

Traditional identification of *V. cholerae* is often achieved through isolation and microbiological and biochemical analysis, which is time-consuming and laborious. Furthermore, the growth medium-dependent methods can detect only the cultivatable bacteria in a particular medium and under certain experimental conditions. To overcome the shortcomings of traditional methods, efforts have been made to develop novel methods characterized by high specificity, sensitivity, and efficiency. Chaivisuthangkura and coworkers [11] reported an immunochromatographic strip test for the detection of *V. cholerae* O1 using two monoclonal antibodies specific to the lipopolysaccharides of the bacterium, and the results showed detection sensitivity of 10^6 cfu/ml and 10^2 to 1 cfu/ml after enrichment for 6–12 h at 36 °C. However, other *Vibrio* spp. and bacterial species often present in seafood samples could reduce the detection ability of this method. Chen and coworkers [12] also developed an immunochromatographic lateral flow device using a new monoclonal antibody pair, named IXiao3G6 and IXiao1D9, for the highly specific and rapid detection of Ogawa. Among the molecular methods, the conventional polymerase chain reaction (PCR) and real-time PCR protocols can detect a single target of *V. cholerae* in one reaction and fail to simultaneously provide the serotype and toxicity information [13]. Multiplex PCR can amplify several genes simultaneously and has the advantages of saving considerable time and cost in the detection of *V. cholerae* [9,13]. However, post-PCR gel electrophoresis detection exhibits poor detection limits and low resolution. A multiplex real-time PCR assay [14] developed by Gubala and Proll targeted four genes of potentially toxigenic *V. cholerae* in environmental water and obtained increased specificity and accuracy for identification of the bacterium and could differentiate between El Tor and Classical biotypes. However, the high cost of Taqman probes has limited its applications in the developing countries. Erler and coworkers [15] expanded the Biotyper database with additional strains for the analysis of *Vibrio* spp. in environmental samples by the technique of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. However, their study did not distinguish intraspecific groups of *V. cholerae*.

Capillary electrophoresis (CE) has the advantages of rapid speed, low sample/reagent consumption, and high resolution. CE combined with highly sensitive laser-induced fluorescence (LIF) detection has exhibited unique features in modern applications of DNA analysis that were accomplished mainly through the use of the non-gel sieving mode [16,17]. The objective of this study was to develop a new method combining multiplex PCR with non-gel sieving capillary electrophoresis coupled with laser-induced fluorescence in order to identify the four serotypes and pathogenicity of *V. cholerae* in marine products. A total of 71 high-risk marine products from Chengdu City in China were detected to initially investigate the prevalence of *V. cholerae* using the proposed method. To our knowledge, there has been no report on the detection of *V. cholerae* in food by a multiplex PCR-based procedure followed by non-gel sieving capillary electrophoresis combined with laser-induced fluorescence.

Materials and methods

Materials

The primers were synthesized by Shanghai Sangon Biotech (Shanghai, China). Agarose gel was purchased from Biowest (France). PCR buffer, deoxynucleoside triphosphates (dNTPs), $MgCl_2$, and Taq DNA polymerase were purchased from Takara Biotechnology (Dalian, China). DL 500 DNA Marker containing fragments of 50, 100, 150, 200, 300, 400, and 500 bp was also obtained from Takara. SYBR Gold was obtained from Life Technologies (Carlsbad, CA, USA). Tris(hydroxymethyl)aminomethane was obtained from Shanghai Analytical Reagent (Shanghai, China). Methylcellulose (MC) was obtained from Sigma–Aldrich (St. Louis, MO, USA).

TBE buffer (Tris–boric acid–EDTA [ethylenediaminetetraacetic acid], pH 8.0) of 0.45 mol/L served as running buffer in capillary electrophoresis and consisted of 0.45 mol/L boric acid, 0.45 mol/L Tris, and 0.45 mol/L EDTA. The stock solution of 1.0% (w/v) MC was prepared in 0.45 mol/L TBE buffer and diluted to 0.4–0.8% (w/v) prior to use.

The water used in the experiment was double distilled and purified to 18.2 MΩ with a MilliQ water purification system (Millipore, Bedford, MA, USA), and all reagents were of analytical reagent grade unless otherwise noted.

Bacterial strains

Non-enterotoxigenic O1, *non-enterotoxigenic O139*, *enterotoxigenic O139*, *non-enterotoxigenic non-O1/non-O139*, and *enterotoxigenic non-O1/non-O139*, provided by the Center for Disease Control and Prevention (Chengdu, China) were used as the reference strains and for preparing standard chromatograms for multiplex PCR–CE assays.

Nine non-target bacterial strains were used for the specificity assays: *Yersinia enterocolitica*, *Staphylococcus aureus*, *Enterobacter sakazakii*, *Bacillus subtilis*, *Citrobacter*, *Staphylococcus*, *Bacillus cereus*, *Escherichia coli*, and *Proteusbacillus vulgaris* (from West China School of Public Health, Sichuan University).

Sample collection and template extraction

The swab specimens were collected from high-risk marine products from the districts (counties) of Chengdu. They were transferred in 10 mL of alkaline peptone water (APW) and incubated at 37 °C for 6 h.

Genomic DNA of *V. cholerae* was prepared by heat procedure. An aliquot of 1.5 mL of APW enrichment broth was transferred to an EP tube and then centrifuged at 13,000 rpm for 30 s. The supernatant was removed. The pellet was resuspended in 200 μL of sterile ultrapure water and heated in a boiling water bath for 15 min, followed by centrifugation at 13,000 rpm for 30 s to eliminate the cell debris. The supernatant containing DNA was stored at −20 °C and used for the subsequent analysis.

PCR amplification

The multiplex PCR mixture contained 2 μL of DNA template, 0.4 μmol/L O139 and VCC primers, 0.8 μmol/L O1 and ctxA primers, 1 × buffer, 0.40 mmol/L dNTPs, 1.0 mmol/L Mg^{2+}, and 2.5 U of Taq DNA polymerase in a final volume of 50 μL. Negative control was performed with 2.0 μL of sterile water instead of DNA template. The multiplex PCR was performed on a thermal cycler (Bio-Rad, Hercules, CA, USA), under the following conditions: initial denaturation at 94 °C for 5 min, 30 cycles of denaturing at 94 °C for 45 s, annealing at 56 °C for 45 s, extension at 72 °C for 45 s, and a final elongation at 72 °C for 10 min. The amplified products of O1, VCC, O139, and ctxA gene were 104, 155, 192, and 304 bp, respectively.

Detection of PCR products by CE–LIF

All CE analyses were carried out on a capillary electrophoresis system equipped with a solid state laser with an excitation wavelength of 473 nm (Beijing Viasho Technology, Beijing, China)