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# Polymerase Chain Reaction-based Detection of Total and Specific *Vibrio* Species

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**Abstract** The polymerase chain reaction (PCR) technique is widely used for efficient detection of food-borne pathogens because of speed and specificity. However, PCR methods have focused mostly on species-specific detection. In the present work, we describe a PCR-based method for the simultaneous detection of all *Vibrio* species because lots of them are notorious food-borne human pathogens. We then combined this total detection method with specific detection of *Vibrio cholerae* pathogen. Using a degenerate primer set based on the sequence of the potassium uptake gene, *trkA*, we were able to successfully detect all *Vibrio* species. Specific detection of *V. cholerae* was also possible using primer sets based on putative flagellin sequence. Importantly, simultaneous total and species-specific *Vibrio* detection was possible using all two primer sets in a multiplexed PCR strategy. Thus, the PCR method we have developed is applicable to both simultaneous and two-step detection of total and specific *Vibrio* species.

**Keywords** Polymerase chain reaction · Multiplex PCR · Total *Vibrio* detection · Species-specific detection · *Vibrio cholerae*

## Introduction

Many pathogens that can cause severe or fatal illness are a threat to public health if food is handled inappropriately [1]. Among the most vicious pathogens are members of the genus *Vibrio*, which includes more than 30 species, 12 of which are human pathogens. Among the many *Vibrio* species is the well-known human pathogen, *Vibrio cholerae*, which causes cholera. Cholera outbreaks are correlated with sea surface temperature, depth, salinity, turbidity, and plankton blooms in endemic areas [2, 3].

Byeong Hee Hwang and Jae Won Lee contributed equally to this paper.

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To date, numerous studies have sought to develop techniques for detecting and diagnosing food-borne pathogens. The conventional method for detecting pathogenic microorganisms in food relies on the enrichment and selective culture of the suspected bacteria in synthetic media [4]. For example, to test for microorganisms in patients with diarrhea and a history of eating raw seafood, clinical specimens are cultured and examined in a recommended medium, such as thiosulfate-citrate-bile salts-sucrose (TCBS) agar [5]. However, the recovery rate of specific *Vibrio* strains on TCBS agar is poor, suggesting that this medium should not be used for the direct plating of clinical specimens. Moreover, culture-based detection methods are often time-consuming and unreliable [6].

A number of molecular based methods, including polymerase chain reaction (PCR; [6–9]) and DNA microarrays [10–13] and immunoassays [14–16], have been developed to overcome the limitations of conventional detection methods. Studies have clearly shown that PCR-based methods are rapid, highly specific, and capable of detecting a number of pathogens. However, these studies have focused mostly on species-specific detection. In the present work, we developed a simultaneous PCR method that combines detection of total *Vibrio* species with specific detection of the important *Vibrio* pathogen, *V. cholerae*. For total *Vibrio* species detection, we newly introduced *trkA* gene which is a well-known potassium uptake protein in bacteria and recently reported to have function on resistance against serum [17].

## Materials and Methods

### Bacteria Stains and Culture Conditions

*Vibrio cholerae* (14035; American Type Culture Collection, Manassas (ATCC), VA, USA) was selected as target *Vibrio* strain for specific detection. *Vibrio vulnificus* (27562; ATCC), *Vibrio parahaemolyticus* (17802; ATCC), *Vibrio mimicus* (33653; ATCC), *Vibrio furnissii* (35016; ATCC), *Vibrio campbelli* (25920; ATCC), *Vibrio cincinnatiensis* (35912; ATCC), *Vibrio diazotrophicus* (33466; ATCC), *Vibrio hollisae* (33564; ATCC), *Vibrio metschnikovii* (700040; ATCC), and *Vibrio ordalii* (33509; ATCC) were used as target strains for total *Vibrio* detection but also as negative controls for specific detection. *Salmonella typhimurium* (12529; Institute for Fermentation, Osaka, Japan), *Salmonella enteritidis* (13076; ATCC), *Salmonella choleraesuis* (13311; ATCC), *Listeria monocytogenes* (15313; ATCC), *Escherichia coli* (25922; ATCC), *Bacillus subtilis* (6633; ATCC), and *Yersinia enterocolitica* (23715; ATCC) were used as negative controls for total and specific *Vibrio* detections. All bacteria were cultured in trypticase soy broth (Difco, Kansan, MO, USA) with 1.5% NaCl at 37°C.

### Design of PCR Primers

The oligonucleotide primer sets for PCR detection of total and specific *Vibrio* strains were designed through multiple alignment and sequence homology analyses (Fig. 1) using a combination of DNAMAN (Lynnon Corporation, Quebec, Canada), Primer Premier 5 (Premier Biosoft International, Palo Alto, CA, USA) and online analysis of the NCBI database. The primer sequences shown in Table 1 were selected to specifically detect 1) total *Vibrio*, using degenerate primers TV-F3 and TV-R5, which amplify a 893-bp DNA fragment from the potassium uptake gene, *trkA* and 2) *V. cholerae*, using primers VC-F2 and VC-R3, which amplify a 427-bp DNA fragment from the putative flagellin gene [18].

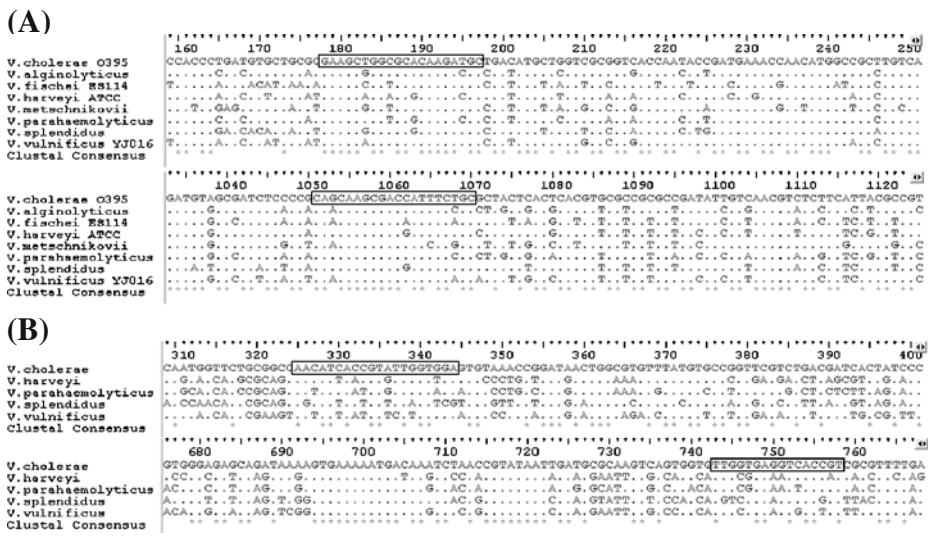


**Fig. 1** Multiple alignments of **a** *trkA* and **b** flagellin genes from various *Vibrio* species. The parts of DNA sequences for primer sets were indicated by a *box*

The thermodynamic properties of these oligonucleotide primers were calculated using Primer Premier 5.

## PCR Conditions for *Vibrio* Detection

For PCR detection, we prepared pathogen DNA samples by DNA purification using a commercially available kit (Wizard genomic DNA purification kit; Promega, Madison, WI, USA). After purification of genomic DNA using this method, the DNA concentration was determined with UV/vis spectrometry (Shimadzu, Kyoto, Japan). Alternatively, DNA samples were prepared by direct boiling. In this case, 1 mL of culture broth was boiled for 10 min and, after brief centrifugation, 1 μL of supernatant was used directly for subsequent PCR reactions.

Single-detection PCR reactions were performed in a total volume of 20 μL. The reaction mixture consisted of 1 μL template DNA (50~250 ng/μL according to bacteria), 1 U Taq DNA polymerase (Bioneer, Daejeon, Korea), 250 nM dNTPs, 1.5 mM $MgCl_2$, 1 μL of each primer (10 pmol), and 2 μL of 10× polymerase buffer. For each reaction, the mixture was pre-denatured at 95°C for 5 min to ensure complete dissociation of the template DNA. The

**Table 1** PCR primers to detect total and specific *Vibrio* species.

| Strain | Primer | Sequence (5′→3′) | Length (bp) | Tm (°C) | Detection principle |
|---|---|---|---|---|---|
| Total *Vibrio* | TV-F3[a] | GAAGCNGGHGCRCAAGAYGC | 20 | 63.1 | *trkA* |
| | TV-R5[a] | GCDGAAATBGTCGCTTGYTG | 20 | 57.6 | |
| *V. cholerae* | VC-F2 | AACATCACCGTATTGGTGGA | 20 | 55.7 | Flagellin |
| | VC-R3 | ACGGTGACCTCACCAA | 16 | 47.9 | |

[a] N is A or T or G or C, H is A or T or C, R is A or G, Y is C or T, D is A or T or G, and B is T or G or C

amplification reaction consisted of 30 cycles of denaturation at 95°C for 30 s, annealing at 56.3°C (determined by gradient PCR for total *Vibrio* detection) or 52°C (for specific *Vibrio* detection) for 30 s, and extension at 72°C for 1 min. A 7-min incubation at 72°C was included after the final cycle to ensure proper product extension. Negative controls using reaction mixtures containing all constituents except template DNA were included in every experiment to control for false positive signals. The PCR products were electrophoresed on 1% (w/v) agarose gels containing 0.2 mg/mL ethidium bromide and visualized by UV detection.

To determine the sensitivity (detection limit) of our proposed PCR detection method, purified genomic DNA from *V. cholerae* was serially diluted with sterile water to yield samples containing DNA amounts ranging from 10 ng to 1 pg. A single colony of *Vibrio* was also utilized to assess detection limits. Accordingly, broth from overnight culture of *V. cholerae* was serially diluted to yield samples containing from $2\times10^9$ to $2\times10^5$ cells/mL, which were then boiled and used as template DNA. PCR and agarose gel electrophoresis were carried out as described above. As a test of the specificity of our detection method, we mixed an equal number of *V. cholerae* cells ($3\times10^8$ cells/mL) with other non-*Vibrio* bacteria in a 1:1 ratio and then boiled the samples to prepare DNA templates. PCR and electrophoresis were performed as described above.

For simultaneous detection of total and specific *Vibrio* strains, multiplex PCR was performed in a total volume of 50 μL using two primer sets. A reaction mixture consisted of 1 μL template DNA, 1 U Taq DNA polymerase, 250 nM dNTPs, 1.5 mM $MgCl_2$, 1 μL of each primer (10 pmol), and 5 μL 10× polymerase buffer. A PCR annealing temperature of 58°C was determined by gradient PCR. The PCR products were visualized on ethidium bromide-containing agarose gels with UV detection.

## Results and Discussion

### Single Detection of Total and Specific *Vibrio* Pathogens

To develop a PCR method specific for total *Vibrio* or for only *V. cholera*, we designed and tested various oligonucleotide primer sets. The representative amplified bands were clearly obtained with the specific primer sets in Table 1. The template DNA dilution, annealing temperature, and number of amplification cycles were varied and optimized (data not shown). The appropriate 893-bp amplification fragment of the potassium uptake gene, *trkA,* was detected in all 11 *Vibrio* strains tested using the TV-F3/TV-R5 degenerate primer set (lanes 1–11, Fig. 2a), and no target band was amplified from any of the six non-*Vibrio* bacteria tested (lanes 12–17, Fig. 2a). In the cases of *Salmonella typhimurium and enteritidis*, there were some nonspecific amplified bands (lanes 12 and 13, Fig. 2a). TrkA is a protein required for the uptake of potassium and the *trkA* gene sequences are very similar in different *Vibrio* strains [17]. Isogenic *trkA* mutants have been shown to be significantly more sensitive to the cytotoxic effects of human serum than wild-type organisms [17] suggesting that TrkA might be important for the survival of *Vibrio* strains in their hosts. This survival advantage likely accounts for the high degree of protein sequence conservation, and enables total *Vibrio* strains to be detected using the *trkA* gene as a marker.

Cell surface bacterial proteins usually exhibit much greater amino acid sequence divergence rates than do intracellular proteins [19]. Thus, genes encoding surface proteins represent good candidate biomarkers for assessing genetic variation in bacterial species.

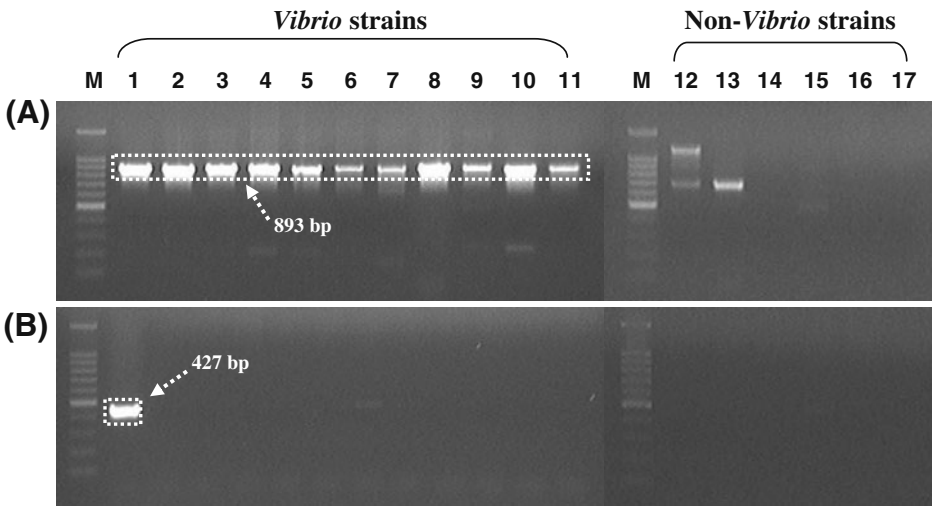


**Fig. 2** PCR-based detection of **a** total *Vibrio* and **b** *V. cholerae*. Lanes: *M*, 100 bp DNA size marker; *1*, *V. cholerae*; *2*, *V. vulnificus*; *3*, *V. parahaemolyticus*; *4*, *V. mimicus*; *5*, *V. furnissii*; *6*, *V. campbelli*; *7*, *V. cincinnatiensis*; *8*, *V. diazotrophicus*; *9*, *V. hollisae*; *10*, *V. metschnikovii*; *11*, *V. ordalii*; *12*, *S. typhimurium*; *13*, *S. enteritidis*; *14*, *S. choleraesuis*; *15*, *L. monocytogenes*; *16*, *E. coli*; *17*, *B. subtilis*. Total *Vibrio* strains were detected using TV-F3/R5 primer set at 56.3°C annealing temperature and *V. cholerae* was detected using VC-F2/R3 primer set at 52°C annealing temperature. The PCR products were electrophoresed on 1% (*w*/*v*) agarose gels and visualized by UV detection

Genes encoding flagellin, a protein that forms the flagellum (the long tail), and which is one of the most abundant proteins in flagellated bacteria, are increasingly being targeted for this purpose [18]. Among all the pathogens tested using the VC-F2/VC-R3 primer set, only *V. cholerae* yielded the correct-sized 427-bp amplification fragment of the flagellin gene (Fig. 2b), demonstrating the utility of this gene for the species-specific detection of *V. cholerae*.

## Sensitivity and Interference for Specific *Vibrio* Detection

The sensitivity of *V. cholerae*-specific detection was investigated by performing PCR reactions using serially diluted chromosomal DNA samples. Based on the range of dilutions tested, the limit for specific *V. cholerae* detection using the VC-F2/VC-R3 primer set was ~1 pg (Fig. 3a). Using boiled cell culture samples serially diluted from an initial density of $2\times10^9$ cells/mL as templates for PCR reactions, we found that the limit for the specific detection of *V. cholerae* was approximately 200~2,000 cells (data not shown). Assuming a chromosome mass of $4.5\times10^{-15}$ g/cell [20], this yields a detection limit estimate (1~10 pg) nearly the same as that arrived at using purified chromosomal samples. Thus, we found that detection sensitivity using boiled cells was not notably different from that obtained using purified chromosomal DNA. Thus, the simple and efficient sample preparation method of direct boiling can be used to detect specific *Vibrio* with our PCR system. This detection limit (1~10 pg) is similar to that reported previously for studies using standard PCR methods [21–23]. We expect that the detection sensitivity of our proposed PCR method could be further enhanced by employing recently developed strategies, such as gold nanoparticles or nested PCR [24].

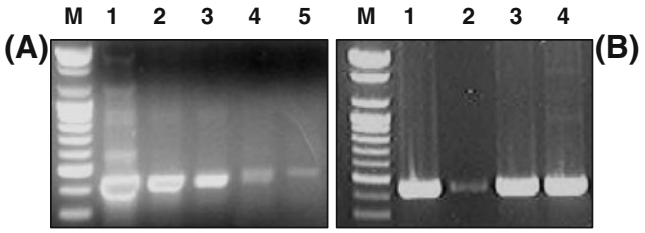


**Fig. 3** **a** Sensitivity of PCR-based detection for *V. cholerae*. Lanes: *M*, 100 bp DNA ladder; *1*, DNA 10 ng; *2*, DNA 1 ng; *3*, DNA 100 pg; *4*, DNA 10 pg; *5*, DNA 1 pg. **b** Interference of PCR-based detection for *V. cholerae* by other bacterial cells. Lanes: *M*, 100 bp DNA ladder; *1*, with *E. coli*; *2*, with *B. subtilis*; *3*, with *S. enteritidis*; *4*, with *Y. enterocolitica*. For interference experiment, equal cell numbers ($3 \times 10^8$ cells/mL) were mixed in a 1:1 ratio. *V. cholerae* was detected using VC-F2/R3 primer set at 52°C annealing temperature. The PCR products were electrophoresed on 1% (*w*/*v*) agarose gels and visualized by UV detection

To investigate the possibility that other contaminating bacteria could interfere with the specific detection of *Vibrio*, we performed PCR using mixed culture samples. *V. cholerae* was mixed with an equal number of *E. coli*, *B. subtilis*, *S. enteritidis*, or *Y. enterocolicica* cells, and the mixtures boiled. In *V. cholerae*-containing mixed culture, only target-amplified band was clearly evident (Fig. 3b), thus confirming that genomic and cellular materials from contaminating bacteria did not interfere with the specific detection of *Vibrio* species. From the experiment using *V. cholera*-contaminated real food sample, we also found no interference of specific *Vibrio* detection (data not shown).

## Simultaneous Detection of Total and Specific *Vibrio* Pathogens

Having successfully demonstrated the separate detection of total and specific *Vibrio* strains, we extended our approach to a multiplex PCR method, using a combination of the designed primer sets, to allow for simultaneous detection. During preliminary optimization experiments using gradient PCR, an annealing temperature of 58°C was found to accommodate the annealing of all primers (data not shown), and was used in subsequent multiplex PCR reaction. For simultaneous detection of total and specific *Vibrio*, we performed multiplex PCR using the two primer sets, TV-F3/TV-R5 and VC-F2/VC-R3, and successfully and specifically amplified bands of the expected sizes (893 bp and 427 bp) without interference (lane D, Fig. 4). Thus, using our proposed PCR method, both simultaneous or two-step

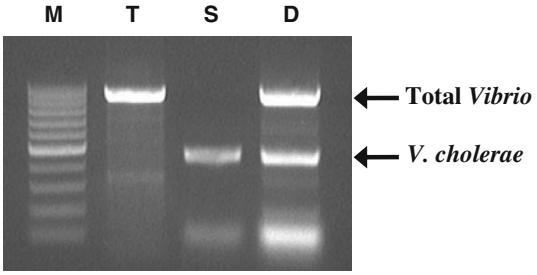


**Fig. 4** Simultaneous detection of *Vibrio* strains by multiplex PCR. Lanes: *M*, 100 bp DNA ladder; *T*, single PCR for total *Vibrio* detection; *S*, single PCR for specific *V. cholerae* detection; *D*, multiplex PCR for both total and specific detection. A PCR annealing temperature of 58°C was used. The PCR products were electrophoresed on 1% (*w*/*v*) agarose gels and visualized by UV detection

detection of total and specific *Vibrio* pathogens is possible. In the case of two-step detection, we might screen for total *Vibrio* strains in a sample in a preliminary step, and then examine species using specific detection as a second step.

## Conclusion

In the present work, we describe a PCR-based method that successfully detected total *Vibrio* species using the degenerate primer sets based on the potassium uptake gene, *trkA*, and specifically detected *V. cholerae* pathogen using the primer set designed with putative flagellin gene information. The detection limit for detecting specific *V. cholerae* was approximately 1~10 pg using purified chromosomal DNA, and 200~2,000 cells using an aliquot of directly boiled culture broth as PCR templates. In addition, other contaminating bacteria did not interfere with specific *V. cholerae* detection. Furthermore, simultaneous total and specific *Vibrio* detection was successful using multiplex PCR.

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