

Application of a Peptide Nucleic Acid-Based Asymmetric Real-Time PCR Method for Rapid Detection of *Vibrio cholerae*

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비브리오 콜레라 신속 검출을 위한 펩티드 핵산 기반 비대칭 real-time PCR 방법의 적용

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Abstract *Vibrio cholerae* is a very important pathogenic bacterium that has to be monitored in seafood and ships' ballast water. Various methods have been developed to identify this bacterium, yet these methods are time-consuming and have limitations for their sensitivity to detect contamination. The purpose of the present study was to develop a robust and reliable method for identifying *V. cholerae*. Peptide nucleic acid (PNA) probes were developed to use for PNA-based asymmetrical real-time PCR techniques. The toxigenic Cholera enterotoxin subunit B (ctxB) gene was selected as a target for detecting *V. cholerae* and the gene was synthesized as a positive template for conventional and real-time PCR. Real-time PCR primers and PNA probes were designed and standard curves were produced for the quantitative analysis. The selected PNA probes reacted specifically to *V. cholerae* without any ambiguity, even among closely related species, and the detection limit was 0.1 cfu/100 mL. Taken together, the PNA probes and asymmetrical qPCR methods developed in this present study could contribute to the rapid, accurate monitoring of *V. cholerae* in marine environments, and as well as in seafood and ships' ballast waters.

요약 비브리오 콜레라는 수산물과 선박평형수 내에서 모니터링되고 있는 중요 병원성 박테리아이다. 이를 검출하기 위한 여러 방법들이 개발되어 왔으나, 시간 소모가 크고 민감도에서 한계가 있었다. 본 연구는 비브리오 콜레라를 보다 정확하게 검출하기 위한 방법을 개발하는 목적으로 수행하였다. PNA 기반 비대칭 real-time PCR 기술에 적용하기 위하여 펩티드 핵산(Peptide nucleic acid, PNA) 프로브를 개발하였다. 독성 유전자인 *Cholera enterotoxin subunit B (ctxB)*를 비브리오 콜레라 검출을 위한 타겟 유전자로 선정하고, conventional PCR과 real-time PCR을 위한 positive template를 합성하였다. Real-time PCR 프라이머와 PNA 프로브를 디자인하여, 정량 분석을 위한 표준곡선 실험을 수행하였다. 선택된 PNA 프로브는 비브리오 콜레라에 특이적으로 반응하였으며, 검출한계는 0.1 cfu/100 mL이었다. 종합해 보면, 본 연구에서 개발된 PNA 프로브와 비대칭 real-time PCR 방법은 수산물과 선박평형수 뿐만 아니라 해양환경에 있는 비브리오 콜레라를 신속하고 정확하게 모니터링할 수 있는 기술로 판단된다.

Keywords : *Vibrio Cholerae*, *Cholera Enterotoxin Subunit B (ctxB)*, Peptide Nucleic Acid, Asymmetrical qPCR, Monitoring

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1. Introduction

Vibrio species belong to Gram-negative facultative bacteria and have a curved rod shape [1]. There are dozens of known species in the genus *Vibrio*, most of which are known to be pathogenic. In particular, *Vibrio cholerae* is an infectious bacterium that causes watery diarrhea and leads to rapid dehydration and death. Coastal waters are an important reservoir for *V. cholerae*, and *V. cholera* is generally transmitted to humans through contaminated seawater or seafood [2, 3]. Due to the rapid infectivity and severe symptoms of *V. cholerae*, monitoring in marine environments and seafood is very important. It is especially necessary to develop an accurate and rapid method for the detection of toxigenic *V. cholerae* with a view to predicting microbes according to the ballast water guidance that has recently become effective [4].

In recent decades, nucleic acid amplification-based tests have been introduced and advanced as genetic techniques. Thereafter, various microbial detection methods have been improved. Ideally, methods for the evaluation of microbial contamination should be fast, sensitive, and specific. To meet these needs, microarrays [5, 6], immunoassays [7], loop-mediated isothermal amplification assays (LAMP) [8], and real-time PCR [9, 10] have been applied to the detection of *V. cholerae*. Despite the technological advances in *V. cholerae* detection, there remains a need for further development of advanced methods in terms of stability and sensitivity. In this regard, peptide nucleic acid-based research methods have been recently applied to improve the stability and sensitivity of DNA probe-based qPCR [11].

Peptide nucleic acid (PNA) is an artificially synthesized DNA analogue [12] with an uncharged peptide backbone. PNA has chemical, thermodynamic, and biological stability due to the peptide bond-linked backbone [13]. It is

more specific than DNA-DNA hybridization and is not degraded by nucleases or proteases [14]. These characteristics ensure that the melting temperature (T_m) of the PNA probes is high, making them available as robust and reliable diagnostic tools [15, 16]. Therefore, multiplex PCR [17], LAMP [18], and fluorescence *in situ* hybridization (FISH) [19] technologies based on PNA have been used for the detection of pathogenic bacteria. However, PNA-based real-time PCR has not yet been developed for the detection of *V. cholerae*. The objective of the present work was to develop highly specific and sensitive molecular methods based on PNA for the rapid identification and monitoring of *V. cholerae* in marine products and in ballast water. Using the synthesized *V. cholerae* *ctxB* gene as a template, an experimental method was established to detect < 1 cfu/100 mL within 2 h. To the best of our knowledge, this is the first study to apply PNA-based asymmetrical qPCR to *V. cholerae* detection. This method has the potential to be used as a generic method for the prediction of waterborne microorganisms that are important to public health issues.

2. Materials and Methods

2.1 Conventional PCR

The species-specific primers for the *V. cholerae* *ctxB* gene were designed using the Primer-BLAST program at NCBI. As a result of the combination of the forward and reverse primers, the size of the amplified PCR product was designed to be 97–242 base pairs (bp). Conventional PCR conditions were constructed such that various sizes of PCR product could be synthesized as follows: 1 μ L template, 10 μ M forward primer, 1 μ L reverse primer, 10 μ M 10 \times PCR buffer, 2 μ L dNTP mix, and 0.5 μ L Taq polymerase. The reaction conditions were

denaturation at 95°C for 10 min, followed by 30 cycles of amplification at 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, followed by 35 cycles and a final reaction at 72°C for 10 min. The PCR product was confirmed by electrophoresis using a 2% agarose gel. The primer combination for the band that was most clearly generated by electrophoresis was selected for detection of the *V. cholerae ctxB* gene. The nucleotide sequences of the selected primers were: forward 5'-ACCACCACACACAAATACATACG-3' and reverse 5'-GCAATCCTCAGGGTATCCTTC-3', and the PCR product size was 190 bp.

2.2 Real-time PCR

A real-time PCR assay for the detection of *V. cholerae* was carried out in a final reaction volume of 20 µL containing 1 µL template, 1 µL 5 pM forward primer, 1 µL 5 pM reverse primer, 10 µL 2× SYBR qPCR mix, and 7 µL distilled water. PCR conditions were optimized as an initial pre-denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 10 sec, and annealing and extension at 60°C for 30 sec. The melting curve analysis was then followed by heating from 60°C to 97°C at 0.1°C per sec.

2.3 PNA-based asymmetrical real-time PCR

To ensure accurate amplification of the *V. cholerae ctxB* gene, 1 µL template, 1 µL PNA probe, 1 µL 4 pM forward primer, 1 µL 20 pM reverse primer, 9 µL 2.5× PCR buffer, and 1 µL *Taq* polymerase were mixed, and the reaction volume was adjusted to 25 µL with distilled water. In the asymmetrical PCR reaction, the ratio of the forward primer to the reverse primer was 1:5. PNA-based asymmetrical qPCR was performed by denaturation at 95°C for 10 min, followed by 45 cycles of 95°C for 10 sec, 60°C for 30 sec, and 72°C for 15 sec. Melting curve analysis was performed following the asymmetrical PCR

reaction by setting denaturation at 95°C for 5 min and at 37°C for 5 min, and measuring the fluorescence while increasing the temperature from 37°C to 80°C at 0.1°C per sec.

3. Results

3.1 Synthesis of the *ctxB* gene as a template

Cholera enterotoxin subunit B (ctxB) was selected as a specific gene for the detection of *Vibrio cholerae*. To select the toxigenic *ctxB* gene sequences of the O1 and O139 serogroups, the nucleotide sequences of the *Vibrio* species registered in the National Center for Biotechnology Information (NCBI, USA) database were compared and analyzed. The nucleotide sequence of the *ctxB* gene was selected as 536 bp, and synthesis by Bioneer, South Korea was requested (Table 1). To confirm whether the synthesized gene was inserted properly into the pBHA vector, the nucleotide sequence obtained from *EcoRI* restriction enzyme digestion and sequencing was compared with the synthesized nucleotide sequence. The synthesized *ctxB* gene was used as a template for the design of primers and PNA probes for the detection of *V. cholerae*.

Table 1. Synthesized *V. cholerae ctxB* gene fragment.

Bacterial gene	Sequences (5'→3')	Size (mer)
<i>V. cholerae ctxB</i>	TGTGGGAATGCTCCAAGATCATCGATGAGT AATACTTGGCGATGAAAAACCCAAAGTCTA GGTGTAATAATTCCTTGACGAATACCAATCT AAAGTTAAAAGACAAATATTTTCAGGCTATC AATCTGATATTGATACACATAATAGAATTA GGATGAATTATGATTAATTAATTTGGTG TTTTTTTTACAGTTTTACTATCTTCAGCATA TGCACATGGAACCTCAAAATATTACTGA TTTGTGTGCAGAATACCACAACACAAAT ACATACGCTAAATGATAAGATATTTTCGTAT ACAGAACTCTAGCTGGAAAAAGAGAGATG GCTATCATTACTTTTAAAGAATGGTGCAACTT TTCAAGTAGAAGTACCAGGTAGTCAACATA TAGATTACAAAAAAAAGCGATTGAAAGGA TGAAGGATACCCTGAGGATTGCATATCTTA CTGAAGCTAAAGTCGAAAAGTTATGTGTAT GGAATAATAAAAACGCCTCATGGCGATTGCCG CAATTAGTATGGCAAATTA	536

3.2 Real-time PCR

For the quantitative detection of the *V. cholerae* *ctxB* gene, real-time PCR was performed using primers selected by conventional PCR. The *ctxB* gene was diluted to 1×10^1 – 1×10^8 copies/mL, real-time PCR was performed using SYBR Green, and a standard curve was created (Fig. 1). The detection limit of the *ctxB* gene obtained from the real-time PCR reaction and standard curves was 1×10^2 copies/mL. To confirm that the selected primers reacted specifically with toxic *V. cholerae*, cross-reactivity analysis with other species (*V. xuii*, *V. sinaloensis*, *V. littoralis*, *V. zureus*, and *V. natriegens*) was performed. PCR results show that the primers for the selected *ctxB* gene did not react with other *Vibrio* species, only with *V. cholerae* (Table 2).

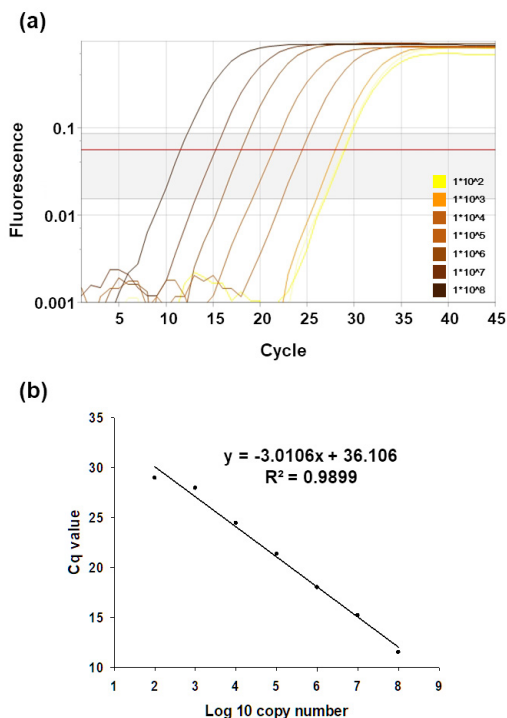


Fig. 1. Real-time PCR and standard curve for *V. cholera* *ctxB* gene. (a) real-time PCR reaction curve. (b) standard curve.

Table 2. Cross reactivity with various *Vibrio* species.

Samples	Cq value	Reactivity
Negative control ^a	30.224	-
<i>V. cholerae</i>	19.029	+
<i>V. xuii</i>	-	-
<i>V. sinaloensis</i>	31.389	-
<i>V. littoralis</i>	30.316	-
<i>V. zureus</i>	-	-
<i>V. natriegens</i>	29.033	-

^a is the no template reaction

3.3 PNA probe design

For optimization of the fusion temperature, PNA probes were designed for the detection of the *V. cholerae* *ctxB* gene using the complementary sequence to that of the *ctxB* gene (Table 3). Probes were covalently labelled at the 5'-end with a dabsyl (dimethylamino-azosulphonic acid) quencher dye, and at the 3'-end with a fluorophore (FAM) reporter dye. All the PNA probes used in the present study were synthesized using an HPLC purification method by Panagene, South Korea. The purity of all synthesized probes was confirmed by mass spectrometry.

Table 3. The nucleotide sequences of the PNA probes for the detection of *ctxB* genes of *V. cholerae*.

Oligonucleotide	Sequences (5' → 3')	Tm (°C)
ctx PNA 1	Dabcyl-ATGGCTATCATTAA-O-K(FAM)	49.7
ctx PNA 2	Dabcyl-CCAGGTAGTCAACA-O-K(FAM)	63.4
ctx PNA 3	Dabcyl-GTAGTCAACATATAG-O-K(FAM)	58.1

3.4 PNA-based asymmetrical real-time PCR

Melting curve analysis using the selected primer sets and PNA probes was performed by MyGo mini real-time PCR (IT-IS Life Science Ltd, Ireland). Asymmetrical PCR was performed using primer sets and PNA probes to generate single-stranded target nucleic acids. Three different PNAs with different Tm values were

used for asymmetrical PCR reactions, and analysis of the melting curve shows that these three PNA probes had a melting peak at each temperature (Fig. 2). The T_m values of PNA1, PNA2, and PNA3 were 53°C, 70.2°C, and 63.2°C, respectively, and subsequent experiments, such as detection limit analysis, were performed using PNA2 with the highest T_m value. Analysis of the melting curve revealed that all three PNA probes for each species could be used to detect *V. cholerae* and also in multiplex PCR form.

The detection limit of real-time PCR of the *V. cholerae* *ctxB* gene-specific PNA probes was analyzed. The limit of detection was determined using the *ctxB* PNA2 probe, which shows the highest T_m value among the selected probes. Asymmetrical PCR analysis revealed that the detection limit of the *V. cholerae* toxin gene *ctxB* was 0.1 cfu/100 mL (Fig. 3).

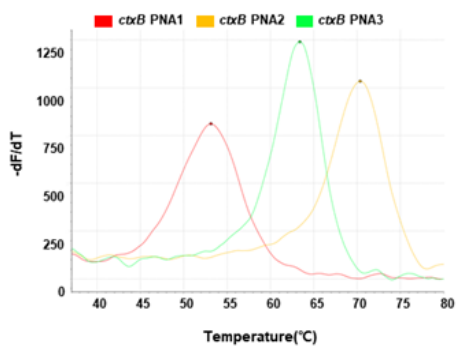


Fig. 2. Melting peak for T_m values of PNA1, PNA2, and PNA3 at each temperature.

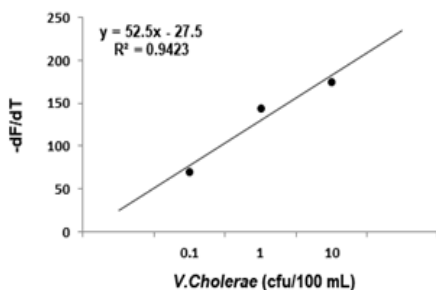


Fig. 3. Standard curve for quantitative analysis of *V. cholerae* using PNA probes.

4. Discussion

Peptide nucleic acid (PNA) is a polyamide analogue of DNA that was first proposed in 1991 [20]. PNA has a structure in which the sugar-phosphate backbone of DNA is replaced with a polyamide consisting of repeated N-(2-aminoethyl) glycine units, but it behaves like DNA and promotes binding and selectivity. PNA has gained attention as a diagnostic tool in several attractive fields [21] and has been successfully applied to the detection of point mutations and single nucleotide polymorphisms (SNPs) in diagnosis, particularly of cancer, by exploiting the high sensitivity of PNA probes to RNA and DNA [22]. PNA probes have been developed and used as sensors and diagnostic kits for the rapid screening of biomedical applications and biological items [23].

In the present study, PNA probes were developed as a diagnostic tool for the monitoring of *V. cholerae* in seafoods and ship ballast water, and quantitative analysis was performed in conjunction with real-time PCR. We selected the *ctxB* gene of toxigenic *V. cholerae* as the target for the development of PNA probes [24, 25]. To obtain the real-time PCR template, a method of synthesizing the *ctxB* gene was selected instead of culturing *V. cholerae*, which is difficult to perform in the general laboratory (Table 1). We designed various primers for conventional and real-time PCR, selected optimal primer sets by evaluating product size and reaction intensity, and finally designed PNA probes containing a fluorescent reporter dye (Table 3). The optimal reaction conditions for asymmetrical real-time PCR with PNA probes were established and the detection limits were calculated as 0.1 cfu/100 mL when applied to the standard curve, which is consistent with seafoods and the detection criteria for ballast water (Fig. 3). The detection limits for multiplex PCR using the *ctxA*, *ctxA*, and *vopF* genes [10] and for the LAMP assay

using the *ctxA* gene [8] were 10 cfu/PCR tube and 10 cfu/mL, respectively. These results show that the sensitivity in the present study using PNA probes is relatively higher than that using non-PNA probes.

To the best of our knowledge, this study is the first application of the PNA-based asymmetrical real-time PCR method for the detection of *V. cholerae*. The PNA-based qPCR method proposed in the present study significantly reduced the detection time as compared with culture-based methods and improved sensitivity as compared with previous gene-based detection methods. The PNA-based asymmetrical real-time PCR method has high specificity and sensitivity for *V. cholerae* detection and can be used as a simple, fast, and robust detection method. This method will be useful for the detection of *V. cholerae* in marine environments as well as in specific samples such as seafood and ship ballast water.

5. Conclusion

Although *Vibrio cholerae* is a critical pathogenic bacterium, diagnostic techniques are time-consuming and have limitations in sensitivity. In this study, a detection method was developed for the identification of *V. cholerae* with PNA-based asymmetrical real-time PCR. We selected the *ctxB* gene as the target for the detection and designed the real-time PCR primers and PNA probes containing a fluorescent reporter dye. Three different PNAs with different T_m values were designed for asymmetrical PCR reactions, and performed using PNA2 with the highest T_m value. The selected PNA probes detected specifically to *V. cholerae* without any ambiguity, even among closely related species and the detection limit was calculated as 0.1 cfu/100 mL, which is more sensitive than non-PNA probes. This study provided useful

diagnostic method for the detection of *V. Cholerae* in seafoods and ship ballast water.

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