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Detection of *rtxA* Gene as a Biomarker of Seafood-Borne Pathogen *Vibrio cholerae* using *In Silico* PCR Assay

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Abstract

Seafood-borne outbreaks caused by *Vibrio cholerae* have led to the increased need for food safety risk assessment of marine products. An *in silico* investigation about the potential of virulence gene of *V. cholerae*, *rtxA*, as a DNA biomarker of the toxigenic bacterium has been carried out. The aim of this study was to use the bacterial DNA biomarker sequence as a tool to facilitate early rapid detection of cholera infection. Five specific pairs of primers were designed from the *rtxA* open reading frame DNA of *V. cholerae* O1 biovar El Tor str. N16961 genomic DNA using Primer3Plus. Next, *in silico* Polymerase Chain Reaction (PCR) assay was carried out using the newly designed primers and 25 genomic DNA of vibrio spp. retrieved from the *in silico* database. One of the five designed pairs of primers, RtxAOF-RtxAOR: '5-CGCAAAACAGTTTCAGCCGA-3' and 5'-AGGTTGGTCTTTTGTGGCCA-3', could result in single DNA amplicon sized 518 bp only from *V. cholerae* species. No amplicon bands were produced from 17 other vibrio genomes studied using similar RtxAF-RtxAR primers. A further check showed that the amplicon was indeed part of the *rtxA* gene of *V. cholerae*. Based on this *in silico* study, *rtxA* gene appeared to be a DNA biomarker of *V. cholerae*, which is potential to facilitate rapid diagnosis of the virulence bacterium using *in silico* PCR assay.

Keywords: seafood-borne infection, *Vibrio cholerae*, DNA biomarker, primer design, *rtxA*

1. Introduction

Seafood products are consumed worldwide, therefore playing a significant role in the economic market (Bonnin-Jusserand et al., 2019). The proportion of globally produced-seafood commodities that is internationally-traded is higher than other products. It is also increasing mostly due to globalization (Guillen et al., 2019). However, several recent outbreaks of human gastroenteritis have been linked to the consumption of contaminated seafood (Elbashir et al., 2018).

Infectious diseases are classified as illnesses caused by pathogenic microorganisms, including bacteria. Such diseases have been the main threat worldwide and have a significant impact on public health and the world's economy (Hwang, Hwang, & Bueno, 2018). Seafood-borne outbreaks caused by bacterium have led to the increased need for food safety risk assessment of marine products. Continuous monitoring of bacterial contamination in aquatic products and identification of risk factors are, therefore, crucial for assuring food safety (Xu, Wu, & Chen, 2019).

Investigation of seafood-borne illnesses caused by bacteria and viruses requires a concrete knowledge about the pathogenicity and virulence properties of the etiologic agents (Elbashir et al., 2018; Iwamoto, Ayers, Mahon, & Swerdlow, 2010). Among pathogenic bacteria- contaminating seafood, *V. cholerae* is the

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main *Vibrio* species responsible for the dramatic increase of seafood-borne infections worldwide (Bonnin-Jusserand et al., 2019). *V. cholerae* is notorious as the causal agent of cholera, a severe diarrheal disease, which could be quickly fatal if untreated. It is commonly transmitted via contaminated water and person-to-person contact (Baker-Austin et al., 2018). Approximately 2.9 million cases of cholera and 95,000 deaths had occurred annually worldwide between 2008 and 2012 (Ali, Nelson, Lopez, & Sack, 2015).

To date, more than 200 serogroups of *V. cholerae* have been recognized, based on variable somatic O antigen composition; O1 was the only known epidemic serogroup of *V. cholerae* up to 1991. In 1992, serogroup O139 was identified as the second epidemic serogroup of *V. cholerae* (Albert et al., 1993; Bhattacharya et al., 1993). Within the *V. cholerae* O1 serogroup, there are two well-established biotypes: classical and El Tor. The El Tor biotype emerged in 1961 and subsequently displaced the classical biotype as a cause of cholera throughout the world (Pradhan, Baidya, Ghosh, Paul,& Chowdhury, 2010). Both classical and El Tor biotypes are differentiated from each other by several properties, including agglutination of chicken red blood cells, susceptibility to polymyxin B and to biotype-specific phages, haemolysis of sheep red blood cells, and Voges–Proskauer reaction (Kaper, Morris Jr, & Levine, 1995). *V. cholerae* strains of El Tor biotype initiated the seventh cholera pandemic, which is continuing to cause outbreaks worldwide with catastrophic effects.

Among the critical virulence factors of *V. cholerae* are the cholera toxin (CTX) genetic element and the repeats in toxin (RTX) cluster (Davis, Moyer, Boyd, & Waldor, 2000). CTX and RTX are the chief virulence gene clusters and are grouped together (Cheng, Zhou, Kan, Wang, & Rui, 2014). The RTX family of toxins generally produced by several pathogenic Gram-negative bacteria. The RTX toxins constitute a family of dominant virulence factors that have widely spread among Gram-negative bacteria. In *V. cholerae*, the RTX toxin gene cluster encodes the presumptive cytotoxin (*rtxA*), an acyltransferase (*rtxC*), and a related ATP-binding cassette transporter system (two proteins for toxin transportation, RtxB and RtxD) (Chou et al., 2001; Lin et al., 1999)

The search for the ideal biomarkers in infectious diseases with high sensitivity, specificity, and predictive capacity must initially be focused on detection and identification of the infectious agent (Mohan & Harikrishna, 2015). *V. cholerae* genomic sequence provides a starting point for understanding how a free-living, environmental organism emerged to become a significant human bacterial pathogen. The complete genomic sequence of the Gram-negative, gamma-Proteobacterium *V. cholerae* El Tor N16961 has been reported to have a length of 4,033,460 base pairs (bp) (Heidelberg et al., 2000). Numerous mutations, however, have been reported in *V. cholerae* O1 strains encompassing genes, which code virulence factors including the repeat in toxins (*rtxA*). In the case of seafood-borne infectious disease, detection and identification of the infectious agent *V. cholerae* are therefore necessary to do. In this study, *rtxA*, a member of RTX chief virulence gene cluster, was tested for its potential as a DNA biomarker of *V. cholerae*. Such biomarker is beneficial to facilitate early rapid diagnosis of *V. cholerae* infection using *in vitro* PCR.

2. Material and Methods

A literature study was initially conducted to obtain information related to the unique phenotypic feature of *V. cholerae*, particularly on specific toxins the species produces. Next, the genotype feature, such as genes associated with proteins underlining the unique phenotype was targeted. Primers were then designed from the internal part of open reading frame (ORF) of the targeted gene sequences in FASTA format as input using Primer3Plus at https://primer3plus.com/ (Untergasser et al., 2007; Ethica et al., 2013; Ethica, 2014; Ethica, Sulistyaningtyas, & Darmawati, 2019; Ethica, Darmawati, Dewi, Nurrahman, & Sulistyaningtyas, 2020). The input of the program is basically a FASTA format of a gene of interest. Pairs of primers having the least possibility of hairpin formation, self-complementarity, and dimerization were selected (Ethica, Semiarti, Widada, Oedjijono, & Raharjo, 2017). The newly designed primers were subsequently used as input for the web-based *in silico* PCR. The virtual PCR work was run from a web-based software at http://insilico.ehu.es/PCR/ using *Vibrio* spp. genomes stored in the program database. *In silico* PCR can be done by choosing “PCR Amplification” from the main page of the website, and once it opens, inserts the primers, then click “amplify” (Bikandi, Millán, Rementeria, & Garaizar, 2004; San Millán, Martínez-Ballesteros, Rementeria, Garaizar, & Bikandi, 2013); Ethica, Sulistyaningtyas, & Darmawati, 2019; Ethica, Darmawati, Dewi, Nurrahman, & Sulistyaningtyas, 2020). The nucleotide database used in the *in silico* PCR website comes from National Center of Bioinformatics Institute (NCBI) database. An analysis was eventually carried out to confirm if the *in silico* PCR products (amplicons) were specific to *V. cholerae* species and were indeed part of genes targeted as DNA biomarkers. This analysis is conducted to check whether the *in silico* PCR amplicons virtually seen on gel electrophoresis were correct according to primer positions. The analysis

was carried out by "clicking" the amplicon band or size shown by the *in silico* PCR result window.

3. Results and Discussion

Severe infection outbreaks originated from fish and other foods often create a diagnostic challenge for clinicians. There is a limited official guidance to help clinicians decide which biomarkers that can aid with the diagnosis of bacterial infections (Rogers et al., 2011). Therefore, the development of bacterial DNA biomarkers to detect *V. cholerae* was carried out in this study. It was expected that in the case of diarrheal infection outbreaks, the involvement of *V. cholerae* could be determined by detecting the presence of their DNA biomarkers.

From a literature study, it is known that *rtxA* gene, a member of RTX gene cluster, is among distinct genotypic features of infectious bacterium *V. cholerae* (Lin et al., 1999). The uniqueness of the gene makes it potential to be targeted as a DNA biomarker of *V. cholerae* species. The *rtxA* gene sequence was then retrieved from the genome sequence of *V. cholerae* O1 biovar El Tor strain N16961 (NCBI Reference Sequence: NC_002505.1). The sequence encodes RTX toxin RtxA [*Vibrio cholerae* O1 biovar El Tor str. N16961 in the NCBI GenBank] (NCBI Reference Sequence: NP_231094.1). The 3-D structure of cholera RtxA protein (UniProt accession number: Q9KS12) as the product of *rtxA* gene is shown in Figure 1. Using primer3Plus (Untergasser et al., 2007), pairs of primers obtained by using *rtxA* DNA sequence as input (Figure 1) are listed in Table 1.

In silico PCR assay using Pair 2 primers and genomic DNA of 25 *Vibrio* species were run on http://insilico.ehu.es/PCR/. The assay resulted in eight single 518-bp DNA bands (Figure 2) belonging to all *V. cholerae* or species number 4 to 11 (Table 2, highlighted). According to Table 2, *V. cholerae* species number 4-11 are *V. cholerae*, *V. cholerae* str. IEC224, *V. cholerae* str. LMA3984-4, *V. cholerae* str. M66-2, *V. cholerae* str. MJ-1236, *V. cholerae* O1 str. 2010EL-1786, *V. cholerae* str. O395, and *V. cholerae* str. O395 chromosome 1. Table 1 shows that though primers were designed from similar *rtxA* sequence, not all primers could detect unique conserved regions of *rtxA*

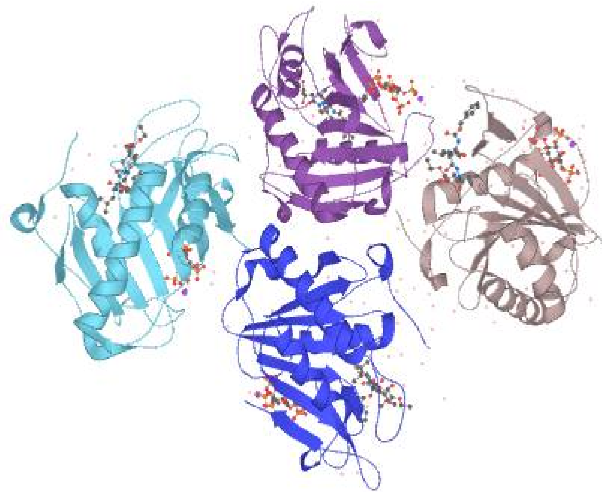

Figure 1. The 3-D structure of cholera RtxA protein (*rtxA* gene product) visualized by Litemol (UniProt Accession Number: Q9KS12)

Table 1. Primers designed using Primer3Plus from *rtxA* DNA sequence

Primer	Forward Primer	Reverse Primer	Amplicon Size (bp)	DNA of *V. cholerae* strains amplified (genome code number)
Pair 1	GCGCGAACGTAATAACCCAC	ACCGACATGCCATCACCAAT	508	4,5, and 7-9
Pair 2	CGCAAAACAGTTTCAGCCGA	AGGTTGGTCTTTTGTGGCCA	518	4-11 (all *V. cholerae*)
Pair 3	GTGGCGCGAACGTAATAACC	AGATGTTGACGTTCCCCACC	597	4,5, and 7-9
Pair 4	TAGCGGTGAAAGCTCAGGTG	GTTATTACGTTCGCGCCACC	585	4,5, and 7-9
Pair 5	GCGCATTTACTGGCTTACGG	TGGTGAAGATGTTACCCGCC	559	4-9

of all strains of *V. cholerae*, which differentiate them from other *Vibrio* species (genome code number 1-3 and 12-25, see Table 2). Also, not all of these primers could detect all *V. cholerae* strains. Only Pair 2 primer that could indicate *rtxA* conserved regions of all *V. cholerae* strains. As seen in Table 1, Primer3Plus by default produces five best pairs of primers a output and automatically eliminates low quality primers that have the potential of mispriming, self-dimer, or cross dimer.

After carrying out the *in silico* PCR assay, it is necessary to check whether the amplicon products that where seen on the virtual gel electrophoresis (Figure 2) were correct, based on the primer positions. The analysis was carried out easily by clicking the amplicon size that appeared on the *in silico* PCR result window. A new tab was then appeared, showing that the *in silico* amplicons were indeed part of the *rtxA* gene fragments (Figure 3). The DNA sequence seen in Figure 3 represents one of eight PCR amplicon run on *in silico* PCR using the selected Pair 2 primer (see Table 1) and a genome of vibrios (species number 4, see Table 2).

"Clicking" step on the amplicon size on *in silico* PCR (Figure 2) was necessary. The result is shown in Figure 3. This is a way to prove that even though

Table 2. List of genomic DNA of *Vibrio* spp. used as *in silico* PCR templates

No.	Complete list of *Vibrio* strains in the database used as *in silico* PCR templates
1	*Vibrio alginolyticus NBRC 15630 = ATCC 17749*
2	*Vibrio anguillarum 775*
3	*Vibrio campbellii ATCC BAA-1116*
4	*Vibrio cholerae*
5	*Vibrio cholerae IEC224*
6	*Vibrio cholerae LMA3984-4*
7	*Vibrio cholerae M66-2*
8	*Vibrio cholerae MJ-1236*
9	*Vibrio cholerae O1 str. 2010EL-1786*
10	*Vibrio cholerae O395*
11	*Vibrio cholerae O395 chromosome 1*
12	*Vibrio fischeri ES114*
13	*Vibrio fischeri MJ11*
14	*Vibrio furnissii NCTC 11218*
15	*Vibrio harveyi ATCC BAA-1116*
16	*Vibrio parahaemolyticus BB22OP*
17	*Vibrio parahaemolyticus O1:K33 str. CDC_K4557*
18	*Vibrio parahaemolyticus O1:Kuk str. FDA_R31*
19	*Vibrio parahaemolyticus RIMD 2210633*
20	*Vibrio sp. EJY3*
21	*Vibrio sp. Ex25*
22	*Vibrio splendidus LGP32*
23	*Vibrio vulnificus CMCP6*
24	*Vibrio vulnificus MO6-24/O*
25	*Vibrio vulnificus YJ016*

Note: Highlighted rows are genomic sequences of *Vibrio cholerae* strains among those of other Vibrios (25 strains) available that were retrieved from the *in silico* database at the time of this study.

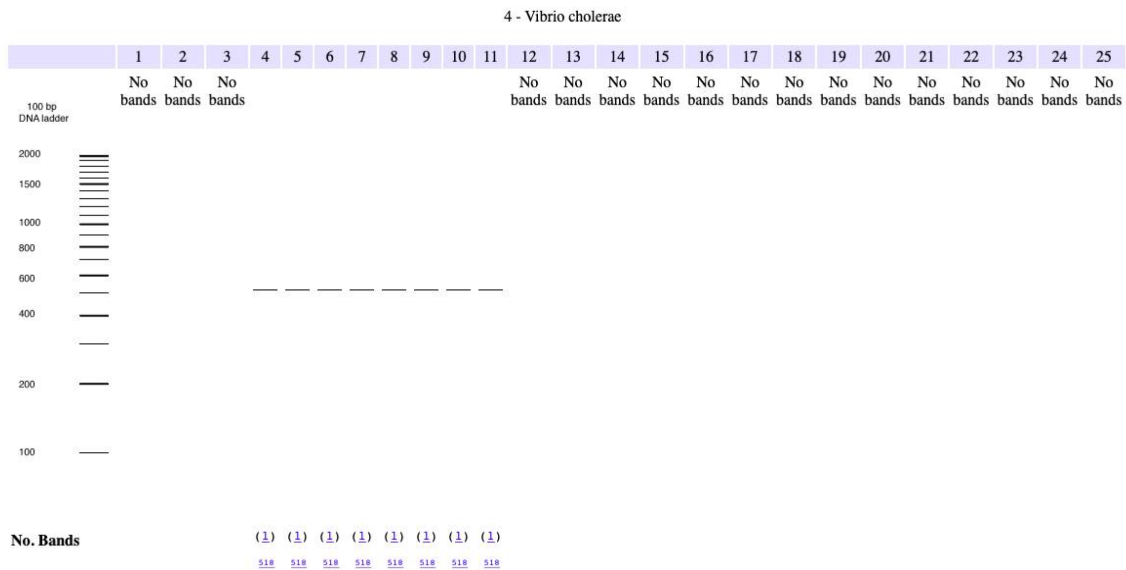

Figure 2. *In silico* PCR assay using Pair 2 primer and 25 *Vibrio* genomic DNA resulted seven single 518-bp DNA bands belonging to all *V. cholerae* strains

Genome: **Vibrio cholerae**
Start position: 1558124
End position: 1558641
Length: 518

DNA sequence

```
>NC_002505, from 1558124 to 1558641 (518 bp); Vibrio cholerae
CGCAAAACAGTTTCAGCCGACTATATGACCAGCTAGAAGCCGCTAACTTGAAAGAGAGTAAGCACCTTTA
TCTGGATCAAAATGGTGACTTTGTTACCAAAGGCAAAGGTAATCTTGCCAATATCGATCTGCTAGGTAGC
CGCGAAGCTGTGCTTGAAAAAGTGAAGTTAACAGTAAGTAACGAGTACGGTCAAACCGTTGCGGATACAA
TTTTTGCTGGATTATCAGCCAAAGATCTTGCCAAAGACGGTAAAGGGGTTGATATCGCGGGTTTGAATAA
AGTACATCAAGCGATTGAACAGCATCTGTCACCTGTCAGCGCCACGTTGTACATTTGGAAACCGAGTGAT
CATAGCGCGCTAGGTCATGCCGCATTGCAAATAGGCCAAGGTCGCACGCAACTTGAAGGTCAAGCTGCAG
CTGATTTTAACCAGCAAAATTACGTAAGCTGGTGGCCACTAGGCAGCAAGTCATCCAATATCAGCAATAT
CTTGAATGTGGCCACAAAAGACCAACCT
```

Translate to protein
Restriction digest
BLAST
Design primers with primer3
Genome related info at NCBI

Gene(s) or part of gene(s) amplified:

ORF. 1550108-1563784 Sequence VC1451 - RTX toxin RtxA

Figure 3. *In silico* PCR amplicon with *V. cholerae* genome sequence as the template using Pair 2 primer designed in this study

Vibrio spp. might have the same *rtxA* gene, our primers recognize specific DNA regions, which is part of *rtxA* of *V. cholerae* only, and not part of *rtxA* of other *Vibrio* species. Primer sequences used in this study were taken from the *rtxA* gene of *V. cholerae* precisely because we targeted all strains of this species. Not all primers designed from the *rtxA* gene could amplify the targeted genome selectively and therefore the *in silico* PCR was conducted. In this case, checking the amplicon size was carried out to confirm if the amplicon was the genuine part of *rtxA*. Occasionally, *in silico* PCR resulted DNA amplicon, which is not the targeted gene. This step also confirmed that primers, other than Pair 2, that were designed from

Pair 2:	NC_002505.1:1550147-1563784 Vibrio cholerae O1 bi								
Left Primer 2:	CGCAAAACAGTTTCAGCCGA								
Start: 7979	Length: 20 bp	Tm: 60.0 C	GC: 50.0 %	Any: 14.5	End: 0.0	TB: 10.0	HP: 0.0	3' Stab: 5.5	Penalty: 0.029
Right Primer 2:	AGGTTGGTCTTTTGTGGCCA								
Start: 8496	Length: 20 bp	Tm: 60.0 C	GC: 50.0 %	Any: 0.0	End: 0.0	TB: 10.0	HP: 0.0	3' Stab: 5.4	Penalty: 0.033
Pair:	Product Size:	518 bp		Any: 0.0	End: 0.0	TB: 20.0			Penalty: 0.061

Send to Primer3Manager Reset Form

Pair 3:	NC_002505.1:1550147-1563784 Vibrio cholerae O1 bi								
Left Primer 3:	GTGGCGCGAACGTAATAACC								
Start: 2273	Length: 20 bp	Tm: 60.0 C	GC: 55.0 %	Any: 12.1	End: 0.0	TB: 12.0	HP: 42.8	3' Stab: 2.9	Penalty: 0.025
Right Primer 3:	AGATGTTGACGTTCCCCACC								
Start: 2869	Length: 20 bp	Tm: 60.0 C	GC: 55.0 %	Any: 0.0	End: 0.0	TB: 11.0	HP: 0.0	3' Stab: 4.6	Penalty: 0.036
Pair:	Product Size:	597 bp		Any: 17.4	End: 18.8	TB: 21.0			Penalty: 0.062

Figure 4. The output of Primer3Plus showing complete parameters of Pair 2 Primer, including HP (hairpin possibility)

the targeted *rtxA* gene of a *V. cholerae* could produce positive *in silico* PCR bands using all of its strains' genome (in this study eight strains).

Primers designed to amplify the *rtxA* gene of *V. cholerae* have been reported before. According to the previous report, a pair of designed primers, rtxA-F 5'-GGGATACAATGCCCTCTGGCA-3' and rtxA-R 5'-TGGGTTGGCGGTTGGATTTTAC-3', was successfully used to detect *rtxA* of *V. cholerae* (Xu et al., 2019; Rivera, Chun, Huq, Sack, & Colwell, 2001). Despite being able to amplify the targeted genes in most *V. cholerae* strains by showing 977 bp amplicon, the primers were failed to amplify that of *V. cholerae* strain LMA 3984-4. If a pair of primers theoretically failed to amplify a targeted DNA sequence in an *in silico* PCR, then they were most likely unable to amplify the same sequence in an *in vitro* PCR.

In this study, the potential of *rtxA* gene as a DNA biomarker for *V. cholerae* was tested by conducting *in silico* PCR assay. To prove the hypothesis, five pairs of primers were successfully designed using web-based Primer3Plus software using *rtxA* full-length gene sequence of *V. cholerae* O1 biovar El Tor str. N16961. Among five pairs of primers obtained, a new pair of primers (Pair 2 based on Table 1) namely RtxAOF-RtxAOR: '5-CGCAAAACAGTTTCAGCCGA-3' and 5'-AGGTTGGTCTTTTGTGGCCA-3' passed the hairpin and other stability test results based on Primer3Plus output as seen in Figure 4. In addition, the selected newly designed specific primer, the RtxAOF-RtxAOR, was able to selectively amplify the internal part of the *rtxA* gene fragments of all genomic sequences of *V. cholerae*, but not those of other Vibrios. It is important to also underline that the *in silico* PCR assay developed in this study for *rtxA* could identify *rtxA*-producing *V. cholerae* strains, not only from the O1, but also the non-O1 serogroup.

Our results infer that the *rtxA* gene fragment of *V. cholerae* flanked by a pair of primers designed in this study that indicated sequences between QNSFSR and ATKDQPD conserved regions of protein sequences coded by the gene appeared as the biomarker of the bacterium. The *rtxA* gene fragment could differentiate the infectious bacterial species from other Vibrios.

The newly designed RtxAOF-RtxAOR (Pair 2 primer resulted from primer design work of Primer3Plus) is potential to be used as prospective primers to detect the presence of *V. cholerae* in bacterial samples using *in vitro* PCR.

Among limitation of this work is that the performance of the obtained primers is only on a theoretical basis, which means the results are predictive. Visual PCR simulation in this study also only utilized resources accessed from http://insilico.ehu.es/PCR. If an update on the genomic sequence in this study is released, results might slightly change due to the alterations on DNA

templates used for *in silico* PCR. Therefore, if a new genomic DNA sequence of *V. cholerae* strain is added on the database, then consequently new *in silico* PCR assay should be conducted. The aim is to check if the selectivity level of these primers stays the same. Moreover, results from this study could be more meaningful if they are applied for an *in vivo* PCR study. Thus, they could support early detection and identification to control *V. cholerae* as cholera infection agent directly from actual samples.

4. Conclusion

Based on *in silico* PCR assay carried out in this study, the *rtxA* gene was theoretically proved to be a DNA biomarker of the seafood-borne pathogenic bacterium *V. cholerae*. The aforementioned gene could be used to differentiate it from other *Vibrio* species. The newly designed primers are potential to facilitate early rapid diagnosis of cholera infection.

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