[squalen] Submission Acknowledgement

From: Dr. Ekowati Chasanah (squalenbulletin@gmail.com)

To: sn_ethica@yahoo.com

Date: Sunday, 15 December 2019, 10:08 GMT+7

Dr. Stalis Norma Ethica:

Thank you for submitting the manuscript, "Detection of Cholera rtxA Gene as a Biomarker of Seafood-Borne Pathogen Vibrio cholera using In Silico PCR Assay" to Squalen Bulletin of Marine and Fisheries Postharvest and Biotechnology. With the online journal management system that we are using, you will be able to track its progress through the editorial process by logging in to the journal web site:

Manuscript URL:
https://www.bbp4b.litbang.kkp.go.id/squalen-bulletin/index.php/squalen/author/submission/417
Username: norma

If you have any questions, please contact me. Thank you for considering this journal as a venue for your work.

Dr. Ekowati Chasanah
Squalen Bulletin of Marine and Fisheries Postharvest and Biotechnology

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Squalen Bulletin of Marine and Fisheries Postharvest and Biotechnology
http://www.bbp4b.litbang.kkp.go.id/squalen-bulletin

Stalis Norma Ethica <norma@unimus.ac.id>

Manuscript Acceptance #417

3 messages

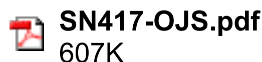
Ms. Merissa Nurasih <squalenbulletin@kkp.go.id> Sun, Aug 23, 2020 at 8:46 PM
To: Stalis Norma Ethica <norma@unimus.ac.id>

Dear Ibu Stalis Norma

We are delighted to inform that article 417, is accepted by editor after reviews. Please kindly proofread your article attached within 2 weeks.

Thank you for your cooperation.

regards
merissa



SN417-OJS.pdf
607K

Stalis Norma Ethica <norma@unimus.ac.id> Sun, Aug 23, 2020 at 9:08 PM
To: "Ms. Merissa Nurasih" <squalenbulletin@kkp.go.id>

Dear Ms. Merissa
Thank you for accepting my article. I have checked all names, institutions and other sections. I don't find any issues that require revision from my part. Also with the content, just maybe the note underneath Table 2 should be written in a smaller font because it should act like footnotes.
I attached the Tabel 2 note that I think the note needs a smaller font.
I hope the publication process goes well

Stalis Norma Ethica
Magister Program of Medical Laboratory Science
Universitas Muhammadiyah Semarang
Semarang, Central Java, Indonesia 50273

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23 Aug - SN417-OJS.pdf
613K

Ms. Merissa Nurasih <squalenbulletin@kkp.go.id> Sun, Aug 23, 2020 at 10:39 PM
To: Stalis Norma Ethica <norma@unimus.ac.id>

Dear Dr. Stalis Norma

Thank you so much in advance, I will forward the points in your email to the editor in chief, for further publication progress of your manuscript.

Regards
Merissa
[Quoted text hidden]

Stalis Norma Ethica <norma@unimus.ac.id>

Manuscript Revision Reminder

5 messages

Ms. Merissa Nurasih <squalenbulletin@gmail.com> Sun, Jul 26, 2020 at 11:15 PM
To: Stalis Norma Ethica <norma@unimus.ac.id>

Dear Author(s),
Dr. Stalis Norma Ethica,

Please remember that the manuscript revision, according to our reviewer suggestions, needs to be submitted before July 31th 2020.

Sincerely,
Merissa

---------- Forwarded message ---------
From: **Ms. Merissa Nurasih** <squalenbulletin@gmail.com>
Date: Mon, 20 Jul 2020 at 10:21
Subject: Manuscript Evaluation
To: Stalis Norma Ethica <norma@unimus.ac.id>

Yth. Dr. Stalis Norma Ethica

Bersama ini disampaikan hasil evaluasi naskah "Detection Of Rtxa Gene As A Biomarker Of Seafood-Borne Pathogen Vibrio Cholera Using In Silico PCR Assay" mohon untuk dapat disesuaikan dan dikembalikan ke sekretariat squalen selambatnya tanggal 31 Juli 2020.

Atas perhatian dan kerjasamanya diucapkan terimakasih.

Salam
Merissa

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Please send your CV to squalenbulletin@kkp.go.id.

Contact us:
Jl. KS Tubun Petamburan VI, Jakarta | INDONESIA 10260 | Tel. +62-21-53650157

3 attachments

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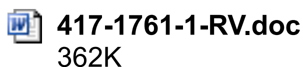
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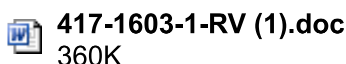
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Stalis Norma Ethica <norma@unimus.ac.id> Mon, Jul 27, 2020 at 5:59 AM
To: "Ms. Merissa Nurasih" <squalenbulletin@gmail.com>

Dear Ms. Merissa,
I am sorry for the delay. Main reason is because there are so many comments from the second reviewer that I find them out of the scope of this study and biases. I have to make as many clarifications and revisions as I could, but at the end, I should focus on the terms set in my study to avoid biases.
I am sending you my response, and within 2 days will send the final revised version combining inputs from both reviewers.

Stalis Norma Ethica
Magister Program of Medical Laboratory Science
Universitas Muhammadiyah Semarang
Semarang, Central Java, Indonesia 50273

[Quoted text hidden]

SNE Combined review for DZ-417.docx
126K

Ms. Merissa Nurasih <squalenbulletin@gmail.com> Mon, Jul 27, 2020 at 10:23 PM
To: Stalis Norma Ethica <norma@unimus.ac.id>

Dear Dr Stalis Norma Ethica

Thank you for your confirmation, we will then wait for your revision within two days as you asked.

Sincerely
Merissa

[Quoted text hidden]

Stalis Norma Ethica <norma@unimus.ac.id> Mon, Jul 27, 2020 at 10:32 PM
To: "Ms. Merissa Nurasih" <squalenbulletin@gmail.com>

Dear Ms. Merissa,
Please find attached my revised article, I have included all revisions based on comments from the 2 reviewers. I have combined them into one revised article to avoid mistakes.
Thank you for your attention.

Regards
Stalis Norma Ethica
Magister Program of Medical Laboratory Science
Universitas Muhammadiyah Semarang
Semarang, Central Java, Indonesia 50273

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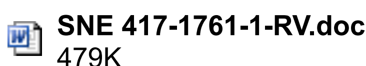
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Detection of *rtxA* Gene as a Biomarker of Seafood-Borne Pathogen *Vibrio cholera* using *In Silico* PCR Assay

A B S T R A C T

Seafood-borne outbreaks caused by *Vibrio cholera* 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 was to use the bacterial DNA biomarker sequence as a tool to facilitate early rapid detection of choleral infection. Five specific pairs of primers were designed from *rtxA* open reading frame DNA of *Vibrio cholerae* O1 biovar El Tor str. N16961 genomic DNA using Primer3Plus. Next, *in silico* PCR (Polymerase Chain Reaction) assay was carried out using the newly designed primers and 25 genomic DNA of vibrios (all species) retrieved from *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 24 other vibrio genomes studied using similar RtxAF-RtxAR primers. Further check showed that the amplicon was truly part of *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 diagnostic of the virulence bacterium using *in silico* PCR assay.

Keywords: Seafood-borne infection, *Vibrio cholera,* DNA biomarker, primer design, *rtxA*

INTRODUCTION

Seafood products play a significant role on the economic market as they are widely consumed all around the world (Bonnin-Jusserand *et al.,* 2019). Per capita, seafood consumption has increased globally during recent decades. Seafood importation and domestic aquaculture farming has also increased. However a number of 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 main threat worldwide and have a great impact on public health and the world's economy (Hwang *et al*., 2018). Seafood-borne infection diseases are a major public health hazard worldwide. Seafood-borne outbreaks caused by the bacterium has 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 *et al*., 2019).

Investigation of seafood-borne illnesses caused by bacteria and viruses require a concrete knowledge about the pathogenicity and virulence properties of the etiologic agents (Elbashir *et al*., 2018; Iwamoto *et al*., 2010). Among poisonous bacteria contaminating seafood, *V. cholera* is the main vibrio species responsible for a 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 *et al.* 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; Shimada *et al*., 1993). Within the *V. cholerae* O1 serogroup there are two well-established biotypes: classical and El Tor. *Vibrio cholerae* strains of the O1 serogroup that typically cause epidemic cholera can be classified into two 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 *et al*., 2010). Both classical and El Tor biotypes are differentiated from each other by a number of properties, including

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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 *et al.*, 1995). *V. cholerae* strains of El Tor biotype initiated the seventh cholera pandemic, which is still continuing. To date, *V. cholerae* O1 strains, which include El Tor biotype has been causing outbreaks worldwide with catastrophic effects.

Among the key virulence factors of *V. cholerae* are the cholera toxin (CTX) genetic element and the repeats in toxin (RTX) cluster (Davis *et al*., 2000). CTX and RTX are the chief virulence gene clusters and are grouped together (Cheng *et al*., 2014). The RTX family of toxins generally produced by several pathogenic Gram-negative bacteria. The RTX toxins represent a family of important virulence factors that have disseminated widely 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 towards 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 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* is therefore necessary to do. In this study, *rtxA*, a member of RTX chief virulence gene cluster, was tested for its potential as DNA biomarkers of *V.cholerae*. Such biomarker is beneficial to facilitate early rapid diagnosis of *V.cholerae* infection using *in vitro* PCR.

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Commented [AL10]: Please the aim should same with abstract which is more general. In this phrase, you already choose rtxA as DNA biomarkers.

MATERIAL AND METHODS

Commented [AL11]: Please write completely and relatively balance between Introduction and Results, so minimalis method. Even you ever write it, please write it the method a little bit detail so the reader who evr read your research result will know, and if they are interest so they will search your publication.

Literature study was initially conducted to obtain information related with the most 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 were targeted. Primers were then designed from internal part of open reading frame of the targeted gene sequences using Primer3Plus at https://primer3plus.com/ (Untergasser *et al*., 2007; Ethica *et al*., 2013; 2014; 2019). Pairs of primers having the least possibility of hairpin formation, self-complementarity, and dimerization were selected (Ethica *et al*., 2017). The newly designed primers were subsequently used as input for the web-based *in silico* PCR run from a web based software at http://insilico.ehu.es/PCR/ using vibrio genomes (all species) stored in the program database (Bikandi *et al*., 2004; San Millán *et al*., 2013; Ethica *et al*., 2019). An analysis was eventually carried out to confirm if the *in silico* PCR products (amplicons) were specific to *V.cholerae* species and were truly part of genes targeted as DNA biomarker.

Commented [AL12]: Where are your database from? E.q. the ACNUC database or the VFDB DNA core dataset

RESULTS AND DISCUSSION

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

Commented [AL13]: Please added Rogers in References

From literature study, it is known that *rtxA* gene, a member of RTX gene cluster, is among distinct genotypic features of infectious bacterium *Vibrio cholerae* (Lin *et al*., 1999). The uniqueness of the gene makes it potential to be targeted as DNA biomarker of *V. cholerae* species. The *rtxA* gene sequence was then retrieved from genbank NCBI (*National Center for Biotechnology Information*) from genome sequence of *Vibrio cholerae* O1 biovar El Tor strain N16961 (NCBI Reference Sequence: NC_002505.1) which encode RTX toxin RtxA [*Vibrio cholerae* O1 biovar El Tor str. N16961] (NCBI Reference Sequence: NP_231094.1). The 3-D structure of cholera RtxA protein (UniProt accession number: Q9KS12) as product of *rtxA* gene is shown in Figure 1.

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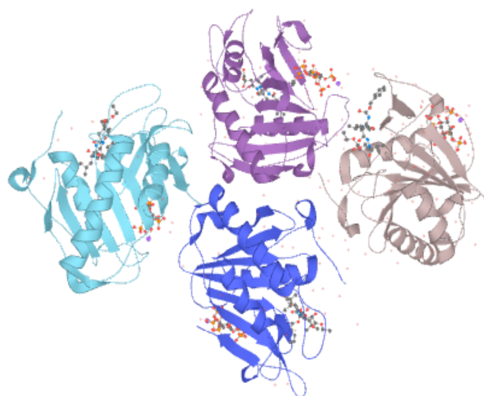

Figure 1. The 3-D structure of cholera RtxA protein (*rtxA* gene product) visualized by Litemol (UniProt Accession Number: Q9KS12)

Using primer3Plus (Untergasser *et al*., 2007), pairs of primers obtained by using *sagC* and *sagD* DNA sequences as input (Figure 1 and 2 respectively) are listed in Table 1.

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

Primer	Forward Primer	Reverse Primer	Amplicon Size (bp)	DNA of *V.cholerae* strains amplified (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

In silico PCR assay using pair 2 primers and genomic DNA of 25 streptococci was run on http://insilico.ehu.es/PCR/. The assay resulted in 8 single 518-bp DNA bands (Figure 2) belonging to *all V.cholerae* or species no 4 to 11 (Table 2, highlighted). According to Table 2, *V.cholerae* species number 4-11 are *Vibrio 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.

Commented [AL14]: Why using 25 Streptococci? I didn't find the explanation of Streptococci in method.
Why from streptococci you identify as Vibrio? Its different Genus. Please explain in method so there is no question in Result.

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Investigation of seafood-borne illnesses caused by norovirus, bacteria and viruses require a concrete knowledge about the pathogenicity and virulence properties of the etiologic agents (Elbashir *et al*., 2018; Iwamoto *et al*., 2010). Among poisonous bacteria contaminating seafood, *V. cholera* is the main vibrio species responsible for a 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 *et al.* 2015).

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MATERIAL AND METHODS

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Commented [MA1]: This statement is not clear, specify what input file to use?

RESULTS AND DISCUSSION

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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 template using pair 2 primer designed in this study.

Primers designed to amplify *rtxA* gene of *V.cholerae* had actually been reported before. According to the previous report, a pair of designed primers., rtxA-F 5'-GGGATACAATGCCCTCTGGCA-3' rtxA-R 5'-TGGGTTGGCGGTTGGATTTTAC-3', was successfully used to detect *rtxA* of *V.cholerae* (Xu et al., 2019; Rivera et al., 2001). However, when tested using *in silico* PCR in this study, the primers failed to amplify *rtxA* gene of *cholerae* strain LMA3984-4 although it could result in 977 bp amplicon with that of other *cholerae* strains. If a pair of primers theoretically failed to amplify a targeted DNA sequence in an *in silico* PCR, then they are most likely failed to amplify the same sequence in an *in vitro* PCR.

In this study, the potential of *rtxA* gene as DNA a DNA biomarker for *V. cholera* was tested by conducting *in silico PCR assay*. To prove the hypothesis, 5 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 5 pairs of primers obtained, a new pair of primers (pair 1 based on Table 1) named as RtxAOF-RtxAOR: '5-CGCAAAACAGTTTCAGCCGA-3' and 5'-AGGTTGGTCTTTTGTGGCCA-3'was selected based on hairpin and other self complementarity test results. In addition, the selected newly designed specific primer, the RtxAOF-RtxAOR, was able to selectively amplify internal part of *rtxA* gene fragments of all genomic sequences of *V. cholera*, but not 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:

1. The *rtxA* gene is a biomarkers for *Vibrio cholera*, which differentiate the infectious bacterial species from other vibrios. As a bacterial biomarker, rtxA gene is potentially beneficial to facilitate early rapid diagnosis choleral infection.
2. The newly designed RtxAOF-RtxAOR is potential to be used as prospective primers to detect the presence of *V. cholera* in bacterial samples using *in vitro* PCR.

CONCLUSION

Based on *in silico* PCR assay carried out ini study, *rtxA* gene was theoretically proved to be DNA biomarker of the seafood-borne pathogenic bacterium *Vibrio cholera*, which could be used to differentiate it from other vibrio species. The newly designed primers designed from the gene bear the potential as *in vitro* PCR components to facilitate early rapid diagnosis of choleral infection.

Commented [MA6]: This explanation is technical, please explain the substantive meaning of the process carried out at this stage. If it's only proof that it's part of the rtxA gene, then that's for sure because the primer sequence is taken from the rtxA gene, right?

Commented [MA7]: This figure only shows part of the V.cholerae genome sequence, not showing that the primer sequence is part of the V.cholerae genome sequence.

Commented [MA8]: Pair 1 or pair 2?

Commented [MA9]: Which data shows that this primer has better hairpin and self complementarity test results than others.

Commented [MA10]: Actually, the biomarker is the rtxA gene or pair 2 primer designed?. If the intended biomarker is the rtxA gene, please prove the difference between the rtxA gene Vibrio cholera and other Vibrio based on the results of the alignment.

Commented [MA11]: Which primer? pair 2? please mention.