Canadian Journal of Microbiology

Loop-Mediated Isothermal Amplification (LAMP) Assay for Rapid Detection of viable but non-culturable *Vibrio cholerae* O1

Journal:	<i>Canadian Journal of Microbiology</i>
Manuscript ID	cjm-2021-0142.R1
Manuscript Type:	Article
Date Submitted by the Author:	22-Oct-2021
Complete List of Authors:	Chamanrokh, Parastoo; University of Maryland College Park, Maryland Pathogen Research Institute Colwell, Rita; University of Maryland at College Park, Maryland Pathogen Research Institute; University of Maryland at College Park, Maryland Institute of Applied Environmental Health; University of Maryland at College Park, CBCB. UMIACS; Johns Hopkins University Bloomberg School of Public Health Huq, Anwar; University of Maryland at College Park, Maryland Pathogen Research Institute
Keyword:	LAMP, PCR, <i>Vibrio cholerae</i> O1, VBNC, coccoid
Is the invited manuscript for consideration in a Special Issue? :	Not applicable (regular submission)

SCHOLARONE™
Manuscripts

Loop-Mediated Isothermal Amplification (LAMP) Assay for Rapid Detection of viable but non-culturable *Vibrio cholerae* O1

Parastoo Chamanrokh¹, Rita R. Colwell^{1,2,3,4} and Anwar Huq^{*1,2}

¹ Maryland Pathogen Research Institute, University of Maryland College Park, MD, USA

² Maryland Institute of Applied Environmental Health, University of Maryland, College Park, MD, USA

³ Center of Bioinformatics and Computational Biology, University of Maryland Institute of Advanced Computer Studies, University of Maryland, College Park, MD, USA

⁴ Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA

Abstract

Vibrio cholerae, an important waterborne pathogen, is a rod-shaped bacterium that naturally exists in aquatic environments. When conditions are unfavorable for growth, the bacterium can undergo morphological and physiological changes to assume a coccoid morphology. This stage in its life cycle is referred to as viable but non-culturable (VBNC) since VBNC cells do not grow on conventional bacteriological culture media. The current study compared polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) to detect and identify VBNC *V. cholerae*. Because it is difficult to detect and identify VBNC *V. cholerae*, the results of the current study are useful in showing LAMP to be more sensitive and rapid than PCR in detecting and identifying non-culturable, coccoid forms of *V. cholerae*. Furthermore, the LAMP method is effective in detecting and identifying very low numbers of coccoid VBNC *V. cholerae* in environmental water samples, with the added benefit of being inexpensive to perform.

Keywords: LAMP; PCR; *Vibrio cholerae* O1; VBNC; coccoid

Word Count:

Correspondence:

Anwar Huq

huq@umd.edu

PH: 301-405-7428

Introduction

Vibrio cholerae O1, an important waterborne pathogen, is the causative agent of epidemic cholera, a severe life-threatening gastrointestinal infection typically transmitted via contaminated water (Baker-Austin 2018; Colwell 1996).

In the aquatic environment, this bacterium can transform from curved bacillus to coccoid morphology, the latter unable to grow in the laboratory on standard culture media and commonly referred to as viable but non-culturable (VBNC). Detection, identification, and quantification of *V. cholerae* in environmental samples are important to determine the health risk of cholera (Chaiyanan 2007; Mazaheri Assadi 2015; Sack 2004; Colwell 1996). More than 200 serotypes of *V. cholerae* have been reported, with *V. cholerae* serotypes O1 and O139 associated with epidemic and pandemic cholera. It is estimated that 2.86 million cases of cholera occur in cholera endemic countries, with 95,000 deaths recorded annually (Ali et al. 2015).

The VBNC form of *V. cholerae* O1 has been found to be capable of causing cholera (Colwell et al. 1996). Furthermore, coccoid *V. cholerae* O1 are common in the natural environment (Sultana et al. 2018; Alam et al. 2007; Huq et al. 2012). Conventional culture methods are ineffective when bacterial cells have entered into the VBNC state. Thus, detection and identification of VBNC *V. cholerae* in environmental water samples are important and require a method that is both reliable and accurate. Traditional methods for detection and enumeration of *V. cholerae* generally employ either culture or conventional biochemical and immunological assays, both of which are time-consuming and laborious, requiring at least three days to accomplish. It is important to note that direct plating of environmental samples on agar media allows enumeration only of the culturable population, as opposed to results obtained employing the fluorescent antibody direct viable counting method (DFA-DVC). The latter allows detection and

enumeration of both culturable and VBNC *V. cholerae* (Bliem 2015; Alam 2010; Huq 2006; Alam 2006; Ceccarelli 2015; Roszak 1987).

Results of laboratory studies have shown that detection and identification of *V. cholerae* inoculated into water samples are best achieved by employing loop-mediated isothermal amplification (LAMP) versus PCR. Several PCR assays have been designed and routinely employed to detect and identify *V. cholerae* in clinical and environmental samples, providing relatively rapid results for *V. cholerae*, compared to relatively long incubation required for conventional culture (Hoshino et al. 1998; Shirai et al. 1991). However, PCR employs gel electrophoresis to detect amplified products, a process both time-consuming and tedious. The real-time PCR assay is not routinely employed for rapid detection and identification of *V. cholerae* in the field, even though it is more sensitive and less time-consuming than culture, since it requires a thermal cycler and fluorescence detector—both of which are expensive and not field adaptable (Lyon 2001). Also, primers of RT-PCR have many pitfalls (Wootton and Federhen 1996). Taking into consideration the limitations of commonly used methods for *V. cholerae* detection and enumeration, LAMP is concluded to offer important advantages (Mazaheri Assadi et al. 2015; Nagamine et al. 2002). LAMP employs nucleic acid amplification and is based on the principle of auto-cycling strand displacement by DNA synthesis, using the Bst DNA polymerase fragment (Mazaheri Assadi et al. 2015; Notomi et al. 2000).

Genes coding for cholera toxin (CT), a significant virulence factor of *V. cholerae*, and genes coding for *V. cholerae* O antigen offer useful targets for specific detection and identification of toxigenic *V. cholerae* O1. In addition, useful targets for toxigenic *V. cholerae* detection are the genes coding for biosynthesis of serotype-specific determinants, located in the *rfb* region of the *V. cholerae* chromosome (Hoshino et al. 1998). The *ompW* sequence is highly conserved among

V. cholerae of various biotypes and/or serogroups and has proven useful for species-specific identification of *V. cholerae* (Colwell 1991; Senoh et al. 2014; Srisuk et al. 2010). By incorporating these specific gene targets, the LAMP test was developed and used to detect *V. cholerae* effectively and efficiently. LAMP is simple to run and cost-effective compared to PCR and RT-PCR. Furthermore, the LAMP method is specific, sensitive, and simple to use to detect VBNC coccoid *V. cholerae* in contaminated water and aquatic products.

Materials and Methods

Culture and DNA extraction methods for V. cholerae strains used in this study

V. cholerae O1 N16961 (ATCC 39315) and *V. cholerae* O1 NIH 1823 (original ID NHCM-0055) were employed as controls for both PCR and LAMP. The *V. cholerae* strains were individually transferred from laboratory stock cultures stored at -80°C to Luria-Bertani (LB) and thiosulfate-citrate-bile salts-sucrose (TCBS) agar (DIFCO Labs), respectively, and incubated at 37°C for 16–18 h. Overnight enrichment broth cultures were plated and single colonies of each strain were individually transferred to alkaline peptone water (APW) adjusted to pH 8.6. Aliquots taken from the microaerophilic surface pellicle layer of the APW were inoculated onto TCBS agar plates and incubated at 37°C for 16–18 h for further analysis (Huq et al. 2012).

Genomic DNA Preparation

Genomic DNA was extracted by thermal lysis, as described previously (Okada et al. 2010). Bacterial genomic DNA from an overnight culture was extracted using the QIAamp DNA mini kit (Cat No: 51306 Qiagen), following manufacturer's instructions, with chemical lysis in buffer

containing chaotropic salt, enzymatic digestion by treatment with proteinase K, and thermal lysis. DNA is bound to a silica-gel membrane in a spin-column, and PCR inhibitors are not retained. The DNA template was used for detection of *V. cholerae* in water samples. Extracted DNA samples were analyzed by agarose gel electrophoresis and visualized under short-wave length UV light (260 nm).

Optimization of Multiplex PCR using *CtxA/rfbN* and *ompW* gene probes

Multiplex PCR was used to detect *rfb* sequences specific to *V. cholerae* O1 serogroup and *ctxA* (Hoshino et al. 1998; Nahar et al. 2011) (Table 2). *Ca.* 25 µL of PCR mixture contained 12.5µl of GoTaq® Green Master Mix (Promega); 1 µl from each primer (F*ctxA* = VCT1, R*ctxA* = VCT2, F-*rfbN*=O1F2-1, R-*rfbN* = O1R2-2); 3.5 µl of nuclease-free water; and 5 µL Target DNA.

Table 2 The *ctxA*, *rfbN*, and *ompW* PCR primers employed in this study as listed follows.

Sequence (5'.....3')	Primer	bp
5' ACAGAGTGAGTACTTTGACC 3'	F- <i>ctxA</i> (VCT1)	308bp
5' ATACCATCCATATATTTGGGAG 3'	R- <i>ctxA</i> (VCT2)	
5'GTTTCACTGAACAGATGGG3'	F- <i>rfbN</i> (O1F2-1)	192bp
5'GGTCATCTGTAAGTACAAC3'	R- <i>rfbN</i> (O1R2-2)	
5' CACCAAGAAGGTGACTTTATTGTG3'	F- <i>ompW</i>	588bp
5' GAACTTATAACCAACCCGCG3'	R- <i>ompW</i>	

Amplification conditions were as follows: 5 min at 94°C for initial denaturation of the DNA and 35 cycles, each lasting for 30 seconds at 94°C, at 55°C, and at 72°C, with a final round of extension for 10 min at 72°C, using the BIO-RAD T100™ Thermal Cycler (Bio-Rad, Inc., Hercules, California). The PCR product was separated by electrophoresis on 2% agarose gel and observed under UV, after staining with 0.5 µg/ml ethidium bromide.

PCR was used to detect outer membrane protein ompW sequences (Table 2). *Ca.* 25 µL of the PCR mixture contained: 12.5 µl of GoTaq® Green Master Mix (Promega); 1 µl from each primer (F-ompW and R-ompW); 5.5 µl nuclease-free water; and 5 µL Target DNA. Amplification conditions included 3 min at 95°C for initial DNA denaturation and 35 cycles, each for 30 seconds at 95°C and at 64°C respectively, 1 minute at 72°C, and a final round of extension for 5 min at 72°C, using the BIO-RAD T100™ Thermal Cycler (Nandi et al. 2000). The PCR product was separated by electrophoresis, employing 2% agarose gel, and visualized under UV, after staining with 0.5 µg/ml ethidium bromide.

PCR sensitivity and specificity

Suspensions of fresh cultures of *V. cholerae* were adjusted to 0.5 McFarland standard 1.5×10^8 CFU/ml, OD = 600 nm, and DNA extracted using the QIAamp DNA Mini Kit (Cat No: 51306 Qiagen). Serial dilutions of extracted DNA were prepared to obtain a range of 10^6 to 5 copies of DNA. Specificity was determined using DNA extracted from control strains, including *Salmonella enterica* CP1094, *Escherichia coli* CP 1120, *Shigella flexneri* CP1129, *Klebsiella pneumoniae* CP1170, and *Vibrio mimicus* MB-451.

Optimization of LAMP

LAMP primers were ctxA (Yamazaki et al. 2008), outer membrane genes (Srisuk , et al. 2010), and rfbN genes, and were designed using Primer Explorer V4 software (<http://primerexplorer.jp/e/>) (Table 3).

Table 3 LAMP assay primers employing ctxA, rfbN, and ompW employed in this study as listed follows.

LAMP Primers	Sequence (5'.....3')
FIP (ctxA)	TCT GTC CTC TTG GCA TAA GAC GCA GAT TCT AGA CCT CCT G
BIP (ctxA)	TCA ACC TTT ATG ATC ATG CAA GAG GCT CAA ACT AAT TGA GGT GGA A
F3 (ctxA)	GCA AAT GAT GAT AAG TTA TAT CGG
B3 (ctxA)	GMC CAG ACA ATA TAG TTT GAC C
LF (ctxA)	CAC CTG ACT GCT TTA TTT CA
LB (ctxA)	AAC TCA GAC GGG ATT TGT TAG G
F3 (ompW)	CGGTAGTACCTAATGACAGTAG
B3 (ompW)	GCAAATGTTTTGTTTCACCAAT
FIP (ompW)	CAAGCGTTAACCCTAAGTGGGTATTTTTTTAAAGTGTTAAACACTCAAA GTGAG
BIP (ompW)	ACATCAGTTTTGAAGTCCTCGCTTTTTATCACCAAGGCTACCTAAC
LB (ompW)	ACATAAGATTCTACCTCTGGTGGT
F3 (rfbN)	ATACGTGGGTAGCGTTGT
B3 (rfbN)	GGCTGCAATAACAAAAAGAGAA
FIP (rfbN)	GATCCCCAGTGATGTATACAAGAAAATGCAATCAAAAAGCTTCACA
BIP (rfbN)	CTGAATGCCCGTCTGAGAAATCGACGGTCAGGTTAAGGAA
LF (rfbN)	ACCGTGTTGAAGTTTCTGAACA
LB (rfbN)	ACATGCAAGTTGGCAATGATGCA

Ca. 25 µL of LAMP reaction solution contained 5.2 µL nuclease-free water; 4 µL betaine (5 M); 3.5 µL dNTP (10 mM); 2.5 µL 10X buffer; 1.8 µL MgSo4 (100 mM); 1 µL Mix I; 1 µL Mix II; 1 µL Bst DNA polymerase enzyme (8,000 U/ml; Lucigen); and 5 µL target DNA, to the final volume of 25 µL. Mix I contained 5µL F3, 5µL B3, 40 µL FIP, 40 µL BIP primer (1.6 µM), and 10 µL nuclease free water, to a total volume of 100 µL. Mix II contained 20 µL LF and 20 µL LB primer (0.8 µM), and 60 µL nuclease free water.

Determination of Specificity and Sensitivity of the LAMP Method

To determine the specificity of LAMP, DNA was extracted from *Salmonella enterica* CP1094, *Escherichia coli* CP 1120, *Shigella flexneri* CP1129, *Klebsiella pneumonia* CP1170, and *Vibrio mimicus* MB-451 and compared with DNA extracted from *V. cholerae* N16961. Amplification of the thermal profile of *ctxA*, *rfbN*, and *ompW* genes was achieved, using a simple block heater, for each of the six bacterial species. After preparation of serial dilutions of the LAMP samples, 0.1% SYBR green was added to each tube and the reaction was observed under 302 nm ultra-violet. Positive tubes fluoresced green, with orange color signifying negative reaction.

Seawater Microcosms of VBNC *V. cholerae*

Single colonies of *V. cholerae* O1 N16961 and O1 NIH 1823 were picked from Luria-Bertani (LB) agar culture plates, transferred to 100 ml of LB broth, and incubated for 18 hours at 35°C. Logarithmic phase cells were harvested from 1 liter of LB broth, incubated for 6 hours at 35°C, and centrifuged at 5000x g for 20 min at 4°C. The cells were washed twice in artificial seawater [Instant Ocean (IO)] and resuspended in 250 ml autoclaved and filtered (0.22 µm) Instant Ocean adjusted to 1% salinity and incubated at 25°C for 18 h to shift the cells to starvation state (Huq et al. 1996).

To induce the VBNC state, flasks containing cell suspensions each of *V. cholerae* N16961 and *V. cholerae* 1823 (1.5×10^8 CFU/ml cells/mL measured via spectrophotometer) were maintained at 4°C for four months (both *V. cholerae* N16961 and 1823) and at 37°C for one year (both *V. cholerae* N16961 and 1823) until cells in the microcosms did not produce colonies on LB and/or TCBS agar.

Sequential filtration was used to collect coccoid VBNC cells from each of the microcosm flasks by first filtering through a 0.45 µm filter, followed by filtration with 0.22 µm pore size

membrane filter. Cells were confirmed present and VBNC by DFA-DVC staining (Chaiyanan et al. 2001).

Cocoid VBNC cells in freshwater

Polypropylene-sterilized bottles were used to collect and transport water samples from the field to the laboratory for processing. Five sample replicates were collected from five locations—Paint Branch, Northwest Branch, Lake Artemesia, Sligo Creek, and the Potomac River in Maryland, USA. A total of 60 samples were divided into 20 replicates of 30 ml autoclaved water; 20 replicates of 30 ml of filter-sterilized (0.22 µM membrane) water, and 20 replicates of 30 ml of untreated water. For each of the 20 replicates of the three water types, *V. cholerae* N16961 was added to one set of water samples (10 tubes) and *V. cholerae* 1823 was added to the remaining set. Each environmental water sample was divided into two groups: one stored at 4°C (total of 30 tubes) and the other at 37°C (30 tubes) After incubation at the respective temperature for four months and one year, respectively, two aliquots of 100 µl from each water sample were streaked on TCBS agar to confirm absence of growth and examined by DFA-DVC to confirm presence of VBNC cells.

Culture and DFA-DVC count

A total of 64 samples, including four microcosms and 60 environmental water samples, was prepared for each strain and tested for culturability. Samples were cultured each week on TCBS agar to enumerate VBNC cells using DFA-DVC (Huq et al. 2012). Viable cells examined by DFA-DVC elongated in the presence of yeast extract and nalidixic acid. Degree of elongation was observed by comparison with non-VBNC DFA-stained cells, i.e., actively growing cultures of *V. cholerae* (Chaiyanan et al. 2001; Nahar et al. 2011).

DNA extraction of VBNC cells for PCR and LAMP Tests

VBNC cells from both microcosms (4 flasks) and inoculated environmental water samples (60 tubes) were obtained by filtration, first by filtering through 0.45 µm filters, followed by filtration using 0.22 µm filters. Cells collected on 0.22 µm filters were centrifuged and washed twice with 1% IO until UV absorption in the supernatant was undetectable (indicating no free nucleic acid) and chromosomal DNA extraction was done using the PowerSoil DNA Isolation Kit (Cat No. 12888-100) (Chaiyanan et al. 2001). PCR and LAMP assays targeting *ctxA*, *ompW*, and *rfbN* were performed in parallel. PCR and LAMP methods were employed as previously described.

Results

The viability of non-culturable cells from microcosms incubated at 4°C and 37°C was determined by DFA-DVC (Fig. 1a). Actively growing cells of *V. cholerae* demonstrate a distinctive curved rod shape, i.e. vibrio morphology, and are readily culturable. However, after incubation at 4°C and 37°C for one year, the cells had entered the VBNC state and did not grow on TCBS or LB agar. Coccoid VBNC cells were, however, detectable by DFA-DVC (Chaiyanan et al. 2001), even in one-year-old microcosms showing no growth on culture media (Fig. 1b). DVC-DFA cells were confirmed using PCR and LAMP.

Optimization of multiplex PCR and LAMP

The *ctxA* (308 base pairs), *rfbN* (192 base pairs), and *ompW* (588 base pairs) amplicons were detected by PCR, employing 2% agarose (Fig. 2. a and b). LAMP was optimized for *ctxA* after 1 hour at 65°C, *ompW* after 75 min at 65°C, and *rfbN* after 1 hour at 66°C.

Specificity and sensitivity of PCR using ctxA/rfbN and ompW

The sensitivity of PCR was determined using serial dilutions of *V. cholerae* N16961 and 1823 DNA. The sensitivity of *V. cholerae* N16961*ctxA/rfbN* was 10 copies and *V. cholerae* 1823 *ctxA/rfbN* 100 copies of DNA (Fig. 3a). PCR proved highly specific (100%) for *V. cholerae*

(Fig. 3.b). Similarly, *V. cholerae* N16961 and 1823 ompW sensitivity was 10 copies of DNA (100% specificity).

Specificity and sensitivity of LAMP using *ctxA*, *rfbN*, and *ompW*

The LAMP reaction was conducted using dilutions of *V. cholerae* DNA extracted after incubation for one hour at 66°C. Sensitivity for *V. cholerae* N16961 *ctxA* proved sufficient, with amplification providing positive results with as few as five copies of DNA, twice as sensitive as PCR. *V. cholerae* 1823 *ctxA* was positive with 10 copies—10 fold more sensitive than PCR, with green color observed even when so few as five or more copies were detected. LAMP provided 100% specificity (Figs. 4 a and b). A positive reaction for *rfbN* was observed with 10 copies of *V. cholerae* N16961 and 1823, hence 10 fold more sensitive than PCR. LAMP sensitivity with *ompW* was five copies, two-fold more sensitive than PCR and 100% specificity. Table 1 lists variables for the PCR and LAMP tests (*ctxA*, *rfbN*, and *ompW* genes) with cells that had been incubated for one year at 4°C and 37°C.

Table 1 PCR and LAMP results for *ctxA*, *rfbN*, and *ompW* after incubation of *V. cholerae* for one year at 4°C and 37°C.

Temperature	<i>ctxA</i> (PCR)	<i>ctxA</i> (LAMP)	<i>rfbN</i> (PCR)	<i>rfbN</i> (LAMP)	<i>ompW</i> (PCR)	<i>ompW</i> (LAMP)
37°C	81%	87%	87%	87%	78%	93%
4°C	72%	81%	72%	81%	75%	93%

Discussion

Conventional culture methods are ineffective when bacterial cells have entered into the VBNC state. Fluorescent monoclonal antibody staining that is routinely employed in microbial ecology studies was successful in detecting viable cells of *V. cholerae* O1 and O139 even when non

culturable (Chowdhury 1995; Pruzzo et al. 2003) and was able to demonstrate persistence of adhesive properties in *V. cholerae* after long-term exposure to seawater, indicating such properties are retained by VBNC cells. Traditional methods used to characterize bacteria include culture and biochemical tests which are time-consuming, relatively expensive, often difficult to perform, and usually labor-intensive (Huq et al. 2006). To overcome these limitations, molecular approaches, notably those employing DNA amplification by PCR, were developed to detect *V. cholerae* in environmental water samples, offering a more sensitive and specific methodology (Huq et al. 2006; Kumar et al. 2010). However, PCR has limitations, mainly requiring an expensive thermocycler for thermo-cycling, a procedure requiring at least three hours for a complete assay. Additionally, *V. cholerae* transforms from rod to coccoid morphology (VBNC) in the aquatic environment making its detection difficult (Chamanrokh et al. 2015; Huq et al. 2006). It should be pointed out that VBNC *V. cholerae* retain potential pathogenicity, hence continue to be a health risk (Colwell et al. 1985). Development of quick, sensitive, and low-cost methods is needed for prompt detection and prevention of disease (Okada et al. 2010).

In contrast to PCR, LAMP can be carried out within an hour and does not require a fluorescent probe or expensive equipment. Another advantage is that LAMP obviates the necessity of DNA denaturation (Chamanrokh et al. 2015; Minami et al. 2006).

Various LAMP assays have been developed for detection and identification of *V. cholerae* and for a variety of other water and foodborne pathogens (Sirsuk et al. 2010). Yamazaki and team reported rapid and sensitive detection of *V. cholerae* using LAMP by targeting the outer membrane protein coding gene *ompW*. Their results showed LAMP to be precise, accurate, and useful for detection of *V. cholerae* (Yamazaki et al. 2008), notably using LAMP to detect cholera toxin-producing *V. cholerae* in fecal samples and achieving approximately five times greater

sensitivity than PCR. Thus, LAMP can be considered a powerful tool for rapid, simple, and sensitive detection of CT-producing *V. cholerae*, with potential to facilitate detection of *V. cholerae* in seafood, and early diagnosis of cholera in humans. Syafirah et al. (2018) developed an ambient temperature stable, ready-to-use LAMP to detect toxigenic *V. cholerae* in low-resource settings (Wang et al. 2008). In addition, LAMP proved more specific and sensitive than PCR based assays for detection of foodborne pathogens. Based on those findings, Bavisetty et al. (2018) proposed a rapid pathogen detection LAMP for seafood safety testing. Thus, LAMP has achieved wide recognition (Law et al. 2015). Law et al. (2016) compared a variety of rapid methods used for detecting foodborne bacterial pathogens, and concluded that LAMP is clearly an important method for rapid detection and control of spread of foodborne pathogens and prevention of foodborne disease outbreaks. Xu et al. (2019) developed their LAMP method and compared it with PCR assay, finding LAMP detected the virulence genes *hapA*, *mshA*, *pilA* and *tlh*, and also the toxigenic genes *ctxA* and *tcpA*, as well as the species-specific gene *lolB* of *V. cholerae*.

These earlier studies referenced here, however, did not investigate sensitivity and specificity of detection of VBNC *V. cholerae* O1. Here we report LAMP is very effective in detecting coccoid VBNC *V. cholerae* in environmental water samples. Moreover, compared to conventional PCR, it is inexpensive, accurate, and rapid in detecting and identifying coccoid VBNC *V. cholerae* O1 in natural water samples, with the significant benefit of being field applicable.

Acknowledgments

The authors gratefully acknowledge funding provided by the National Institute of Health (NIH) Grant No. 2R01A1039129-11A2.

Conflict of interest: The authors declare that the research has been conducted in the absence of any commercial or financial relationships that can be construed as a potential conflict of interest.

References

- Alam, M., Sadique, A., Nur-A-Hasan Bhuiyan, N.A., Nair, G.B., Siddique, A.K., Sack, D.A., Ahsan, S., Huq, A., et al. 2006. Effect of transport at ambient temperature on detection and isolation of *Vibrio cholerae* from environmental samples. *Applied and Environmental Microbiology*, **72**(3): 2185-2190. <https://doi.org/10.1128/AEM.72.3.2185-2190.2006>.
- Alam, M., Sultana, M., Nair, G.B., Siddique, A.K., Hasan, N.A., Sack, R.B., Sack, D.A., Ahmed, K.U., et al. 2007. Viable but nonculturable *Vibrio cholerae* O1 in biofilms in the aquatic environment and their role in cholera transmission. *Proceedings of the National Academy of Sciences*, **104**(45): 17801-17806. <https://doi.org/10.1073/pnas.0705599104>.
- Alam, M., Hasan, N.A., Sultana, M., Nair, G.B., Sadique, A., Faruque, A.S.G., Endtz, H.P., Sack, R.B., et al. 2010. Diagnostic limitations to accurate diagnosis of cholera. *Journal of Clinical Microbiology*, **48**(11): 3918-3922. <https://doi.org/10.1128/JCM.00616-10>.
- Ali, M., Nelson, A.R., Lopez, A.L., and Sack, D.A. 2015. Updated global burden of cholera in endemic countries. *PLoS Neglected Tropical Diseases*, **9**(6), p.e0003832. <https://doi.org/10.1371/journal.pntd.0003832>.
- Baker-Austin, C., Oliver, J.D., Alam, M., Ali, A., Waldor, M.K., Qadri, F., and Martinez-Urtaza, J. 2018. *Vibrio* spp. infections. *Nature Reviews Disease Primers*, **4**(1): 1-19.
- Bavisetty, S.C.B., Vu, H.T.K., Benjakul, S., and Vongkamjan, K. 2018. Rapid pathogen detection tools in seafood safety. *Current Opinion in Food Science*, **20**: 92-99.

- Ceccarelli, D., Chen, A., Hasan, N.A., Rashed, S.M., Huq, A., and Colwell, R.R. 2015. Non-O1/non-O139 *Vibrio cholerae* carrying multiple virulence factors and *V. cholerae* O1 in the Chesapeake Bay, Maryland. *Applied and Environmental Microbiology*, **81**(6): 1909-1918.
- Chaiyanan, S., Chaiyanan, S., Huq, A., Mangel, T. and Colwell, R.R. 2001. Viability of the nonculturable *Vibrio cholerae* O1 and O139. *Systematic and Applied Microbiology*, **24**(3): 331-341. <https://doi.org/10.1078/0723-2020-00032>.
- Chaiyanan, S., Chaiyanan, S., Grim, C., Mangel, T., Huq, A. and Colwell, R.R. 2007. Ultrastructure of coccoid viable but non-culturable *Vibrio cholerae*. *Environmental Microbiology*, **9**(2): 393-402. <https://doi.org/10.1111/j.1462-2920.2006.01150.x>.
- Chamanrokh, P., Shahhosseiny, M.H., Assadi, M.M., Nejdassattari, T. and Esmaili, D. 2015. Three tests used to identify non-culturable form of *Helicobacter pylori* in water samples. *Jundishapur Journal of Microbiology*, **8**(4): e16811. [https://doi.org/10.5812/jjm.8\(4\)2015.16811](https://doi.org/10.5812/jjm.8(4)2015.16811).
- Chowdhury, M.A.R., Xu, B., Montilla, R., Hasan, J.A.K., Huq, A. and Colwell, R.R. 1995. A simplified immunofluorescence technique for detection of viable cells of *Vibrio cholerae* O1 and O139. *Journal of Microbiological Methods*, **24**(2): 165-170.
- Colwell, R. 1991. Viable but non-culturable bacteria in the aquatic environment. *Culture* **12**: 1-4.
- Colwell, R.R. 1996. Global climate and infectious disease: the cholera paradigm. *Science*, **274**(5295): 2025-2031.
- Colwell, R., Brayton, P., Herrington, D., Tall, B., Huq, A., and Levine, M. 1996. Viable but non-culturable *Vibrio cholerae* O1 revert to a cultivable state in the human intestine. *World Journal of Microbiology and Biotechnology*, **12**(1): 28-31.

- Colwell, R.R., Brayton, P.R., Grimes, D.J., Roszak, D.B., Huq, S.A., and Palmer, L.M. 1985. Viable but Non-Culturable *Vibrio-Cholerae* and Related Pathogens in the Environment - Implications for Release of Genetically Engineered Microorganisms. *Bio-Technology*, **3**(9): 817-820. <https://doi.org/10.1038/nbt0985-817>.
- Hoshino, K., Yamasaki, S., Mukhopadhyay, A.K., Chakraborty, S., Basu, A., Bhattacharya, S.K., Nair, G.B., Shimada, T., et al. 1998. Development and evaluation of a multiplex PCR assay for rapid detection of toxigenic *Vibrio cholerae* O1 and O139. *Fems Immunology and Medical Microbiology*, **20**(3): 201-207. <https://doi.org/10.1111/j.1574-695X.1998.tb01128.x>.
- Huq, A., Grim, C., Colwell, R.R., and Nair, G.B. 2006. Detection, isolation, and identification of *Vibrio cholerae* from the environment [Research Support, N.I.H., Extramural Research Support, U.S. Gov't, Non-P.H.S.]. *Current protocols in microbiology* Chapter 6: Unit6A 5. <https://doi.org/10.1002/9780471729259.mc06a05s02>.
- Huq, A., Xu, B., Chowdhury, M., Islam, M.S., Montilla, R., and Colwell, R.R. 1996. A simple filtration method to remove plankton-associated *Vibrio cholerae* in raw water supplies in developing countries. *Applied and Environmental Microbiology*, **62**(7): 2508-2512.
- Huq, A., Haley, B.J., Taviani, E., Chen, A., Hasan, N.A. and Colwell, R.R. 2012. Detection, isolation, and identification of *Vibrio cholerae* from the environment. *Current Protocols in Microbiology*, **26**(1): 6A-5. <https://doi.org/10.1002/9780471729259.mc06a05s26>.
- Kumar, P., Peter, W.A. and Thomas, S. 2010. Rapid detection of virulence-associated genes in environmental strains of *Vibrio cholerae* by multiplex PCR. *Current Microbiology*, **60**(3): 199-202. <https://doi.org/10.1007/s00284-009-9524-6>.

- Law, J.W.F., Ab Mutalib, N.S., Chan, K.G., and Lee, L.H. 2015. Rapid methods for the detection of foodborne bacterial pathogens: principles, applications, advantages and limitations. *Frontiers in Microbiology*, **5**: 770.
- Law, J.W.F., Letchumanan, V., Chan, K.G., Goh, B.H. and Lee, L.H. 2016. Insights into detection and identification of foodborne pathogens. *Food Borne Pathogens and Antibiotic Resistance*.
- Lyon, W.J. 2001. TaqMan PCR for detection of *Vibrio cholerae* O1, O139, non-O1, and non-O139 in pure cultures, raw oysters, and synthetic seawater. *Applied and Environmental Microbiology*, **67**(10): 4685-4693. <https://doi.org/10.1128/AEM.67.10.4685-4693.2001>
- Mazaheri Assadi, M., Chamanrokh, P., Whitehouse, C.A., and Huq, A. 2015. Methods for Detecting the Environmental Coccoid Form of *Helicobacter pylori*. *Front Public Health*, **3**: 147. <https://doi.org/10.3389/fpubh.2015.00147>.
- Minami, M., Ohta, M., Ohkura, T., Ando, T., Torii, K., Hasegawa, T. and Goto, H. 2006. Use of a combination of brushing technique and the loop-mediated isothermal amplification method as a novel, rapid, and safe system for detection of *Helicobacter pylori*. *Journal of Clinical Microbiology*, **44**(11): 4032-4037. <https://doi.org/10.1128/JCM.00898-06>.
- Nagamine, K., Hase, T. and Notomi, T.J.M.C.P. 2002. Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Molecular and Cellular Probes*, **16**(3): 223-229. <https://doi.org/10.1006/mcpr.2002.0415>.
- Sultana, M., Nahar, S., Naser, M.N., Nair, G.B., Watanabe, H., Ohnishi, M., Yamamoto, S., Endtz, H., et al. 2012. Role of shrimp chitin in the ecology of toxigenic *Vibrio cholerae* and cholera transmission. *Frontiers in Microbiology*, **2**: 260. <https://doi.org/10.3389/fmicb.2011.00260>.

- Nandi, B., Nandy, R.K., Mukhopadhyay, S., Nair, G.B., Shimada, T. and Ghose, A.C. 2000. Rapid method for species-specific identification of *Vibrio cholerae* using primers targeted to the gene of outer membrane protein OmpW. *Journal of Clinical Microbiology*, **38**(11): 4145-4151. <https://doi.org/10.1128/JCM.38.11.4145-4151.2000>.
- Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. and Hase, T. 2000. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research*, **28**(12): e63-e63. <https://doi.org/10.1093/nar/28.12.e63>.
- Okada, K., Chantaroj, S., Taniguchi, T., Suzuki, Y., Roobthaisong, A., Puiprom, O., Honda, T., and Sawanpanyalert, P. 2010. A rapid, simple, and sensitive loop-mediated isothermal amplification method to detect toxigenic *Vibrio cholerae* in rectal swab samples. *Diagnostic Microbiology and Infectious Disease*, **66**(2): 135-139.
- Pruzzo, C., Tarsi, R., del Mar Lleò, M., Signoretto, C., Zampini, M., Pane, L., Colwell, R.R. and Canepari, P. 2003. Persistence of adhesive properties in *Vibrio cholerae* after long-term exposure to sea water. *Environmental Microbiology*, **5**(10): 850-858. <https://doi.org/10.1046/j.1462-2920.2003.00498.x>.
- Roszak, D.B. and Colwell, R. 1987. Survival strategies of bacteria in the natural environment. *Microbiological Reviews*, **51**(3): 365-379.
- Sack, D.A., Sack, R.B. and Nair, G.B. 2004. Siddique Ak. *Cholera Lancet*, **363**: 223-233.
- Senoh, M., Ghosh-Banerjee, J., Mizuno, T., Shinoda, S., Miyoshi, S., Hamabata, T., Nair, G.B., and Takeda, Y. 2014. Isolation of viable but nonculturable *Vibrio cholerae* O1 from environmental water samples in Kolkata, India, in a culturable state. *Microbiology Open*, **3**(2): 239-246. <https://doi.org/10.1002/mbo3.164>.

- Shirai, H.I.R.O.M.A.S.A., Nishibuchi, M.I.T.S.U.A.K.I., Ramamurthy, T., Bhattacharya, S.K., Pal, S.C. and Takeda, Y. 1991. Polymerase chain reaction for detection of the cholera enterotoxin operon of *Vibrio cholerae*. *Journal of Clinical Microbiology*, **29**(11): 2517-2521.
- Srisuk, C., Chaivisuthangkura, P., Rukpratanporn, S., Longyant, S., Sridulyakul, P. and Sithigorngul, P. 2010. Rapid and sensitive detection of *Vibrio cholerae* by loop-mediated isothermal amplification targeted to the gene of outer membrane protein ompW. *Letters in Applied Microbiology*, **50**(1): 36-42. <https://doi.org/10.1111/j.1472-765X.2009.02749.x>.
- Sultana, M., Nusrin, S., Hasan, N.A., Sadique, A., Ahmed, K.U., Islam, A., Hossain, A., Longini, I., et al. 2018. Biofilms comprise a component of the annual cycle of *Vibrio cholerae* in the Bay of Bengal estuary. *MBio*, **9**(2): e00483-18. <https://doi.org/10.1128/mBio.00483-18>.
- Syafirah, E.E.N., Najian, A.N., Foo, P.C., Ali, M.R.M., Mohamed, M. and Yean, C.Y. 2018. An ambient temperature stable and ready-to-use loop-mediated isothermal amplification assay for detection of toxigenic *Vibrio cholerae* in outbreak settings. *Acta Tropica*, **182**: 223-231. <https://doi.org/10.1016/j.actatropica.2018.03.004>.
- Wang, L., Shi, L., Alam, M., Geng, Y., and Li, L. 2008. Specific and rapid detection of foodborne *Salmonella* by loop-mediated isothermal amplification method. *Food Research International*, **41**(1): 69-74. <https://doi.org/10.1016/j.foodres.2007.09.005>.
- Wootton, J.C., and Federhen, S. 1996. [33] Analysis of compositionally biased regions in sequence databases. *Methods in Enzymology*, **266**: 554-571. [https://doi.org/10.1016/S0076-6879\(96\)66035-2](https://doi.org/10.1016/S0076-6879(96)66035-2).
- Xu, M., Fu, H., Chen, D., Shao, Z., Zhu, J., Alali, W.Q., and Chen, L. 2019. Simple Visualized Detection Method of Virulence-Associated Genes of *Vibrio cholerae* by Loop-Mediated

Isothermal Amplification. *Frontiers in Microbiology*, **10**: 2899.
<https://doi.org/10.3389/fmicb.2019.02899>.

Yamazaki, W., Seto, K., Taguchi, M., Ishibashi, M. and Inoue, K. 2008. Sensitive and rapid detection of cholera toxin-producing *Vibrio cholerae* using a loop-mediated isothermal amplification. *BMC Microbiology*, **8**(1): 1-7. <https://doi.org/10.1186/1471-2180-8-94>.

Draft

Figure 1a Typical *V. cholerae* cells stained by DVC-DFA and viewed using Zeiss Axio Observer Florescent microscope after growth on TCBS and LB agar and incubation for 24 hr at 37°C.

Figure 1b Typical coccoid *V. cholerae* cells stained by DVC-DFA and viewed by Zeiss Axio Observer Florescent microscopy after incubation for one year at 37°C. No growth was obtained on either TCBS or LB agar.

Figure 2a Optimized Multiplex PCR assay employing the *V. cholerae* ctxA/rfbN gene. M = Hyper ladder IV (Bio line); 1 = Negative control; 2 = *V. cholerae* O1 N16961; 3 = *V. cholerae* O1 NIH 1823. The top arrow shows ctxA gene (308 bp), the down arrow shows rfbN gene (192 bp).

Figure 2b Optimized PCR employing *V. cholerae* ompW gene (588 bp). M = Hyper ladder IV (Bioline); 1 = *V. cholerae* O1 N16961; 2 = *V. cholerae* O1 NIH1823; 3 = Negative control.

Figure 3a PCR employing serial dilutions showing sensitivity of *V. cholerae* N16961 DNA. M = Hyper ladder IV (Bio line); 1 = Positive control (10^6 copies of DNA per reaction); 2 = 10^5 ; 3 = 10^4 ; 4 = 10^3 ; 5 = 10^2 ; 6 = 10; 7 = 5 copies of DNA; 8 = Negative control.

Figure 3b PCR showing specificity of *V. cholerae* ctxA/rfbN. M = Hyper ladder IV (Bio line); 1 = *Salmonella enterica* CP1094; 2 = *Escherichia coli* CP 1120; 3 = *Shigella flexneri* CP1129; 4 = *Klebsiella pneumonia* CP1170; 5 = *Vibrio mimicus* MB-451; 6 = *V. cholerae* N16961.

Figure 4a LAMP showing sensitivity of *V. cholerae* ctxA. 1 = positive control (A = 1823 and B = N16961 and 10^6 copies of DNA); 2 = 10^5 ; 3 = 10^4 ; 4 = 10^3 ; 5 = 10^2 ; 6 = 10; 7 = 5 copies of DNA; DW = negative control.

Figure 4b LAMP showing specificity of *V. cholerae* ctxA. 1 = *Shigella flexneri* CP1129; 2 = *Klebsiella pneumonia* CP1170; 3 = *Salmonella enterica* CP1094; 4 = *Escherichia coli* CP 1120; 5 = *Vibrio mimicus* MB-451 study.

Table 1 PCR and LAMP results for ctxA, rfbN, and ompW after incubation of *V. cholerae* for one year at 4°C and 37°C.

Temperature	ctxA (PCR)	ctxA (LAMP)	rfbN (PCR)	rfbN (LAMP)	ompW (PCR)	ompW (LAMP)
37°C	81%	87%	87%	87%	78%	93%
4°C	72%	81%	72%	81%	75%	93%

Draft

Table 2 The ctxA, rfbN, and ompW PCR primers employed in this study as listed follows.

Sequence (5'.....3')	Primer	bp
5' ACAGAGTGAGTACTTTGACC 3'	F-ctxA(VCT1)	308bp
5' ATACCATCCATATATTTGGGAG 3'	R-ctxA(VCT2)	
5'GTTTCACTGAACAGATGGG3'	F-rfbN (O1F2-1)	192bp
5'GGTCATCTGTAAGTACAAC3'	R-rfbN (O1R2-2)	
5' CACCAAGAAGGTGACTTTATTGTG3'	F-ompW	588bp
5' GAACTTATAACCACCCGCG3'	R-ompW	

Table 3 LAMP assay primers employing ctxA, rfbN, and ompW employed in this study as listed follows.

LAMP Primers	Sequence (5'.....3')
FIP (ctxA)	TCT GTC CTC TTG GCA TAA GAC GCA GAT TCT AGA CCT CCT G
BIP (ctxA)	TCA ACC TTT ATG ATC ATG CAA GAG GCT CAA ACT AAT TGA GGT GGA A
F3 (ctxA)	GCA AAT GAT GAT AAG TTA TAT CGG
B3 (ctxA)	GMC CAG ACA ATA TAG TTT GAC C
LF (ctxA)	CAC CTG ACT GCT TTA TTT CA
LB (ctxA)	AAC TCA GAC GGG ATT TGT TAG G
F3 (ompW)	CGGTAGTACCTAATGACAGTAG
B3 (ompW)	GCAAATGTTTTGTTTCACCAAT
FIP (ompW)	CAAGCGTTAACCCTAAGTGGGTATTTTTTTAAAGTGTTAAACACTCAAA GTGAG
BIP (ompW)	ACATCAGTTTTGAAGTCCTCGCTTTTTATCACCAAGGCTACCTAAC
LB (ompW)	ACATAAGATTTCTACCTCTGGTGGT
F3 (rfbN)	ATACGTGGGTAGCGTTGT
B3 (rfbN)	GGCTGCAATAACAAAAAGAGAA
FIP (rfbN)	GATCCCCAGTGATGTATACAAGAAAATGCAATCAAAAAGCTTCACA
BIP (rfbN)	CTGAATGCCCGTCTGAGAAATCGACGGTCAGGTAAAGGAA
LF (rfbN)	ACCGTGTTGAAGTTTCTGAACA
LB (rfbN)	ACATGCAAGTTGGCAATGATGCA

Caption : Figure 1a Typical *V. cholerae* cells stained by DVC-DFA and viewed using Zeiss Axio Observer Fluorescent microscope after growth on TCBS and LB agar and incubation for 24 hr at 37°C.

Caption : Figure 1b Optimized Multiplex PCR assay employing the *V. cholerae* ctxA/rfbN gene. M = Hyper ladder IV (Bio line); 1 = Negative control; 2 = *V. cholerae* O1 N16961; 3 = *V. cholerae* O1 NIH 1823. The top arrow shows ctxA gene (308 bp), the down arrow shows rfbN gene (192 bp).

Caption : Figure 2a Typical coccoid *V. cholerae* cells stained by DVC-DFA and viewed by Zeiss Axio Observer Florescent microscopy after incubation for one year at 37°C. No growth was obtained on either TCBS or LB agar.

Caption : Figure 2b Optimized PCR employing *V. cholerae* ompW gene (588 bp). M = Hyper ladder IV (Bioline); 1 = *V. cholerae* O1 N16961; 2 = *V. cholerae* O1 NIH1823; 3 = Negative control.

Caption : Figure 3a PCR employing serial dilutions showing sensitivity of *V. cholerae* N16961 DNA. M = Hyper ladder IV (Bio line); 1 = Positive control (106 copies of DNA per reaction); 2 = 105; 3 = 104; 4 = 103; 5 = 102; 6 = 10; 7 = 5 copies of DNA; 8 = Negative control.

Caption : Figure 3b PCR showing specificity of *V. cholerae* ctxA/rfbN. M = Hyper ladder IV (Bio line); 1 = *Salmonella enterica* CP1094; 2 = *Escherichia coli* CP 1120; 3 = *Shigella flexneri* CP1129; 4 = *Klebsiella pneumonia* CP1170; 5 = *Vibrio mimicus* MB-451; 6 = *V. cholerae* N16961.

Caption : Figure 4a LAMP showing sensitivity of *V. cholerae* ctxA. 1 = positive control (A = 1823 and B = N16961 and 106 copies of DNA); 2 = 105; 3 = 104; 4 = 103; 5 = 102; 6 = 10; 7 = 5 copies of DNA; DW = negative control.

Caption : Figure 4b LAMP showing specificity of *V. cholerae* ctxA. 1 = *Shigella flexneri* CP1129; 2 = *Klebsiella pneumoniae* CP1170; 3 = *Salmonella enterica* CP1094; 4 = *Escherichia coli* CP 1120; 5 = *Vibrio mimicus* MB-451 study.