Int J Enteric Pathog. 2014 November; 2(4): e20997. DOI: 10.17795/ijep20997

Published online 2014 November 9. **Research Article**

Vibrio cholerae Detection in Water and Wastewater by Polymerase Chain Reaction Assay

Behnaz Barzamini [1]; Majid Moghbeli [1,*]; Nazila Arbab Soleimani [1]

[1]Biology Department, Islamic Azad University, Damghn Branch, Damghan, IR Iran

*Corresponding author: Majid Moghbeli, Biology Department, Islamic Azad University, Damghn Branch, Damghan, IR Iran. Tel: +98-2335256002, Fax: +98-2335236813, E-mail: moghbeli552@gmail.com

Received: June 10, 2014; **Revised:** September 20, 2014; **Accepted:** September 22, 2014

Background: *Vibrio cholerae* is a significant human pathogen worldwide and annually causes some cases of deaths. Contaminated water plays an important role in transmission of this pathogen, which indicates the importance of early diagnosis.
Objectives: The current study aimed to perform Polymerase Chain Reaction (PCR) on water and wastewater samples to determine the detection limit for *Vibrio cholerae*.
Materials and Methods: PCR was performed on the DNA extracted from *Vibrio cholerae* of the contaminated water and wastewater using *ctx*A gene specific primers. The accuracy of PCR method to detect these bacteria was also assessed.
Results: The result of PCR performed on the extracted DNA showed a specific 241 base pair band. The limit of bacterial detection for water and wastewater were 40 cfu/mL and 81 cfu/mL, respectively.
Conclusions: In the current study, PCR performance using the *ctx*A gene specific primers to detect *Vibrio cholerae* was found highly accurate and specific.

Keywords:Vibrio cholera; PCR; Detection Limit

1. Background

Vibrio cholerae is a Gramm-negative, comma-shaped bacterium belonging to the family of Vibrionaceae and the genus Vibrio; it is the causative agent of cholera which produces symptoms such as rice-water diarrhea and vomiting (1, 2). Approximately 206 serogroups of *V. cholerae* are identified with the classification based on variations in the heat-stable somatic O antigens of the strains (1, 3, 4). Only serogroups O1 and O139 are associated with cholera, which polluted water is the most common source of it (5, 6). *Vibrio cholerae* O1 is classified into two biotypes, classical and ElTor and each of the biotypes contains three serotypes named Ogawa, Inaba and Hikojima (7). Cholerae is spread throughout the world in seven large pandemics (8). It is an acute life-threatening diarrhoeal disease which causes dehydration and electrolyte imbalance, and death in some cases. It is estimated that three to five million people worldwide are affected every year, and it is responsible for around 100'000 to 130'000 deaths (1, 9, 10). Most cholera cases occur in poor communities and Developing countries (6). The pathogenesis of *V. cholerae* is due to its cholerae toxin (7, 11). This toxin is an exotoxin and the major pathogenic determinant of *Vibrio cholerae* which belongs to the larger family of AB toxins and can be transmitted by lysogenic bacteriophage called CTXφ (4, 12, 13). Although the knowledge on human health has grown, *Vibrio cholerae* is still a serious threat to human health (7). The high incidence of *V. cholerae* in water samples indicate the natural inhabitant of the organism in aquatic environments, hence water plays an important role in the transmission of cholerae. It is necessary to determine the prevalence of *V. cholerae* in aquatic environments (14). The conventional methods currently used to identify *V. cholerae* are time-consuming and laborious. These protocols are not adequate to detect low numbers of bacteria. Since under certain conditions, such as starvation and physical stress, *V. cholerae* organism may enter VBNC state (viable but non-culture able) as a survival strategy, the conventional culture methods failed to isolate these organisms in unhygienic water and contaminated foods (15-17).

2. Objectives

The current study mainly aimed to detect *Vibrio cholerae* in water and wastewater by PCR, and determine the accuracy of this method.

3. Materials and Methods

3.1. Preparation of Bacteria

Vibrio cholerae was obtained from the clinical laboratories. Then the enrichment of water samples in alkaline

peptone water (APW) was done for 16 to 18 hours at 37°C followed by culture on selective thiosulfate-citrate-bile salts-sucrose (TCBS) medium, afterward yellow *V. cholerae* colonies were cultured on BHI medium and biochemical tests such as oxidase, TSI, VP, motility, indole, and string of pearls test were performed (18).

3.2. Preparation of Spring Water and Wastewater

Water samples were selected from a spring, located in a village around Ramian city in Golestan Province, Iran, and the wastewater was taken from wastewater treatment system located in Damghan city, Iran, and transported to the laboratory immediately on ice.

3.3. Preparation of Polluted Water and Wastewater

About 1.35×10^5 cfu of *V. cholerae*, *Salmonella enterica*, *Shigella dysenteriae* and *Escherichia coli* were added to each milliliter of water and wastewater, separately.

3.4. Separation of Total DNA

Water and wastewater samples were centrifuged after one hour inoculation by bacteria and total DNA was extracted from sediments by phenol/chloroform method (19).

3.5. PCR Optimization on Vibrio cholerae

Specific primers F: 5-CAAATGATGATAAGTTATATCGG-3´and R: 5´-GACCAGACAATATAGTTTGACC- 3´were used which had been designed based on *ctx*A gene sequences (20) and constructed by Sigma company. These primers were used to detect and amplify 241 base pair fragment of *ctx*A gene. The total volume of 25µL contains a final concentration of 0.2 mM dNTP, 0.4 µM for each primer, 2 mM MgCl2, and 1 U Taq DNA Polymerase (CinnaGen, Iran); 1µL of the extracted *Vibrio cholerae* DNA was used as a template for each reaction. Extracted genomic DNA of *Salmonella enterica*, *Escherichia coli*, *Shigella dysenteriae* and water and wastewater samples with no *Vibrio cholerae* were used as the negative control. The PCR protocol includes an initial denaturation step at 94°C for five minutes, followed by 30 amplification cycles (denaturation for one minutes at 94°C, annealing for one minute at 51°C, extension for 30 seconds at 72°C) and the final extension for seven minutes at 72°C. Then, 5µL of the PCR product along with the marker (GeneRuler 50 bp DNA Ladder, Thermo scientific, USA) was analyzed by 2% agarose gel electrophoresis.

3.6. Evaluation of the Detection Limits of PCR

In order to assess the accuracy of PCR, 10, 100, 1000 and 10'000 cfu of *V. cholerae* were added to 1mL of water and wastewater and isolated DNA samples were diluted to 1/10, 1/30, 1/50, 1/100 and then PCR was performed on DNA samples with the conditions in the previous step.

4. Results

4.1. Identification of a Bacterial Sample

Vibrio cholerae produced smooth yellow colonies with two to four mm in diameter, an opaque center and transparent periphery on TCBS agar, after 16 to 18 hours of incubation. The results of oxidase, TSI, VP, motility, indole, and string of pearls test were positive. The bacteria were capable of fermenting carbohydrate on TSI media with no gas or hydrogen sulfide production. *Vibrio cholerae* was confirmed by studying the test results.

4.2. Separation of Total DNA

Separated DNA from the pure *V. cholerae*, polluted water, and wastewater were electrophoresed on 1% gel agarose and one power band was observed.

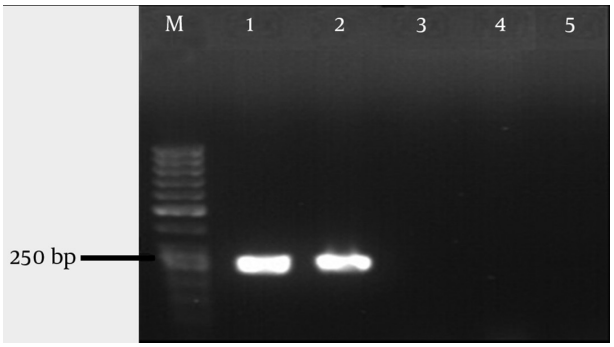
4.3. PCR optimization on Vibrio cholera

Polymerase chain reaction (PCR) assay was carried out on the DNA extracted from the *V. cholerae*, polluted water, and the negative control samples with primers designed for *ctx*A and 241bp band was observed (Figure 1).

4.4. Detection of Vibrio cholerae in Water and Wastewater, and Evaluation of the Detection Limits

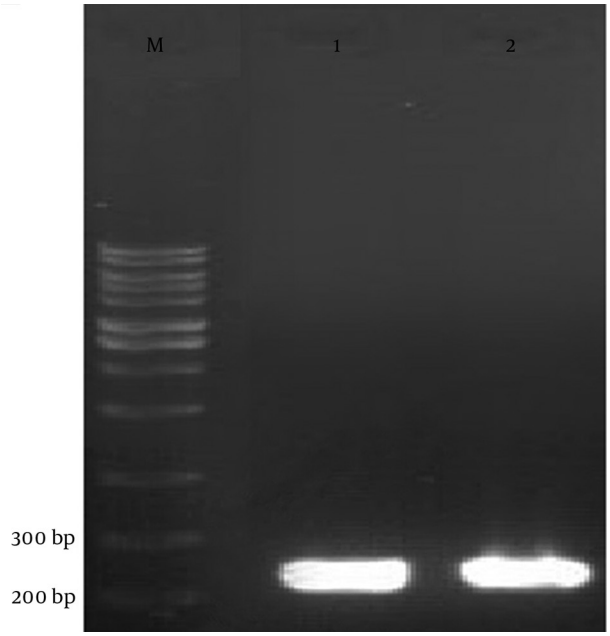
DNA samples extracted from water and wastewater generated the PCR product of 241 bp size which did not show up in the negative control (water and wastewater with no *V. cholerae*); indicating the specificity of the PCR assay (Figure 2). The detection limits of the PCR assay for water and wastewater samples were 40 cfu/mL and 81 cfu/mL, respectively, according to the results of the bacterial counts and dilution.

Figure 1. Gel Electrophoresis of the PCR Products From *ctx*A gene



Line M, 50 bp ladder; Line 1, PCR on *V. cholerae* (positive control); Line 2, PCR on the polluted water; Line 3, PCR on *Salmonella enterica* (negative control); Line 4, PCR on *Shigella dysenteriae* (negative control); Line 5, PCR on *E. coli* (negative control).

Figure 2. Gel Electrophoresis of the PCR Products From *Vibrio cholera*



Line M, 100 bp marker; Line 1) 241 bp PCR product on wastewater (contains *V. cholerae*); Line 2) 241 bp PCR product on water (contains *V. cholerae*).

5. Discussion

The conventional methods currently used to identify *V. cholerae* are time-consuming and laborious; therefore, detection methods such as PCR and other molecular techniques are suitable alternatives for bacterial culture and microscopic examination, especially for environmental samples (15, 16) because of high accuracy, specificity and speed. Thus, the PCR technique can be used to rapidly detect and identify *Vibrio cholera* due to its high accuracy and specificity (14). Koch et al. used PCR method to detect *V. cholerae* in food samples. They used primers specific for the *ctx*AB gene and the detection limits were about 100 bacteria in 10 gr of the contaminated food samples. Specific primers for the *ctx* gene were used in the current study, except that two sets of primers based on cholerae toxin subunit A (*ctx*A) were used (21). Maheshwari et al., used the two methods, microbiological and PCR, on 245 food and drinking water samples, including 35 samples of the contaminated water to identify *Vibrio cholerae*. In this regard, 34.29% and 63.16% of the samples in the cultivation technique and PCR assay (based on specific primers for the *ompW* gene) were detected as samples contaminated with *Vibrio cholerae*, respectively. This study demonstrated the accuracy of PCR to detect *Vibrio cholerae* compared to the conventional cultivation methods. Agata et al. reported detection limits of 1.4 cfu/mL for *Vibrio cholerae* by real-time PCR assay (1). PCR assay was performed on various genes of *Vibrio cholerae* O1, including *zot*, ace, *tcp*A and *ctx*A, using specific primers. The presence of virulence genes were analyzed by Leal et al. ; they revealed that all of the toxigenic *V. cholerae* strains possess these genes (22). Molecular epidemiological study of the *Vibrio cholerae* isolated from the infected patients in Teheran, Iran, was done by Pourshafie et al. . Results of this study showed that all of the toxigenic *V. cholerae* strains possessed *ctx*A genes (23). PCR using specific primers for the *ctx* gene was used in the current study, similar to the study by Yamazaki et al., and detected all cholerae toxin-producing *V. cholerae*. *Vibrio cholerae* were detected in water and wastewater by PCR assay and the detection limits were 40 cfu/mL and 81 cfu/mL for water and wastewater samples, respectively. The accuracy of this method was higher than those of the other studies. The results of the current study showed that specifically about 80 *Vibrio cholerae* per milliliter in swage can be identified even when the pollution and infection is too much. Cholera is a major public health problem for the developing countries and contaminated water plays an important role in transmission of this pathogen, which indicates the importance of its early diagnosis. The current study provided a fast and accurate method to detect *Vibrio cholerae* in water and wastewater with a detection limit of approximately 40 cfu/mL and 81 cfu/mL for water and wastewater, respectively.

Acknowledgements

Authors wish to thank the research council of Islamic Azad University, Damghan Branch for supporting the project.

References

1. Agata BD, Mirski T, Michal B, Piotr C, Anna R, Aleksander M. Development of Real-time PCR Assay for Detection of Vibrio cholerae. *Pol J Environ.* 2012;**21**(2):279–88.
2. Taneja N, Sangar G, Chowdhury G, Ramamurthy T, Mishra A, Singh M, et al. Molecular epidemiology of Vibrio cholerae causing outbreaks & sporadic cholera in northern India. *Indian J Med Res.* 2012;**136**(4):656–63.
3. Farajzadeh SA, Goodarzi H, Aslani S. Identification of Vibrio cholerae pathogenicity island (ctxA, OmpW and tcpA) in non-O139 and non-O1 V. cholerae strains isolated from Karun River in Ahvaz, Iran. *Afr J Microbiol Res.* 2012;**6**(6):1185–9.
4. Reidl J, Klose KE. Vibrio cholerae and cholera: out of the water and into the host. *FEMS Microbiol Rev.* 2002;**26**(2):125–39.
5. Hill VR, Cohen N, Kahler AM, Jones JL, Bopp CA, Marano N, et al. Toxigenic Vibrio cholerae O1 in water and seafood, Haiti. *Emerg Infect Dis.* 2011;**17**(11):2147–50.
6. Wong E, Vaaje-Kolstad G, Ghosh A, Hurtado-Guerrero R, Konarev PV, Ibrahim AF, et al. The Vibrio cholerae colonization factor GbpA possesses a modular structure that governs binding to different host surfaces. *PLoS Pathog.* 2012;**8**(1).
7. Taneja N, Biswal M, Tarai B, Sharma M. Emergence of Vibrio cholerae O1 Biotype El Tor serotype Inaba in north India. *Jpn J Infect Dis.* 2005;**58**(4):238–40.
8. Patrick GB, Nishibuchi M, Tunung R, Son R. Molecular characterization of clinical isolate of Vibrio cholerae isolated from outbreaks cases in Malaysia. *Int Food Rese J.* 2012;**19**(3):1267–74.
9. Mrityunjoy AK, Fahmida J. Prevalence of Vibrio cholerae in different food samples in the city of Dhaka Bangladesh. *Int Food Res J.* 2013;**20**(20):1017–22.
10. Bogard RW, Davies BW, Mekalanos JJ. MetR-regulated Vibrio cholerae metabolism is required for virulence. *MBio.* 2012;**3**(5).

11. Ubong A, Tunung R, Noorlis A, Elexson N, Tuan Zainazor TC, Ghazali FM. Prevalence and detection of Vibrio spp. and Vibrio cholera in fruit juices and flavored drinks. *Int Food Rese J.* 2011;**18**(3):1163–9.
12. Chinnapen DJ, Chinnapen H, Saslowsky D, Lencer WI. Rafting with cholera toxin: endocytosis and trafficking from plasma membrane to ER. *FEMS Microbiol Lett.* 2007;**266**(2):129–37.
13. Harris JB, LaRocque RC, Qadri F, Ryan ET, Calderwood SB. Cholera. *Lancet.* 2012;**379**(9835):2466–76.
14. Maheshwari M, Krishnaiah N, Ramana DBV. Evaluation of Polymerase Chain Reaction for the detection of Vibrio cholerae in Contaminants. *Ann Biol Res.* 2011;**2**(4):212–7.
15. Lyon WJ. TaqMan PCR for detection of Vibrio cholerae O1, O139, non-O1, and non-O139 in pure cultures, raw oysters, and synthetic seawater. *Appl Environ Microbiol.* 2001;**67**(10):4685–93.
16. Hasan JA, Huq A, Tamplin ML, Siebeling RJ, Colwell RR. A novel kit for rapid detection of Vibrio cholerae O1. *J Clin Microbiol.* 1994;**32**(1):249–52.
17. Lipp EK, Rivera IN, Gil AI, Espeland EM, Choopun N, Louis VR, et al. Direct detection of Vibrio cholerae and ctxA in Peruvian coastal water and plankton by PCR. *Appl Environ Microbiol.* 2003;**69**(6):3676–80.
18. Choopun N, Louis V, Huq A, Colwell RR. Simple procedure for rapid identification of Vibrio cholerae from the aquatic environment. *Appl Environ Microbiol.* 2002;**68**(2):995–8.
19. Kochl S, Niederstatter H, Parson W. DNA extraction and quantitation of forensic samples using the phenol-chloroform method and real-time PCR. *Methods Mol Biol.* 2005;**297**:13–30.
20. Yamazaki W, Seto K, Taguchi M, Ishibashi M, Inoue K. Sensitive and rapid detection of cholera toxin-producing Vibrio cholerae using a loop-mediated isothermal amplification. *BMC Microbiol.* 2008;**8**:94.
21. Koch WH, Payne WL, Wentz BA, Cebula TA. Rapid polymerase chain reaction method for detection of Vibrio cholerae in foods. *Appl Environ Microbiol.* 1993;**59**(2):556–60.
22. Leal NC, Sobreira M, Leal-Balbino TC, de Almeida AM, de Silva MJ, Mello DM, et al. Evaluation of a RAPD-based typing scheme in a molecular epidemiology study of Vibrio cholerae O1, Brazil. *J Appl Microbiol.* 2004;**96**(3):447–54.
23. Pourshafie M, Grimont F, Kohestani S, Grimont PA. A molecular and phenotypic study of Vibrio cholerae in Iran. *J Med Microbiol.* 2002;**51**(5):392–8.