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Rapid Identification of *Vibrio cholerae* Bacteria using LAMP and PCR methods in Water and Wastewater

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Vibrio cholerae **serogroup 01 is known as the cause of epidemic cholera. Cholera is a pandemic or epidemic infectious disease that only occurs in humans and if it is not paid attention, it will lead to death in 6 hours or less a week. Recent epidemics of cholera around the world make the rapid and reliable detection of Vibrio cholerae necessary more than ever. Therefore, its early, accurate and on-time detection seems essential to prevent its epidemic and the devastating effects. There are several methods for detecting** ***Vibrio cholerae*****, such as culturing, immunoassay, PCR and Real-time PCR. All these methods are time consuming and require large numbers of bacteria in the initial sample, expensive laboratory equipment and professionals in this field. In this study, PCR method was studied and compared with LAMP method to detect** ***Vibrio cholerae*****. Bacterial DNA was extracted according to standard methods and detected using specific primers in PCR machine based on thermal cycle program. In contrast, the specific primer sets were amplified by LAMP method, at a single temperature in a thermal block and created products were identified through the turbidity examined visually and using gel electrophoresis. The results of both methods in molecular detection of** ***Vibrio cholerae*** **showed that LAMP method is faster, cheaper and more specific and Vibrio can be detected in a shorter time, using a very simple and inexpensive heat block and at a single temperature of 62 °C, as well as, observing the turbidity resulted from gene amplification process in new ways compared with electrophoresis method in examining PCR products, instead of expensive PCR machine and multi-temperature thermal cycle program. This method can be used for quick, accurate and affordable molecular detection of** ***Vibrio cholerae*** **with wide application in diagnostic laboratories, laboratory in** ***underdeveloped regions*** **and mobile laboratories.**

Key words: Detection, LAMP, Vibrio cholera, PCR.

Vibrionaceae family has three genera, *Vibrio*, *Aeromonas* and *Plesiomonas*, that are clinically significant for humans and widely distributed in nature. *Vibrio* is found in pond water and salt water and separated *from Enterobacteriaceae by positive oxidase reaction* and the presence of polar flagella (1). *Vibrio cholerae* causes epidemic and endemic cholera considered as a cause of illness and death in many parts of the world, especially Asia and Africa (2). Although the disease is easily preventable, it is one of the main causes of deaths in the world, so the World Health Organization (*WHO)* refers to it as disaster tragedy. Its annual incidence is estimated to be about 5 million (3) and a large increase occurred in cholera cases in 2005. This report included 131943 patients with cholera, including 2272 deaths from 52 countries (4). Since *Vibrio cholerae* is one of the most well-known

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infectious agents worldwide, so its early detection is of particular importance (5, 6). Therefore, a rapid and sensitive method for the early detection and control of disease is required. Methods used in microbiology laboratories to detect cholera depend on the specific features and immunological evaluation of its poison (7). But these methods require a long time, a lot of costs and restrictions on the number of cases diagnosed. Although PCR method is a rapid and sensitive test with many advantages, it also possesses some limitations, such as high thermal cycles, use of expensive thermocycler machine and some methods to detect and identify product and for this reason, it needs much time and can't be used in mobile laboratories (8, 9). Loop- Mediated Isothermal Amplification of DNA (LAMP) is a new, easy method with high performance introduced by Notomi et al. 2000 that lacks these limitations. In this method, DNA is amplified specifically and with high speed and performance in isothermal conditions. In this way, four primers are used (two internal primers and two external primers), that, in total, identify six gene regions of target DNA and target region is amplified during a continuous process and the formation of hairpin regions at a temperature of 65-60 °C and using a thermostable DNA polymerase enzyme (10). This method has many advantages; such that LAMP is able to amplify DNA with high performance under isothermal conditions and can initiate to detect with a small number of DNA.

LAMP is even able to identify less than 6 copies of DNA in the reaction mixture. LAMP method is simple and easy to do and only requires a primer, DNA polymerase and reaction mixture, and no special equipment is needed and the reaction can be done in a water bath or heat block (10). Reaction products are a mixture of DNA hairpin structure with different sizes and cauliflower-like structure with multiple rings. That it is possible to reveal the final result of reaction based on the turbidity of reaction environment which can be seen by eyes or a device (11). This method doesn't need to denature the initial DNA and even to extract the initial DNA from some biological samples (12, 13). In this study, PCR and LAMP methods were investigated to detect *Vibrio cholera* in water and west water and it was shown that LAMP is easier, faster and more accurate than PCR.

MATERIALS AND METHODS

Preparation of Bacteria

In this study, the strain used was prepared from the Ministry of Health Reference Laboratory and confirmed using culturing, biochemical and immunological techniques.

How to prepare samples

50 ml of desired sample were taken and cultured on 5.1 ml of LB broth and incubated at 37 °C for 24 h. then, they were prepared for DNA extraction.

The DNA of cultured *Vibrio cholerae* was extracted by phenol-chloroform standard method (14, 15) and then the presence of their genome was confirmed on a 1% agarose gel. Prior to testing LAMP to confirm the *Vibrio cholerae* genome, conventional PCR method using the following primers and PCR optimization method were used for molecular diagnosis of *Vibrio cholerae* (Table 1).

PCR detection limit

Needed tests were done on clean water and urban wastewater samples for PCR detection limit. 1.35´10^{7} CFU/ml bacteria were detected, of which 100 ml were inoculated into 10 ml spring water and wastewater. As a result, contaminated water and wastewater were diluted at a ratio of 1:100, that the number of bacteria reaches 1.35´ 10^{7} CFU/ml. PCR detection limit was calculated according to this amount.

LAMP Test

6 specific primers, designed by Lockman et al. based on *Vibrio cholerae ctxA* gene and synthesized by Sigma Co., were used for LAMP reaction. Sequences of primers are shown in Table (3).

All factors, affecting the LAMP reaction were optimized to reproduce the desired fragment well. After applying all effective factors, the amount of materials and thermal cycle in LAMP reaction are as follows (Table 4).

Agarose gel electrophoresis

DNA sample extracted from *Vibrio cholerae* was reproduced based on the optimized LAMP method. Then, the product was analyzed on 2% agarose gel electrophoresis. Various factors affecting the replication process, such as the amount of different materials and thermal

conditions were optimized to obtain the best results.

Detection of LAMP products using fluorescent

SYBR Green has a high affinity for double-stranded DNA and emits green light after exciting the fluorescent dye. Upon completion of LAMP amplification reaction, , the results were assessed according to the color changes by adding 1 ml of diluted SYBR green solution to the reaction mixture. In the case of the presence of target gene (*ctxA*), green color (positive result) is created due to the accumulation of grate products and binding of SYBR green. Finally, Fluorescence intensity increases by UV light and thus, positive samples should be seen as bright green. But observing no changes in the original color of SYBR green from orange to green indicates that there is no target gene and amplified products (negative result).

RESULTS

Confirmation of DNA Extraction

Electrophoresis was performed after extracting DNA from *Vibrio cholerae*. The image of desired DNA is visible at the left of gene ruler (O Gene ruler bp plus-VIVANTIS)

DNA Confirmation: PCR reaction was performed in 30 cycles after preparation of DNA, the mixture of PCR and primers F3 and B3. Electrophoresis was performed.

Finally, a single sharp band was formed with the size of 240 bp (in the expected range), that indicates the complete accuracy of PCR conditions, from the number of cycles to concentrations in PCR reaction. The image of the desired PCR product band is visible in the range of 240 bp at the right of gene ruler (O Gene ruler kb plus-VIVANTIS).

PCR detection limit

PCR was performed for DNA samples extracted from spring water and wastewater in different dilutions. After adding 100 µl of bacteria to 10 ml of spring water, and wastewater, 1 ml of it was removed and the extraction was performed. DNA obtained was then dissolved in l0µl of distilled water and its PCR result was positive with l ì1 and showed a sharp band in the desired range. Thus, the DNA, dissolved in lì40 of H2O, was diluted at the rate of 1:100 and its PCR result was

Table 1. Primer Sequences

Primer	Primer Sequence
(F3)	GCA AAT GAT GAT AAG TTA TAT CGG
(R3)	GAC CAG ACA ATA TAG TTT GAC C

Table 2. PCR Cycle

Cycle	Time	Temp	Stage
1	Min5	Cº 94	Initial denaturation
30	Min1	Cº 94	Denaturation
	Min1	Tm -3 (51 ºC)	Annealing
	Sec30	Cº 72	Extension
1	Min5	Cº 72	Final extension

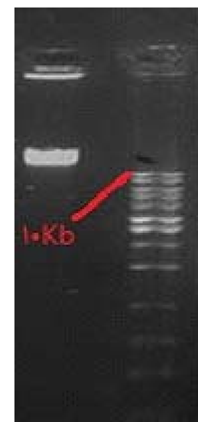


Fig. 1. DNA extracted from *Vibrio cholerae*

Table 3. LAMP primer sequences

Sequence (5' to 3')	Primer
TCTGTCCTCTTGGCATAAGACGCAGATTCTAGACCTCCTG	CtxA-FIP
TCAACCTTTATGATCATGCAAGAGGCTCAAACTAATTGAGGTGGAA	CtxA-BIP
GCAAATGATGATAAGTTATATCGG	CtxA-F3
GACCAGACAATATAGTTTGACC	CtxA-B3
CACCTGACTGCTTTATTTCA	CtxA-LF
AACTCAGACGGGATTTGTTAGG	CtxA-LB

positive with 1ì1. PCR was performed for genomic DNA in different dilutions to determine the detection limit. PCR samples were reported positive (Figure 3). Finally, bacteria detection limit was obtained 33CFU/ml.

LAMP Confirmation

LAMP reaction was performed after a positive PCR and the confirmation of DNA accuracy and the presence of *ctxA* gene. The reaction was performed in different amounts of materials. LAMP product was centrifuged for 2 min at rpm 10000 after completion of the reaction. Finally, turbidity was visually observed my magnesium pyrophosphate (Figure 4). 1 µl SYBR green was added to LAMP products to confirm them and seen by UV spectrometry and the result reported was positive (Figure 5).

The resulted sample was confirmed by electrophoresis to confirm LAMP reaction accuracy (Figure 6).

DISCUSSION

Cholera is an ancient disease and has been remained endemic in many parts of the developing world and yet, causes many deaths annually (16). *Vibrio cholerae* is a water-borne pathogen, which causes gastrointestinal symptoms such as diarrhea like-rice and vomiting. Epidemic cholera is created by toxigenic strains of serotypes O1 and O139. These strains are often found in water contaminated with the bacteria (17).

Virulence of *Vibrio cholerae* is due to having cholerae toxin genes (*ctx*) (18). So, because *Vibrio*

Table 4. Concentrations in LAMP reaction

Material	Stoke concentration	The final concentration of each reaction
dNTP	mM10	mM1/0- mM4/0
LAMP buffer	X10	X1
BSM Polymerase Enzyme	U/ìl1	U8
$MgSO_4$	mM100	mM2-mM8
FIP,BIP Primer	ìM10	ìÌ 8/0
LF,LB Primer	ìM10	ìÌ8/0
B3,F3 Primer	ìM10	ìM2/0
Betaine	M5	M4/0

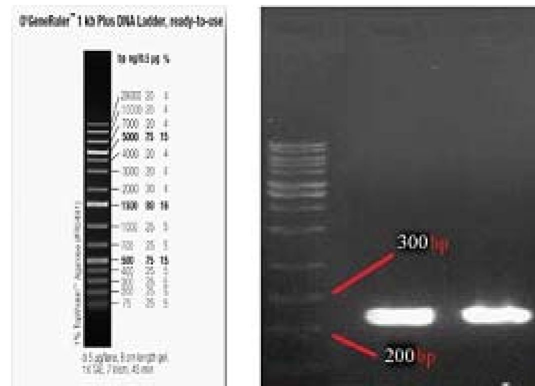
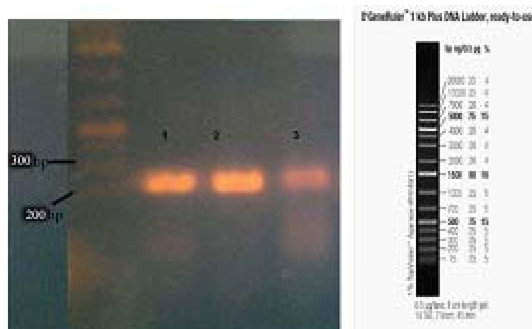


Fig. 2. PCR product of *Vibrio cholerae* 01 *ctxA* gene



Line 1: PCR-positive sample diluted 1:20
Line 2: PCR-positive sample diluted 1:50
Line 3: PCR-positive sample diluted 1:100

Fig. 3. PCR product samples extracted in different dilutions

Sample 1: positive sample, observation of turbidity caused by the accumulation of white precipitate of magnesium pyrophosphate
Sample 2: negative sample, with no turbidity

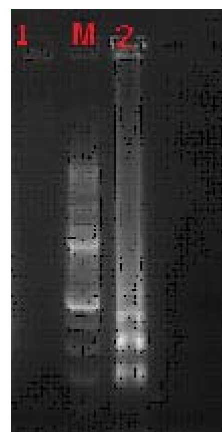
Fig. 4. detection by visual turbidity

cholerae is one of the most important globally recognized infectious factors and cholera disease is always considered as pandemic or endemic, therefore, its early detection has a special importance and it is required to find a method able to test many samples in a short time with a high sensitivity (19, 20). Traditional detection methods used to identify *Vibrio cholerae* are time consuming and laborious. In addition, traditional methods have low sensitivity for patient samples (especially in patients who have previously received antibiotic treatment). It is also difficult to identify it in cases with low number of bacteria in the sample. *Vibrio cholerae* cells may also enter the VBNC state (viable but uncultivable) in poor diet and physical stress. This could be an explanation for the failure of traditional cultivation methods to isolate this organism from contaminated waters and food samples during an outbreak through food (21, 22). Detection methods, based on nucleic acid such as polymerase chain reaction (PCR) and other molecular techniques, are proper alternatives for culturing and microscopy, especially for environmental samples. However, detection can be performed more quickly by these molecular techniques. The range of sensitivity and the specificity of molecular methods are comparable or even better than conventional culturing methods (19). In this study, PCR method was firstly used as a rapid diagnostic method using specific primers designed based on *Vibrio cholerae ctxA* gene. The primers used to detect all pathogenic serotypes of *Vibrio cholerae*, because all pathogenic serotypes have *Vibrio cholerae* toxin genes. Given that PCR was able to detect *Vibrio cholerae* samples, but it had some disadvantage, such as its duration compared with LAMP molecular methods and the use of expensive equipment, as well as the use of substances harmful to humans. This study, with the aim of resolving the disadvantages of the methods mentioned, attempted to provide a rapid and sensitive method for the identification of the bacteria. So, LAMP method was used to detect *Vibrio cholerae* in water and wastewater as a detection method more superior than PCR. This method is faster, easier and with more features compared to conventional PCR methods. Moreover, because a large amount of DNA is produced in LAMP method, far more byproducts are also produced. One of the byproducts is pyrophosphate that makes a white precipitate of magnesium pyrophosphate with magnesium ions present in the reaction mixture and causes the reaction mixture becomes turbid. Thus, the amplified product can be detected from the created turbidity (23).

Yamazaki et al. examined the sensitivity of LAMP method for detection of toxin-producing strains of *Vibrio cholerae* O1 compared to conventional PCR methods. They prepared serial dilutions using phosphate buffer from strains grown in APW. In their study, LAMP reaction was

Microtube 1: positive sample after adding SYBR green
Microtube 2 negative sample after adding SYBR green

Fig. 5. Detection by SYBR green under UV,



Line 1: negative control
Line M: marker 1kb (1kb Plus O 'Gene ruler, Fermentas).
Line 2: LAMP laddering band

Fig. 6. LAMP product

positive upto 0.1 CFU, but conventional PCR was positive upto 1.4 CFU. They concluded that LAMP method is easier and faster and more sensitive than conventional PCR methods in detecting toxin-producing Vibrio cholerae (23).

Okada et al. also used LAMP method to identify *Vibrio cholerae* with *ctx*A gene in clinical samples. They also used different times from the method, but reported 60 minutes as the best time for 64 ^{o}C. In this way they were able to detect 0.54 CFU in each reaction using purified DNA. While the sensitivity of PCR assay for the identification of this gene was lower than the LAMP method in their study and PCR was able to detect only 54 CFU in each reaction (24).

Various optimizations were conducted in this study and showed that the sensitivity of both methods is the same in the detection of bacteria in water and domestic wastewater and detection limits of PCR and LAMP for *Vibrio cholerae* was 33 CFU/ml. But 4 to 5 hours is required for gel electrophoresis and gel staining in the conventional PCR method for DNA amplification, while there is only a two-step DNA extraction of sample and amplification in LAMP method, which is done in 35 to 70 min. Additionally, DNA amplification in LAMP method is confirmed visually, without the need for electrophoresis.

CONCLUSION

Due to the high sensitivity and specificity of LAMP, as well as the lack of the need for skilled personnel and expensive equipment, and also because this method is simpler and less costly and time saving, can be a good alternative to conventional PCR methods to identify *Vibrio cholerae* and other infectious agents.

REFERENCES

1. Feachem R, Miller C, Drasar BS. Environmental aspects of cholera epidemiology. II. Occurrence and survival of Vibrio cholerae in the environment. *Tropical Diseases Bulletin* 1981; **78**: 865-880.
2. Jiang SC, Matte M, Matte G, Huq A, and Colwell RR. Genetic diversity of clinical and environmental isolates of Vibrio cholera determined by amplified length polymorphism fingerprinting. Appl Environ Microbiol 2000; 66:148-153.
3. Guidelines for drinking water quality: Microbiological agents in drinking water. WHO Publications, 2002; 2nd Ed. Geneve,Switzerland.
4. Cholera 2005. WHO Annual subscription SW.fr./ Fr.s. 334-05 2006; Geneve, Switzerland
5. Dalsgaard, A., et al. "Cholera in Vietnam: changes in genotypes and emergence of class I integrons containing aminoglycoside resistance gene cassettes in Vibrio cholerae O1 strains isolated from 1979 to 1996." *Journal of clinical microbiology* 37.3 (1999): 734-741.
6. Theron, J., et al. "Detection of toxigenic Vibrio cholerae from environmental water samples by an enrichment broth cultivation–pit stop semi nested PCR procedure." *Journal of applied microbiology* 89.3 (2000): 539-546.
7. Minami, A., et al. "Cholera enterotoxin production in Vibrio cholerae O1 strains isolated from the environment and from humans in Japan." *Applied and environmental microbiology* 57.8 (1991): 2152-2157.
8. Forbes BA, Sahm DF, Weissfeld AS. Bailey& Scott's Diagnostic Microbiology, 11th Ed. Mosby Inc., St. Louis, USA. 2002; pp.: 423-433.
9. Albert MJ, Islam D, Nahar S, Qadri F, Falklind S, and Weintraub A. Rapid detection of Vibrio cholerae O139 Bengal from stool specimens by PCR. . J Clin Microbiol 1997; 35: 1633-1635.
10. Notomi T, Okayama H, Masubuchi H, Yonekawa T. Loop-mediated isothermal amplification of DNA. J Nuc Acids Res 2000; 28: 63.
11. Mori Y, Magamine K, Tomita N, Notomi T. Detection of loop-mediated isothermal amplification by turbidity derived from magnesium pyrophosphate formation. Biochem Biophys Res Commun 2001; 289: 150-4.
12. Kaneko H, Kawana T, Fukushima E, Suzutani T. Tolerance of loop-mediated isothermal amplification to a culture medium and biological substances. J Biochem Biophys Methods 2007; 70: 499-501
13. Nagamine K, Watanabe K, Ohtsuka K, Notomi T. Loop-mediated isothermal amplification reaction using a nondenatured template. J Clin Chem 2001; 47: 1742-3.
14. Kochl S, Niederststter H, Parson W. DNA extraction and quntitation of forensic samples using the phenol-chloroform method and real-time PCR. J Methods Mol BIol 2005; 297: 13-30.
15. Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim van, Dillen PM. Rapid and simple method for purification of nucleic acids. J Clin

Microbiol 1990; 28: 495-503.

16. Gil, Ana l, Louis. R Valeri,Rivera Irma N.G.,et al. Occurrence and distribution of*Vibrio cholerae in* the coastal environment of Peru. Environmental Microbiology (2004)6(7), 699–706
17. Vincent R. Hill, Nicole Cohen, Amy M. Kahler, et al., Toxigenic *Vibrio cholerae* O1 in Water and Seafood, Haiti. Vol. 17, No. 11, November 2011
18. Ubong, A., Tunung, R., Noorlis, A.,Elexson, N.,et al. Prevalence and detection of *Vibrio* spp. and *Vibrio cholerae* in fruit juices and flavored drinks. *International Food Research Journal 18(3): 1163-1169 (*2011)
19. Mori Y, Nagamine K, Tomita N, Notomi T. Detection of loopmediated isothermal amplification reaction by turbidity derivedfrom magnesium pyrophosphate formation. Biochem Biophys Res Commun 2001;289:150–4.
20. Mani Maheshwari,N. Krishnaiah,and D. B. V. Ramana.Evaluation of Polymerase Chain Reaction for the detection of Vibriocholerae in Contaminants.Annals of Biological Research, 2011, 2 (4) :212-217
21. JAFRUL A. K. HASAN, ANWARUL HUQ, MARK L. TAMPLIN, et al. A Novel Kit for Rapid Detection of Vibrio cholerae 01. JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1994, p. 249-252.
22. Erin K. Lipp,Irma N. G. Rivera,Ana I. Gil et al.,Direct Detection of Vibrio cholerae and ctxA in Peruvian Coastal Waterand Plankton by PCR.APPLIED AND ENVIRONMENTALMICROBIOLOGY, June 2003, p. 3676–3680 .
23. WataruYamazaki,KazukoSeto, Masumi Taguchi et al.,Sensitive and rapid detection of cholera toxin-producing Vibriocholerae using a loop-mediated isothermal amplification.BMC Microbiology 2008, 8:94 doi:10.1186/1471-2180-8-94.
24. Kazuhisa Okada,SiripornChantaroj,Tooru Taniguchi et al.,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 (2010) 135–139.