

A Rapid and Robust DNA Extraction Method for PCR-Based Diagnosis of *V. cholerae*

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Abstract

Cholera, caused by *V. cholerae*, needs rapid diagnosis because of its threat for rapid spread. Recent molecular diagnosis by PCR assay is more advantageous than the conventional methods. The bottleneck in PCR diagnosis lies in the delay in template DNA extraction of *V. cholerae*, which is the basic requirement. This study describes a simple, less expensive, and rapid template DNA extraction method to lessen the total time for PCR diagnosis of *V. cholerae*. To obtain template DNA, our method involves boiling of *V. cholerae* suspension in distilled water (takes 18-24 hours) instead of boiling the suspension in broth medium, which is a lengthy process (takes 72 hours). The validation of our template DNA was conducted using 40 *V. cholerae* O1 strains that were confirmed previously, and its usefulness was checked on 20 clinical strains of *V. cholerae* O1 isolated from acute diarrhea patients; results were compared with those of conventional template DNA. The analysis of our template DNA in simplex and quadruplex PCR assays revealed the presence of *ctxA*, *tcpA* (El Tor), *rfb* (O1), *ToxR*, *ctxB*, *zot*, *ace*, *rst*, and *OmpW* genes in *V. cholerae* O1. The comparison of the results of PCR assays using the template DNA from both sources showed equal results and matched correctly. The sensitivity of our template DNA was 1.5×10^3 CFU per assay. Our template DNA extraction method is simple, less expensive, and rapid, and it can be employed for early diagnosis of *V. cholerae* during outbreaks, in research laboratories, and in hospital setups where infrastructures are available.

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Introduction

Cholera is a long-standing diarrhoeal disease caused by *V. cholerae* O1 and *V. cholerae* O139 serogroups with epidemic and pandemic potential, particularly in developing countries. Among more than 206 serogroups of *V. cholerae* identified till today^[1], only *V. cholerae* O1 and O139 cause cholera due to the presence of cholera toxin^[2]. Because untreated cholera leads to serious health consequences, it needs rapid diagnosis during outbreaks and surveillance for timely implementation of public health measures. The long-standing conventional method of diagnosis is the gold standard for detection of *V. cholerae*. In order to confirm the *V. cholerae* serogroup, a series of biochemical tests, toxin assays, and slide agglutination with specific antiserum have to be done in conventional methods^[3]. However, this method of diagnosis is tedious, time-consuming, requires expertise, and reveals low sensitivity. Moreover, culturing *V. cholerae* in rural hospitals may not be possible due to the lack of advanced laboratory facilities and technical expertise. To overcome these difficulties in conventional diagnosis, several PCR methods have been developed with high sensitivity and specificity to confirm the serogroups, toxigenic potential, and other characteristics of *V. cholerae* with the detection of specific genes^{[4][5][6][7][8][9][10][11]}. PCR-based tests developed so far rely on detection of genes that encode toxins (*ctxA*, *ctxB*, *zot*, *ace*), surface antigens (*wbe* and *wbf*), biotype (*tcpA* classical/EI Tor), and other housekeeping genes of *V. cholerae*. The primary requirement for performing PCR-based tests is template DNA, which has to be extracted by the cetyltrimethylammonium bromide (CTAB)^[12] / phenol / chloroform method or boiling method as described elsewhere^[10]. The steps involved in template DNA extraction, either by CTAB or the boiling method, delay the entire process and take at least 3 days to obtain PCR results, which contrasts with the early diagnosis of cholera. In this study, an attempt has been made to establish an easy, rapid, and less expensive template DNA extraction method. Our rapid DNA extraction method facilitates the early detection of *V. cholerae* by PCR assay in one day instead of three days. The application of this new DNA extraction method will be easy for the rapid diagnosis of *V. cholerae* by PCR assay, which is urgently needed for the early implementation of control measures to stop the spread of cholera outbreaks.

Materials and Methods

In order to validate the amplification potential of our template DNA, a total of 44 *V. cholerae* O1 strains from laboratory stocks were included in this study. Among these strains, 30 were *V. cholerae* O1 EI Tor biotype, 9 were EI Tor variants with *ctxB1*, and 5 were Haitian variants carrying *ctxB7*. These strains were confirmed earlier at the Regional Medical Research Center, Bhubaneswar, for the presence of different housekeeping genes^{[13][14]}. *V. cholerae* strains were sub-

cultured on Thiosulphate citrate bile salt sucrose (TCBS, BD, USA) agar plates and incubated at 37°C overnight. From the typical yellow colonies on the TCBS culture plate, a bacterial suspension was prepared in 500µl-1000µl of sterile distilled water in a 1.5ml Eppendorf tube, having a bacterial population equivalent to the McFarland standard of 0.5 ($\approx 10^6$ CFU/ml). The culture/suspension was vortexed and boiled for 10 minutes in a water bath followed by quick transfer of the tubes to -20°C until use. The crude lysate was used as a source of template DNA in the PCR master mix. Our method of template DNA extraction usually takes 18-24 hours. For comparison, the *V. cholerae* DNA from the same samples, template DNA, was extracted by the previous boiling method as described previously^[10]. Briefly, a subculture of *V. cholerae* from the TCBS plate was made on a Luria Agar (LA, BD, USA) plate and incubated at 37°C overnight. From the pure culture on LA, a subculture was made in Luria Bertani Broth (LB, BD, USA) and incubated at 37°C. Extraction of template DNA was done by boiling the bacterial suspension in LB for 10 minutes and instantly preserved at -20°C till use. The total time taken to extract the template DNA by this method was 72 hours (3 days).

Quadruplex and monoplex PCR assays were conducted to detect different genes of *V. cholerae* O1 using the template DNA extracted by both methods. The genes of *V. cholerae* O1 *ctxA*, *tcpA* (classical/EI Tor), *wbe*, and *ToxR* were detected by using the Quadruplex PCR assay as described elsewhere^[11]. Briefly, the Quadruplex PCR was conducted with a preparation of 35µl master mix constituting 10x PCR buffer (100 mM Tris [pH 9.0]; 500 mM KCl, 0.1% gelatin (Bangalore Genei, India), 2.5 µl dNTP mix containing 2.5mM of each nucleotide (Bangalore Genei), 90 pmol of primer pair for *tcpA* EI Tor^[6], *tcpA* classical^[6], 68 pmol of primer pair *ctxA*^[6], and *wbe*^[5], 60 pmol of primer *ToxR*^[15], 0.3µl (3U/µl) of Taq DNA Polymerase (Bangalore Genei). Final adjustment was done with Milli-Q water, followed by addition of template DNA (5.5µl). Amplification was done in a Thermocycler (G-storm, England) with 94°C initial denaturation for 4 minutes, followed by subsequent 30 cycles of 1.5 minutes at 94°C, 1.5 minutes at 55°C, and 1.5 minutes at 72°C, and 1 cycle of final round extension was done for 7 minutes. The ethidium bromide-stained 2% agarose gel loaded with 12µl of amplicon was visualized under a UV transilluminator after electrophoresis in EDTA buffer at 100 volts for 45 minutes. Simplex PCR assays were conducted to detect *ctxB1*^[16], *ctxB7*^[17], *zot*^[18], *ace*^[19], *rst*^{ET}^[20] and *OmpW*^[21] genes. The experiment was repeated twice after the samples were blinded. An automated thermocycler (England) was employed for the simplex PCR assay through 30 cycles following the conditions as described in Table 1.

Table 1.

Target gene	Primer sequences (5'-3')	Amplicon size (bp)	References
<i>ctxA</i>	CTCAGACGGGATTTGTTAGGCACG	301	6
	TCTATCTCTGTAGCCCTATTACG		
	CACGATAAGAAAACCGTCAAGAG		
	ACCAAATGCAACGCCGAATGGAG		
<i>tcpA</i> classical	GAAGAAGTTTGTAAGAAGAAGAACAC	617	6
<i>tcpA</i>	GAAGGACCTTCTTTCACGTTG		
El Tor	GTTTCACTGAACAGATGGG	471	6
<i>wbe</i> (O1)	GGTCATCTGTAAGTACAAC		
<i>wbf</i> (O139)	GGTCATCTGTAAGTACAAC	192	5
<i>ToxR</i>	GTCAAACCCGATCGTAAAGG		
<i>ctxB</i> , Fw-con	GGGATCCATGTTCCGGATTAGGACAC	449	5
Rv- clas	GGGATCCTACTCACACACTTTGATGGC		
Rv- elt-	ACTATCTTCAGCATATGCACATGG	203	16
<i>ctxB</i> , Rvcon	CCTGGTACTTCTACTTGAAACG		
<i>ctxB1</i> -F	CCTGGTACTTCTACTTGAAACG	191	17
<i>ctxB7</i> -F	CCTGGTACTTCTACTTGAAACG		
<i>zot</i>	GTTTTACTATCTTCAGCATATGCGA	947	18
<i>ace</i>	GTTTTACTATCTTCAGCATATGCGC		
<i>rst</i> ^{ET}	TCGCTTAACGATGGCGGTTTT AACCCCGTTTCACTTCTACCCA	289	19
	TAAGGATGTGCTTATGATGGACACCC		
<i>OmpW</i>	CGTGATGAATAAAGATACTCATAGG	588	21
	GCACCATGATTTAAGATGCTC		
	TCGAGTTGTAATTCATCAAGAGTG		
	CACCAAGAAGGTGACTTTATTGTG		
	GAACTTATAACCACCCGCG		

To assess the specificity of our template DNA, monoplex and multiplex PCR assays were performed using our template DNA (cell lysate) in *V. cholerae* O1 and non-O1 *V. cholerae*. To check the specificity the strains of Enterobacteriaceae such as *E. coli* (ETEC), *S. typhi*, *Sh. flexneri*, and *A. hydrophila* were included and their DNA were extracted following our method for PCR assays.

For sensitivity assessment of our DNA, culture suspension was prepared in sterile distilled water from fresh typical yellow colonies on TCBS culture plates up to McFarland standard 1, carrying the bacterial suspension equivalent to 3×10^8 CFU/ml, which was subjected to serial dilution of 10-fold with sterile distilled water. The above bacterial suspension

was boiled for 10 minutes to extract the template DNA, followed by subsequent cooling at -20°C until ready for use. The extracted template DNA was used in monoplex and multiplex PCR assays to detect different genes of *V. cholerae* O1 using the extracted template DNA. The sensitivity was calculated based on the optimum PCR results.

The practical utility of our DNA extraction method was checked on 20 *V. cholerae* O1 strains and 5 non-O1 and non-O139 *V. cholerae* strains of clinical origin isolated during 2014-15 from hospitalized consented diarrhoea patients in Puri, India. Rectal swab samples from diarrhoea patients were inoculated on TCBS plates and incubated at 37°C overnight. Monoplex and quadruplex PCR assays were employed to detect different genes of *V. cholerae* O1 using the extracted template DNA from typical yellow colonies in distilled water as described above in our method.

Results and Discussion

The template DNA of *V. cholerae* extracted by both methods showed the same results in PCR assays, amplifying all genes (Figure 1). The monoplex and quadruplex PCR assays detected *ctxA*, *tcpA*, *wbe*, *toxR*, *ace*, *zot*, *rst* genes in all of the *V. cholerae* O1 strains, while *ctxB1* in the El Tor variant and *ctxB7* in Haitian variants amplified from both template DNA without showing any non-specific bands.

The specificity of our template DNA was confirmed with the detection of *V. cholerae* genes only. The results of PCR assays with target primer sequences encoding genes of *V. cholerae* revealed that our extracted template DNA is specific for the amplification of *V. cholerae* genes only and no amplicons were generated from other species.

The lower numbers of CFU of *V. cholerae* strains required for optimum amplification of target genes by PCR assays were calculated. The calculations revealed that 1.5×10^3 CFU per assay demonstrated satisfactory sensitivity for amplification of genes. The analysis of the practical usefulness of our template DNA revealed that the amplification of genes on both DNAs was the same, which confirmed that the strains were *V. cholerae* O1, harbouring other housekeeping genes. The results of both PCR assays detected that all the tested *V. cholerae* O1 strains harbored *tcpA*, *wbe*, *toxR*, *zot*, *ace*, *ctxB*, and *rst* genes, while non-O1 and non-O139 strains carried only the *toxR* gene. The analysis revealed that both the template DNAs amplified in PCR assays and detected all genes, yielding equal results.

Currently, advanced molecular tools have been largely employed for detection of microbiological organisms. However, rapid, simple, and less expensive molecular diagnosis has higher importance. In nucleic acid-based bacterial detection, sample processing is considered one of the major steps in DNA extraction. The present study highlights that DNA extraction is the central dogma for PCR analysis. Different methods are available for extraction of DNA from microbiological samples. The methods can be enzymatic, chemical, or thermal lysis, mechanical disruption of the cell wall using beads or sonication, or a combination of all said methods [22][23][24][25]. But these methods have several drawbacks. The weakness of the enzymatic lysis method is that commercially available enzymes may be contaminated with microbial DNA. The recent advanced nucleic acid-based techniques for identification of bacteria need good quality DNA extraction reagents that are free from contaminants for the harvest of good bacterial nucleic acid. Moreover, high cooling facilities

such as refrigeration and buffer storage are required for preservation of enzymes. In chemical lysis, some chemicals may be aggressive and toxic, which are injurious and may cause health hazards. These chemicals are restricted for DNA extraction use where laboratory safety conditions are absent. In laboratory methods that use a large size and heavy weight centrifuge machine for centrifugation, this is again considered one of the disadvantages. Another alternative method to obtain template DNA is by lysing the bacterial cell wall by the boiling method through bacterial culture in microbiological liquid (broth) medium (TSB/LB). However, the bottleneck of this method is the procurement of microbiological media, which is expensive, and broth culturing to obtain bacterial suspension is time-consuming. Moreover, overgrowth of bacteria in the broth medium, either due to delay in processing or heavy inoculation, may yield a hyper harvest of DNA content that will interfere in amplification during the PCR process, which may lead to errors or non-specific results.

The weakness of these methods of DNA extraction motivated us to make an attempt to develop a simple, less expensive, and rapid method with high sensitivity. Our DNA extraction method has several advantages: (a) template DNA can be extracted in 24 hours; (b) the pellet purification steps involved in phenol/chloroform or by commercial kits are avoided here; (c) RNAs are not digested with RNase; and (d) our DNA isolation method is rapid and robust. (e) This method is cost-effective compared to the broth method, since it only uses sterile distilled water instead of broth culture medium. Early diagnosis using this method of DNA extraction will be very useful during cholera outbreak/surveillance investigations to implement control measures.

Statements and Declarations

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Authors' Contributions

Hemant Kumar Khuntia and Manoranjan Ranjit designed the experiments. Hemant Kumar Khuntia and Madhusmita Bal performed the experiments. Hemant Kumar Khuntia drafted the manuscript. Manoranjan Ranjit, Madhusmita Bal, and Sanghamitra Pati reviewed and supervised the manuscript. All authors read and approved the manuscript.

Conflict of Interest

The authors declare that there exists no conflict of interest.

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Data Availability

All relevant data are within the paper and its supporting information files.

Ethics Statement

Not applicable.

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