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Original Article

A Novel and Rapid Isothermal Nucleic Acid Based Detection Assay of *Vibrio cholerae* by Polymerase Spiral Reaction (PSR) in Emergency Situations

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Abstract

Background: The purpose of this survey was to develop a novel and rapid isothermal nucleic acid based detection assay of *Vibrio cholerae* by polymerase spiral reaction (PSR) in emergency situations.

Methods: The current study was conducted in Baqiyatallah University of Medical Sciences, Tehran, Iran in 2021. The conserved *ctxA* gene sequence of *V. cholerae* was used as a target of designed two pairs of primers. Amplification of nucleic acids performed under isothermal temperature of 65 °C in 55 min by using *Bst* DNA polymerase. PSR amplified products were real-time visualized under UV transilluminator and also on agarose gel electrophoresis.

Results: Seven non- *V. cholerae* bacteria were negative for detection, which indicated the specificity of PSR assay was 100%. A 10- fold serial dilution of *V. cholerae* genomic DNA was subjected to conventional polymerase chain reaction (PCR) and real-time PCR to compare their sensitivities with PSR. The detection limit of PSR was 3×10^{-5} ng/ µL within 60 min, which 100-fold higher than that of PCR (3×10^{-3} ng/µL), but the sensitivity of real-time PCR was found as same as it.

Conclusion: The PSR assay developed in this study can provide a simple, cost-effective, rapid, and precise diagnosis technique in endemic cholera outbreaks, especially in low-income with limited access provinces.

Keywords: *Vibrio cholerae*; Polymerase spiral reaction (PSR); Isothermal nucleic acid amplification; Isothermal

Introduction

Vibrio cholerae, the Gram-negative, non-spore-forming curved rod, oxidase-positive bacterium, colonizes on the mucosal surface of small intestine and causes acute secretory diarrheal disease cholera (1, 2). Only O1 and O139 serogroups of *V. cholerae* produce cholera toxin and are associated with epidemics of cholera. The bacteriophage CTXΦ encodes the genes for the two subunits of cholera toxin (*ctxA* and *ctxB*) that is structurally and functionally similar to the heat-labile enterotoxin of *Escherichia coli* (3).

Seven major pandemics of cholera have occurred since 1816, resulting in thousands of deaths and major socioeconomic changes. It is estimated

that 3 to 5 million cases of cholera and 100,000 deaths occur worldwide each year (4). Therefore, accurate and well-timed diagnosis is necessary to control disease outbreaks and for appropriate treatment.

Two major diagnostic methods have been established for *V. cholerae*, including microscopy and culture methods (5). Large numbers of organisms are typically present in the stools of patients with cholera, so the direct microscopic examination of stool specimens can provide a rapid, but not precise, presumptive diagnosis in endemic cholera outbreaks. In addition, culture methods need to provide good quantity of specimens, transport, and selective mediums, selective biochemical tests, and at least 24 hours (6). Thus, although culture of a stool specimen remains the gold standard for the laboratory diagnosis of *V. cholerae* (7), but a simple, cost-effective, and rapid detection method is needed.

Polymerase spiral reaction (PSR) is a novel rapidity, high sensitivity, and high specificity isothermal nucleic acid testing method, which is different from PCR performed under isothermal conditions (8). Therefore, the PSR doesn't require any special instrumentation, and its features qualifies it for point of care test (9).

In this study, the sensitivity and specificity of PSR for detection of *V. cholerae* were determined by designed primers targeting the *ctxA* gene in optimized conditions. This technique can be used as a rapid alternate detection method of *V. cholerae* in emergency situations. To the best of our knowledge, this is the first report of PSR assay for detection of *V. cholerae*.

Materials and Methods

Bacterial strains and DNA extraction

The current study was conducted in Baqiyatallah University of Medical Sciences, Tehran, Iran in 2021. Three *Vibrio* spp. (*V. cholera* ATCC 14033, *V. parahaemolyticus* IBRC-M 10706, *V. vulnificus* ATCC 27562) were obtained from Pasteur Institute of Iran and Baqiyatallah University of Medical Sciences and used for PSR assay optimization. Non-*Vibrio* bacterial such as *Shigella dysenteriae* PTCC 1188, *Salmonella Typhi* PTCC 1609, *E. coli* ATCC 43888, *Pseudomonas aeruginosa* MTCC 1688, *Staphylococcus aureus* ATCC 29213 available in Baqiyatallah University of Medical Sciences were used for checking the specificity of the test.

All of the genomic DNAs were extracted by Genomic DNA Extraction Kit (Beta Bayern Biotech, Germany). Until further use, the isolated DNAs were stored at -20 °C.

Primer design

The primers for the PSR assay including the forward primer (Ft) and the reverse primer (Rt), were designed to target the *ctxA* gene from *V. cholerae* with NCBI GenBank Accession NO. AF452584 (Table 1) (10).

Table 1: The PSR primer design for the detection of *V. cholera*

Primer	*Sequence (5'-3')*	*Position within the ctxA gene*[a]
Ft	*acgattcgtacatagaagtatag*-TGCAAGAGGAACTCAGACGG	186-205
	Nr[b] F	
Rt	*gatatgaagatacatgcttagca*-TTGGAGCATTCCCACAACCC	628-609
	N[b] R	
IF	ATGTTGGGTGCAGTGGCTAT	335-316
IR	GGTTTCCCTCCGGAGCATAG	550-569

[a] The primer position is based on the sequence of the *V. cholera* with NCBI GenBank Accession NO. AF452584.1.
[b] Nr and N sequences were abstracted from a botanic gene [1]

The uppercase sequences at the 3'-end of the forward primer (F) and reverse primer (B) are complementary to the *ctxA* target gene sequence (position 186-205 and 628-609, respectively). The

lowercase sequences at the 5'-end of the forward primer (Nr) is reverse to the lowercase sequences at the 5'-end of the reverse primer (N), abstracted from a botanic gene (8). Furthermore, two auxiliary primers IF and IR were included in this study to improve the reaction velocity (positions 335-316 and 550-569, respectively).

Optimization of the PSR assay

The PSR was evaluated using different time (15-90 min), temperatures (60-65 ºC), and concentration of *Bst* DNA polymerase, primers, dNTPs, $MgSO_4$, and Betaine. Each optimized PSR reaction tube contained 25 µl reaction mixture composed of 2.5 µl of 10X Thermopol reaction buffer (including 10 mM KCl, 20mM Tris-HCl, 2 mM $MgSO_4$, 0.1% Tween 20, and10mM $(NH_4)_2SO_4$), 50-100 ng of template DNA, 1M Betaine (Sigma-Aldrich, USA), 1.4mM dNTPs mix (Thermo Scientific, USA), 4 mM MgSO4 (New England Biolabs, USA), 30 µM each of primers, 8U of *Bst* DNA polymerase large fragment (New England Biolabs, USA), and nuclease-free water to complete the final volume. The PSR amplified products were visualized for the appearance of laddering pattern on 2.5% agarose gel electrophoresis and also under UV transilluminator by addition of 1µl of 1000X concentrate of SYBR Green-I (Sigma-Aldrich, USA).

Sensitivity and specificity of PSR

A 10-fold serially diluted *V. cholerae* ATCC 14033 genomic DNA (3×10^1 to 3×10^{-8} ng/µL) were used to analyze sensitivity of PSR. The trials with conventional PCR and real-time PCR were also tested by forward primer 5'- TCAAACTA-TATTGTCTGGTC-3' and reverse primer 5'-CGCAAGTATTACTCATCGA-3', for comparison their sensitivity (10).

PCR was performed using DreamTaq PCR Master Mix (Ampliqon, Denmark). Each PCR tube contained 25 µl reaction mixture composed of 12.5 µl of the master mix, 1.5 µl of each forward and reverse primer solution, 5 µl of DNA and nuclease-free water to complete the final volume. The following reaction conditions were used: 35 cycles at 94 °C for 45 sec, 55 °C for 2 min, and 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. The PCR were analyzed by 2% agarose gel electrophoresis. Real-time PCR reaction was done by combined forward and reverse primer (0.5 µl), template DNA (2 µl), SYBR Green Master Mix (12 µl) (Takara, Japan) and DNA free water (5 µl). Real-time PCR parameters were set for 10 min at 94 °C and 40 cycle of 94 °C for 5 sec and 59 °C or 30 sec by a Rotor-gene Q instrument. For negative control, nuclease-free sterile water was used.

The PSR assay specificity was evaluated using genomic DNA from *Vibrio* spp. (*V. parahaemolyticus* IBRC-M 10706, *V. vulnificus* ATCC 27562) as well as non*Vibrio* organisms (*Escherichia coli* ATCC 43888, *S. Typhi* PTCC 1609, *S. dysenteriae* PTCC 1188, *P. aeruginosa* MTCC 1688, *S. aureus* ATCC 29213).

Results

When the trial was performed using reagents optimum concentration for duration of 60 min at a temperature of 65 ºC, appreciable amplification of PSR was obtained. The negative samples remained orange in color while the amplified product, which intensified under UV-transilluminator, showed bright green fluorescence. Moreover, a positive PSR assay was visualized through a ladder of multiple bands in 2.5% agarose gel (Fig.1).

Fig. 1: Agarose gel electrophoresis and visual detection by SYBR green I with green fluorescence of PSR amplified products. M: 100 bp DNA ladder; 1: *V. cholera*; 2: negative control

By serial dilution of *V. cholerae* genomic DNA, sensitivity of PSR was assessed, which the lowest limit of detection by PSR was found to be 3×10^{-5} ng/ µL (30 fg/µL). Besides, the PSR sensitivity was compared with conventional and real-time PCRs. Although, sensitivity of real-time PCR was found to be equivalent to PSR, but limit was found to be 3×10^{-3} ng/µL (3 pg/µl) of *V. cholerae* genomic DNA in classic PCR detection (Fig.2).

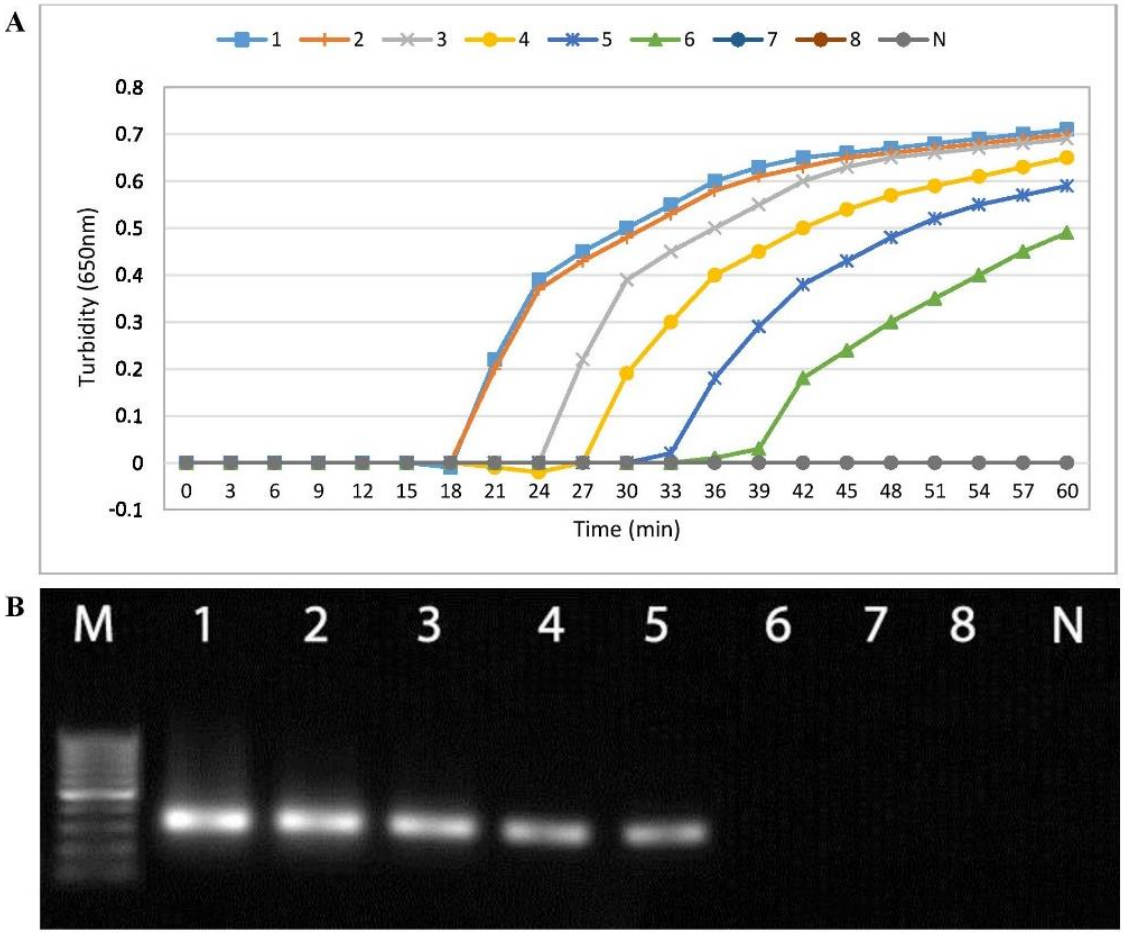

Fig.2: The PSR assay sensitivity. Sensitivity of the PSR method (A, B) versus conventional PCR for the detection of *V. cholera*. 1-7: 10-fold serial dilution of genomic DNA extracted from *V. cholera* ATCC 14033 (30 ng/µL to 0.003 pg/µl). The expected PCR product size was 380 bp

PSR specifically detected *V. cholerae* whereas non-*V. cholerae* bacterial pathogens weren't detected (Fig. 3). Both real-time monitoring of fluorescence signals and gel electrophoresis detection correctly identified *V. cholerae*. Double-distilled water were used as the negative control.

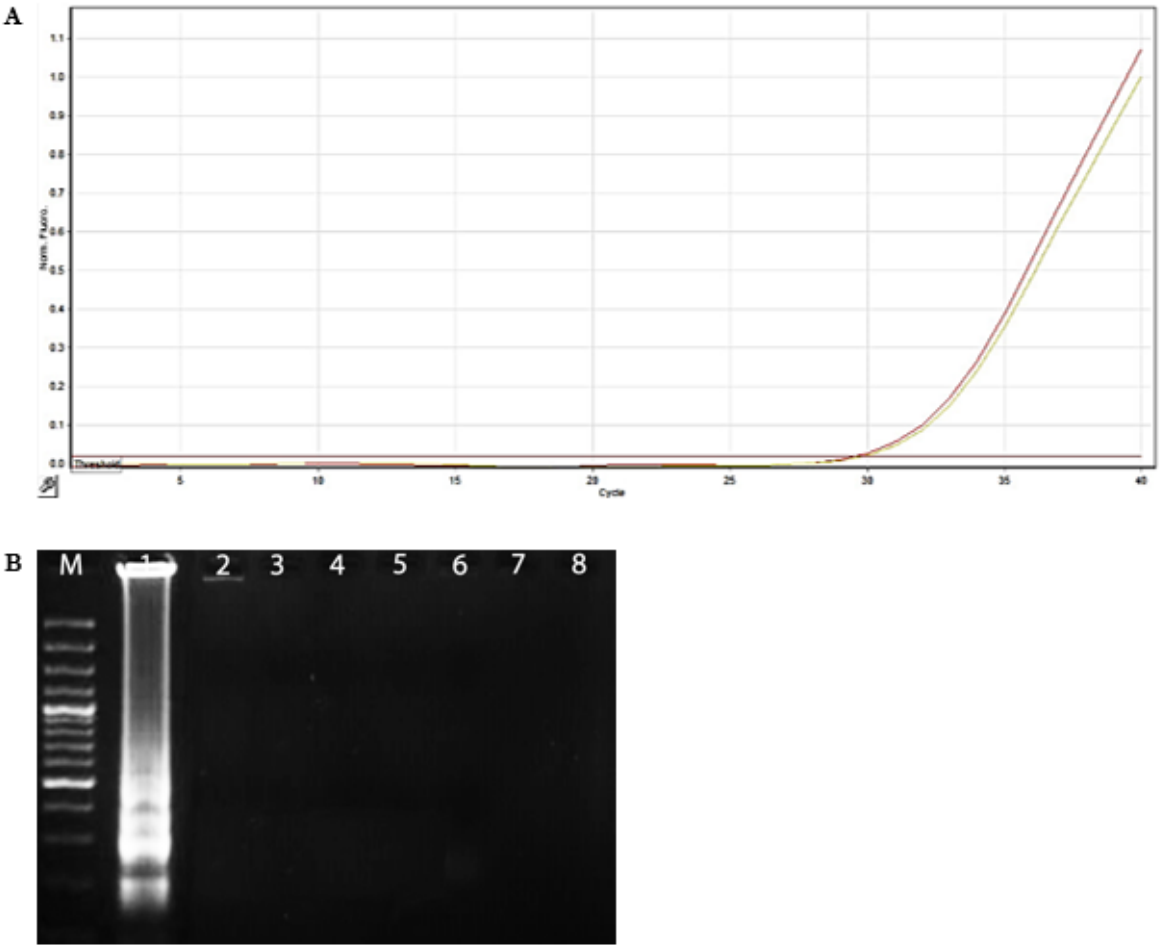

Fig.3: The PSR assay specificity. Specificity of the PSR method for detecting *V. cholera* by real-time turbidimeter (A) or gel electrophoresis (B). 1: *V. cholera* ATCC 14033; 2: *V. parahaemolyticus* IBRC-M 10706; 3: *V. vulnificus* ATCC 27562; 4: *E. coli* ATCC 43888; 5: *S. Typhi* PTCC 1609; 6: *S. dysenteriae* PTCC 1188; 7: *P. aeruginosa* MTCC 1688; 8: *S. aureus* ATCC 29213

Discussion

Cholera, caused by the bacteria *V. cholerae*, is a life-threatening watery diarrhea associated with the rapid onset of dehydration and hypovolemia (11). Notwithstanding, the routine treatment (fluid and electrolyte replacement) is employed for the patients with secretory diarrhea regardless of etiology (12), but rapid identification of the agent can affect the subsequent course of a potential epidemic outbreak, profoundly.

Isolation of *V. cholerae* by a stool specimen culture remains the gold standard for confirmatory diagnosis (13), but it suffers from certain drawbacks like: transport media, requirement of incubation period, and selective and differential media. Nowadays, for the direct detection of *V. cholerae*, various molecular tests have been found to be an alternative (10, 14). Despite the good sensitivity and specificity of some of these tests, but each has its challenges. For instance, when conventional PCR applied directly on clinical samples, may give false negative reaction due to presence of PCR inhibitors; or, in spite of skilled personnel and costly equipment which makes this test unsuitable for extensive application at the fact that real-time PCR assays are very sensitive

and of course rapid method, but it requires highly field (10).

The development of novel isothermal nucleic acid testing methods like PSR, has greatly facilitated on-site diagnosis and clinical screening (15). The PSR assay is a simple, rapid, sensitive and specific method for detection of *Brucella* spp. (16), *Candida albicans* (15), Canine parvovirus-2 (CPV-2) (17), *P. aeruginosa* (18), and *Mycobacterium tuberculosis* (9) that may improve diagnostic potential in clinical laboratories or can be used at diagnostic laboratories with minimal infrastructure. In this study, the PSR method was used to detect *V. cholerae* targeting the *ctxA* gene. The *ctxA* gene has been used as the target of many nucleic acid sequence-based amplification protocols for diagnosis of *V. cholerae,* previously (10). Additionally, to improve the reaction velocity, two auxiliary primers were designed. In contrast classic PCR which involves temperature cycling, just a thermostatic water bath or even a vacuum cup is sufficient to perform the PSR reaction.

The novel PSR developed in this study was found specific, as it did not detect any other bacterial pathogens. The PSR detection sensitivity was comparable to that of real-time PCR and 100 times higher than that of conventional PCR for the same set of template concentration and primers.

PSR and other isothermal reaction like LAMP utilizes Bst polymerase enzyme which shows high resistance to known Taq polymerase inhibitors such as haemoglobin, bile salts, N-acetyl cysteine, EDTA, NaCl, etc. (19). PSR uses only one main pair of primer and amplification takes places utilizing Bst polymerase enzyme, it overcomes limitations of both LAMP as well as PCR. The result of PSR can be obtained within 60 min, can be seen visually and needs no sophisticated instrumentation.

Conclusion

We established a novel approach for rapid detection of *V. cholerae*, which focuses on using the ASSURED (affordable, sensitive, specific, user friendly, rapid, equipment free, and deliverable) criteria as a framework for developing diagnostic techniques. In samples, the PSR requires 55 min for measurable detection and has sensitivity down to 30 fg/µL. Because of simplicity, this method has great potential as an accurate and rapid way for quantitation and identification of *V. cholerae* in disease outbreaks, especially in low-income limited access provinces. By and by, the PSR may become a novel method for detecting other epidemic potential bacteria/virus such as COVID-19 and demonstrate even greater potency.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

Authors declare that there is no conflict of interest.

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