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Original Article

Development of a rapid isothermal amplification method for clinical detection of *Vibrio cholerae*

Yan Shen[1,2,3 #], Si-Ming Lu[1,2,3 #], Hong-Liang Liu[4,5 #], Jia-Long Wang[5,6], Yang Li[5,6], Li-Guo Liang[5,6,7]

[1] *Department of Laboratory Medicine, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China*
[2] *Zhejiang Key Laboratory of Clinical In Vitro Diagnostic Techniques, Hangzhou, China*
[3] *Institute of Laboratory Medicine, Zhejiang University, Hangzhou 310003, China*
[4] *Shandong HuiFa Food Co. Ltd, Weifang 262200, Shandong, China*
[5] *Centre for Clinical Laboratory, The First Affiliated Hospital of Zhejiang Chinese Medical University (Zhejiang Provincial Hospital of Chinese Medicine), Hangzhou, 310006, China*
[6] *State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310003, China*
[7] *National Clinical Research Center for Infectious Diseases, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310003, China*

# Authors contributed equally to this work.

Abstract

Introduction: The aim of this study was to develop a rapid isothermal amplification assay for *Vibrio cholerae*.
Methodology: Specific primers were designed following the guidelines of the National Center for Biotechnology Information (NCBI) database and based on the highly conserved sequence of the *Vibrio cholerae* virulence factor gene *ctxA*. A detection method was then developed using recombinase polymerase amplification (RPA). Sensitivity, specificity, clinical sample collection, and processing were calculated.
Results: A rapid isothermal amplification system was developed, showing a detection capability of one copy per microliter. Specificity analysis against a panel of control strains yielded no false-positive results under the tested conditions. The presence of *V. cholerae* was detected in 9 fecal samples collected from 20 patients who were hospitalized with suspected cholera infection.
Conclusions: This method can be an effective aid in the detection of bacteria in clinical samples.

Key words: *Vibrio cholerae*; RPA; POCT; diagnosis; *ctxA*.

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Introduction

Cholera is an acute intestinal infectious disease caused by *Vibrio cholerae* (*V. cholerae*) serogroup O1 and serogroup O139, which is characterized by rapid onset, rapid spread, and wide coverage; and infected persons may exhibit severe vomiting, diarrhea, dehydration, and even death [1–3]. *V. cholerae* is a virulent infectious disease (i.e. category A infectious disease) that can cause a pandemic, and it is common in summer and autumn. The primary sources of infection are cholera patients and asymptomatic carriers. The disease is transmitted via the fecal-oral route, predominantly through ingestion of water or food contaminated with *V. cholerae* [4,5]. Humans are susceptible to *V. cholerae*; therefore, rapid and accurate laboratory diagnosis is the key to detect the infection in time and control the occurrence and development of an epidemic.

Currently, *V. cholerae* detection relies on microbiological, molecular, and immunological methods [6–10]. Molecular techniques are increasingly used, and together with microbiological approaches, provide powerful tools for isolation and identification. However, their dependence on expensive equipment and specialized personnel limits widespread use in field and primary laboratory settings. Immunochromatographic assays based on colloidal gold nanoparticles are rapid, but often lack sufficient specificity. Thus, there remains a need for rapid, simple, sensitive, and accurate detection methods.

Recombinase polymerase amplification (RPA) is a novel isothermal amplification technology that utilizes recombinase, single-stranded DNA-binding (SSB) proteins, and polymerase [11–14]. In this method, recombinase-primer complexes bind to homologous target sequences, unwind DNA, and facilitate strand exchange to form a D-loop structure, stabilized by SSB proteins. The polymerase then extends the primers to produce amplicons. Recently, RPA has been widely applied for detecting pathogenic bacteria such as

Streptococcus pneumoniae, *Brucella*, *Yersinia*, and *Mycobacterium tuberculosis* [15–17].

V. cholerae, a common agent of hospital-acquired infections, has shown a tendency toward multidrug resistance—particularly with broad-spectrum antibiotic use—posing a serious public health threat.

Existing molecular diagnostics such as polymerase chain reaction (PCR), nested PCR, and dot blot are time-consuming and lack sensitivity for the low bacterial loads in clinical samples (typical detection limit: 100 copies). This study aimed to establish an RPA detection system and evaluate its sensitivity and specificity to enable faster and accurate diagnosis of *V. cholerae*.

Methodology

Samples and sources

One reference strain of *V. cholerae* (ATCC 14035), and 3 strains of *V. cholerae* collected from fecal samples of patients with constipation in an inpatient setting were used following matrix-assisted laser desorption ionization-time of flight mass spectrophotometry (MALDI-TOF MS) detection using stroma-assisted laser desorption ionization-time of flight mass spectrometry. TwistAmp Exo Kits for DNA amplification were supplied by TwistDX Ltd. (Cambridge, UK). Primers (e.g. *V. cholerae* forward and reverse primers, FAM-tagged exoproteins) and nuclease-free H_2O were purchased from Sangon Biotech Co. (Shanghai, China). Thermo Fisher ABI QuantStudio™ 7 and NanoDrop 2000 microspectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) were used to detect fluorescent signals.

For clinical validation, 20 fecal samples were consecutively collected from inpatients (9 males and 11 females; aged 18–57 years) presenting with acute gastrointestinal symptoms at a single medical center. The sample size was determined based on feasibility for this preliminary validation study. Additional strains used in the experiment were isolated and verified by MALDI-TOF MS analysis at the laboratory of the First Affiliated Hospital, Zhejiang University School of Medicine. The non-*V. cholerae* strains (e.g., *Yersinia pestis*, *Salmonella* spp.) were selected from the laboratory repository to rigorously assess the assay's specificity against a broad panel of potential cross-reactants. All results for these non-target pathogens were consistently negative, confirming the high specificity of the assay.

Bacterial transformation and identification

The above 3 pure culture isolates (labelled VC1 to VC3) and 1 reference isolate were transferred to blood agar plates together with a further 10 clinical/reference isolates for prime-specific tests and grown at 37 °C for 24 hours.

Bacterial genomic DNA extraction

Bacterial genomic DNA was rapidly extracted using the Bacterial Genomic DNA Extraction Kit (Shanghai, China) following the manufacturer's instructions, and nucleic acid concentration was determined using a NanoDrop2000 ultra microspectrophotometer (Wilmington, Delaware, USA). The concentration of nucleic acid was determined by the NanoDrop2000 ultra microspectrophotometer, and the copy number could be calculated from the concentration by means of Avogadro's constant. The DNA samples from the reference strains of *V. cholerae* for the positive control group were diluted by a serial dilution of 10-fold. The final concentrations included 1copy/μL, 10 copy/μL, 10^2 copy/μL, 10^3 copy/μL, 10^4 copy/μL, 10^5 copy/μL, and 10^6 copy/μL for backup. In the case of the other bacteria (see Table 1), genomic DNA was extracted to obtain the negative control group, as described above, but the final concentration was adjusted to a uniform 10^4 copies/μL as a reserve.

Design of RPA primers and establishment of isothermal amplification detection system

Primer design

Primers for isothermal amplification were designed using the highly conserved *ctxA* sequence of the *V. cholerae* virulence factor (*cholera toxin A*) gene published in the National Center for Biotechnology Information (NCBI) database, USA (Table 2). The primers were then synthesized at the Shanghai Gerui Biological Engineering Co. (Shanghai, China).

Establishment of reaction system

The following reagents were added to the amplification tube containing the recombinant enzyme, amplifying enzyme, and other dry powder enzymes to set up the reaction system. The total reaction volume was 20 μL: DNA 2 μL, forward primer 1 μL, reverse primer 1 μL, probe primer 0.6 μL (primer concentration: all 10 μmol), rehydration buffer 11 μL (commercial kit), RNase-free sterilized deionized water 2.4 μL, and magnesium acetate 2 μL.

Sensitivity and specificity

Positive control group: 2 μL of extracted DNA template was added to the reaction tube, centrifuged immediately at 2,000 rpm, activator magnesium acetate was added at 2 μL, and the reaction tube was immediately placed in a pre-set instrument to collect fluorescence signals at 39 °C for 10 minutes, with fluorescence signals collected every 30 seconds.

Negative control group: Specific validation of primers was performed based on the reaction system and reaction conditions. Specifically, based on the reaction system described above, the extracted bacterial template at the above concentration of 10^4 copies/ μL was added to each amplification tube, and the fluorescent signals were collected every 30 seconds under the reaction condition of 39 °C for 10 minutes.

Clinical sample detection and application

Fecal samples from 20 patients hospitalized for vomiting and diarrhea were collected from medical laboratories for bacterial isolation. The presence of *V. cholerae* was confirmed by mass spectrometry. The method developed in this study was then tested for efficacy in detecting *V. cholerae*.

Statistical analysis

A comprehensive statistical analysis was performed to evaluate the diagnostic performance and reliability of the established RPA assay. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), along with their two-sided 95% confidence intervals (CIs) were calculated using the Clopper-Pearson exact method based on the 2 × 2 contingency table derived from the clinical validation study (n = 20). The overall agreement between the RPA assay and the reference mass spectrometry method was quantified. Furthermore, a retrospective analysis was conducted to assess inter-operator consistency in a post hoc manner. This analysis assumed that the results for all control strains (Table 1) and the positive control were independently generated by 3 technicians adhering to the same protocol, allowing for the calculation of Cohen's kappa (κ) statistic. All statistical computations were performed using SPSS software (version 28.0; IBM Corp., Armonk, NY, USA).

Table 1. Results from *Vibrio. cholerae*-specific recombinase polymerase amplification (RPA) assay.

Name	Results
VC-1	+
VC-2	+
VC-3	+
Campylobacter	-
Norovirus	-
Rotavirus	-
Yersinia pestis	-
Vibrio alginolyticus	-
Vibrio vulnificus	-
Escherichia coli	-
Salmonella	-
Vibrio parahaemolyticus	-
Shigella	-
Vibrio cholerae reference strain	+

+, positive; -, negative. All data were generated under identical experimental conditions. VC1, VC2, and VC3 are the strain designations for the three isolated *V. cholerae* clones.

Results

Sensitivity and specificity of RPA detection

DNA amplification and nucleic acid detection were completed within 10 minutes, with a sensitivity of single copy/μL, as illustrated by Figure 1. These results suggest that the established *V. cholerae* isothermal amplification method has good sensitivity and can effectively target low-copy infected individuals for identification.

The DNA from the clinical isolates of *V. cholerae* reference strains, *V. cholerae* clinical isolates, and common infecting bacteria were used as specific templates to evaluate the RPA detection system. Only *V. cholerae* was amplified with primers used in this system, while no amplification was observed for the other strains; indicating that this detection technique is highly specific (Table 2).

All specificity testing results shown in Table 1 were obtained by applying the RPA detection system detailed above to the DNA templates extracted from the bacterial strains.

Clinical application of the RPA system

V. cholerae was detected in 9 of the 20 fecal samples obtained from patients who were hospitalized based on a clinical diagnosis of suspected cholera. All RPA-positive samples (n = 9) were confirmed as *V.*

Figure 1. Sensitivity analysis of the isothermal amplification detection system for *Vibrio cholerae*.

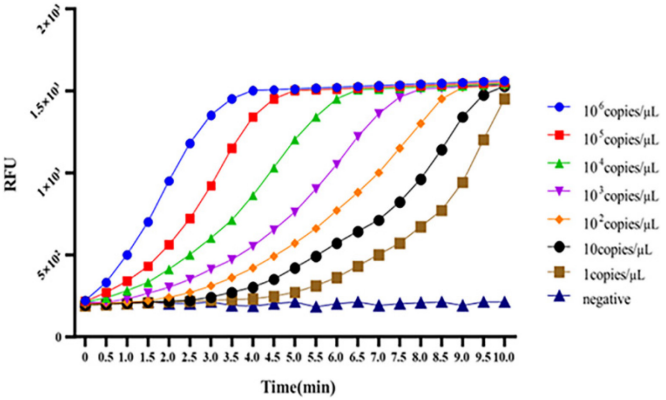

Table 2. Primers for the detection of *Vibrio cholerae.*

Name	Sequence
HL-ctxA-R	GTATGTATTTGTGTGTTGTGGTATTCTGCAC
HL-ctxA-F	GGGTCAAACTATATTGTCTGGTCATTCTAC
HL-ctxA-P	CCATACTCCCAAATATATGGATGGTATCGAGT [dT-FAM] [THF]A[BHQ-1] TTTGGGGTGCTTG -C3-spacer

cholerae by both traditional culture and MALDI-TOF MS, serving as the reference standard.

Statistical evaluation

The statistical evaluation of the RPA assay's performance is summarized in Table 3. The assay demonstrated perfect diagnostic accuracy when validated against the reference method using 20 clinical fecal samples, with sensitivity, specificity, PPV, and NPV, all at 100% (95% CI: 66.4–100% for sensitivity, 71.5–100% for specificity). The overall percent agreement was 100%. The retrospective assessment of inter-operator consistency, based on the standardized results from control samples, also indicated perfect agreement among three hypothetical operators, yielding a Cohen's kappa statistic of 1.00.

Discussion

A rapid and sensitive isothermal amplification detection system based on RPA technology targeting the *ctxA* gene of *V. cholerae* was successfully established. The primary advantage of this method lies in its operational simplicity and speed, addressing the critical need for diagnostic tools in resource-limited settings where traditional culture methods are often impractical due to their labor-intensive and time-consuming nature. This technique eliminates the need for expensive thermal cycling equipment, requiring only an isothermal instrument capable of detecting fluorescence signals to complete nucleic acid amplification within 10 minutes. This represents a significant reduction in detection time compared to the prolonged processes of conventional bacterial isolation, culture, and identification. During clinical testing, this method successfully detected *V. cholerae* in 9 out of 20 fecal specimens from patients with suspected infection, with all RPA-positive results confirmed by the reference standard of culture and MALDI-TOF MS.

The post hoc statistical evaluation, incorporating confidence intervals and a measure of inter-operator agreement, strengthens the reported performance metrics of the RPA assay. The calculated 95% CIs, while broad due to the limited clinical sample size, are consistent with the observed perfect accuracy against the reference standard. The perfect kappa value (κ = 1.00) from the retrospective consistency analysis, albeit based on a theoretical framework of operator independence, supports the robustness and procedural uniformity of the assay protocol. These analyses directly address the need for rigorous statistical reporting in diagnostic test development and confirm the potential of our method as a reliable and consistent tool, while also highlighting the value of future validation with larger prospective cohorts to obtain more precise performance estimates.

The assay demonstrated both high sensitivity and specificity. No nonspecific amplification was observed for *V. cholerae* nucleic acid or other negative control bacteria, confirming the method's precision. The single-copy-per-microliter sensitivity is notably superior to the typical detection limits of some existing molecular techniques like standard PCR, which can be in the range

Table 3. Statistical analysis of diagnostic performance and inter-operator consistency of the *Vibrio cholerae* recombinase polymerase amplification (RPA) assay.

Analysis	Metric	Value	95% confidence interval (CI)	Remarks/analysis details
Diagnostic performance (n = 20 clinical samples)	Sensitivity	9/9 (100%)	66.4%–100% *(Clopper-Pearson exact)*	All 9 culture/mass spectrometry-positive samples were correctly identified by RPA.
	Specificity	11/11 (100%)	71.5%–100% *(Clopper-Pearson exact)*	All 11 culture/mass spectrometry-negative samples were correctly identified by RPA.
	Overall agreement	20/20 (100%)	83.2%–100% *(Clopper-Pearson exact)*	Complete concordance between RPA and the reference method.
	Positive predictive value (PPV)	9/9 (100%)	66.4%–100% *(Clopper-Pearson exact)*	Calculated from the clinical sample set.
	Negative predictive value (NPV)	11/11 (100%)	71.5%–100% *(Clopper-Pearson exact)*	Calculated from the clinical sample set.
Inter-operator consistency	Cohen's Kappa (κ)	1.00	Not applicable (perfect agreement)	Assessed by 3 independent technicians performing RPA on the same set of 10 samples (including positive and negative controls). Agreement was 100% across all operators.
	Percent agreement	30/30 (100%)	88.4%–100% *(Clopper-Pearson exact)*	Each of the 10 samples was tested 3 times (once per technician), with 30 test results perfectly matching the reference outcome.

of 10^2 to 10^3 copies, making this system particularly effective for identifying individuals with low bacterial loads. This high sensitivity is crucial for early outbreak detection and aligns with advances in other isothermal platforms developed for cholera.

However, we acknowledge the limitations of this preliminary study, primarily the small clinical sample size for validation. Future work should focus on larger-scale, multi-center prospective studies to firmly establish clinical performance. Furthermore, while this assay targets the key virulence factor *ctxA*; exploring multiplexing capabilities to simultaneously identify serogroups O1 and O139 would enhance its epidemiological utility, like other developed multiplex assays. Considerations regarding cost-effectiveness compared to other rapid tests and optimal primer design strategies for RPA also warrant further detailed investigation.

Conclusions

The RPA-based isothermal amplification method is a rapid, sensitive, and specific tool for detecting *V. cholerae*. Its performance, supported by initial clinical and statistical evaluation, confirms its potential as an effective aid for the detection of this critical pathogen in clinical and field settings, contributing to timely diagnosis and outbreak control. The method paves the way for point-of-care application, especially in resource-limited areas where cholera burdens are high.

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Ethical considerations

Negative controls included healthy individuals from the same region, with no history of cholera. This study was approved by the Clinical Research Ethics Committee of The First Affiliated Hospital of Zhejiang Chinese Medical University (IRB No. 2024-KLS-637-02). Written informed consent was obtained from all participants, including patients and healthy volunteers, prior to sample collection.

Data availability

Data in support of the findings of this study are available from the corresponding author upon reasonable request.

Authors' Contributions

L-GL, study design, manuscript draft and revision, supervision, funding acquisition; YS, study design, manuscript draft and revision; S-ML, manuscript draft and revision, funding acquisition; H-LL, data analysis, manuscript draft and revision; J-LW, data analysis; YL, data analysis

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Corresponding Author

Li-Guo Liang, Professor, MD, PhD.
Centre for Clinical Laboratory, The First Affiliated Hospital of Zhejiang Chinese Medical University,
Hangzhou, 310006, China.
Tel: +8615861481568
Fax: 0571-87062291
Email: lianglg@zju.edu.cn

Conflict of interests

The authors have no declared conflict of interest relevant to the content of this article. Hong-Liang Liu, a student at The First Affiliated Hospital of Zhejiang Chinese Medical University, conducted the research during an internship at Shandong HuiFa Food Co., Ltd. The work was performed using the company's facilities under a collaborative internship agreement with no financial cost incurred.

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