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Original Article

Establishment and application of a rapid visualization method for detecting *Vibrio parahaemolyticus* nucleic acid

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ABSTRACT

Background: Swift and accurate detection of *Vibrio parahaemolyticus*, which is a prominent causative pathogen associated with seafood contamination, is required to effectively combat foodborne disease and wound infections. The *toxR* gene is relatively conserved within *V. parahaemolyticus* and is primarily involved in the expression and regulation of virulence genes with a notable degree of specificity. The aim of this study was to develop a rapid, simple, and constant temperature detection method for *V. parahaemolyticus* in clinical and nonspecialized laboratory settings.

Methods: In this study, specific primers and CRISPR RNA were used to target the *toxR* gene to construct a reaction system that combines recombinase polymerase amplification (RPA) with CRISPR–Cas13a. The whole-genome DNA of the sample was extracted by self-prepared sodium dodecyl sulphate (SDS) nucleic acid rapid extraction reagent, and visual interpretation of the detection results was performed by lateral flow dipsticks (LFDs).

Results: The specificity of the RPA-CRISPR/Cas13a-LFD method was validated using *V. parahaemolyticus* strain ATCC-17802 and six other non-parahaemolytic *Vibrio* species. The results demonstrated a specificity of 100%. Additionally, the genomic DNA of *V. parahaemolyticus* was serially diluted and analysed, with a minimum detectable limit of 1 copy/μL for this method, which was greater than that of the TaqMan-qPCR method (10^2 copies/μL). The established methods were successfully applied to detect wild-type *V. parahaemolyticus*, yielding results consistent with those of TaqMan-qPCR and MALDI-TOF MS mass spectrometry identification. Finally, the established RPA-CRISPR/Cas13a-LFD method was applied to whole blood specimens from mice infected with *V. parahaemolyticus*, and the detection rate of *V. parahaemolyticus* by this method was consistent with that of the conventional PCR method.

Conclusions: In this study, we describe an RPA-CRISPR/Cas13a detection method that specifically targets the *toxR* gene and offers advantages such as simplicity, rapidity, high specificity, and visual interpretation. This method serves as a valuable tool for the prompt detection of *V. parahaemolyticus* in nonspecialized laboratory settings.

1. Introduction

Vibrio parahaemolyticus, which is a commonly encountered Gram-negative bacterium, is a halophilic *Vibrio* species that can be detected not only in various marine products, such as fish, shrimp, and shellfish [1], but also in ready-to-eat food items [2]. Infection with *V. parahaemolyticus* not only gives rise to gastrointestinal diseases characterized by symptoms such as watery diarrhoea, nausea, vomiting, and abdominal cramps, but can

Abbreviation: RPA, Recombinase polymerase amplification; CRISPR/Cas, Clustered regularly interspaced short palindromic repeats and CRISPR associated protein; crRNA, CRISPR RNA; ssRNA, Single-Stranded Ribonucleic Acid; Taqman-qPCR, Taqman Real-time quantitative Polymerase chain reaction; *V. parahaemolyticus*, *Vibrio parahaemolyticus*; MALDI-TOF MS, Matrix-assisted laser desorption ionization time-of-flight mass spectrometry; LAMP, Loop-mediated isothermal amplification; LFD, Lateral flow dipstick; SDS, sodium dodecyl sulphate; ddH_2O, Double distilled water.

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also lead to wound infections. In severe cases, these infections can result in sepsis, shock, and even mortality [3]. Reports of *V. parahaemolyticus* gastroenteritis outbreaks have been documented in multiple countries worldwide, thus highlighting its status as a prevalent and significant public health concern [4–6]. In China, *V. parahaemolyticus* is the second most common cause of infectious diarrhoea [7]. Therefore, the establishment of sensitive and rapid detection methods holds crucial significance for providing effective diagnoses, controlling *V. parahaemolyticus* infections, and safeguarding public health. *V. parahaemolyticus* carries various virulence factors, among which the *toxR* gene encodes a transmembrane protein involved in bacterial membrane formation, transport, and the expression of specific virulence genes. The *toxR* gene is relatively conserved within *V. parahaemolyticus* species and has a high degree of specificity, making it a common target for nucleic acid detection [7,8].

Currently, there are several commonly used methods for detecting *V. parahaemolyticus*, including pathogen-based diagnosis, immunological diagnosis, and molecular diagnosis [9]. Among these methods, isolation and culture identification are considered the 'gold standard' for *V. parahaemolyticus* detection [3]; however, this approach is time-consuming and labor-intensive and does not meet the requirements for rapid testing [1]. Immunological diagnosis techniques, such as enzyme-linked immunosorbent assays (ELISAs) and colloidal gold detection, require the preparation of high-quality antigens and specific antibodies, resulting in high costs and lengthy procedures. Moreover, they are prone to interference, leading to false-positive results due to cross-reactivity or false-negative results during the window period due to insufficient sensitivity. With the rapid development of molecular biology, nucleic acid detection techniques, including quantitative PCR and multiplex PCR, have been widely applied for the rapid detection of *V. parahaemolyticus* [10,11]. However, PCR methods typically require specialized and expensive equipment, complex testing procedures, and skilled operators, making rapid testing inconvenient in nonspecialized laboratories or non-laboratory settings [12]. Isothermal amplification techniques, such as recombinase polymerase amplification (RPA) and loop-mediated isothermal amplification (LAMP), are increasingly being used for the rapid nucleic acid detection of pathogens in non-laboratory settings because they do not require specialized equipment or skilled personnel [13–15]. These isothermal amplification techniques have their own advantages and disadvantages. Compared with LAMP, RPA offers the advantages of relatively simple primer design and the ability to achieve significant amplification of nucleic acids within 30 min at a constant temperature of 37–42°C [16]. However, the sample preprocessing and nucleic acid extraction processes of RPA still require specific equipment, such as a centrifuge, and are time-consuming [16]. To some extent, this limitation restricts the application of RPA in the development of nucleic acid detection methods. Therefore, improving existing nucleic acid extraction methods would be beneficial for increasing the application of RPA for on-site testing.

Clustered regularly interspaced short palindromic repeats-associated protein (CRISPR–Cas), which is an adaptive immune system found in prokaryotes, exhibits specific cleavage activity when a specific CRISPR RNA (crRNA) recognizes DNA or RNA targets [17]. Currently, three types of CRISPR–Cas systems have been identified, with Type II systems requiring only one nucleic acid endonuclease, one guide RNA, and one adjacent protospacer motif for target cleavage [18]. Cas13a, also known as C2c2, is a single-effector RNA-guided RNA enzyme in the Type II CRISPR–Cas system. Upon specific recognition of its single-stranded RNA (ssRNA) target, this enzyme is activated and exhibits nonspecific endonuclease activity. By cleaving nontarget ssRNAs (linked to fluorescent quenching groups or biotin reporter groups), this enzyme achieves signal amplification, thereby enhancing the sensitivity and specificity of the detection system [19]. Cas13a can cleave specific nucleic acid sequences without temperature cycling, suggesting that combining the CRISPR/Cas13a system with isothermal amplification methods can enhance the sensitivity of isothermal amplification detection methods [20]. Zhang et al. combined the RPA technique with the CRISPR–Cas13a system to establish the specific high-sensitivity enzymatic reporter unlocking (SHERLOCK) nucleic acid detection system, which has been successfully applied for the rapid detection of *Zika* virus, *dengue* virus, and other pathogens. This technology offers high sensitivity and excellent specificity, making it highly valuable in rapid pathogen nucleic acid detection [21]. RPA can be performed with CRISPR–Cas13a detection in the same reaction tube, thus simplifying the detection process and reducing the risk of contamination. Additionally, the Cas13a protein can be used to cleave ssRNA probes to allow for detection through fluorescence or visualization of the results on lateral flow dipstick (LFD) test strips. LFD test strips are portable and can be visually read, thus eliminating the need for fluorescence detection equipment and making them more suitable for on-site testing and testing in resource-limited areas.

This study aimed to establish a simple, efficient, and portable rapid nucleic acid detection method for *V. parahaemolyticus* based on the RPA-CRISPR/Cas13a reaction system and using the *toxR* gene as the target. Detection was completed within 50 min under isothermal conditions at 40°C. This method provides a convenient means for the rapid detection of pathogenic *V. parahaemolyticus* in non-laboratory settings.

2. Materials and methods

2.1. Strains and bacterial genome extraction

A total of seven standard strains were used in this study, namely, *V. parahaemolyticus* (ATCC 17802), *Vibrio alginolyticus* (ATCC 17749), *Vibrio fluvialis* (ATCC 27562), *Vibrio metschnikovii* (ATCC 700040), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli O157:H7* (ATCC 43888), and *Pseudomonas aeruginosa* (ATCC 27853), all of which were preserved in our laboratory. The nine wild-type *V. parahaemolyticus* strains were isolated from coastal seawater in China in previous studies (Table S1). The strains were retrieved from a −80°C freezer and individually inoculated onto blood agar plates for recovery. Further identification was performed using Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) mass spectrometry. After recovery, single colonies were picked and used to prepare bacterial suspensions with a turbidity of 0.5 McFarland standard (approximately 1×10^8 colony-forming units (CFU)/mL). Equal volumes of the bacterial suspensions (20 μL) were subjected to genomic DNA extraction using either the magnetic bead-based method (hereafter referred to as the 'magnetic bead method') (Tiangen Biotech Co., Ltd, Beijing, China) or a self-prepared rapid DNA extraction method. The concentration and purity of the extracted template DNA were determined using a Tecan Infinite 200 PRO microplate reader.

The composition of the SDS rapid DNA extraction reagent was as follows: 20 μL of SDS lysis solution (0.04 μL of EDTA (0.5 M, pH 8.0; Mei5bio, China), 4 μL of 10% SDS (Beyotime, China), 2 μL of Tris (0.5 M, pH 8.0; Mei5bio), and 13.96 μL of ddH_2O) mixed with 20 μL of the bacterial suspension. The mixture was incubated at room temperature for 10 min, after which the supernatant was used as the amplification template.

2.2. Acquisition of the toxR gene sequence and alignment of toxR gene sequences from similar Vibrio species

The nucleic acid sequences of the *toxR* gene were downloaded from the GenBank database. A relatively conserved region was selected as the detection target based on sequence alignment with other *Vibrio* species. The alignments were performed with ClustalX software with the default parameter settings. The alignment images were produced with ESPript software (http://espript.ibcp.fr).

2.3. Design of primers, probes, crRNAs, and RNA reporters

RPA and TaqMan qPCR-amplified primers and probes were designed with Primer Premier 7.0 software (Premier Biosoft International, CA, USA). The species specificity of the primer and probe sequences was preliminarily validated using Primer BLAST on the NCBI website. The primers, probes, crRNAs, and RNA reporters were synthesized by Shanghai Sangon Biotech, as detailed in Table S2.

2.4. PCR system and amplification conditions

The PCR mixture consisted of 10 μL of 2 × Mix of PCR Buffer (Tiangen Biotech Co., Ltd, Beijing, China), 0.6 μL of forward primer (10 μM), 0.6 μL of reverse primer (10 μM), 0.4 μL of TaqMan fluorescent probe (10 μM), 1 μL of template, and 7.4 μL of ddH_2O. The amplification program was set as follows: 95°C for 5 min, then 95°C for 30 s and 58°C for 40 s (fluorescence collection) for 35 cycles.

2.5. RPA reaction system and amplification conditions

One RPA lyophilized reagent (TwistAmp Basic Kit, TwistDx, UK) was used for the detection of five samples. First, 29.5 μL of the rehydration buffer provided in the TwistAmp Basic Kit was added to rehydrate one lyophilized RPA pellet. Then, in the rehydrated RPA pellet mixture, 2.1 μL of forward primer (10 μM), 2.1 μL of reverse primer (10 μM), 1.2 μL of ddH_2O, 3 μL of T7 RNA polymerase (50 U/μL; Lucigen, USA), and 4 μL of nucleotide mixture (25 mM; NEB, USA) were added and the sample was mixed thoroughly. The mixture was then divided equally into five 200 μL Eppendorf tubes, and 1 μL of the test sample was added to each tube. Then, 0.5 μL of magnesium acetate (280 mM) was added to each reaction tube to initiate the RPA amplification reaction at 40°C, which lasted for 20 min.

2.6. RPA-CRISPR/Cas13a-LFD

Following the same procedure described above, after completion of the RPA reaction, the CRISPR/Cas13a reaction system ((2.0 μL of Tris (400 mM, pH 7.4; Sigma, USA), 1 μL of $MgCl_2$ (120 mM; Sigma), 1 μL of LwaCas13a (20 ng/μL; GenScript, China; Jiangsu East-Mab Biomedical Technology Co., Ltd, China), 1 μL of RNase inhibitor (40 U/μL; Takara, Japan), 1 μL of crRNA (10 ng/μL), and 1 μL of LFD Reporter (10 μM)) was set up. The CRISPR/Cas13a reaction system and LFD reporter group on the caps of the reaction tubes were mixed with the RPA reaction products by brief centrifugation. The mixture was then incubated at 37°C for 25 min. After the reaction, 80 μL of HybriDetect assay buffer was added to each reaction tube. Subsequently, test strips (Milenia Biotech) were placed into each reaction tube, and after allowing the reaction mixture to flow onto the strip, the results were read.

2.7. Infection of mice with V. parahaemolyticus

Six- to eight-week-old female specific-pathogen-free BALB/c mice ((20 ± 5) g) were purchased from Hua-

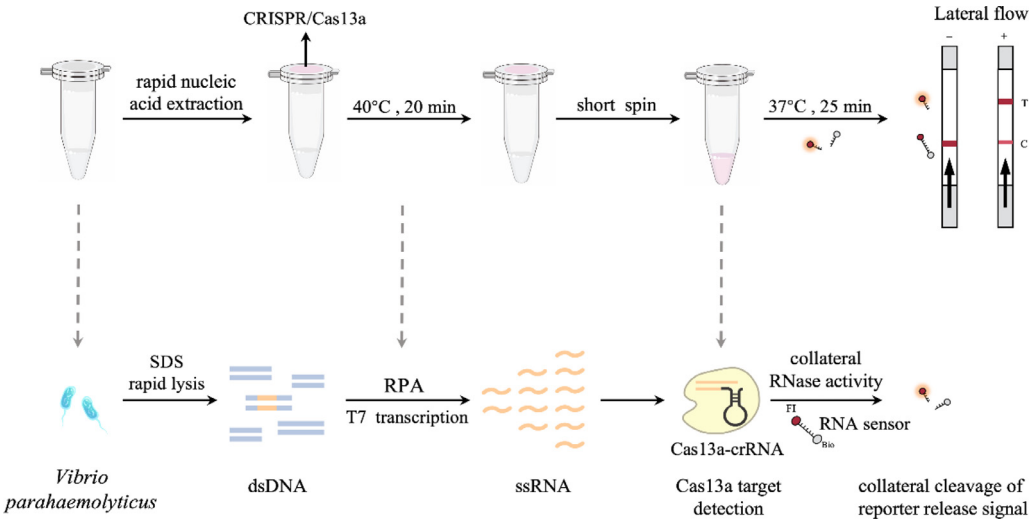

Fig. 1. RPA-CRISPR/Cas13a method for the detection of *V. parahaemolyticus*.

fukang Biotechnology Co., Ltd (Beijing, China). This study was approved by the Ethical Committee on Animal Experimentation of the Sixth Medical Center of the PLA General Hospital (IACUC-DWZX-2023-P520). Mice were housed in stainless steel cages and allowed to acclimatize to the environmental conditions for 5 days before the experiment. To simulate *V. parahaemolyticus* infection, six randomly selected mice were assigned to the experimental group, and six strains of wild-type *V. parahaemolyticus* were randomly gavaged into the peritoneal cavity of the mice; each mouse was gavaged with a suspension of *V. parahaemolyticus* (1×10^8 CFU/mL) at 0.2 mL/10 g body weight. The three control mice were gavaged with PBS. The clinical condition and mortality of each group of mice were observed, and 24 h later, blood samples were collected from the heart of each mouse. The whole blood specimens were used for RPA-CRISPR/Cas13a-LFD and PCR detection.

3. Results

3.1. RPA-CRISPR/Cas13a detection of V. parahaemolyticus

RPA was first used for large-scale amplification of *toxR* DNA fragments, after which the amplified fragments were transcribed into ssRNAs by T7 RNA polymerase *in vitro*. The complementary binding of crRNA to the target ssRNA activated the incidental cleavage activity of the Cas13a protein to RNA reporter cleavage and combined with LFD to complete the detection process (Fig. 1).

3.2. Rapid nucleic acid extraction of V. parahaemolyticus genomic DNA by SDS versus magnetic bead extraction

To evaluate the efficiency of the self-prepared SDS nucleic acid rapid extraction method, two indicators, 'concentration' and 'purity', of the extracted nucleic acid templates were compared with those extracted by a commercial kit (magnetic bead method). As shown in Fig. 2A and B, for the same concentration of bacterial solution, there was no significant difference in the nucleic acid extraction efficiency between the two methods ($P > 0.05$).

To assess whether the self-prepared nucleic acid rapid extraction reagents have an impact on the subsequent amplification efficiency, TaqMan-qPCR and RPA amplification were performed simultaneously using the two extraction methods with genomic templates. As shown in Fig. 2C and D, the Ct values of the TaqMan-qPCR products for both sample groups were similar. During RPA amplification, at approximately 7 min, both groups exhibited fluorescence signals almost simultaneously, forming similar 'S-shaped curves' with comparable peak fluorescence values. By contrast, the negative control showed no signal, indicating that the template extracted using the self-prepared reagents did not affect the subsequent nucleic acid amplification and detection.

3.3. Establishment and optimization of the RPA-CRISPR/Cas13a assay for the V. parahaemolyticus toxR gene

First, the *toxR* gene sequences of *V. parahaemolyticus* strains were aligned with those of seven other *Vibrio* species (*V. alginolyticus, V. harveyi, V. vulnificus, V. cholerae, V. anguillarum, V. mediterranei*, and *V. shilonii*) (Fig. S1a). The alignment results indicated that a 213 bp region of the *toxR* coding sequence at positions 318 to 530 exhibited significant sequence diversity among different *Vibrio* species, suggesting that this region is suitable for the design of specific primers and crRNAs. Three pairs of RPA candidate primers were designed to target the *toxR* gene, and the amplification products were analysed via gel electrophoresis. The results showed that the F1/R1 primer pair produced the strongest amplification band

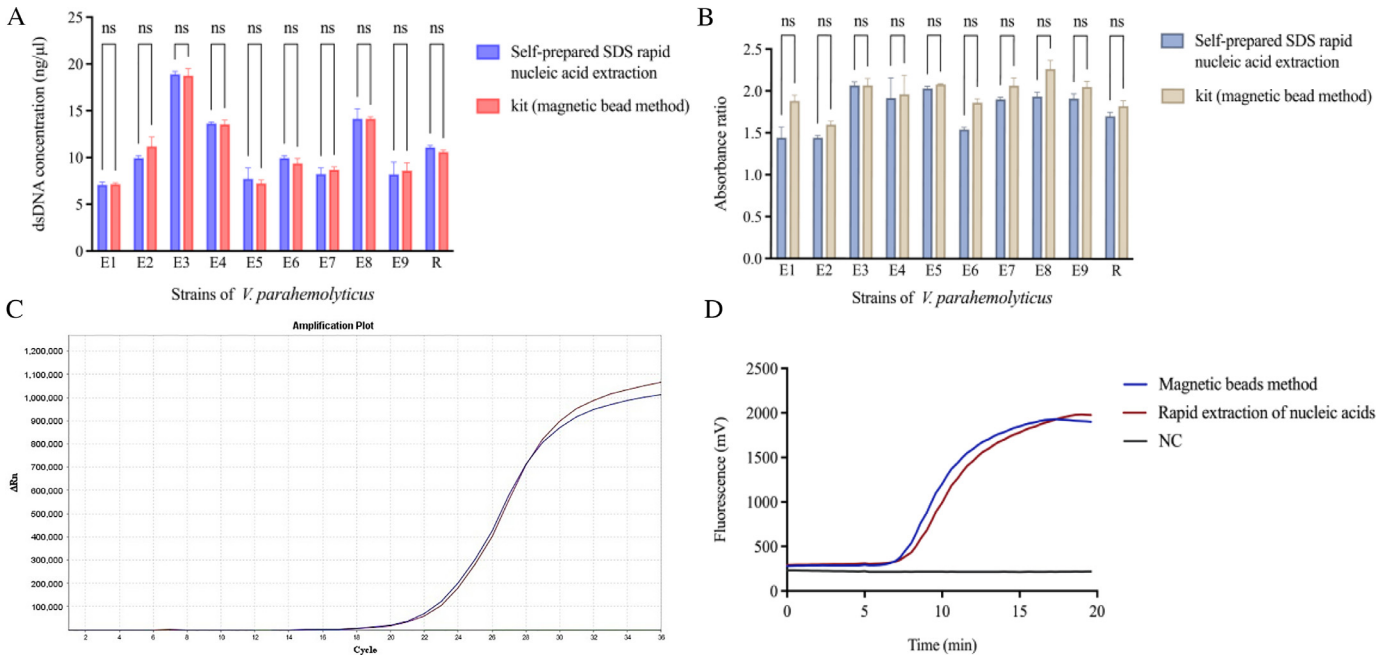

Fig. 2. Comparison of rapid SDS nucleic acid extraction of *Vibrio parahaemolyticus* genomic DNA with magnetic bead method. A. Genomic DNA concentration of *V. parahaemolyticus* extracted by SDS rapid nucleic acid extraction or a kit (magnetic bead method). B. Genomic DNA purity of *V. parahaemolyticus* extracted by SDS rapid nucleic acid extraction or a kit (magnetic bead method). C. The results of TaqMan-qPCR amplification of *V. parahaemolyticus* extracted by SDS rapid nucleic acid extraction or a kit (magnetic bead method). D. The results of RPA amplification detection of *V. parahaemolyticus* extracted by SDS rapid nucleic acid extraction or a kit (magnetic bead method). E, environmental strain; R, reference strain ATCC17802; NC, negative control.

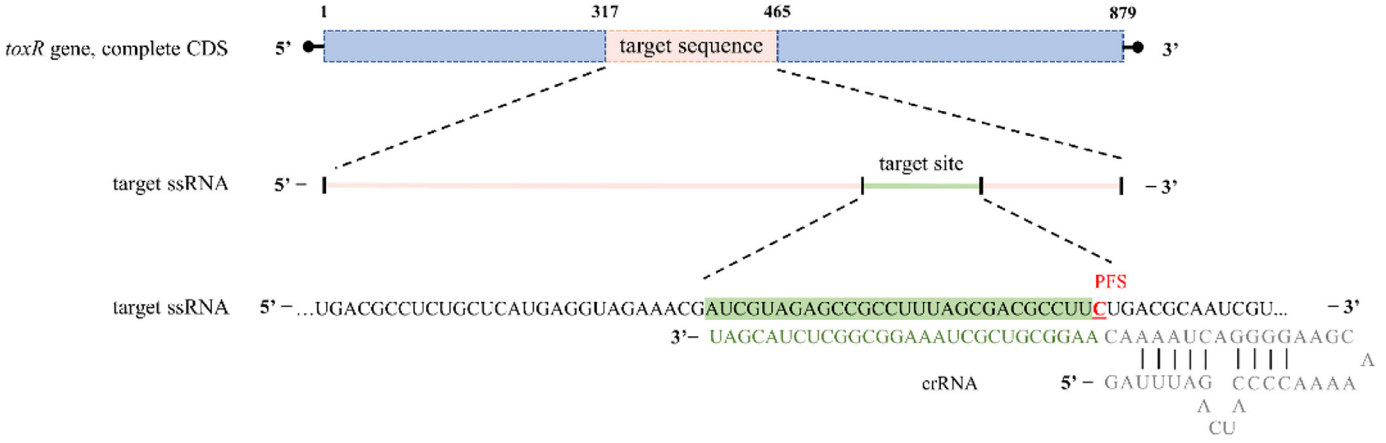

Fig. 3. Design of crRNA. The first horizontal line represents the complete CDC sequence of the *V. parahaemolyticus toxR* gene. The target sequence (amplification area) is highlighted in pink. The second and third horizontal lines represent the ssRNA substrate being targeted by the crRNA. The target site is highlighted in green, and PFS is indicated by the magenta bar. PFS: protospacer flanking site.

(Fig. S1b), indicating a significantly greater amplification efficiency than that of the other primer pairs. Therefore, the F1/R1 primer pair was selected for subsequent experiments, and crRNAs were designed within the region covered by this primer pair (Fig. 3).

3.4. Specificity of the RPA-CRISPR/Cas13a method for V. parahaemolyticus detection

To evaluate the specificity of the detection system, four *Vibrio* species (*V. parahaemolyticus, V. alginolyticus, V. fluvialis*, and *V. metschnikovi*) and three common diarrhoea-causing organisms (*S. aureus, E. coli O157:H7*, and *P. aeruginosa*) were selected for testing; the results showed that the amplification of all of the other tested *Vibrio* species and diarrhoea-causing organisms, except for *V. parahaemolyticus*, was negative, and there was no cross-reactivity (Fig. 4), which indicated that the RPA-CRISPR/Cas13a-LFD assay has high specificity for *V. parahaemolyticus*.

3.5. Sensitivity of the RPA-CRISPR/Cas13a method for V. parahaemolyticus detection

To verify the sensitivity of the two methods, *V. parahaemolyticus* genomic DNA was serially diluted,

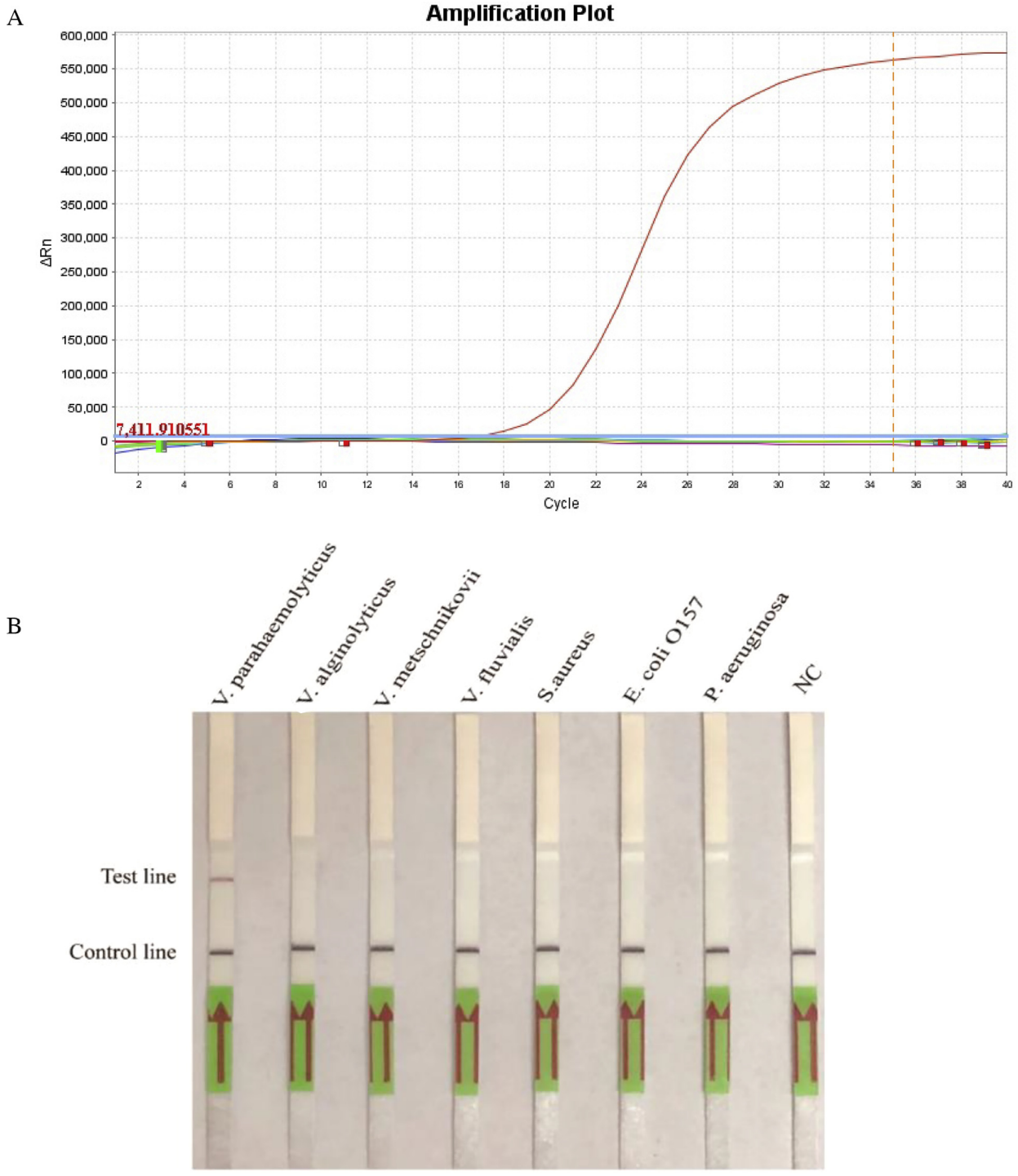

Fig. 4. Evaluation of the specificity of the RPA-CRISPR/Cas13a assay for *V. parahaemolyticus* detection. A. TaqMan-qPCR method; B. RPA-CRISPR/Cas13a-LFD method. NC, negative control.

with concentrations ranging from 10^9 copies/μL to 1 copy/μL. The amplification results showed that the detection limit of the RPA-CRISPR/Cas13a-LFD method was 1 copy/μL, which was higher than the sensitivity of the TaqMan-qPCR method (10^2 copies/μL) (Fig. 5).

3.6. Evaluation of the efficacy of the RPA-CRISPR/Cas13a system for detecting wild-type strains of V. parahaemolyticus

To validate the effectiveness of the RPA-CRISPR/Cas13a-LFD method for detecting wild-type *V. parahaemolyticus* strains, the genomic DNA of nine

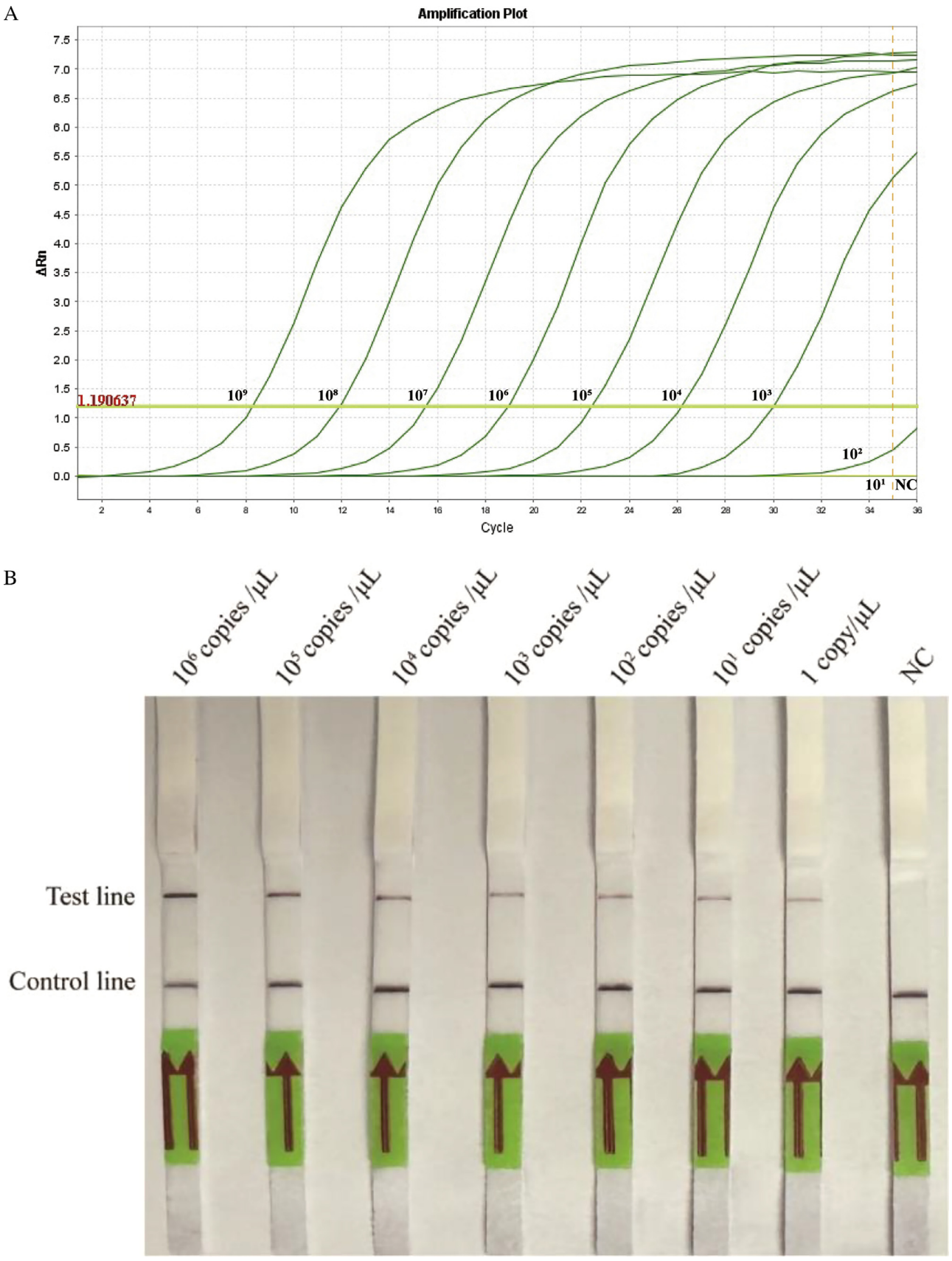

Fig. 5. Sensitivity of the RPA-CRISPR/Cas13a assay for *V. parahaemolyticus* detection. A. TaqMan-qPCR method; B. RPA-CRISPR/Cas13a-LFD method. NC, negative control.

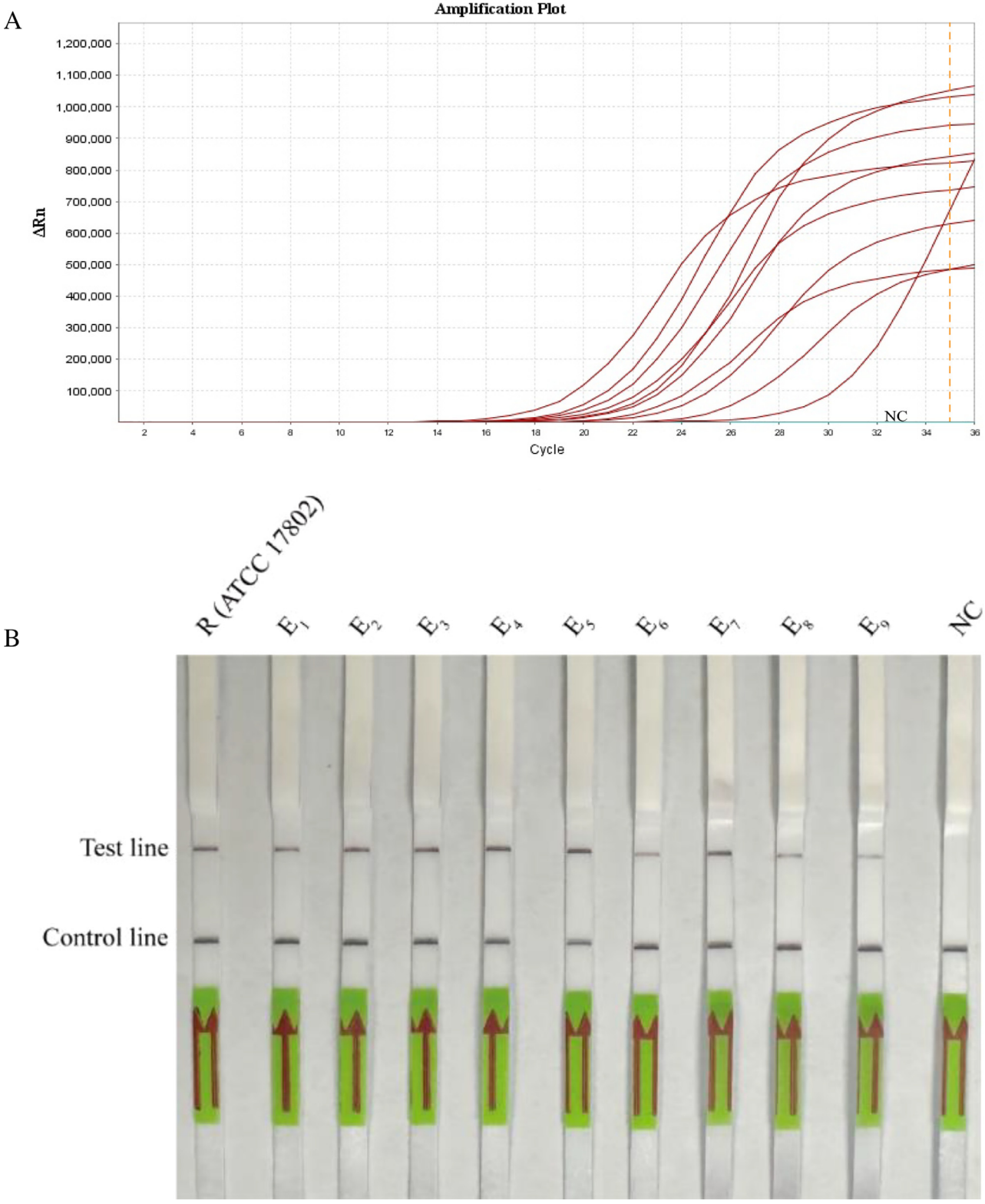

Fig. 6. Validation of the RPA-CRISPR/Cas13a assay for *V. parahaemolyticus* detection using environmental strains. A. TaqMan-qPCR method; B. RPA-CRISPR/Cas13a-LFD method. E, environmental strain; NC, negative control.

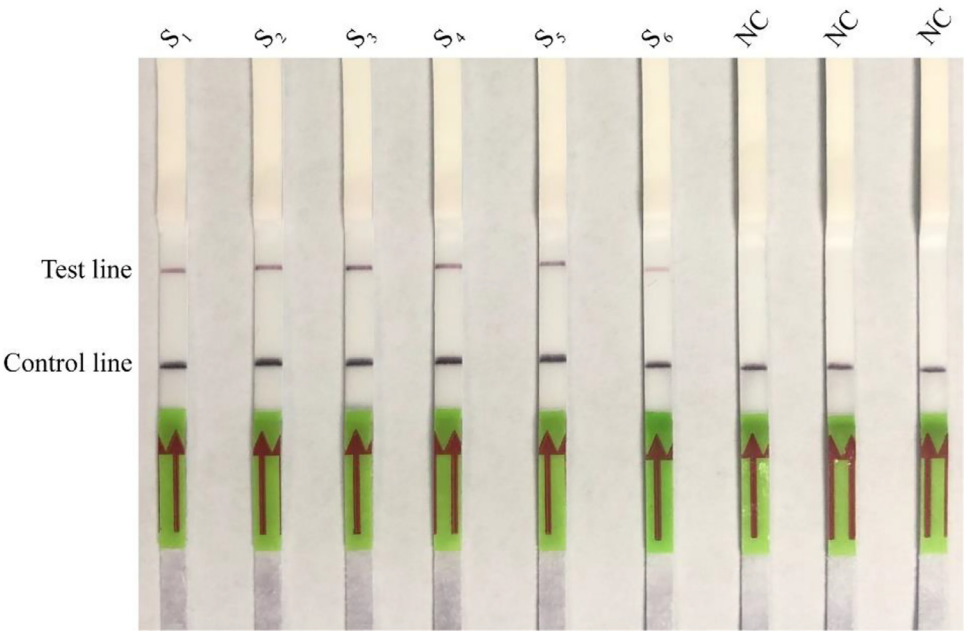

Fig. 7. Detection of *V. parahaemolyticus* by RPA-CRISPR/Cas13a-LFD in whole blood specimens from infected mice. S, sample; NC, negative control.

V. parahaemolyticus wild-type strains was simultaneously tested using the CRISPR/Cas13a-LFD method and the TaqMan-qPCR method. The results of both methods were consistent (Fig. 6).

3.7. Detection of V. parahaemolyticus infection in mice by the RPA-CRISPR/Cas13a-LFD method

The RPA-CRISPR/Cas13a-LFD method was used to detect *V. parahaemolyticus* in whole blood specimens from infected mice and to compare the detection accuracy with that of the traditional PCR method. The results of the RPA-CRISPR/Cas13a-LFD method in whole blood specimens from infected and control mice were consistent with the PCR results (Fig. 7 and Table S3).

4. Discussion

V. parahaemolyticus is a prominent foodborne pathogen that has become an increasingly common cause of epidemics worldwide. *V. parahaemolyticus* poses a significant threat to human health and seafood safety. Currently, the traditional bacterial culture identification method is the gold standard for *V. parahaemolyticus* detection and identification. However, this method is time-consuming, requires skilled personnel, and is not suitable for rapid testing in nonspecialized laboratory settings. Therefore, there is a need to establish a rapid, accurate, cost-effective, and field-applicable detection method that can effectively address the challenges of rapid *V. parahaemolyticus* testing in resource-limited areas with a shortage of skilled professionals.

In this study, a rapid visual method for the detection of *V. parahaemolyticus* nucleic acid was established based on a combination of RPA isothermal amplification and the CRISPR/Cas13a system. In research on different rapid nucleic acid detection methods, achieving simplified nucleic acid extraction under nonspecialized laboratory conditions is a crucial factor that limits their application. In this study, we attempted to develop a rapid nucleic acid extraction reagent and compared it with a commercial magnetic bead extraction reagent. The results showed that the rapid nucleic acid extraction reagent exhibited comparable efficacy in extracting nucleic acids from an equal amount of bacterial suspension, and the difference was not significant ($P > 0.05$). However, rapid nucleic acid extraction only requires simple manual inversion and a 10-minute incubation at room temperature to complete the extraction of sample nucleic acids; thus, this method has a shorter processing time and is easier to perform, making it more suitable for on-site applications.

To further improve the sensitivity of RPA detection, in this study, the T7 promoter sequence was introduced into the forward primer for RPA amplification and subsequently transcribed into a large amount of ssRNA by T7 RNA polymerase, which activated the nonspecific nucleic acid endonuclease activity of Cas13a to increase the sensitivity of detection. The results showed that the RPA-CRISPR/Cas13a-LFD method was more sensitive than the TaqMan-qPCR (10^2 copies/μL) method, and its detection limit was 1 copy/μL. The sensitivities reported by different research teams based on the SHERLOCK nucleic acid detection system are slightly different. Zhou et al. achieved a sensitivity of 1 copy/μL for *S. aureus* using the RPA-CRISPR/Cas13a-fluorescence assay, whereas Miao et al. achieved a sensitivity of only 10^3 copies/μL for *Nipah* virus using the same method [20–22]. Although these detection methods are based on the same principle, the compositions of the detection systems constructed for different pathogens are different, and the corresponding

detection sensitivities therefore also differ. This difference may be due to the different excision efficiencies of the designed crRNAs in combination with the ssRNA that is synthesized by *in vitro* transcription of the corresponding pathogen genomes. In addition, the different sequences of fluorescent reporters used in different studies have different incidental cleavage efficiencies for crRNAs, which in turn affects the strength of the final fluorescent signal released, leading to differences in detection sensitivity. For this reason, we will explore these differences in depth in future studies to improve the sensitivity of the RPA-CRISPR/Cas13a-LFD assay.

The design of crRNA for CRISPR–Cas13a is relatively simple and requires only 24 or more nucleotides to complementarily pair with the ssRNA; however, the crRNA possesses high target specificity, which further enhances detection specificity [14]. The results showed that the RPA-CRISPR/Cas13a-LFD method and the TaqMan-qPCR method exhibited a specificity of 100% for detecting *V. parahaemolyticus*, and there was no cross-reactivity with the other seven pathogenic bacteria tested. Finally, we tested nine wild-type *V. parahaemolyticus* strains isolated from the coastal waters of China to verify the feasibility of the RPA-CRISPR/Cas13a system and found that the RPA-CRISPR/Cas13a-LFD method produced results consistent with those of the TaqMan-qPCR method and MALDI-TOF MS mass spectrometry identification, all yielding positive results with a detection rate of 100%. Finally, the established RPA-CRISPR/Cas13a-LFD method was applied to the whole blood specimens of mice infected with *V. parahaemolyticus*, and the detection rate of *V. parahaemolyticus* by this method was consistent with that of the conventional PCR method. Therefore, the RPA-CRISPR/Cas13a system established in this study provides sufficient sensitivity and specificity for the detection of *V. parahaemolyticus*.

In this study, all reaction components were added to a single Eppendorf tube before the reaction was initiated. The RPA isothermal amplification and T7 *in vitro* transcription systems were added to the bottom of the tube, while the CRISPR/Cas13a system was added to the tube cap. After the RPA thermostatic amplification and T7 *in vitro* transcription system reaction were completed at the bottom of the Eppendorf tube, the tube was briefly centrifuged to mix the CRISPR/Cas13a system on the tube cap with the transcribed products and initiate the reaction, thereby activating the targeted and collateral cleavage of the Cas13a protein, which induced the fluorescence signal emission. Therefore, in this study, we achieved a 'one-pot reaction' and completed the entire detection process in the same tube without the need to transfer reaction components, thereby reducing potential cross-contamination that may be caused by amplification products and avoiding false positives during subsequent detection. Moreover, the CRISPR/Cas13a system reacted with all of the transcribed products, thereby improving the detection sensitivity. Furthermore, compared with traditional PCR-based methods, the detection method based on the RPA-CRISPR/Cas13a system has cleavage activity towards the transcribed target ssRNA, which is more easily degraded than DNA, thereby reducing the risk of cross-contamination. RPA amplification at 40°C also reduces the generation of aerosols during high-temperature PCR, thereby reducing the chance of nucleic acid fragment contamination. Additionally, compared with previous studies on *V. parahaemolyticus* nucleic acid detection, the detection method established in this study does not require pre-enrichment, which allows for a lower limit of detection but also a shorter detection time. The RPA-CRISPR/Cas13a-LFD method can even provide visual results with the naked eye, making this method simple and convenient.

5. Conclusion

We established a rapid, sensitive, and portable detection method for *V. parahaemolyticus*, namely the RPA-CRISPR/Cas13a-LFD method. This method can be applied for the rapid detection of *V. parahaemolyticus* in field settings and resource-limited remote epidemic areas. This new approach may have significant implications for human health and food safety.

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Author contributions

C.G.C., Y.C.H., and Y.N.W. designed the research. Y.C.H., X.P.L., Y.N.W., L.G., L.Y.W., W.R.X., Y.Q.Z., J.C., W.W.X., and C.G.C. conducted the research. Y.C.H. wrote the manuscript. C.G.C. directed the project. All of the authors have read and approved the manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data available statement

The data that support the findings of this study are available on request from the corresponding author.

Ethics statement

This study was approved by the Ethical Committee on Animal Experimentation of the Sixth Medical Center of the PLA General Hospital (IACUC-DWZX-2023-P520).

Informed consent

Not applicable.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.imj.2024.100111.

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