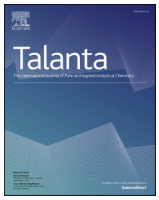
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Engineering of an adaptive tandem CRISPR/Cas12a molecular amplifier permits robust analysis of *Vibrio parahaemolyticus*

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ABSTRACT

Seeking new molecular diagnostic method for pathogenic bacteria detection is of utmost importance for ensuring food safety and protecting human health. Herein, we have engineered an adaptive tandem CRISPR/Cas12a molecular amplifier specifically designed for robust analysis of vibrio parahaemolyticus (*V. parahaemolyticus*), one of the most harmful pathogens. Our strategy involves the integration of three crucial processes: recombinase polymerase amplification (RPA) for copy number amplification, terminal deoxynucleotidyl transferase (TdT) for template-free strand elongation, and CRISPR/Cas12a-mediated *trans*-cleavage of a reporter molecule. By combining these processes, the target genomic DNA extracted from *V. parahaemolyticus* is able to activate many CRISPR/Cas12a units (CRISPR/Cas12a^{n}) simultaneously, resulting in a greatly amplified target signal to indicate the presence and concentration of *V. parahaemolyticus*. This unique model offers more advantages compared to traditional amplification models that use one RPA amplicon to activate one CRISPR/Cas12a unit. Under optimized conditions, our method enables the detection of target *V. parahaemolyticus* within a linear range of 1×10^2–1×10^7 CFU/mL, with an impressive limit of detection as low as 12.4 CFU/mL. It is conceivable that the adaptive tandem CRISPR/Cas12a molecular amplifier could be adapted as routine diagnostic kits in future for in-field detection of pathogens.

1. Introduction

The widespread presence of an increasing number of pathogenic bacteria has raised significant concerns among the public regarding food safety and the potential for disease outbreaks [1,2]. One prominent example is *Vibrio parahaemolyticus* (*V. parahaemolyticus*), a Gram-negative, rod-shaped, and motile bacterium belonging to the Vibrio species within the Vibrionaceae family [3–5]. This bacterium is widely distributed in various environments such as soil, marine and estuarine waters, and the intestinal tracts of animals, which has been commonly found in seafood, food-processing facilities, manufacturing environments, as well as in water contaminated with fecal matter [6–8]. As a result of its prevalence, *V. parahaemolyticus* has emerged as a primary cause of seafood-associated gastroenteritis in humans. Although considerable efforts have been made to control and prevent its contamination, the infection risk with *V. parahaemolyticus* is still high. Factors contributing to this risk include the abundance of the pathogen at the time of harvest, environmental conditions during processing, and the potential for cross-contamination [5,9]. This situation stresses the need of analytical tools for identification of pathogenic bacteria to ensure the safety of foods and to protect the human health.

Up to now, a variety of detection methods for *V. parahaemolyticus* have been developed [7,10–12]. The most traditional method of detection relies on bacterial culture, which has been officially exploited for the analysis of *V. parahaemolyticus* over the years [13,14]. However, this method is slow, laborious, and often requires several days to accomplish the entire detection procedure, involving steps such as pre-enrichment, selective enrichment, biochemical screening and

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serological confirmation. The results of such tests are not readily available within the desired timeframe in clinical laboratories. Alternatively, molecular testing method using PCR or advanced real-time PCR has been proven to be very useful for identification of *V. parahaemolyticus*. [15] These techniques can rapidly amplify specific nucleic acid segments to billions of copies in vitro within hours [16]. Although these methods show a very high sensitivity to even detect very limited amounts of target pathogen, their application is limited to well-equipped laboratories with skilled operators expertise in molecular biology and expensive instruments to perform thermal cycling for denaturation, annealing, and extension of DNA strands. These shortcomings impede their use in point-of-care testing and low-resource settings. In response to the challenges associated with PCR-based methods, considerable effort is now directed towards seeking isothermal nucleic acid amplification [17,18]. These approaches enable nucleic acid replication to occur at constant temperatures, eliminating the need for complex thermal cycling procedures and breaking through the traditional laboratory boundaries. As valuable and powerful as PCR, the most well established representative isothermal methods includes rolling circle amplification (RCA) [4], recombinase polymerase amplification (RPA) [19], loop-mediated isothermal amplification (LAMP) [20], and strand displacement amplification (SDA) [21,22]. Among them, RPA has gained significant attention over the past decade and has been widely applied in various fields, including bacterial detection [23–25]. Its popularity stems from its simplicity, high sensitivity and selectivity, rapid amplification, user-friendliness, and suitability for point-of-care testing in low-resource settings. Especially, the recent research hot spot of CRISPR and CRISPR-associated protein (CRISPR--Cas) has provided a promising opportunity of developing RPA integrated CRISPR/Cas12a diagnostic tools, exhibiting great potential in the qualitative and quantitative analysis of infectious pathogens [26–30]. At present, the most common method for the combination of RPA with CRISPR/Cas12a is as follows: first, RPA is used to amplify the target DNA containing a protospacer adjacent motif (PAM) recognition site; second, the significantly amplified target DNA is specifically bound with the crRNA to form crRNA-DNA hybrid, which activates the *trans*--cleavage activity of the Cas protein to indiscriminately degradation of any available single-stranded DNA (ssDNA) molecules into single/-double nucleotides for signal output [31–33]. However, in this classical case, one double-stranded DNA (dsDNA) amplicon obtained from RPA is only able to activate one CRISPR-Cas12a unit, compromising the signal amplification ability for detecting very low amounts of target DNA. Additionally, this approach requires the design of primers that can create a PAM site on the dsDNA amplicons, limiting its universality and applicability. This raises the question: How can we overcome the restriction imposed by the PAM and efficiently activate multiple CRISPR/Cas12a units during a single RPA amplification event to maximize the amplification of positive signals?

The terminal deoxynucleotidyl transferase (TdT) is an exceptional enzyme that can rapidly catalyze the repetitive addition of deoxyribonucleotides to the 3′-OH of oligodeoxyribonucleotides and single-stranded, or double-stranded DNA to several kilobases without relying on the presence of template [34–37]. Moreover, the synthesized DNA sequences produced by TdT is highly dependent on the substrate deoxyribonucleotide triphosphate (dNTP) pool. Consequently, by manipulating the extension of TdT using RPA amplicons as primers, it becomes possible to generate an elongated DNA chain. This, in turn, allows for the binding of multiple crRNAs along the DNA chain, thereby activating numerous CRISPR/Cas12a units. We can expect a significantly improved amplification signal attributed to the RPA--CRISPR/Cas12a^{n} (n »1) model rather than the conventional RPA-CRISPR/Cas12a model to detect interested targets via the facile integration of RPA, TdT, and CRISPR/Cas12a together.

Taken abovementioned into account, in this work, we designed a new pathogen detection method towards *V. parahaemolyticus* via the combination of RPA for isothermal amplification, TdT for template-free elongation, and CRISPR/Cas12a for *trans*-cleavage-based signal reporting. In this strategy, RPA amplifies the target DNA extracted from *V. parahaemolyticus*. The 3′-OH of the obtained isothermal RPA amplicons is then bound with TdT for bidirectional chain elongation to form new poly-A rich chains at two sides of the amplicon, allowing the capturing of multiple crRNAs designed with a poly-U segment along its strand to activate CRISPER/Cas12a^{n} (n »1). This activation finally *trans*-cleave lots of single-stranded reporter probe (RP), resulting in the emission of fluorescence. Of note, to control any background signal produced by the extension of polymerization primers by TdT, the Exonuclease I (Exo I) was applied to specifically degrade single-stranded primer pairs used for RPA. Based on this principle, even the target pathogen is present in trace amounts, we can expect to achieve a significantly improved signal to analyze and identify *V. parahaemolyticus* with high sensitivity and accuracy.

2. Experimental section

2.1. Materials and reagents

The DNA amplification primers (Forward primer:5′-GCGAA AGTGCTTGAGATGA-3′; Reverse primer: 5′-ACAGACGATGAGCG GTTGA-3′) and the crRNA strand (5′- UAAUUUCUACUAAGUGUA-GAUUUUUUUUUUUUUUUUUUUUUUUU-3′) were synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China). The Exo I with its buffer (670 mM Glycine-KOH, 67 mM $MgCl_2$, 10 mM DTT; pH = 9.5), Tris-EDTA (TE, 10 mmol/L Tris-HCl, 1 mmol/L EDTA; pH = 8.0), agarose, dATP (10 mM), sterile deionized water, Luria-Bertani (LB), 25–500 bp DNA Marker, and 6 × DNA loading buffer were purchased from Sangon Biotechnology Co., Ltd. (Shanghai, China). The TdT and 5 × TdT reaction buffer (500 mM HEPES, 40 mM $MgCl_2$, 0.5 mM DTT, pH = 7.2) were purchased from Takara Bio Inc. The CRISPR/Cas12a was offered from New England Biolabs (USA) Ltd. (Beijing, China). The basic nucleic acid isothermal amplification kit of RPA was purchased from the Synda Gene Technology Co., Ltd. (Suzhou, China).

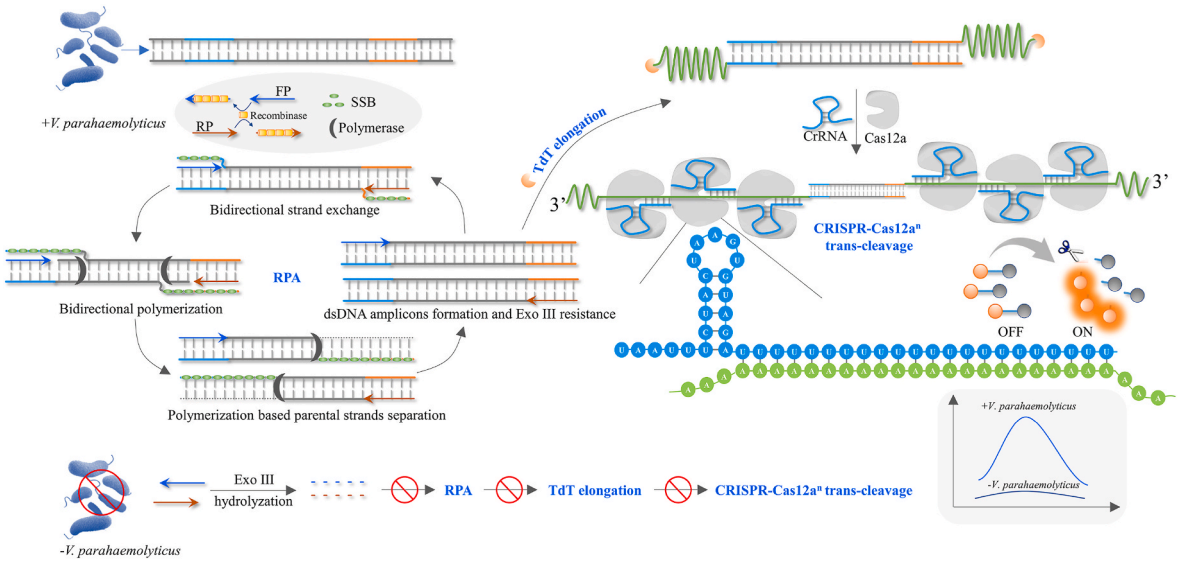
2.2. Apparatus

The Fluorescent spectra were measured using a F97 Pro fluorescence spectrophotometer (Shanghai Cold 116 Light Technology Co., Ltd.). The excitation wavelength was set to 490 nm, and the emission spectra were collected in the range of 500–650 nm. Both the excitation and emission slits were set to 10 nm, and the photomultiplier tube (PMT) voltage was set at 650 V.

The gel electrophoresis analysis (2% agarose prepared with 4s red plus) was performed by mixing 5 μL of oligonucleotide sample and 2 μL of 6 × loading buffer using 1 × tris-borate-EDTA (TBE) (18 mM Tris, 18 mM boric acid, 2 mM EDTA, pH = 8.0) as the electrophoretic buffer. The assay was then carried out at a constant voltage at 120 V for 30 min using a gel electrophoresis instrument (Beijing Liuyi biotechnology Co., Ltd.). Final imaging of the agarose gel was performed on an image system (Hangzhou Dragon Gene Scientific Instrument Co., Ltd, China).

2.3. Bacterial culture and DNA extraction

The *V. parahaemolyticus* were offered from the Guangdong Microbial Culture Collection Center (GDMCC) and cultured in Luria-Bertani (LB) medium at 37 °C for 18 h. The bacterial colony count was determined using the conventional plate counting method. To extract the genomic DNA, 1 mL of V. parahaemolyticus with a specific concentration was centrifuged at 8000 rpm for 5 min to remove the culture medium. Subsequently, 100 μL of 1 × TE was added, and the mixture was sonicated for 5 min to lyse the bacteria. After that, the solution was centrifuged at 8000 rpm for 5 min to collect the supernatant (about 100 μL), which contains the extracted bacterial DNA. The resulting precipitate was resuspended in 900 μL of 1 × TE and subsequently diluted to



Scheme 1. Schematically illustration of the adaptive tandem CRISPR/Cas12a molecular amplifier for amplified detection and identification of *V. parahaemolyticus* via the integration of RPA amplification, TdT elongation, and CRISPR/Cas12a *trans*-cleavage.

indicated various concentrations.

2.4. Tandem CRISPR/Cas12a molecular amplifier-based V. parahaemolyticus detection

To carry out the tandem CRISPR/Cas12a molecular amplifier-based *V. parahaemolyticus* detection, the RPA was firstly performed using a commercially purchased basic nucleic acid isothermal amplification kit. In brief, 24.4 μL of dd-H_2O, 20 μL of dissolving agent, 0.8 μL of 8 mM forward primer (FP), and 0.8 μL of 8 mM reverse primer (RP) were completely mixed and dissolved the amplification powder reagent containing polymerase, recombinase, SSB, and dNTPs. For the amplification of genomic DNA, 1 μL of DNA template and 1 μL of 250 mM magnesium acetate (MgAc) were injected into 23 μL of the pre-mixed amplification solution. The RPA reaction was performed by incubating the mixture at 37 °C for 20 min, followed by termination at 80 °C for 10 min. In the negative control, 1 μL of H_2O was used instead of the DNA template. Subsequently, a droplet of 3 μL of RPA products, 2 μL of 10 × Exo I buffer, 0.5 μL of Exo I, and 14.5 μL of dd-H_2O were mixed and reacted at 37 °C for 20 min to degrade the residual amplification primers. Followed by incubating at 80 °C for 15 min to inactivate Exo I, the resultant mixture was added with 5 μL of 10 × TdT buffer, 2 μL of dATP (10 mM) and 0.5 μL of TdT (14 U/μL) and reacted at 37 °C for 1 h to perform the TdT based stand elongation. Next, the reaction solution was annealed at 80 °C for 10 min to end the activity of TdT and added with 2 μL of 10 × NEB buffer 2.1, 2 μL of reporter probe (5 μM), 1 μL of Cas12a (1 μM), and 0.5 μL of crRNA (2.5 μM). Finally, the CRISPR/Cas12a based strand cleavage was exerted at 37 °C for 1 h for fluorescence emission.

3. Results and discussion

3.1. Principle of V. parahaemolyticus adaptive tandem CRISPR/Cas12a molecular amplifier

To construct the adaptive tandem CRISPR/Cas12a molecular amplifier, as schematically shown in Scheme 1, a pair of amplification primers was firstly designed to combine with polymerase, recombinase, and SSB to perform the RPA amplification in the dNTPs pool according to the instructions given in the RPA kit. In detail, the binding of recombinase with DNA primers promotes the binding of primers to the target sequence and enable the subsequent strand displacement and amplification steps. The SSBs stabilize the displaced single-stranded DNA during the amplification process, preventing reannealing of the amplified products. A DNA polymerase with strand displacement activity synthesizes new DNA strands using the displaced single-stranded DNA as a template. Due to the high amplification efficiency of RPA at a constant temperature of 37 °C, this step allows for exponential amplification of the target sequence, resulting in the exhaustion of lots of the primers and generation of numerous dsDNA amplicons against the degradation by Exo I, an enzyme that only specifically degrades single-stranded DNA. The 3′ ends of the dsDNA amplicons are then exposed and available for rapid template-free incorporation of nucleotides by terminal deoxynucleotidyl transferase (TdT) in the presence of a dATP pool. This process accurately generates a long synthetic poly-A rich chain along both sides of the dsDNA amplicons. We have termed these new products as Poly-A/dsDNA/Poly-A. Based on the complementary base pairing between "A" and "U," the crRNA with a poly-U rich segment can specifically hybridize to the long poly-A chain. This enables the simultaneous activation of multiple CRISPR/Cas12a units, forming what we refer to as the adaptive tandem CRISPR/Cas12a molecular amplifier (RPA-CRISPR/Cas12a). Once activated, the CRISPR/Cas12a complex indiscriminately cleaves the single-stranded RP. This cleavage event leads to the physical separation of a fluorophore and a quencher molecule, resulting in the recovery of fluorescence. The intensity of the fluorescence is dependent on the concentration of the target *V. parahaemolyticus*. In the absence of *V. parahaemolyticus* DNA, as shown in the lower part of Scheme 1, the single-stranded amplification primers are subjected to hydrolysis by Exo I. This hydrolysis serves to prevent the formation of RPA products or any false RPA products, such as primer dimers. Moreover, it also inhibits any further elongation by TdT. Consequently, in this case, the CRISPR/Cas12a system remains inactive. This property ensures the suppression of background signals and increases the signal-to-noise ratio (S/N). In addition, it is important to highlight the distinction between the conventional approach, as depicted in Scheme S1, and our proposed method of combining RPA with CRISPR/Cas12a. In the conventional approach, one RPA amplicon is only capable of activating a single CRISPR/Cas12a unit. However, in our novel approach, we have achieved a significant advancement. By utilizing TdT as a bridge, a single RPA amplicon can activate multiple CRISPR/Cas12a units in parallel. This enhancement holds the potential for a higher signal gain, thereby improving the sensitivity of *V. parahaemolyticus* detection. This design also eliminates the need to consider the presence of a PAM site when designing primers, thus

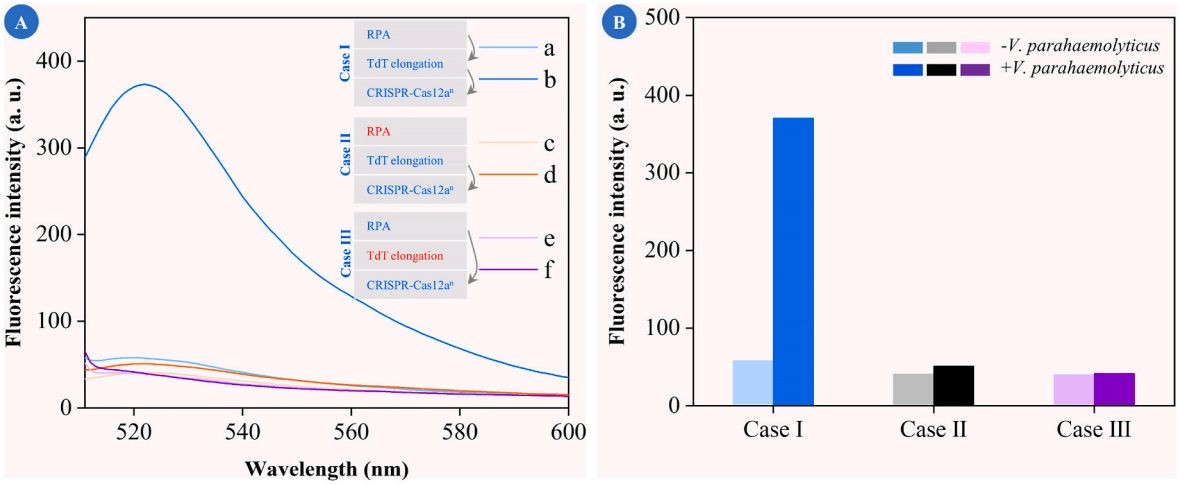


Fig. 1. Typical fluorescence spectra collected from (a) RPA/TdT elongation/CRISPR/Cas12a^n *trans*-cleavage, (b) DNA template/RPA/TdT elongation/CRISPR/Cas12a^n *trans*-cleavage, (c) TdT elongation/CRISPR/Cas12a^n *trans*-cleavage, (d) DNA template/TdT elongation/CRISPR/Cas12a^n *trans*-cleavage, (e) RPA/CRISPR/Cas12a^n *trans*-cleavage, and (f) DNA template/RPA/CRISPR/Cas12a^n *trans*-cleavage. Line b vs a, line d vs c, and line f vs e are defined as Case I, II, and III, respectively. The concentrations of FP, RP, TdT, dATP, and *V. parahaemolyticus* are 8 μM, 8 μM, 7 U, 1 mM and 1 × 10^7 CFU/mL.

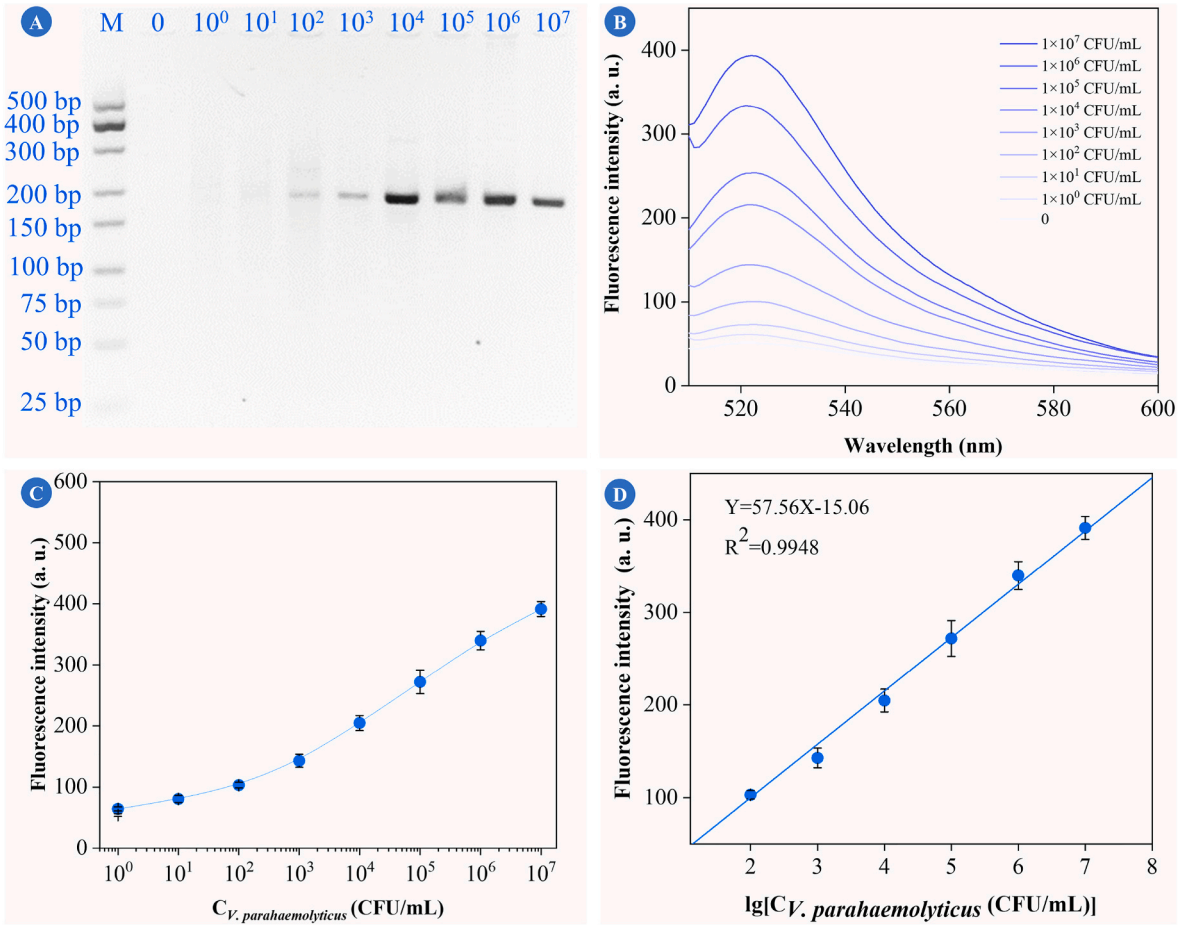


Fig. 2. Sensitivity evaluation of the adaptive tandem CRISPR/Cas12a molecular amplifier for *V. parahaemolyticus* detection. (A) Gel electrophoresis analysis showing the visualization of *V. parahaemolyticus* samples at different concentrations using the RPA method. (B) Fluorescent measurement of target concentrations (0, 1 × 10^0, 1 × 10^1, 1 × 10^2, 1 × 10^3, 1 × 10^4, 1 × 10^5, 1 × 10^6 and 1 × 10^7 CFU/mL) using the adaptive tandem CRISPR/Cas12a molecular amplifier. (C) Plot showing the dependence of the peak fluorescence value on the target concentration in the range of 1 × 10^2–1 × 10^7 CFU/mL. From low to high *V. parahaemolyticus* concentration, the RSDs are 4.52%, 7.35%, 6.01%, 7.10%, 4.45%, and 3.15%, respectively. (D) The correlation equation between fluorescence intensity and concentration of *V. parahaemolyticus* in the range of 1 × 10^2–1 × 10^7 CFU/mL. Error bars show the standard deviation of three experiments.

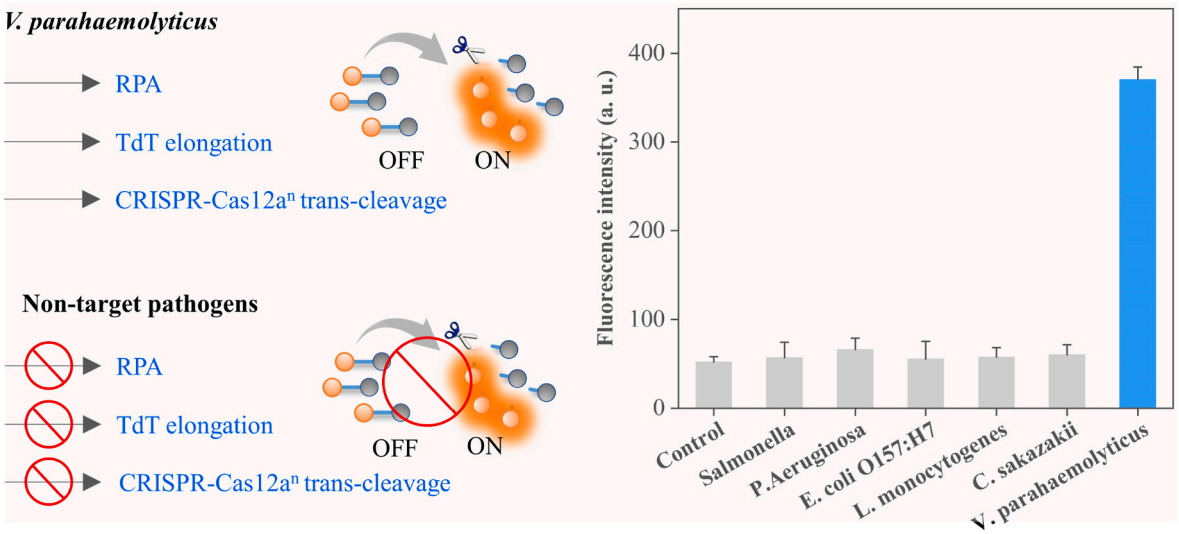


Fig. 3. Fluorescent responses of the adaptive tandem CRISPR/Cas12a molecular amplifier induced by target *V. parahaemolyticus* (1×10^7 CFU/mL) and non-targets (1×10^8 CFU/mL) including *Salmonella*, *P. Aeruginosa*, *E. coli O157:H7*, *L. monocytogenes*, and *C. sakazakii*. Error bars show the standard deviation of three experiments. The concentrations of FP, RP, Exo I, TdT, dATP, and *V. parahaemolyticus* are 8 μM, 8 μM, 10 U, 7 U, 1 mM, and 1×10^7 CFU/mL.

enhancing the universality and applicability of our method for a broad range of applications.

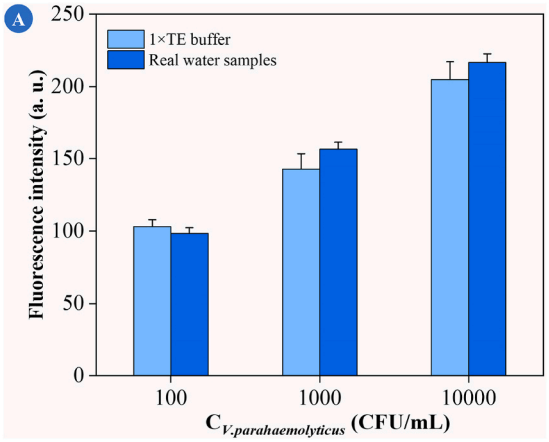
3.2. Feasibility demonstration

After demonstrating the effectiveness of RPA and TdT-based strand elongation (Fig. S1), we proceeded to evaluate the feasibility of the adaptive tandem CRISPR/Cas12a molecular amplifier for fluorescence detection of *V. parahaemolyticus*. As shown in Fig. 1, introduction of the target DNA template extracted from the *V. parahaemolyticus* to carry out the RPA, TdT elongation, and CRISPR/Cas12a^n *trans*-cleavage in sequence, the biosensing system outputted a remarkably enhanced fluorescence compared to the control without DNA template. This detection scenario is referred to as Case I. The substantial difference in fluorescence signals demonstrates the feasibility of this amplifier for *V. parahaemolyticus* detection. Moreover, we also demonstrated the necessity of both RPA and TdT elongation. As observed in line d compared to line c, the target DNA template without RPA (Case II) can only induce a very slightly enhanced signal over its control negative. This is attributed to the limited amount of DNA template available without isothermal amplification, resulting in insufficient –OH groups for strand elongation. Likewise, we tested the signal of the target DNA template without the process of TdT elongation. One can find that in this case (Case III), the target sample in line f still showed no signal enhancement over its control in line e since no poly-A chain can be produced to bind with the crRNA for the activation of CRISPR/Cas12a. The comparison between Case I, II, and III strongly emphasizes the importance of both the RPA and TdT elongation processes for amplified signal emission.

3.3. Investigation of assay performance

As a newly developed molecular detection method, the sensitivity should be firstly interrogated to evaluate its assay performance. Under optimized (Fig. S2) and identical conditions, a series of *V. parahaemolyticus* with concentrations ranging from 0, 1×10^0, 1×10^1, 1×10^2, 1×10^3, 1×10^4, 1×10^5, 1×10^6 and 1×10^7 CFU/mL were prepared and subjected to the adaptive tandem CRISPR/Cas12a molecular amplifier assay. The results, visualized by gel electrophoresis in Fig. 2A, indicate that the RPA method can only detect *V. parahaemolyticus* down to a concentration of 100 CFU/mL. Higher concentrations of *V. parahaemolyticus* cannot be differentiated based on the gel electrophoresis results. In contrast, when using the proposed adaptive tandem CRISPR/Cas12a molecular amplifier, as shown in Fig. 2B, the fluorescence intensity of the reaction system gradually increases with the increasing concentration of *V. parahaemolyticus*. This is due to the generation of more RPA amplicons with long poly-A tails, which activate multiple CRISPR/Cas12a units. The *trans*-cleavage of RPs by the CRISPR/Cas12a^n system leads to the observed increase in fluorescence intensity. Fig. 2C demonstrates the dependence of the peak fluorescence value on the target concentration within the range of 1×10^2 to 1×10^7 CFU/mL. A linear relationship is observed, as shown in Fig. 2D, which can be described by equation F = 57.56 l g [$C_{V.\ parahaemolyticus}$/(U/mL)] - 15.07 ($R^2 = 0.9948$). Based on a 3σ/S rule, the limit of detection (LOD) was deduced to be as low as 12.4 CFU/mL σ represents the standard deviation of the blank sample, and S represents the slope of the standard curve. Table S1 provides a comparison of our method with previously reported methods for *V. parahaemolyticus* detection. The data clearly demonstrate the improvement of our method in terms of the LOD and linear detection range for pathogen detection. For example, one of the previously reported methods utilized magnetic nanobead-based immune-separation and quantum dot-based immunofluorescence, achieving only a LOD of 10^2 CFU/mL and a linear detection range of 10^2–10^6 CFU/mL [38]. Similarly, another reported method employing a bacteriophage-displayed nanobody biosensor demonstrated a LOD of 10^3 CFU/mL and a linear detection range of 10^3–10^6 CFU/mL [39]. While it is true that our method's LOD surpasses those listed in Table S1, and the linear detection range also exhibits some improvement, we must acknowledge that our linear detection range remains narrower compared to a few other reported studies [40,41]. However, it is essential to note that some of these methods involve complex electrochemical interface construction using nanomaterials [40] or utilize advanced and expensive digital PCR techniques [41]. From this perspective, our method still demonstrates clear methodological advantages.

Accurate and specific detection of pathogens is crucial for ensuring food safety and disease diagnosis. In addition to sensitivity, specificity is a critical parameter for evaluating the proposed method. To assess the specificity, we selected several other bacterial pathogens, including *Salmonella*, *Pseudomonas aeruginosa* (*P. Aeruginosa*), and *Escherichia coli O157:H7* (*E. coli O157:H7*), *Listeria monocytogenes* (*L. monocytogenes*), and *Cronobacter sakazakii* (*C. sakazakii*) as non-target analytes to challenge the reaction system alongside the target *V. parahaemolyticus*. The results depicted in Fig. 3 demonstrate that all non-target pathogens exhibited negligible fluorescence signals compared to the negative



B

Samples	Added (CFU/mL)	Found (CFU/mL)	Recovery (%)	RSD (%)
1	10^2	9.33×10^1	93.3	3.80
2	10^3	9.62×10^2	96.2	2.99
3	10^4	1.06×10^4	106.4	2.67

Fig. 4. Analysis of *V. parahaemolyticus* in real samples using the adaptive tandem CRISPR/Cas12a molecular amplifier. (A) Fluorescence signals generated in positive samples spiked with V. parahaemolyticus in Feicui Lake water. (B) Evaluation of recovery rates for V. parahaemolyticus detection in real samples.

control without any pathogenic DNA. In contrast, the presence of the target *V. parahaemolyticus* resulted in a significantly intensified fluorescence signal that was approximately six times higher than the non-target signals. These comparative findings confirm the excellent specificity of our study and establish a solid foundation for the practical analysis of real samples using our method.

3.4. Application of the molecular amplifier in real sample analysis

On the basis of the above demonstrated assay features, we finally applied the adaptive tandem CRISPR/Cas12a molecular amplifier to analyze *V. parahaemolyticus* from real samples. The real samples were prepared by spiking *V. parahaemolyticus* DNA to water collected from the local Feicui Lake. As measured in Fig. 4A, similar to the results obtained from the detection of *V. parahaemolyticus* in 1 × TE buffer, the fluorescence signals were generated in the positive samples and the fluorescence intensity is proportional to the target concentration. To further assess the practicality of the method, we evaluated the recovery rates in Fig. 4B. The recovery rates ranged from 93.3% to 106.4%, confirming the promising potential of this molecular amplifier for practical applications. Meanwhile, we have also utilized scallop, a widely consumed seafood, as a real food matrix to further demonstrate the applicability of our proposed bioassay. Of note, the scallop samples were purchased from a local seafood market and pre-treated with 75% ethanol for 10 min to disinfect them. They were then washed three times with deionized water to remove any irrelevant impurities. Subsequently, 5 g of scallop meat were weighed and ground. Next, different V. parahaemolyticus standard solutions with concentrations of 1×10^3, 1×10^4, and 1×10^5 CFU/mL were added to the scallop samples and incubated for 2 h. The recovery rates in Table S2 ranged from 88.2% to 108.6%, which were still acceptable to a certain extent. However, it is also evident that real food samples may have potential matrix effects that can influence the accuracy of detection. Additionally, considering that in both the lake water sample and the scallop all the relative standard derivations (RSDs) are within 8.0%, these results also suggest the acceptable repeatability and reproducibility.

4. Conclusion

In conclusion, in this work, we proposed an adaptive tandem CRISPR/Cas12a molecular amplifier by integrating the amplification by RPA, TdT elongation, and CRISPR/Cas12a *trans*-cleavage and applied it as the analytical tool for the robust detection of *V. parahaemolyticus*. Compared with already reported existing CRISPR/Cas12a amplification amplifier usually activated by one RPA amplicon-one CRISPR/Cas12a manner, the newly developed adaptive tandem CRISPR/Cas12a molecular amplifier contains lots of activated CRISPR/Cas12a units to amplify the reporting signal, benefiting the signal gain of the target-presented positive sample. Moreover, the method overcame the limitation of PAM restriction, making it universally applicable. The use of Exo I effectively suppressed the background signal by degrading amplification primers in the absence of target genomic DNA. This treatment ensures that no oligonucleotides can be elongated by TdT, resulting in a well suppressed background signal value in the sensing system. The total effect is that the adaptive tandem CRISPR/Cas12a molecular amplifier based biosensing system owns a high sensitivity to detect *V. parahaemolyticus* down to 12.3 CFU/mL. The method also exhibited excellent specificity and practicality for *V. parahaemolyticus* detection. This study paves the way for the development of promising molecular diagnostic technologies in food safety monitoring and biomedical diagnosis.

CRediT authorship contribution statement

Yubo Peng: Validation, Formal analysis, Writing – original draft. **Pengpeng Xue:** Formal analysis, Validation, Investigation. **Renjing Wang:** Formal analysis, Investigation. **Huijie Shang:** Validation, Formal analysis. **Bangben Yao:** Formal analysis. **Zhi Zheng:** Formal analysis. **Chao Yan:** Formal analysis. **Wei Chen:** Conceptualization, Funding acquisition, Supervision. **Jianguo Xu:** Conceptualization, Writing – review & editing, Resources, Funding acquisition, Supervision.

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 availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2023.125061.

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