Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology) 2023 24(8):749-754
www.jzus.zju.edu.cn; www.springer.com/journal/11585
E-mail: jzus_b@zju.edu.cn

Correspondence

https://doi.org/10.1631/jzus.B2200705

Check for updates

Rapid visual detection of *Vibrio parahaemolyticus* by combining LAMP-CRISPR/Cas12b with heat-labile uracil-DNA glycosylase to eliminate carry-over contamination

Fang WU[1], Chen LU[1,2], Wenhao HU[1], Xin GUO[3], Jiayue CHEN[1], Zhidan LUO[1,2]✉

[1]*Jiangsu Key Laboratory of Marine Biological Resources and Environment, Jiangsu Key Laboratory of Marine Pharmaceutical Compound Screening, Jiangsu Ocean University, Lianyungang 222005, China*
[2]*Co-Innovation Center of Jiangsu Marine Bio-industry Technology, Jiangsu Ocean University, Lianyungang 222005, China*
[3]*BestEnzymes Biotech Co., Ltd., Lianyungang 222005, China*

Vibrio parahaemolyticus is a major pathogen frequently found in seafood. Rapid and accurate detection of this pathogen is important for the control of bacterial foodborne diseases and to ensure food safety. In this study, we established a one-pot system that combines uracil-DNA glycosylase (UDG), loop-mediated isothermal amplification (LAMP), and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 12b (Cas12b) for detecting *V. parahaemolyticus* in seafood. This detection system can effectively perform identification using a single tube and avoid the risk of carry-over contamination.

V. parahaemolyticus is a common bacterial pathogen in seawater, which is widely distributed in marine, estuarine, and aquaculture environments, and frequently isolated from seafood (Su and Liu, 2007). On one hand, it can be transmitted to humans through contaminated, raw, or undercooked seafood, causing severe human seafood-borne gastroenteritis or even death. On the other hand, in aquatic farms and the mariculture industry, *V. parahaemolyticus* infection can cause severe gastroenteritis in aquatic animals, and quickly lead to massive mortality and huge economic losses (He et al., 2015). Thus, the development of accurate and convenient detection methods of this pathogen is of particular urgency (Ward and Bej, 2006; Zhang et al., 2017).

Recently, due to the independence of expensive thermal cyclers and fast reading of results, several isothermal amplification techniques such as LAMP and recombinase polymerase amplification (RPA) have emerged for the rapid detection of *V. parahaemolyticus* (Nemoto et al., 2011; Yang et al., 2020). To increase the specificity and sensitivity of detection, CRISPR/Cas nuclease can be applied in combination with isothermal amplification. These methods are based on the *trans*-cleavage activity of Cas nucleases, including the single-stranded DNA (ssDNA)-targeting *trans*-cleaving Cas12 or the RNA-targeting *trans*-cleaving Cas13 (Gao et al., 2022). Combined with isothermal amplification procedures, Cas12 and Cas13 have been employed to develop rapid nucleic acid detection systems for pathogen detection (Lv et al., 2022).

However, the above methods are prone to generating false-positive results caused by carry-over contamination with DNA products from previous positive amplification reactions in laboratories that frequently amplify similar samples. UDG has been widely used to eliminate carry-over contamination (Hsieh et al., 2014; Tang et al., 2016). During DNA amplification, deoxythymidine triphosphate (dTTP) is substituted by deoxyuridine triphosphate (dUTP), and the amplified DNA will contain uracil. In subsequent rounds, UDG is added to digest the possible carry-over previous amplicon containing uracil. Then, the UDG is denatured by high temperature or a UDG inhibitor before amplification.

✉ Zhidan LUO, lzd@jou.edu.cn
Zhidan LUO, https://orcid.org/0009-0001-7855-2572
Chen LU, https://orcid.org/0000-0001-7619-8891

Received Jan. 8, 2023; Revision accepted Mar. 10, 2023; Crosschecked June 20, 2023

The denaturation temperature of commonly used heat-labile UDG (HL-UDG) is above 50 ℃ (Lanes et al., 2002), which is higher than the inactivated temperature of RPA reagent and Cas12a/Cas13 nuclease. Therefore, the current technology combining UDG, LAMP, and CRISPR/Cas12a is divided into several steps and needs to add the Cas detection system into the amplification product after amplification, which increases the operational complexity and may cause cross-contamination among samples (Zhang et al., 2021).

Unlike Cas12a, Cas12b has a wider temperature adaptation range (48–60 ℃) and better specificity (Li et al., 2019; Teng et al., 2019). In this study, we established a one-tube rapid nucleic acid detection system for *V. parahaemolyticus*, that combines LAMP-CRISPR/Cas12b technology with HL-UDG to eliminate carry-over contamination. The schematic diagram of this system is shown in Fig. 1. Firstly, *V. parahaemolyticus* genomic DNA is extracted from seafood samples. The detection system contains all components in a single tube for carry-over contamination removal, such as LAMP and CRISPR/Cas12b reaction, including HL-UDG, Bst DNA polymerase, LAMP primers, dNTP mixture (a certain proportion of dTTP substituted by dUTP), Cas12b nuclease, *V. parahaemolyticus* virulence *TL* gene (GenBank accession No. M36437.1) specific small guide RNA (sgRNA), and a non-specific single-strand DNA probe labeled by fluorophore and quencher. If the DNA template is contaminated by carry-over LAMP amplicon containing uracil from the amplification of the previous sample, the contaminated DNA will be digested by HL-UDG at 25 ℃ for 5–10 min before LAMP amplification. HL-UDG will be completely inactivated during subsequent LAMP amplification and CRISPR/Cas12b cleavage at 60 ℃, without affecting the true DNA template and subsequent reactions. At the same time and temperature of the LAMP reaction, the sgRNA specifically binds to the true positive amplicon to activate the *trans*-cleavage activity of Cas12b nuclease. The activated Cas12b cleaves the non-specific ssDNA probes and

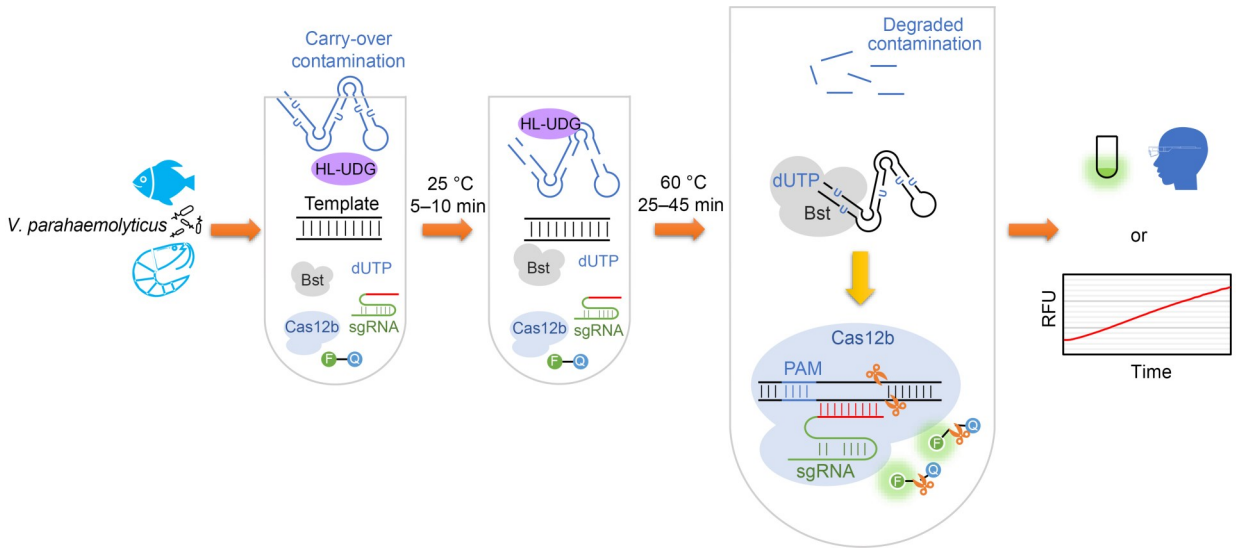

Fig. 1 Schematic diagram of *Vibrio parahaemolyticus* detection in seafood using UDG-LAMP-CRISPR/Cas12b. The detection system contains all components in a single tube for carry-over contamination removal, LAMP amplification, and Cas12b cleavage. If the DNA template is contaminated by carry-over LAMP amplicon containing uracil from amplification of the previous sample, the contaminated DNA will be digested by heat-labile UDG (HL-UDG) at 25 ℃ for 5–10 min before LAMP amplification. HL-UDG will be completely inactivated during subsequent LAMP amplification at 60 ℃. At the same time and temperature as the LAMP reaction, the sgRNA specifically binds to the true positive amplicon to activate the *trans*-cleavage activity of Cas12b nuclease. The activated Cas12b cleaves the non-specific single-stranded DNA probes and separates the fluorophore from the quencher. After running the LAMP and CRISPR/Cas12b reaction for 25–45 min, the released fluorescence signal can be easily detected by a fluorescence meter or observed by naked eye under blue light. UDG: uracil-DNA glycosylase; LAMP: loop-mediated isothermal amplification; CRISPR: clustered regularly interspaced short palindromic repeats; Cas12b: CRISPR-associated protein 12b; sgRNA: small guide RNA; Bst: Bst DNA polymerase; dUTP: deoxyuridine triphosphate; PAM: protospacer adjacent motif; RFU: relative fluorescence units.

separates the fluorophore from the quencher. After 25–45 min of the LAMP and CRISPR/Cas12b reaction, the released fluorescence signal can be easily detected by a fluorescence meter or observed by naked eye under blue light.

The LAMP primer sequence has a great influence on the amplification efficiency. Three LAMP primer sets from the *TL* gene (Table S1) were designed and screened to obtain the most suitable LAMP primer sets (Fig. S1a). After agarose gel verification (Fig. S1b), primer set No. 3 was found to have the highest amplification efficiency and used for subsequent experiments. To establish a decontamination system using UDG, it was necessary to verify whether the incorporation of dUTP affected LAMP amplification. Interestingly, the incorporation of less than 50% dUTP significantly improved the LAMP amplification efficiency in this study, and the highest amplification efficiency was achieved at the dTTP/dUTP ratio of 7:3 (Fig. 2a). However, LAMP amplification was completely inhibited when the substitution ratio of dUTP was higher than 60% (Fig. 2a).

The dUTP-incorporated dNTP mixture not only influenced the amplification efficiency but also affected the recognition and binding of the substrate by the Cas12b nuclease. We next investigated whether six candidate sgRNAs located in different regions of the initial LAMP amplicon (Fig. 2b) would affect the cleavage efficiency of the one-step LAMP-CRISPR/Cas12b reaction in the presence of dUTP incorporation. All combinations of sgRNA and dTTP/dUTP ratios were tested (Fig. 2c). The analysis of variance (ANOVA) result showed that both dTTP/dUTP ratio and different sgRNAs significantly affected the efficiency of LAMP-CRISPR/Cas12b. Among the sgRNAs in the variable regions of LAMP amplicons, sgRNA4 presented the highest cleavage efficiency (Fig. 2d). When the ratio of dTTP/dUTP was gradually changed from 10:0 to 7:3, the cleavage efficiency of LAMP-CRISPR/Cas12b progressively improved, and the best performance occurred at a dTTP/dUTP ratio of 7:3 (Fig. 2e).

The incorporation of dUTP may cause the change of several optimal protospacer adjacent motifs (PAMs)

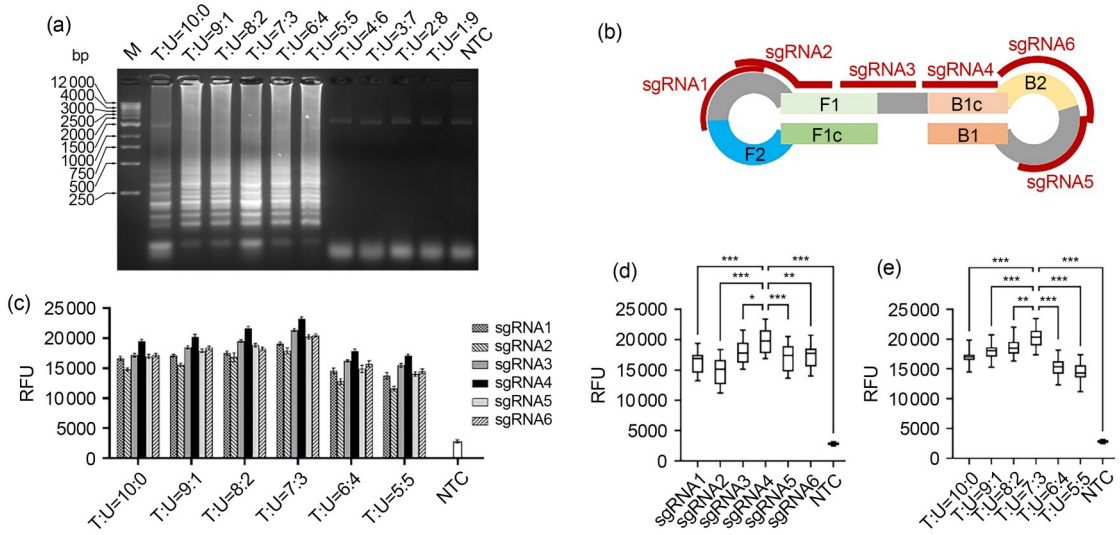

Fig. 2 Screening of sgRNA and dTTP/dUTP ratio for LAMP-CRISPR/Cas12b. (a) LAMP amplification results with different dTTP/dUTP concentration ratios. (b) Illustration of the initial LAMP stem-loop amplicon and sgRNA-binding position. (c) Endpoint fluorescence signals of all combinations of sgRNAs and dTTP/dUTP ratios. Data are expressed as mean±standard deviation (SD), *n*=3. The NTC bar integrates the negative controls without DNA template in all experimental groups, and the fluorescence signal values of all NTC were around 2500–3000. (d) Endpoint fluorescence signals of different sgRNAs from panel (c). Each box represents all data for the same sgRNA at different dTTP/dUTP ratios, and the significance was determined by one-way analysis of variance (ANOVA). (e) Endpoint fluorescence signals of different dTTP/dUTP ratios from panel (c). Each box represents all data for the same dTTP/dUTP ratio with different sgRNAs, and the significance was determined by one-way ANOVA. * *P*<0.05, ** *P*<0.01, and * *P*<0.001 indicate significant differences between two groups. dTTP: deoxythymidine triphosphate; dUTP: deoxyuridine triphosphate; LAMP: loop-mediated isothermal amplification; CRISPR: clustered regularly interspaced short palindromic repeats; Cas12b: CRISPR-associated protein 12b; sgRNA: small guide RNA; T:U: molar ratio of dTTP to dUTP; RFU: relative fluorescence units; NTC: no template control; M: marker.**

(TTN) into suboptimal PAMs (UUN). Because Cas12-mediated double-stranded DNA (dsDNA) cleavage competes with LAMP amplification in this one-pot system, the decreased binding affinity of Cas12 with suboptimal PAM leads to the subdued *cis*-cleavage activity, and prompts the reaction equilibrium to shift to LAMP. Therefore, suboptimal PAMs cause the dsDNA substrate to accumulate at the early stages of the LAMP reaction, and the accumulated amplicons can efficiency increase subsequent CRISPR/Cas12b cleavage (Lu et al., 2022). Finally, we chose the sgRNA4 and a dTTP/dUTP ratio of 7:3 as the optimal combination for subsequent experiments.

In order to verify the performance of HL-UDG in eliminating carry-over contamination, the pre-amplified LAMP product containing uracil was added into the LAMP-CRISPR/Cas12b system at 1/1000, 1/10 000, and 1/100 000 of the normal amount of template (1 ng) to simulate the carry-over contamination. Under blue light, no fluorescent signal was observed in the reaction system containing UDG (Fig. 3a). Compared with the group of LAMP-CRISPR without UDG, all proportions of simulated contaminated U-containing DNA were digested completely, and no obvious fluorescence amplification bands appeared in the reaction (Fig. 3b).

The limit of detection (LOD) of the UDG-LAMP-CRISPR/Cas12b system was tested in both pure culture of *V. parahaemolyticus* and artificially contaminated shrimp samples. The template was diluted at a tenfold gradient dilution with the first concentration containing 1×10^5 CFU (CFU: colony forming units). When the concentration of the bacteria in pure culture was 1×10^2 CFU/mL or 1×10^2 CFU/g in shrimp samples, the fluorescence signal generated by the UDG-LAMP-CRISPR/Cas12b method was still significantly different from those in the NTC group (Figs. 3c–3f). These results showed that the LOD was as low as 1×10^2 CFU/mL in the pure cultures and 1×10^2 CFU/g in the shrimp samples.

The specificity of the UDG-LAMP-CRISPR/Cas12b assay was evaluated by testing the culture solution for the presence of several bacterial strains, including six other *Vibrio* species and six other food pathogens. None of the other *Vibrio* species and common pathogenic bacteria were detected (Fig. 3g), confirming the specificity of the UDG-LAMP-CRISPR/Cas12b assay.

In order to verify the performance of the UDG-LAMP-CRISPR/Cas12b assay on actual seafood samples, ten shrimps and ten fish purchased from a local seafood market were tested using both the UDG-LAMP-CRISPR/Cas12b and quantitative real-time polymerase chain reaction (qPCR) methods (Fig. 3h). Among the twenty samples, seven tested positive *V. parahaemolyticus* using the UDG-LAMP-CRISPR/Cas12b assay, which was completely consistent with the results of the qPCR method. These results demonstrated that the UDG-LAMP-CRISPR/Cas12b assay performed stably for *V. parahaemolyticus* detection in actual seafood.

In summary, we successfully established a one-pot UDG-LAMP-CRISPR/Cas12b assay to rapidly detect *V. parahaemolyticus* (within 40 min). This assay could effectively remove carry-over contamination and avoid false positive results. The LOD of this method was as low as 1×10^2 CFU/mL in pure cultures and 1×10^2 CFU/g in shrimp samples. Moreover, it was able to distinguish *V. parahaemolyticus* from other *Vibrio* species and other aquatic pathogens. Our assay only needs a simple heating equipment and a blue light instrument, and can serve as an on-site rapid detection system.

Materials and methods

Detailed methods are provided in the electronic supplementary materials of this paper.

Acknowledgments

This work was supported by the Priority Academic Program Development of Jiangsu Higher Education Institutions of China, the Postgraduate Research & Practice Innovation Program of Jiangsu Province (No. KYCX2021-038), and the Lianyungang Science and Technology Program (No. CG2232). We thank Mr. Xiang ZHOU (Lianyungang Institute of Food and Drug Control, Lianyungang, China) and Dr. Juan LI (Wuhan Institute for Food and Cosmetic Control, Wuhan, China) for kindly providing the samples used in this study. We also thank Dr. Yong XUE (Jiangsu Ocean University, Lianyungang, China) for his critical review of this manuscript.

Author contributions

Zhidan LUO conceived and designed the experiments. Fang WU, Wenhao HU, Xin GUO, and Jiayue CHEN performed the experimental research. Fang WU and Chen LU analyzed the data. Zhidan LUO and Chen LU wrote this manuscript. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

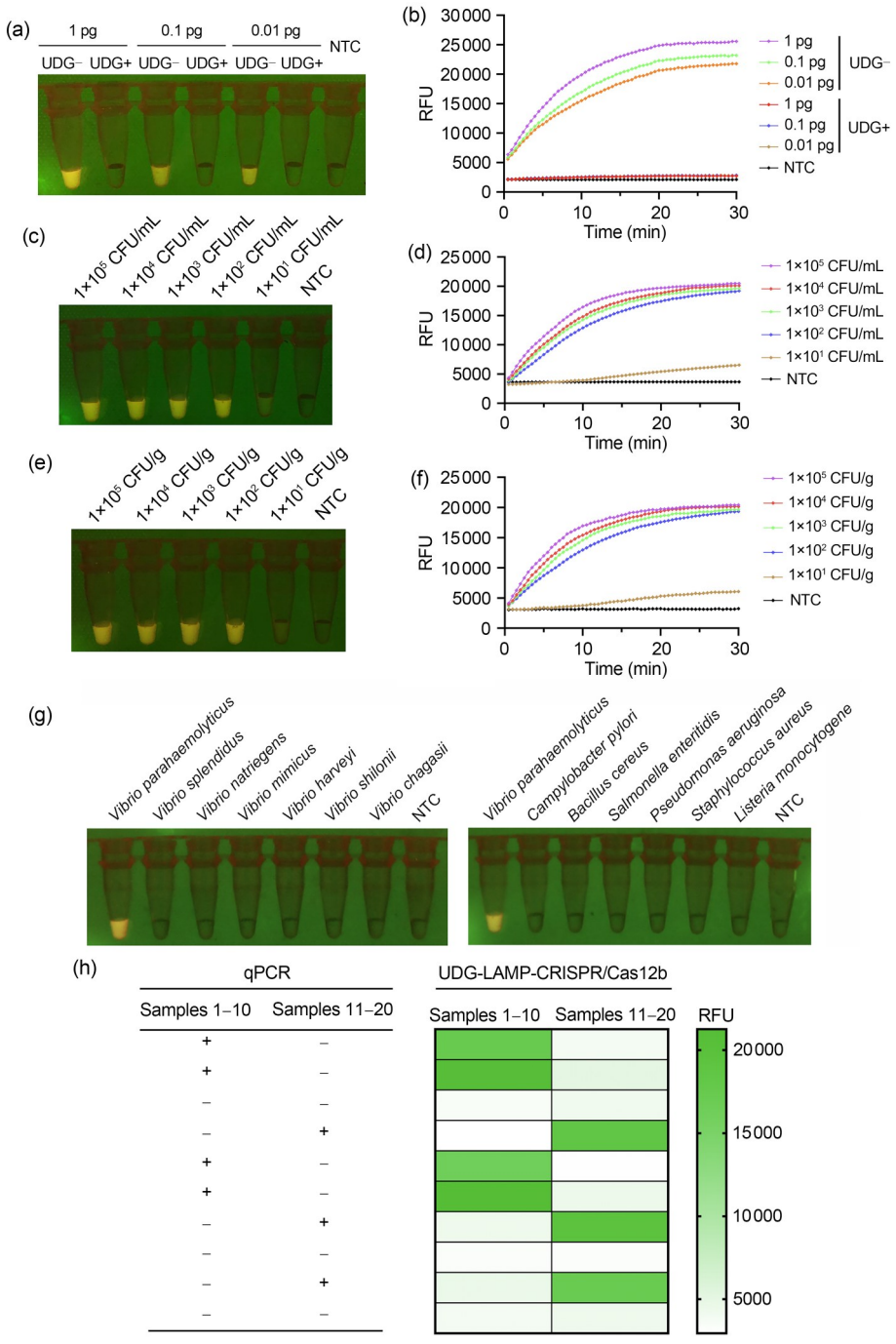

Fig. 3 Performance of the UDG-LAMP-CRISPR/Cas12b assay in *Vibrio parahaemolyticus* detection. (a) The results of treatment with or without UDG at different concentrations of artificially contaminated templates before the LAMP-CRISPR/Cas12b reaction. (b) Fluorescence curve corresponding to each tube in panel (a). (c) Sensitivity analysis based on *V. parahaemolyticus* genomic DNA extracted from a tenfold gradient dilution of pure culture under blue light. (d) Fluorescence curve corresponding to each tube in panel (c). (e) Sensitivity analysis based on DNA extracted from shrimp samples contaminated with tenfold gradient dilutions of *V. parahaemolyticus* under blue light. (f) Fluorescence curve corresponding to each tube in panel (e). (g) Specificity assessment of the UDG-LAMP-CRISPR/Cas12b assay in *V. parahaemolyticus* detection. (h) Detection of *V. parahaemolyticus* in 20 actual seafood samples using the qPCR (left) and UDG-LAMP-CRISPR/Cas12b assays (right). UDG: uracil-DNA glycosylase; LAMP: loop-mediated isothermal amplification; CRISPR: clustered regularly interspaced short palindromic repeats; Cas12b: CRISPR-associated protein 12b; NTC: no template control; RFU: relative fluorescence units; CFU: colony forming units; qPCR: quantitative real-time polymerase chain reaction.

Compliance with ethics guidelines

Fang WU, Chen LU, Wenhao HU, Xin GUO, Jiayue CHEN, and Zhidan LUO declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

References

Gao H, Shang Z, Chan SY, et al., 2022. Recent advances in the use of the CRISPR-Cas system for the detection of infectious pathogens. *J Zhejiang Univ-Sci B (Biomed & Biotechnol)*, 23(11):881-898. https://doi.org/10.1631/jzus.B2200068

He Y, Wang H, Chen LM, 2015. Comparative secretomics reveals novel virulence-associated factors of *Vibrio parahaemolyticus*. *Front Microbiol*, 6:707. https://doi.org/10.3389/fmicb.2015.00707

Hsieh K, Mage PL, Csordas AT, et al., 2014. Simultaneous elimination of carryover contamination and detection of DNA with uracil-DNA-glycosylase-supplemented loop-mediated isothermal amplification (UDG-LAMP). *Chem Commun (Camb)*, 50(28):3747-3749. https://doi.org/10.1039/c4cc00540f

Lanes O, Leiros I, Smalås AO, et al., 2002. Identification, cloning, and expression of uracil-DNA glycosylase from Atlantic cod (*Gadus morhua*): characterization and homology modeling of the cold-active catalytic domain. *Extremophiles*, 6(1):73-86. https://doi.org/10.1007/s007920100225

Li LX, Li SY, Wu N, et al., 2019. HOLMESv2: a CRISPR-Cas12b-assisted platform for nucleic acid detection and DNA methylation quantitation. *ACS Synth Biol*, 8(10): 2228-2237. https://doi.org/10.1021/acssynbio.9b00209

Lu SH, Tong XH, Han Y, et al., 2022. Fast and sensitive detection of SARS-CoV-2 RNA using suboptimal protospacer adjacent motifs for Cas12a. *Nat Biomed Eng*, 6(3):286-297. https://doi.org/10.1038/s41551-022-00861-x

Lv XR, Cao WW, Zhang H, et al., 2022. CE-RAA-CRISPR assay: a rapid and sensitive method for detecting *Vibrio parahaemolyticus* in seafood. *Foods*, 11(12):1681. https://doi.org/10.3390/foods11121681

Nemoto J, Ikedo M, Kojima T, et al., 2011. Development and evaluation of a loop-mediated isothermal amplification assay for rapid and sensitive detection of *Vibrio parahaemolyticus*. *J Food Prot*, 74(9):1462-1467. https://doi.org/10.4315/0362-028X.JFP-10-519

Su YC, Liu CC, 2007. *Vibrio parahaemolyticus*: a concern of seafood safety. *Food Microbiol*, 24(6):549-558. https://doi.org/10.1016/j.fm.2007.01.005

Tang Y, Chen H, Diao YX, 2016. Advanced uracil DNA glycosylase-supplemented real-time reverse transcription loop-mediated isothermal amplification (UDG-rRT-LAMP) method for universal and specific detection of Tembusu virus. *Sci Rep*, 6:27605. https://doi.org/10.1038/srep27605

Teng F, Guo L, Cui TT, et al., 2019. CDetection: CRISPR-Cas12b-based DNA detection with sub-attomolar sensitivity and single-base specificity. *Genome Biol*, 20:132. https://doi.org/10.1186/s13059-019-1742-z

Ward LN, Bej AK, 2006. Detection of *Vibrio parahaemolyticus* in shellfish by use of multiplexed real-time PCR with TaqMan fluorescent probes. *Appl Environ Microbiol*, 72(3): 2031-2042. https://doi.org/10.1128/AEM.72.3.2031-2042.2006

Yang XH, Zhao PP, Dong Y, et al., 2020. An improved recombinase polymerase amplification assay for visual detection of *Vibrio parahaemolyticus* with lateral flow strips. *J Food Sci*, 85(6):1834-1844. https://doi.org/10.1111/1750-3841.15105

Zhang T, Zhao W, Zhao W, et al., 2021. Universally stable and precise CRISPR-LAMP detection platform for precise multiple respiratory tract virus diagnosis including mutant SARS-CoV-2 spike N501Y. *Anal Chem*, 93(48): 16184-16193. https://doi.org/10.1021/acs.analchem.1c04065

Zhang ZH, Lou Y, Du SP, et al., 2017. Prevalence of *Vibrio parahaemolyticus* in seafood products from hypermarkets in Shanghai. *J Sci Food Agric*, 97(2):705-710. https://doi.org/10.1002/jsfa.7715

Supplementary information

Materials and methods; Table S1; Fig. S1