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Rapid detection of *Vibrio vulnificus* in *Penaeus (Litopenaeus) vannamei* by loop-mediated isothermal amplification with lateral flow dipstick technique

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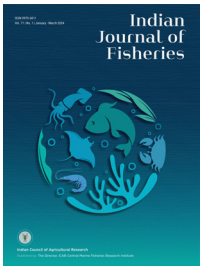

Abstract

Vibrio vulnificus is a is a highly virulent bacterial pathogen that poses significant risks to both humans and aquatic animals, necessitating the development of rapid and sensitive diagnostic methods. In this study, we designed four specific loop-mediated isothermal amplification (LAMP) primers, including a biotin labelled forward internal primer (FIP) along with a fluorescein isothiocyanate (FITC)-labelled probe targeting the *RtxC* gene sequence of *V. vulnificus* and established a novel LAMP combined with lateral flow dipstick (LFD) assay for the rapid visual detection of the pathogen. The optimized LAMP was run at 62°C for 15 min and the LFD could specifically and visually detect *V. vulnificus* within 5 min. The assay exhibited high specificity, showing no cross-reactivity against 24 non-target bacterial strains. It also exhibited higher sensitivity than conventional PCR methods, with a detection limit as low as 2.0×10^{-5} ng μl^{-1} of genomic DNA. We further validated the LAMP-LFD method using *Penaeus (Litopenaeus) vannamei* samples infected with *Vibrio*. Notably, the assay accurately detected *V. vulnificus* with high accuracy and the presence of host shrimp DNA did not interfere with the detection, highlighting the robustness of the assay for field applications in aquaculture. The developed LAMP-LFD assay offers a rapid, highly specific, sensitive, and reliable visual detection method for *V. vulnificus,* suited for field based surveillance and disease management in aquaculture.

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Introduction

Members of the genus *Vibrio* are widely distributed in the aquatic environments and play diverse ecological roles, that are vital to the marine ecosystems (Zhang *et al*., 2018; Leng *et al*., 2019; Laverty *et al*., 2020). However, several *Vibrio* species are pathogenic and pose significant threats to both human and aquatic animal health, raising public health concerns (Leng *et al*., 2019; Mok *et al*., 2019). Among these, *V. vulnificus* is a highly virulent pathogen that affects humans as well as aquatic animals (Heng *et al*., 2017). In humans, the bacterium can cause gastroenteritis, wound infections, necrotizing skin and soft tissue infections and primary septicemia, with mortality rates up to 50% in immunocompromised patients (Abeyagunawardena and Priyankara, 2023). *V. vulnificus* is an opportunistic human pathogen that causes life-threatening infections following contaminated seafood consumption or wound exposure. Among the toxic substances produced by this pathogen, the MARTX toxin (multifunctional autoprocessing repeats-in-toxin) is the major virulence factor that greatly contributes to disease progression by promoting the dysfunction and death of host cells, which allows the bacteria to disseminate and colonise the host (Kim *et al*., 2020). Most clinical cases of *V. vulnificus* infection are associated with exposure to contaminated aquatic products or aquaculture water environments, highlighting the need for effective monitoring and rapid detection methods (Haley *et al*., 2004; Sakihara *et al*., 2023; Xu *et al*., 2023).

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Global demand for the whiteleg shrimp *Penaeus* (*Litopenaeus) vannamei*, a leading aquaculture species, has surged over the past three decades, with production increasing at an average rate of 10% annually (Wang *et al*., 2020a, b). The sustainability of whiteleg shrimp production, a vital industry in many countries, is often hampered by disease outbreaks, with vibriosis caused by *V. vulnificus* being a particularly severe threat (Prabina, 2023). Whiteleg shrimp infected with *V. vulnificus* exhibit characteristic clinical signs, including the presence of pyogranulomatous nodules composed of haemocytes in the lymphoid organs, heart, and the loose connective tissues (De Souza Valente and Wan, 2021; Elias *et al*., 2023). Therefore, the development of rapid, sensitive and reliable detection methods for *V. vulnificus* is essential for the early detection and effective management of infections in order to safeguard the health of aquatic animals and to support sustainable aquaculture practices.

Traditional detection techniques such as pathogen isolation and culture as well as antigen-antibody based immunodiagnostic assays are often labour intensive, time-consuming and technically demanding. hence these methods are gradually being replaced by nucleic acid amplification techniques such as conventional polymerase chain reaction (PCR), and real-time quantitative PCR (qPCR). Among these, qPCR has been widely used for the detection of bacteria, viruses and fungi because of high sensitivity, specificity and quantitative capabilities (Yossa *et al*., 2023; Cui *et al*., 2024). However, qPCR has certain limitations, including reliance on expensive instrumentation, relatively long assay times, and the need for trained personnel, limiting its application in field settings. To overcome these challenges and to expand the application of molecular diagnostics, researchers have developed a variety of isothermal nucleic acid amplification techniques that do not require sophisticated instrumentation, time-saving, and can be performed under constant temperature conditions (Cao *et al*., 2019). Among the various isothermal amplification methods, loop-mediated isothermal amplification (LAMP) (Notomi, 2000) has emerged as a highly promising technique for rapid field detection and early disease diagnosis. LAMP offers several advantages, including high sensitivity, specificity, rapid amplification and simple instrumentation requirements. Because the LAMP reaction does not require expensive instruments and has low cost, this technology has been widely adopted for pathogen detection and is increasingly recognised as an important molecular diagnostic technology with broad applications in clinical and aquaculture disease surveillance.

LAMP has been successfully used to detect a variety of aquatic pathogens, including detection of *Vibrio parahaemolyticus* in shrimp (Cao *et al*., 2019), rapid and specific detection of Chinook salmon bafinivirus (CSBV) in flatfish (*Paralichthys olivaceus*) (Su *et al*., 2024), a practical screening method for virulent *Aeromonas* species affecting cultured eels (*Anguilla anguilla*) (Xiong *et al*., 2020) and rapid identification of hepatocyte protozoa in shrimp (Ma *et al*., 2019). For easier detection and field application, LAMP is often combined with a lateral flow dipstick (LFD). Kiatpathomchai *et al*. (2008) first applied LAMP-LFD technology for detecting Taura syndrome virus in shrimp. In this approach, FITC labeled probes specifically hybridise with biotinylated LAMP amplification products and the resulting complexes are captured by colloidal gold labeled anti FITC antibodies to form ternary complexes, which are then immobilised on the detection line of the lateral flow test strip through biotin-streptavidin interactions. The appearance of a visible test band indicates the presence of target amplification product enabling rapid visual interpretation of the results. LAMP-LFD method is safe, fast, efficient, and requires minimal equipment, offering a practical and effective diagnostic tool particularly in resource-limited settings.

Currently, LAMP-LFD technology has been successfully applied for fungi, bacteria, viruses and parasites of plant and animal diseases. In 2014, Rigano *et al*. (2014) established the LAMP-LFD detection method for the detection of citrus huanglongbing. Zhang *et al*. (2019) established a rapid detection method targeting EF-TU gene for the detection of *Mycoplasma pneumoniae* in sheep, employing a biotin labelled FIP primer to facilitate lateral flow-based visualisation. Similarly, Wachiralurpan *et al*. (2017) developed a LAMP-LFD assay for the rapid screening of *Listeria monocytogenes* in raw chicken samples. Wasin *et al*. (2024) established a LAMP-LFD for the detection of echinococcus tapeworms in edible snail samples, which is visible to the naked eye within 70 min at 65°C.

In this study, we focused on the development of a LAMP-LFD assay for rapid, sensitive and visual detection of *V. vulnificus*. LAMP-LFD assay has significant potential for field-based applications. Beyond its utility in aquatic animal health management and disease prevention, the LAMP-LFD platform could also serve as a valuable tool for food safety monitoring, clinical diagnostics and public health surveillance, thereby contributing to the effective control of *V. vulnificus*-associated infections.

Materials and methods

Bacterial strains and shrimp samples

A total of 26 reference bacterial strains were used to evaluate the specificity of the assay, including 2 *V. vulnificus* strains, 8 other *Vibrio* species, and 16 non-*Vibrio* strains. Details of the strain designations and their sources are provided in Table 1. Healthy white-leg shrimp with an average body weight of approximately 8 g, were procured from an aquaculture farm in Donghai island of Zhanjiang City, China, prior to initiating the experiment, the shrimps were acclimated for two weeks in aerated tanks maintained at 28°C under laboratory conditions.

Genomic DNA extraction

All 26 strains were revived and cultured on appropriate media. The genomic DNA of all strains was extracted according to the instructions of the bacterial genomic DNA extraction kit (Tiangen, Beijing, China) and the concentration of the genomic DNA was determined using NanoDrop 2000 (Thermo Fisher Scientific, Inc.) and stored at -20°C as the templates for LAMP and PCR reactions.

Design and screening of LAMP primers and detection probe

LAMP primers were designed based on the sequence of RTX toxin-activated lysine acyltransferase (*RtxC*) gene of *V. vulnificus* (GenBank Acc. No. 66967455). Four sets of candidate primers (Table 2) were designed using PrimerExplorer V5 (https://primerexplorer.jp/lampv5e/index.html), and sequence comparison analysis showed that these primers had good specificity for *V. vulnificus*. The selected LAMP primer sets consisted of the forward inner primer (FIP),

Table 1. The bacterial species and strains used in this study

Species	Strain ID	Source
Vibrio vulnificus	10383	CICC
Vibrio vulnificus	1.1758	CGMCC
Vibrio harveyi	ZJ1603	GDOU
Vibrio harveyi	14126	ATCC
Vibrio alginolyticus	HY9901	GDOU
Vibrio parahaemolyticus	ZJ1502	GDOU
Vibrio fluvialis	ZJ1502	GDOU
Vibrio campbellii	1.8844	CGMCC
Vibrio furnissii	BH1902	GDOU
Vibrio cholerae	1.8676	CGMCC
Enterococcus faecalis	ZJ20133	GDOU
Klebsiella trevisan	1.6366	CGMCC
Enterobacter hormaechei	1.10608	CGMCC
Bacillus licheniformis	ZJ1906	GDOU
Acinetobacter radioresistens	ZJ201001	GDOU
Proteus mirabilis	20049	CICC
Escherichia coli	DH5α	GDOU
Aeromonas hydrophila	10868	CICC
Staphylococcus aureus	1.12409	CGMCC
Staphylococcus warneri	1.2824	CGMCC
Bacillus cereus	ZJ2328	GDOU
Klebsiella pneumoniae	1.839	CGMCC
Salmonella enteritidis	ZJ21005	GDOU
Pseudomonas aeruginosa	1.1785	CGMCC
Streptococcus agalactiae	ZQ0901	GDOU
Streptococcus iniae	ZQ1008	GDOU

CICC: China Center of Industrial Culture Collection
CGMCC: China General Microbiological Culture Collection Center
ATCC: American Type Culture Collection, Manassas, USA
GDOU: Guangdong Ocean University, Zhanjiang, China

backward inner primer (BIP), forward outer primer (F3) and backward outer primer (B3), which were synthesised by Sangon Biological Engineering Co. Ltd. For the LAMP-LFD assay, the 5' ends of the RtxC-FIP and DNA hybridization probe were labeled with biotin and FITC respectively, to facilitate visual detection using the lateral flow dipstick.

Table 2. Summary of primers and probes used in the study

Primer	Length	Sequence (5'– 3')
RTXC-F	18	GCGTATTCTGCCTGCTTT
RTXC-R	18	AGACCTTCTGCCCTTTCC
RTXC-F3	19	TTGGGCGTTTGTCTCTGAG
RTXC-B3	18	ATCCACTTTGCCGCGAA
RTXC-FIP[a]	45	(Biotin) CTGAACGCCAGTCGGATGGAGTTTTCAGAACCGAGATGAGCTGTT
RTXC-BIP	44	TGATTGCTCCATTCGGGCATGGTTTTCTTCTGCCCTTTCCACGG
DNA probe[b]	15	(FITC) GCATATCTACATTCC

LAMP reaction

The LAMP assay was performed in a total reaction volume of 25 µl containing 2.5 µl of 10×*Bst* reaction buffer, 5 µl of $MgSO_4$ (50 mmol l^{-1}), 3.5 µl of dNTPs (10 mmol l^{-1}), 1.6 µl each of internal primers (20 mmol l^{-1}), 0.2 µL each of external primers (10 mmol l^{-1}), 1 µl of *Bst* DNA polymerase (8 U), 1 µl of DNA template, and nuclease free DEPC-treated water to make up the final volume to 25 µl. The LAMP reaction tube was kept at 60°C for 1 h and then inactivated at 80°C for 2 min (except for LFD assay). The amplification products were analysed by 1.5% agarose gel electrophoresis.

Optimisation of LAMP reaction system

The LAMP reaction conditions were optimised by varying one parameter at a time while keeping other conditions unchanged. The effects of $MgSO_4$ concentration (0, 1, 2, 3, 4, 5, 6, 7 and 8 mM), betaine concentration (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 1 mM), dNTP concentration (0, 0.2, 0.4, 0.6, 0.8, 1, 1.2, 1.4, 1.6 and 1.8 mM), reaction temperature (58~67°C), reaction time (0, 1, 3, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 min), and the concentration ratios of the internal to external primers (F3:FIP and B3:BIP; 1:1, 1:2, 1:4, 1:8, and 1:16) were systematically evaluated to determine the optimal reaction conditions. The reaction products were analysed by 1.5% agarose gel electrophoresis.

Establishment of the visual LAMP-LFD assay for *V. vulnificus*

The visual LAMP–lateral flow dipstick (LAMP-LFD) assay was established using a 25 µl LAMP reaction mixture was performed under the optimised amplification conditions, by adding biotin-labeled *RtxC*-FIP primers in place of the normal *RtxC*-FIP primer. At the end of LAMP reaction, 5' FITC-labelled DNA probe (Table 2) was added directly to the reaction mixture and allowed to hybridise with the amplification products for 5 min at 62°C without prior heat inactivation of *Bst* DNA polymerase. Subsequently, 5 µl of LAMP reaction product was transferred to a new tube containing 80 µl of LFD buffer and the mixture was then applied to the sample pads on the LFD test strips. The test results were observed by naked eyes after 5 min. A test was judged positive when both test (T) line and control (C) line turned red, while when only the C line turned red, the test was judged negative.

Specificity evaluation of the LAMP-LFD assay for *V. vulnificus*

To evaluate the specificity of the developed LAMP-LFD assay for *V. vulnificus*, genomic DNA extracted from the 26 bacterial strains (detailed in Table 1) was used as the template for amplification. The LAMP amplification products were analysed by agarose gel electrophoresis and independently evaluated using the LFD assay to assess the specificity of target detection.

Sensitivity evaluation of the LAMP-LFD assay for *V. vulnificus*

To determine the analytical sensitivity of the LAMP–LFD assay, genomic DNA of *V. vulnificus* was serially diluted 10-fold to obtain concentrations ranging from 20.0 to 2.0×10^{-9} ng μl^{-1}. LAMP-LFD amplification was carried out, and the reaction products were detected by electrophoresis and LFD for sensitivity analysis. For evaluating the sensitivity of LAMP-LFD compared to that of conventional PCR, PCR reaction was performed in total of 20 µl reaction volume, including 10 µl of Premix rTaq, 1 µl each of primers *RtxC*-F1 and *RtxC*-R1, 1 µl of the diluted DNA and 7 µl of DEPC treated water. The PCR conditions were as follows: 95°C for 3 min, 35 cycles of 95°C for 15 s, 52°C for 20 s, 72°C for 40 s, and finally 72°C for 5 min. The amplified products were analysed by 1.5% agarose gel electrophoresis.

Applicability of the visual LAMP-LFD assay

To evaluate the applicability of the developed visual LAMP–LFD assay, healthy whiteleg shrimps were randomly divided into five

groups (I-IV) (with 10 shrimps per group) and placed in tanks containing well oxygenated water at 25-28°C, respectively. The shrimp were fed twice daily with commercial shrimp feed. Shrimp in groups I-IV were intraperitoneally injected with 0.1 ml bacterial suspension (1.0×10^7 CFU ml^{-1}) of *V. vulnificus*, *V. cholerae*, *V. harveyi* and *V. parahaemolyticus*, respectively. Group V was intraperitoneally injected with PBS as the negative control. At 8 h post-injection, 0.1 g of hepatopancreas tissue was collected from shrimp in each group for genomic DNA extraction using a bacterial genomic DNA isolation kit. The extracted DNA were subjected to PCR and LAMP-LFD assay as templates, to assess the performance of the method in experimentally infected shrimp.

The LAMP-LFD detection method was employed to detect *V. vulnificus* in 84 samples of diseased whiteleg shrimp with symptoms of vibriosis collected from coastal aquaculture areas in South China. Each sample was tested by LAMP-LFD for three times. In addition, conventional PCR was used as a comparative method to analyse these clinical samples. A paired analysis of the LAMP-LFD and PCR results was conducted to assess the presence of *V. vulnificus* in the shrimp samples. The comparison aimed to validate the performance of the LAMP-LFD method against the PCR results, providing a comprehensive evaluation of its diagnostic accuracy for detecting this pathogen in aquaculture products.

Results

Optimisation of the LAMP reaction

The LAMP reaction was optimised by setting the different concentrations of Mg^{2+}, betaine, dNTPs, reaction temperature, reaction time, betaine and the ratio of internal and external primers. The optimisation experiments indicated that the optimal LAMP reaction mixture consisted of: 1 µL of *Bst* DNA polymerase (8U), 2.5 µl of 10× *Bst* DNA polymerase buffer, 1 mM of dNTPs, 1.6 µM of the internal primers BIP/FIP, 0.2 µM of the external primers F3/B3, 1 µl DNA template, and DEPC treated water added to achieve a final reaction volume of 25 µl (Fig. 1).

Analysis of LAMP-LFD specificity

To evaluate the specificity of LAMP-LFD, genomic DNA from 10 *Vibrio* strains and 16 non-*Vibrio* strains was respectively tested as the template for LAMP using the optimal reaction conditions. The results of LAMP reaction were showed by LFD test strip and 1.5% agarose gel electrophoresis. The results indicated that only the products derived from *V. vulnificus* genomic DNA were successfully detected as positive samples, while other *Vibrio* and non-*Vibrio* strains yielded negative results in the LFD test strip as well as in agarose gel electrophoresis (Fig. 2).

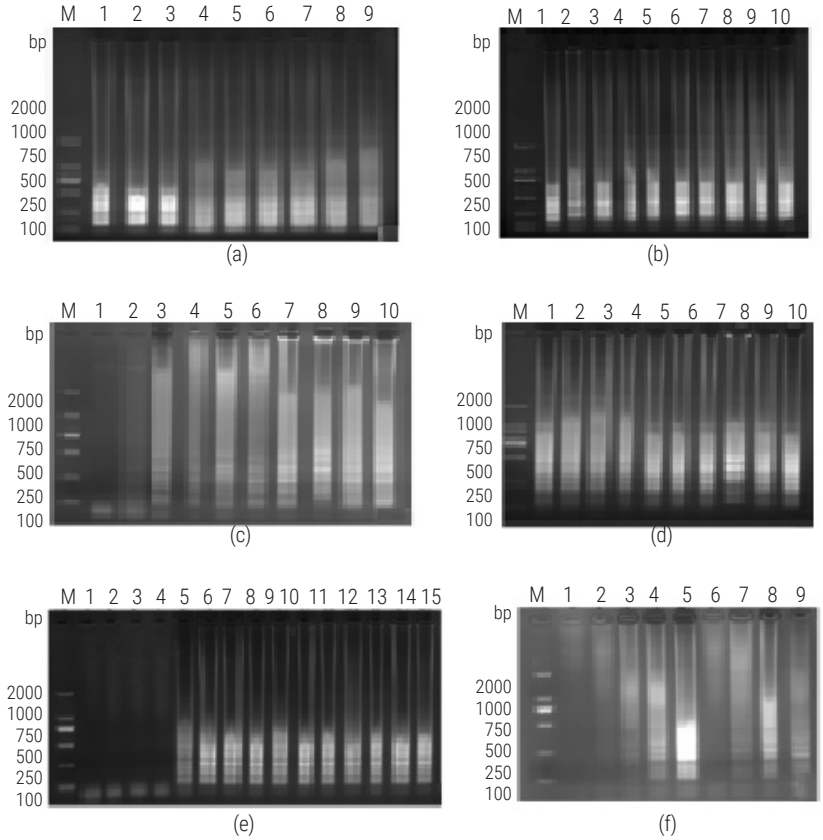

Fig. 1. Optimisation results of LAMP reaction conditions. (a) Mg^{2+} concentration. Lane M: DL 2000 DNA Marker, lane 1~9: 0, 1, 2, 3, 4, 5, 6, 7 and 8 mM; (b) Betaine concentration. Lane M: DL 2000 DNA Marker, lane 1~10: 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 1 mM; (c) dNTPs concentration. Lane M: DL 2000 DNA Marker, lane 1~10: 0, 0.2, 0.4, 0.6, 0.8, 1, 1.2, 1.4, 1.6 and 1.8 mM; (d) Reaction temperature. Lane M: DL 2000 DNA Marker, lane 1~10: 58, 59, 60, 61, 62, 63, 64, 65, 66 and 67°C; (e) Reaction time. Lane M: DL 2000 DNA Marker, lane 1~15: 0, 1, 3, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 min; (f) External/internal primer ratio. lane 1~5: 1:1, 1:2, 1:4, 1:8 and 1:16 (the final concentration of the external primer of 0.2 µM), lane 6~9: 1:1, 1:2, 1:4 and 1:8 (the final concentration of the external primer of 0.4 µM).

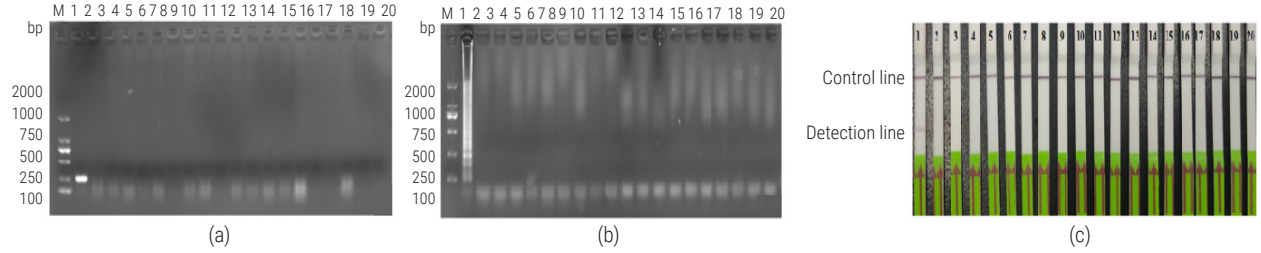

Fig. 2. *V. vulnificus* LAMP-LFD specific assay. (a) Agarose gel electrophoresis of PCR products; (b) agarose gel electrophoresis of LAMP products; (c) LAMP-LFD. Lane M: DL2000 DNA Marker, lane 1~20: *V. vulnificus*, *V. harveyi*, *V. alginolyticus*, *V. parahaemolyticus*, *V. fluvialis*, *V. campbellii*, *V. furnissii*, *V. cholerae*, *E. faecalis*, *K. trevisan*, *E. hormaechei*, *B. licheniformis*, *A. radioresistens*, *P. mirabilis*, *E. coli*, *A. hydrophila*, *S. aureus*, *B. cereus*, *P. aeruginosa*, *S. agalactiae*

Analysis of LAMP-LFD sensitivity

The extracted *V. vulnificus* genomic DNA was serially diluted in 10-fold increments and amplified using both LAMP and PCR primers, utilising the same template. The results indicated that after LAMP amplification, the minimum detectable template concentration for agarose gel electrophoresis and LFD detection was 2.0×10^{-5} ng μl^{-1}. In contrast, after PCR amplification, the minimum detectable concentration was 2.0×10^{-3} ng μl^{-1}. Therefore, the LAMP-LFD method demonstrated a sensitivity 100 times greater than that of PCR (Fig. 3).

Applicability of the LAMP-LFD

In this study, LAMP-LFD and PCR methods for detecting *V. vulnificus* were developed for clinical application. The results demonstrated that LAMP-LFD could accurately detect the presence of *V. vulnificus* in the DNA extracted from the hepatopancreas of infected shrimp (*P. (L.) vannamei*), with the results of LAMP-LFD aligning with those of PCR (Fig. 4), indicating that the LAMP-LFD method established is suitable for clinical diagnosis. The LAMP-LFD method was used to detect *V. vulnificus* in 84 samples from the coastal aquaculture area

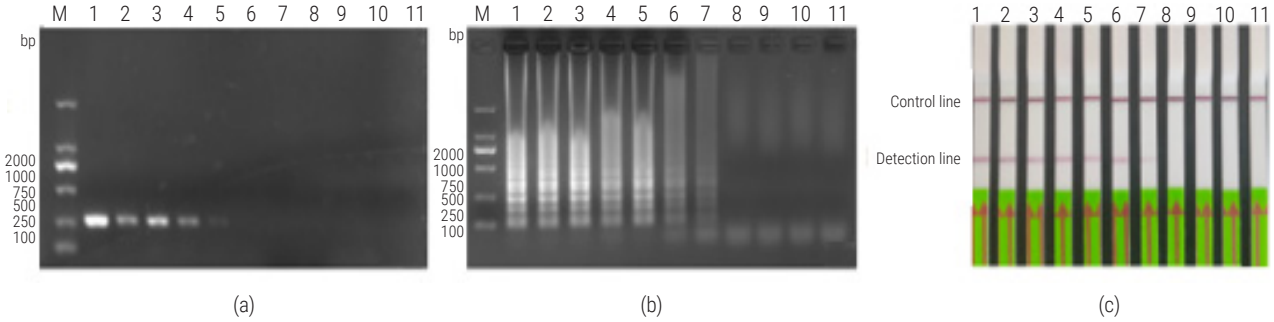

Fig. 3. Comparison of sensitivity for the detection of *V. vulnificus* between PCR (a) LAMP; (b) LAMP-LFD; (c) Lane M: DL2000 DNA Marker, lane 1~11: 20.0 ng μL^{-1}~2.0×10^{-9} ng $\mu L^{-1}$for 10-fold dilution.

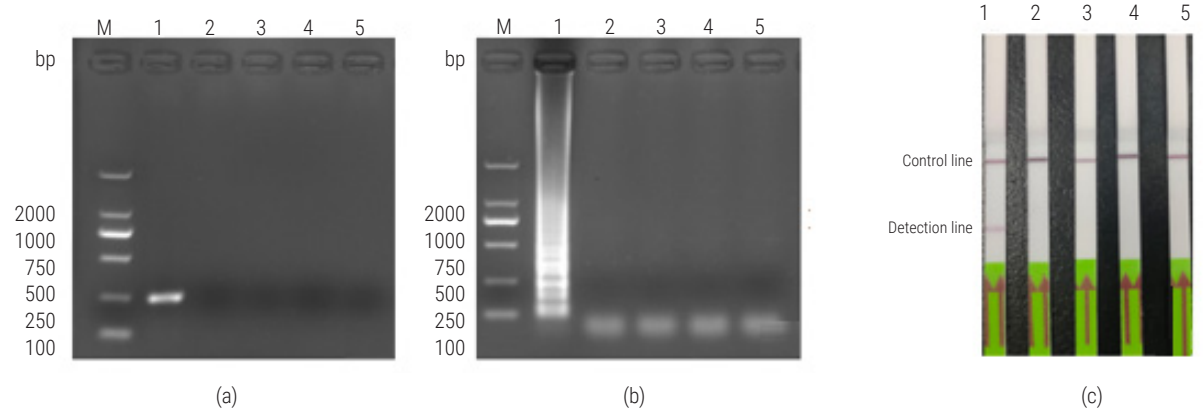

Fig. 4. LAMP-FLD detection in shrimp injected with different *Vibrio* strains. (a) Agarose gel electrophoresis of PCR products; (b) Agarose gel electrophoresis of LAMP products; (c) LAMP-LFD. Lane M: DL2000 DNA Marker, Lane 1: *V. vulnificus* infection, Lane 2: *V. cholerae* infection, Lane 3: *V. harveyi* infection, Lane 4: *V. parahaemolyticus* infection, Lane 5: PBS control.

of South China, and PCR was used for comparative detection, and the detection results are shown in Table 3. LAMP-LFD assay identified as positive for all samples infected by *V. vulnificus* and negative for the samples infected by the other strains of non-*V. vulnificus*, which was 100% consistent with the results of specificity of PCR assay.

Table 3. Results of LAMP-LFD and PCR methods employed to detect *V. vulnificus* in *P. (L.) vannamei*

Farms	Species	Number	Number of positive samples		Positive rate		Consistency rate
			LAMP-LFD	PCR	LAMP-LFD	PCR	
1	Whiteleg shrimp	22	19	19	86.36%	86.36%	100%
2	Whiteleg shrimp	16	13	13	81.25%	81.25%	100%
3	Whiteleg shrimp	18	17	17	94.44%	94.44%	100%
4	Whiteleg shrimp	13	11	11	84.62%	84.62%	100%
5	Whiteleg shrimp	15	15	15	100%	100%	100%

Discussion

V. vulnificus is a pathogenic bacterium commonly found in marine environments that has garnered significant attention in shrimp aquaculture in recent years (Prabina, 2023). As a thermophilic zoonotic pathogen, it not only poses a threat to shrimp health but also has the potential to impact human health. The bacterium exhibits polymorphism and strong drug resistance, making infections prone to recurrence and difficult to treat, which significantly hinders the development of the aquaculture industry (El-Zamkan *et al*., 2023). Therefore, the development of a rapid, sensitive, and accurate detection method for *V. vulnificus* is essential for the timely diagnosis and and effective control of infections, thereby minimising its impact on human health, aquatic animals and the aquaculture industry. Nucleic acid amplification methods for pathogen detection can be broadly classified into two categories: thermal cycling-based methods and thermostatic methods. Thermal cycling methods are primarily represented by PCR and qPCR (Cui *et al*., 2024), while thermostatic methods mainly involve nucleic acid isothermal amplification techniques, such as LAMP and recombinase-aided amplification (RAA) (Tian *et al*., 2022). However, thermal cycling assays not only rely on expensive real-time fluorescence PCR instruments but also have long processing times and require a high level of technical expertise from the operator, making them less suitable for use in field settings. These limitations reduce the practicality and broad applicability of thermal cycling methods for routine testing. Therefore, establishing a rapid, simple, sensitive, specific and visual detection method for *V. vulnificus* is essential for field level detection and comprehensive management of *V. vulnificus* infections.

The MARTX family of toxins, a key virulence factor of *V. vulnificus*, is highly homologous and conserved across many serotypes of the species (Hernández-Cabanyero *et al*., 2023). Based on this target gene, the present study aimed to establish an efficient and specific LAMP-LFD method for detecting *V. vulnificus* by targeting the MARTX family toxin genes. This approach is of great significance for the diagnosis and prevention of diseases caused by *V. vulnificus*. In this study, four LAMP-specific primers were designed based on the *RtxC* gene sequence within the MARTX toxin family. After optimising the reaction conditions, a probe was introduced for LAMP-LFD detection. The results indicated that nucleic acid amplification *via* LAMP at 62°C, followed by LFD visualisation, required a minimum of 20 min for detection by the naked eye. Wang *et al*. (2020a) developed a LAMP-LFD method for detecting *Alexandrium catenella*, with the reaction completed at 61°C in 75 min. Similarly, Wachiralurpan *et al*. (2017) established a LAMP-LFD method for detecting *Listeria monocytogenes* in raw chicken meat, with optimal temperature ranges of 60-65°C and 60-63°C for amplifying *plcB* and *hly* genes, respectively, and results could be visualised within 90 min. Cui X *et al*. (2024) found that the qPCR method for detecting *V. vulnificus* required thermal cycling for over 1 h. Compared with the qPCR method, the LAMP-LFD assay features a shorter assay time, relies only on an isothermal heating device, requires no expensive instrumentation, and enables direct visual detection.

The *V. vulnificus* LAMP-LFD assay developed in this study demonstrated high specificity, showing no cross-reactivity with 8 other *Vibrio* strains and 16 non-*Vibrio* strains. LAMP-LFD had a minimum detection concentration of 2.0×10^{-5} ng μl^{-1}, with a sensitivity 100 times higher than that of PCR. Furthermore, the results of the LAMP-LFD assay could be directly visualised by the naked eye in just 25 min, underscoring that *V. vulnificus* LAMP-LFD method is a highly suitable protocol for field diagnosis in aquaculture. This rapid, efficient detection process makes it ideal for on-site testing, allowing for timely and accurate diagnosis.

Wang *et al*. (2020a) reported that the lowest detection limit of the LAMP-LFD method for detecting *A. catenella* was 4.63×10^{-4} ng μl^{-1}, while Kiatpathomchai *et al*. (2008) established a LAMP-LFD method for detecting shrimp Taura syndrome virus with a minimum detection limit of 1×10^{-7} ng μl^{-1}. In this study, the sensitivity of LAMP-LFD was 2.0×10^{-5} ng μl^{-1}, which is consistent with the results reported in the above studies. This confirms the high sensitivity of the LAMP-LFD assay, aligning with similar detection limits observed by other researchers.

Currently, common methods for detecting LAMP amplification products include agarose gel electrophoresis, turbidimetry and use of fluorescent dyes. Ren *et al*. (2009) established a LAMP method targeting *hemolysin* gene sequence of *V. vulnificus*, using a reaction at 65°C for 60 min, with amplification products detected *via* SYBR Green I visualisation and agarose gel electrophoresis. However, these methods are susceptible to aerosol contamination, potentially leading to misclassification of results (Zhang *et al*., 2019). In contrast, LAMP technology combined with LFD test strips utilises FITC-labeled probes that specifically hybridise with biotin-labeled amplification products. This allows for detection by the naked eye within a short time, avoiding the drawbacks of traditional detection methods while maintaining high specificity and sensitivity in the assay.

LAMP-LFD was used to detect *V. vulnificus* in artificial *Vbirio*-infection in whiteleg shrimp and clinic samples of whiteleg shrimp with symptoms of vibriosis. The results demonstrated that only *V. vulnificus*-infected shrimp yielded positive results, which were fully consistent with the PCR findings. This confirms that the established LAMP-LFD method for detecting *V. vulnificus* is rapid, accurate, sensitive, and

simple, making it a direct and reliable tool for field applications. This offers a more reliable and convenient method for the rapid diagnosis and early detection of diseases caused by *V. vulnificus*.

This study has certain limitations that should be acknowledged for future optimisation. Firstly, the current LAMP-LFD assay lacks an internal amplification control (Higgins *et al*., 2018). Without an internal amplification control, the risk of false-negative results cannot be entirely ruled out, as potential PCR inhibitors present in complex biological matrices like shrimp hepatopancreas could impede the amplification process. Future iterations of this assay should incorporate an internal amplification control (*e.g.*, co-amplifying a highly conserved host housekeeping gene) to validate the reliability of negative results. Second, while LAMP offers robust amplification, its application is limited by the complexity of primer design, which requires a set of four to six specific primers recognising distinct regions of the target gene (Becherer *et al*., 2020). Additionally, the extremely high sensitivity of LAMP increases the risk of aerosol contamination, especially when reaction tubes must be opened for downstream LFD detection. This contamination could potentially lead to false positives in field conditions (Liu *et al*., 2024). Therefore, strict physical separation between the amplification and LFD detection steps is highly recommended. In practical aquaculture settings, false negatives might also occur if the target pathogen titre is below the detection limit during the early stages of infection, whereas false positives could arise from non-specific cross-reactivity with unknown environmental microflora in aquaculture water.

In conclusion, we successfully established a LAMP-LFD technique based on the *RtxC* gene of *V. vulnificus* in this study. This method is highly specific for detecting *V. vulnificus* and requires only 25 min for the results of the detected samples. The method has excellent sensitivity and specificity, with accurate detection of *V. vulnificus* in infected *P.* (*L.*) *vannamei*, making it ideal for field applications.

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