



Development of a loop-mediated isothermal amplification (LAMP) assay for rapid visual detection of snakehead vesiculovirus (SHVV) in snakehead

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Abstract

Infections caused by snakehead vesiculovirus (SHVV) have seen frequent outbreaks in recent years, inflicting significant losses on the snakehead aquaculture industry. Early detection is therefore essential for effective prevention and control of pathogenic infections and reduction of economic losses caused by infections. There is an urgent need for a simple, rapid, specific, sensitive, and intuitive method to monitor snakehead infected with SHVV. The aim of the present study was to develop and evaluate a loop-mediated isothermal amplification (LAMP) assay for the rapid visual detection of SHVV in snakehead. Three pairs of primers were designed according to the conserved region of phosphoprotein (P) gene sequences of SHVV and were applied for the detection of SHVV from fish samples. Time and temperature conditions for the amplification of SHVV were optimized at 65 °C and 55 min. The LAMP assay demonstrated high specificity, with no cross-reactivity with seven other viruses. Amplification results were visualized by a color change after the addition of hydroxynaphthol blue (HNB) dye. Sensitivity test results showed that the minimum detection volume with this method was 1.76×10^2 copies/ μ L, which was 100 times more sensitive than RT-PCR assay. We used the established LAMP system to test 50 clinical samples and detected 32 positive responses, whereas 22 positive samples out of 50 samples were detected by RT-PCR. The establishment of a visual LAMP assay further shortens the virus detection process and allows visual reading of positive responses through color changes; it is suitable for use in quarantine and field detection. Therefore, this proposed method provides a sensitive, specific, and user-friendly method for the rapid diagnosis of SHVV in snakehead farming.

Keywords Snakehead vesiculovirus · Visualization · Loop-mediated isothermal amplification · Rapid detection

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Introduction

Snakehead vesiculovirus (SHVV) is a negative-sense single-stranded RNA virus that belongs to the family Rhabdoviridae, genus *Perhabdovirus*, and is the main pathogen causing snakehead (*Channa argus*) virus disease (Cao et al. 2021). Fish infected with SHVV often exhibit physical signs such as darker skin pigmentation, pale gills that lack blood, reduced appetite, weak swimming ability, occasionally frenzied behavior, and a tendency to gather in areas with slow water flow; liver, spleen, and kidney enlargement with ascites can be observed at necropsy (Liu et al. 2015a). SHVV infections can result in large-scale mortality of snakeheads, inflicting substantial economic losses on the snakehead aquaculture industry in China (Zhang et al. 2019a; Sun et al. 2020). Effective disease management planning requires early and

proper screening that identifies the pathogenic factors of the disease (Sarkes et al. 2020). Therefore, early detection plays a crucial role in preventing and controlling pathogenic infections and minimizing economic losses. Currently, techniques such as reverse transcription polymerase chain reaction (RT-PCR), real-time PCR, and electron microscopy are used for SHVV detection. However, these methods have drawbacks, including long test cycles, cumbersome operation, and low specificity (Ward and Harper 2012; Rigano et al. 2014). Therefore, the development of a fast, efficient, and sensitive detection method targeting the phosphoprotein (P) gene carried by SHVV is of great practical importance. Such a method would enable prompt detection, efficient intervention, and rapid control of SHVV-caused diseases in aquaculture production.

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method, with advantages over RT-PCR in that the reaction system can be continuously and rapidly amplified at a constant temperature without repeated thermal denaturation and warming and cooling. It is economical and convenient, and the reaction system is stable and reliable (Soroka et al. 2021). Identification against the six loci of the gene provides high sensitivity and specificity, and enables rapid detection of pathogens independently of contaminated samples and interfering fragments (Zhang et al. 2019b; Li et al. 2018). In 2008, the first visual LAMP assay was developed to visualize the significant fluorescence changes during the reaction, and due to the high sensitivity of the signal recognition, the system was able to visualize the results without costly specialized equipment (Tomita et al. 2008). Thanks to the simplicity, rapidity, efficiency, and affordability of the LAMP reaction, it has been widely adopted for the detection of several pathogens, including viruses (Jones et al. 2022), bacteria, and parasites (Garg et al. 2021; Mugambi et al. 2015), as well as some bacteria in the environment (Okai et al. 2022).

The mortality rate of fish infected with SHVV is very high. So far, there are no effective diagnostic tests or effective prevention and control methods for this virus. Therefore, a test is urgently needed for the early detection of SHVV infection. In this study, a specific ring-mediated isothermal

amplification primer was designed for the detection of the P gene of SHVV, and the complementary DNA (cDNA) of SHVV was used as the template to establish a LAMP detection reaction system. The specificity and sensitivity of the LAMP assay were then verified, and it was possible to visualize the results with the aid of hydroxynaphthol blue (HNB) dye indicators. A visual LAMP assay that is specific, sensitive, user-friendly, and reliable for the rapid detection of SHVV was developed.

Materials and methods

Virus and samples

The SHVV were isolated from the infected snakeheads and kept in our lab. A negative control group with infectious spleen and kidney necrosis virus (ISKNV), largemouth bass ranavirus (LMBV), red sea bream iridovirus (RSIV), red-spotted grouper neuron necrosis virus (RGNNV), grass carp reovirus (GCRV), and cyprinid herpesvirus 2 (CyHV-2) were also stored in our lab.

Primer design and synthesis

Multiple sets of LAMP primers were designed by Primer-Explorer V4 software based on the published SHVV phosphoprotein (P) gene as the target sequence in GenBank (GenBank accession number: OQ211331.1), and the primers were compared and screened by pre-experiment. Finally, one set of LAMP primers were identified (Table 1): outer primer (SHVV-F3/SHVV-B3), inner primer (SHVV-FIP [forward inner primer]/SHVV-BIP [backward inner primer]), and the loop primer (SHVV-LF/SHVV-LB). All primers were synthesized by Tsingke Biotechnology (Nanjing) Co., Ltd.

Preparation of the viral template cDNA

Total RNA was isolated using TRIzol Reagent (Beijing Aomi Jiade Pharmaceutical Technology Co., Ltd.) according to instructions provided by the manufacturer. The

Table 1 Primer sequences used for LAMP

Primer name	Sequence (5'–3')	Position
<i>SHVV-F3</i>	CCCGACTGAGCTGATCTTGA	595–614
<i>SHVV-B3</i>	CACCTCGAGAGGGATGTACT	831–840
<i>SHVV-FIP</i>	GGCACATGGCTTGAGGATAGCTCTGTGCTCTGG GCTTCTG	632–649, 684–705
<i>SHVV-BIP</i>	GCAAGTAGACATCGGGGCCGCTGGGAGTTTTTCG CCAACAT	732–749, 781–800
<i>SHVV-LF</i>	GGTCTTCTTCTCGACTACCCTTAC	652–675
<i>SHVV-LB</i>	GTAAGGGTAGTCGAGAAGAAGACC	652–675

concentration and quality of total RNA were measured using an ultra-micro spectrophotometer (Tiangen Biotech Co., Ltd.) and verified by agarose gel electrophoresis. cDNA synthesis was performed using the PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara Biotech Co., Ltd.) according to instructions provided by the manufacturer.

Establishment and optimization of LAMP reaction system

A 25- μ L system was used in the LAMP reaction, with 2.5 μ L 10 \times ThermoPol buffer, 5 μ L betaine (5 M), 1 μ L template cDNA (SHVV), F3 (10 M), B3 (10 M), FIP (100 M), BIP (100 M), LF (10 M), LB (10 M), 1 μ L Bst polymerase (8 U/ μ L), 3 μ L dNTPs (10 mM), and 1 μ L MgSO₄ (100 mM). Finally, diethyl pyrocarbonate (DEPC)-treated water was added to a volume of 25 μ L to establish the LAMP reaction system. The amount of external, internal, and ring primer, and the temperature and time of the reaction were optimized to determine the optimal reaction conditions for the system.

Specificity assay of LAMP amplification

The specificity of LAMP amplification was tested using ISKNV, LMBV, RSIV, RGNNV, GCRV, and CyHV-2 genomic cDNA as templates under optimized conditions. The LAMP products were examined by agarose gel electrophoresis to determine the results.

Hydroxynaphthol blue (HNB) detection of LAMP amplification products

Apart from gel electrophoresis, a color development reaction was applied to assess the results of LAMP amplification. In the optimized LAMP assay system of SHVV, 2 μ L of HNB was added, but the total system was kept at 25 μ L. After water-bath heating, the positive samples showed a change from purple to blue under natural light, while the negative samples exhibited no color change.

Sensitivity testing of LAMP amplification

The pMD19T-P2 plasmid was amplified and recovered from the P2 primer, and the plasmid concentration was determined by a NanoDrop 2000 spectrophotometer and converted to copy number through equations. The plasmid samples were diluted tenfold using the limited dilution method, and the diluted samples were used as templates for LAMP reactions. Sensitivity testing was conducted according to the established visual LAMP assay. The samples were then observed with the naked eye for color changes, and after the reactions, agarose gel electrophoresis was performed. The sensitivities were compared with the normal RT-PCR assay.

Clinical application

The LAMP assay and the RT-PCR assay were used to test 50 clinical samples simultaneously, and the positive samples were sequenced and identified to compare and validate the reliability and clinical effectiveness of the LAMP assay.

Results

Results of reaction condition optimization

The optimization results for the amount of outer, inner, and loop primers in the LAMP reaction showed obvious stepped bands in the PCR products when the amount of outer primers was 0.4 μ L, the amount of loop primers was 1 μ L, and the amount of inner primers was 0.4 μ L. Therefore, 0.4 μ L, 1 μ L, and 0.4 μ L were confirmed as the most applicable for the outer, inner, and loop primers, respectively, for the reaction (Fig. 1A–C). LAMP reaction temperature and time optimization results showed that 65 °C was the optimal reaction temperature among six temperature gradients (Fig. 1D). Among the five time gradients, 55 min was the optimal time for reaction (Fig. 1E).

After optimization, the optimal parameters of the LAMP reaction system were determined to be 2.5 μ L of 10 \times ThermoPol buffer, 5 μ L of betaine (5 M), 1 μ L of template cDNA (SHVV), 0.4 μ L of F3 (10 M), 0.4 μ L of B3 (10 M), 0.4 μ L of FIP (100 M), 0.4 μ L of BIP (100 M), LF (10 M) 1 μ L, LB (10 M) 1 μ L, Bst polymerase (8 U/ μ L) 1 μ L, dNTPs (10 mM) 3 μ L, MgSO₄ (100 mM) 1 μ L, and finally DEPC water was added to 25 μ L; the reaction conditions for LAMP were held at 65 °C for 55 min, followed by termination of the reaction at 85 °C for 5 min.

Specificity of LAMP for SHVV

The established LAMP assay had no cross-reaction with ISKNV, LMBV, RSIV, RGNNV, GCRV, and CyHV-2. The results of LAMP amplification indicated that SHVV amplifies ladder-like bands, while the other six pathogens failed to show any amplified bands (Fig. 2). The results indicated that the established LAMP assay for SHVV is highly specific.

Hydroxynaphthol blue (HNB) detection of LAMP amplification products

The results indicated that adding HNB directly to the reaction system does not affect the LAMP reaction. The reaction solution of the positive samples gradually deepened in blue color as the reaction time increased, while the control samples remained unchanged in purple (Fig. 3A). Agarose gel electrophoresis revealed that the amplification products

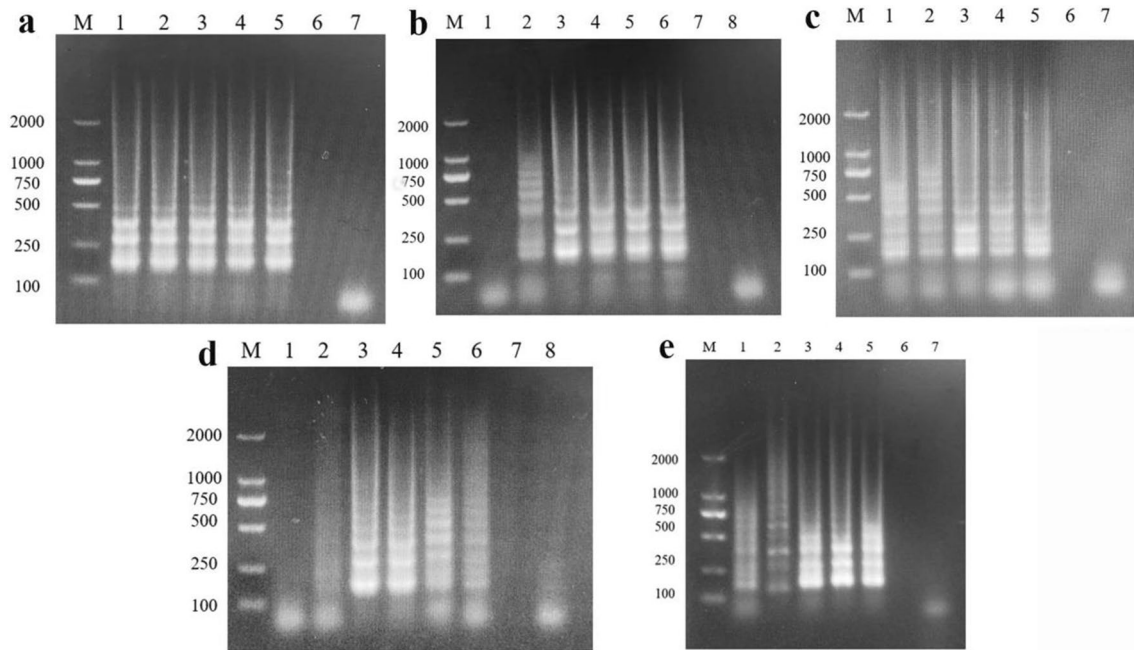


Fig. 1 Optimal reaction conditions of the LAMP assay for detecting SHVV. **a** Outer primers optimization (M: DL2000; 1–5: P2 outer primers of 0.2 μL, 0.4 μL, 0.6 μL, 0.8 μL, 1 μL; 6: blank; 7: control). **b** Loop primer optimization (M: DL2000; 1–6: P2 loop primers of 0.2 μL, 0.6 μL, 1 μL, 1.4 μL, 1.8 μL, 2.2 μL; 7: blank; 8: control). **c**

Inner primer optimization (M: DL2000; 1–5: P2 inner primers of 0.2 μL, 0.4 μL, 0.6 μL, 0.8 μL, 1 μL; 6: blank; 7: control). **d** Temperature optimization (M: DL2000; 1–6: 59 °C, 61 °C, 63 °C, 65 °C, 67 °C, 69 °C; 7: blank; 8: control). **e** Time optimization (M: DL2000; 1–5: 35 min, 45 min, 55 min, 65 min, 75 min; 6: blank; 7: control)

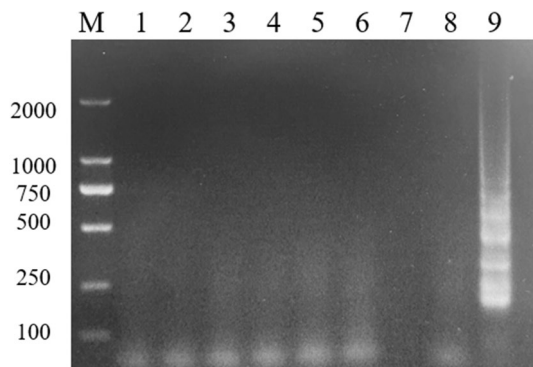


Fig. 2 Specificity of the SHVV LAMP assay. (M: DL2000; 1: ISKNV; 2: LMBV; 3: RSIV; 4: RGNNV; 5: GCRV; 6: CyHV-2; 7: Blank; 8: control; 9: SHVV)

of the positive reaction tubes formed a ladder pattern, while the negative control had no bands, which also verified the reliability of the visualization results (Fig. 3B).

The optimal reaction system for visualized LAMP is 25 μL, and it was detected by 2.5 μL 10×ThermoPol buffer, 5 μL betaine (5 M), 1 μL template cDNA (SHVV), 0.4 μL F3 (10 M), 0.4 μL B3 (10 M), 0.4 μL FIP (100 M), 0.4 μL BIP (100 M), 1 μL LF (10 M), 1 μL LB (10 M), 2 μL HNB (1.5 mM), 1 μL Bst polymerase (8 U/μL), 3 μL dNTPs (10 mM), and 1 μL MgSO₄ (100 mM). Finally, DEPC water was added to a volume of 25 μL, and the LAMP reaction

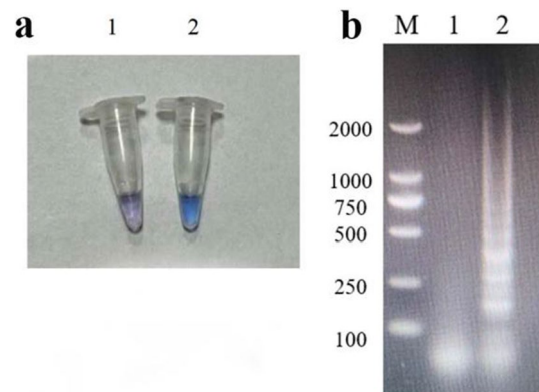


Fig. 3 HNB staining and agarose gel detection of LAMP reactions. **a** Visual results by adding HNB. (1: HNB, control; 2: HNB, SHVV). **b** Results of agarose gel electrophoresis staining with DNA_{GREEN}(UV) (M: DL2000; 1: HNB, control; 2: HNB, SHVV)

conditions were held at 65 °C for 55 min, followed by termination of the reaction at 85 °C for 5 min.

Sensitivity of SHVV LAMP

A tenfold serial dilution of the pMD19T-P2 plasmid was adopted as the template for the LAMP amplification reaction using the optimized method. The results demonstrated

a blue color for all positive tubes (Fig. 4A), and clear step-wise bands were amplified at concentrations ranging from 10^{-1} to 10^{-10} (Fig. 4B), which suggests that the sensitivity of the LAMP assay for SHVV is 1.76×10^2 copies/ μ L. In addition, RT-PCR amplification reactions were performed using a tenfold serial dilution of the pMD19T-P2 plasmid as a template with outer primers. The findings indicated that clear bands can be amplified at plasmid concentrations from 10^{-1} to 10^{-8} (Fig. 4C) with sensitivity of 1.76×10^4 copies/ μ L. This study indicated that the LAMP assay developed in this experiment is more sensitive than the RT-PCR assay.

Clinical application

The assay of the 50 samples showed 22 positive samples detected by RT-PCR and 32 positive samples detected by LAMP, with a positive rate of 64%, thus yielding a higher positive rate than that of RT-PCR (Table 2). All positive products were identified as SHVV-positive through

Table 2 Results of LAMP assay and common PCR assay

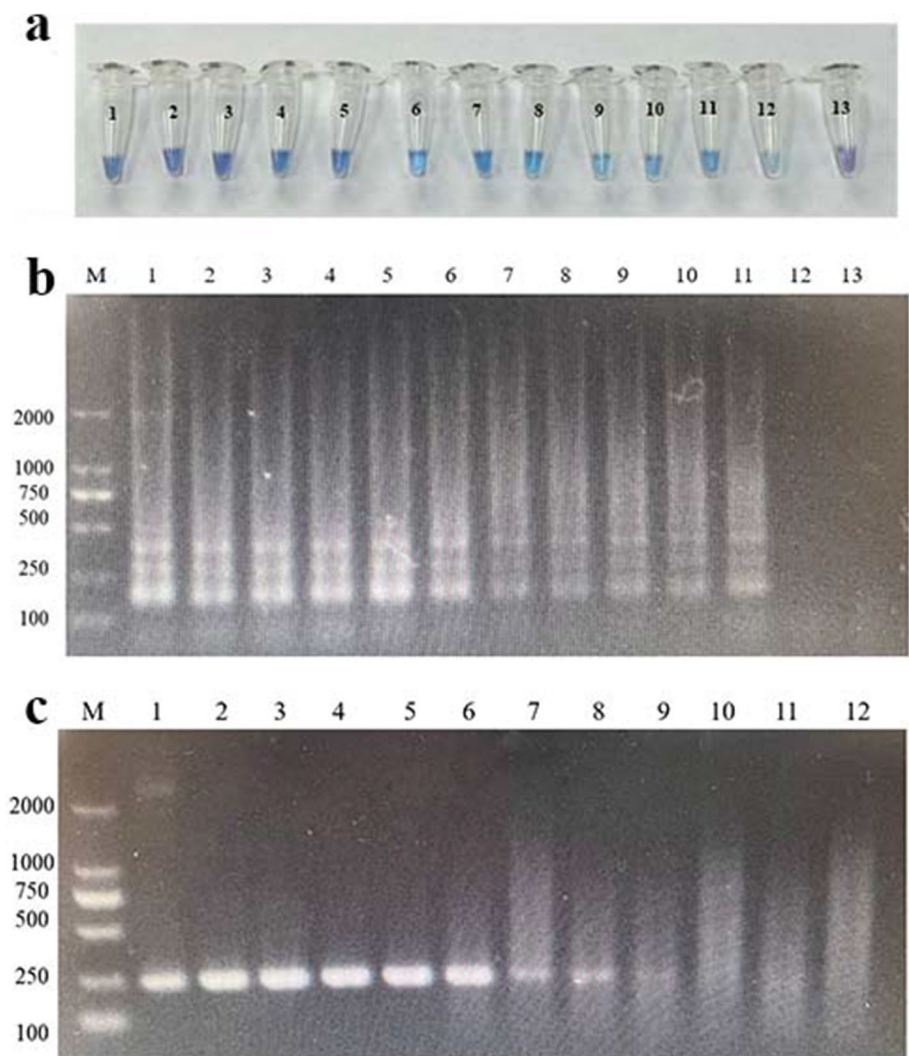
Assay results	LAMP	RT-PCR
Number of positive samples	32	22
Number of negative samples	18	28
Positive rate	64%	44%

sequencing. The results indicate that the LAMP assay outperforms the RT-PCR assay and provides better clinical detection results.

Discussion

Snakehead vesiculovirus (SHVV) has a wide range of hosts and can infect fish such as *Siniperca chuatsi* (Perciformes: Percichthyidae), in addition to *Channa*. When fish are infected with SHVV, they develop a darker body surface

Fig. 4 Comparison of the sensitivity of LAMP and RT-PCR for the detection of SHVV. **a** Sensitivity of the SHVV LAMP assay and visual results by adding HNB. **b** Sensitivity of the SHVV LAMP assay and results of agarose gel electrophoresis staining with DNA_{GREEN}(UV). **c** Sensitivity of the SHVV RT-PCR assay and results of agarose gel electrophoresis staining with DNA_{GREEN}(UV) (M: DL2000; 1–12: 1.76×10^{12} copies/ μ L– 1.76×10^1 copies/ μ L of the recombinant plasmid at tenfold gradient dilution, respectively; 13: control)



and pale gills, and necropsy shows ascites and no food in the gastrointestinal tract. The mortality rate of fish infected with this virus is extremely high, and there is a lack of diagnostic and effective prevention and control methods for this virus.

LAMP is a novel technique developed by Notomi et al. (2000) for the in vitro thermostatic amplification of nucleic acid fragments, and has been widely used in recent years to detect pathogens, as it is highly specific, efficient, and responsive (Yu et al. 2013; Arunrut et al. 2020). Yamamoto et al. used LAMP to detect *Candida auris* (Yamamoto et al. 2018), and Ou et al. developed a LAMP method based on real-time fluorescence and visual observation for the detection of *Salmonella enterica*, a zoonotic pathogen with a conserved *invA* gene (Ou et al. 2021). Leptospirosis is a genetically zoonotic disease that afflicts both humans and animals worldwide, and Hsu et al. reported a novel LAMP method for the pathogenic *Leptospira lipL32* gene (Hsu et al. 2017). Recently, LAMP assays have also been widely applied to detect pathogens in the aquaculture industry. For example, Liu et al. developed a ring-mediated isothermal amplification (LAMP) method for the visual detection of *Plesiomonas shigelloides* (Liu et al. 2019). Cai et al. reported a LAMP assay to monitor streptococcal disease in fish during culture (Cai et al. 2012). Xu et al. established a rapid, specific, and sensitive method based on LAMP to better control outbreaks of RGNNV (Xu et al. 2010).

The genome of SHVV contains nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), RNA-dependent RNA polymerase (L) five structural proteins (Hegazy et al. 2021; Sun et al. 2017). Phosphoprotein (P) is a key factor that promotes genomic transformation from the transcription state to replication state, changes its structure through high phosphorylation, initiates the binding of the nucleocapsid to viral genomic RNA, and plays an important role in the process of RNA and protein complex formation (Basak et al. 2003; Leyrat et al. 2011). In this study, a LAMP method was established to detect SHVV in snakehead for the P gene of SHVV.

Currently, the methods commonly used to identify LAMP reaction products at home and abroad include dye, turbidity, and gel electrophoresis (Liu et al. 2015b; Lu et al. 2015; Sriworat et al. 2015). Our LAMP detection can be performed simply through agarose gel electrophoresis to observe the amplification products of stem ring structure with various fragment lengths, which are shown as stepped strips starting from the sample holes in the electrophoretic map, or HNB dye indicator can be added to the amplification tube (Fig. 3). Color changes are used to determine the amplification process over time, ensuring high sensitivity while making the results more phenomenal and visual, without the need for tedious electrophoresis processes. Specificity is an important indicator in LAMP detection technology. In this study, the P gene was selected as the target gene sequence to design

LAMP-specific primers that effectively amplified SHVV with no amplification of any other viruses, showing high specificity (Fig. 2). Sensitivity is another important indicator in LAMP detection technology. Many studies have shown that the sensitivity of LAMP detection is generally higher than that of corresponding PCR detection (Meng et al. 2017). The sensitivity of the LAMP detection method in this study was compared with that of RT-PCR. The LAMP assay for SHVV demonstrated minimum detection of 1.76×10^2 copies/ μL , 100 times more sensitive than the RT-PCR assay (Fig. 4), which is unmatched by conventional PCR assays. Liang established a TaqMan real-time fluorescence quantitative PCR method for *Siniperca chuatsi* rhabdovirus detection. Sensitivity tests showed that conventional PCR assay had a detection limit of 1×10^4 copies/ μL , whereas the minimum detection limit in this study was 1.76×10^4 copies/ μL . The detection limit of the real-time PCR was 100 copies/ μL , whereas the minimum detection limit of the LAMP assay for SHVV was 1.76×10^2 copies/ μL (Liang et al. 2019). Therefore, the LAMP detection method established in this study is highly sensitive and specific, with a short reaction time, and it can therefore be considered the optimal method for the screening of SHVV infection in aquaculture.

The rapid LAMP assay for SHVV developed in this study excels in the accurate detection of SHVV. It is a new method for rapid screening of SHVV and is characterized by easy operation, short time consumption, high sensitivity, and high specificity, and requires no sophisticated instruments or expensive consumables. It is therefore highly effective in the rapid primary screening of clinical samples and can provide effective technical support for time-efficient diagnosis of SHVV pathogens in snakehead aquaculture sites and in labs.

Author contributions MG: methodology, investigation, writing—original draft preparation, data curation. ZZ: language polishing. SX: data curation. VNV: writing—review. WK: writing—review & editing, supervision, project administration. XL: conceptualization, methodology, data curation, funding acquisition.

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Data availability Data will be made available on request.

Declarations

Conflict of interest There are no conflicts of interest declared by the authors.

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