*Turk. J. Fish.& Aquat. Sci. 22(10), TRJFAS21519*
https://doi.org/10.4194/TRJFAS21519

RESEARCH PAPER

Turkish Journal of FISHERIES and AQUATIC SCIENCES

# Visual LAMP Method for Detection of White Spot Syndrome Virus in Shrimp and Crayfish

**Chao Ma[1,#], Zhuo Tian[2,#], Lu Bai[2], Lili Yang[1], Jijuan Cao[1,*]**

[1]Dalian Minzu University, Key Laboratory of Biotechnology and Resource Utilization Ministry of Education, 116600, Dalian, China.
[2]Dalian Customs Technology Center, 116001, Dalian, China.
[#]These authors share the first authorship.

**How to cite**

Ma, C., Tian, Z., Bai, L., Yang, L., Cao, J. (2022). Visual LAMP Method for Detection of White Spot Syndrome Virus in Shrimp and Crayfish. *Turkish Journal of Fisheries and Aquatic Sciences, 22(10), TRJFAS21519.* https://doi.org/10.4194/TRJFAS21519

**Article History**
Received 19 March 2022
Accepted 09 June 2022
First Online 17 June 2022

**Corresponding Author**
Tel.: +8615140588808
E-mail: caojijuan@dlnu.edu.cn

**Keywords**
White spot syndrome virus
Loop-mediated isothermal amplification (LAMP)
Visualization
Rapid detection

## Abstract

White spot syndrome virus (WSSV) has caused large economic losses to the aquaculture industry, and thus far there are no efficient therapeutic treatments available against this lethal virus. Therefore, rapid detection of white spot syndrome virus (WSSV) in shrimp is crucial. This study was conducted to establish a rapid visual detection method for WSSV in shrimp and on the basis of loop-mediated isothermal amplification (LAMP). The color change of the amplification products was observed using a fluorescence amplification curve (with the SYTO-9 fluorescent dye) and LAMP visualization (with calcein), and the method's specificity, sensitivity, and reproducibility were analyzed. It showed that this method can specifically detect WSSV, with a detection sensitivity of 1 fg/μL and has good reproducibility and reliability. This method was used to analyze cultured shrimp and crayfish samples; among 329 suspected clinical samples, the positive diagnostic rate of WSSV was 27.96%. The infection activity of WSSV was the highest at temperatures of 20–26°C. The LAMP-based method for rapid visual detection of WSSV does not need complex experimental instruments, and an equipment with a stable heat source is sufficient for the reaction. It is easy to use and can be used at shrimp breeding sites.

## Introduction

White spot disease (WSD) is a highly contagious viral infection of decapod crustaceans, the causative agent of WSD is *white spot syndrome virus* (WSSV), which is a large, non occluded, enveloped, rod-shaped to elliptical DNA virus with a tail-like extension at one end. WSD was first discovered in China Taiwan in 1992 (Zhu et al., 2019). In the following 10 years, the disease swept the world and broke out successively in South Korea, Japan (Boonyakida et al., 2021), Southeast Asia, India (Patil et al., 2021), North America (Lightner, 2011), Europe and other countries and regions, causing immeasurable losses to the world shrimp breeding industry (Han et al., 2018). In 1995, the world organization for animal health (OIE), Food and Agriculture Organization (FAO) and Network of Aquaculture Centres in Asia-Pacific (NACA) listed it as one of the important aquatic animal viral diseases to be reported (Durand et al., 1997). WSSV hosts a wide range, including all kinds of cultured shrimp and most wild shrimps and crabs in the world. WSSV has strong infection ability and high mortality. The mortality of shrimp can reach more than 70% or even 100% within 3 ~ 10 days after infection (Escobedo-Bonilla et al., 2008). To date, there is no effective drug to control a WSD

epidemic ; therefore, early prevention and monitoring is crucial to eliminate the source of infection and cut-off the route of transmission (Ding et al*.,* 2015; Sanchez-Paz, 2010).

The detection of WSSV in shrimp culture is an arduous task. The traditional diagnosis assay mainly relies on histopathology and electron microscope observation, mainly staining histopathology detection method, to observe whether the nucleus is swollen and eosinophilic amphoteric pigmented lesions in the nucleus. These assays can intuitively locate the replication location of WSSV, reveal the pathological characteristics of WSSV, and display and compare the size and morphology of virus particles and nucleocapsids of different WSSV isolates. However, due to the limitations of time, experimental equipment and personnel, it is not suitable for on-site detection. At the same time, this kind of detection assay has cumbersome operation, low sensitivity and easy to miss detection. Have developed and applied a clonal antibody to WSSV and detected it by enzyme-linked immunosorbent assay (ELISA) (Poulos et al., 2001). ELISA is based on antigen antibody reaction and has fast detection speed. It can be applied to the detection of a large number of samples. The disadvantage is that the preparation of polyclonal antibody or monoclonal antibody is cumbersome and takes a long time. Using gene analysis technology to detect WSSV is a widely studied and applied assay. Some scholars used real-time fluorescent polymerase chain reaction (qPCR) (Durand & Lightner, 2002; Zhu & Quan, 2012), nested PCR (Li et al., 2009), real-time fluorescent loop mediated isothermal amplification (LAMP) to detect the specific genes of WSSV (Li et al., 2015; Zhang et al., 2020). The gene detection assays are not affected by the virus strain and different periods of the virus, which provides an effective assay for the study of early infection and transmission route of the virus. However, these assays are time-consuming and cumbersome, so it is not suitable to be used in the breeding site. This means that the on-site rapid detection technology of WSSV is facing an interesting challenge.

Visual LAMP assay based on LAMP technology (Notomi et al., 2000) uses the fluorescent chelator calcein as the reaction indicator, which can efficiently amplify the target gene under constant temperature conditions (60 ℃ ~ 65 ℃), realize intuitive and visual detection (Tomita et al., 2008). The detection process can be completed in a water bath or an ordinary metal bath, and the reaction results can be observed directly under ultraviolet light. This assay has low requirements for instruments and equipment and simple operation method (Gong et al., 2021; Mori & Notomi, 2009). Therefore, in this study, visual LAMP assay for WSSA detection were developed, verified the clinical performance of the assay, and further compared their effects with qPCR to detect clinical samples, trying to find an effective and simple method to detect WSSA in shrimp breeding site.

## Materials and Methods

### Test Material

WSSV infected shrimp (*Penaeus vannamei*) samples were provided by Dalian Customs Technology Center (Dalian, China), and WSSV infected crayfish *(Procambarus clarkii)* samples were provided by DHelix Co., Ltd. (Guangzhou, China). In total, 329 suspected clinical samples of shrimp and crayfish have shown symptoms, including 256 shrimp and 73 crayfish, were collected from four shrimp farms in Liaoning Province and three shrimp farms and three crayfish farms in Zhejiang Province (China). A total of 6 other non-WSSV-infected diseased shrimp samples or porcine genomic DNA were used for the specificity assay in this study, including *Infectious hypodermal and hematopoietic necrosis virus* (IHHNV), *Hepatopancreatic parvovirus* (HPV), *Enterocytozoon hepatopenaei* (EHP), *Acute hepatopancreatic necrosis disease* (AHPND), *Necrotizing hepatopancreatitis bacteria* (NHPB), and *Decapod iridescent virus 1* (DIV1) were purchased from Beijing Laboratory Biology Technology Co., Ltd. (Beijing, China). WSSV plasmid (concentration 100 ng/μL) was obtained from TaKaRa Co., Ltd. (Dalian, China).

### DNA Extraction

For each clinical sample, the tissue of the gill filament, hepatopancrea, abdominal limb was isolated. DNA was extracted from 10 mg of the tissue sample with a genomic DNA was extracted using genomic DNA extraction kit (No. 9766, TaKaRa Co., Ltd. [Dalian, China]) according to the manufacturer's instructions. The extracted template DNA was stored at -80°C.

### Primer Design

The gene information of 19 WSSV isolates with different genotypes from different countries was obtained using GenBank (Table 1). Using the gene sequence of the WSSV2 isolate (GenBank: KF976716.1) as the target sequence, the sequence homology among 19 WSSV isolates was analyzed using software DNAStar, and highly conserved regions were identified to facilitate primer design. Phylogenetic tree of 19 WSSV isolates using software DNAStar. Six groups of primers were designed for screen the best primer using LAMP PrimerExplorer v5 (Eiken Chemical Co., Ltd., Japan). Each set comprised five primers, including two outer primers (OF and OB), two inner primers (IF and IB), and one loop primer (LB) (Table 2). Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to assess the specificity of the primer sequences. Finally, the best group of primer with good specificity and the earliest peaking time was screened out by fluorescence LAMP curve analysis. Primers and probe of qPCR (Fw/Rev/Probe) was based on previous report

(Zhu & Quan, 2012). Fw, 5'-TTGGTTTCATGCCCGAGATT-3'; Rev, 5'-CCTTGGTCAGCCCCTTGA-3'; Probe, 5'-FAM-TGCTGCCGTCTCCAA-BHQ1-3'. These primers and probe were synthesized by TaKaRa Co., Ltd. (Dalian, China).

**LAMP System Assays**

Fluorescence LAMP assay (with SYTO-9 fluorescent dy) was performed in a 25 µL reaction volume containing 12.5 µL 2× RM reaction solution, 1.0 µL Bst DNA polymerase, 0.5 µL SYTO-9 fluorescent dye (No. 051011M, DHelix Co., Ltd. [Guangzhou, China]), 1.0 µL of each primer (0.4–1.6 µM for inner primers, 0.1–0.2 µM for outer primers, and 0.1–0.8 µM for loop primers), 2 µL DNA template, and double-distilled water. CFX96 Real-Time PCR System (Bio-Rad, USA) for fluorescence LAMP assay was used to obtain the fluorescence amplification curves (with SYTO-9 fluorescent dye) using the following reaction conditions: 63°C for 15 s, followed by 45 cycles at 63°C for 45 s.

Visual LAMP assay (with calcein) was performed in a 25 µL reaction volume containing 12.5 µL 2× RM reaction solution, 1.0 µL Bst DNA polymerase, 1.0 µL of visual $MnCl_2$-calcein stock solution (No. SLP221, Rongyan Biotechnology Co., Ltd. [Beijing, China]), 1.0 µL of each primer (the concentration is the same as above), 2 µL DNA template, and double-distilled water. Thermostatic heater (DB1, JOANLAB, China) for visual LAMP assay, using the following reaction conditions: 65°C for 30 min, followed at 95°C for 2 min or on ice for 2 min. The result was observed under UV light (350–370 nm). Samples that turned green were considered positive for WSSV, whereas samples that remained orange were considered negative.

**qPCR System Assay**

qPCR assay ware performed on CFX96 (BIO-DL, USA). The reactions were prepared as a 25 µl reaction volume containing 12.5 µl probe qPCR mix containing enzyme (Code391A, TaKaRa Co., Ltd., Dalian, China), 0.5 µl (0.2 µM) forward primers, 0.5 µl (0.2 µM) reverse primers, 1 µl (0.4 µM) probe and 2 µl extracted DNA of samples templates. qPCR thermal cycling program ware: 30 s at 95℃, followed by 40 cycles of 5s at 95℃ and 30s at 60℃; Fam fluorescence signals were collected at 60℃.

**Specificity of LAMP Assays**

To verify the specificity of the fluorescent LAMP assay (with SYTO-9 fluorescent dye) and visual LAMP assay (with calcein), WSSV plasmid DNA and DNA samples were extracted from WSSV infected shrimp and WSSV infected crayfish were used, and the genomic DNA were extracted from 6 other virus-infected diseased shrimp for the specificity assays, including IHHNV, HPV, EHP, AHPND, NHPB, and DIV1. The assays were repeated twice for each DNA samples ($n$ = 2).

**Sensitivity of LAMP Assays**

In order to compare the sensitivity of fluorescent LAMP assay and visual LAMP assay for WSSV detection, tenfold serial dilutions of WSSV plasmid from 1ng/ µ L to 0.1 fg/ µ L were used as templates to estimate the limit of detection for LAMP assays. The assays were repeated twice for each dilution ($n$ = 2). Using ultrapure water and genomic DNA from healthy shrimp as negative control. The standard curve was created according to the

**Table 1.** Information of 19 isolates of *white spot syndrome virus* (WSSV) selected from GenBank

| Strain name | GenBank | Time | Area | Host |
|---|---|---|---|---|
| WSSV2 | KF976716.1 | 2014.05.16 | Saudi Arabia | Fenneropenaeus indicu |
| 3 | MF537411.1 | 2017.09.18 | Australia | Penaeus monodon |
| 7 | MF348242.1 | 2017.09.18 | Australia | shrimp |
| BAN_SH_AL-1_2014 | KJ773998.1 | 2014.06.22 | Bangladesh | shrimp |
| BAN_ST-3_2013 | KJ719075.1 | 2014.06.22 | Bangladesh | shrimp |
| CAS07 | JN165706.1 | 2016.07.25 | India | Litopenaeus vannamei |
| CAS08 | JN165707.1 | 2016.07.25 | India | Litopenaeus vannamei |
| chantaburi-2 | FJ356711.1 | 2016.07.26 | Thailand | black tiger shrimp |
| chantaburi-3 | FJ356712.1 | 2016.07.26 | Thailand | black tiger shrimp |
| chantaburi-5 | FJ356714.1 | 2016.07.26 | Thailand | black tiger shrimp |
| CM-VN1 | JX564899.1 | 2012.11.07 | Viet Nam | Penaeus monodon |
| G85 | KF981443.1 | 2014.12.31 | Germany | Cherax quadricarinatus |
| HQY_1 | MF101757.1 | 2018.05.11 | China | Procambarus clarkii |
| IR-Chabahar-Naseri-1 | KF032716.1 | 2013.08.14 | Iran | penaeus indicus |
| IWV-MS1 | KT894028.3 | 2017.09.06 | Iran | shrimp |
| KK-Lv-VIET1 | MN481520.1 | 2020.06.07 | Viet Nam | Penaeus vannamei |
| SA 2 | KU531723.1 | 2016.06.26 | Saudi Arabia | Litopenaeus vannamei |
| SISTAN_GH02 | MT108485.1 | 2020.12.22 | Iran | Penaeus indicus |
| WSSV-Mx-F-2001 | KT964173.1 | 2017.11.09 | Mexico | Litopenaeus vannamei |

concentration gradient of diluted plasmid analyzed by fluorescent LAMP assay, and the Ct value were calculated and were expressed as the mean ± standard deviation (SD).

**Stability Test of Minimum Limit of Detection**

On the basis of the sensitivity test results, the minimum limit of detection for WSSV plasmid DNA was determined using the fluorescent LAMP assay for 20 repeated tests (*n* = 20), and the reliability of its reproducibility and stability was analyzed.

**Practical Application of the Clinical Samples**

To evaluate the accuracy of the visual LAMP assay established in this study in clinical samples, a total of 329 suspected clinical shrimp and crayfish samples (*N* = 329, Table 3) including 256 shrimp samples (*n* = 256), 73 crayfish samples (*n* = 73), were analyzed using the visual LAMP assay and real-time qPCR assay (Zhu & Quan, 2012). These suspected clinical samples were collected from 4 shrimp farms in Liaoning Province, China, and 3 shrimp farms and 3 crayfish farms in Zhejiang Province, China.

**Table 2.** Sequences of LAMP primers for *white spot syndrome virus* (WSSV)

| Group | Primer | Sequence (5′–3′) |
|---|---|---|
| | WSSV-N1-OF | TGTAGTAGGGGCTTCTGTGG |
| | WSSV-N1-OB | TTCCCTGAACGGGTGCTT |
| Group 1 | WSSV-N1-IF | CGTTGAGATGTGCCCCTCTGGGGCCGGGTTTTTAGGTCC |
| | WSSV-N1-IB | CACACCAGCCCTAAAGGTGTCACTTAGCCCATCCGGTGTTTC |
| | WSSV-N1-LB | TTGTGGACTTTTCTTGTGCAGA |
| | WSSV-N2-OF | TGTAGTAGGGGCTTCTGTGG |
| | WSSV-N2-OB | TTCCCTGAACGGGTGCTT |
| Group 2 | WSSV-N2-IF | ACGTTGAGATGTGCCCCTCTGCCGGGTTTTTAGGTCCAGC |
| | WSSV-N2-IB | CACACCAGCCCTAAAGGTGTCACTTAGCCCATCCGGTGTTTC |
| | WSSV-N2-LB | TTGTGGACTTTTCTTGTGCAGA |
| | WSSV-N3-OF | CGGAGGACCCAAATCGAAAT |
| | WSSV-N3-OB | CCTCTGCAACATCCTTTCCT |
| Group 3 | WSSV-N3-IF | GGTCGTCGAATGTTGCCCAAGACTACGCACCAATCTGTGGAA |
| | WSSV-N3-IB | AAGGACAATCCCTCTCCTGCGAAAGAACGGAAGAAACTGCCT |
| | WSSV-N3-LF | TCTCCTTGAGCAGCATCTTTTG |
| | WSSV-N4-OF | GCGATAAAGGGATGTCCACT |
| | WSSV-N4-OB | GGATTGAGGCGTGTAGCAG |
| Group 4 | WSSV-N4-IF | GGCGTCCTCTGCAACCTCAAAAAAGGCAGTTTCTTCCGTTCT |
| | WSSV-N4-IB | TTGGCGAGCAAGGCAATTTCAGCAAATCCAAGAGGCACTCCA |
| | WSSV-N4-LF | GCAACATCCTTTCCTATGGACAAA |
| | WSSV-N5-OF | ACGGAGGACCCAAATCGA |
| | WSSV-N5-OB | CCTCTGCAACATCCTTTCCT |
| Group 5 | WSSV-N5-IF | GGTCGTCGAATGTTGCCCAAGACTACGCACCAATCTGTGGAA |
| | WSSV-N5-IB | AAGGACAATCCCTCTCCTGCGAAAGAACGGAAGAAACTGCCT |
| | WSSV-N5-LF | TCTCCTTGAGCAGCATCTTTTG |
| | WSSV-N6-OF | CGGAGGACCCAAATCGAAAT |
| | WSSV-N6-OB | CCTCTGCAACATCCTTTCCT |
| Group 6 | WSSV-N6-IF | CGGGTCGTCGAATGTTGCCCCTACGCACCAATCTGTGGAA |
| | WSSV-N6-IB | AAGGACAATCCCTCTCCTGCGAAAGAACGGAAGAAACTGCCT |
| | WSSV-N6-LF | TCTCCTTGAGCAGCATCTTTTG |

**Table 3.** Information on 329 suspected clinical samples of shrimp and crayfish

| Sampling time | Shrimp and crayfish farm area/number of suspected clinical samples (cases) | | | | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Farm No.1 | Farm No.2 | Farm No.3 | Farm No.4 | Farm No.5 | Farm No.6 | Farm No.7 | Farm No.8 | Farm No.9 | Farm No.10 | |
| April | 6 | 8 | 8 | 8 | 8 | 8 | 7 | 5 | 4 | 4 | 66 |
| May | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 7 | 8 | 7 | 85 |
| June | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 7 | 7 | 6 | 90 |
| July | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 8 | 5 | 5 | 88 |
| Total | 35 | 37 | 37 | 37 | 37 | 37 | 36 | 27 | 24 | 22 | 329 |

Farm No.1, Haicheng City, Liaoning, China (shrimp). Farm No.2, Gaizhou City, Liaoning, China (shrimp). Farm No.3, Yingkou City, Liaoning, China (shrimp). Farm No.4, Donggang City, Liaoning, China (shrimp). Farm No.5, Xiangshan County, Zhejiang, China (shrimp). Farm No.6, Cangnan County, Zhejiang, China (shrimp). Farm No.7, Hangzhou City, Zhejiang, China (shrimp). Farm No.8, Yuyao City, Zhejiang, China (crayfish). Farm No.9, Longgang City, Zhejiang, China (crayfish). Farm No.10, Shaoxing City, Zhejiang, China (crayfish).

## Results and Discussion

### Target Sequence Homology Analysis

According to the gene information of 19 different WSSV isolates selected in GenBank, the gene sequence of wssv2 isolate (GenBank: KF976716.1) was used as the target. The Homology analysis performed using DNAStar showed 98.6–100% homology between the target sequence of the WSSV2 isolate and 18 other WSSV isolates (Figure 1A). The phylogenetic tree of the genomes of these different isolates (Figure 1B) showed that the tevolutionary relationship of WSSV2 isolate is only close to that of one isolate from Thailand, but is relatively far from that of 17 isolates from other countries. It indicated that the selection of the gene sequences of WSSV2 isolates to design primers was more conducive to the detection of WSSV isolates from different countries.

### Screening of LAMP Primers

The six groups of primers designed for WSSV detection (Table 1) were screened using fluorescence LAMP assay. The results showed, group 1 of primers had the earliest positive peak time and high fluorescence value (Figure 2), which was the primer with the best amplification efficiency. However, the binding ability of group 2, group 3 and group 4 of primers were not strong, resulting in low amplification efficiency; group 5 of primers caused nonspecific amplification on account of the secondary structure between primers. thus, group 1 of primers with the best screening effect was selected for the analysis of specificity and sensitivity.

### LAMP Specificity Analysis

The specificities of the fluorescence LAMP assay and visual LAMP assay for WSSV were assessed using

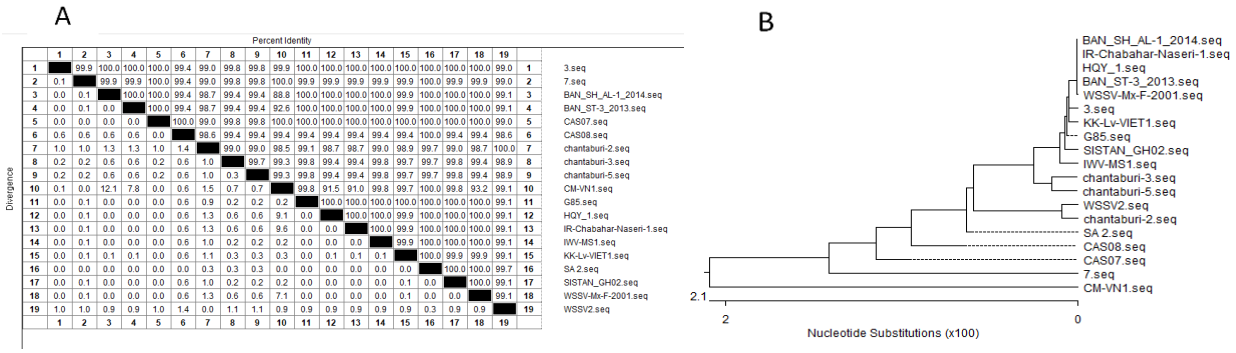


**Figure 1.** Target Sequence Homology Analysis. A, Homology analysis between *white spot syndrome virus* (WSSV2) isolate and other 18 isolates using DNAStar software. B, Phylogenetic tree analysis of *white spot syndrome virus* (WSSV2) isolate and 18 other isolates.

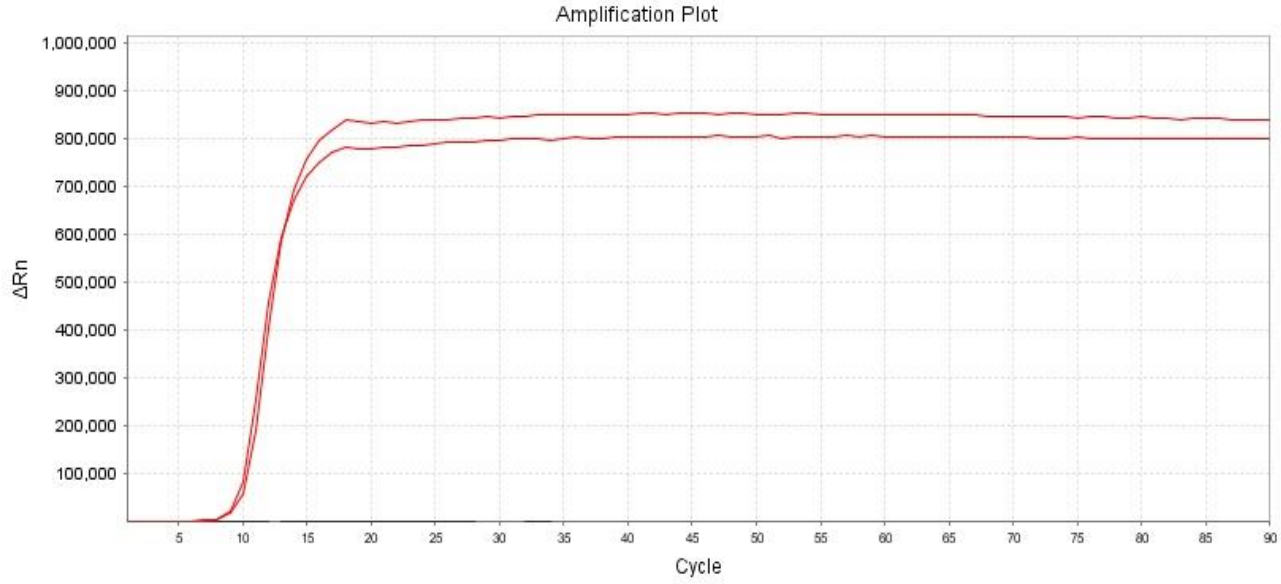


**Figure 2.** Screening results of group 1 of primers designed for WSSV detection

WSSV plasmid DNA, the genomic DNA of clinical shrimp and clinical crayfish samples positive for WSSV, and with shrimp samples containing six other viruses, including IHHNV、HPV、EHP、AHPND、NHPB、DIV1. Using fluorescence LAMP assay, the results showed that only the WSSV plasmid DNA (Figure 3A) and the two positive clinical samples (figure not included) completed the typical positive fluorescence amplification curve. Using visual LAMP assay, only the WSSV plasmid DNA (Figure 3B) and the two positive clinical samples (figure not included) showed the change in color and turbidity of the LAMP products under ultraviolet light. The 6 other viral samples were negative. Thus, the established fluorescence LAMP assay and visual LAMP assay for WSSV detection have good specificity.

**LAMP Sensitivity Analysis**

Continuous dilution of WSSV plasmid DNA was used to evaluate the sensitivity of the newly established fluorescent LAMP assay and visual LAMP assay. If the repeated tests of the minimum concentration were positive, the concentration was the limit of detection (LOD). The results indicated that fluorescent LAMP assay

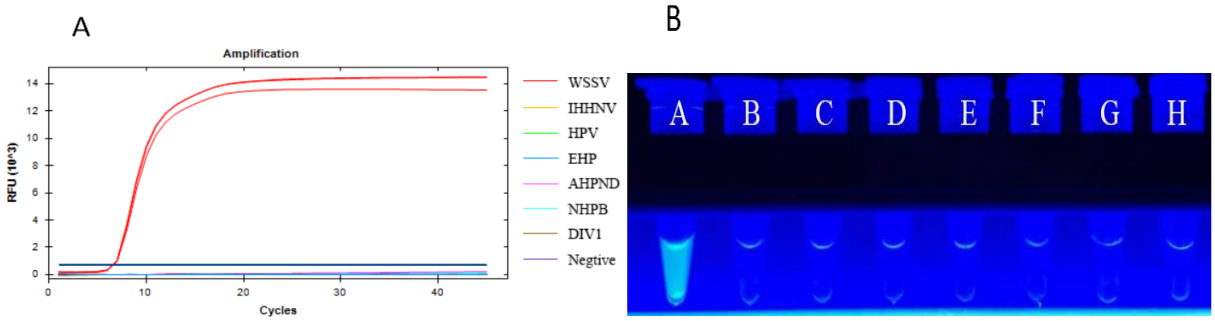


**Figure 3.** Specificity analysis of LAMP assays for the detection of WSSV. A, Specificity of fluorescence LAMP assay for WSSV. B, Specificity of visual LAMP assay for WSSV under ultraviolet light (A, WSSV; B, IHHNV; C, HPV; D, EHP; E, AHPND; F, NHPB; G, DIV1; H, negative control).

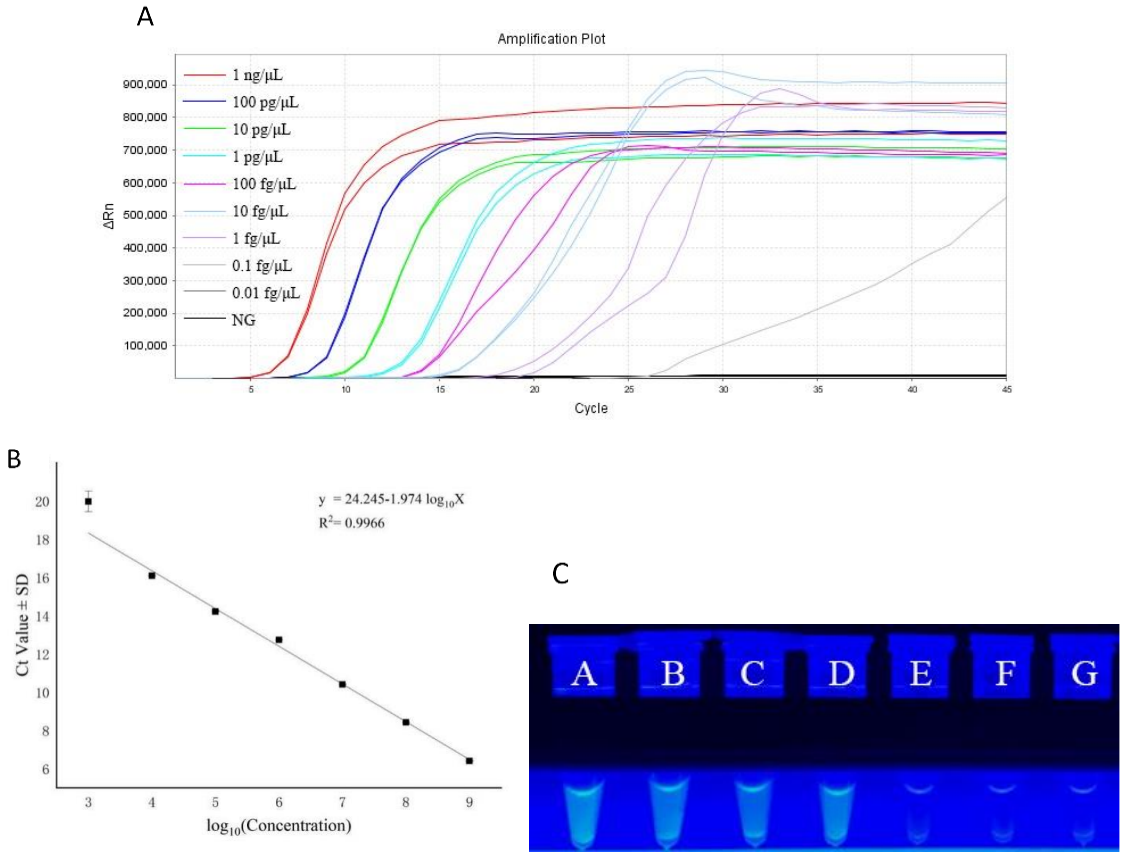


**Figure 4.** Sensitivity analysis of LAMP assays for detection of WSSV. A, Sensitivity of fluorescence LAMP assay for WSSV. B, Sensitivity linear analysis of fluorescence LAMP assay for WSSV (A, 1 pg/μL; B, 100 fg/μL; C, 10 fg/μL; D, 1 fg/μL; E, 0.1 fg/μL; F, 0.01 fg/μL; G, negative control). C, Sensitivity of visual LAMP assay for WSSV under ultraviolet light.

and visual LAMP assay could effectively detect low levels of WSSV (Figure 4). For WSSV plasmid DNA concentrations of 1 fg/µL and above, fluorescence amplification curves were observed using fluorescent LAMP assay (Figure 4A), and the results were stable, the LOD of fluorescent LAMP assay was 1fg/µL. The linear regression value ($R^2$) of the fluorescent LAMP assay was 0.9966, indicating a good correlation (Figure 4B). The LOD based on visual LAMP assay also was 1fg/µL (Figure 4C), it was observed that the amplified product showed typical positive turbid fluorescence under ultraviolet light, and the LOD of visual LAMP assay was the same as that of fluorescent LAMP assay. In general, both assays have high sensitivity and can achieve effective detection for WSSV with a sensitivity of 1 fg/µL.

**Stability of Minimum Limit of Detection**

WSSV plasmid DNA concentration of 1 fg/µL was used as the minimum limit of detection sensitivity, and the fluorescence amplification curve obtained with fluorescent LAMP assay was used for WSSV detection (*n* = 20). The DNA sample at the concentration of 1 fg/µL could be detected stably after 20 repeated tests; the CT value was 17.50 ± 1.70, and the relative standard deviation (RSD) was 9.69% (Figure 5). Thus, the LAMP assays established in this study exhibited good reproducibility and stability.

**Analysis of Clinical Samples with Visual LAMP Assay**

329 suspected clinical samples of shrimp (*Penaeus vannamei*) and crayfish were analyzed by visual LAMP assay. The results of clinical samples detected by qPCR assay published previously were used to evaluate the accuracy of the visual LAMP assay (Table 4). The results of 329 suspected clinical samples detected by visual LAMP assay in this study were consistent with the qPCR assay published previously, the positive diagnostic rate of WSSV was 27.96% (92 / 329), of which the positive

**Table 4.** Suspected clinical samples (*N* = 329) analyzed by visual LAMP assay and qPCR assay.

| Sampling time | Assay | Positive diagnostic rate of clinical samples, % | | |
|---|---|---|---|---|
| | | Liaoning Province, China | Zhejiang Province, China | |
| April | Visual LAMP | 0% (0/30) [a] | 91.3% (21/23) [b] | 84.62% (11/13) [c] |
| | qPCR | 0% (0/30) | 91.3% (21/23) | 84.62% (11/13) |
| May | Visual LAMP | 91.67% (33/36) | 44.44% (12/27) | 45.45% (10/22) |
| | qPCR | 91.67% (33/36) | 44.44% (12/27) | 45.45% (10/22) |
| June | Visual LAMP | 7.5% (3/40) | 3.33% (1/30) | 5% (1/20) |
| | qPCR | 7.5% (3/40) | 3.33% (1/30) | 5% (1/20) |
| July | Visual LAMP | 0% (0/40) | 0% (0/30) | 0% (0/18) |
| | qPCR | 0% (0/40) | 0% (0/30) | 0% (0/18) |
| Total | | 27.34% (70/256) | | 30.14% (22/73) |
| | | 27.96% (92/329) | | |

a, Positive rate of shrimp samples, % (Number of positive/total number of shrimp samples).
b, Positive rate of shrimp samples, % (Number of positive/total number of shrimp samples).
c, Positive rate of crayfish samples, % (Number of positive/total number of crayfish samples).

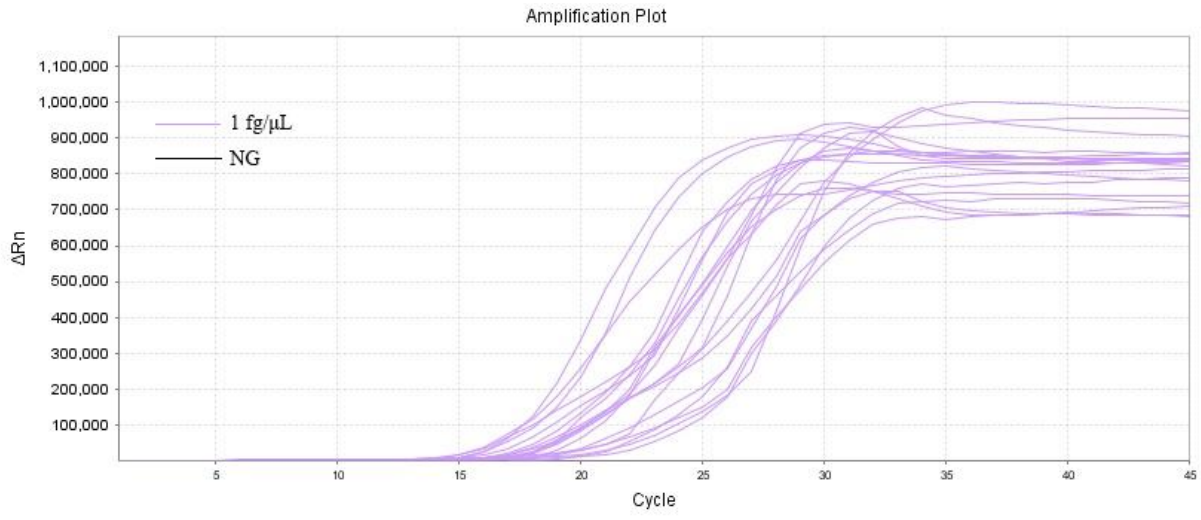


**Figure 5**. Stability of Minimum Limit of Detection. Fluorescence LAMP assay of WSSV plasmids DNA with 1 fg/µL for minimum limit of detection (*n* = 20).

diagnostic rate of suspected clinical shrimp and crayfish samples were 27.34% (70 / 256) and 30.14% (22 / 73), respectively. The analysis data calculated by SPSS statistical software 22.0.0.0 (International Business Machines Corporation, USA) showed that the diagnostic sensitivity (DSe), diagnostic specificity (DSp), positive predictive value (PPV) and negative predictive value (NPV) of visual LAMP assay for the detection of WSSV suspected clinical samples were 100%. The visual LAMP assay had good accuracy in the detection of clinical samples for WSSV.

## Conclusion

WSD have posed a serious threat to China and the global shrimp breeding industry. On site diagnosis of WSSV can timely block the spread of WSD epidemic and avoid causing major economic losses. The traditional technology based on ELISA assay and qPCR assay usually requires complex operation and expensive laboratory tools, which is difficult to meet the needs of on-site rapid detection. Constant temperature nucleic acid amplification technology based on visualization has the advantages of fast and easy operation, and has become a popular trend for field rapid detection.

This is the first study of fluorescent LAMP assay and visual LAMP assay for WSSV identification, which were used for WSSV detection of clinically suspected samples. In this study, on the basis of the LAMP technology, optimal primers for LAMP detection of WSSV were designed and screened, the use of loop primers in addition to inner and outer primers greatly shortened the reaction time, the amplification test could be completed in 30 minutes. The comparative analysis of the two assays showed that the sensitivity and specificity of fluorescent LAMP assay and visual LAMP assay were consistent, which were very effective for WSSV detection. Visual LAMP assay did not need complex experimental instruments, the equipment with stable heat source was sufficient for reaction, and the operation was simple. Visual LAMP assay was more suitable for rapid detection with simple equipment in shrimp breeding site, which was a good choice for shrimp farms.

## Ethical Statement

All experimental samples were taken after natural death of illness, which did not involve ethical issues.

## Funding Information

This study was supported by the High-Level Talent Innovation Project of Liaoning (XLYC2002106) and (2019CT09).

## Author Contribution

First Author: Experiment operation, thesis writing and modification; Second Author: Data collation and analysis, investigation and writing; Third Author: Supervision, Writing - review and editing; and Fourth Author: Funding Acquisition, Project Administration, Resources, Writing -review and editing.

## Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

## Acknowledgements

We would like to thank Guangzhou DHelix Co., Ltd. for the positive samples of crayfish infected with WSSV. We would also like to thank Ma Lidan for providing 183 shrimp and crayfish samples in Zhejiang Province and Dr. Tian Zhuo for his contribution in the application of practical sample detection.

## References

Boonyakida, J., Xu, J., Satoh, J., Nakanishi, T., Mekata, T., Kato, T., & Park, E. Y. (2021). Identification of antigenic domains and peptides from VP15 of white spot syndrome virus and their antiviral effects in Marsupenaeus japonicus. *Sci Rep*, *11*(1), 12766. https://doi.org/10.1038/s41598-021-92002-8

Ding, Z., Yao, Y., Zhang, F., Wan, J., Sun, M., Liu, H., . . . Zhao, Z. (2015). The first detection of white spot syndrome virus in naturally infected cultured Chinese mitten crabs, Eriocheir sinensis in China. *J Virol Methods*, *220*, 49-54. https://doi.org/10.1016/j.jviromet.2015.04.011

Durand, S., Lightner, D. V., Redman, R. M., & Bonami, J.-R. (1997). Ultrastructure and morphogenesis of White Spot Syndrome Baculovirus (WSSV). *Diseases of Aquatic Organisms*, *29*, 205-211. https://doi.org/10.3354/dao029205

Durand, S. V., & Lightner, D. V. (2002). Quantitative real-time PCR for the measurement of white spot syndrome virus in shrimp. *Journal of fish diseases*, *25*, 381-389. https://doi.org/10.1046/j.1365-2761.2002.00367.x

Escobedo-Bonilla, C. M., Alday-Sanz, V., Wille, M., Sorgeloos, P., Pensaert, M. B., & Nauwynck, H. J. (2008). A review on the morphology, molecular characterization, morphogenesis and pathogenesis of white spot syndrome virus. *Journal of fish diseases*, *31*, 1-18. https://doi.org/10.1111/j.1365-2761.2007.00877.x

Gong, H. Y., Li, Q. Y., Zhang, H., Ye, L., Shi, L., & Feng, Y. H. (2021). Development and comparison of qPCR and qLAMP for rapid detection of the decapod iridescent virus 1 (DIV1). *J Invertebr Pathol*, *182*, 107567. https://doi.org/10.1016/j.jip.2021.107567

Han, L., Wang, X., Yang, B., Wan, X., Wu, H., Zhang, Q., & Huang, J. (2018). Pathogen analysis of an outbreak death of Penaeus japonicus. *Journal of Fisheries*, *42*(03), 431-441.

Li, H., XiaoJiang, Liu, Z., Zhao, Z., Ren, C., Hu, C., . . . Wang, G. (2015). Establishment and application of real-time fluorescent lamp detection method for white spot syndrome virus. *Journal of aquatic biology*, *39*(01), 142-148. https://kns.cnki.net/kcms/detail/42.1230.q.20150126.1447.004.html

Li, W., Zhou, G., Zhu, F., Wang, W., & Zhou, G. (2009). Establishment and preliminary application of nested PCR for detection of Procambarus clarkii white spot syndrome virus (WSSV). *Journal of Nanjing Normal University (NATURAL SCIENCE EDITION)*, *32*(02), 98-102.

Lightner, D. V. (2011). Virus diseases of farmed shrimp in the Western Hemisphere (the Americas): a review. *J Invertebr Pathol*, *106*(1), 110-130. https://doi.org/10.1016/j.jip.2010.09.012

Mori, Y., & Notomi, T. (2009). Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. *J Infect Chemother*, *15*(2), 62-69. https://doi.org/10.1007/s10156-009-0669-9

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., & Hase, T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res*, *28*(12). https://doi.org/10.1093/nar/28.12.e63

Patil, P. K., Geetha, R., Ravisankar, T., Avunje, S., Solanki, H. G., Abraham, T. J., Vijayan, K. K. (2021). Economic loss due to diseases in Indian shrimp farming with special reference to Enterocytozoon hepatopenaei (EHP) and white spot syndrome virus (WSSV). *Aquaculture*, *533*. https://doi.org/10.1016/j.aquaculture.2020.736231

Poulos, B. T., Pantoja, C. R., Bradley-Dunlop, D., Aguilar, J., & Lightner, D. V. (2001). Development and application of monoclonal antibodies for the detection of white spot syndrome virus of penaeid shrimp. *47*(1), 13-23. https://doi.org/10.3354/dao047013

Sanchez-Paz, A. (2010). White spot syndrome virus: an overview on an emergent concern. *Vet Res*, *41*(6), 43. https://doi.org/10.1051/vetres/2010015

Tomita, N., Mori, Y., Kanda, H., & Notomi, T. (2008). Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nat Protoc*, *3*(5), 877-882. https://doi.org/10.1038/nprot.2008.57

Zhang, N., Huang, L., & Si, Z. (2020). Establishment and clinical application of double lamp detection method for shrimp white spot syndrome virus and infectious subcutaneous and hematopoietic organ necrosis virus. *Aquatic Science and technology information*, *47*(02), 68-72. https://doi.org/10.16446/j.cnki.1001-1994.2020.02.002

Zhu, F., & Quan, H. (2012). A new method for quantifying white spot syndrome virus: Experimental challenge dose using TaqMan real-time PCR assay. *J Virol Methods*, *184*(1-2), 121-124. https://doi.org/10.1016/j.jviromet.2012.05.026

Zhu, F., Twan, W. H., Tseng, L. C., Peng, S. H., & Hwang, J. S. (2019). First detection of white spot syndrome virus (WSSV) in the mud shrimp Austinogebia edulis in Taiwan. *Sci Rep*, *9*(1), 18572. https://doi.org/10.1038/s41598-019-54837-0