Validation of qPCR from a crude extract for the rapid detection of white spot syndrome virus

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Abstract
White spot disease (WSD) has posed a serious threat to the China and the global shrimp aquaculture. In order to diagnose white spot syndrome virus (WSSV) early and prevent the spread and outbreak of WSD, it is necessary to establish a highly sensitive WSSV diagnosis method suitable for shrimp farming sites. In this study, a pre-amplification qPCR assay from the crude extract of samples heated lysis was established, which was further compared with the universal qPCR assay to verify the shrimp samples. The limit of detection (LOD) of pre-amplification qPCR assay and universal qPCR assay was 2.80 copies and 20.57 copies per reaction at 95\% CI, respectively. It had good WSSV specificity and did not show cross-detection of infectious hypodermal and hematopoietic necrosis virus (IHHNV), hepatopancreatic parvovirus (HPV), Enterocytozoon hepatopenaei (EHP), acute hepatopancreas necrosis disease (AHPND), necrotizing hepatopancreatitis bacteria (NHPB), and decapod iridescent virus 1 (DIV1). A total of 36 shrimp samples were detected as WSSV DNA positive by pre-amplification qPCR with crude extract from samples heated lysis and universal qPCR with DNA extraction. The diagnostic sensitivity and specificity were 97.22\% (85.5~99.9\%, 95\% CI) and 100\% (81.5~100\%, 95\% CI), respectively. The agreement Kappa value was 0.959 (0.879~1, 95\% CI), and the analysis results were basically consistent. Eliminating the tedious steps of extracting DNA and using pre-amplified qPCR to detect WSSV in shrimp, it is a good choice for aquaculture farms.

Keywords White spot syndrome virus (WSSV) · qPCR · Crude extract · Validation · Rapid detection

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Chao Ma and Zhuo Tian contributed equally to this work.

Highlights - Validation for the effect of pre-amplification qPCR with crude extract from shrimp samples heated lysis for the rapid detection of white spot syndrome virus.
- The pre-amplification qPCR assay with crude extract was fast, sensitive, and easy to operate, which was more suitable for the detection of white spot syndrome virus in remote areas and laboratory with limited conditions.

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Introduction

White spot syndrome virus (WSSV) is an important pathogen endangering shrimp culture (Lei et al. 2017). White spot disease (WSD) caused by the virus has brought great harm and serious economic losses to shrimp breeding industry in China and all over the world (Millard et al. 2021). In 1921, WSD was first discovered in China Taiwan (Zhu et al. 2019), and has spread to other Asian countries (Cui et al. 2020; Boonyakida et al. 2021). In 1995, WSD was also found in a shrimp farm in southern Texas (Sanchez-Paz 2010). The genome of WSSV is huge, and the structural proteins mainly include nucleocapsid protein, envelope protein, and envelope protein (Li et al. 2019; Lu et al. 2019; Boonyakida et al. 2020; Shine et al. 2020). The infection mechanism of WSSV is complex (Liu et al. 2021). WSSV can spread in a wide range through the water area, with the characteristics of wide host range, strong infection ability, and high mortality rate. Crustacean aquatic animals such as shrimp and crab can be infected (Ding et al. 2015). The mortality of aquatic animals is more than 70% within 3–10 days after infection, and that of shrimp are almost 100% (Wen 2017). Due to the rapid and vast spread of WSSV in China, the aquaculture industry has responded promptly to outbreaks, and the government has issued emergency plans, such as expert consultation and target surveillance programs. The World Organization for Animal Health (OIE) and the Network of Aquaculture Centers in Asia Pacific (NACA) have listed WSD as a compulsory notification of aquatic animal diseases (Xie et al. 2022). China has included it as a class II animal epidemic disease in the list of quarantine diseases of imported animals of the People’s Republic of China (2014). So far, there is no effective drug to treat WSSV; hence, rapid detection and monitoring on the breeding site are the key measure to control virus transmission (Verbruggen et al. 2016).

Antibody-dependent immunological assays are fast and have been used for rapid screening of WSSV (Kulabhusan et al. 2017; Tang et al. 2018). However, the success of immunoassay of viruses depends on the high affinity and specificity of antibodies (Mangal et al. 2016). Immune antibodies usually have low sensitivity and specificity to some mutant viruses, and are easily affected by matrix interference, which will limit the stable expression of target virus antigen, and then affect the accuracy of detection (Su et al. 2021). Polymerase chain reaction (PCR) is typical and advanced nucleic acid amplification technology, which have the advantages of simplicity and high efficiency (Tang et al. 2013; Thamizhvanan et al. 2019). Nested PCR have high specificity and sensitivity, and are used to detect WSSV (Chang et al. 2020). However, nested PCR assays are cumbersome and difficult to popularize. Real-time quantitative PCR (qPCR) have good specificity and sensitivity, and have been applied to detect WSSV (Meng et al. 2010; Jang et al. 2011). Loop-mediated isothermal amplification (LAMP) based on thermostatic amplification has been used for rapid detection of WSSV in the field, and its simple equipment, faster speed, and amplification of targets at constant temperature (Li et al. 2015; Wu 2019; Gong et al. 2021). However, LAMP primers (4–6 primers are required) are more difficult to design than qPCR primers. Compared with qPCR, LAMP assays have slightly higher false positive rate and slightly lower sensitivity (Xia et al. 2014). Recombinase polymerase amplification (RPA) and recombinase-mediated amplification (RAA) can complete the rapid amplification of nucleic acid at 37 °C for about 30 min, which have been used in the study of rapid detection of WSSV (Huang et al. 2022). However, the stability of RPA and RAA is poor, and the reagent price is relatively expensive. In contrast, qPCR assays are more widely and stably applied to the detection of WSSV (Qiu et al. 2018), and its reagents are easier to obtain. Its specificity and sensitivity are higher than those of LAMP, RPA, and RAA assays, which are more suitable for the analysis of low concentration virus clinical samples.
DNA extraction in qPCR detection process is cumbersome and time consuming, which limits the application of qPCR assays in the field. In this study, we aim to establish a pre-amplified qPCR assays for detecting WSSV, with crude extract from shrimp samples heated lysis, so as to reduce the operation links, and verify the effect of pre-amplified qPCR in detecting shrimp samples compared with universal qPCR. This paper attempts to find a convenient and highly sensitive method for detecting WSSV in county and district laboratories and medium-sized shrimp farm laboratories in China.

Materials and methods

Samples and WSSV standard plasmid

In total, 54 Penaeus vannamei shrimp samples suspected of WSSV infection were collected from four shrimp farms in Liaoning Province (China). WSSV-infected dead shrimp samples were obtained from Guangzhou Double Helix Gene Technology Co., Ltd. (Guangzhou, China). A total of 6 other non-WSSV-infected diseased shrimp samples used for the specificity assay in this study, including DNA reference material of infectious hypodermal and hematopoietic necrosis virus (IHHNV, GSB 11–3528-2018) and decapod iridescent virus 1 (DIV1, GSB 11–3627-2019), were obtained from Beijing Lambri Biotechnology Co., Ltd. (Beijing, China). DNA-positive samples of hepatopancreatic parovirus (HPV), Enterocytozoon hepatopenaei (EHP), acute hepatopancreas necrosis disease (AHPND), and necrotizing hepatopancreatitis bacteria (NHPB) were obtained from Guangzhou Double Helix Gene Technology Co., Ltd. (Guangzhou, China). All of the samples for the specificity assay were validated by sequencing and a published PCR assay. The synthesis of the standard plasmid was completed by TaKaRa Co., Ltd. (Dalian, China). 887-bp target sequence of the WSSV VP664 gene (GenBank accession number MN481520.1) was inserted into the commercial vector pMD19 (TaKaRa Co., Ltd., Dalian, China) to construct WSSV standard plasmid (9.8 ng/μl). Using the formula: Copies/μl = \( \frac{6.02 \times 10^{23} \text{ (copies/mol)} \times \text{DNA Concentration (ng/μl)} \times 10^{-9}}{\text{DNA length} \times 660 \text{ (g/mol)}} \), the calculations showed that the copy number of WSSV plasmid was 1.0 × 10^{10} copies/μl. The WSSV plasmid was diluted as standard molecule for sensitivity analysis.

Samples heated lysis with crude extract preparation

Took 0.1~0.2 g of shrimp tissue samples (including gills, stomach, swimming feet or walking feet, and hemolymph) and placed in a grinding tube containing phosphate buffer solution (PBS). After 6000×g shaking grinding for 45 s, about 10% of tissue homogenate were prepared and centrifuged at 5000×g for 5 min. Took 5~20 μl supernatant and added 100 μl MightyPrep reagent (Code 9182, TaKaRa Co., Ltd., Dalian, China), mixed by oscillator. Heated at 95°C for 10 min and centrifuged at 12,000×g for 2 min. The supernatant of the crude extract was directly used for pre-amplification qPCR assays.

Sample DNA extraction

DNA was extracted with magnetic bead virus DNA extraction kit (DP438-T2K, TianGen Biochemical Technology Co., Ltd., Beijing, China) according to the manufacturer’s
instructions. The operations were as follows: 10-mg shrimp tissues (including gills, stomach, swimming feet or walking feet, and hemolymph) were taken for low-temperature grinding, and 200 μl PBS. Joined 200 μl VGB buffer, 20 μl proteinase K, and 1.0 μl carrier RNA, fully mixed in 56°C water bath for 10 min. Joined 200 μl of 96–100% ethanol, fully mixed. Placed the spin column on the collection tube, transferred the solution to the spin column, centrifuged at 12,000×g for 2 min, and discarded the filtrate. Added 500 μl RWA buffer to spin column, 12,000×g centrifuged for 1 min, and discarded the filtrate. Added 700 μl buffer RWB to spin column, 12,000×g centrifuged for 1 min, and discarded the filtrate. Joined RWB buffer, placed the spin column on the collection tube, 12,000×g centrifuged for 2 min, placed the spin column on a new 1.5-ml RNase free collection tube, and added 30–50 μl RNase free dH₂O, standing at room temperature for 5 min. 12,000×g centrifuged for 2 min to elute DNA. The DNA templates were used for qPCR assays.

**qPCR primers and probe design**

Information on the WSSV sequence was obtained from published articles, and the WSSV VP664 gene (GenBank accession no. MN481520.1) was selected to design primers targeted to specific conserved DNA fragments in the present work. The forward and reverse primers and probes were designed for qPCR using Primer Express software 5.0 (Applied Biosystems, Foster City, CA, USA) for twenty strains of WSSV (Supplementary Table S1), and were evaluated the secondary structure and the presence of possible primer dimers. Forward primer, 5'-CGATCTTGGAAAGTTATC-3'; Reverse primer, 5'-GAGCTTAGTCTATCAATCA-3'; Probe, FAM-5'-CGGCACCATCGCTGAATCTGT-3'-BHQ1. The primer-BLAST of the National Center for Biotechnology Information (NCBI) was used to assess the specificity of the primers and probes based on the NCBI nucleotide database (www.ncbi.nlm.nih.gov/nucleotide). The primers and probe were synthesized from TaKaRa Co., Ltd. (Dalian, China).

**qPCR assay**

qPCR assays were 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, 2 μl extracted DNA of samples templates for universal qPCR, or 2 μl supernatant of the crude extract for pre-amplification qPCR assay. Universal qPCR thermal cycling program was as follows: 30 s at 95°C, followed by 40 cycles of 5 s at 95°C, and 30 s at 60°C; Fam fluorescence signals were collected at 60°C. Pre-amplification qPCR thermal cycling program was as follows: first, pre-amplification step (15 cycles of 10 s at 95°C and 10 s at 50°C), and Fam fluorescence signal was not collected; next, 1 min at 95°C, followed by 35 cycles of 10 s at 95°C, and 30 s at 55°C; Fam fluorescence signals were collected at 55°C.

**Analytical sensitivity and specificity of pre-amplification qPCR and universal qPCR**

To determine the limit of detection (LOD) for pre-amplification qPCR and qPCR, serial dilutions of WSSV standard plasmid for concentration including 1.0×10<sup>5</sup>, 1.0×10<sup>4</sup>, 1.0×10<sup>3</sup>, 1.0×10<sup>2</sup>, 1.0×10<sup>1</sup>, 5, 1, and 0.5 copies/μl were prepared. Each concentration
was assayed in six replicates by pre-amplification qPCR and qPCR. The analytical specificity of WSSV by pre-amplification qPCR and qPCR was evaluated in WSSV-infected shrimp samples and other pathogens DNA (IHHNV, HPV, EHP, AHPND, NHPB, DIV1). Healthy shrimp were used as negative control.

**Comparison of pre-amplification qPCR with crude extract and universal qPCR with DNA extraction using shrimp samples**

A total of 54 *Penaeus vannamei* shrimp samples suspected of WSSV infection symptoms were collected from four shrimp farms in Liaoning Province (China). To explore the performance of pre-amplification qPCR assay with crude extract in the detection of specimen, the performance of pre-amplification qPCR assay with crude extract was compared to that of universal PCR assay with DNA extraction. The degree of agreement between pre-amplification qPCR with crude extract and qPCR with DNA extraction assay results was measured with kappa value by using MedCalc software (MedCalc Software bvba, Ostend, Belgium).

**Statistical analysis**

Data in this study were presented as mean ± standard deviation. For the determination of WSSV for pre-amplification qPCR assay and universal qPCR assay analytical sensitivity, a semi-log regression analysis (PRISM, Graphpad Software Inc., San Diego, CA, USA). The probit regression analysis using MedCalc Software (MedCalc Software bvba, Ostend, Belgium) was performed for the pre-amplification qPCR assay and universal qPCR assay at a 95% probability level.

**Results and discussion**

**Sensitivity and specificity of WSSV pre-amplification qPCR and qPCR**

The sensitivities of the newly established pre-amplification qPCR and universal qPCR assays were evaluated using continuous dilutions of the WSSV standard plasmid DNA. The results indicated that the pre-amplification qPCR and universal qPCR assays could efficiently detect low levels of WSSV (Fig. 1). The LOD determination should be done by probit analysis at 95% probability of the result data of n = 6 replicates of testing the standard curve (Fig. 1). The LOD of pre-amplification qPCR assay and universal qPCR assay were 2.80 copies (Fig. 1A, 1C) and 20.57 (Fig. 1B, 1D) copies per reaction at 95%, respectively. The specificities of the pre-amplification qPCR and universal qPCR assays of WSSV were assessed using WSSV-infected shrimp samples, and with DNA samples containing 6 other viruses, including IHHNV, HPV, EHP, AHPND, NHPB, DIV1, and healthy shrimp. The results indicated that only the WSSV-infected shrimp samples completed the index amplification; the 6 other viral samples and healthy shrimp had no amplification curves and were negative (Fig. 1E, 1F). Thus, the pre-amplification qPCR and universal qPCR assays and WSSV detection method have good specificity.

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Fig. 1  The sensitivity and specificity for WSSV. WSSV DNA molecules after series dilution were detected by pre-amplified qPCR (A) and universal qPCR (B). Probit regression analysis using MedCalc Software was performed on data of 6 replicates from serial dilutions by pre-amplified qPCR (C) and universal qPCR (D). Specificity test result of pre-amplified qPCR (E) and universal qPCR (F) on detecting WSSV, IHNV, HPV, EHP, AHPND, NHPB, DIV1, and healthy shrimp.

Performance of WSSV pre-amplification qPCR with crude extract on shrimp samples and its comparison with universal qPCR with DNA extraction testing

The purpose of this study was to detect WSSV by pre-amplification qPCR with crude extract in shrimp farms. In order to evaluate the practical application of WSSV pre-amplification qPCR with crude extract in this work, 54 shrimp samples suspected of WSSV infection symptoms were tested and the results were compared with that universal qPCR.
with DNA extraction (Supplementary Table S2). By the detection of universal qPCR with DNA extraction, 36 samples were confirmed as WSSV DNA positive (Ct value, ranging from 18.32 to 38.79) while 18 as negative (Ct value, undetermined). By the detection of pre-amplification qPCR with crude extract in this work, 35 samples were identified as WSSV DNA positive (Ct value, ranging from 11.61 to 23.19) and 19 as negative (Ct value, undetermined). Linear correlation analysis revealed that, as the Ct value of universal qPCR with DNA extraction increased, the Ct value by pre-amplification qPCR with crude extract presents a growing trend correspondingly (Fig. 2). Agreement analysis based on the detection of shrimp samples showed that the kappa value between pre-amplification qPCR with crude extract and universal qPCR with DNA extraction was 0.959 (0.817~1, 95% CI). Additionally, in comparison to universal qPCR with DNA extraction, the specificity and the sensitivity of pre-amplification qPCR assay with crude extract for identification of WSSV were 100% (81.5~100%, 95% CI) and 97.22% (85.5~99.9%, 95% CI), respectively, indicating an excellent diagnostic agreement between pre-amplification qPCR with crude extract and universal qPCR with DNA extraction (Table 1).

Conclusions

WSD have posed a serious threat to China and the global shrimp breeding industry (Millard et al. 2021). Considering the prevention of heavy losses in shrimp farming, it is very important to establish a simple and sensitive diagnostic method for early diagnosis of WSSV in shrimp farms. Traditional cell culture for virus detection and ELISA assays are time-consuming and complex operations (Ozer et al. 2020), and the assays are difficult to popularize basic level laboratories and medium-sized aquaculture farm laboratories in China. qPCR assays are the “gold standard” for the detection of most viruses, with the advantages of strong specificity, high sensitivity, and high efficiency, which have been widely used in various research fields of aquatic products (Ozer et al. 2020, Meng et al. 2010, Jang et al. 2011, Chen et al. 2020, Qiu et al. 2018). These qPCR assays for detecting pathogens in aquatic products were based on cumbersome DNA extraction procedures, and also have different LODs such as 4 copies, 19 copies, and even 10^3 copies. qPCR analytical instruments have been widely deployed in China’s basic laboratories and laboratories of medium-sized aquaculture farms. However, in China, the operators of grass-roots
Table 1 Diagnostic performance comparison between pre-amplification qPCR with crude extract and universal qPCR with DNA extraction

| Shrimp samples | Universal qPCR with DNA extraction | Pre-amplification qPCR with crude extract | Performance characteristics (%) |
|----------------|-----------------------------------|------------------------------------------|----------------------------------|
|                | Positive  | Negative | Positive  | Negative | Sensitivity | Specificity |
| Positive       | 36        | 0        | 35        | 1        | 97.22% (85.5~99.9%, 95% CI) | 100% (81.5~100%, 95% CI) |
| Negative       | 0         | 18       | 0         | 18       |              |              |
| Total          | 54        | 54       | 54        | 54       |              |              |

Agreement Kappa value: 0.959 (0.879~1, 95% CI)
laboratories and medium-sized aquaculture laboratories lack the experience of tedious DNA extraction, which is a problem that needs to be solved.

Based on these considerations, in this study, we have developed a pre-amplified qPCR assay based on the crude extract of shrimp samples heated lysis, and carried out a comparative evaluation with qPCR assay based on DNA extraction. Simplifying the process of nucleic acid extraction is getting more and more concerned by researchers. Solution-based direct lysis method is to destroy pathogens and release DNA and RNA by adding efficient lysis buffer into samples. In 1987, Chomzynski et al. proposed for the first time a one-step method for extracting RNA by splitting cells with guanidine isothiocyanate in a low pH phenol single-phase solution (Chomczynski and Sacchi 1987). The simplified nucleic acid extraction based on the crude extract of thermal lysis has been well applied in the detection of clinically *Acinetobacter baumannii* strains (Ranjbar et al. 2018), and clinical usefulness of extraction-free PCR assay to detect SARS-CoV-2 (Fabio et al. 2021, Yu and Soon 2021). The rapid nucleic acid extraction reagent based on nucleic acid lysate has good consistency in the application of influenza virus detection (Zhao et al. 2019). Studies have shown that pre-amplified qPCR can overcome the problem of insufficient copy number of target gene in the reaction (Korenkova et al. 2015). In this study, the MightyPrep reagent used to obtain the crude extract by heating and cracking was based on the efficient cracking buffer including guanidine hydrochloride, which releases nucleic acids, proteins, polysaccharides, and other biological macromolecules from the virus. Then the nucleic acid was separated and purified by density gradient centrifugation to obtain the crude extract. The purity of DNA in the crude extract was not high enough and the amount of template was low, maybe contain RNA, protein, and other impurities, which usually affected the sensitivity of qPCR detection (Zhang et al. 2019; Chen et al. 2019). In this study, we developed a pre-amplified qPCR assay. First, we accelerated the thermal denaturation and renaturation of template DNA through 15 cycles of 10 s at 95°C and 10 s at 50°C, and did not collect fluorescent signals. More templates were accumulated for subsequent qPCR analysis, which was more conducive to the interpretation of high Ct value for low copy detection. Through the LOD verification of WSSV plasmid series dilution, the LOD of pre-amplified qPCR was 2.80 copies per reaction at 95% CI, which was more sensitive than the LOD of universal qPCR assay which was 20.57 copies, and was more suitable for the detection of low concentration template DNA.

The pre-amplified qPCR assay from crude extract of samples heated lysis showed good sensitivity and specificity. The comparative analysis of shrimp samples showed that pre-amplified qPCR assay from crude extract for WSSV detection was in good agreement with universal qPCR assay with DNA extraction. It can be used in county and district level laboratories and medium-sized shrimp farm laboratories in China, to carry out rapid detection of shrimp samples.

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**Author contribution** Chao Ma and Lili Yang finished experiment operation. Chao Ma and Zhuo Tian wrote the main manuscript text and prepared figures and tables. Jijuan Cao completed the fund acquisition, supervision, and editing. All authors reviewed the manuscript. Chao Ma and Zhuo Tian contributed equally to this work.
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**Data availability** The data that support the findings of this study are available from the corresponding author on reasonable request.

**Declarations**

**Ethical approval and consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Human and animal ethics** Not applicable.

**Competing interests** The authors declare no competing interests.

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