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ORIGINAL ARTICLE

The establishment and application of isothermal cross-priming amplification techniques in detecting penaeid shrimp white spot syndrome virus

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Significance and Impact of the Study: White spot syndrome, caused by white spot syndrome virus (WSSV), is the major disease threatening the shrimp aquaculture industry and leads to tens of billion dollars of economic losses in the world each year. This study established a CPA-based method for detecting WSSV, which is rapid, sensitive and specific. It is anticipated that this novel assay will be instrumental for diagnosis and surveillance of WSSV.

Keywords
Cross-priming amplification, detect, polymerase chain reaction, white spot syndrome virus.

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Abstract

White spot syndrome virus (WSSV) is one of the main pathogens seriously threatening penaeid shrimp farming in the world. This study designed and synthesized five specific primers based on the conserved sequences of WSSV genome, optimized the reaction system and conditions, and finally established a cross-priming amplification (CPA)-based detection method for WSSV (WSSV-CPA). The results indicated that the optimized reaction temperature and time of WSSV-CPA were 63°C and 60 min, respectively. The detection limit of the WSSV-CPA assay was as low as 10 copies μl^{-1} and shared same sensitivity with the WSSV-qPCR assay. Due to dispensing with expensive thermal cycler, time- and cost-saving, and ease of use in field, it is anticipated that the WSSV-CPA method developed in this study will be instrumental for the diagnosis and surveillance of WSSV.

Introduction

White spot syndrome virus (WSSV), of which the earliest possible origin was considered from a common ancestor in the Southeast Asia or South China Sea (Dieu *et al.* 2004; Shekar *et al.* 2012), can infect a wide range of aquatic crustaceans especially decapod with a very high mortality (Maeda *et al.* 2000). As white spot syndrome was reported in southern China in 1992 (Huang *et al.* 1995), the disease rapidly spread in shrimp farms globally and became the major disease threatening the shrimp aquaculture industry causing tens of billion dollars of economic losses each year. The disease has been listed by the World Organization of Animal Health (OIE) and the QAAD (Quarterly Aquatic Animal Disease) reporting system by the Network of Aquaculture Centres in Asia-Pacific (NACA) as one of the viral epidemic diseases of aquatic animals to be reported. Early detection of WSSV is important in the shrimp farming industry for an effective health management and control strategies. Therefore, more rapid and simplified detection techniques of WSSV are developed for field diagnosis.

Due to the lack of method for cell culture with isolated virus, the diagnosis methods based on direct detection of the WSSV DNA become recommended confirmative protocols in the 'Manual of Diagnostic Tests for Aquatic Animals' of OIE. Many laboratory-based techniques have been developed for detection of WSSV DNA, such as PCR (Kim *et al.* 1998), *in situ* PCR (Jian *et al.* 2005), nucleic acid hybridization (Durand *et al.* 1996; Wang *et al.* 1998),

real-time quantitative PCR (Durand *et al.* 2003), etc. The molecular detection techniques such as PCR, nested PCR, etc., developed by Lo *et al.*(Lo *et al.* 1996a,b) have been widely applied globally and recognized by the OIE as a standard method for WSSV detection (OIE 2013). Although these methods are highly specific and sensitive, they have shortcomings such as complex operation, longer detection time, higher cost, more stringent requirement for space, equipment, personnel quality, etc., and thus cannot meet the need for rapid field detection.

In recent years, a series of new isothermal nucleic acid amplification techniques appeared in the field of molecular biology, either based on the new discoveries in the mechanism of DNA/RNA biosynthesis or using polymerases with special functions to achieve the amplification of the target nucleic acids under isothermal conditions (Gill and Ghaemi 2008). These isothermal amplification technologies are simpler and cheaper than PCR methods in the field of nucleic acid detection and diagnosis; they thus become a widely used replacement method of PCR technology (Craw and Balachandran 2012). Among them, the loop-mediated isothermal amplification (LAMP) has already been applied in the detection of WSSV (Kono *et al.* 2004) and adopted by the latest version of the OIE standards.

Cross-priming amplification (CPA), a recently developed and novel isothermal amplification technique, utilizes multiple primers, one or more of which is a cross-primer, to rapidly amplify the target DNA sequence at a constant temperature with the chain displacement activity of *Bst* DNA polymerase (Fang *et al.* 2009; Yulong *et al.* 2010; Xu *et al.* 2012; Ke *et al.* 2013). As it does not require any denaturation step or additional nicking enzyme, CPA technology became another option for the high-sensitive diagnostic detection method which may be more suitable for farm-based usage. This study aimed to establish a CPA-based detecting method for WSSV and compare it with qPCR and LAMP methods to provide a novel method for rapid and sensitive filed detection of WSSV.

Results and discussion

Optimization of the WSSV-CPA reaction

Based on the initially established WSSV-CPA protocol, we further optimized the reaction temperature, Mg^{2+}, dNTPs and incubation time. The results showed that the optimized WSSV-CPA conditions were as follows: Mg^{2+}, 6 mmol l^{-1} (Fig. 1a); dNTPs, 1·4 mmol l^{-1} (Fig. 1b); reaction temperature, 63°C (Fig. 1c); and reaction time, 60 min (Fig. 1d). Therefore, the finalized optimal 25 μl WSSV-CPA reaction mixture contained 6 mmol l^{-1} Mg^{2+}, 1·4 mmol l^{-1} dNTPs, 1·2 mol l^{-1} betaine, primers 1s, 2a, 3a, 4s, and 5a at 1·6, 0·8, 0·8, 0·2, and 0·2 μmol l^{-1}, respectively, 8 U *Bst* DNA polymerase, 2·5 μl 10× ThermoPol buffer, and 1 μl template. The reaction was carried out by incubation at 63°C for 55 min.

CPA is a novel isothermal amplification technology emerged in recent years, which has relatively broad

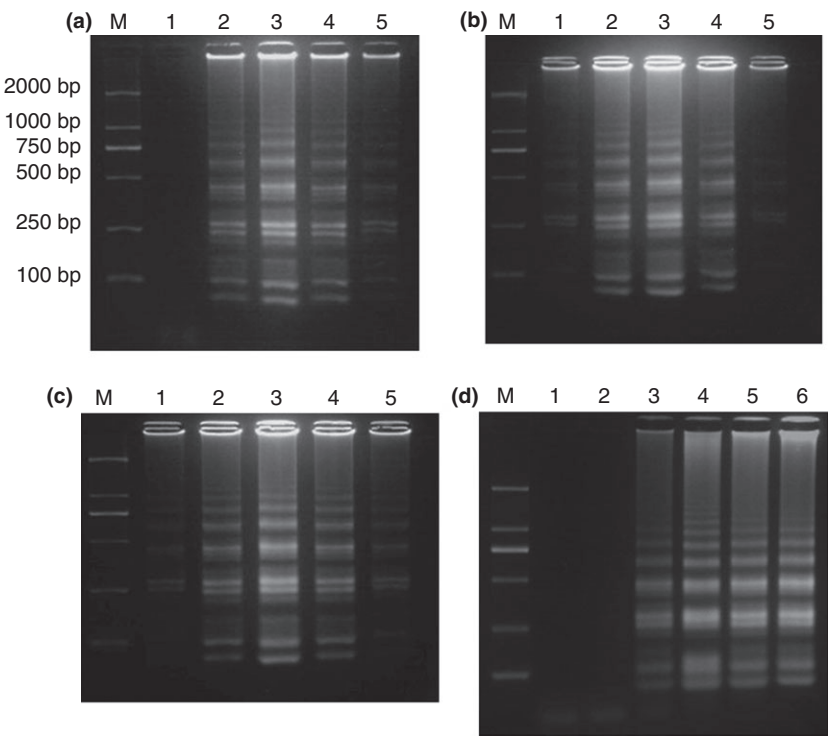

Figure 1 Optimization of the reaction and conditions of white spot syndrome virus (WSSV)-cross-priming amplification (CPA). (a) Lanes 1–6, Mg^{2+} concentrations of the CPA reaction at 2, 4, 6, 8 and 10 mmol l^{-1}, respectively; (b) Lanes 1–6, concentrations of dNTPs in the CPA reaction at 0·8, 1·2, 1·4, 1·6 and 2·0 mmol l^{-1}; (c) Lanes 1–5, temperatures of the CPA reaction at 58, 61, 63, 65 and 68°C, respectively; and (d) Lanes 1–6, time of the CPA reaction at 0, 15, 30, 45, 60 and 75 min, respectively. M, DL2000 DNA marker.

application prospects in the field of pathogen detection. Currently, several CPA-based techniques have already been introduced globally to detect infectious pathogens in human beings, animals and plants, as well as food (Cui *et al.* 2012; Zhang *et al.* 2012). However, the components of a CPA reaction are relatively complex, wherein the Mg^{2+} concentration and temperature are factors mostly affecting the reaction. While Mg^{2+} largely affects the primer annealing temperature and *Bst* DNA polymerase activity, temperature primarily affects the activity of *Bst* DNA polymerase. To establish a CPA detection method for WSSV, this study optimized the reaction parameters of WSSV-CPA and the result showed that the optimal Mg^{2+} concentration and temperature were 6 mmol l^{-1} and 63°C, respectively, which is consistent with the results of previous studies (Xu *et al.* 2012; Ke *et al.* 2013).

Verification of the WSSV-CPA products with restriction enzyme digestion

The WSSV-CPA amplification products were further analysed by Pst I restriction enzyme digestion. The results showed that WSSV-CPA products were digested into five fragments with four around 230, 200, 150 and 80 bp, respectively, and a very small primers-like band (Fig. 2a).

As the majority of the amplified products were from primer pairs 1s/3a and 1s/2a; they should be sensitive to *Pst* I digestion. Although the sizes of the digested amplification products were somehow inconsistent with the estimated sizes, which may be due to the formation of complicated secondary structures of the amplicons, these results confirmed that our WSSV-CPA products were tandem copies of the amplicons from the target gene, not the shrimp genomic DNA fragments, suggesting a good specificity of this WSSV-CPA detection method.

The specificity of WSSV-CPA assay

The specificity of the newly developed WSSV-CPA method was tested using respective infectious hypodermal and hematopoietic necrosis virus (IHHNV), hepatopancreatic parvovirus (HPV), Taura syndrome virus (TSV), yellow head virus (YHV) and *Vibro parahaemolyticus* as templates. Only the amplification with template

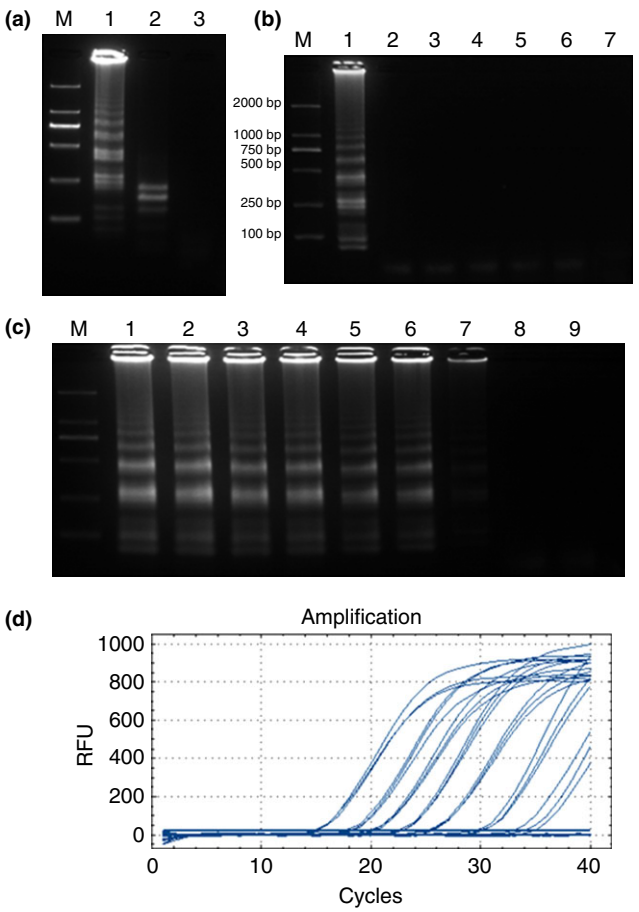

Figure 2 (a) White spot syndrome virus (WSSV)-cross-priming amplification (CPA) products digested by restriction enzyme *Pst* I. Lane 1, undigested CPA products. Lane 2, CPA products after *Pst* I digestion. Lane 3, negative control. The CPA products were electrophoresed on 2% agarose gels and stained with GeneFinder. (b) The specificity test of WSSV-CPA. Lanes 1-6, WSSV, IHHNV, HPV, TSV, YHV and *Vibro parahaemolyticus*, respectively. Lane 7, negative control. M, DL2000 DNA marker. (c, d) Comparison of the sensitivity between WSSV-CPA and qPCR. (c) WSSV-CPA products, Lanes 1–8, concentrations of WSSV DNA at 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 and 10^0 copies μl^{-1}, respectively. Lane 9, negative control. M, DL2000 DNA Marker. (d) Sensitivity of qPCR for detection of WSSV, Numbers 1–8, concentrations of WSSV DNA at 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 and 10^0 copies μl^{-1}, respectively. Lane 9, negative control. M, DL2000 DNA Marker.

containing WSSV appeared positive, whereas all other pathogens displayed negative results, indicating no cross-reactions (Fig. 2b). Sequence analysis showed that the CPA primers used in this study were located in the conserved regions of WSSV genome with no homology with other pathogenic species. The specificity test with five other penaeid shrimp pathogens demonstrated that the WSSV-CPA primers used in this study were highly specific to WSSV sequences, with no cross-reaction with the most common pathogens of shrimp.

Comparison of the sensitivity of WSSV-CPA and quantitative PCR

To test the sensitivity of WSSV-CPA, 10-fold serially diluted WSSV DNA (10^7–10^0 copies μl^{-1}) was detected using CPA and quantitative PCR (qPCR), respectively. The results showed that the detection limit of WSSV-CPA was up to 10^1 copies μl^{-1} (Fig. 2c), while that of the qPCR in the OIE Manual of Diagnostic Tests for Aquatic Animals was up to 10^1 copies μl^{-1} (Fig. 2d), too. Therefore, the present WSSV-CPA method has the same sensitivity with the qPCR in detecting WSSV.

Comparison of the sensitivity between WSSV-CPA and WSSV-LAMP

To test the sensitivity of WSSV-CPA, 10-fold serially diluted WSSV DNA (10^7–10^0 copies μl^{-1}) was detected using CPA and LAMP-based Highly Sensitive and Rapid Detection Kit for WSSV, respectively. The results showed that the detection limit of WSSV-CPA and WSSV-LAMP was both up to 10^1 copies μl^{-1} (Fig. 3).

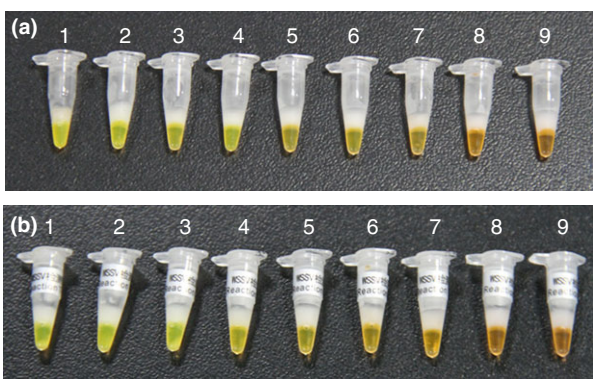

Figure 3 Comparison of the sensitivity between white spot syndrome virus (WSSV)-cross-priming amplification (CPA) and loop-mediated isothermal amplification (LAMP). (a)WSSV-CPA products; (b) LAMP produces. Numbers 1–8 in (a, b), concentrations of WSSV DNA at 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 and 10^0 copies μl^{-1}, respectively. Number 9, negative control.

Clinical samples test by WSSV-CPA assay

A total of 40 samples collected from shrimp farms were detected by the methods of WSSV-CPA, qPCR and LAMP-based Highly Sensitive and Rapid Detection Kit for WSSV, respectively. The result of qPCR assay indicated that 20 of the 40 tissue samples were positive for WSSV. The WSSV-CPA assay showed that the DNA templates from the 20 WSSV-positive samples (determined by qPCR) produced positive results. Furthermore, the 20 WSSV-negative samples in qPCR assay yielded negative results in WSSV-CPA (Table 1). Therefore, the diagnostic sensitivity and specificity of the WSSV-CPA method (compared to the qPCR method in the OIE standards) were both 100%. The results of LAMP-based Highly Sensitive and Rapid Detection Kit for WSSV were consistent with those of WSSV-CPA assay and qPCR assay; that is, an equal number of 20 samples were detected as WSSV positive and negative, respectively (Table 1). Similarly, comparing with LAMP and qPCR assay, both the diagnostic sensitivity and specificity of the WSSV-CPA assay were 100%.

In conclusion, to avoid the large-scale outbreak of white spot syndrome and death of penaeid shrimp, it is critical to quarantine and early detect WSSV with the seed shrimp. As a convenient detection method, WSSV-CPA is highly specific, sensitive, simple to perform and without the need of expensive equipment; it is thus suitable for field use in the shrimp aquaculture industry, with a high clinical value. Thus, this study provides a whole new and easier WSSV detecting technique to both the shrimp farming industry, and the inspection and quarantine departments.

Materials and methods

Samples

Healthy and diseased *Litopenaeus vannamei* (Lab Code: 040831JN) were from Jiaonan of Qingdao, in Shandong Province of China. Samples of penaeid shrimp infected with WSSV, HPV, IHHNV, TSV and YHV, respectively,

Table 1 The results of cross-priming amplification (CPA) and qPCR and loop-mediated isothermal amplification (LAMP) detection of unknown samples

Comparison of the methods	LAMP +	LAMP –	qPCR +	qPCR –	Total
CPA +	20	0	20	0	20
CPA –	0	20	0	20	20
Total	20	20	20	20	40

as well as cultured *Vibrio parahaemolyticus* isolates were collected or prepared by our laboratory and preserved at −80°C.

Extraction of DNA templates

DNA samples were extracted from 200 mg gill tissue of *Litopenaeus vannamei* using Marine Animal Genomic DNA Extraction Kit (Tiangen, China) according to the manufacturer's instruction. The extracted samples were used as detection templates for both qPCR and CPA.

Determination of WSSV-positive template

The templates were detected according to the standard WSSV detection methods recommended in the Manual of Diagnostic Tests for Aquatic Animals of the OIE. Deionized water and the templates extracted from gill tissue DNA of healthy *Litopenaeus vannamei* were used as blank and negative controls, respectively.

Design of WSSV-CPA primers

After analysis of the genomic sequence of WSSV (GenBank: AF332093), a conserved region (17441–17220 bp) was selected as the detection target of WSSV-CPA. A set of CPA primers was designed according to the conserved region to cover a 143-bp sequence (Fig. 4). A recognition sequence of restriction enzyme *Pst* I (CTGCAG) was introduced into the primer 1s which can be used in the specificity test for WSSV-CPA product.

The initial protocol of WSSV-CPA reaction

The initially established CPA reaction system contained 4 mmol l^{-1} $MgSO_4$, 1·2 mmol l^{-1} betaine (Sigma-Aldrich, St. Louis, MO), 1·0 mmol l^{-1} dNTPs each, 1·6 μmol l^{1} primer 1, 0·8 mmol l^{-1} primer 2a/3a each, 0·2 μmol l^{-1} primer 4s/5a each, 2·5 μl 10× ThermoPol buffer, 8 U Bst DNA polymerase (NEB, Beverly, MA) and an appropriate amount of DNA template, with a total volume adjusted to 25 μl with ddH_2O. The reaction was incubated at 65°C for 60 min and inactivated at 85°C for 5 min. The amplification products were analysed on a 2% agarose gel.

Optimization of the reaction conditions of WSSV-CPA

The reaction conditions of WSSV-CPA in the initial protocol were optimized successively on the reaction temperature, Mg^{2+}, dNTPs and incubation time. Each optimization test used previously optimized parameters as the initial protocol. All tests ran with three repeats. Determination of optimal reaction temperature was tested with the initial protocol above-mentioned by setting the amplification temperature at 58, 61, 63, 65 and 68°C, respectively. Optimal Mg^{2+} concentration was determined by setting the Mg^{2+} concentration at 2, 4, 6, 8 and 10 mmol l^{-1}, respectively, following the initial protocol except the reaction temperature which was under the optimal condition. Optimal concentration of dNTPs was determined by setting the final concentration of dNTPs at 0·8, 1·2, 1·4, 1·6 and 2·0 mmol l^{-1}, respectively, following the changed protocol using the optimal reaction temperature and Mg^{2+}. The incubation time was determined by setting the amplification time at 0, 15, 30, 45, 60 and 75 min, respectively, following the changed protocol with the optimized reaction temperature, Mg^{2+} and dNTPs.

Verification of the WSSV-CPA products by enzyme digestion

The WSSV-CPA products were digested with *Pst* I restriction enzyme to verify existence of the specific sequence and length in the amplicons. A 25-μl restriction enzyme digestion system contained 5 μl CPA product, 2·5 μl 10× buffer H and 40 U *Pst* I enzyme (Promega, Madison, WI). The reaction was incubated at 37°C for 4 h, and 5 μl products from the reaction were analysed by 2% agarose gel electrophoresis and documented under a gel imager.

Figure 4 Target sequence, cross-priming amplification primers location and primer design. Note that CTGCAG is the recognition site of restriction enzyme *Pst* I.

The specificity of WSSV-CPA

The specificity of WSSV-CPA was tested using the nucleic acid of IHHNV, HPV, TSV, YHV and *Vibro parahaemolyticus* as templates. The WSSV nucleic acid and the genomic nucleic acid of the healthy shrimp were used as positive and negative controls, respectively. The CPA reaction was triplicated with each template.

Comparison of the sensitivity of WSSV-CPA and qPCR

WSSV-DNA samples (10^7 copies μl^{-1}) were serially 10-fold diluted until 10^0 copy μl^{-1}, which were used as templates to perform the detection with qPCR following the protocol recommended by the OIE Aquatic Manual (OIE 2013) and optimized CPA, respectively. The WSSV-CPA products were analysed by 2·0% agarose gel electrophoresis.

Comparison of the sensitivity of WSSV-CPA and WSSV-LAMP

The 10-fold serially diluted WSSV DNA (10^7–10^0 copies μl^{-1}) was used as templates to perform the detection with LAMP-based Highly Sensitive and Rapid Detection Kit for WSSV (Yellow Fisheries Research Institute, China; Chinese Patent: ZL200810139949.4) and optimized CPA, respectively. The LAMP assay was performed according to the three steps in the protocol of the kit, that is (i) collect about 100 mg tissue and quickly grind into paste and pain the tissue paste on the sampling membrane; (ii) add 2–3 drops of Buffer A onto the sampling membrane and then wash the sampling membrane for 3–4 min; and (iii) denature the sampling membrane at 95°C for 4 min and then incubate it in the LAMP reaction tube at 57–60°C for 50 min. Both LAMP and CPA detections used the anti-contamination sealed fluorescence development system following the protocol of the kit. After the amplification incubation, the reaction tubes were heated at 95°C to melt the in-tube-sealed fluorescence dye for observation of amplified DNA.

Clinical samples test by WSSV-CPA assay

The optimized WSSV-CPA was used to detect 40 DNA samples extracted from actual penaeid shrimp collected from farms with unknown status of WSSV infection. As a comparison, paralleled detections with qPCR (OIE 2013) and the LAMP-based Highly Sensitive and Rapid Detection Kit for WSSV (Yellow Fisheries Research Institute, China) were also performed. The diagnostic sensitivity and specificity (DSp) of the WSSV-CPA to the qPCR method were calculated according to the formula recommended in Chapter 1·1·2 of the OIE Aquatic Manual (OIE 2013).

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Conflict of interest

The authors declare no conflict of interest.

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