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Rapid detection of giant salamander iridovirus by cross-priming amplification

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ABSTRACT

Giant salamander iridovirus (GSIV) belongs to the epizootic genus *Ranavirus*, and is the cause of epidemic diseases associated with high mortality and great losses to artificial breeding and farming. Here, we established a simple, accurate, and reliable cross-priming amplification (CPA) method to detect GSIV. The CPA assay targets the major caspid protein gene of the GSIV genome to design crossing primer pairs, and the reaction conditions were optimized, including optimal concentrations of the primers, betaine, dNTPs, Mg^{2+}, and Bst DNA polymerase, and reaction conditions. The sensitivity was shown to be 10 times higher than that of conventional polymerase chain reaction (PCR), and the specificity was 100%. The results were identified on nucleic acid strips within 3–5 min. Application of the CPA and PCR to 54 samples of giant salamander showed a positive rate of 72.22% and 74.07%, respectively, demonstrating high coincidence (94.44%, kappa = 8.7, $P < 0.0001$). The sensitivity of the CPA assay was 97.50% and the specificity was 92.86%. Thus, the CPA assay is as effective as conventional PCR, but with added practical advantages of simplicity and an almost instrument-free platform, which will be useful for both laboratories and giant salamander farms.

1. Introduction

Andrias davidianus (giant salamanders) is classified as a second-grade animal under state protection in China (Desnitskiy and Litvinchuk, 2015; Li, 2017; Chen et al., 2018). In recent years, giant salamanders have experienced outbreaks of severe diseases due to increasing trade and intensive aquaculture. In particular, *Giant salamander iridovirus* (GSIV) is an emerging pathogen and contributor to high mortality rates of giant salamanders (Du et al., 2016).

GSIV, belonging to the genus *Ranavirus* in the family *Iridoviridae*, is a linear double-stranded DNA virus (Geng et al., 2011), and the virus particle is 140 nm in diameter (Meng et al., 2014). GSIV was first reported in 2010 in Shanxi Province, and subsequently in Shaanxi, Sichuan, Jiangxi, and Hubei Provinces (Jiang et al., 2015). In addition, large-scale deaths of giant salamanders were reported in 2011 across China (Perlin et al., 2009; Geng et al., 2010; Wu et al., 2011; Meng et al., 2013; Jiang et al., 2015). Giant salamanders infected by GSIV develop very serious symptoms such as reduced appetite, multiple organ hemorrhagic lesions, and limbs dropsy (Meng et al., 2014).

There are currently some methods available for identifying GSIV. However, the various polymerase chain reaction (PCR) techniques are restrictive owing to the need for expensive laboratory instrumentation and skilled professionals. Antibody serological detection is effective, but is a time-consuming and laborious process (Dias et al., 2014; Abdella et al., 2015). Although loop-mediated isothermal amplification (LAMP) methods can detect the virus within only 2 h without the need for specific equipment (Meng et al., 2013), the results need to be analyzed by agarose gel electrophoresis and restriction endonuclease

Abbreviations: CCV, *Channel catfish virus*; CPA, cross-priming amplification; CTIV, *Chinese turtle iridovirus*; GSIV, *Giant salamander iridovirus*; HGC, hemorrhage of grass carp; LAMP, loop-mediated isothermal amplification; MCP, major capsid protein; PCR, polymerase chain reaction; RSIV, R*ed sea bream iridovirus*; SCPA, single-crossing cross-priming amplification; SVCV, *Spring viraemia of carp virus*; TFV, *Tiger frog iridovirus*; TRBI, *Turbot reddish body iridovirus*

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digestion. Moreover, aerosol pollution can contribute to false positive results using the LAMP assay.

As an alternative method that overcomes the above limitations, cross-priming amplification (CPA) shows good potential for rapid and convenient GSIV detection. CPA is a novel nucleic acid amplification method, in which nucleic acids are amplified using multiple cross-reacting primers and probes that are designed based on a conserved region of the virus genome (Xu et al., 2012). In contrast to conventional PCR, the temperature is kept constant in CPA (Gao et al., 2018; Gou et al., 2018; Liu et al., 2018; Wang et al., 2018). A disposable amplicon cross-contamination-proof device, nucleic acid strip, is used to measure the labeled amplicons in 3–5 min (Perlin et al, 2009). Therefore, the entire procedure, from specimen processing to result reporting, can be completed within 1–1.5 h.

One-cross primers (i.e., single-crossing CPA, SCPA) targeting the major caspid protein (MCP) gene were previously used to detect GSIV in clinical samples (Li et al., 2014; Zhou et al., 2015). In the present study, we optimized the CPA reaction conditions for GSIV detection, including the concentration of each primer pair; concentrations of Mg^{2+}, betaine, and dNTPs; amount of Bst DNA polymerase; and the reaction temperature and time (Fang et al., 2009; Bai et al., 2015). We further compared the results, sensitivity, and specificity of the optimized CPA assay with conventional PCR for GSIV detection in clinical salamander samples. These results could help promote the use of the CPA assay coupled with the nucleic acid test strip to establish a convenient visual detection method for rapidly and sensitively detecting GSIV from both clinical and environmental sources.

2. Materials and methods

2.1. Design, synthesis, and optimization of GSIV-SCPA primers

GSIV-specific primers for SCPA were designed using Primer Premier 5.0 software to target the conserved sequence of the GSIV MCP gene. Five groups of primers with higher scores were selected from the software and synthesized by Sangon Biotech (Shanghai, China). Using these primers, isothermal amplifications were conducted at the optimized temperature and time (63 °C for 40 min) with nuclease-free water as a negative control, and amplicons were detected by 2.0% agarose gel electrophoresis. At the same time, cross-primers were screened by conventional PCR.

2.2. Construction of the MCP gene standard plasmid

The MCP gene (GenBank accession no. AET51835.1) was purchased from Sangon (Shanghai, China). The plasmid was extracted from the inoculum, which was obtained from a scale-up culture, using the plasmid extraction kit. The concentration of the plasmid was determined by an ultraviolet spectrophotometer and adjusted to the optimum concentration for follow-up experiments as the template and positive control. Moreover, the plasmid copy number (N) was calculated by the following formula: $N\ (mL) = [6.02 \times 10^{23}\ (copies/mol) \times C\ (g/mL)]/MW\ (g/mol)$, where C is the concentration, and MW is the average molecular weight (double-stranded DNA) calculated as the number of bases (B) × 660 (g/mol) bases. B was determined by adding the number of bases in the plasmid and in the insert.

2.3. Establishment and optimization of the basic GSIV-CPA detection reaction system

The basic reaction system (Table 1) for GSIV-CPA was developed according to the methods of Zhao and Sun (2014). After amplification with the basic reaction system, amplicons were identified by restriction enzyme digestion and DNA sequencing. In addition, cross-primers of different concentrations (Table 2) were screened out by conventional PCR and CPA, respectively. The concentrations of betaine, dNTPs, and Mg^{2+}; the amount of Bst DNA polymerase; and the reaction temperature and reaction time were then optimized, respectively.

Table 1
Basic reaction system for GSIV-CPA.

Reaction system component	Final concentration	Volume (μL)
10× ThermoPol® Reaction Buffer	1×	2.5
10 mM dNTPs	1.0 mM	2.5
100 mM Mg^{2+}	8.0 mM	2
5 M betaine	0.7 M	3.5
8 U/μL Bst DNA polymerase	8 U	1
10 μM 2R1F	1.6 μM	4
10 μM 2R	0.8 μM	2
10 μM 3R	0.8 μM	2
10 μM 4 F	0.24 μM	0.6
10 μM 5R	0.24 μM	0.6
DNA template		1
Nuclease-free water		3.3

Table 2
Different concentration combinations of primers tested.

Primer combination	Primer concentration (μM)				
	2R1F	2R	3R	4 F	5R
1	1.0	0.5	0.5	0.2	0.2
2	1.0	0.5	0.5	0.4	0.4
3	1.0	0.5	0.5	0.6	0.6
4	1.0	0.5	0.5	0.8	0.8
5	1.0	0.5	0.5	1.0	1.0
6	1.0	0.5	0.5	1.2	1.2

The CPA assay was performed using betaine concentrations of 0.1 M, 0.5 M, 0.7 M, 0.9 M, 1.1 M, and 1.5 M. To determine the optimal Mg^{2+} concentration, Mg^{2+} concentrations of 2–12 mM were tested. The optimum concentration of dNTP was selected among trials using 0.6 mM, 0.8 mM, 1.0 mM, 1.2 mM, 1.4 mM, and 1.6 mM. The optimal temperature for an isothermal amplification assay must balance binding of the primer to the template strand with appropriate elongation activity of the DNA polymerase. Accordingly, the CPA reaction was conducted under different temperatures, from 55 °C to 65 °C, with the appropriate primers for 20–120 min. Amplicons were analyzed by 2.0% agarose gel electrophoresis and observed on an electrophoretic imager to determine the optimal reaction conditions based on the bands produced.

2.4. Specificity of the GSIV-CPA assay

The specificity of the GSIV-CPA assay was evaluated by CPA of genomic DNA extracted from *Turbot reddish body iridovirus* (TRBI), *Red sea bream iridovirus* (RSIV), *Tiger frog iridovirus* (TFV), *Chinese turtle iridovirus* (CTIV), *Spring viraemia of carp virus* (SVCV), hemorrhage of grass carp (HGC), and *Channel catfish virus* (CCV), which are all maintained in our laboratory. The MCP gene was used as the positive control template and nuclease-free water was used as the negative control template.

2.5. Sensitivity of the GSIV-CPA assay

The sensitivity of the CPA assay was further evaluated by detecting GSIV with 10-fold serial dilutions of the quantified GSIV MCP gene plasmid (10^{0}–10^{-10}) in nuclease-free water. Each dilution was used for both CPA and PCR as templates. After the reaction, the products were detected by 2% agarose gel electrophoresis.

2.6. Visualization detection with strips

After amplicons of the CPA reaction were obtained, the nucleic acid test strip was placed directly into the reaction tube for visual detection. There are two primers involved in CPA: one labeled with biotin and the other with FAM. As a result, the amplified products are labeled simultaneously during amplification. One end of the amplicon labeled with biotin binds to avidin-conjugated color particles for visualization, and the other end labeled with FAM is captured by an anti-FAM antibody located on the test line of the strip. Accordingly, the test results appear as a red line visible to the naked eye within 3–5 min. Positive results are indicated by two red lines at the test line and control line positions, respectively. In negative reaction strips, only the control red line is visible.

2.7. Agreement between the CPA assay and conventional PCR for GSIV detection

The degree of agreement between the CPA assay and PCR for GSIV was determined by detecting 54 giant salamander samples collected from several provinces in China, including Guangdong, Shaanxi, Jiangsu, and Henan. The 54 clinical samples harbored different GSIV isolates such as CGSIV-HN1104 (KF512820.1), GSIV-LY201112 (KF023635.1), and ADIV2010001 (KC465189.2). DNA from each of the samples was amplified using both the CPA assay and conventional PCR assay with the MCP gene standard plasmid as the positive control and nuclease-free water as the negative control.

3. Results

3.1. Selection of GSIV-CPA primers

A set of optimal primers with trapezoidal bands was obtained using PCR and CPA, separately, as shown in Table 1. PCR was performed using the CPA primers 4 F and 5R to screen out primers that could be matched with the target gene and used for amplification. As shown in Fig. 1, target genes could be obtained by PCR amplification with all five groups of primers. The clearest and brightest band (Fig. 1, lane 3) demonstrating the best effect of CPA was then selected, as shown in Fig. 2. Additionally, there were no bands in the negative control lane (lane 8), confirming that the CPA primers used in lane 3 were the optimal primers (Table 3).

3.2. Digestion and sequencing of the amplified products

The GSIV-CPA product was digested by the endonuclease HindIII,

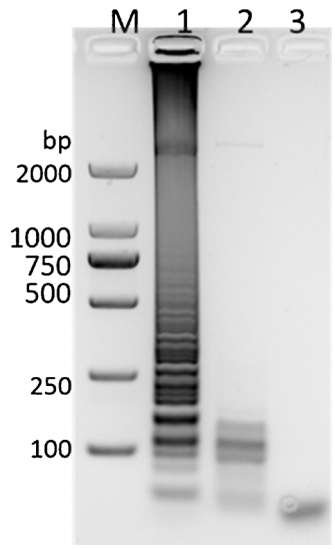

Fig. 2. Enzyme digestion analysis of MCP-CPA amplification products. M: DL2000; lane 1, amplification product without digestion; lane 2, amplification product after digestion; lane 3, negative control.

which produced a clear band of ˜142 bp (Fig. 2). The sequencing result proved that the correct target gene was amplified. Multiple sequence alignment covering the primers used in this study was conducted, and the results are shown in Fig. 3 in Supplementary File.

3.3. Optimization of GSIV-SCPA reaction conditions

The primer concentration, betaine concentration, Mg^{2+} concentration, dNTP concentration, reaction temperature, and reaction time are the major factors that determine successful amplification in the CPA reaction. We found that the optimal primer concentration was primer combination 3 (Table 2), which produced the brightest and strongest bands of the amplified products (Fig. 3a, lane 5). Otherwise, the individual primer concentrations were as follows: 1.0 μM for 2R1F, 0.5 μM for both 2R and 3R, and 0.6 μM for both 4 F and 5R. The optimal betaine concentration was 0.7 M (Fig. 3b, lane 3), and optimal bands were produced with a dNTPs concentration of 1.2 mM (Fig. 3c) and an Mg^{2+} concentration of 8 mM (Fig. 3d). The best multiple ladders of CPA amplification appeared at a reaction temperature of 63 °C (Fig. 3e). In terms of reaction time, the brightest strips were observed in lane 3 and lane 4, corresponding to 60 min and 80 min, respectively (Fig. 3f). However, because of the need for rapid detection, a reaction time of

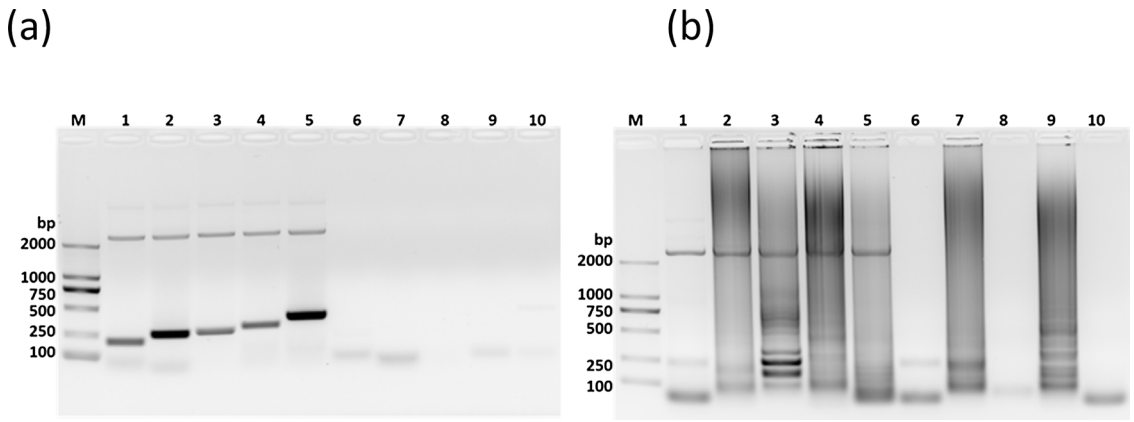

Fig. 1. Results of conventional PCR and CPA primer screening. (a) Conventional PCR primer screening. M: DL2000; lanes 1–5 are different primers; lanes 6–10 are negative controls for the corresponding primers. (b) Conventional CPA primer screening. M: DL2000; lanes 1–5 are different primers; lanes 6–10 are negative controls for the corresponding primers.

Table 3
. Primers used for cross-priming amplification in this study.

Primer	Primer sequence (5′-3′)	Features
1R1F	TGTGACGTTCTGCACCATAAAAGCTTGCCATGAGCAGCACAGTCAGG-3'	Cross-primer (underlined Hind) III endonuclease site)
2R	TGTGACGTTCTGCACCATAA	Specific primer
3R	GAAGGTGGCCGCGTTCCTGG	
4 F	ACCGTCGAGGCCAACGTC	Stripping primer
5R	CAGGACCGTGTTGCCGAC	
2R-Bio	(Biotin)-TGTGACGTTCTGCACCATAA	Detection primer
3R-FAM	(FAM)-GAAGGTGGCCGCGTTCCTGG	

40 min (Fig. 3f, lane 2) was considered to be optimal.

In summary, the optimized reaction conditions were established as follows: reaction mixture containing 1.0 μM 2R1F, 0.5 μM each of 2R and 3R, 0.6 μM each of 4 F and 5R, 2.5 μL of 10× ThermoPol® Reaction Buffer, 0.7 M betaine, 8.0 mM Mg^{2+}, 1.2 mM of dNTP mix, 8 U/μL of Bst DNA polymerase (New England Biolabs, USA), along with 1 μL template DNA for a final volume of 25 μL with nuclease-free water. The reaction was carried out at 63 °C for 40 min.

3.4. Specificity of the CPA assay

The specificity of the CPA assay was evaluated using a recombinant plasmid of *Brucella, Mycobacterium tuberculosis*, and classical swine fever virus as templates. The optimized CPA reaction system could

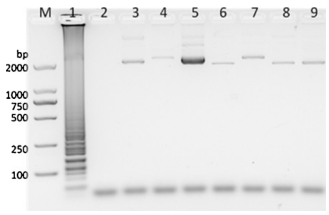

Fig. 4. Specific detection of CPA. M: 2 K Plus; Lane 1 is a positive control; Lane 2 is a negative control; Lanes 3–11 correspond to amplification products of *Turbot reddish body iridovirus* (TRBI), *Red sea bream iridovirus* (RSIV), *Tiger frog iridovirus* (TFV), *Chinese turtle iridovirus* (CTIV), *Spring viraemia of carp virus* (SVCV), hemorrhage of grass carp (HGC), and *Channel catfish virus* (CCV).

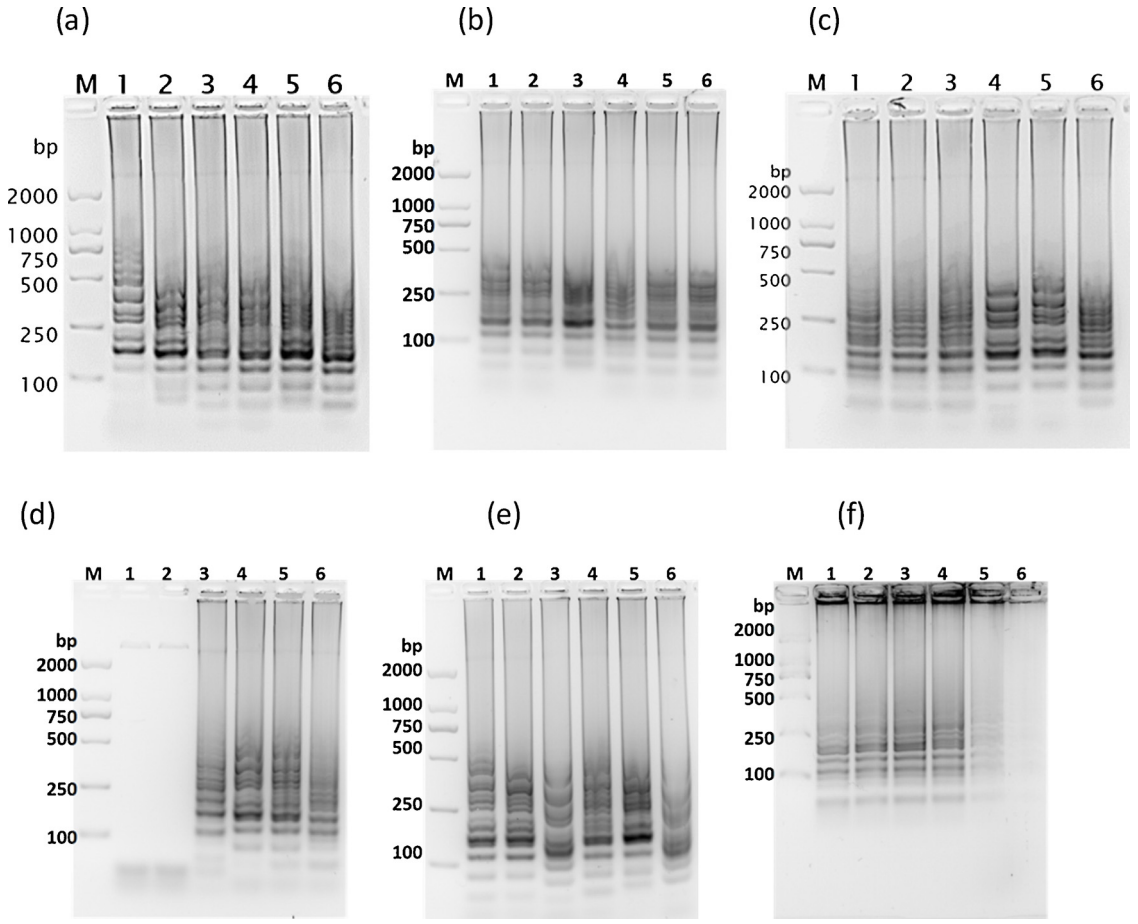

Fig. 3. Optimization of GSIV-SCPA reaction conditions. (a) Effect of different primer concentrations and ratios on amplification. M: DL2000; lanes 1–6 correspond to primer concentration combinations 1, 2, 3, 4, 5, 6 (see Table 2). (b) Effect of different betaine concentrations on amplification. M: DL2000; lanes 1–6 correspond to 0.1, 0.5, 0.7, 0.9, 1.1, 1.5 M betaine. (c) Effect of different dNTPs concentrations on amplification results. M: DL2000; lanes 1–6 correspond to 0.6, 0.8, 1.0, 1.2, 1.4, and 1.8 mM dNTPs. (d) Effect of different Mg^{2+} concentrations on amplification. M: DL2000; lanes 1–6 correspond to 2.0, 4.0, 6.0, 8.0, 10.0, and 12.0 mM Mg^{2+}. (e) Effect of reaction temperature on amplification. M: DL2000; lanes 1–6 correspond to 55 °C, 57 °C, 59 °C, 61 °C, 63 °C, and 65 °C. (f) Effect of reaction time on amplification. M: DL2000; lanes 1–6 correspond to 20 min, 40 min, 60 min, 80 min, 100 min, and 120 min.

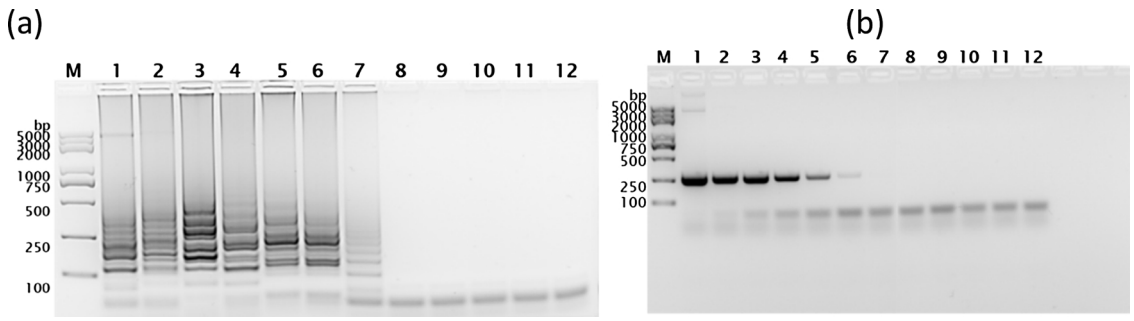

Fig. 5. Sensitivity of GSIV detection by CPA and a conventional PCR assay. (a) Sensitivity of the detection of CPA. M: 2 K Plus; Lanes 1–11 correspond to the 10^{0}–10^{-10} dilution of amplification products, respectively; Lane 12 is the negative control. (b) Sensitivity of the conventional PCR assay. M: 2 K Plus; Lanes 1–11 correspond to the 10^{0}–10^{-11} dilutions of amplification products, respectively; Lane 12 is the negative control.

detect the MCP gene of GSIV, producing a ladder-like pattern of bands (Fig. 4, lane 1). However, no such pattern was produced for spring viremia when using TRBI, RSIV, TFV, CTIV, SVCV, HGC, and CCV as templates (Fig. 4, lanes 3–9). In addition, nuclease-free water used as a negative control template did not produce bands (Fig. 4, lane 2). This result confirmed that CPA can detect GSIV with relatively high specificity.

3.5. Sensitivity of the CPA assay

The sensitivity of the optimized CPA reaction system was also determined using 10-fold serial dilutions of GSIV DNA as templates, which showed that the template DNA could be detected at a 10^{-6} dilution (Fig. 5a), corresponding to a lower detection limit of 0.1 pg/μL template DNA. These results demonstrated that the sensitivity of CPA is ten times higher than that of PCR, with a lower limit of detection of a 10^{-5} dilution (Fig. 5b).

3.6. Visual detection of CPA

The test results of CPA appeared as a colored line visible to the naked eye within 3–5 min on a rapid nucleic acid test strip. Furthermore, the strips could detect the plasmid template at a 10^{-5} dilution, with a lower detection limit of 1.0 pg/μL template DNA. Therefore, the sensitivity of CPA characterized using the strips was 10 times lower than that of agarose gel electrophoresis (Fig. 6).

3.7. Agreement between the CPA assay and PCR for GSIV detection

To determine the practical application of the optimized CPA assay, we compared the results obtained by CPA and conventional PCR for the diagnosis of 54 giant salamander samples. The positive rate of the CPA assay was 72.22% (39/54) and that of conventional PCR was similar at 74.07% (40/54). The sensitivity of the CPA assay was 97.50% (39/40) and the specificity was 92.86% (13/14). Furthermore, the coincidence rate of the two assays was 94.44% (51/54), with a kappa value of 0.870 ($P < 0.0001$). Therefore, the CPA assay developed in this study shows comparable performance with the conventional PCR assay in detecting different strains of GSIV (Table 4).

Table 4
Agreement between the CPA assay and conventional PCR for GSIV detection.

		CPA assay		summary
		positive	negative	
Conventional PCR	positive	38	1	39
	negative	2	13	15
summary		40	14	54

4. Discussion

The CPA assay coupled with visualization strips provides a simple, cost-effective, and visual format for molecular identification (Xu et al., 2012). Optimization of CPA assay conditions is critical for the success of the reaction. Here, we succeeded in optimizing the conditions of heat, time, salt, betaine to DNA templates degeneration, and the composition of primer binding and elongation for obtaining true isothermal amplification of target DNA sequences. Use of an appropriate temperature could allow for the highest activity of the Bst DNA polymerase (Xu et al., 2012). Hence, the melting temperature of the crossing primers was set to 60–65 °C for GSIV detection and maintained with a heating block during the reaction in 200-μL tubes. Multiple bands were obtained at both 61 °C and 63 °C although the bands were clearest and brightest at 63 °C, which is similar to the optimal reaction temperature of the LAMP method (Meng et al., 2013) and is the same as the temperature of CPA with a nucleic acid test strip used to detect *Porcine epidemic diarrhea virus* (Wang et al., 2016). Moreover, the Mg^{2+} concentration is an important factor to regulate the activity of Bst DNA polymerase. When the concentration of dNTPs is too high, the redundant dNTPs will bind with Mg^{2+} to reduce the Mg^{2+} concentration, and thus decrease the activity of Bst DNA polymerase. Thus, the

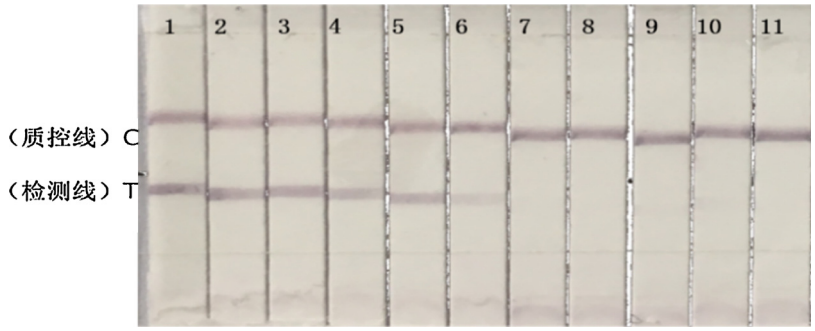

Fig. 6. Sensitivity of CPA with nucleic acid test strips. Label 1–11 correspond to the 10^{0}–10^{-10} dilutions of the amplification products.

reaction conditions are interdependent and must be considered as a whole. Here, we determined the optimal dNTPs and Mg^{2+} concentration to be 1.2 mM and 8.0 mM, respectively, which is lower than that reported for the CPA assay to identify *Porcine epidemic diarrhea virus* (Wang et al., 2016). In addition, betaine can facilitate opening of the double-helix structure of DNA and can slow down its rate of decomposition in the water. Therefore, we regularly refreshed the betaine solution to ensure maintaining the optimal concentration of 0.7 M. The reaction time directly determines the yield of the reaction products. If the amplification time is too short or too long, for instance less than 20 min or more than 80 min, amplicons will be produced at a low yield. When the reaction time ranged from 40 min to 80 min, the yield remained about the same. To achieve rapid detection, we chose 40 min as the optimal reaction time.

Frazczyk et al. (2016) reported a CPA assay test for detection of *African swine fever virus* in pig and wild boar blood and sera samples within 100 V/50 min, and Huo et al. (2017) demonstrated that detection of *Prunus necrotic ringspot virus* by the CPA assay took less than 90 min. Therefore, the method developed in this study is relatively time-efficient. With regard to the sensitivity of the CPA reaction, we found that a 10^{-5} dilution with a lower detection limit of 1.0 pg/μL template DNA was the detection limit, which had ten times higher sensitivity compared to that of PCR. This result is in line with the sensitivity previously reported for the detection of *Vibrio parahaemolyticus* by the CPA assay (Xu et al., 2015). Our optimized CPA assay was also confirmed to be capable of specifically detecting GSIV.

Although there are various detection methods available to identify GSIV, such as conventional PCR, nested-PCR, TaqMan real-time PCR, LAMP, and serologic examination (Meng et al., 2013; Ma et al., 2014), these detection methods are limited for wide application owing to the need for expensive instrumentations and professionals. In order to overcome some of the technical and cost barriers associated with the methods above, we sought to provide a more rapid and convenient test of GSIV utilizing a CPA mechanism involving one-cross primers (SCPA) to detect GSIV in clinical samples (Xu et al., 2012). Although the sensitivity obtained in this study (0.1 pg/μL template DNA) is lower than that of TaqMan real-time PCR (1.1×10^{-3} pg/μL DNA) and the LAMP assay (0.01 pg/μL) (Meng et al., 2013), the only equipment needed for CPA amplification is a heat block or water bath and it can be performed in an on-limits system without any equipment to avoid false-positive results (Ou et al., 2014; Xu et al., 2015). The whole process of amplification and detection can be completed within 1 h, and visualization nucleic acid strips used to monitor the results offer high specificity, efficiency, simplicity, and rapidity.

Many iridoviruses are responsible for severe systemic diseases in amphibians and fish, and have attracted global attention (Granzow et al., 1997; Zhang et al., 2001; Geng et al., 2010). Studies on the morphological features and genome of GSIV have confirmed that the virus is part of the family *Iridoviridae*. GSIV causes universal symptoms such as systemic hemorrhage and serious swelling with a high mortality rate, contributing to huge economic losses. Moreover, the majority of giant salamander farms are in remote areas, which do not have the facilities for professional testing technologies such as PCR and TaqMan real-time PCR. Hence, early detection of GSIV pathogens is essential to best control the spread of emerging diseases and provide help to giant salamander farmers. The neoteric nucleic acid amplification technology CPA has been applied to detect many pathogens to date (Zhang et al., 2012; Wen et al., 2013; Ou et al., 2014); however, this is the first application for its detection of GSIV. Our optimized CPA assay represents a novel and significant tool for the fast and precise diagnosis of GSIV infection in any salamander farm or laboratory.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.113678.

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