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Development of a loop-mediated isothermal amplification assay for rapid detection of iridovirus in the Chinese giant salamander

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A B S T R A C T

The Chinese giant salamander (*Andrias davidianus*) iridovirus (GSIV) is an emerging infectious pathogen responsible for severe hemorrhagic disease and high mortality in cultured Chinese giant salamanders. A loop-mediated isothermal amplification (LAMP) assay based on the major caspid protein (MCP) gene has been developed to detect this virus. Primer pairs for the LAMP assay were designed based on the GSIV MCP gene sequence. Amplification results indicate that under optimized conditions the LAMP assay has the ability to specifically detect the virus in both diseased animals and infected *epithelioma papilloma cyprinid* (EPC) cells. The assay was shown to be 10-fold more sensitive than nested PCR and was able to detect concentrations of 10^{-9} (approximately 0.01 pg/μL). The LAMP assay is relatively easy to perform in situ and the amplification products can be observed directly under UV light or via staining with SYBR Green I. The LAMP assay is also rapid and cost-effective. This study establishes the use of a LAMP assay for rapid detection of GSIV, which is a novel and important tool for the diagnosis of GSIV infection in laboratory or farmed Chinese giant salamanders.

1. Introduction

The Chinese giant salamander (*Andrias davidianus*), family *Cryptobranchidae*, is the largest extant amphibian species in the world. It has previously been endemic and widely distributed throughout mainland China (Zhao, 1998). Because of hunting, fragmentation, and loss of natural habitats, this endangered amphibian has now been listed in annex I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and class II of the national list of protected animals in China. The Chinese giant salamander has significant economic value as an edible delicacy and in medicine. At present, approximately two million Chinese giant salamanders are bred annually in China. However, increasing trade and intensive aquaculture have led to the emergence of severe diseases (Geng et al., 2011; Jiang et al., 2011; Meng et al., 2009; Wang et al., 2010). The Chinese giant salamander iridovirus (GSIV) is an emerging pathogen responsible for high mortality of Chinese giant salamanders. This pathogen causes a disease characterized by symptoms including rotting limbs, skin ulcers, and hemorrhaging (Dong et al., 2011; Geng et al., 2011; Jiang et al., 2011). The disease has been prevalent in the major cultivated populations of Chinese giant salamanders. According to investigations, GSIV was responsible for economic losses of ~300 million RMB (48 million USD) in 2010. There is currently no effective way to control GSIV; therefore the development of a rapid method to detect the virus early is critical. Conventional polymerase chain reaction (PCR) has a wide range of applications as a fundamental molecular biological tool for iridovirus detection (Chinchar and Mao, 2000; Jeong et al., 2006). PCR and TaqMan real-time PCR have been used in prior studies to detect GSIV (Zhou et al., 2012a,b). However, loop-mediated isothermal amplification (LAMP) (Notomi et al., 2000) displays many characteristics that render it suitable for the rapid and simple detection of nucleic acids in samples (Karanis and Ongerth, 2009). The LAMP method has been optimized as a rapid diagnostic tool for several diseases. In this study, the LAMP assay was developed to detect GSIV in viral culture as well as in infected animal tissues. LAMP and nested PCR were compared for their detection sensitivities.

2. Materials and methods

2.1. Virus and cell line

The Chinese giant salamander iridovirus was isolated in 2010 and stored in our laboratory. The *epithelioma papilloma cyprinid* (EPC) cell line was obtained from the China Center for Type Culture Collection (CCTCC), Wuhan University.

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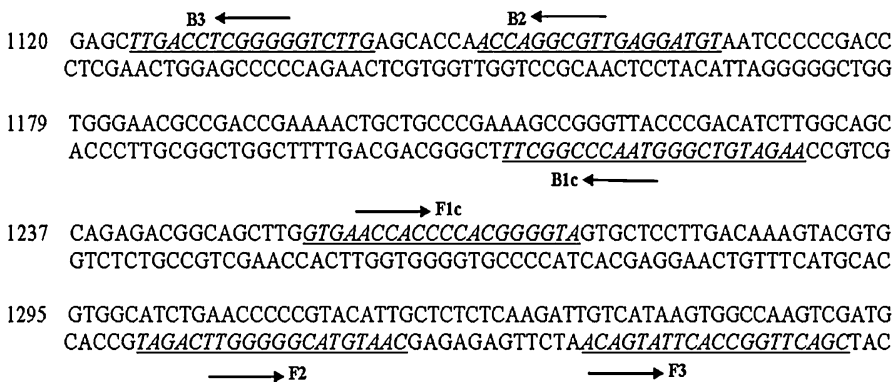

Fig. 1. The nucleotide sequence of MCP gene of GSIV and the primers (underlined) used for LAMP.

Table 1
Primers used for loop mediated isothermal amplification (LAMP) and nested PCR in this study.

Name	Sequence (5′ → 3′)
FIP(F1c + TTTT + F2)	GTGAACCACCCCACGGGGTA-TTTT-CAATGTACGGGGGTTCAGAT
BIP(B1c + TTTT + B2)	AAGATGTCGGGTAACCCGGCTT-TTTT-ACCAGGCGTTGAGGATGT
F3	CGACTTGGCCACTTATGACA
B3	TTGACCTCGGGGGTCTTG
MCP-OF	GACTTGGCCACTTATGAC
MCP-OR	GTCTCTGGAGAAGAAGAA
MCP-IF	TCGCTGGTGTTGCCTATCAT
MCP-IR	CTGCCAAGATGTCGGGTAAC

2.2. Viral culture and genomic DNA extraction

The virus, at a titer of $10^{9.5-10.5}$ $TCID_{50}$/mL, was incubated in EPC cells cultured in minimal essential medium (MEM; Sigma, St. Louis, MO, USA) containing 2% fetal bovine serum at 25 °C. The infected cell suspensions were collected when a cytopathic effect (CPE) was detected. Cell suspensions were freeze-thawed in triplicate, then centrifuged at 5000 rpm for 30 min. The resulting supernatant was used for viral purification via ultracentrifugation with 100,000 rpm for 1 h at 4 °C (Beckman-coulter, Optima LX80, USA). The precipitate was collected to extract viral genomic DNA, using a viral DNA extraction kit (Qiagen, Hilden, Germany). The concentration of the purified DNA was calculated using a BioPhotometer plus (Eppendorf, Hamburg, Germany) and the samples were stored at −20 °C.

2.3. Design of LAMP primers

All LAMP primers were designed using Primer Explorer Version 4.0 (http://primerexplorer.jp/elamp4.0.0/index.html) to target the Chinese giant salamander iridovirus MCP gene (GenBank accession no. JN516141). Details of the primers are shown in Fig. 1 and Table 1. All primers were synthesized by GeneCore BioTechnologies (Shanghai, China).

2.4. Optimization of LAMP reaction conditions

The LAMP reaction was performed using a heating block set at 60, 61, 62, 63, 64 and 65 °C, respectively, for 1 h or 45 min, followed by 80 °C for 5 min to terminate the reaction. To determine the optimal Mg^{2+} concentration, Mg^{2+} concentrations of 2–10 mM were tested. In addition, the LAMP reaction was carried out at 65 °C for 30, 45 or 60 min. The LAMP reaction mixture contained 0.8 μM each of the inner primers FIP and BIP, 0.1 μM each of the outer primers F3 and B3, 1.0 mM dNTP mix (Promega, Madison, WI, USA), 0.5 M betaine (Sigma, St. Louis, MO, USA), 8 mM $MgSO_4$, 8 U *Bst* DNA polymerase (large fragment; New England Biolabs, MA, USA), and template DNA for a final volume of 25 μL. The products were analyzed via 2% agarose gel electrophoresis.

2.5. Visualization of LAMP products

LAMP products were electrophoresed in 2% agarose gel with ethidium bromide (EB, 1 μg/mL) and visualized under UV light. Visual inspection of the LAMP amplification in the reaction tube was performed by adding SYBR Green I. The color of the solution differed between positive and negative samples and could be observed under UV light.

2.6. Nested PCR

LAMP was compared with nested PCR for molecular detection of GSIV. Based on the GSIV MCP gene sequence (GenBank accession no. JN516141), Nested PCR primers were designed using Primer 5.0 (http://www.premierbiosoft.com, Canada). Primers are shown in Table 1. Amplified products were ~530 bp and 320 bp in length. The PCR reaction was carried out using PCR Core System II (Promega, Wisconsin, Madison, USA) and the reaction mixture consisted of 5.0 μL of 10× reaction buffer, 0.4 μL of 10 μM dNTPs, 2.0 U of Taq DNA polymerase, 2.0 μL of 10 μM each of primers and 1 μL of the DNA template for a total reaction volume of 50 μL. To evaluate the sensitivity of the detection limit, nested PCR was carried out using 2 μL GSIV DNA with 10-fold serial dilutions of template DNA. PCR amplifications were detected by 1.5% agarose gel electrophoresis and visualized under UV light.

2.7. Evaluation of the detection sensitivity of the LAMP assay

The sensitivity of LAMP was further evaluated by detecting GSIV in diseased Chinese giant salamanders. Total DNA from spleens, livers and kidneys of diseased Chinese giant salamanders were extracted. On ice, the tissues were homogenized in sterile phosphate-buffered saline (PBS, pH 7.2, Sigma) at a ratio of 1:10 (w/v). Total DNA was extracted from mixture of these tissues using DNA purification kit (Promega, Madison, WI, USA) following the manufacture's protocol. Ten-fold serial dilutions (10^{-1} to 10^{-10} dilutions) of genomic DNA extracted from infected Chinese giant salamanders were used as templates for the LAMP assay. After the reaction, products were detected by 2% agarose gel electrophoresis and observed via the addition of SYBR Green I.

2.8. Evaluation of the specificity of LAMP assay

The specificity of the LAMP assay was evaluated using independent DNA extractions of Koi herpesvirus (KHV), lymphocystis

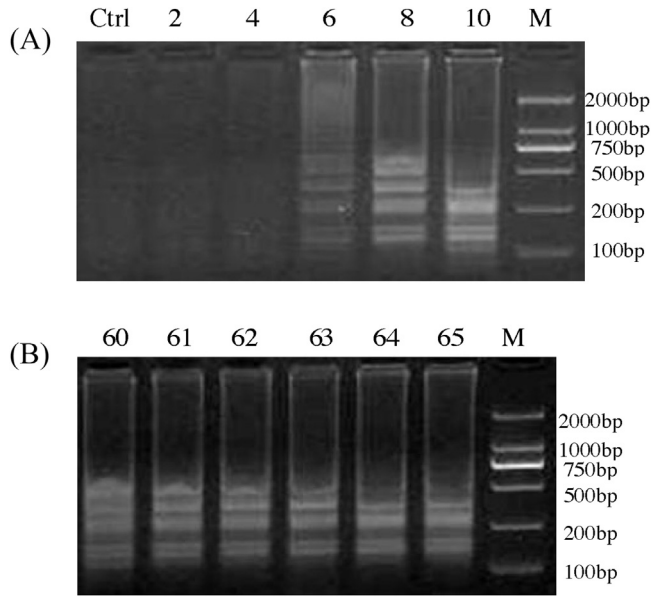

Fig. 2. Optimization of the LAMP conditions for GSIV detection. Different Mg^{2+} concentrations and temperatures were tested in LAMP and the PCR products were loaded on agarose gel for electrophoresis. (A) Determination of the optimal Mg^{2+} concentration for the LAMP assay. M: molecular marker; 2–10: different Mg^{2+} concentrations; Ctrl: negative control. (B) Determination of the optimal temperature for LAMP assay. M: DNA marker; 60–65: reaction at 60 °C, 61 °C, 62 °C, 63 °C, 64 °C and 65 °C, respectively.

disease virus (LCDV), *Aeromonas hydrophilia*, *Citrobacter freundii* and EPC cells. DNA from healthy Chinese giant salamanders was used as a control template. The products were analyzed via electrophoresis on 2% agarose gel.

2.9. Application of LAMP assay for clinical diagnosis of GSIV infection in Chinese giant salamanders

To validate the application of LAMP assay for clinical diagnosis of GSIV infection, LAMP assay was compared with nested PCR for detecting GSIV in Chinese giant salamanders with typical symptoms of GSIV infection as well as in animals with suspicious infections. In total 48 diseased Chinese giant salamanders from different zones were included in this study. Total DNA was isolated from livers, spleens, and kidneys of Chinese giant salamanders as mentioned above and used in LAMP and nested PCR assays, respectively.

3. Results

3.1. Optimal LAMP conditions

Mg^{2+} concentration, reaction temperature and reaction time were the major factors determining successful amplification in the LAMP reaction. Mg^{2+} concentrations of 2–10 mM were tested. The results showed that no amplification occurred at Mg^{2+} concentrations below 6 mM, the characteristic ladder of multiple bands appeared at 6 mM, and LAMP reaction products decreased at Mg^{2+} concentrations above 10 mM (Fig. 2A). The optimal Mg^{2+} concentration was thus determined to be 8 mM. The reaction temperature was set at 60–65 °C, based on the primers' reference temperature. The best multiple ladder of LAMP amplification appeared at 65 °C (Fig. 2B). LAMP reaction typically used a reaction time of 45 or 60 min. In this study, a reaction time of 60 min was used in a block heater, followed by 80 °C for 5 min, which produced optimal results. Overall, the optimized reaction conditions were as follows: reaction mixture containing 1.6 μM each of FIP and BIP, 0.2 μM each of F3 and B3, 1.0 mM of dNTP mix, 0.5 M betaine, 8 mM $MgSO_4$, 8 U of *Bst* DNA polymerase (Promega, Madison, WI, USA) along with template DNA in for a final volume of 25 μL. The reaction was carried out at 65 °C for 60 min then terminated by increasing the temperature to 80 °C for 5 min.

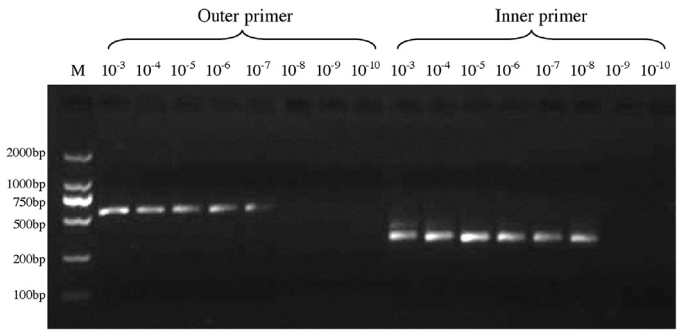

Fig. 3. GSIV detection using nested PCR. GSIV DNA with 10-fold serial dilution (10 μg/mL) was used as template. M: DNA marker.

3.2. Nested PCR assay

Outer and inside primer pairs were amplified, respectively. The outer primers produced a 531 bp fragment. The first amplification product was used as a template for inside primer amplification, which produced a clear band of ~320 bp (Fig. 3). Controls were negative in PCR reaction. In order to determine the sensitivity of the nested PCR assay, viral DNA was diluted at 10-fold serial dilutions to a concentration of 10^{-10}. The first PCR amplification indicated a light band at 10^{-6} and a weak band at 10^{-7}, but a clear band in the second PCR amplification at 10^{-8} (Fig. 3). Nested PCR was thus able to detect the virus at a concentration of up to 10^{-8}.

3.3. Sensitivity of LAMP assay

The sensitivity of the LAMP reaction was also determined using 10-fold serial dilutions of GSIV DNA as the template. These results indicated that the LAMP assay could detect template DNA at a 10^{-9} dilution (Fig. 4A), corresponding to a lower detection limit of 0.01 pg template DNA. These results indicate that LAMP has higher sensitivity than nested PCR. The results were not clear when the amplification products of the differently diluted DNA templates were observed directly, but the addition of 2.0 μL diluted SYBR Green I to the reaction tubes rendered positive amplification products green, while negative reaction remained orange (Fig. 4B). Meanwhile, products dyed with SYBR Green I appeared bright under UV light (Fig. 4C). The sensitivity of the LAMP assay was further evaluated by detecting total DNA in mixture of spleens, livers and kidneys of diseased Chinese giant salamanders. It was found that LAMP could detect GSIV at 10^{-7} dilution of total DNA (Fig. 5).

3.4. Specificity of LAMP assay

The LAMP reaction was able to detect viral DNA isolated from infected Chinese giant salamanders, producing a ladder-like pattern of bands (Fig. 6). However, no such pattern was produced for KHV, LCDV, EPC cells, or the two bacterial strains *A. hydrophilia* and *C. freundii*, isolated from Chinese giant salamanders (Fig. 6). In addition, DNA from healthy Chinese giant salamanders used as a negative control template did not produce bands (Fig. 6). These results suggest that LAMP can detect GSIV of relatively high specificity.

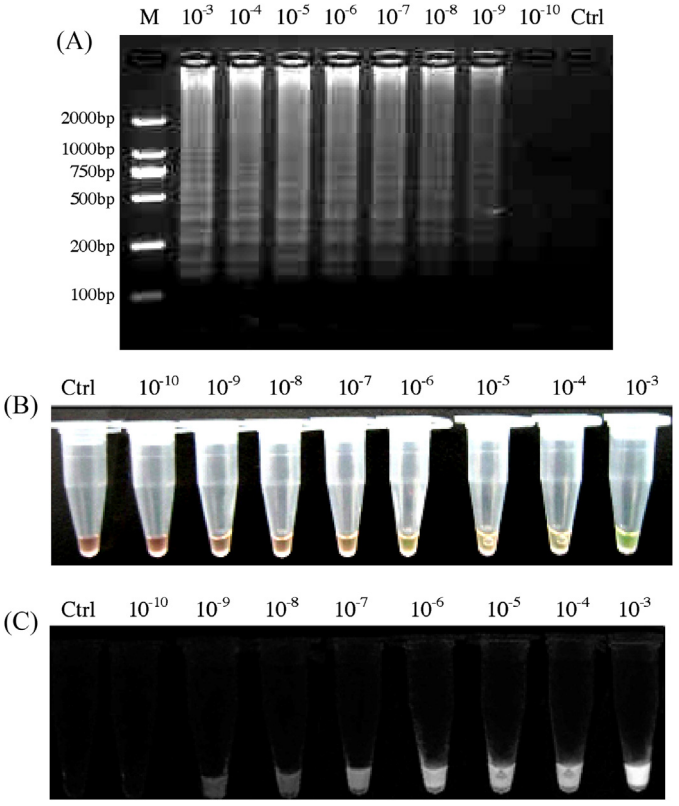

Fig. 4. GSIV detection using LAMP assay. GSIV DNA with 10-fold serial dilution (10 μg/mL) was used as templates. Visualization of amplification products were conducted under different conditions. By agarose gel electrophoresis (A); after SYBR Green I staining (B); under UV light (C). M: DNA marker; Ctrl: negative control; lane 2–9: different dilutions of viral genome DNA.

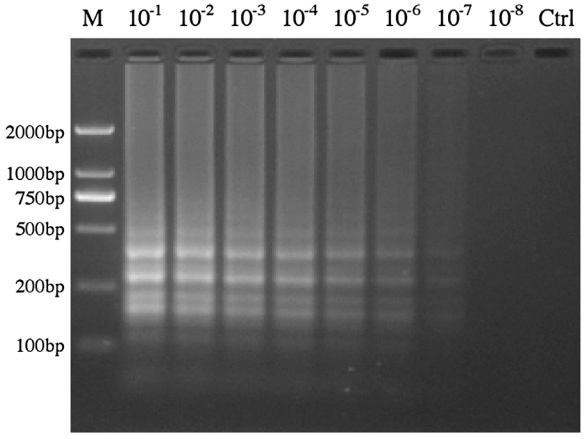

Fig. 5. Evaluation of the sensitivity of LAMP assay for GSIV detection in diseased Chinese giant salamanders. Total DNA isolated from mixture of spleens, livers and kidneys of diseased Chinese giant salamanders was diluted serially of 10-fold and used as template. Visualization of amplification products was conducted via 2% agarose gel electrophoresis. M: DNA marker; Ctrl: negative control; lanes 2–8: serial dilutions of total DNA.

3.5. LAMP assay used for clinical diagnosis of GSIV infection

LAMP and nested PCR were compared for the detection of GSIV in specimens from 48 Chinese giant salamanders in which 34 had typical syndrome of GSIV infection. Both LAMP and nested PCR were positive of GSIV in 34 samples from animals of typical syndrome of GSIV infection LAMP and nested PCR were positive of GSIV in 11 and 9 samples from animals of atypical syndrome, respectively

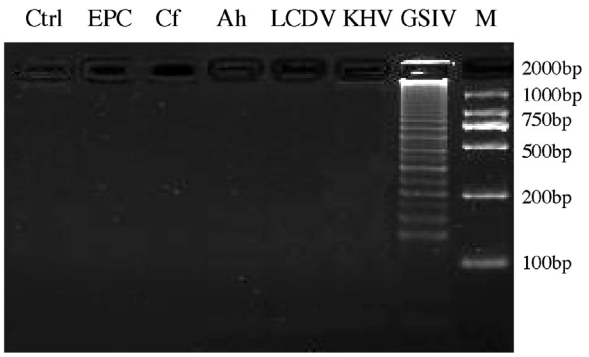

Fig. 6. Evaluation of the specificity of LAMP assay for GSIV detection. M: DNA marker; GSIV: Chinese giant salamander irodivirous; KHV: Koi herpesvirus; LCDV: lymphocystis disease virus; Ah: *Aeromonas hydrophlla*; Cf: *Citrobacter freundii*; EPC: EPC cell; Ctrl: nagetive control.

(Table 2). GSIV infection of the 11 samples that were positive by LAMP was confirmed by EPC cell infection. These results suggest that LAMP is more sensitive than nested PCR for GSIV detection.

4. Discussion

Chinese giant salamander iridovirus is highly infectious and results in high mortality in Chinese giant salamanders. Symptoms include skin and limb ulcers. The disease has been common in farmed Chinese giant salamanders in the past three years, and a ranavirus, of the family *Iridoviridae*, was identified as the infectious agent (Geng et al., 2011; Jiang et al., 2011). Several publications have suggested that ranaviruses are partially responsible for global amphibian declines (Greer et al., 2005; Jancovich et al., 2005; Pearman et al., 2004). *Ranavirus* infections have been added to the list of notifiable diseases by the World Organization for Animal Health (OIE), which reflects a global concern for the health of farmed amphibians and declines in wild populations (OIE, 2008). Conventional PCR has been used to confirm iridovirus infections and displays accurate results (Allan et al., 1995; Ellen et al., 2009; Matthew et al., 2011). However, nested PCR amplification is time-consuming and requires expensive equipment, costly consumables, and relatively large sample volumes (Albert et al., 2011). Since the advent of LAMP, which is a powerful and innovative gene amplification technique (Notomi et al., 2000), it has emerged as a simple rapid diagnostic tool for the early detection and identification of microbial diseases (Liu et al., 2011; Manmohan et al., 2008). Compared with traditional PCR amplification, LAMP amplifies DNA with higher specificity, efficiency, simplicity, and rapidity under isothermal conditions (Mori and Notomi, 2009; Notomi et al., 2000). Several LAMP-mediated diagnostic methods have been developed for viral pathogens in aquaculture (Arunruta et al., 2011; Caipang et al., 2004; Sung and Lu, 2009; Mekata et al., 2006; Nimitphak et al., 2008; Sun et al., 2006, 2010; Yeh et al., 2005).

In this study, a LAMP assay for the detection of GSIV was successfully developed. Optimization of the LAMP reaction conditions are critical for the success of the assay, and the melting temperature and Mg^{2+} concentration during hybridization of the four primers to the target DNA during the initial step were therefore critical factors. A higher or lower temperature would reduce the activity of the *Bst* DNA polymerase (Notomi et al., 2000). Therefore, the melting temperature of the synthesized primers was set to 60–65 °C for GSIV detection. The device incorporated a heating block that maintained the temperature at the optimal 60–65 °C for the duration of the reaction, which were performed in 0.2 mL tubes. Although multiple bands were obtained at 64 °C and 65 °C, the bands were clearer at 65 °C for a given Mg^{2+} concentration. This demonstrated that the reaction conditions were interdependent, and must be considered

Table 2
The GSIV detection results in Chinese giant salamanders based on LAMP and nested PCR.

Samples	Collected regions	Sample numbers	Detection results	
			Nested PCR (positive/negative)	LAMP (positive/negative)
Typical symptoms of GSIV infection	Hubei, Hunan, Zhejiang, Shanxi, Fujian	34	34/0	34/0
Suspicious samples	Hubei, Hunan, Zhejiang, Shanxi, Fujian	14	9/5	11/3

as a system. Mg^{2+} concentration influenced the destabilizing of the DNA and was thus another important factor for LAMP amplification. The optimal Mg^{2+} concentration was determined to be 8 μM, as is typical for the majority of LAMP amplifications.

LAMP has attracted considerable attention as a potentially rapid, accurate, and cost-effective nucleic acid amplification method (Albert et al., 2011). In this study, LAMP was compared with nested PCR for the detection of GSIV. Nested PCR could detect template DNA (10 μg/mL) at a 10^{-8} dilution, which was similar to the reported sensitivity in other studies (Matthew et al., 2011). The sensitivity of the LAMP assay, however, was at a 10^{-9} dilution using the 10 μg/mL template DNA, which represented a 10-fold increase in sensitivity relative to nested PCR. In addition, the LAMP assay was accomplished in just 1 hour with an isothermal cycle. In terms of sensitivity and rapidity, nested PCR thus demonstrated a disadvantage even over LAMP.

An alternative method was tested to simplify the extraction of viral DNA; the suspension was incubated in boiling water for 10 min then used as the template for LAMP and nested PCR amplification. However, no amplification occurred unless the boiled suspension was centrifuged at 5000 rpm for 5 min. The same result was also obtained in an earlier study of detecting the red seabream iridovirus (RSIV) by LAMP method (Caipang et al., 2004). It is possible that this effect could be due to the presence of inhibitors or impurities in the template. Previous studies have indicated that ranavirus could be released into the blood (Xie et al., 2002). The Chinese giant salamander is a protected and rare species, and it was therefore preferable to detect the virus without killing the animal. However, the results of viral detection using blood samples from diseased Chinese giant salamanders in this study were negative. The mechanism of GSIV infection in Chinese giant salamanders, the viral dose in blood and the similarity with ranavirus infection in frogs remains unknown. Nonetheless, GSIV cannot currently be detected in the blood.

LAMP produced ladder-like patterns of bands by gel agarose electrophoresis. However, LAMP yields not only multiple DNA bands, but also the byproduct, pyrophosphate ions. This results in a white precipitate of magnesium pyrophosphate in the reaction mixture, usually visible with the naked eye. However, this was not observed in any samples in the current study. Under these conditions, a fluorescent intercalating dye SYBR Green I or EB can be used (Manmohan et al., 2008). SYBR Green I belongs to a fluorescent dsDNA intercalating dye, which will turn from orange to green under natural light following positive amplification. This color change is permanent. In the absence of amplification production, the dye remains orange. In the GSIV LAMP assay, no white precipitate was visible when the template DNA was diluted more than 10^{-5}. However, it produced a green color after the addition of 2 μL SYBR Green I to the tubes, and the results could also be seen clearly under UV light. The presence of fluorescence can be used to detect the target gene. This represents a further advantage of LAMP as a suitable screening assay in the field.

The early detection of viral pathogens is essential for controlling the spread of emerging diseases. A rapid detection method for GSIV in Chinese giant salamanders was thus urgently required. The results of the current study demonstrate that LAMP is superior to nested PCR for the detection of GSIV in terms of rapidity, sensitivity, and ease of visual observation. In conclusion, LAMP detection represents a suitable assay for rapid screening of GSIV in Chinese giant salamanders.

Acknowledgements

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