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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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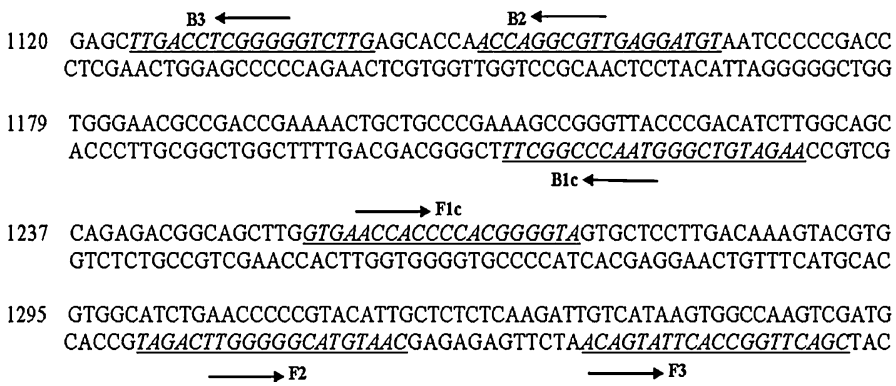

Fig. 1. The nucleotide sequence of MCP gene of GSIV and the primers (underlined) used for LAMP.

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