Arch Virol (2013) 158:2121–2126
DOI 10.1007/s00705-013-1724-5

ORIGINAL ARTICLE

A loop-mediated isothermal amplification method for the detection of members of the genus *Ranavirus*

Zhang Min · Jing Hongli · Zhang Lifeng · Wang Na · Wu Shaoqiang · Lin Xiangmei

Received: 11 November 2012 / Accepted: 8 April 2013 / Published online: 12 May 2013

Abstract A loop-mediated isothermal amplification (LAMP) method was developed for detection of members of the genus *Ranavirus*. The optimum reaction mixture contained 2.5 μL of each inner primer, RV-FIP (20 pmol/μL) and RV-BIP (20 pmol/μL), 0.5 μL of each outer primer, RV-F3 (10 pmol/μL) and RV-B3 (10 pmol/μL), 1.25 μL of each loop primer, RV-LF (20 pmol/μL) and RV-LB (20 pmol/μL), 3.5 μL dNTP mix (10 mM each), 8 μL $MgSO_4$ (25 mM), 1 μL of Bst DNA polymerase (8 U/mL, large fragment; New England Biolabs Inc., Beverly, MA, USA), 2.5 μL 10 × supplied buffer, and 1 μL of template DNA in a final volume of 25 μL. The optimum reaction conditions were 63 °C for 60 min. This LAMP method could detect Andrias davidianus iridovirus (ADIV), soft-shelled turtle iridovirus (STIV), and epizootic hematopoietic necrosis virus (EHNV), all of which belong to the genus *Ranavirus*, but it could not detect other viruses such as koi herpes virus (KHV), channel catfish virus (CCV), infectious spleen and kidney necrosis virus (ISKNV) and white spot syndrome virus (WSSV). The detection limit of the LAMP method was 100 copies of STIV DNA segment, and the sensitivity was 10 times higher than that of the polymerase chain reaction (PCR) assay. The results could be estimated visually by eye when calcein was added.

Z. Min (✉) · J. Hongli · W. Na · W. Shaoqiang · L. Xiangmei (✉)
The Institute of Animal Quarantine, Chinese Academy of Inspection and Quarantine, No.241 Building, Huixinxijie, Chaoyang District, Beijing 100029, China
e-mail: zhmbiohunter@hotmail.com

L. Xiangmei
e-mail: xiangmei8lin@yahoo.com.cn

Z. Lifeng
Beijing Exit-Entry Inspection and Quarantine Bureau, No.6 Building, Tianshuiyuanjie, Chaoyang District, Beijing 100026, China

Introduction

The members of genus *Ranavirus* belong to the family *Iridoviridae* and have icosahedral particles with a diameter of 150-170 nm, containing 150-170 kb of a double-stranded DNA genome [4]. Because of the severe losses of wild and farmed amphibian populations, ranaviruses are receiving increasing attention [5]. In 2009, infection with ranaviruses was added to the list of 'notifiable' diseases by the World Organization for Animal Health (OIE; http://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2012/).

The hosts of ranaviruses include fish, amphibians and reptiles. EHNV was isolated in Australian in 1986 [7], and the susceptible hosts of this virus mainly include redfin perch (*Perca fluviatilis*) and rainbow trout (*Oncorhynchus mykiss*) [8, 9]. STIV was isolated from soft-shelled turtle (*Trionyx sinensis*) with 'red neck disease' in China in 1997 [3]. Multiple alignment and phylogenetic trees of various STIV major capsid protein (MCP) genes have shown that STIV belongs to the genus *Ranavirus* [15]. The complete sequence of the STIV genome has been determined (GenBank accession no. EU627010). ADIV is a newly discovered ranavirus that was isolated from the spleens, kidneys and livers of sick cultured giant salamanders (*Andrias davidianus*) from a farm in Shanxi Province, China, in 2010 [11]. Electron microscopy showed numerous hexagonal viral particles with typical iridovirus morphology.

According to Manual of Diagnostic Tests for Aquatic Animals (2011), the detection methods for ranaviruses included histopathology examination, isolation of viruses, ELISA detection and PCR. In this study, a loop-mediated

isothermal amplification (LAMP) assay was developed for the detection of ranaviruses. The LAMP assay is a novel method of gene amplification that rapidly amplifies nucleic acids with high specificity and sensitivity under isothermal conditions [13].

In this LAMP assay for ranaviruses, four primers and two loop primers were designed according to the published sequence of STIV and applied also for the detection of EHNV, STIV and ADIV. The LAMP assay was shown to be specific and sensitive. It would provide a convenient and efficient method for the clinical diagnosis of ranavirus infection.

Materials and methods

Virus strains and cell lines

EHNV, STIV, ADIV and the bluegill fry cell line (BF-2) were stored at the Institute of Animal Quarantine.

Genomic DNA of other viruses

The genomic DNA of KHV, CCV, ISKNV, and WSSV were also stored at the Institute of Animal Quarantine.

Cell culture and DNA extraction

BF-2 cells were maintained at 25 °C in medium 199 supplemented with 10 % fetal calf serum (FCS) to propagate EHNV, STIV and ADIV. Infected cell cultures were collected when CPE was complete. Total DNA was extracted from viral suspensions using a Tianamp Genomic DNA Kit (Tiangen Biotech CO., LTD, Beijing, China).

Construction of recombinant plasmids

Target sequences were amplified by PCR using primers (forward, 5′-GCATGTCTTCTGTAACTGG-3′; reverse, 5′-CGTTACAAGATTGGGAATCC-3′) that were designed according to the published sequence of STIV MCP gene (GenBank accession no. EU627010.1). After the 1392-bp PCR product was ligated to pGEM-T Easy Vector (Promega, USA), the ligation product was introduced into DH5α competent cells by transformation (Tiangen Biotech) followed by blue-white colony selection. The plasmid DNA was purified using a TIANprep Mini Plasmid Kit (Tiangen Biotech), followed by PCR detection and spectrophotometric analysis at 260/280 nm. The recombinant plasmid was serially diluted tenfold to 10^7-10^1 copies/μL.

Design of primers for LAMP

LAMP primers for the detection of ranaviruses were designed according to the published sequence of the STIV MCP gene using Primer Explorer version 3 (http://primerexplorer.jp/lamp3.0.0/index.html). The details of the primers are given in Table 1 and Fig. 1.

Table 1 LAMP primers used to detect the STIV MCP gene

Primer name	Genomic position	Sequence 5′-3′
RV-F3	751-768	ATGACCGTCGCCCTCATC
RV-B3	949-968	CAGGTGTAATTGGAGCCGAC
RV-FIP (F2 + F1c)	835-854/775-793	TTGACCATGTGGACTGGGGC-TTTT-GACGAGAGACAGGCCATGA
RV-BIP (B2 + B1c)	862-882/921-940	AACGCAACCACCTTCCACACC-TTTT-GTGTGACGTTCTGCACCATA
RV-LF	794-811	TGTCCCTGACTGTGCTGC
RV-LB	883-901	GACATGCGGTCCTCACACG

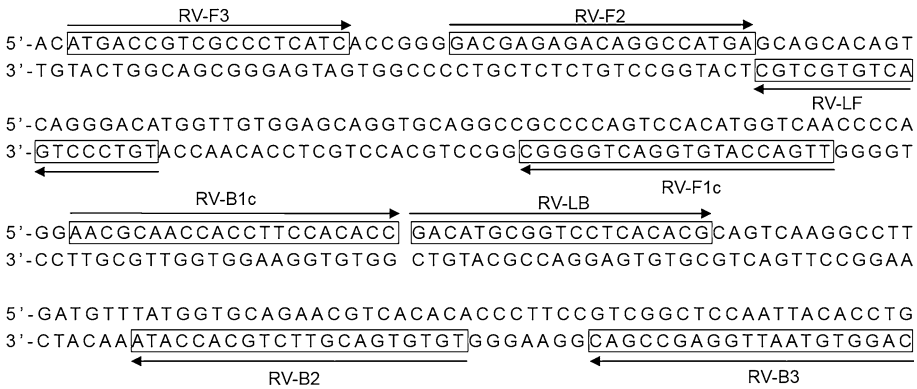

Fig. 1 Nucleotide sequence of a portion of the STIV MCP gene used to design primers for LAMP. The nucleotide sequences and the positions of the primer sequences are indicated by *boxes* and *arrows*

Optimization of the LAMP mixture and conditions

To determine the optimum Mg^{2+} concentration for the reaction, the LAMP reaction was carried out in a series of mixtures containing 8, 7, 6, 5, 4, 3, and 2 mM $MgSO_4$, at 63 °C for 90 min; in a series of reaction mixtures containing 1.4, 1.2, 1.0, 0.8, 0.6, 0.4, 0.2 mM dNTP to determine the optimum dNTP concentration; and 0.9, 0.6, 0.3, 0 M betaine to optimize its concentration.

The optimum reaction mixture contained 2.5 μL of each inner primer, RV-FIP (20 pmol/μL) and RV-BIP (20 pmol/μL), 0.5 μL of each outer primer, RV-F3 (10 pmol/μL) and RV-B3 (10 pmol/μL), 1.25 μL of each loop primer, RV-LF (20 pmol/μL) and RV-LB (20 pmol/μL), 3.5μL dNTP mix (10 mM each), 8 μL $MgSO_4$ (25 mM), 1 μL of Bst DNA polymerase (8 U/mL, large fragment; New England Biolabs Inc., Beverly, MA, USA), 2.5 μL 10 × supplied buffer, and 1 μL of template DNA in a final volume of 25 μL.

The LAMP reactions were carried out at 59, 60, 61, 62, 63, 64, and 65 °C for 1.5 h to determine the optimal temperature, and for 30, 45, 60 and 90 min at 63 °C to determine the optimum reaction time. The reaction mixture without template was included as a negative control.

Detection limits of LAMP

Tenfold serial dilutions (10^1 to 10^7 copies/μL) and twofold serial dilutions (100 to 12.5 copies /μL) of recombinant plasmid containing the STIV MCP gene were used as the template for the LAMP assay. The products were analyzed by 1 % agarose gel electrophoresis and using a Loopamp real-time turbidimeter (Eiken Chemical Co., LTD, Japan).

PCR detection of ranaviruses

Tenfold serial dilutions (10^1 to 10^7 copies/μL) of recombinant plasmid containing the STIV MCP gene was then amplified by PCR using primers (forward, 5′-AACCCGG CTTTCGGGCAGCA-3′; reverse, 5′-CGGGGCGGGGTT GATGAG-3′) according to the OIE manual of diagnostic tests for aquatic animals (2011). The 321-bp segment was detected by 1 % agarose gel electrophoresis following ethidium bromide staining and viewing on an ultraviolet (UV) transilluminator.

Cross-reaction of LAMP detection

The cross-reaction of LAMP was examined using STIV, EHNV, KHV, CCV, ISKNV and WSSV genomic DNA as the template.

Fluorescence LAMP for detection of ranaviruses

Using optimized conditions, fluorescence LAMP was carried out in a reaction mixture containing 1 μL calcein (Eiken Chemical CO., LTD, Japan). Tenfold serial dilutions (10^1 to 10^7 copies) of recombinant plasmid were used as the template. The reaction was heated using a drying oven (Yiheng, China), and the products were analyzed visually.

Results

Optimization of the reaction mixture for ranavirus detection

The LAMP reaction was carried out using recombinant plasmid as a template in order to determine the optimal concentration of Mg^{2+}, dNTP, and betaine. LAMP products were detected earliest when using 8 mM Mg^{2+} (Fig. 2A) and 1.4 mM dNTP (Fig. 2B). At all concentrations of betaine, the LAMP products were detected at almost the same time (Fig. 2C), so this LAMP could be carried out without betaine. According to these data, the reaction mixture was optimized as described in section 2.6.

Optimization of reaction temperature and time for ranavirus detection

At 63-65 °C, LAMP products could be detected in 25 min, earlier than at 59-61 °C (Fig. 2D). For the optimization of the reaction time, the reaction was carried out at 63 °C for 30, 45, 60, and 90 min, and all LAMP products were detected (Fig. 2E). Therefore, a reaction time of 60 min was selected as the optimal reaction time.

Detection limit of LAMP

To compare their detection limits, LAMP and PCR were carried out using various concentrations of recombinant plasmid containing the STIV MCP gene as template. LAMP was able to detect template at 10^2 copies (Fig. 3A), but the template was almost undetectable at 50 copies (Fig. 3B). The PCR assay, using the conditions specified in the OIE manual, was able to detect template at 10^3 (Fig. 3C) copies. Thus, the detection limit of the LAMP method was 100 copies, 10 times lower than that of the PCR.

Cross-reaction of LAMP detection

ADIV, STIV and EHNV gave positive results in the LAMP assay. No positive results were observed by this method

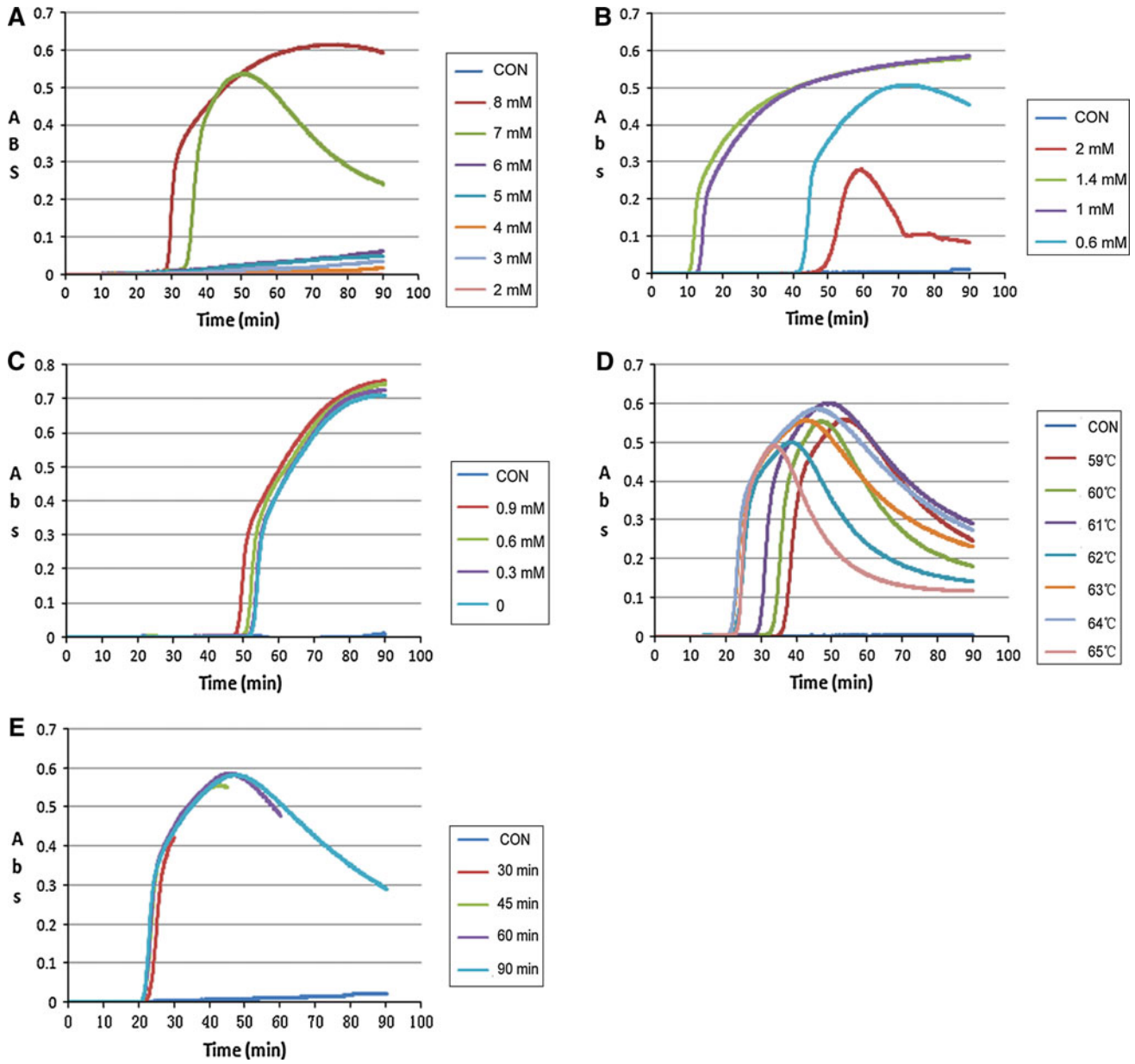

Fig. 2 Determination of optimal LAMP conditions at various concentrations of Mg^{2+} (**A**), dNTP (**B**), betaine (**C**), temperature (**D**) and time (**E**). CON, negative control

when using nucleic acids from KHV, CCV, ISKNV and WSSV (Fig. 3D). This suggests that the LAMP method is highly specific for members of the genus *Ranavirus*.

Fluorescence LAMP for ranavirus detection

Fluorescence LAMP was carried out at 63 °C for 1 h. Yellow-green fluorescence was observed in positive samples, and negative samples were brown in color and did not show any fluorescence. According to the result shown in Fig. 4, the detection limit of fluorescence LAMP for ADIV was 10^3 copies of target DNA.

Discussion

The LAMP assay is being increasingly applied for the detection of viruses in aquatic animals, such as Taura syndrome virus (TSV) [12], infectious hematopoietic necrosis virus (IHNV) [6], red seabream iridovirus (RSIV) [2], and WSSV [10]. The LAMP mixture typically utilizes a pair of outer primers (F3, B3) and a pair of inner primers (FIP, BIP). The ranavirus LAMP mixture additionally contains a pair of loop primers (LF, LB), which can reduce the reaction time by half [14]. Furthermore, the Mg^{2+}, dNTP, and betaine concentrations were also optimized.

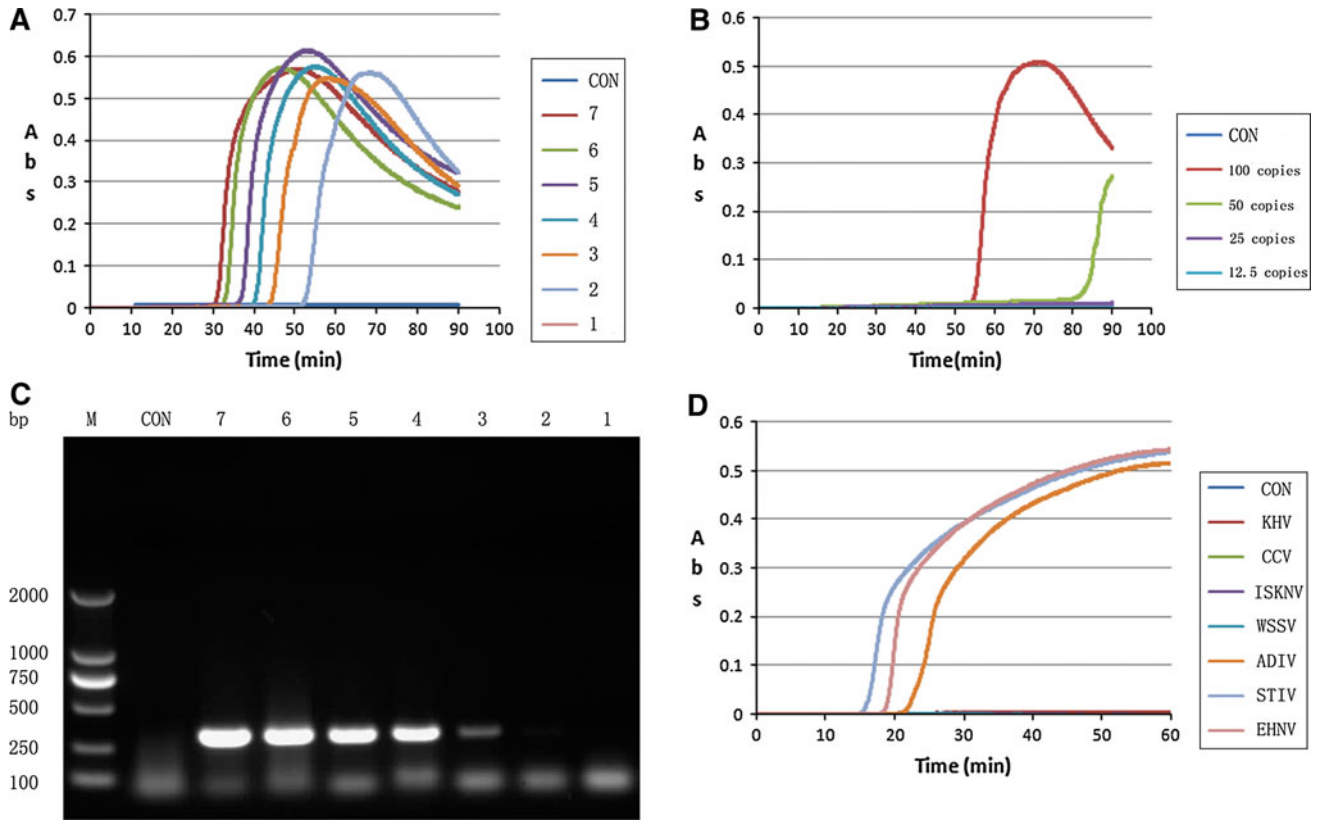

Fig. 3 Comparison of the detection limits of the LAMP and PCR assays and cross-reaction of the LAMP assay for the detection of ranaviruses. **A.** Detection limits of LAMP in the range of 10^1 to 10^7 copies. **B.** Detection limits of LAMP in the range of 10 to 100 copies. **C.** Detection limits of PCR in the range of 10^1 to 10^7 copies. **D.** Cross-reaction of the LAMP assay. M, molecular marker; CON, negative control; *lanes* 1-7, 10^1 to 10^7 copies of recombinant plasmid

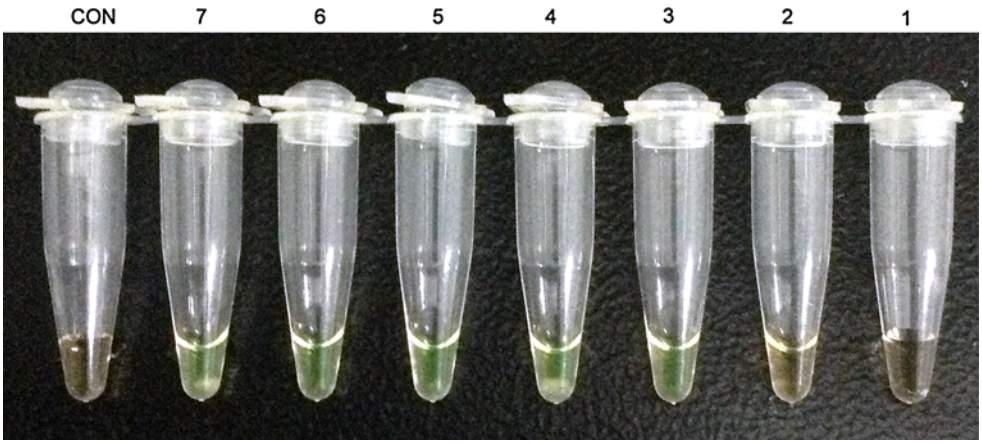

Fig. 4 Fluorescence LAMP for the detection of ranaviruses. CON, negative control; *lanes* 1-7, 10^1 to 10^7 copies of recombinant plasmid

The optimum concentration of Mg^{2+} and dNTP were determined to be 8 and 1.4 mM, respectively. Interestingly, the LAMP assay was unaffected by the presence or absence of betaine, although betaine is thought to improve the stability of DNA polymerase and reduce the melting temperature required for primer annealing [1]. This may be explained by the fact that the supplied Bst DNA polymerase buffer contains 0.1 % Triton X-100, which also enhances polymerase stability.

The MCP gene sequence of seven ranaviruses including EHNV (GenBank accession no. AY187045. 1), STIV (GenBank accession no. EU627010.1), Bohle iridovirus (BIV, GenBank accession no. FJ358613.1), frog virus 3 (FV3, GenBank accession no. FJ459783.1), European

catfish virus (ECV, GenBank accession no. FJ358608.1), tiger frog virus (TFV, GenBank accession no. AF389451.1), and Ambystoma tigrinum stebbensi virus (ATV, GenBank accession no. AY150217) were compared, and the results of sequence analysis was indicated that the ranaviruses share more than 96 % nucleotide sequence identity with each other. This suggested that primers designed based on the STIV MCP gene could detect these ranaviruses specifically. Because the sequences of ranavirus MCP genes are highly conserved, this LAMP assay can detect ADIV, STIV and EHNV, as shown in Fig. 3, but no reaction is observed with non-ranaviruses. Thus, this LAMP method is specific for members of the genus *Ranavirus*.

Compared with the standard PCR detection method, the LAMP assay for ranavirus detection has four advantages: (1) because it uses an isothermal reaction, the LAMP assay can be performed in a drying oven or water bath and does not require specialized equipment. (2) LAMP takes only 1 h for the reaction, compared to PCR, which takes approximately 2 h to complete; (3) the detection limit of the LAMP assay appears to be 10 times lower than that of PCR; and (4) detection of amplification products from the LAMP assay is carried out using a Loopamp real-time turbidimeter and does not require electrophoresis. Moreover, upon addition of calcein to the reaction mixture, products could be observed by eye. However, this causes the limit of detection to be reduced to 10^3 copies of target DNA, similar to that of the PCR assay. As a new method of virus detection, the LAMP assay has some shortcomings. If the reaction time is too long, false positive can be produced easily. Also, it is not a quantitative detection method, as real-time PCR is. Primarily, the LAMP method developed in this study is applicable for identification of ranaviruses after virus isolation.

Acknowledgments This study was supported by the Public Welfare Project of General Administration of Quality Supervision, Inspection and Quarantine of P. R. C (AQSIQ) (201010020), and the Fundamental Research Funds for the Public Research Institutes (2010JK003).

References

1. Boubourakas IN, Fukuta S, Kyriakopoulou PE (2009) Sensitive and rapid detection of peach latent mosaic viroid by the reverse transcription loop-mediated isothermal amplification. J Virol Method 160:63–68
2. Caipang CM, Haraguchi I, Ohira T, Hirono I, Aoki T (2004) Rapid detection of a fish iridovirus using loop-mediated isothermal amplification (LAMP). J Virol Method 121:155–161
3. Chen ZX, Zheng JC, Jiang YL (1999) A new iridovirus isolated from soft-shelled turtle. Virus Res 63:147–151
4. Chinchar VG, Essbauer S, He JG, Hyatt A, Miyazaki T, Seligy V, Williams T (2005) International committee on taxonomy of viruses, ICTV, vol 8. Academic Press. San Diego, pp 145–162
5. Cullen BR, Owens L (2002) Experimental challenge and clinical cases of Bohle iridovirus (BIV) in native Australian anurans. Dis Aquat Organ 49:83–92
6. Gunimaladevi I, Kono T, Lapatra SE, Sakai M (2005) A loop mediated isothermal amplification (LAMP) method for detection of infectious hematopoietic necrosis virus (IHNV) in rainbow trout (Oncorhynchus mykiss). Arch Virol 150:899–909
7. Langdon JS, Humphrey JD, Williams LM, Hyatt AD, Westbury HA (1986) First virus isolation from Australian fish: an iridovirus-like pathogen from redfin perch, Perca fluviatilis L. J Fish Dis 9:263–268
8. Langdon JS, Humphrey JD (1987) Epizootic hematopoietic necrosis a new viral disease in Redfin perch Perca fluviatilis L. in Australia. J Fish Dis 10:289–297
9. Langdon JS, Humphrey JD, Williams LM (1988) Outbreaks of an EHNV-like iridovirus in cultured rainbow trout, Salmo gairdneri Richardson, in Australia. J Fish Dis 11:93–96
10. Jaroenram W, Kiatpathomchai W, Flegel TW (2009) Rapid and sensitive detection of white spot syndrome virus by loop-mediated isothermal amplification combined with a lateral flow dipstick. Mol Cell Probes 23:65–70
11. Jiang YL, Zhang M, Jing HL, Gao LY (2011) Isolation and characterization of an iridovirus from sick giant salamander (Andrias davidianus). Bing Du Xue Bao 27:274–282
12. Kiatpathomchai W, Jareonram W, Jitrapakdee S, Flegel TW (2007) Rapid and sensitive detection of Taura syndrome virus by reverse transcription loop-mediated isothermal amplification. J Virol Method 146:125–128
13. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T (2000) Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28:E63
14. Ushikubo H (2004) Principle of LAMP method: a simple and rapid gene amplification method. Uirusu 54:107–112
15. Zhao Z, Teng Y, Liu H, Lin X, Wang K, Jiang Y, Chen H (2007) Characterization of a late gene encoding for MCP in soft-shelled turtle iridovirus (STIV). Virus Res 129:135–144