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Development of a reverse transcription loop-mediated isothermal amplification assay for rapid detection of grass carp reovirus

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A B S T R A C T

Reverse transcription loop-mediated isothermal amplification (RT-LAMP) is a gene amplification method that can amplify the RNA template by isothermal incubation. This paper reports a rapid and sensitive RT-LAMP method which was developed for the detection of grass carp reovirus (GCRV). The present study concluded the optimal conditions for the LAMP reaction of which the Mg^{2+} concentrations in the reaction mixtures, the incubation temperature, and the reaction time are at 8 mM, 64 °C, and 30 min, respectively. The analytical sensitivity of the RT-LAMP method was revealed as low as 7 copies of viral templates and 100-fold more sensitive than the published RT-PCR method. A visual inspection of in-tube LAMP products stained with a DNA fluorescent dye demonstrated that the positive and negative reactions exhibit distinct and different colors in daylight, which means that gel electrophoresis is not necessary to judge the positive or negative results. As the application of the method is rapid, easy, and no complicated instrument required, the GCRV-RT-LAMP method established in this study has great potential for the detection of GCRV in both the laboratory and the farm.

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1. Introduction

Grass carp, *Ctenopharyngodon idella*, is one of the four major fish species that are crucial to freshwater aquaculture in China (Ke et al., 1990; Ding et al., 1991; Wang et al., 1994). Grass carp farming in China has been suffering disease problems due to infection of bacteria, virus and protozoa. One of the most prevalent viral pathogens is grass carp reovirus (GCRV), which causes hemorrhage disease and the mortality up to 80% in farmed grass carp (Wang et al., 2002). GCRV considered being the most pathogenic aquareovirus is a tentative member in the genus Aquareovirus of family Reoviridae (Rangel et al., 1999; Mertens et al., 2005). In addition to threating the grass carp farming, GCRV is also a fatal pathogen to many other aquatic animals such as black carp (*Mylopharyngodon piceus*), topmouth gudeon (*Pseudorasbora parva*) and rare gudgeon (*Gobiocypris rarus*) in the world.

In an attempt to control the spread of GCRV, many diagnostic methods including the immunological methods based on the antigen-antibody reactions (Ye et al., 1989; Yang et al., 1991; Dopazo et al., 1992; Shao et al., 1996), the molecular methods based on nucleic acid hybridization (Subramanian et al., 1993, 1997; Rangel et al., 1999) and reverse transcription-polymerase chain reaction (RT-PCR) methods (Li et al., 1997; Seng et al., 2004; Zhang et al., 2010) have been developed. However, all of the methods based on conventional immunological reaction, nucleic acid hybridization, and RT-PCR have to be confronted with some inherent disadvantages, such as insufficient specificity, time consuming or the need for a rapid thermal cycler (Mori et al., 2001; Savan et al., 2005). To avoid these limitations in viral diagnosis, a loop-mediated isothermal amplification (LAMP) reaction was developed for the detection of GCRV.

LAMP is an isothermal replication method for rapid and, specific amplification of nucleic acids (Notomi et al., 2000; Mori et al., 2001; Nagamine et al., 2002). This method can achieve very high specificity in viral detection in a short time without expensive equipment. LAMP detection methods for many aquaculture animal viruses such as nervous necrosis virus (NNV), spring viraemia of carp virus (SVCV), red seabream iridovirus (RSIV), Singapore grouper iridovirus (RGIV) and turbot reddish body iridovirus (TRBIV) have been established (Xu et al., 2010; Shivappa et al., 2008; Caipang et al., 2004; Mao et al., 2008; Zhang et al., 2009). However, this method has not been applied to the specific detection of GCRV. This paper describes a highly sensitive and rapid diagnostic protocol for GCRV detection in grass carp. In addition, LAMP and conventional RT-PCR method for GCRV detection are compared.

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Table 1
Primers used for reverse transcriptase loop-mediated isothermal amplification.

Primer	Sequence
F3	TCTCCACTGGTTCTCTTCCA
B3	GAACGTTTTCGGCGACCG
FIP (F1c + TTTT + F2)	TGAGATGGGCACCAAGCAAAGTTTTGCCCGATACCCCAGTGTT
BIP (B1c + TTTT + B2)	AGGTATCACTTGGCGAGACCCATTTTCTGGGGGTGGTTGAATGG
LF	ACAACGATGTTGCGTGATGC
LB	TTCACTAGCTCCAGTCCCTG

2. Materials and methods

2.1. Preparation of virus and viral template RNA

The virus was purified from the livers and spleens of the diseased grass carp with hemorrhagic symptom from Nanchang County in Jiangxi Province. Total RNA was extracted from the tissue of above samples using a commercial RNA Rapid Extraction Kit (Bio Teke, Beijing, China) according to the manufacturer's instructions. cDNA was synthesis by the PrimeScript II 1st Strand cDNA Synthesis Kit (TAKARA, Dalian, China).

2.2. Design of RT-LAMP primers

Three pairs of pathogen-specific RT-LAMP primers including a pairs of loop primers were designed to amplify the GCRV genome sequence as described by Nagamine et al. (2002). Firstly, the VP6 gene of core protein from the segment 8 of GCRV genome (GenBank accession number AF403414) was submitted to the NCBI website for alignment with other genes of closely related viruses using the BLASTn program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Then, a set of RT-LAMP primers was designed using Primer Explorer software, version 4.0 (http://primerexplorer.jp/elamp4.0.0/index.html) (Table 1 and Fig. 1).

2.3. Optimization of RT-LAMP reactions

The RT-LAMP reaction was conducted as described by Zhang et al. (2009), with minor modification. The reaction was carried out in a 25 μL reaction mixture containing 1.6 μM each of FIP and BIP; 0.2 μM each of F3 and B3; 1× ThermoPol reaction buffer (20 mM Tris–HCl, 10 mM KCl, 6 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100); 1 M betaine; 1.4 mM each deoxynucleoside triphosphate (dNTP); 2 U M-MLV reverse transcriptase; 8 U *Bst* DNA polymerase; and 1 μL of target RNA. The mixture was incubated at 65 °C for 60 min and then the reaction was terminated by heating at 80 °C for 5 min. A 3 μL portion of RT-LAMP products was taken for electrophoresis on a 2% agarose gel. To optimize the RT-LAMP conditions, the reactions with different Mg^{2+} concentrations, reaction temperatures, and reaction times were performed.

Fig. 1. Target nucleotide sequence in the VP6 gene of GCRV (GenBank accession number AF403414) used for design of the RT-LAMP primers set. Nucleotide sequences used for primer design are indicated by boxes and arrows, and digest position of *Msp* I is shown as a gray background.

2.4. Analytical specificity of the GCRV-RT-LAMP assay

To verify the specificity of the GCRV-RT-LAMP assay, a 2 μL aliquot of LAMP products was digested in 20 μL reaction mixtures with 5 U restriction enzyme of *Msp* I for 3 h at 37 °C. Then, a 2 μL portion of the digested product was electrophoresed on a 2% agarose gel. The specificity of the GVRV-RT-LAMP assay was also tested by RT-LAMP reactions that used the nucleic acids from GCRV and three other fish viruses, including SVCV, IHNV, and TRBIV, as templates.

2.5. Analytical sensitivity of the GCRV-RT-LAMP assay

The detection limit of the assay was analyzed with two sources of tamplates. One of the templates was prepared with the total RNA extracted from the livers and spleens of the diseased grass carp. Another template was prepared with a plasmid pMD19-GCRV containing the target fragment from the GCRV genome segment 8. A 10-fold serial dilutions of the total RNA and the plasmid pMD19-GCRV (10^7–10^0 copies) was used as the template for RT-LAMP under the pre-determined conditions (refer to Section 2.3). After the reaction, the RT-LAMP products were electrophoresed on a 2% agarose gel and documented using a LAS3000 Gel Imaging System (Fuji, Japan).

2.6. Analytical sensitivity of GCRV by the RT-PCR

As a comparison of the detection limit of GCRV-LAMP, the templates above-mentioned (in Section 2.5) were tested by the RT-PCR method following the procedure reported by Zhang et al. (2010). The RT-PCR products were analyzed on a 1.5% GeneFinder™ (BIOV, Xiamen, China) stained agarose gel.

2.7. Evaluation of GCRV-RT-LAMP assay using grass carp samples

The validity of the GCRV-RT-LAMP assay was tested by applying the method to 24 grass carp samples, weighing 30–300 g, which were obtained from different farms in Zhejiang and Anhui Province. The RNAs of the samples were extracted as described in Section 2.1. RT-LAMP was performed with the optimized conditions (Section 2.3). Meanwhile, RT-PCR detections were also performed as described by Zhang et al. (2010). All amplification products were electrophoresed and analyzed on a 2% agarose gel. The calculation of diagnostic sensitivity (DSe) and diagnostic specificity (DSp) (OIE, 2011) between the two methods were based on the following formula: DSe = TP/(TP + FN) and DSp = TN/(TN + FP), while judged by the reference method, TP means the true positive cases, TN means the true negative cases, FP means false positive cases, and FN means false negative cases.

2.8. Detection of RT-LAMP products with fluorescent dyes

Visual inspection of the GCHV-RT-LAMP products in the reaction tubes was performed by adding 0.5 μL fluorescent dye GeneFinder™ (Bio-V, Xiamen, China) into the cap of the tubes prior to the incubation. The fluorescent dye was then mixed with the reaction mixtures after the incubation finished without reopening

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