

Full Length Research Paper

Rapid and sensitive detection of iridovirus by loop-mediated isothermal amplification (LAMP)

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Red seabream iridovirus (RSIV), a member of the Iridoviridae family, is the causative pathogen of some of the most explosive epidemics of emerging viral diseases in many Asian countries, leading to huge economic losses in aquaculture. Rapid molecular detection for surveillance or diagnosis has been a critical component in reducing the prevalence of RSIV infection. In the present study, a novel and highly specific loop-mediated isothermal amplification (LAMP) assay for the sensitive and rapid detection of RSIV infection in fishes was developed. Using a set of synthesized primers matching a specific region of the RSIV genome (GenBank accession no.: AB666336.1), the efficiency and specificity of the LAMP assay were optimized in terms of the reaction temperature and DNA polymerase concentration, as they are the main determinants of the sensitivity and specificity of the LAMP assay. In particular, we demonstrated that our assay could be applied to efficiently detect RSIV infection in red sea bream. Our results provide a simple and convenient method for the detection of viral infection in aquatic organisms.

Key words: Red sea bream, Iridovirus, polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP).

INTRODUCTION

Iridoviridae are largely divided into five genera: *Chloriridovirus*, *Iridovirus*, *Lymphocystivirus*, *Megalocytivirus* and *Ranavirus* (Regenmortel et al., 2000). Red seabream iridovirus (RSIV) has caused significant economic losses in aquaculture. Recently, this

virus has attracted attention because of their effects on the marine aquaculture industry in various regions (Caipang et al., 2004). Iridoviruses diseases affecting marine aquaculture have been reported in several countries like Korea, Japan, China, Taiwan, Thailand and

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and caused huge economic losses (Inoue et al., 1992; Chua et al., 1994; Kasornchandra and Khongpradit, 1997; Miyata et al., 1997; Jung and Oh, 2000). The RSIV was first isolated from the cultured red sea bream *Pagrus major* in 1992 (Imajoh et al., 2007; Inoue et al., 1992; Caipang et al., 2006), and has been found to infect more than 30 species of cultured fishes since then. In particular, RSIV caused serious disease in *P. major* in Asian countries (Kawakami and Nakajima, 2002). Fish infected with RSIV exhibit severe anemia, enlargement of splenic tissue or cells, necrosis in renal and splenic hematopoietic tissues, and gill petechiae (Inoue et al., 1992).

Several detection techniques have been established and applied to detect RSIV, including quantitative real-time PCR (Caipang et al., 2003), conventional PCR (Oshima et al., 1996; Oshima et al., 1998; Kurita et al., 1998), Giemsa staining (Inoue et al., 1992) and immunofluorescence assay using a monoclonal antibody (Nakajima and Sorimachi, 1995). Among these methods, real-time PCR is effective for detection and quantification of the virus. However, this method has limitations due to the expensive reagents and equipment required for the handling of bulk samples. Conventional PCR, although inexpensive, has several disadvantages, including the lengthy reaction time and the inability to quantify viral particles (Dhar et al., 2001). The Giemsa staining and immunofluorescence methods are biochemical methods of virus detection, but sometimes fail to detect viral antigens in fish during the early stages of infection (Kurita et al., 1998).

Loop-mediated isothermal amplification (LAMP) was developed to amplify nucleic acids with high sensitivity and specificity, and easy to perform under isothermal conditions (Notomi et al., 2000; Mori et al., 2001). This method, performed with *Bst* DNA polymerase and a set of specific primers containing a total of six distinct sequences from the target DNA, makes the amplification of the target sequence highly selective. The forward inner primer (FIP), which contains sequences of the sense and anti-sense strands of the target DNA, synthesizes the first strand and thereby initiates the LAMP process. The outer forward primer anneals to the target DNA and initiates strand displacement DNA synthesis, releasing the FIP-linked complementary strand, which has a stem-looped DNA structure at one end. This strand initiates a process in which a backward inner primer (BIP) hybridizes to the other end of the target DNA, resulting in strand displacement by a backward outer primer to form a light bulb shape. This process continues in subsequent LAMP cycles, resulting in multiple stem-looped DNAs (Notomi et al., 2000).

In this study, we developed a novel and highly specific LAMP assay for diagnosing RSIV infection. LAMP is a specific nucleic acid amplification method, which is easy to perform and can amplify nucleic acid at isothermal

conditions at 60–65°C in a 1 h incubation period, also, this is the first study of detection of RSIV in infected fish using synthesized RSIV sequences, not DNA extracted from infected fish, and provides new insight into methods for the detection of viral infections.

MATERIALS AND METHODS

Virus isolates and fish samples

RSIV isolates from liver samples of infected, red seabream from jeonnam prefecture, Korea, were used. Apparently healthy juvenile red seabream (5–10 g) were used for the challenge tests.

RSIV gene synthesis

The capsid protein (CP) gene of RSIV was utilized for RSIV detection using LAMP (GenBank accession no.: AB666336.1). CP is one of the unifying features of virus group. This is the predominant polypeptide produced upon viral infection and has been estimated to account for up to 45% of all virion protein. The complete CP genes from a number of both vertebrate and invertebrate iridoviruses have been sequenced and their coding regions were found to have much sequence similarity. This gene was synthesized by the Bioneer Corporation of South Korea. Finally, RSIV gene cloning to pGEMT-easy vector.

Construction of LAMP primers

The LAMP method requires a set of four specially designed primers (B3, F3, BIP and FIP) that recognize a total of six distinct sequences (B1, B2, B3, F1, F2 and F3) in the target DNA. Primers for RSIV-6 LAMP were designed against the RSIV gene sequences in GenBank (NCBI) by using Primer Explorer V4 (<http://primerexplorer.jp/e>) software. Primer details are listed in Table 1. The primer sequences and their respective binding sites are shown in Figure 1.

Optimization of LAMP conditions

The LAMP reaction mixture contained 1 µL of 10x *Bst* DNA polymerase reaction buffer (final concentration: 20 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, 0.1% Triton X-100), 10 mM dNTPs, 1.6 µM each of the inner FIP and BIP primers, 0.4 µM each of the outer F3 and B3 primers, and 1 µL of template DNA (various concentrations) in a final volume of 20 µL, with 4–16 U of *Bst* DNA polymerase (New England Biolabs, Ipswich, USA) and extra MgSO₄ (final concentration: 5–40 mM). The LAMP reaction was performed at 54, 56, 58, 60, 62, 64, 66, 67 and 68°C for various reaction times (15, 30, 45, 60, 70 and 90 min). LAMP amplification products were analyzed by electrophoresis on a 1% agarose gel followed by staining with ethidium bromide. Fluorescence was visualized by adding 1-µL diluted SYBR Green I (Invitrogen, New York, USA), and observing the sample under natural and ultraviolet (UV) light.

Amplification of wild-type sample

Infected and non-infected fish sample

To test our primers on wild-type RSIV, liver samples were collected

Table 1. Specific LAMP primers.

Name	Sequence (5' - 3')	Size
RSIV F3	CGACAATGCCGTGACCTAC	19
RSIV B3	GCGAATGTAGCTGTTCTCCT	19
RSIV FIP (F1c – F2)	GCCGAAATTAGCATGGCCAGTC - AGACCGTGCGTAGTTCCTG	41
RSIV BIP(B1 – B2c)	TTAGTGTGACTGTGGCAAGGGG - GACGTGATGGAGGGGATCT	41

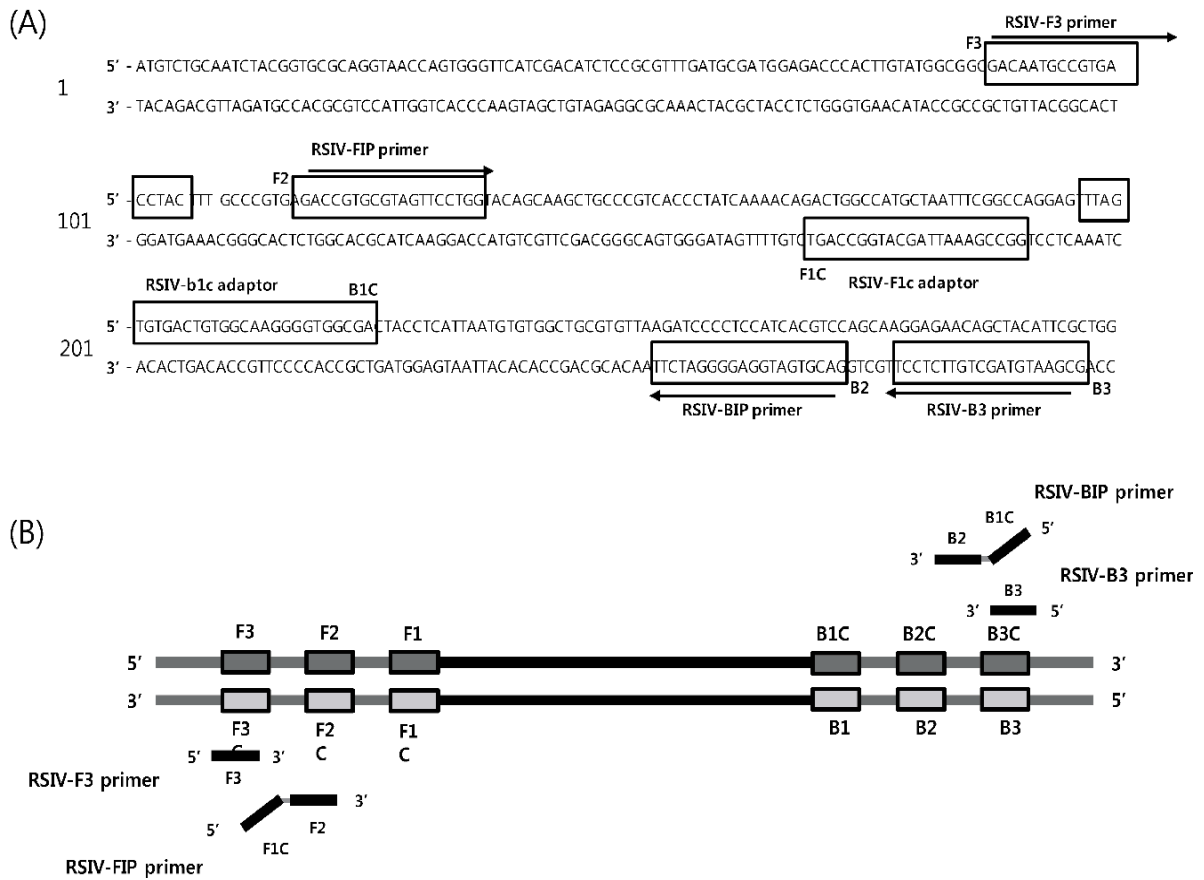


Figure 1. (A) Nucleotide sequence of RSIV DNA used for the inner and outer primers. DNA sequences used for primer design are shown by boxes and arrows. (B) Schematic diagram of two-outer (F3 and B3) and two-inner (FIP and BIP) primers for LAMP.

from infected and non-infected fish (*Pagrus major*) from the Tongyeong bay area, Korea. In preliminary investigations, the thick PCR product appeared from liver samples, although a thin band was amplified from some organs including stomach, intestine and kidney. Therefore, the liver of diseased red sea bream were chosen for DNA extraction in this study

Virus DNA extraction

This tissue was homogenized in a 4-fold volume of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and centrifuged at 2500 \times g for 10 min at 4°C. A 20 μ l aliquot of Proteinase K (1 mg/ml; TaKaRa)

was added to 200 μ l of the supernatant. The mixture was incubated at 55°C for 2 h. DNA was isolated using phenol and chloroform. Nucleic acids were precipitated with isopropanol, resuspended with distilled water and stored at -20°C until use.

PCR amplification

To test our primers on wild-type RSIV, liver samples were collected from infected and non-infected fish from the Tongyeong bay area, Korea. DNA amplification was conducted as follows: a set of primers (F: TACAACATGCTCCGCCAAGA and R: GCGTTAAAGTAGT GAGGGCA) designed based on RSIV gene

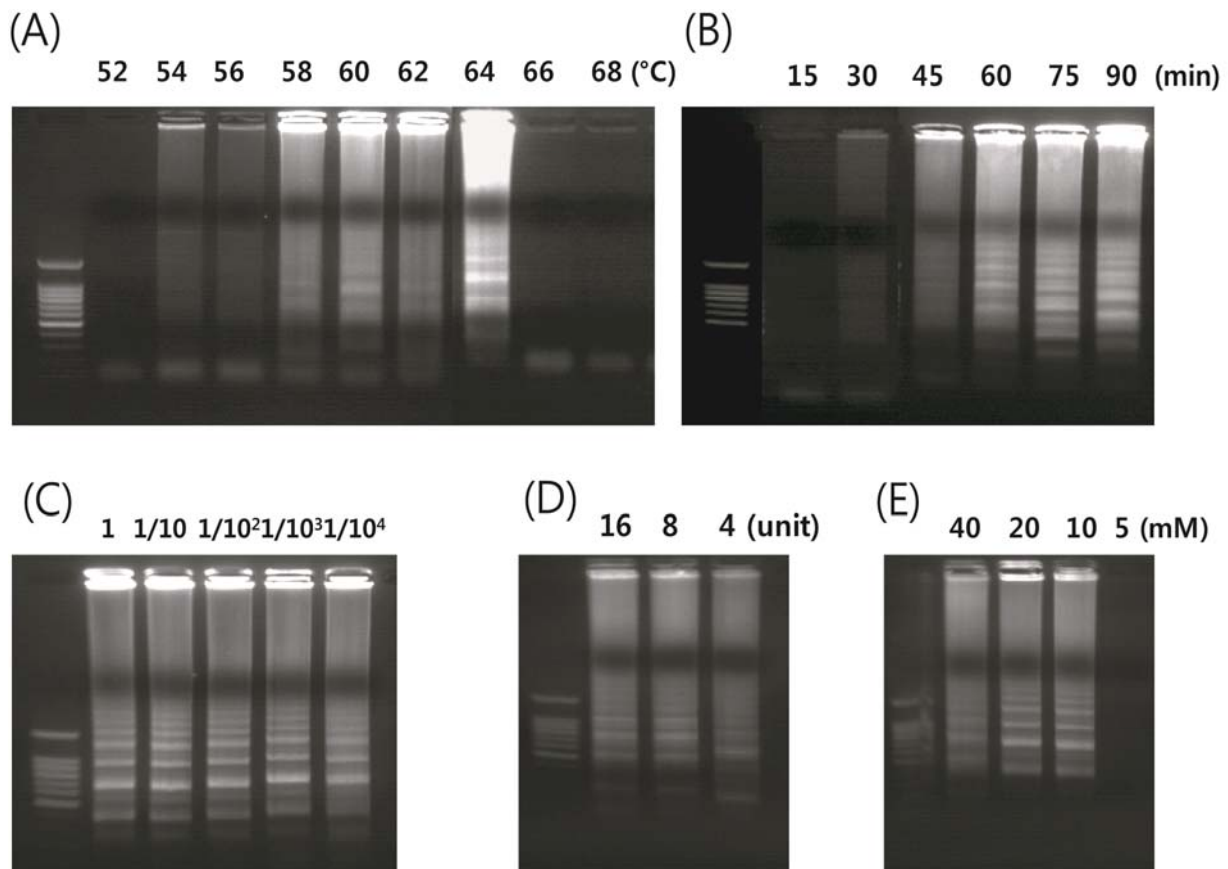


Figure 2. LAMP assay optimization test for detecting RSIV. (A) and (B), Effects of reaction time and temperature on the LAMP reaction; (C), Effect of template concentration on the LAMP reaction; (D), Effect of *Bst* DNA polymerase concentration on the LAMP reaction; (E), Effect of $MgSO_4$ concentration on the LAMP reaction

sequence obtained from NCBI was used to amplify a 1300 bp fragment of viral DNA. The PCR reactions contained 4 μ L dNTPs (2.5 mM), 2 μ L 10x buffer with $MgCl_2$ (25 mM), 1 μ L of each of forward and reverse primers (10 μ M), 2 μ L template DNA (0.02 μ g), 1 μ L Taq (5 U/ μ L), and distilled water to a final volume of 20 μ L. Amplification conditions were as follows: 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min, followed by a final elongation at 72°C for 5 min (Kurita et al., 1998). The amplified DNA fragments were analyzed by electrophoresis in a 1.5% EtBr-stained agarose gel in Tris–acetic acid–EDTA (TAE) buffer (pH 8.0).

RESULTS

Optimal LAMP conditions

To optimize the LAMP assay against RSIV, LAMP was performed under various conditions using different temperatures, times, and $MgSO_4$ concentrations, all of which are major factors that affect the efficiency of the LAMP assay (Caipang et al., 2004). The distinct ladder-like patterns of LAMP products were observed at reaction

temperatures ranging from 54 to 64°C (Figure 2A); the pattern was not visible at temperatures < 52 or > 66°C. Among the temperatures tested, we observed the most clear and distinct ladder pattern at 64°C. In addition, the LAMP reaction functioned efficiently with a 60-min reaction time, while DNA ladders were not visible or exhibited very weak signals for reaction times < 60 min (Figure 2B). One of the major determinants of a LAMP reaction, $MgSO_4$, was tested at concentrations of 5 to 40 mM. While amplification did not occur at 5 mM $MgSO_4$, the characteristic DNA ladder appeared at concentrations \geq 10 mM (Figure 2D). To investigate the quantity of DNA polymerase required for amplification, the LAMP reaction was performed using 4, 8, and 16 U *Bst* DNA polymerase. Interestingly, amplification occurred at all DNA polymerase concentrations, with no visible differences among them (Figure 2E). Overall, the optimized conditions for the LAMP reaction were as follows: master mixture containing 1.6 μ M each of FIP and BIP, 0.4 μ M each of F3 and B3, 10 mM dNTP mix, 40 mM $MgSO_4$ and 8 U of *Bst* DNA polymerase in a final

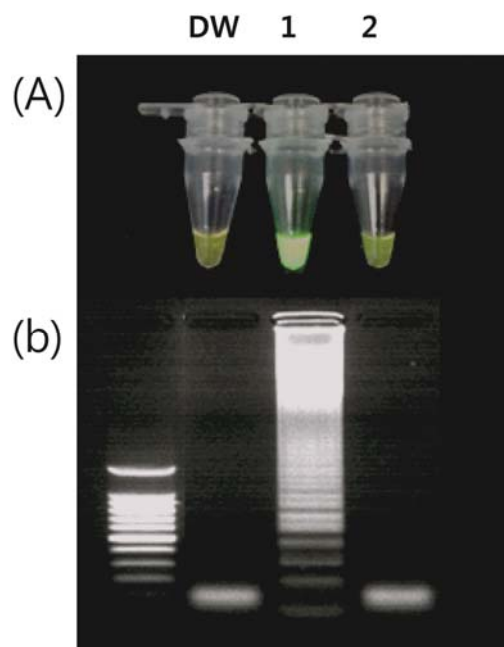


Figure 3. LAMP results of RSIV infected (1, infected fish; 2, not infected fish) sample using specific primer set. (A) Electrophoresis with EtBr. (B) Direct visual inspection with SYBR Green I.

volume of 20 μ L. The reaction was carried out at 64°C for 60 min and then terminated by increasing the temperature to 80°C for 5 min. The sensitivity of the LAMP assay for RSIV was analyzed using template DNA serially diluted from 1×10^4 to 1×10 copies/mL of RSIV-pGEM-T Easy clone. Interestingly, the distinct ladder-like patterns were visible at all template concentrations (Figure 2C), indicating that the LAMP assay has high sensitivity for virus detection.

Amplification of wild-type virus

To determine whether the synthesized detection primers could be used to detect wild-type RSIV in infected samples, DNA was extracted from RSIV-infected and non-infected fish (*P. major*). The isolated DNAs were used as templates for the LAMP assay using specific LAMP primers. DNA ladders were evident *via* gel electrophoresis (Figure 3B) and green fluorescence was observed in the tubes containing RSIV DNA, but not the negative control (Figure 3A); the green color was visible even under natural light.

DISCUSSION

Viral infections pose a serious threat to the marine

aquaculture industry and responsible for significant financial losses (Caipang et al., 2004). Because of this risk, it is clearly important to rapidly identify various agents of fish diseases to prevent further disease transmission or outbreaks (Bruchhof et al., 1995). RSIV has caused serious fish losses, which result in severe economic losses. The presence of the virus in fish has been reported from Asian regions including Korea, Japan, China, Taiwan and Thailand (Inoue et al., 1992; Chua et al., 1994; Kasornchandra and Khongpradit, 1997; Miyata et al., 1997; Jung and Oh, 2000).

Several detection methods have been developed to monitor and control RSIV in fish culture. Recently, a real-time PCR approach has been found to be a useful technique for RSIV diagnosis (Min et al., 2011). However, PCR is technically demanding and time consuming for complete diagnosis. In this study, a novel method called LAMP was used as a detection technique for RSIV in fish. LAMP assay has many advantages that include high sensitivity, specificity, no expensive equipment and potential application in fieldwork. LAMP method can amplify a few copies of DNA to a magnitude of 10^9 CFU in less than 1 h under isothermal condition (Notomi et al., 2000). This method is convinced for a rapid diagnosis in case of RSIV infection.

Our data, optimization of the LAMP conditions was critical for the success of the assay. In particular, in the initial step, the melting temperature and $MgSO_4$ concentration during hybridization of the four primers to the target DNA functioned as critical effectors. Consistent with a previous study (Notomi et al., 2000), our data showed that higher or lower temperatures reduced the activity of the *Bst* DNA polymerase; the melting temperature of detection primers must be 58–64°C. Also, extremely low amount virus less than 10^4 copies was detected by LAMP method. However, we could not see difference level in several DNA concentration using serial dilutions of a purified RSIV DNA fragment. But other study showed high correlation between the amount of input viral DNA copy and its corresponding turbidity at the end of the LAMP reaction (Caipang et al., 2004; Mori et al., 2001). These results have implications particularly in diagnosing infectious diseases when both qualitative and quantitative diagnoses are required.

Conclusion

This study demonstrated that the synthesized primers can be utilized for the detection of RSIV in infected organisms. RSIV is believed to have a wide geographical range and RSIV strains isolated in Korea share high similarity with iridovirus isolates from Taiwan and Japan. This raises the possibility that the LAMP assay developed in this work may be useful for the detection and diagnosis of RSIV infections in other countries with highly similar

iridoviruses. Furthermore, we demonstrated that the synthesized sequences could be efficiently applied to detect wild-type RSIV in red sea bream. Together, these data indicate that our approach to the development of LAMP primers based on genetic information available in a public database may provide an easy and simple method of detecting viral infections in organisms.

Conflict of interest

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENT

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