

Research Article

Low-cost, specific and sensitive detection of infectious spleen and kidney necrosis virus by loop-mediated isothermal amplification

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Abstract

Infectious spleen and kidney necrosis virus (ISKNV) has caused high mortality of mandarin fish (*Siniperca chuatsi*) cultured in Asian countries. The current research priority is to develop a low-cost, rapid, specific and sensitive diagnosis method which can be widely in the fish farm. In this report, we have established a loop-mediated isothermal amplification (LAMP) which met the above criteria. Based on the open reading frame 72R (ORF72R) in the genome of ISKNV, a set of four primers were designed. The LAMP reaction conditions were optimized and the detection was ISKNV-specific. First of all, the detection sensitivity of the LAMP assay was 100-fold higher than the established PCR detection method of ISKNV. Secondary, the LAMP method was time saving and low-cost. Thirdly, ISKNV from kidney tissues of mandarin fish was included for test. The results showed that the established LAMP assay was applicable in the clinical samples. Taken together, the established LAMP assay has a potential to be widely used in the field and will be a powerful measure for the prevention of the ISKNV infection.

Keywords: Infectious Kidney and Spleen Necrosis Virus (ISKNV); Loop-Mediated Isothermal Amplification (LAMP); *Siniperca chuatsi*

Introduction

Infectious kidney and spleen necrosis virus (ISKNV) is the type species of the genus *Megalocytivirus* which is the most recently established genus in the family *Iridoviridae*. The genome of ISKNV is a single dsDNA with the length of 111,362 bp, which encodes 125 potential open reading frames (ORFs). ISKNV is the causative agent of mandarin fish infectious spleen and kidney necrosis (ISKNV) and has caused high mortality and great economic loss in mandarin fish (*Siniperca chuatsi*) cultured in Asian countries. Clinical signs of ISKN include anorexia, abnormal swimming, darkened colour, ulcerative gills, red spotted livers, swollen spleens and kidneys [1-3]. Spleen and

kidney are two major target organs of the virus. Rapid and specific detection methods are very critical for the prevention of ISKNV. Several methods have been developed to detect ISKNV, such as histopathology [1,2,4,5] cell culture [6-9], ELISA [10,11], and PCR [12-16]. Among these methods, PCR is a routine method in almost all laboratories worldwide. However, the method has several disadvantages, such as the time consuming and the requirement for expensive equipment as well as skilled operator. Hence, a current research priority is to develop a low-cost, rapid, specific and sensitive diagnosis method which can be widely in the fish farm. Loop-

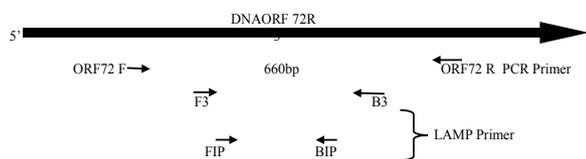
isothermal amplification (LAMP) is such a method which meets the above criteria. LAMP has been developed to amplify specific nucleic acid sequences using a designated primer set and a DNA polymerase with high strand displacement activity [17]. With the LAMP method, we can amplify specific sequences of DNA under isothermal conditions [18]. Thus; it requires only simple and inexpensive equipment which is available in fish farms. In addition, LAMP is highly specific since its reaction mixture contains six independent primers which recognize the target sequences specifically [17]. Furthermore, since there is no time loss for a thermal change, the amplification efficiency of the LAMP method is extremely high [19]. The amplified products are a unique mixture of stem-loop DNAs with several inverted repeats and cauliflower-like loops. When the LAMP products are visualized by agarose gel electrophoresis, many bands of different sizes up are observed [17]. This method has been applied already for the diagnosis of bacterial, viral, fungal and parasitic diseases in animals and plants [20-23]. Recently, an LAMP-LFD method which combines LAMP with a lateral flow dipstick (LFD) has been established for ISKNV detection [24]. However, the application of the LAMP-LFD assay was still limited by the high-cost of the primers which must be labeled with biotin and FITC. In this report, we have established a specific, rapid and lower-cost LAMP method which will be widely used for ISKNV detection in the field.

MATERIAL AND METHODS

LAMP primers design

The ISKNV genome encodes 125 potential ORFs. To design primers which were specific for ISNV, first of all, we have screened the unique sequences of ISKNV for primers design. The results showed that the sequences of ORF72R are specific for ISKNV with very low similarity at the nucleotide sequences level of other genes of Iridoviridae. Based on the ISKNV ORF 72R gene sequences (NCBI Acc. No. NC 003494), primers were designed using Primer 5.0 software. The primer sequences and locations were shown in figure 1.

Fig 1: The location and the graphic aliasing; specific location right; used in LAMP



Name	Primer sequences	Location
ISKNV-F3	GCCTGGAAGACATACCCGA	326-345
ISKNV-B3	CATGTCCGCGTACATGACG	521-540
ISKNV-FIP	CGCAGGCAATCCATAAGCGCT-CATACCGCCGGAATCTTACC	391-411/349-368
ISKNV-BIP	CCGGAGCCGTGCGCAATAAAT-CGAGGAACGACACGACTGT	483-502/432-451
ISKNV-ORF72F	TGTTTCGAGTTTGGCGTGGATTCC	49-72
ISKNV-ORF72R	TTTCCAGTCTCGCCGAAAGACG	628-649

Viral DNAs or RNAs extraction and cDNA synthesis

Viral DNAs or RNAs of ISKNV and other five viruses, including channel catfish virus (CCV), spring viraemia of carp virus (SVCV), infectious hematopoietic necrosis virus (IHNV), viral hemorrhagic septicemia virus (VHSV), and infectious pancreatic necrosis virus (IPNV) were extracted from the viruses infected cells or from mandarin fish kidney tissues using extraction kits (Ding guo, Beijing, China) according to the manufacturer's instructions. Subsequently, viral cDNAs were synthesized from extracted RNAs using ReverTra Dash kit (Thermo Fisher, Massachusetts, USA) following the manufacturer's protocol. All above viruses were stored in our laboratory.

Optimization of LAMP reaction conditions

LAMP reactions were conducted as described previously [17]. The amplification reaction mixture contained different concentrations of the following components: inner primers, outer primers, dNTPs, Bst polymerase (New England Biolabs, Ipswich, MA, USA), buffer, betaine (Promega, Madison, WI, USA), MgSO₄, DNA and distilled water. The mixture was incubated at different temperatures for a period of time. Subsequently, the reaction was terminated by heating at 80 °C for 5 min. Thereafter, 5 µl of the amplified products were visualized in an agarose gel (1%) or in the reaction tube stained with SYBR Green I. In addition, the products were digested with Hind III and were subjected to electrophoresis to confirm the structure of the amplified DNA.

Sensitivity and specificity analysis of the LAMP assay

To determine the sensitivity of the LAMP assay, 1 µg of ISKNV DNA was 10-fold serially diluted and subjected to LAMP and PCR assays. The amplified products were analyzed as described above. To test the specificity of the assay, viral DNA or cDNAs synthesized above were used as templates. The kidney tissues of mandarin with or without ISKNV infection were used to evaluate the application of the established LAMP assay in the clinical samples.

RESULTS

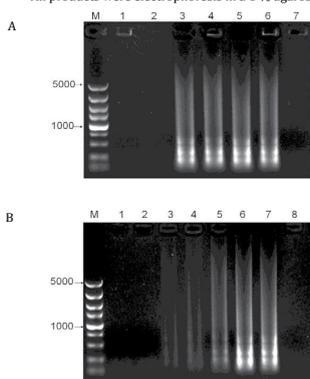
Optimization of LAMP reaction conditions for ISKNV detection

When the DNA of ISKNV was used as a positive control, a ladder-like pattern of DNA bands were observed in the agarose gel stained with SYBR Green I. However, there was no DNA band appeared in the negative control. The LAMP assay for ISKNV detection was optimized and standardized by using a set of four primers as shown in figure 1. The optimal temperature for the reaction was studied by incubating the reaction mixture at temperatures of 45, 55, 58, 60, 63 and 65 °C, respectively. The products amplified at 63 °C

exhibited slightly larger amounts of DNA compared to other temperatures (Fig. 2A). Subsequently, the reaction mixture was incubated at 63°C for 10, 20, 30, 40, 50, 60 and 70 min. The product at 60 min was maximal (Fig. 2B). Based on the above, incubation at 63°C for 60 min was routinely used for the following experiments to optimize the other reaction conditions unless otherwise indicated. First of all, the effect of free Mg²⁺ on the LAMP reaction was determined by the addition of Mg²⁺ at the final concentrations of 2, 4, 6, 8, 10 and 12 mM. The results showed that 10 mM of Mg²⁺ yielded the optimal amplification (Fig. 2C). Secondly, the LAMP reaction in the presence of betaine with different concentrations (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 M). As shown in Fig. 2D, the reaction took place at all the tested concentrations, but 1.2 M gave the maximal amplification efficiency. Thirdly, the LAMP reaction was performed in the presence of various concentrations of dNTPs (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 mM dNTPs). The results showed that the reaction at 1.4 mM generated the maximal reaction product (Fig. 2E). Fourthly, the effect of ratio of outer and inner primers on the LAMP reaction were determined. As shown in Fig. 2F, the cDNA target was amplified in the presence of different ratios of outer primer versus inner primer (1:1, 1:2, 1:4, 1:6, 1:8 and 1:10). The ratio of 1:10 was optimal for the reaction.

Fig. 2 Optimization of LAMP reaction conditions for ISKNV detection.

(A) Temperature: M, marker DL 5000; lanes 1-6, LAMP carried out at 45, 55, 58, 60, 63 and 65°C, respectively; lane 7, negative control. (B) Reaction time: M, marker DL 5000; lanes 1-7, LAMP carried out for 10, 20, 30, 40, 50, 60 and 70 min, respectively; lane 8, negative control. (C) Mg²⁺ concentration: M, marker DL 5000; lanes 1-6, 7 negative LAMP reaction system contained 2, 4, 6, 8, 10 and 12 mM Mg²⁺, respectively; lane 7, negative control. (D) Betaine concentration: M, marker DL 5000; lanes 1-6, LAMP reaction system contained 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 M betaine, respectively; lane 7, negative control. (E) dNTPs concentration: M, marker DL 5000; lanes 1-8, LAMP reaction system contained 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 mM dNTPs, respectively; lane 9, negative control. (F) Primer ratio: M, marker DL 5000; lanes 1-6, ratio of outer to inner primers was 1:1, 1:2, 1:4, 1:6, 1:8 and 1:10, respectively; lane 7, negative control. All products were electrophoresis in a 1% agarose gel and stained with SYBR Green I.



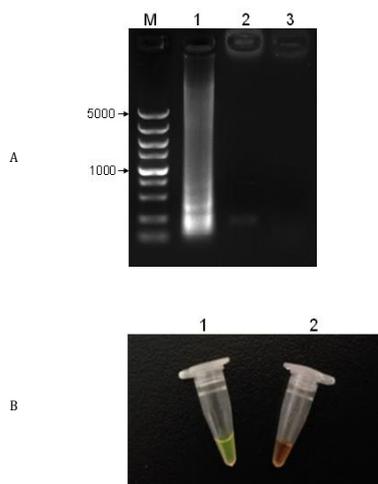
The structure of the LAMP products

The amplified LAMP products are a unique mixture of stem-loop DNAs with several inverted repeats and cauliflower-like loops. When the LAMP products were digested with Hind III, the digested products were linearized and the fragment size was the same to the target gene which was in good agreement with the predicted size

(Fig. 3A). When color changes were noted on visual inspection of LAMP reaction tubes after addition of diluted SYBR Green I, positive samples turned green, while negative samples remained orange (Fig. 3B). These observations agreed well with the gel electrophoresis results (Fig. 3A), indicating that the amplified DNAs were indeed originated from LAMP assay.

Fig. 3 The structure of the amplified DNA products.

(A) LAMP reaction and Hind III digestion: M, marker; lane 1, LAMP products; lane 2, Hind III-digested products; lane 3, negative control. (B) Visual detection of ISKNV LAMP products using SYBR Green I stain. Tube 1, positive LAMP reaction turned green. Tube 2, negative LAMP reaction remained orange.

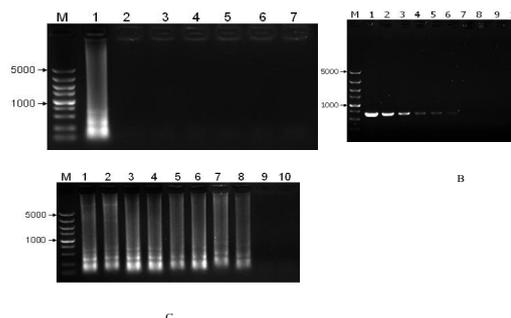


The specificity and sensitivity of the LAMP assay

The specificity of the reaction for ISKNV was determined by checking the cross-reactivity of the assay with the different sources of RNA/DNA virus and the extracted DNA from kidney tissues of mandarin fish without ISKNV infection. As shown in figure 4A, the positive result was only observed in the DNA of ISKNV. Comparative analysis of sensitivity of ISKNV detection by LAMP and PCR was carried out using 10-fold serial dilutions of ISKNV DNA. The PCR detected ISKNV specific signal at the template of 10⁻⁶ dilution (Fig.4B), while LAMP detected the virus specific signal at 10⁻⁸ dilution (Fig. 4C). Hence, the sensitivity of the detection limit by LAMP was about 100-fold higher than that of PCR.

Fig. 4 The specificity and sensitivity of the LAMP assay.

(A) M, marker; lane (tube) 1, positive control; lanes (tube) 2-6, SVCV, IHNV, CCV, VHSV and IPNV. Lane 7, negative control. (B) M, marker; lanes (tubes) 1-9, PCR was carried out using 10-fold serial dilutions of template DNA (10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸ and 10⁻⁹). Lane 10 is negative control. (C) M, marker; lanes (tubes) 1-9, LAMP was carried out using 10-fold serial dilutions of template DNA (10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸ and 10⁻⁹). Lane 10 is negative control.

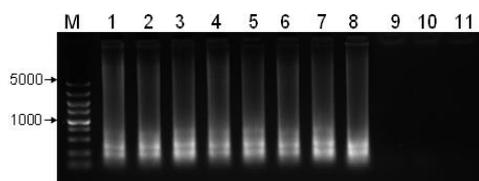


Applicability of the LAMP assay

With the optimized reaction conditions in above, the LAMP assay was used to detect ISKNV in mandarin fish. A total of seven ISKNV-infected (positive) and two non-infected (negative) mandarin fish (confirmed by PCR) were used for the test. All seven infected samples were positive for ISKNV in LAMP assay, while none of the non-infected samples showed positive signal (Fig. 5). This result indicated that the LAMP assay can be used as a diagnostic method for ISKNV infection in the field.

Fig. 5 Applicability of the LAMP assay.

M, marker; lane 1, positive control; lanes 2-8, infected samples; lanes 9 and 10, healthy samples; lane 11, negative control.



Discussion

In the past decade, ISKN has broken out frequently and has emerged as a major constraint on the culture of mandarin fish. In order to improve the disease management, several methods have been reported for the detection of ISKNV. Of these, virus isolation is performed traditionally for laboratory confirmation of virus infection, but the sensitivity of virus isolation is low because of poor conditions of sample transport and inadequate timing of sample collection [25]. Several PCR methods were established for the virus detection. However, these methods are time consuming and require expensive equipment and other costly consumables [26]. Therefore, lower-cost, specific and more sensitive detection methods are urgently required. The present paper describes a lower-cost, highly sensitive and specific diagnostic method for ISKNV detection using the LAMP assay. The primers used in our assay are not labeled with biotin and FITC, therefore it is much more cheaper than that of LAMP-LFD assay. The sensitivity of our LAMP for ISKNV detection was similar as that of LAMP-LFD assay and was 100-fold higher than that of PCR assays. The most significant advantage of our LAMP assay is its ability to amplify specific sequences at isothermal conditions at 63°C during incubation, and the results can be observed by the naked eye, which facilitates the application of LAMP in a field test. The established LAMP assay will be a powerful hygiene measure for the prevention of the ISKNV infection.

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