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Development and evaluation of a loop-mediated isothermal amplification assay for diagnosis of Cyprinid herpesvirus 2

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Goldfish Haematopoietic Necrosis caused by Cyprinid herpesvirus 2 (CyHV-2) is a severe fish disease with high level of mortality. This is the first study on detection of this disease by loop-mediated isothermal amplification (LAMP). A set of six primers targeting terminase gene (accession no. EU349285.1) was determined after a serial of tests. Detection limit was 1.09×10^{-4} μg/μL, which was superior to conventional PCR and real-time PCR. No cross reaction with 28 other viruses or bacteria commonly found in fish was observed. The application of commercial kit and instrument for the LAMP assay could reduce the risk of cross contamination, which is suitable for detection of infection under field conditions.

1. Introduction

Goldfish Haematopoietic Necrosis Virus (GFHNV), namely Herpesviral Haematopoietic Necrosis Virus (HVHNV), the second herpesvirus isolated from Cyprinid, is named Cyprinid herpesvirus 2 (CyHV-2) according to the rules of International Committee on Taxonomy of Viruses (9th report, 2012, www.ictvonline.org). The first outbreak of this disease was reported in Japan, with a mortality rate of almost 100% (Jung and Miyazaki, 1995). Fatal outbreaks were also reported in other countries such as the USA, Australia, and UK (Groff et al., 1998; Goodwin et al., 2006a,b; Stephens et al., 2004; Waltzek et al., 2009; Philbey, 2006). In China, serious losses had been caused by this virus from 2011 to 2012 (Wang et al., 2012). However, studies and inspection protocols on this disease are rare, thus the spread of it is a threat to worldwide ornamental goldfish industry.

To date, there is no available vaccine against CyHV-2, so early detection is vital in preventing the spread of this disease. Sensitive cell line for isolation is still unknown (Gilad et al., 2004; Jeffrey et al., 2007). Real-time PCR and conventional PCR with high sensitivity and specificity had been developed (Goodwin et al., 2006a,b; Waltzek et al., 2009). However, the requirement of a rapid thermal cycler limits the detection of CyHV-2.

Loop-mediated isothermal amplification (LAMP) can amplify nucleic acids under isothermal conditions. This reaction employs a DNA polymerase with strand displacement activity and two pairs of primers recognizing six distinct sequences on target DNA (Notomi et al., 2000). This method is also superior to conventional PCR in its rapidity and sensitivity. Therefore, detection methods for CyHV-2 based on LAMP assay are important for disease control.

2. Materials and methods

2.1. Fish samples, virus and bacteria

Crucian carps infected with CyHV-2 were collected from central China in September 2011. Water temperature was 21–25 °C. Brain, spleen and kidney tissues were then collected and stored at −80 °C.

Virus koi herpesvirus (KHV), *Rana grylio* virus (RGV) strain 9506 and 9507, soft-shelled turtle iridovirus (STIV) strain 9701, spring viraemia of carp virus (SVCV), viral nervous necrosis virus (VNNV), hirame rhabdovirus (HRV) were from the laboratory of Shenzhen Exit and Entry Inspection and Quarantine Bureau of China. Channel catfish virus (CCV) was purchased from ATCC. Yellowtail ascites virus (YAV), infectious pancreatic necrosis virus (IPNV), infectious haematopoietic necrosis virus (IHNV), Bohle iridovirus (BIV) were provided by Institute of Hydrobiology, Chinese Academy of Sciences. Infectious salmon anaemia virus (ISAV) was provided by National Veterinary Institute, Norway. Viral haemorrhagic septicemia virus (VHSV) was provided by Centre for Environment, Fisheries and Aquaculture Science of UK.

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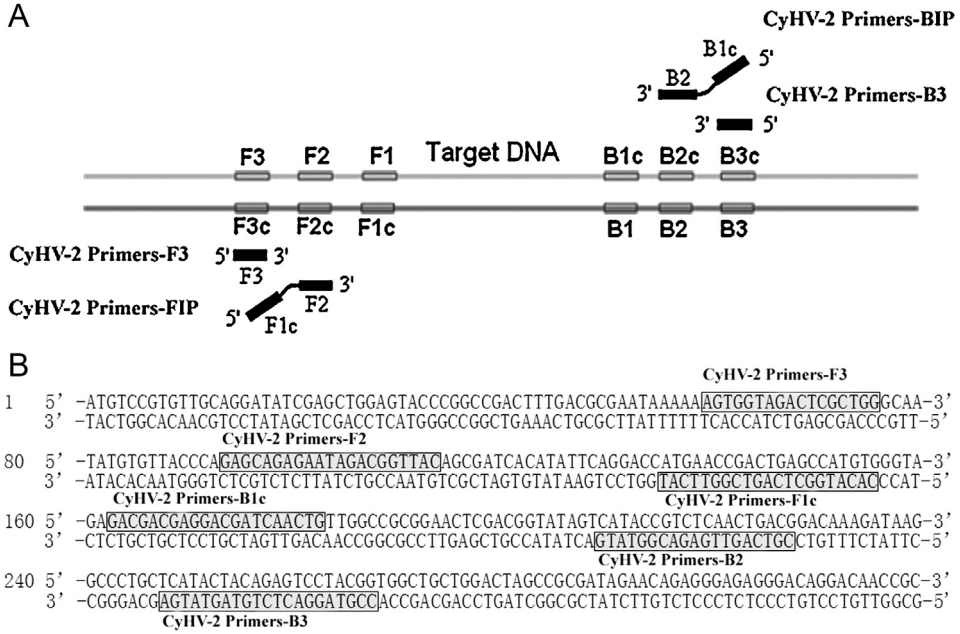

Fig. 1. Primers for the detection of CyHV-2 by LAMP assay. (A) A schematic diagram indicating the positions at which the primers attach for target gene amplification. (B) DNA sequence of terminase gene (accession no. EU349285.1) showing target region of the primers.

Bacteria *Staphylococcus aureus* (IAM1011), *Aeromonas hydrophila* (ATCC19570), *Pseudomonas fluorescens* (IAM12022), *Pseudomonas putida* (IAM12354T), *Yersinia ruckeri* (ATCC29473), *Vibrio splendidus* (IAM14411), and *Vibrio anguillarum* (NCIMB829) were provided by Dr. Wakabayashi, University of Tokyo. *Streptococcus iniae* (ATCC29178) was purchased from ATCC. *Stapylococcus xylosus/leutus* (20020296), *Aeromonas sobria* (20010836), *Enterobacter cloacae* (20010243), *Proteus mirabilis* (20010925), *Vibrio parahaemolyticus* (20090853), *Vibrio vulnificus* (20080191) were isolated by the laboratory of Shenzhen Exit and Entry Inspection and Quarantine Bureau of China.

2.2. DNA extraction

Brain, spleen and kidney of diseased fish were homogenized and used for CyHV-2 DNA extraction. DNA or RNA of CyHV-2 and other viruses in Section 2.1 were extracted by MiniBEST Viral RNA/DNA Extraction Kit Ver 3.0 (Takara, Dalian, China) according to the recommendation of the manufacturer. For bacteria DNA extraction, single colony was suspended in ddH_2O to match a 1.0 McFarland Standard, placed in a boiling water bath for 10 min, and centrifugated at 12,000 rpm/min for 10 min. Then the supernatant was used as DNA template.

2.3. Design of primers for CyHV-2 LAMP assay

Based on the sequence of terminase gene (accession no. EU349285.1), two outer primers (F3 and B3), two inner primers (FIP and BIP) and two loop primers (loop F and loop B) were designed by using software LAMP Designer. The sequences and location of them were shown in Table 1 and Fig. 1, respectively.

2.4. Optimization of LAMP reaction temperature

Based on the recommendation of manufacture, the reaction was performed in a 25 μL mixture containing 1.6 μM each of FIP and BIP; 0.2 μM each of F3 and B3; 0.8 μM loop F and loop B, respectively; 15 μL 1× Isothermal Master Mix (OptiGene, Horsham, UK) and 4 μL DNA template. The mixture was incubated at 62, 63, 64, 65, 66, 67, 68, 69 °C for 30 min, respectively, and then terminated at a range from 98 °C to 80 °C, with a decline rate of 0.05 °C per second. The result was analyzed on Instrument GENIE II (OptiGene, Horsham, UK). The optimal temperature was determined and used in the subsequent tests.

Table 1
Sequences of designed primers.

Primers	Sequence (5′ → 3′)
F3	AGTGGTAGACTCGCTGG
B3	CCGTAGGACTCTGTAGTATGA
FIP (F1c + F2)	CACATGGCTCAGTCGGTTCATGAGCAGAGAATAGACGGTTAC
BIP (B1c + B2)	GACGACGAGGACGATCAACTGCGTCAGTTGAGACGGTATG
Loop F	GGTCCTGAATATGTGATCGCT
Loop B	GCGGAACTCGACGGTATAG

2.5. Sensitivity tests

Comparison among methods of real-time PCR, conventional PCR and LAMP was carried out. A 10-fold serial dilution of extracted CyHV-2 DNA (10^{-1}–10^{-8}) was used as template.

Real-time PCR was performed on 7500 real-time PCR system (Life Technologies, New York, USA), using Platinum qPCR SuperMix-UDG (Life Technologies, New York, USA). The primers, probes and amplification protocol designed by Goodwin et al. (2006a,b) were involved in this study.

For conventional PCR, TaKaRa rTaq (DR001B, including loading buffer and dNTP) was used. The primers and amplification protocol described by Waltzek et al. (2009) were employed in this research. Then products were electrophoresed on 1.5% agarose gel and documented by Gel Logic 4000 imaging system. LAMP assay was carried out based on Section 2.4. Then detection limits of these three methods were assessed.

2.6. Specificity tests

According to manufacturer's instruction, Isothermal Master Mix could also be used to detect RNA virus. Therefore, as mentioned in Section 2.1, 14 fish virus and 14 common bacteria found in fish