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Designment and evaluation of the primers for cyprinid herpesvirus 2 and spring viremia of carp virus duplex RT-PCR detection

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ABSTRACT

To develop the rapid method for detecting of Cyprinid herpesvirus 2(CyHV2) and Spring viremia of carp virus (SVCV), 2 pairs of specific primers for duplex reverse transcriptase polymerase chain reaction (RT-PCR) and 5 overlapping oligo primers were designed according to the nucleotide sequence information of CyHV2 and SVCV published in GenBank, and a DNA fragment about 228 bp of the SVCV G gene was synthesized *in vitro* by overlap extension PCR to construct the recombinant plasmid pMD19-T-SVCVG. Then, the 2 pairs of specific primers were evaluated via a serial of tests, including reaction temperature optimization test, sensitivity and specificity tests. The results showed that the 2 pairs of designed specific primers are suitable for CyHV2 and SVCV duplex RT-PCR detection which is a rapid method with high specificity and sensitivity, the detection limits for CyHV2 and SVCV detection were both approximately 88 copies of the cloned viral genomic fragments, as well as resulted in no cross-reaction for GCRV, *Aeromonas veronii*, *Pseudomonas fluorescens*, and *Streptococcus* isolated from fish, and the DNA (cDNA) of the normal *Carassius auratus gibelio* samples.

KEYWORDS

Cyprinid herpesvirus 2; SVCV; Overlap extension PCR; RT-PCR; Duplex RT-PCR.

INTRODUCTION

The Goldfish (*Carassius auratus*) Haematopoietic Necrosis (GFHN), caused by Cyprinid herpesvirus 2(CyHV2) belonging to the genus *Cyprinivirus,* family Alloherpesviridae, and order Herpesvirales, is also commonly referred to as the CyHV2 infection[1]. Since its first reported outbreak in Japan with a nearly 100% mortality rate, the CyHV2 infection has also been found with a high mortality level in many countries such as Australia, the UK, and the USA[2-6]. Recently, CyHV2 has also been detected in other fish species, the prussian carp (*C. gibelio*) and the crucian carp (*C. carassius*)[7], and the CyHV-2 infection had also led to serious economic losses when it emerged in cultured gibel carp (*Carassius auratus gibelio*) with a severe mortality rate from 2009 to 2012 in China[8,9]. Spring viremia of carp (SVC) caused by SVCV, a rhabdovirus, is an acute hemorrhagic infectious disease with significant morbidity and mortality strongly affecting the farming of many cyprinids fish species such as common carp (Cyprinus carpio), prussian carp and so on[10]. Because of its significant risk and harmfulness, SVC has been registered in the list of notifiable contagious diseases to the Office International des Epizooties (OIE),and it is also designated as one of class I diseases in China[11]. Obviously, the CyHV2 infection and SVC are both the important contagious and serious diseases which highly impact of the economy and ecology of gibel carp farming throughout the world. while there are no very effective available vaccines that can control these diseases, and in addition Gibel carp as a major aquaculture species (*Carassius auratus gibelio*) has been widely cultured in nearly all of China. Therefore, it is both critical and important to study rapid detection and diagnosis techniques for preventing them. In this paper we describe and evaluate a double reverse transcription polymerase chain reaction (RT-PCR) assay for the simultaneous detection of CyHV2 and SVCV in one tube based upon the artificial *in vitro* synthesis of the glycoprotein (G) gene sequences of SVCV and previous researches about the CyHV2 infection dectecion.

MATERIALS AND METHODS

Reagents and samples

DH5α competent cells, Pfu DNA polymerase, TIANprep Mini Plasmid Kit (DP103), TIANgel Midi Purification Kit (DP209), and 2×Taq PCR Master Mix were provided by TIANgen Biotech (Beijing) Co.,Ltd., RNAiso Plus, Primerscript RT reagent Kit (BK1901),pMD19-T Vector kit and DNA Maker DL2000 were provided by the TaKaRa, Biotechnology (Dalian) Co.,Ltd. recombinant plasmid pMD19-T-CyHV2, Grass carp reovirus (GCRV vaccine), *Aeromonas veronii*, *Pseudomonas fluorescens,* and *Streptococcus* isolated from fish were provided by Sichuan Agricultural University's animal quarantine lab.

Primer preparation

Using DNAStar software, 4 specific primers and 5 overlapping oligo primers (listed in TABLE 1) were carefully designed according to the sequences data of the CyHV2 DNA-dependent DNA polymerase gene[12] and SVCV glycoprotein gene published in GenBank (accession no: HM014349, EU177782). All of these primers were prepared by the TaKaRa Biotechnology Co., Ltd.

TABLE 1 : Primers used for CyHV2 and SVCV detection

Tasks and Primer name	Sequence (5′–3′)	Products Size(bp)
PCR primers		
P1c	AGTCCATAGTGTCTAGGAGCGA	413
P2c	AGTGTGTTTTACAGCGTTCTCG	
P3s	CGGATGGGCATCTGTCACAAC	228
P4s	CCGTACATAATTCCTTCAACTTC	
Overlap primers		
SF1	GGGCATCTGTCACAACAGTGTCAAATACTAATTACAAGGTAGTACCCCATTCTGTTCAT	
SR1	CCATTGAAGTCATGATCGATCCAGTGTCCTCCGTACGGCTCCAAATGAACAGAATGGGG	
SF2	ATCATGACTTCAATGGGGGCGAATGCAGAGAAAAAGTGTGTGAAATGAAAGGGAACCAC	
SR2	TTTCACATTCATGCTGCACGGTCTCATCTGTGATCCAAATAGAGTGGTTCCCTTTCATT	
SF3	CAGCATGAATGTGAAAAGCACATAGAGGAAGTTGAAGGAATTA	

Templates preparation of target DNA fragments

In vitro synthesis of SVCV target DNA fragments: The conserved sequence fragment of the SVCV G gene was synthesized *in vitro* using an overlap extension PCR method and was then cloned into the pMD9-T vector in order to construct the recombinant plasmid named pMD19-T-SVCVG, as described in reported literature[13].

For the primary extension procedure, a 50 μL reaction volume containing 5μL 10×Pfu DNA polymerase buffer,4μL of dNTP (10 mmol/L of each nucleotide), 1μL (10μmol/L) of each of the overlapping oligo primers (SF1/SR1, SF2/SR2, or SF3/SR2),1μL of Pfu DNA polymerase, and 38μL ddH_2O was used. The reaction conditions were 95℃ for 30s and 72℃ for 15min. Then, the secondary extension reactions were carried out until the full-length target DNA fragment production had been synthesized in the 50 μL volume reaction including 1μL 10×Pfu DNA polymerase buffer, 4μL of dNTP (10 mmol/L of each nucleotide), 1μL of Pfu DNA polymerase, and 22μL of each of the two overlap primary/previous extension reaction products. The reaction conditions were kept the same as the above-mentioned primary extension procedure.

From the last secondary extension reaction, the full-length target DNA fragments were amplified by PCR using the primers P3s and P4s in a 50 μL reaction volume containing 25μL 2×Taq PCR Master Mix, 1μL (10μmol/L) of each of the primers,3μL of template(full-length target DNA fragments), and 20 μL ddH_2O. The PCR was conducted as follows: denaturing at 95 ℃ for 5 min; followed by 35 cycles at 95 ℃ for 40 s, 56 ℃ for 35 s, and 72 ℃ for 30 s; and then terminated by an elongation at 72 ℃ for 8 min. 5μL of PCR products were analyzed in a 1.0% agarose in TAE Buffer gel containing 0.5 mg/mL GoldView through electrophoresis.

The PCR products were subsequently separated and purified according to the TIANgel Midi Purification Kit(DP209)instructions and were then directly cloned into the pMD19-T Vector followed by a transformation into the DH5α competent cells. Then, the recombinant plasmids were extracted using a TIANprep Mini Plasmid Kit and were subsequently identified by PCR (using P3s/P4s and RV-M/M13-47 as primers) and by sequencing analysis[13].

Identification of CyHV2 target DNA fragments: The recombinant plasmids pMD19-T-CyHV2 were cultured, then extracted using a TIANprep Mini Plasmid Kit and were identified by PCRs (using P1c/P2c and RV-M/M13-47 as primers)[13].

Optimization of reaction temperature

The optimum annealing degree for specific primers P1c, P2c, P3s and P4s was determined by a double PCR assay in one tube which used the two recombinant plasmids above as the templates in a 25μL reaction volume containing 12.5 μL 2×Taq PCR Master Mix, 1μL (10μmol/L) of each of the 4 specific primers, 1μL of each of the templates, and 6.5 μL ddH_2O. The reaction conditions are same as the PCR above but different from a gradient of annealing degree from 51℃ to 62 ℃. In all, 5μL of PCR products were analyzed.

Specificity and Sensitivity tests

To assess the sensitivity, the two recombinant plasmids constructed above were estimated by a ND-1000 ultraviolet spectrophotometer (Nano Drop Co.,Ltd,USA) and were then amplified in a 10-fold serial dilution[12] by duplex RT-PCR using the optimum reaction system and conditions as determined above. The specificity of the duplex RT-PCR with the 4 specific primers was assessed through comparing the recombinant plasmids to GCRV, *Aeromonas veronii*, *Pseudomonas fluorescens,* and *Streptococcus* isolated from fish and to a negative tissue sample of Gibel carp. The RNA extraction of GCRV and tissue sample using the method in reference SN/T 1152-2002, and RT reaction using Primerscript RT reagent Kit. The samples DNA templates were prepared by using TIANgel Midi Purification Kit(DP09-02).

RESULT

Identification and synthesis of templates DNA fragments

To develop a duplex RT-PCR for SVCV and CyHV2 detection, a conserved sequence fragment of the SVCV G gene about 228 bp was synthesized in vitro using an overlap extension PCR method (see Figure 1 lane1),and was cloned into the pMD9-T vector to construct the recombinant plasmid named pMD19-T-SVCVG. And then, pMD19-T-SVCVG and pMD19-T-CyHV2 were identified by two types of PCR methods and sequencing analysis as described above, the results(see Figure 2)showed the PCR products of the 2 recombinant plasmids from the pMD19-T vector usual primers(lane2,lane4) were both longer than the PCR products from the special primers (P3s/P4s,P1c/P2c) (lane1,lane3). Blast analysis of the pMD19-T-SVCVG sequencing result (see Figure 3) revealed that it had a 100% homology with the reference sequences (DQ097384) in GenBank. All these results are indicative that the templates DNA fragments were prepared successfully.

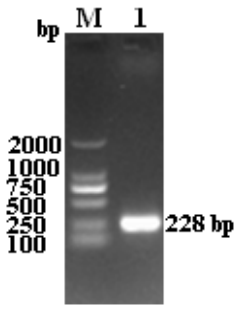

Figure 1 : Synthesis result of target DNA fragments on electrophoresis. M: DNA marker DL2000, 1: target DNA fragments of SVCV

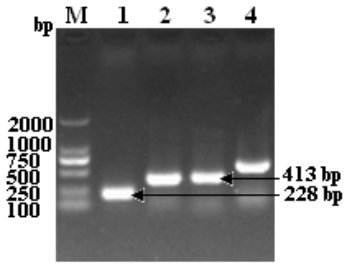

Figure 2 : The PCRs identification results of the 2 recombinant plasmids. M: DNA marker DL2000;

1: PCR(P3c/P4c as primer) products of pMD19-T-SVCVG,
2: PCR(RV-M/M13-47 as primers) products of pMD19-T- SVCVG
3: PCR(P1c/P2c as primer) products of pMD19-T-CyHV2,
4: PCR(RV-M/M13-47 as primers) products of pMD19-T-CyHV2

CGGATGGGCATCTGTCACAACAGTGTCAAATACTAATTACAAGGTAGTACCCCATTCTG
TTCATTTGGAGCCGTACGGAGGACACTGGATCGATCATGACTTCAATGGGGGCGAATG
CAGAGAAAAAGTGTGTGAAATGAAAGGGAACCACTCTATTTGGATCACAGATGAGAC
CGTGCAGCATGAATGTGAAAAGCACATAGAGGAAGTTGAAGGAATTATGTACGG

Figure 3 : Sequencing results of synthesized SVCV target DNA fragments

Optimization of reaction conditions.

The two recombinant plasmids were used in the gradient duplex PCR to determine an ideal annealing degree. The results show that the PCR products of SVCV and CyHV2 in lane 5 (58℃) are both present in a greater quantity than in the other lanes (see Figure 4), which indicates that 58℃ is the ideal annealing degree in the amplification cycles for creating a high specificity and amplification efficiency.

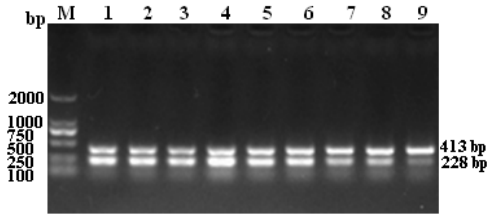

Figure 4 : The optimization results of annealing degree determination. M: DNA marker DL2000,1:52℃, 2:53℃,3:55℃, 4:57℃, 5:58℃, 6:59℃,4:60℃, 5:61℃, 6:62℃

Specificity and sensitivity of duplex RT-PCR

The sensitivity and specificity of the duplex RT-PCR were assessed in the tests that were previously described above. There were two clear electrophoretic bands located at about 413 bp and 228 bp separately for CyHV2 and SVCV detection could be both observed (lane 4) when almost 88 copies of the target DNA fragments in each of the 2 recombinant plasmids were detected(see Figure 5). For the specificity(see Figure 6), the two expected PCR products that were only observed for the recombinant plasmids detection (lane1,lane3 and lane4), and no specific amplification products were observed for the detection of GCRV, *Aeromonas veronii*, *Pseudomonas fluorescens*, and *Streptococcus* isolated from fish, and the DNA(cDNA) of the normal *Carassius auratus gibelio* samples.

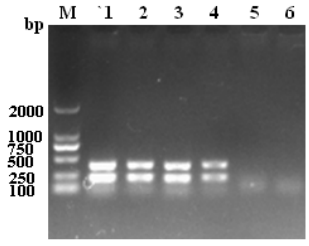

Figure 5 : The sensitivity assay results of CyHV2-SVCV duplex RT-PCR M: DNA marker DL2000; 1: 8.8×10^4 copies,2:8.8×10^3 copies,3. 880 copies, 4: 85copies, 5. 8.8 copies, 6: Negative control

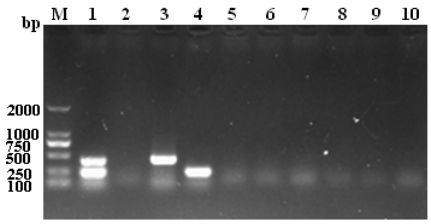

Figure 6 : The specificity assay results of CyHV2-SVCV duplex RT-PCR; M: DNA marker DL2000, 1:Positive control, 2:Negative control, 3:pMD19-T-CyHV2, 4: pMD19-T-SVCVG, 5:GCRV, 6:*Aeromonas veronii*, 7: *Pseudomonas fluorescens*,8: *Streptococcus*, 9: Tissues DNA of healthy *Carassius auratus gibelio*, 10: Tissues RNA of healthy *Carassius auratus gibelio*.

DISSCUSS AND CONCLUSIONS

Viral infections remain one of the most devastating diseases in fish farming, especially the high risk and harmfulness diseases including SVC and GHFN in recent years in China, which have caused serious economic losses in the fish farming since their first outbreaks. SVCV and CyHV2 has been the important pathogens which negatively impact of the ecology and environment of warm-water fish farming throughout the world and become the significant diseases to prevent and control in gibel carp farming now. As there is no ideal vaccine, developing a rapid detection and diagnosis technology has important significance for SVCV and CyHV2 control with the development of international trade. Currently, several available methods have been reported to detect SVCV or CyHV2 including ELISA[14], (RT-)PCR[4,15], real-time PCR[16] and (RT-) LAMP[17,18], but there is seldom reports on the duplex PCR method for SVCV and CyHV2 detection in one tube simultaneously. Considering that the specificity of SVCV and the strict requirements for pathogen operation, the duplex PCR templates of SVCV was selected according to the national SVCV detection method(SN/T 1152-2002) and synthesized *in vitro* by a overlapping PCR technology to avoid the spread risk of the high pathogen. In this paper, two pairs of specific duplex PCR primers for SVCV and CyHV2 detection were designed and evaluated after set of serial tests, including artificial synthesis of target gene fragments, optimization of reaction conditions, sensitivity and specificity tests. This is the first report on the duplex PCR method for SVCV and CyHV2 detection in Gibel carp in China.

In conclusion, a 228 bp DNA fragment of SVCV G gene conserved sequence was synthesized in vitro, and the duplex RT-PCR have been proven to be a high sensitive and specific method for CyHV2 and SVCV rapidly detecting in Gibel carp primary. The detection limits of this method described here could reach as few as about 88 copies of the each virus target gene fragments, and no cross reaction was observed with other common main pathogens, including GCRV, *Aeromonas veronii*, *Pseudomonas fluorescens*, and *Streptococcus* isolated from fish and the normal *Carassius auratus gibelio* sample as detected by this method. In brief, this study supplied a new, simple, rapid, and useful detection and diagnostic tool for the molecular epidemiology investigation of CyHV2 and SVCV in China, and also supplied materials for the studies on the detection of SVCV.

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