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A PCR Method for the Detection of Cyprinid Herpesvirus 2

Yang Zexiao[1]*, Li Guili[1], Xu Qiumei[2], Wang Yin[1], Yao Xueping[1], Zhu Ling[1], Xu Zhiwen[1], Chen Defang[3] and Wang Kaiyu[1]*

[1]College of Veterinary Medicine, Sichuan Agricultural University, Yaan Sichuan - 625014, China.
[2]College of Basic Sciences, Sichuan Agricultural University, Yaan Sichuan - 625014, China.
[3]College of Animal Science and Technology, Sichuan Agricultural University, Yaan Sichuan - 625014, China.

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Cyprinid herpesvirus 2(CyHV2) is a fatal pathogen for some species of the Cyprininae fish and, furthermore, is responsible for negatively impacting the goldfish industry worldwide. In order to develop a polymerase chain reaction (PCR) capable of detecting CyHV2, 2 specific primers and 9 overlapping oligo primers were designed based upon the nucleotide sequence information of CyHV2 published in GenBank (accession no:HM014349), and a 413 bp DNA fragment of the CyHV2 DNA-dependent DNA polymerase gene was synthesized *in vitro* by using an overlap extension PCR to construct the recombinant plasmid,pMD19-T-CyHV2. Then, a primary PCR method was established after set of serial tests, including reaction conditions optimization test, sensitivity test, and specificity test. The final results indicate that this developed PCR assay is a rapid method that maintains both a strong specificity and a high sensitivity for detecting CyHV2. The PCR detection limit could reach approximately 62 copies of the cloned viral genomic fragments (pMD19-T-CyHV2) as well as resulted in no amplifications for *Aeromonas veronii*, *Aeromonas hydrophila*, *Pseudomonas fluorescens* and *Streptococcus*, which are common pathogens isolated from fish by this detection approach.

Key words: Cyprinid herpesvirus 2, PCR, overlap extension PCR.

The Cyprinid herpesvirus 2(CyHV2) is an important member of the genus *Cyprinivirus,* family Alloherpesviridae, and order Herpesvirales. It is also commonly referred to as the Goldfish Haematopoietic Necrosis (GFHNV) because it is the causative pathogen of the Goldfish(Carassius auratus) Haematopoietic Necrosis Virus(GFHN), whose first reported outbreak was in Japan with a nearly 100% mortality rate[1].Since this first outbreak in Japan, the CyHV2 infection has also been found with a high mortality level in some European and Asian countries, such as Australia, the UK, and the USA[2-6], which gives reason to believe that it can be distributed globally.

Recently, CyHV2 has also been detected in other fish species, such as the prussian carp (*C. gibelio*) and the crucian carp (*C. carassius*)[7]. From 2009 to 2012 in China, the CyHV-2 infection led to serious economic losses when it emerged in cultured gibel carp (*Carassius auratus gibelio*) with a severe mortality rate[8-9]. A 2014 study that focused on the susceptibility of the Japanese Cyprininae fish species to CyHV2 revealed that the cumulative mortalities for the intraperitoneally injected Edonishiki, ginbuna, and vagabond were 100%, 20%, and 10%, respectively; however, no mortality was observed in another variety of goldfish (the Ranchu) and with immersed ginbuna and nagabuna. The CyHV2 DNA was detected, and the virus was re-isolated from all the dead fish, as well as from some of the surviving Carassius

* To whom all correspondence should be addressed.
Tel.: 15008305405;
E-mail:kywangsicau@126.com

spp. These results indicate that the Japanese indigenous Cyprininae fish species' susceptibility to CyHV2 is different, and the ability for CyHV2 to replicate might be different among the Carassius fish species[10]. As a major aquaculture species, Gibel carp (*Carassius auratus gibelio*) has been widely cultured in nearly all of China. In addition there is no available vaccine that can control the CyHV2 infection. Therefore, it is both critical and important to investigate the distribution and epidemiological situation of CyHV2 to use rapid detection techniques for preventing its spread. In this study we describe a conventional polymerase chain reaction (PCR) assay based upon the artificial *in vitro* synthesis of the DNA-dependent DNA polymerase gene sequences of CyHV2, and we completed the evaluation of this method.

MATERIALS AND METHODS

Reagents and Samples

The following were provided by the Biotech (Beijing) Co., Ltd: DH5± competent cells, pMD19-T Vector kit, Pfu DNA polymerase, TIANprep Mini Plasmid Kit (DP103),and TIANgel Midi Purification Kit (DP209), DNA Maker DL2000 and TaKaRa rTaqTM were provided by the TaKaRa Biotechnology(Dalian) Co., Ltd. *Aeromonas veronii*, *Aeromonas hydrophila*, *Pseudomonas fluorescens,* and *Streptococcus* isolated from fish were provided by Sichuan Agricultural University's animal quarantine lab.

Primer Preparation

Two specific primers and 9 overlapping oligo primers (listed in table 1) were carefully designed using DNAStar according to the sequence data of the CyHV2 DNA-dependent DNA polymerase gene published in Gen Bank (accession no:HM014349). All of these primers were prepared by the TaKaRa Biotechnology (Dalian) Co., Ltd.

Table 1. Primers used for CyHV2 PCR detection

Tasks and Primer name	Sequence (52 –32) Size(bp)	Products
PCR		413
P1	AGTCCATAGTGTCTAGGAGCGA	
P2	AGTGTGTTTTACAGCGTTCTCG	
Overlap PCR		
F1	ATAGTGTCTAGGAGCGACCCGTTCTGTCTCGAGTATGTCAGAAA CTGCGTGCTGCTCGA	
F2	CCTTGATCTCTTCCATGTTACTGGCGGCCGGTATCTTTTTCCAAT CGAGCAGCACGCAG	
F3	TGGAAGAGATCAAGGAATACCCGCACAGCGAAGACCTGTACACGATC CTGTGCTACAAG	
F4	GAAGCGGTGTAGGTCACAAACCGAGTCCAACCGACCTCTCGGTTCTT GTAGCACAGGAT	
F5	GACCTACACCGCTTCCAGTCTGGGCCACTACCTCTCTATGAGATCTCAG TACAAGAAAC	
R1	ATCATAGTACGCCTTGAGACTCGCGTCTTTCTCGGTCTTGATGCGTTTC TTGTACTGAG	
R2	AAGGCGTACTATGATCAGATGCAGGGTGAGATGAAAGTATGCGCCAAC TCTCACTACGG	
R3	GTCCGGACCAAGTAGTCAGATGCTGACAGAGACTCTGGCTCACGCCG TAGTGAGAGTTG	
R4	CTACTTGGTCCGGACGCCAAAAGATTCTGCTGGTCGAGAACGCTGTAAAAC	

In vitro synthesis of target DNA fragments

The conserved sequence fragment of the CyHV2 DNA polymerase gene (target DNA) 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-CyHV2, as described in reported literature[11].

Overlap extension reaction

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 (F1/R1, F2/R2, F3/R3, F4/R4, or F5/R4),1μL of Pfu DNA polymerase, and 38μL ddH_2O was used. The reaction conditions were 94°C for 30s and 72°C 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.

Amplification and identification of target DNA fragments

The full-length target DNA fragments, from the last secondary extension reaction, were amplified by PCR using the primers P1 and P2 in a 50 μL reaction volume containing 5μL 10×TaKaRa rTaq™ DNA polymerase buffer, 4μL of dNTP (10 mmol/L of each nucleotide), 1μL (10μmol/L) of each of the primers (P1/P2),1μL of TaKaRa rTaq™ DNA polymerase, 5μL of template(full-length target DNA fragments), and 33μL ddH_2O. The PCR was conducted as follows: denaturing at 95 °C for 5 min; followed by 30 cycles at 94 °C for 40 s, 56 °C for 40 s, and 72 °C for 45 s; and then terminated by an elongation at 72 °C for 8 min. 5μL of PCR products were analyzed in a 1.0% agarose in TAE Buffer gel containing 0.5 mg/mL GreenView through electrophoresis, which was then photographed under a UV imaging system(Bio-Rad).

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 DH5± competent cells. Then, the recombinant plasmids were extracted using a TIANprep Mini Plasmid Kit and were subsequently identified by PCR (using P1/P2 and RV-M/M13-47 as primers) and by sequencing analysis[12].

Optimization of reaction conditions

A PCR assay for determining the optimum reaction conditions incorporated the recombinant plasmids that contained the overlap extension PCR products of the CyHV2 DNA polymerase gene and P1/P2 as the templates and specific primers, respectively. The reaction conditions were determined to be as follows: denaturing at 95 °C for 5 min; followed by 35 cycles at 94 °Cfor 30 s, a gradient of annealing degree from 57°C to 62 °C for 30 s, and 72 °Cfor 30 s; and then terminated by an extension at 72 °C for 8 min. In all, 5μL of PCR products were analyzed.

Specificity of CyHV2 PCR

The test's specificity was assessed through comparing the recombinant plasmids that contained the overlap extension PCR products of CyHV2 to the *Aeromonas veronii, Aeromonas hydrophila, Pseudomonas fluorescens,* and *Streptococcus* isolated from fish and to a normal sample of *Carassius auratus gibelio*. DNA templates were extracted using a commonly reported method from literature[12].

Sensitivity of CyHV2 PCR

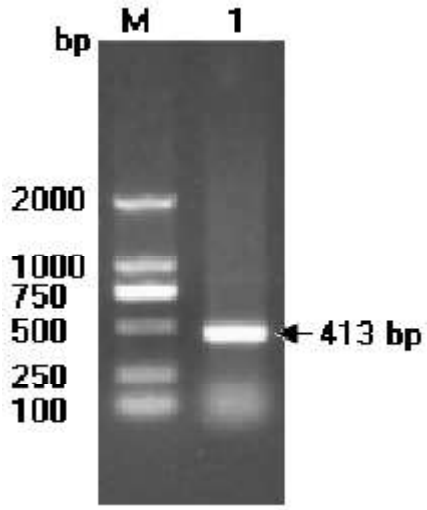
To assess the CyHV2 PCR sensitivity, the recombinant plasmids that contained the overlap extension PCR products of CyHV2 were estimated by a ND-1000 ultraviolet spectrophotometer (Nano Drop Co.,Ltd,USA) and were then amplified in a 10-fold serial dilution[11] by using the optimum reaction system and conditions for PCR as determined above.

RESULTS

Synthesis of the CyHV2 DNA-dependent DNA polymerase gene

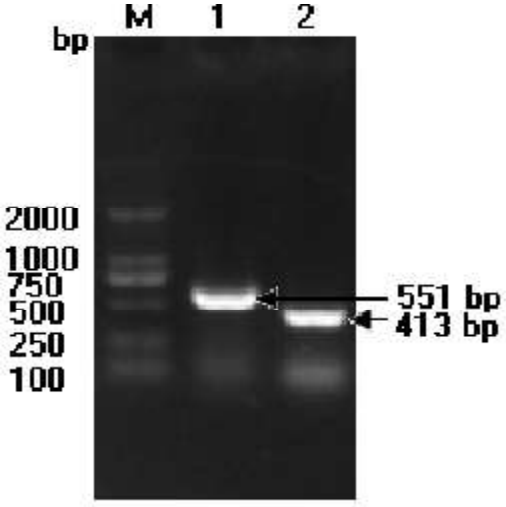
To develop a CyHV2-detectable PCR, a 413 bp-length target DNA fragment of the DNA-dependent DNA polymerase gene of CyHV2 was synthesized *in vitro* using an overlap extension PCR as described above, and then, this target fragment was cloned into a pMD19-T vector in order to construct the recombinant plasmid. Next, the recombinant plasmid was identified by two types of PCR methods and sequencing analysis. The results showed that the target DNA fragment of about 413 bp-length of the CyHV2 DNA-dependent DNA polymerase gene was successfully synthesized (see Fig. 1). The PCR products of the recombinant plasmid from the pMD19-T vector usual primers were 551 bp, which were about 138 bp longer than the PCR products from the special primers (P1/P2) (see Fig.2). This is

highly indicative that the recombinant plasmid was constructed, and, after this confirmation, the plasmid was named pMD19-T-CyHV. Blast analysis of the sequencing result of the target DNA fragment of CyHV2 (see Fig.3) revealed that it had a 100% homology with the reference sequences (HM014349, JQ815364,etc) that are published in GenBank.



M: DNA marker DL2000,1: target DNA fragments

Fig. 1. Synthesis result of target DNA fragments on electrophoresis



M: DNA marker DL2000;
1: PCR(P1/P2as primer) products of pMD19-T-CyHV2,
2: PCR(RV-M/M13-47 as primers) products of pMD19-T-CyHV2

Fig. 2. The PCRs identification results of pMD19-T-CyHV2 construction

ATGCCTGCAGGTCGACGATTAGTGTGTTTTACAGCGTTCTCGACCAGCAGAATCTTTT
GGCGTCCGGACCAAGTAGTCAGATGCTGACAGAGACTCTGGCTCACGCCGTAGTGAG
AGTTGGCGCATACTTTCATCTCACCCTGCATCTGATCATAGTACGCCTTGAGACTCGC
GTCTTTCTCGGTCTTGATGCGTTTCTTGTACTGAGATCTCATAGAGAGGTAGTGGCCC
AGACTGGAAGCGGTGTAGGTCACAAACCGAGTCCAACCGACCTCTCGGTTCTTGTAG
CACAGGATCGTGTACAGGTCTTCGCTGTGCGGGTATTCCTTGATCTCTTCCATGTTACT
GGCGGCCGGTATCTTTTTCCAATCGAGCAGCACGCAGTTTCTGACATACTCGAGACAG
AACGGGTCGCTCCTAGACACTATGGACTAATCTCTAGAGGATCCCCGGG

Fig. 3. Sequencing results of synthesized target DNA fragments

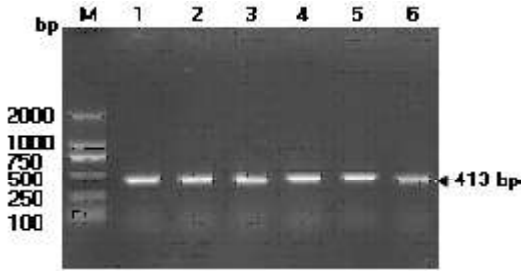
Optimization of reaction conditions

The specific primers P1/P2 and pMD19-T-CyHV2 were used in the gradient PCR so as to determine an ideal annealing degree and reaction time. The results (as shown in Fig.4) show that the PCR products present in lane 4 (60°C) are present in a greater quantity than in the other lanes, which indicates that the optimum conditions for creating a high specificity and amplification efficiency are 60°Cas the ideal annealing degree along with a 30 s reaction time for amplification cycles.

Specificity and sensitivity of CyHV2 PCR

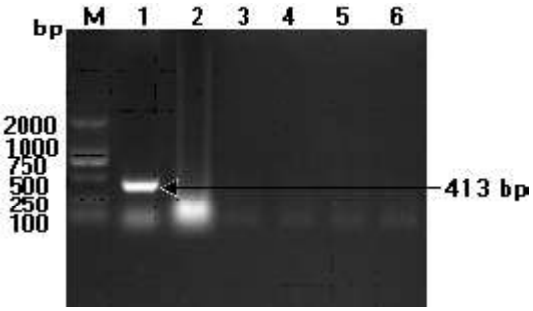
The specificity and sensitivity of the CyHV2 PCR were assessed in the tests that were previously described in the methods section. There was a clear electrophoretic band located at approximately 413 bp that was only observed for the pMD19-T-CyHV2 detection (lane1), and no specific amplification products were obtained for the detection of the *Aeromonas veronii*,

Aeromonas hydrophila, *Pseudomonas fluorescens*, and *Streptococcus* isolated from fish, nor was there from the normal sample of *Carassius auratus gibelio* (as shown in Fig.5). For the sensitivity, the expected PCR products' size of 413 bp could be observed (lane 4) when almost 62 target copies (pMD19-T-CyHV) were detected (as shown in Fig.6).



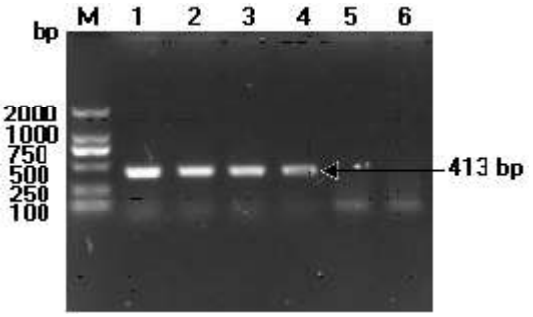
M: DNA marker DL2000,1:57°C, 2:58°C,3:59°C, 4:60°C, 5:61°C, 6:62°C

Fig. 4. The optimization results of annealing degree determination



M: DNA marker DL2000, 1: pMD19-T-CyHV, 2: tissues of healthy *Carassius auratus gibelio*,, 3: *Aeromonas veronii*,4: *Aeromonas hydrophila*, 5. *Pseudomonas fluorescens* 6: *Streptococcus*

Fig. 5. The specificity assay results of CyHV2 PCR



M: DNA marker DL2000;
1: 6.2×10^4 copies,2:6.2×10^3 copies,3. 620 copies, 4: 62 copies, 5. 6.2 copies, 6: Negative control

Fig. 6. The sensitivity assay results of CyHV2 PCR

DISCUSSION

To our knowledge, there are no current high-performance measures capable of controlling a CyHV2 infection. Therefore, enhancing CyHV2 quarantining through both diagnosis and detection are still the main methods for preventing the spread of CyHV2. Cell culture lines and molecular techniques are the classical approaches to studying most fish diseases; however, culturing CyHV2 in cell strains or lines is a very difficult task, and as a result, there have been many sensitive and specific molecular detection methods developed for this task, such as PCR, real-time PCR, and LAMP[4,6,13,14]. Each of these methods has their own inherent advantages and disadvantages. For instance, both real-time PCR and LAMP are more sensitive, but both of their target fragments are shorter. Furthermore, real-time PCR requires more expensive apparatus, and identifying LAMP products remains difficult. Therefore, in this study, a conventional PCR assay based upon the DNA-dependent DNA polymerase gene was established for CyHV2 detection with a specific product of about 413 bp. Most notably, a new, useful approach for genetic engineering methods, the overlap extension PCR, was used for the *in vitro* synthesis of target DNA fragments of the CyHV2 DNA-dependent DNA polymerase gene because of the lack of CyHV2 DNA, and this is the first account of using overlapping PCR to develop a CyHV2-detectable PCR method which is based on DNA polymerase gene.

To summarize, the aforementioned PCR assays have been proven to be a sensitive yet specific approach for rapidly detecting CyHV2. As few as approximately 62 copies of the target gene could be detected, and no cross reaction was observed with other common main pathogens, including *Aeromonas veronii*, *Aeromonas hydrophila*, *Pseudomonas fluorescens*, and *Streptococcus* isolated from fish and a normal sample of *Carassius auratus gibelio* as detected by this method. In brief, this study supplied a simple, rapid, and useful detection and diagnostic tool for the molecular epidemiology investigation of CyHV2 in China.

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