Development of nested PCR for direct detection and diagnosis of cyprinid herpesvirus 3 infection in carp

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Cyprinid herpesvirus 3 (CyHV3), also known as koi herpesvirus (KHV), is a double-stranded DNA virus that causes a severely progressive disease in common carp and koi carp (both *Cyprinus carpio*) worldwide. In this present study, a specific and sensitive method for definitive diagnosis of CyHV3 infection was developed using nested polymerase chain reaction (nested PCR)-based technique. Two primer sets were designed and used to detect 1,322 and 919 bp of the CyHV3 genome in the nested PCR assay. The primers were shown to be exclusively specific for CyHV3 DNA because the amplicon was not detected using DNA extracted from either healthy carp or animals naturally infected by other animal herpesviruses, i.e. pseudorabies virus and duck plaque virus. Using 10-fold serial dilutions of CyHV3 DNA, the detection limit of the developed nested PCR was 12 zg and shown to be greater than (10^9 times) that of IQ2000™ KHV Detection and Prevention System, a commercial nested PCR test kit widely used in laboratories. Additionally, in field application, the developed nested PCR was able to detect the presence of CyHV3 in carp specimens collected from different locations in the Bangkok area in 2009. In conclusion, the developed nested PCR can, therefore, be used as a specific and sensitive method for direct detection and diagnosis of CyHV3 infection in carp.

Keywords : carp, CyHV3, KHV, nested PCR

Introduction

Koi herpesvirus disease (KHVD), which affects common carp (*Cyprinus carpio*) and its varieties such as koi carp and ghost carp worldwide, is caused by a double-stranded DNA virus, koi herpesvirus (KHV) (OIE, 2009a, b). Being a member of the family *Herpesviridae*, this virus was newly classified and renamed as cyprinid herpesvirus 3 (CyHV3), following the nomenclature of other cyprinid herpesviruses, i.e. CyHV1 (carp pox virus or fish papilloma virus) and CyHV2 (goldfish haematopoietic necrosis virus). The estimated size of the CyHV3 genome is now confirmed as 295 kb (Hutoran *et al*., 2005; Aoki *et al*., 2007). Comparisons of the viral genomes of different isolates collected from many locations by nucleotide sequence analysis have suggested them to be genetically identical (Aoki *et al*., 2007; Bigarre *et al*., 2009). Fish infected with CyHV3 develop gross signs that

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are primarily characterized by nephritis, necrosis of the gills, and abundant mucus production on the body. Histopathological lesions are largely resulted from inflammation and necrosis of gill tissues, consisting of hyperplasia and hypertrophy of branchial epithelium, fusion of secondary lamellae, and adhesion of gill filaments. Prominent nuclear swelling, margination of chromatin, and pale diffuse eosinophilic intranuclear inclusions may also be seen within branchial epithelial cells and leucocytes, whereas inflammation and necrosis of other organs, e.g. kidney, spleen, liver, is commonly observed.

Diagnosis of KHVD in infected fish can also be accomplished by a number of laboratory methods. Although virus isolation in cell culture is likely, it is considered to be unreliable since CyHV3 can be propagated in a limited number of cells; moreover, these cells are very difficult to handle (OIE, 2009b). Immunodiagnostic methods, such as immunofluorescence (IF) tests and enzyme-linked immunosorbent assays (ELISAs) can be suitable for rapid identification and diagnosis of KHVD, but they have been scarcely devised and validated (Perelberg *et al*., 2008). Fortunately however, molecular methods, in particular polymerase chain reaction (PCR) followed by sequence analysis of the PCR products, have become broadly accepted to date to be the most reliable and sensitive tool for direct detection of CyHV3 in fish tissues. Not only can these techniques be used for clinically diseased fish, but they also allow differentiation of subclinical levels of virus especially from apparently healthy fish (Gilad *et al*., 2003; St-Hilaire *et al*., 2005; Bercovier *et al*., 2005). In the present study, we develop a new protocol based on nested PCR principle and compare this protocol to a commercial nested PCR test kit.

Materials and methods

Virus and specimens

Positive control samples for CyHV3 were kindly provided by Faculty of Veterinary Medicine, Chulalongkorn University, whereas DNA of 2 other herpesviruses, i.e. pseudorabies virus (PRV) and duck plague virus (DPV) extracted from their host animals and used in the specificity test, were provided by courtesy of Virology Section, National Institute of Animal Health (NIAH). Healthy carp samples were used as negative control, whereas carp (8 totals), suspicious of KHVD originated from farms as well as residences in the Bangkok area in 2009 and submitted to NIAH for routine diagnosis, were used as test specimens. The latter exhibited clinical signs, including anorexia, decreased body movement, increased mucus production, and necrosis of gill and skin, and their mortality was approximately at 70–80%.

Identification of CyHV3 by nested polymerase chain reaction (nested PCR)

Primer Express software (Applied Biosystems) was used to design external and internal primers from the CyHV3 genome to amplify a hypothetical protein (protein ID:

Table 1. Specific primers used in nested PCR analysis of CyHV3

Primers	Nucleotide sequences (5' > 3')	Amplicon size (bp)	Nucleotide positions*
KHE1	CAG CAG CAC CAG CGT CTC TT	1,322	92,723 – 94,044
KHE2	GCA GGA ACG GAC ACC ACA TC		
KHI3	TCG CGC ATA GTA GAC CGA GT	919	92,936 – 93,854
KHI4	GTC GCA GTC GTT CAA GAA GC		

*CyHV3 Genome (GenBank Accession No.: AP008984)

BAF48865.1) from nucleotide positions 92,723 to 94,044 (1,322 bp) and 92,936 to 93,854 (919 bp) (Table 1). DNA was extracted from gill tissues collected from affected fish using QIAamp DNA Mini Kit (Qiagen) following the manufacturer's instruction. The reaction mixture for the first step-PCR contained 3 µl of DNA sample, 12.5 µl of 2x GoTaq Green Master Mix (Promega), 300 nM each of sense (KHE1) and antisense (KHE2) primers in a final volume of 25 µl. The PCR profile was 5 min at 95°C for pre-denaturation followed by 35 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1.5 min for amplification, and a final extension of 72°C for 7 min. The amplified product was analyzed by gel electrophoresis on a 1.5% agarose gel, which was then submerged for 15 min in an ethidium bromide-containing buffer (0.3 ng/l), read and recorded on a UV transilluminator. The second (nested) step of PCR was nearly identical to that of the first step as herewith described except that primer KHI3, primer KHI4, and the first step's PCR product (3 µl) were instead utilized.

Specificity and sensitivity tests

Specificity of the developed nested PCR was assessed using various animal herpesviruses, including CyHV3, PRV, and DPV, while its sensitivity was determined using 10-fold serial dilutions of CyHV3 DNA (1200 ng – 12000 yg).

Comparison of the developed nested PCR to IQ2000 KHV Detection and Prevention System

Sensitivity of a commercial nested PCR test kit, IQ2000 Detection and Prevention System (Farming IntelliGene, Taiwan), was determined using the consecutive 10-fold serial dilutions of CyHV3 DNA (1200 ng – 12 ag) following the manufacturer's instruction manual.

Results

A specific and sensitive nested PCR analysis was developed for direct detection and diagnosis of CyHV3 infection in carp. Two sets of primers were designed and used to amplify specific regions of the CyHV3 genome (Table 1). Using CyHV3 DNA, the first and second steps of nested PCR yielded amplified products of 1,322 bp and

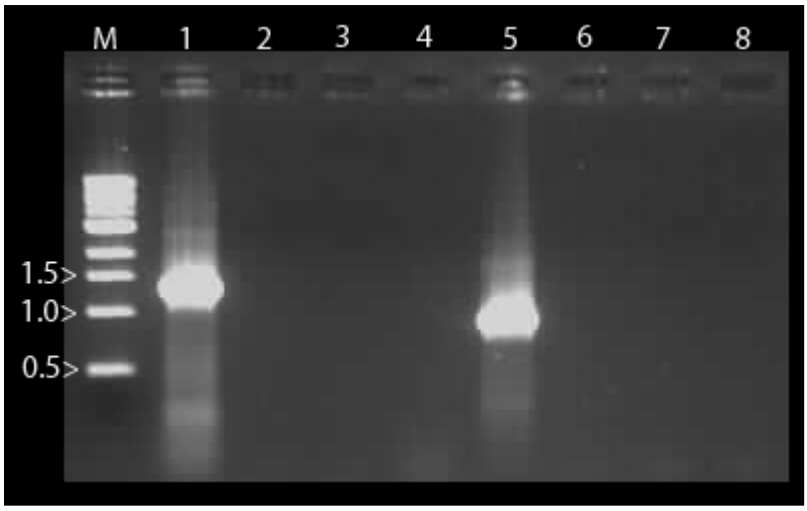

Fig. 1. Specificity of the nested PCR. Included in the first (lanes 1 – 4) and second (lanes 5 – 8) steps of the analysis was DNA extracted from host animals infected by various herpesviruses, i.e. CyHV3 (lanes 1, 5), pseudorabies virus (lanes 2, 6), and duck plague virus (lanes 3, 7) as well as DNA from healthy carp (lanes 4, 8).

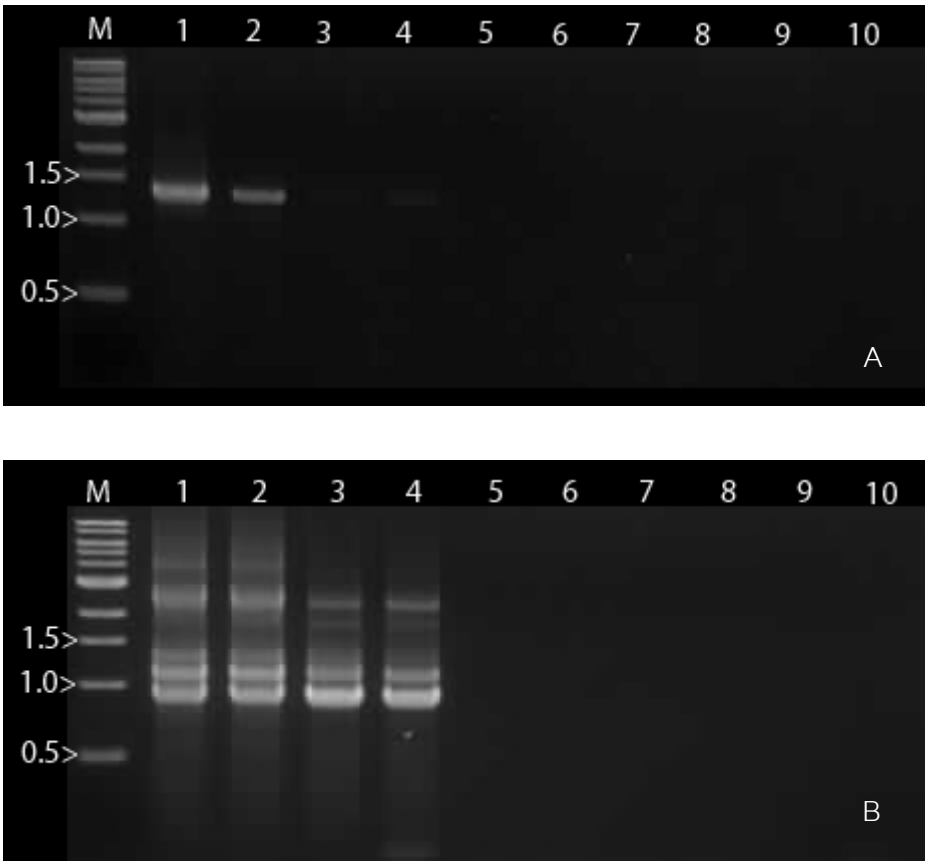

Fig. 2. Sensitivity of the (A) first and (B) second steps of the nested PCR using 10-fold serial dilutions of CyHV3 DNA (lanes 1 – 10: 12 ag – 12000 yg). Note that all of the tests were performed in duplicate.

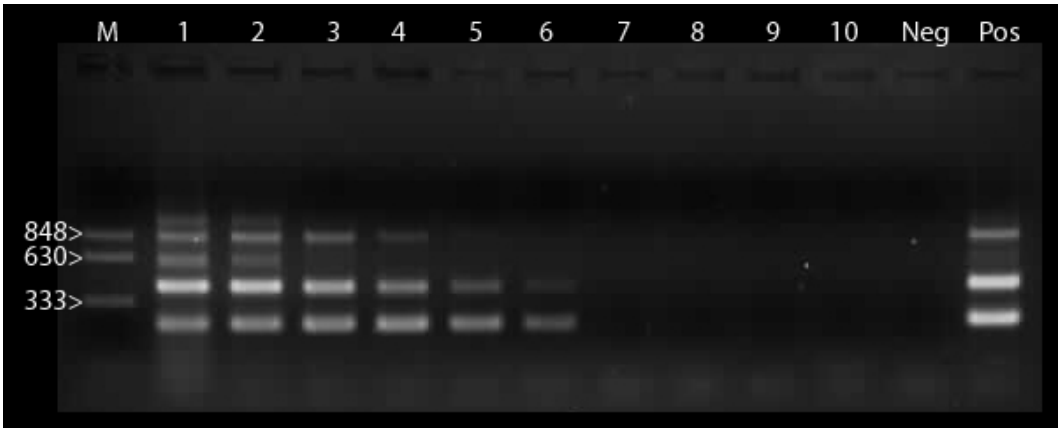

Fig. 3. Sensitivity of a commercial nested PCR test kit, IQ2000 KHV Detection and Prevention System, using 10-fold serial dilutions of CyHV3 DNA (1200 ng – 12 ag). Note that all of the tests were performed in duplicate.

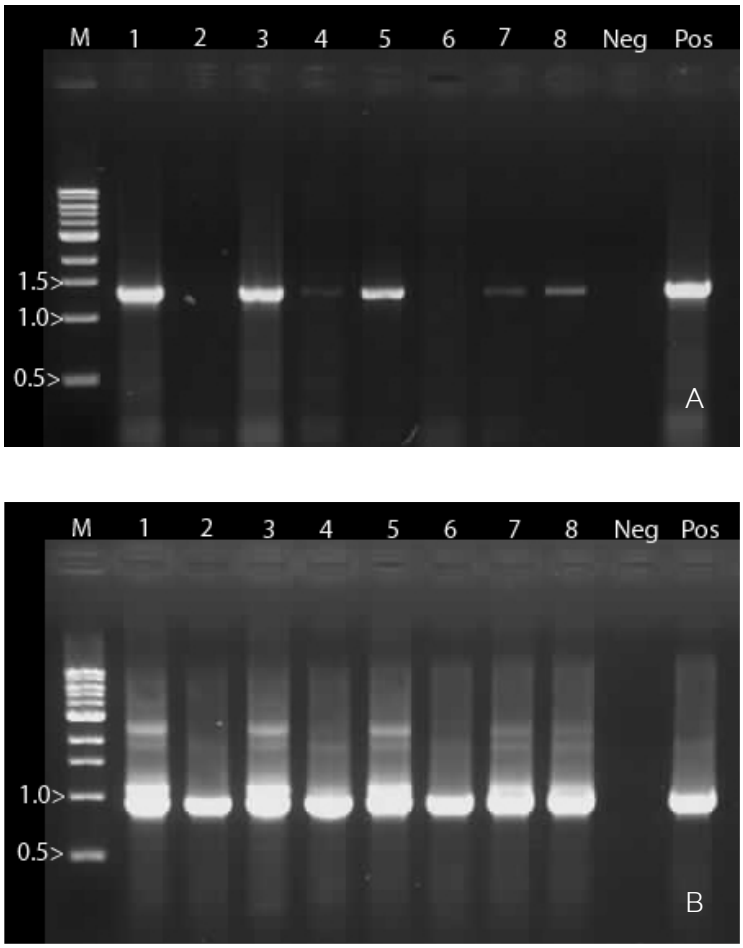

Figure 4. Nested PCR assay of CyHV3 in field carp specimens (lanes 1 – 8). PCR products at approx. 1,322 bp (A) and 919 bp (B) long were shown after analyzed by gel electrophoresis on a 1.5% agarose gel submerged in ethidium bromide (0.3 ng/l).

919 bp (Fig. 1). On the contrary, the amplified product was not found in PCR assays using DNA extracted from healthy carp nor animals infected by other herpesviruses, including PRV and DPV. Using 10-fold serial dilutions of CyHV3 DNA, the detection limit of the developed PCR was shown to be at 12 zg (Fig. 2). In order to compare to that of our developed nested PCR, the sensitivity of a commercial nested PCR test kit, IQ2000 KHV Detection and Prevention System, was also determined using the consecutive 10-fold serial dilutions of the CyHV3 DNA. The results showed that the detection limit of the test kit equals 12 pg; thus, our developed PCR is billion (10^9) times more sensitive than the test kit (Fig. 3). In field application, the PCR protocol was shown to be able to detect the presence of CyHV3 in 8 carp specimens collected from different locations in the Bangkok area in 2009 (Fig. 4).

Discussion

Diagnostic tests with high analytical sensitivity are required to detect CyHV3 in carriers that have been implicated in the dissemination of KHVD (El-Matbouli and Soliman, 2007). In this present study, a nested PCR was newly invented for direct detection and diagnosis of CyHV3 infection in carp. The nested PCR protocol was optimized for sufficient concentrations, temperatures and operation time to obtain the best yield of amplification products. Specifically, its specificity was monitored using different herpesviruses to disprove if the gene could be found within other members of the family *Herpesviridae*. The results revealed that the primers designed were highly specific to CyHV3, while they were not be able to amplify genes of other herpesviruses examined as well as those of healthy carp (Fig. 1). Sensitivity of the nested PCR being developed was investigated and affirmed that a negligible amount of template DNA is required for the amplification reaction to commence (Fig. 2). The results also showed that the nested primers, applied in the second step of the PCR, considerably augmented the sensitivity by up to thousand (10^3) times when compared to that of the first step. More importantly, the sensitivity of the nested PCR has surpassed that of IQ2000 Detection and Prevention System, a commercial nested PCR test kit that is imported and routinely used at NIAH as well as in many other laboratories (Fig. 3). Similar results from a previous study recently found that the diagnostic sensitivity of molecular tools used for detection of CyHV3 may have been different (Bergmann *et al.*, 2010). The study also suggested that a modified real-time PCR (Gilad *et al.*, 2004) combined with an internal control system be used if the virus concentration is to be reliably determined. The nested PCR protocol should, however, be an alternate method for the identification of CyHV3 for definitive diagnosis. Practicability of the nested PCR was substantiated through the application of field specimens of carp naturally afflicted with KHVD (Fig. 4). In summary, the nested PCR protocol developed in the present study can be used as a specific and sensitive method for direct detection and diagnosis of CyHV3 infection in carp.

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การพัฒนาวิธีทดสอบและวินิจฉัยเชื้อ cyprinid herpesvirus 3 ในปลาคาร์พด้วยวิธี nested PCR

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Cyprinid herpesvirus 3 (CyHV3) (koi herpesvirus, KHV) เป็นไวรัสที่มี DNA สายคู่ ก่อโรคระบาดรุนแรงในปลาไนและปลาคาร์พ (*Cyprinus carpio*) พบโรคนี้ได้ทั่วโลก การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อพัฒนาวิธีทดสอบ CyHV3 ที่มีความจำเพาะและความไวสูง โดยอาศัยหลักการ nested polymerase chain reaction (nested PCR) primers 2 คู่ได้รับการออกแบบขึ้นเพื่อใช้เพิ่มจำนวนยีนส์จำเพาะของ CyHV3 ขนาด 1,322 bp และ 919 bp ในปฏิกิริยา nested PCR โดยพบว่า primers ที่พัฒนาขึ้นนี้มีความจำเพาะสูงต่อ CyHV3 และไม่จำเพาะต่อ DNA จากปลาคาร์พปกติ รวมทั้งต่อ herpesviruses ชนิดอื่น ซึ่งได้แก่ pseudorabies virus และ duck plague virus ผลการทดสอบความไวโดยการใช้ 10-fold serial dilutions ของ CyHV3 DNA พบว่าวิธี nested PCR ที่พัฒนาขึ้นนี้มีความไวเท่ากับ 12 zg ไวมากกว่าชุดทดสอบสำเร็จรูป IQ2000 Detection and Prevention System (10^9 เท่า) ซึ่งใช้กันอยู่ทั่วไปในห้องปฏิบัติการหลายแห่ง นอกจากนี้วิธีที่พัฒนาขึ้นยังสามารถใช้ทดสอบ CyHV3 จากปลาคาร์พป่วยที่ได้จากแหล่งเลี้ยงต่างๆ ในเขตกรุงเทพมหานครในปี 2552 อีกด้วย ดังนั้นจึงสรุปได้ว่าวิธี nested PCR ที่ได้พัฒนาขึ้นมีความจำเพาะและความไวสูงต่อ CyHV3 และสามารถใช้ในการทดสอบและวินิจฉัยการติดเชื้อ CyHV3 ในปลาคาร์พได้

คำสำคัญ : ปลาคาร์พ, CyHV3, KHV, nested PCR