Research article

First Report of Occurrences of Two Cyprinid Herpesvirus 3 Variants, I⁺⁺ II⁻ and I⁺⁺ II^{+Δ}, in China

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ABSTRACT—Cyprinid herpesvirus 3 (CyHV-3) is the cause of a lethal disease named as koi herpesvirus disease (KHVD) in carp worldwide. Even though CyHV-3 has been found in China, little is known about the variety of CyHV-3 genotypes distributing in this area. In the present study, to improve the sensitivity of current genotyping approaches, a nested-PCR method was constructed based on the duplex PCR reported previously. The nested-PCR targeting the markers I/II loci was used in genotype identification of CyHV-3 infected fish in Guangdong Province in 2010–2015 and Henan Province in 2013. We detected 3 genotypes including an Asian dominant type (I⁺⁺ II⁺) and two emerging genotypes in East Asian and South-East Asian regions (I⁺⁺ II⁻ and I⁺⁺ II^{+Δ}). I⁺⁺ II⁻ was the predominant genotype in this study. Two CyHV-3 variants, ZS410 and HZ419 isolates, were successfully isolated in cell culture and exhibited the genotype of I⁺⁺ II⁻ and I⁺⁺ II^{+Δ}, respectively. This is the first report of both I⁺⁺ II⁻ and I⁺⁺ II^{+Δ} genotypes isolated in China. These data can contribute consideration and exploration of mode of CyHV-3 spread in China.

Key words: cyprinid herpesvirus 3, KHV, genotype, variant, epidemiology, China, nested-PCR

Cyprinid herpesvirus 3 (CyHV-3), also known as koi herpesvirus (KHV), is the cause of the lethal disease named as koi herpesvirus disease (KHVD) in carp *Cyprinus carpio* and the varieties. In the last two decades, CyHV-3 has emerged as a serious threat to the production of carp worldwide (Hedrick *et al.*, 2000; Michel *et al.*, 2010; Gotesman *et al.*, 2013). This virus has caused massive economic losses in Asia specifically (Sano *et al.*, 2004; Tu *et al.*, 2004; Bondad-Reantaso *et al.*, 2007; Lio-Po *et al.*, 2009; Pikulkaew *et al.*, 2009; Han *et al.*, 2013). Recently, CyHV-3 has also been detected in China (Dong *et al.*, 2011, 2013; Zhou *et al.*, 2014a, 2014b). CyHV-3 represents a member of genus *Cyprinivirus* belonging to the family of *Alloherpesviridae* and has a conserved 295 kb genome. Genome sequences of three CyHV-3 isolated from Israel (CyHV-3-I), the USA (CyHV-3-U), and Japan (CyHV-3-J) reveal that these three strains share more than 99% sequence identity (Aoki *et al.*, 2007; Davison *et al.*, 2009; Avarre *et al.*, 2011). The little genetic variation lays the foundation for discrimination among different CyHV-3 lineages.

At present, three CyHV-3 genotyping approaches have been constructed mainly based on polymerase chain reaction (PCR). The first PCR detection method focuses on the sequence variety on two regions: marker I (located between *ORF29* and *ORF30*) and marker II (located around the initiation codon of *ORF133*) (Bigarré *et al.*, 2009). These two markers show differences in the length of sequence between CyHV-3-J and CyHV-3-U/I. For example, the PCR product of marker I is a 168 bp fragment (I⁺⁺) in J strain, while the corresponding fragment is 130 bp in U/I strain (I⁻) due to the absence of two domains. The amplification of marker II produces a 352 bp fragment in J strain (II⁺) but a 278 bp fragment in U/I strain (II⁻). Besides the above two major genotypes (I⁺⁺ II⁺ and I⁻ II⁻), two additional lineages have been also reported, namely I⁺⁺ II⁻ from Poland and Netherlands, and I⁺⁺ II⁻ from Indonesia (Sunarto *et al.*, 2011). Recently, a novel genotype which carries a 13 bp deletion in marker II has been reported (I⁺⁺ II^{+Δ}) (Chen *et al.*, 2014). The second genotyping method targets three genomic regions including enlarged 9/5, enlarged SphI5 and enlarged thymidine kinase gene regions (Kurita *et al.*, 2009). Compared with the first method, the advantage of this genotyping approach is that the enlarged

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sequences differ in ten variable areas among different CyHV-3 lineages and enable the classification in a more detailed manner. Therefore, based on these variations, the CyHV-3 can be divided into two Asian variants (A1 and A2) and seven European genotypes (E1 to E7) (Kurita *et al.*, 2009).

The third genotyping method focuses on the differences in the variable number of tandem repeats (VNTR) among CyHV-3 lineages and has been recently applied to discriminate among various isolates. Eight loci are chosen for the multiple locus VNTR analysis (MLVA) and the number of alleles in each VNTR loci varies from 2 to 8, which demonstrates considerable genetic polymorphisms. Based on the distinct VNTR molecular markers, the genotypes of two CyHV-3 isolates can be easily distinguished from each other efficiently (Avarre *et al.*, 2011).

In most cases these three genotyping approaches have been used independently to characterize a new isolate. In general, the first and second genotyping methods (Bigarré *et al.*, 2009; Kurita *et al.*, 2009) are preferentially chosen due to their simplicity for targeting 2 or 3 variable regions (Bigarré *et al.*, 2009; Kurita *et al.*, 2009; Sunarto *et al.*, 2011; Kim and Kwon, 2013). To further improve the efficiency of duplex PCR for marker I/II, we optimized the approach with a newly designed nested-PCR.

Although European genotype of CyHV-3 has been identified in China (Dong *et al.*, 2013), the distribution of other genotypes in this area is still unclear. Guangdong Province is the most important koi carp culture zone in China. Henan Province located at the central part of China is the major common carp producing area in China. In the present study, genotypes of 27 samples collected from Guangdong Province and Henan Province in China were analyzed by the marker I/II genotyping system and two novel CyHV-3 isolates, ZS410 and HZ419, have been isolated from infected koi carp. In addition, a nested-PCR was constructed based on the previously reported duplex PCR (Bigarré *et al.*, 2009) in order to improve the sensitivity for genotyping. The nested-PCR results showed that I⁺⁺ II⁻ was the dominant genotype in the regions and together with another two genotypes identified in this study, I⁺⁺ II^{+Δ} and I⁺⁺ II⁺, were first reported in China.

Materials and Methods

CyHV-3 infected samples and CyHV-3 isolation

Total 27 CyHV-3 infected sample collected from different fish farms in Guangdong Province 2010–2015 and Henan Province in 2013 (Table 1). DNA was extracted from the brain tissue of the diseased fish with a TIANamp Genomic DNA Kit (Tiangen Biotech Co., Ltd.), and then used as the template for the diagnosis of CyHV-3 as described previously (Yuasa *et al.*, 2005).

The genomic DNA of KHV-U and KHV-I (Hedrick *et al.*, 2000) were kindly provided by Dr. Ronald P. Hedrick through National Research Institute of Aquaculture (NRIA), Fisheries Research Agency, Japan. The genomic DNA of CyHV-3-NRIA0301 was kindly provided by Dr. Kei Yuasa, NRIA.

Koi fin cell (KFC) (Ronen *et al.*, 2003) was used in this study for the virus isolation. Diseased koi carps (S14 and S17 in Table 1) for CyHV-3 isolation from two fish farms located in Zhongshan and Huizhou, Guangdong Province, China in 2011 and 2012, respectively. The gills, kidney and brain were collected and pooled. The mixed tissues were homogenized with MEM medium (Gibco) containing penicillin-streptomycin (1,000 U/mL) at a ratio of 1:9 (w/v), and then stored overnight at 4°C. The supernatant was obtained by centrifugation at 3,000×g at 4°C for 20 min, then 400 µL of supernatant was inoculated onto the KFC cells grown in 25 cm² cell culture flasks with 8 mL MEM (10% FBS, Gibco). The cultures were incubated at 25°C for observation of the cytopathic effect (CPE). Finally, two CyHV-3 isolates isolated from S14 and S17 samples were designated as ZS410 and HZ419, respectively.

The KFC cells infected with ZS410 and HZ419 isolates and the tissue culture supernatant were collected and examined by transmission electronic microscopy (TEM) (Hitachi, Ltd.), according to the method described previously (Sunarto *et al.*, 2011).

Nested-PCR

Primer pairs of oPVP53/oPVP54 and oPVP55/oPVP56 targeting the marker I and marker II regions, respectively, were used for the first round of amplification as described previously (Bigarré *et al.*, 2009). Two new primer pairs SF (5'-AGGAGCCATCATCGGCAA-3') and SR (5'-AAGGTGCCGAGGTGGTTGT-3') and LF (5'-GTTAGTGCAACTAAGTAAATG-3') and LR (5'-CGC-CGCCTGTTGACCTTA-3') were designed and bound to the interior sites in the first PCR products of markers I and II. The primer sets SF/SR and LF/LR were used in the second round of amplification for a nested-PCR.

In the second round of PCR reaction, the products obtained from the first PCR were diluted by 100-fold with distilled water, and 1 µL of the dilution was used as template. The reaction mixture contained 1.25 U of Ex Taq polymerase (TaKaRa, Japan), 5 µL of 10×PCR buffer (Mg²⁺ plus), 4 µL of a 2.5 mM dNTP mixture, 0.4 µM primer SF, 0.4 µM primer SR, 0.4 µM primer LF, 0.4 µM primer LR. Total volume of reaction mixture was made up to 50 µL with distilled water. The PCR program was established as follows: 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 20 s and a final extension at 72°C for 1 min.

The sensitivity of the nested-PCR was compared with that of duplex PCR (Bigarré *et al.*, 2009). Specifically, 40 ng, 8 ng, 1.6 ng, 0.32 ng and 0 ng of the total

Table 1. The samples used in this study for genotyping of CyHV-3 by the markers land II classification system

sample number	isolate	area	fish	year	genotype
S1	JM110901	Jiangmen, Guangdong	Koi carp	2010	I ⁺⁺ II ⁻
S2	JM110902	Jiangmen, Guangdong	Koi carp	2010	I ⁺⁺ II ⁻
S3	JM110903	Jiangmen, Guangdong	Koi carp	2010	I ⁺⁺ II ⁻
S4	JM110904	Jiangmen, Guangdong	Koi carp	2010	I ⁺⁺ II ⁻
S5	JM110905	Jiangmen, Guangdong	Koi carp	2010	I ⁺⁺ II ⁻
S6	JM110906	Jiangmen, Guangdong	Koi carp	2010	I ⁺⁺ II ⁻
S7	JM110907	Jiangmen, Guangdong	Koi carp	2010	I ⁺⁺ II ⁻
S8	JM110908	Jiangmen, Guangdong	Koi carp	2010	I ⁺⁺ II ⁻
S9	JM110909	Jiangmen, Guangdong	Koi carp	2010	I ⁺⁺ II ⁻
S10	JM110910	Jiangmen, Guangdong	Koi carp	2010	I ⁺⁺ II ⁻
S11	FC1001	Guangzhou, Guangdong	Koi carp	2010	I ⁺⁺ II ⁻
S12	MY1002	Guangzhou, Guangdong	Koi carp	2010	I ⁺⁺ II ^{+Δ}
S13	DM1101	Zhuhai, Guangdong	Koi carp	2011	I ⁺⁺ II ⁻
S14	ZS410	Zhongshan, Guangdong	Koi carp	2011	I ⁺⁺ II ⁻
S15	DM1201	Dongguan, Guangdong	Koi carp	2012	I ⁺⁺ II ⁻
S16	ZS1202	Zhongshan, Guangdong	Koi carp	2012	I ⁺⁺ II ⁻
S17	HZ419	Huizhou, Guangdong	Koi carp	2012	I ⁺⁺ II ^{+Δ}
S18	GZ1301	Guangzhou, Guangdong	Koi carp	2013	I ⁺⁺ II ⁺
S19	GZ1302	Guangzhou, Guangdong	Koi carp	2013	I ⁺⁺ II ⁻
S20	ZS1301	Zhengzhou, Henan	Common carp	2013	I ⁺⁺ II ⁻
S21	HE1302	Zhengzhou, Henan	Common carp	2013	I ⁺⁺ II ⁻
S22	XY1303	Zhengzhou, Henan	Common carp	2013	I ⁺⁺ II ⁻
S23	LE1304	Zhengzhou, Henan	Common carp	2013	I ⁺⁺ II ⁻
S24	SS1305	Zhengzhou, Henan	Common carp	2013	I ⁺⁺ II ⁻
S25	HN1306	Zhengzhou, Henan	Common carp	2013	I ⁺⁺ II ⁻
S26	DG1401	Dongguan, Guangdong	Koi carp	2014	I ⁺⁺ II ⁺
S27	ZS1504	Zhongshan, Guangdong	Koi carp	2015	I ⁺⁺ II ⁺

DNA from koi carp infected with NR1A0301 were adjusted to 100 ng with DNA from uninfected koi carp, and mixed DNA were used as templates for comparison of the sensitivity. The nested-PCR was conducted with representative 5 isolates, NR1A0301, KHV-U, KHV-I, ZS410 and HZ419 to observe the positions of amplified bands in gel. In addition, the bands were cloned in pMD18-T vector (TaKaRa) for sequencing with an ABI PRISM377 DNA Sequencer (Applied Biosystems) to confirm each size of amplicon.

Genotyping analysis

The genotypes of the samples S1–S27 were analyzed by the nested-PCR. The genotypes of ZS410 and HZ419 isolates were further determined by the Asian/European (A/E) classification system (Kurita *et al.*, 2009).

Results

Isolation of CyHV-3

CPE on KFC cells was induced by the tissue extracts from fish samples S14 and S17 at about 14 days post infection (d.p.i.). Severe vacuolation could be observed in the infected KFC cells and the isolated viruses were designated as ZS410 and HZ419 isolates, respectively. The isolates were passaged in KFC cells for 10 times and CPE was usually observed at 5 d.p.i.

(Fig. 1, A). The typical herpesvirus hexagonal capsid configuration and envelope could be demonstrated by the TEM (Fig. 1, B and C).

Nested-PCR

The sensitivity of the nested-PCR was nearly 25 times higher than that of duplex PCR (Fig. 2), which would improve accuracy and capability of genotype identification. The electrophoretic pattern of the nested-PCR was similar to that of duplex PCR (Bigarré *et al.*, 2009) (Fig. 3). The products of marker I⁺⁺ (e.g. in NR1A0301, HZ419, ZS410 isolates) and I⁻ (e.g. in U and I strains) obtained from the nested-PCR were 141 bp and 103 bp, respectively. The products of marker II⁺ (e.g. in NR1A0301 isolate), II^{+Δ} (e.g. in HZ419 isolate) and II⁻ (e.g. in U, I, ZS410 isolates) by the nested-PCR were 296 bp, 283 bp and 222 bp, respectively.

Genotyping

Genotypes of 27 samples were determined with the analysis of markers I/II regions by the nested-PCR (Table 1). The result indicated that most of the samples (22/27) including the ZS410 isolate exhibited the I⁺⁺ II⁻ genotype, which was first reported in Indonesia (Sunarto *et al.*, 2011). In addition, only three isolates showed the I⁺⁺ II⁺ genotype and two isolates including the HZ419 isolate carried the recently identified genotype I⁺⁺ II^{+Δ} (Chen *et al.*, 2014) (Fig. 4).

Fig. 1. Isolation and identification of the CyHV-3 isolates. (A) Cytopathic effect on KFC cells caused by the infection of HZ419 isolate. (B) Many capsids (white arrow) were observed in the nucleus of the infected KFC cells by transmission electronic microscopy. (C) The relatively intact virus with an envelope (black arrow).

Fig. 2. The sensitivity of the nested-PCR and duplex PCR targeting marker I/II. Lane 1 (40 ng), 2 (8 ng), 3 (1.6 ng), 4 (0.32 ng) and 5 (0 ng). The genomic DNA from a koi carp infected with CyHV-3-NRIA0301 were adjusted to 100 ng with DNA from an uninfected koi carp.

Fig. 3. The electrophoresis patterns of Markers I and II fragments obtained by the nested-PCR. Lanes 1: CyHV-3-NRIA0301, 2: CyHV-3-U, 3: CyHV-3-I, 4: ZS410, 5: HZ419, 6: negative control (uninfected koi carp DNA). M: DL1000 DNA ladder.

Fig. 4. Alignment of the markers I (A) and II (B) sequences in representative CyHV-3 isolates by the MUSCLE program. The primers binding sites of SF/SR (A) and LF/LR (B) were denoted and boxed. The 70 bp repeated motifs in marker II region of J strain could be divided into two tandem-repeated sequences of 18 bp and 52 bp which were underlined by solid and dotted lines, respectively (B). The start codon of ORF133 was highlighted in bold and italic (B). The G shown in grey may be replaced by A in some isolates with the genotype I⁺⁺ II^{++Δ} (Chen *et al.*, 2014).

J: Japan, I: Israel, U: United States, NL: Netherlands, PL: Poland, MY: Malaysia, ID: Indonesia.

According to the A/E classification standard, HZ419 and ZS410 isolates belong to A1 variant, which had been detected in the East and Southeast Asia, including Japan, Indonesia, Philippines, Korea and Taiwan China, (Kurita *et al.*, 2009; Kim and Kwon, 2013). This results were consistent with that of genotypic analysis with markers I/II, which revealed a closely genetic relationships between the two isolates and other isolates from Asia region. The thymidine kinase gene sequences of HZ419 and ZS410 isolates had been submitted to NCBI database with GenBank accession number KM277943 and KM277944, respectively.

Discussions

The markers I and II classification system constructed previously is a simple genotyping method independent of DNA sequencing (Bigarré *et al.*, 2009). However, in our study, sometimes PCR amplification encounters failure, especially when the DNA templates were prepared from fish at an early infection stage without obvious clinical signs of the disease. Thus, a nested-PCR was designed and improved the sensitivity 25 times higher than that of duplex PCR. The rate of successful amplification (all of the markers I and II fragments can be obtained) by the nested-PCR increased from 30% to 100% during the genotype investigation of S1–S10 samples (data not shown).

Guangdong Province is located in the south China and has a subtropical climate with an annual temperature ranging from 18°C to 33°C. This kind of environment is very suitable for the propagation of CyHV-3. The trades of direct and indirect (via Hong Kong) between Guangdong and other East and Southeast Asian countries, such as Japan, Korea, Philippines and Indonesia may associate with the increase of prevalence of CyHV-3 in this area. In fact, the first outbreak of KHVD in

China was reported in Guangdong Province (Liu *et al.*, 2002). In addition, three genotypes, I⁺⁺ II⁺, I⁺⁺ II⁻ and I⁺⁺ II^{++Δ}, have been identified in Guangdong Province in this study, but just the I⁺⁺ II⁻ has been detected in Henan Province. The latter is relatively isolated in term of carp trade with other regions and this disease was reported in Henan Province 11 years later from the first occurrence (Liu *et al.*, 2002). Thus, we speculated that I⁺⁺ II⁻ genotype maybe first emerge in Guangdong Province and then spread to Henan Province. However, the actual route of the spread of CyHV-3 in China is still unclear and needs further study. Among the three samples with I⁺⁺ II⁺ genotypes, two of them (S18 and S26) could attribute the infection to fish originated from Japan, indicating that accurate documentation of fish transfer is required for further analysis of the prevalence of CyHV-3. Furthermore, latency of CyHV-3 and carrier fish (e.g. *Carassius auratus*) may increase the difficulty in the detection of CyHV-3 during fish transfer and contribute for the distribution of the virus. (Eide *et al.*, 2011; El-Matbouli and Soliman, 2011; Fabian *et al.*, 2013; Reed *et al.*, 2014).

The first outbreak of koi herpesvirus disease in East Asian and Southeast Asian can be traced back to 1998 in Korea (Lee *et al.*, 2012), 2002 in Indonesia (Sunarto *et al.*, 2005) and 2003 in Japan (Sano *et al.*, 2004). The first detection of CyHV-3 from imported fish in China was reported in 2002 (Liu *et al.*, 2002). The authors indicated the infected fish were imported without demonstrating the exact origin of the infected fish. No outbreak of KHVD had been reported in the following two years and the outbreak was rare until 2008 in China (Zhou *et al.*, 2005). Thus, the source of CyHV-3 distributing in Southeast Asia could be difficult to determine based on the time of emerging of the disease in the relevant countries or regions. Sunarto *et al.* (2005) mentioned the first KHV infection case in Indonesia occurred among fish

imported from China via Hong Kong. Based on the genotype of the emerging virus, both genotypes of I⁺⁺ II⁺ and I⁺⁺ II⁻ are widely distributed in Indonesia from 2002 to 2007, even the mixture of alleles (I⁺⁺ II⁻, I⁺⁺ II⁺) could be detected, which exhibited the prevalence and severity in some degree. However, the present study revealed that just the I⁺⁺ II⁻ was diagnosed as a predominant genotype in 2010 in China. Thus, Indonesian CyHV-3 originated from China seems to be suspicious, but more evidences are needed for the origination of the virus.

Based on marker I and II classification system, five genotypes (I⁺⁺ II⁺, I⁺⁺ II⁻, I⁺⁻ II⁺, I⁺⁻ II⁻ and I⁺⁺ II^{+Δ}) have been reported. However, among them only I⁺⁺ II⁻ has been detected in China (Li *et al.*, 2015). In this study, another three genotypes, I⁺⁺ II⁺, I⁺⁺ II⁻, I⁺⁺ II^{+Δ}, were identified from the 27 samples. Our data showed that the predominant genotype in Guangdong Province and Henan Province, China was I⁺⁺ II⁻, which has been reported to be widely distributed in Indonesia (Sunarto *et al.*, 2011). This observation suggests that distribution of CyHV-3 in China has a close relationship with that in Southeast Asia. Previous study on the evolutionary pathway has speculated that the evolution of the various genotypes of CyHV-3 shows a linear manner, starting from I⁺⁻ II⁻, I⁺⁻ II⁺, I⁺⁺ II⁻, then finally to I⁺⁺ II⁺ (Sunarto *et al.*, 2011). The emergence of genotype I⁺⁺ II^{+Δ} may represent an intermediate lineage between I⁺⁺ II⁻ and I⁺⁺ II⁺. The marker I⁺⁺ II^{+Δ} region of the HZ419 isolate maintained genetic stability after 10 passages in KFC cells. This genotype was also observed in another fish farm in the distance of 150 km (Table 1) and there was no direct association (i.e. transportation of fish) between the two farms, indicating that this genotype raised independently.

Marker I and II which are located in the non-coding sequences do not seem to be associated with the virulence of the virus, therefore the selective pressure on markers I and II loci is still unclear. Taken together, our study in isolation and genotypic analysis of CyHV-3 variants in China may inspire us for further consideration and exploration of the emergence, evolution, spread and prevalence of CyHV-3.

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