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RESEARCH PAPER

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Host Range and Vertical Transmission of Cyprinid herpesvirus 2

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Abstract

In recent years, an epizootic causing severe mortality among the *Allogynogenetic crucian carp* (ACC), designated as haemorrhagic disease of ACC gill, occurred in the Yancheng city of Jiangsu province of China. A herpesvirus, Cyprinid herpesvirus 2 (CyHV-2) was found in diseased ACCs and regarded as a pathogen, but the host range and vertical transmission of CyHV-2 remained unknown. In present study, we conducted loop-mediated isothermal amplification (LAMP) assay, PCR and real-time PCR to detect CyHV-2 in the eggs of diseased *Carassius auratus*, and specific products could be amplified from the extracted DNAs from eggs of the diseased fish. RT-PCR result indicated that *helicase* gene of CyHV-2 could express in eggs. Moreover, the CyHV-2 virus-like particles, ranged between 170 to 220 nm in diameter, could be observed by electron microscopy in eggs of the diseased fish. These results suggested that CyHV-2 probably transmitted vertically to offspring. Additionally, instead of the usual host, the goldfish, *Carassius auratus* and *Carassius auratus gibelio*, CyHV-2 was also detected in *Aristichthys nobilis*, *Erythroculter ilishaeformis, Hypophthalmichthys molitrix* and *Mylopharyngodon piceus* with gill haemorrhagic disease characterized by LAMP assay and electron microcopy examination. Therefore, our experimental results indicated that CyHV-2 can cross-infection among the different species of fishes.

Introduction

Cyprinid herpesvirus 2 (CyHV-2) is a DNA virus, also known as goldfish haematopoietic necrosis virus (GFHNV) or herpesviral haematopoietic necrosis virus (HHNV). It is a lethal pathogen of goldfish and *Carassius auratus* (Goodwin *et al*., 2006). In 1992, first time it was reported in Japan as a fatal pathogen of goldfish and later named as Cyprinid herpesvirus 2 (Jung & Miyazaki, 2010). CyHV-2 is a member of the genus *Cyprinivirus* of family Alloherpesviridae, which also includes carp pox virus (CyHV-1) and cyprinid herpesvirus 3 (CyHV-3) (Hanson, Dishon , & Kotler, 2011). To date, CyHV-2, regarded as a pathogen, has been reported worldwide, including Japan (Ito *et al*, 2013), USA (Goodwin *et al*., 2006a; Goodwin, Merry, & Sadler, 2006b; Groff, LaPatra, Munn, & Zinkl, 1998) Taiwan (Chang, Lee, Chiang, & Jong, 1999), Australia (Stephens, Raidal, & Jones, 2004), UK (Ito *et al*., 2013), China (Xu *et al*., 2013) and France (Boitard *et al*., 2016), and responsible for huge economic loss of aquaculture industry.

CyHV-2 infection usually outbreaks in spring and autumn season when the water temperature ranges from 14–15 °C to 22–25 °C, and fades away with the temperature falling below 10°C or rising above 30°C. The disease can cause severe mortality to all sizes of fish. The infected fishes showed unique feature of lethargy, inappetence, petechial and eccymotic hemorrhages in gills and around the base of the fins (Wu *et al*., 2013). The outbreak of haemorrhagic disease of *Allogynogenetic crucian* carp gill caused by CyHV-2 infection was firstly noticed in the Yancheng city of Jiangsu province of China (Wang *et al*., 2012), currently the epidemic disease was extended to other provinces

of China. Owing to serious outbreak of CyHV-2 infection and enormous financial losses, studies involved in CyHV-2 have been given additional attention. So far, researchers were mainly focused in the identification and detection of CyHV-2(Goodwin *et al*., 2009; Waltzek, Kurobe, Goodwin, & Hedrick, 2009; Wang *et al*., 2012; Wu *et al*., 2013; Zhou *et al*., 2015; Zhu *et al*., 2015), gene function of CyHV-2(Du *et al*., 2015), genomic DNA sequence of CyHV-2(Davison *et al*., 2013;Li *et al*., 2015), protective immunity in gibel carp and *Carassius auratus gibelio* of the truncated ORF25, ORF25C, and ORF25D of CyHV-2 expressed in *Pichia pastoris* (Zhou *et al*., 2015). Determining the host range and transmission of pathogens are also extremely important for the prevention of infectious diseases, however, literatures are scanty related to host range and vertical transmission of CyHV-2. In the present investigation, the loop-mediated isothermal amplification (LAMP) assay, PCR, real-time PCR and electron microscopy were used to detect the diseased fish with gill hemorrhage and found CyHV-2 in the eggs of the diseased *Carassius auratus* and in the tissues of the diseased bighead carp (*Aristichthys nobilis*), *Culter alburnus* (*Erythroculter ilishaeformis*), silver carp *(Hypophthalmichthys molitrix),* black carp *(Mylopharyngodon piceus*). To the best of our knowledge, it was reported for the first time that CyHV-2 can transmit vertically to offspring and can infect the other species besides gold fish, *Carassius auratus* and *Carassius auratus gibelio*.

Materials and Methods

Fish Samples

The diseased fish with gill haemorrhage including bighead carp (*Aristichthys nobilis*), culter alburnus (*Erythroculter ilishaeformis*), silver carp (*Hypophthalmichthys molitrix*), black carp (*Mylopharyngodon piceus*), *Hemibarbus maculatus Bleeker* and *Carassius auratus* were collected from commercial grow-out ponds in the Kunshan city of Jiangsu province of China between August to September in 2014 and May to June in 2015.

Detection of CyHV-2 with LAMP

The sequences of FIP, BIP, F3, B3, and loop primers (LF and LB) used in the LAMP assay were the same as that in our previous report (Zhu *et al*., 2015). The template DNA for the LAMP assay was prepared using a boiling lysis according our earlier study (Zhu *et al*., 2015). Briefly, hepatic tissue about the size of a grain of rice was added into 1.5ml microcentrifugation tube equipped with 100μl LAMP reaction buffer (NEB, MA, USA), and incubated in a boiling water bath for 10 min. After boiling, samples were centrifuged at 12 000 ×g for 1 min, subsequently, the supernatant was used as a template for LAMP assay, whereas the plasmid pMD-hel containing *helicase* gene sequence of CyHV-2 and distilled water were respectively used as a positive and negative control. The LAMP reaction protocol was carried out following our previous investigation (Zhu *et al*., 2015). Amplification products of LAMP were not only analyzed by electrophoresis through 1% agarose gels with Gelred, but also observed directly under UV stained with SYBR Green I (Zhu *et al*., 2015). To explore, whether CyHV-2 can pass to offspring by vertical transmission, eggs of the diseased *Carassius auratus* were also detected by LAMP assay following our previous methods (Zhu *et al*., 2015).

Standard Curve for Amplifying CyHV-2 DNA

The concentration of recombinant plasmid pMD-hel DNA was determined by (NanoDrop 2000, Thermo, USA), then serially diluted from 10^5 down to 10 copies per reaction. The PCR efficiency and correlation coefficient were calculated from the standard curve (CFX96^{TM} Real-Time system, Bio-Rad). Real-time PCR for CyHV-2 was performed in 20 μl reactions containing 1 μl of template, 1 μl of mixture of primers CyHV2-Qhel-1 and CyHV2-Qhel-2 (1 μM each) (Table 1), 10μl of SYBR Green PCR Master Mix (TransGen Biotech, Beijing, China), and 8μl of double distilled water. The PCR thermal profile consisted of an initial incubation of 2 min at 95°C followed by 35 cycles of 45 s at 58°C, 45 s at 72°C, and 30 s at 95°C, and a final extension of 2 min at 72°C.

Detection of CyHV-2 in Eggs

In order to further confirm whether the eggs carry CyHV-2, we first disinfected the eggs by SDS 1%(Zhu *et al*., 2015), then extracted the genomic DNA of eggs, and then detected the DNA fragment of CyHV-2 helicase gene by PCR with primers CyHV2-h1and CyHV2-h2 (Table 1), and by real-time PCR with primers CyHV2-Qhel-1 and CyHV2-Qhel-2(Table 1). To prove that the

Table 1 The primers used in this study

Name of gene	Primers	Sequence (5'-3')
CyHV-2 *Helicase*	CyHV2-h1	ATGCTCACGGGTCCCATGCTG
	CyHV2-h2	CGCTCGTCCGGGTTCTGCACG
CyHV-2 *Helicase*	CyHV2-Qhel-1	GGGTGAGGACTTGCGAAGAG
	CyHV2-Qhel-2	CGCTCGTCCGGGTTCTGCACG
Gibel carp *actin*	Cara-actin-q-F2	TGTCCCTGTATGCCTCTGGT
	Cara-actin-q-R2	GCTGTAGCCTCTCTCGGTCA

fish eggs were thoroughly disinfected, we also examined the presence of CyHV-2 in 1% SDS after washing fish eggs by PCR. Moreover, we also detected CyHV-2 in a single egg by real-time PCR. Firstly, 40 eggs from the fish with obvious hemorrhagic symptoms or no obvious symptoms were collected separately and then were disinfected by 1% SDS (Zhu *et al*., 2015). Then, the individual egg was placed in a microcentrifuge tube containing 6µl LAMP reaction buffer and mashed by toothpick before incubation in a boiling water bath for 10 min, and followed by centrifugation at 12 000 ×g for 1 min, and the supernatant was collected as the template for real-time PCR .Real-time PCR products amplified from the eggs were detected by agarose gel electrophoresis, and the recovered PCR products from the gel were cloned into a T-vector (Takara, Dalian, China) for sequencing by Sangon Biotech (Shanghai) Co., Ltd. Then, the obtained sequence is aligned with the sequence of CyHV-2 *helicase* gene (Accession number: JQ815364) using the BLAST program online (http://www.ncbi.nlm.nih.gov/BLAST). The viral load was estimated by the prepared standard curve. Additionally, to detect the expression level of CyHV-2 helicase gene in the eggs relative to actin gene by real-time PCR, 0.15 grams of eggs were collected from normal fish and diseased fish, respectively. Total RNA from the eggs were isolated using RNAiso plus (Takara, Dalian, China), and cDNA was synthesized with the TransScript^{R}One-Step gDNA Removal and cDNA Synthesis SuperMix (Transgen Biotech, Beijing, China) from 2µg of RNA. The primers Cara-actin-q-F2 and Cara-actin-q-R2 were used to detect actin gene expression.

Electron Microscopy Examination

The kidney, spleen, liver and egg were collected from the spontaneous diseased moribund fish, fixed in 2.5% glutaraldehyde and rinsed for 6 hr in 0.1 M sodium cacodylate by replacing every 2 h, and then post-fixed for 2 h in 1% osmium acid. The specimens were washed twice with 0.1 M sodium cacodylate buffer, followed by dehydration through a graded series of ethanol, and embedded in the epoxy resin (Epon 812). The ultrathin section of 50–60 nm thickness was prepared using microtome machine (Leica UC7, Germany) and mounted on uncoated copper grids, and stained with 2% uranyl acetate and Reynolds's lead citrate (Zhu *et al*., 2015). Grids were observed with a transmission electron microscope (HITACHI-H7650, Japan).

Results

Clinical Examination

The moribund fishes collected from commercial grow-out ponds in the Kunshan city of Jiangsu province of China, showed symptoms of hemorrhages on the body surface with various levels (Figure 1A-E). Bighead carp displays slight hemorrhages around the base of pelvic fin (Figure 1B). Silver carp (Figure 1D), *Carassius auratus* (Figure 1A), *Culter alburnus* (Figure 1E) and black carp (Figure 1C) besides the bases of fin, emerges more hemorrhagic symptom including the opercula, anterior abdominal area (Figure 1A, 1C-E). Internally, gills of *Carassius auratus* were extreme pale and covered with excess mucus, severe haemorrhage was recorded in the visceral organs; there were petechiae on the liver surface (Figure 1F). According to the clinical examination, we suspected that all the sampled fishes might have infected by CyHV-2.

LAMP Assay

In order to confirm our speculation, we performed LAMP assay to rapidly detect CyHV-2 in the fishes. Hepatic tissue collected from silver carp, *Culter alburnus*, *Carassius auratus* and black carp were positive by LAMP assay, which confirmed that there were CyHV-2 in these diseased fish, whereas *Hemibarbus maculatus Bleeker* was negative (Figure 2A, C), indicating the diseased fish was not caused by CyHV-2 infection. Products of LAMP assay could be visualized by staining with SYBR Green I (Figure 2B), the results were consistent with the those of electrophoresis detection (Figure 2B). The two *Carassius auratus* with hemorrhagic symptom were inspected with LAMP assay, the one was positive and another was negative, which demonstrated that one of the diseased fishes with hemorrhagic symptom were infected by other pathogens besides CyHV-2.

Detection of CyHV-2 in Eggs by PCR and Real-Time PCR

To further identify whether eggs of the diseased fish carried CyHV-2, the genomic DNA extracted from the eggs was used as a template to amplify with primers CyHV2-h1/CyHV2-h2 (Table 1). The results showed that a specific product could be amplified (Figure 3A), and sequencing result showed that the sequences were consistent with the sequences of CyHV-2 *helicase* gene. In addition, 1% SDS after washing fish eggs was also amplified as a template, and the results showed that no specific product in 1% SDS, suggesting that there were CyHV-2 in the eggs from diseased fish. Subsequently, a standard curve for amplifying CyHV-2 DNA was made with logX (X was initial copy number of plasmid pMD-hel DNA) and PCR cycle threshold (Ct) values (Figure 3B). Only one of 40 eggs from the fish without hemorrhagic symptoms was suspected of carrying CyHV-2, while five of 40 eggs from the fish with obvious hemorrhagic symptoms were suspected of carrying CyHV-2 by real-time PCR and agarose gel electrophoresis(Figure 3C, D), and then the PCR products from the eggs which were suspected of carrying CyHV-2 according to the standard curve were sequenced and the results showed that the sequences of the products shared 100% identity to

helicase gene(Accession number: JQ815364) sequences of CyHV-2(Figure 3E), suggesting eggs from the diseased fish carry CyHV-2. Additionally, the copies of CyHV-2 DNA in a single egg was determined by the standard curve and found there were about 5.69 copies of CyHV-2 in a single egg. Moreover, the CyHV-2 *helicase* gene could express in fish eggs, and the expression level of CyHV-2 *helicase* gene relative to *actin* gene in the diseased fish eggs was 359 times higher than that of normal fish eggs. (Figure 3F).

Electron Microscopy Observation

CyHV-2 nucleocapsids-like particles ranged between 115–117 nm in diameter and obvious pathological changes were found in liver tissues from the diseased silver carp, *Culter alburnus* and black carp by electron microscopy observation (Figure 4A-C). In kidney and spleen cells of the infected *Carassius auratus,* the virion morphology and size consistent with CyHV-2 were observed. The developing virion showed an outer membrane and an electron lucent centre, while mature virion illustrated an electron dense core particle (Figure 5A, B). It's noteworthy that some of virus-like particles were displayed in the eggs of diseased *Carassius auratus* (Figure 5C, D), which suggesting CyHV-2 may be transmitted to offspring by eggs.

Discussion

At present, gill haemorrhagic disease caused by CyHV-2 is a serious epidemic threat to aquaculture in the Jiangsu province of China. Finding out the ways of prevention, treatment and cure of CyHV-2 infection is

Figure 1. Clinical signs in different kinds of fish infected with CyHV-2. External signs showed hemorrhages on the body surface, including fin bases, opercula, anterior abdominal and the anterior abdominal. (black arrow). A. *Bighead carp* (*Aristichthys nobilis*). B. *Carassiusauratus;* C. black carp *(Mylopharyngodon piceus*); D. silver carp *(Hypophthalmichthys molitrix);* E. culter alburnus (*Erythroculter ilishaeformis*). F. Internal signs of *Carassiusauratus* showed pale gills, severe haemorrhage in visceral organs and petechiae on the liver surface.

still pressing. To date, the gill hemorrhagic disease of *Carassius auratus gibelio* is not controlled effectively and occurs as usual even with mixed farming mode and low-density culture. Eliminating infections source and cut off the route of transmission play a significant role in controlling epidemic of the infectious disease. Epidemiological investigation suggesting hemorrhagic disease of gill can cause severe mortality among tiny size

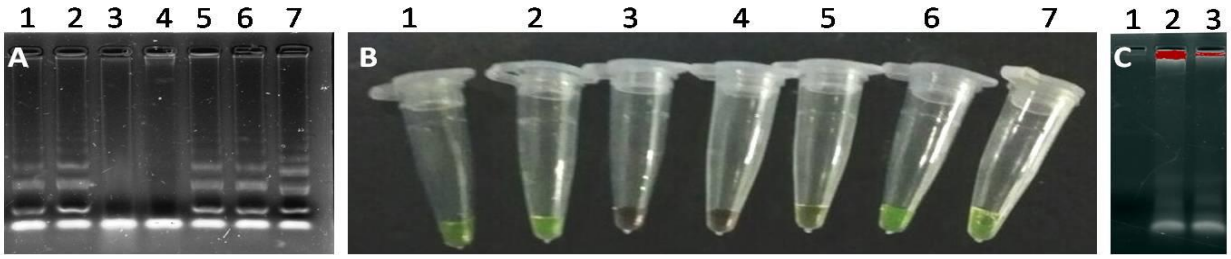

Figure 2. LAMP detection of CyHV-2 in the fish from commercial farm in Kunshan city.
A, Electrophoresis of LAMP products from different kinds of fish. Lanes A1–A6 represent *hypophthalmichthys molitrix, Bighead carp, Hemibarbus maculatus Bleeker, Carassiusauratus, Carassiusauratus, Erythroculter ilishaeformis.* Lane A7 as positive control [plasmid pMD-hel]. B, LAMP products stained with SYBR Green under UV. Tubes B1–B6 represents the LAMP products for *hypophthalmichthys molitrix, Bighead carp, Hemibarbus maculatus Bleeker, Carassiusauratus, Carassiusauratus, Erythroculter ilishaeformis.* Tube B7 as positive control [plasmid pMD-hel].C, Electrophoresis of LAMP products from *mylopharyngodon piceus.* Lane C1 represents negative control [distilled water], Lanes C2 and C3 represent *mylopharyngodon piceus.*

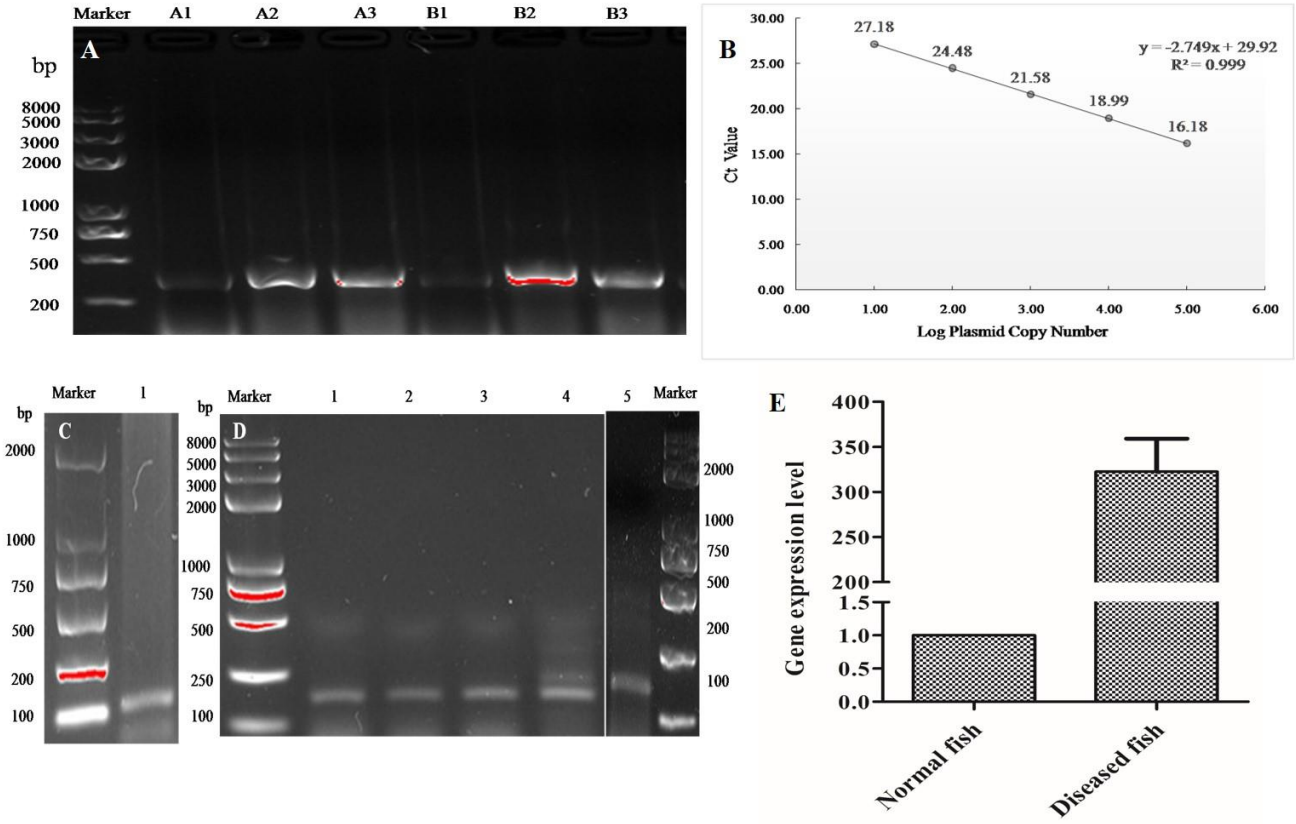

Figure.3. Detection of CyHV-2 in eggs by PCR and real-time PCR.
A, detection of CyHV-2 by PCR. Lane A1 represents the PCR product of 1% SDS after washing eggs of CyHV-2 diseased fish. Lane A2 represents the PCR product of the eggs of CyHV-2 diseased fish treated by 1% SDS. Lane A3 represents the PCR product of 1% SDS after washing eggs of CyHV-2 non-diseased fish. Lane A4 represents the PCR product of the eggs of CyHV-2 non-diseased fish treated by 1% SDS. B, standard curve for amplifying CyHV-2 DNA. X axis represents initial copy number of plasmid pMD-hel DNA; Y axis represents PCR cycle threshold (Ct) values. C and D, detection of CyHV-2 by real-time PCR. Lane 1 represents amplification products from the fish without hemorrhagic symptoms(C). Lanes 1-5 represent amplification products from the fish with hemorrhagic symptoms(D). E, the gene expression level of CyHV-2.

of *Carassius auratus* even in good quality water, so we suspected that the *Carassius auratus* fry carry CyHV-2?

It was reported that channel catfish Ictalurid herpesvirus 1 could be transmitted to offspring by egg (Thompson, Khoo, Wise, & Hanson, 2005). Previous study showed that CyHV-2 could be found in the offspring seed, breeding fish, disinfected eggs and fry of goldfish, suggesting that CyHV-2 transmit vertically to offspring in the goldfish (Goodwin *et al*., 2009). In our present report, the CyHV-2 can be found in the egg of the diseased *Carassius auratus* with hemorrhagic disease by LAMP assay, PCR, real-time PCR and electron microscopy examination. Sequences of the PCR products s shared 100% identity to *helicase* gene (Accession number: JQ815364) sequences of CyHV-2. This result tells us the virus strain, which provides a theoretical basis for the further use of molecular methods to control the disease. Moreover, the expression of CyHV-2 helicase gene could be also found in eggs, suggesting the gill hemorrhagic disease of *Carassius auratus* can be vertically transmitted to offspring by eggs carrying CyHV-2. Therefore, it is necessary that the female parent fish must be inspected in advance to supply diseased free fry without CyHV-2 for *Carassius auratus* industry. In addition, it was found that one diseased fish with hemorrhagic symptom was not infected by CyHV-2, and hemorrhage may be caused by other causes. In aquaculture, there are four kinds of hemorrhagic diseases in fish: stress hemorrhage, toxic hemorrhage, bacterial infection hemorrhage and viral hemorrhage. In the process of fish disease prevention and treatment, it is important to find out the cause of the disease, and then to find a reasonable solution.

Previous work revealed that the gold fish (Goodwin *et al*., 2006a, 2006b, 2009; Jeffery *et al*., 2007), *Carassiu sauratus* (Fichi *et al*., 2013) and *Carassius auratus gibelio* (Li, Luo, Liu, Gu, & Yuan., 2013; Zhu *et al*., 2015) could be infected by CyHV-2, and some infectious disease could be spread by cross transmission between different species. In the spring season of 2015, we noted that diseased fishes including silver carp, black carp, bighead carp, *Hemibarbus maculatus bleeker* and culter alburnus occurred in the Kunshan city of Jiangsu province of China, also have similar clinical features such as those were suffering from gill hemorrhagic disease of *Carassius auratus gibelio*. We suspected that these diseased fishes were caused by infection of CyHV-2. Both LAMP assay and electron microscopy examination confirmed that silver carp, *Culter alburnus*, *Carassius auratus* and black carp were positive, whereas *Hemibarbus maculatus bleeker* was negative. These results indicating the infection of CyHV-2 is not limited to the gold fish, *Carassius auratus* and *Carassius auratus gibelio*, whereas, silver carp *culter alburnus* and black carp can also be infected by CyHV-2. It is not suitable to polyculture *Carassius auratus* with silver carp, *Culter alburnus* and black carp. It has been confirmed by quantitative PCR that CyHV-2 has incubative infection among goldfish (Goodwin *et al*., 2009). In our prior finding, we exposed that CyHV-2 can be detected in the *Allogynogenetic crucian* carp without clinical symptoms of haemorrhagic disease of the gill from commercial farms of the Dafeng City, Jiangsu province, China, which is an epidemic area of CyHV-2. This indicates CyHV-2 might have incubative infection among *Allogynogenetic crucian* carps (Zhu *et al*., 2015), consequently, decreasing the stress reaction of fish may improve the occurrence of gill haemorrhagic disease in the aquaculture industry.

As there are no effective drugs or vaccines are available for the gill haemorrhagic disease caused by CyHV-2, therefore, focus should be on the prevention of this disease rather than to cure. The results of this study provide new perspectives for us to prevent the gill

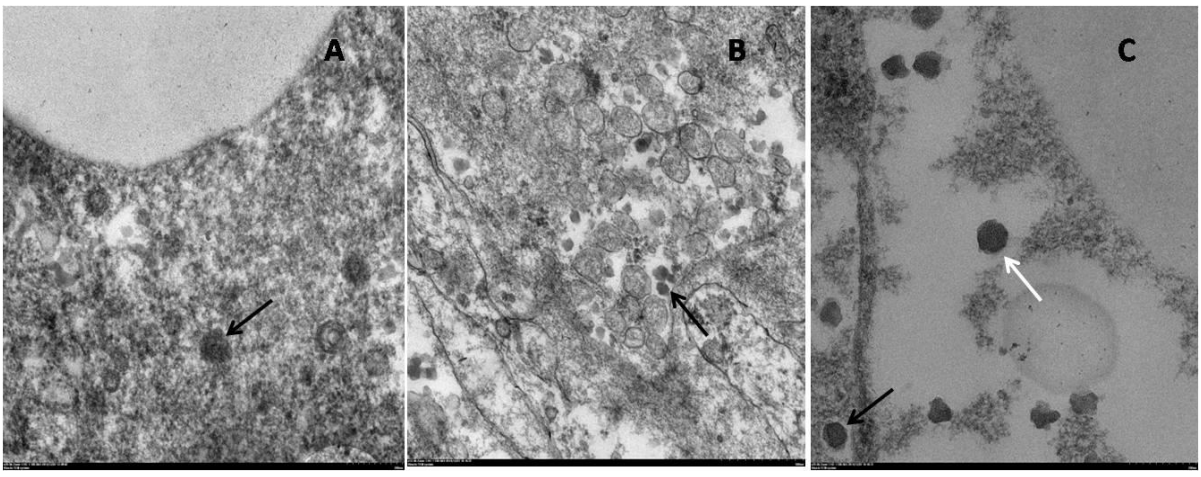

Figure 4. Electron micrograph of infected liver from diseased fish.
Fully formed virions had an outer membrane and electron dense core (black arrow). Non-enveloped virus particles dispersedly located in the cytoplasm (white arrow). A, Culter alburnus. B, *Bighead carp* C, black carp.

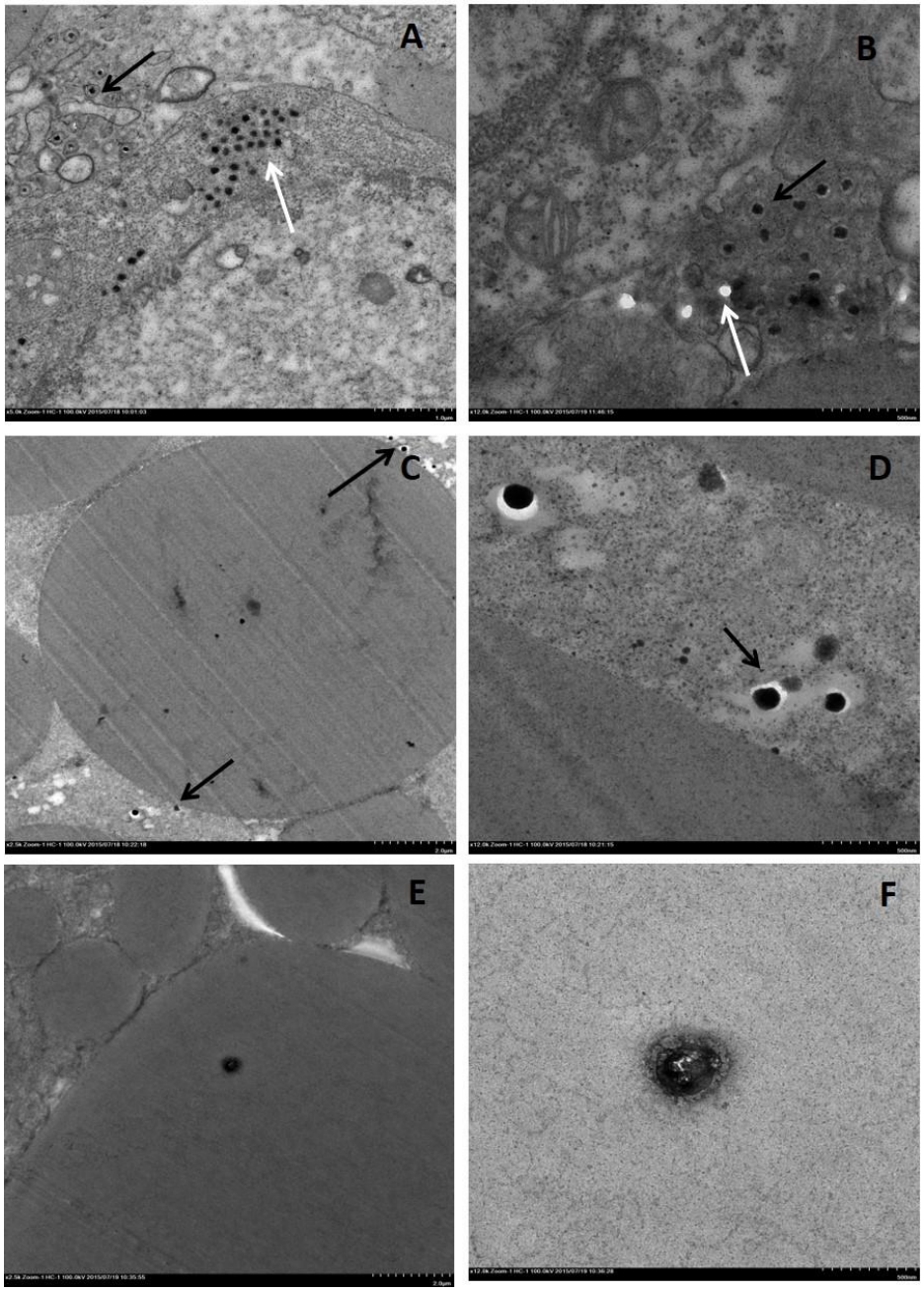

Figure 5. Electron micrograph of infected tissues and eggs from a naturally infected *Carassiusauratus*.
A, kidney, enveloped virus had an outer membrane and electron dense core in the cytoplasm (black arrow) and non-enveloped virus particles aggregated in the cytoplasm (white arrow). B, spleen, fully formed virions had an outer membrane and electron dense core (black arrow) while developing virions had an electon lucent centre (white arrow). C. D, in diseased fish eggs, the virions with outer membrane and electron dense core (black arrow) scattered. E, Electron micrograph of uninfected eggs.

haemorrhagic disease and save economic losses of aquaculture industry of China.

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Conflicts of Interest

The authors declare no conflicts of interest.

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