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Recombinant baculovirus BacCarassius-D4ORFs has potential as a live vector vaccine against CyHV-2

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ABSTRACT

Cyprinid herpesvirus II (CyHV-2) is highly contagious and pathogenic to *Carassius auratus gibelio* (gibel carp), causing enormous economic losses in aquaculture in Yancheng city, Jiangsu province, China; however, to date, there is no effective way to protect *C. auratus gibelio* from CyHV-2 infection. In this study, a recombinant baculovirus vector vaccine, BacCarassius-D4ORFs, containing a fused codon-optimized sequence D4ORFs comprising the ORF72 (region 1–186 nt), ORF66 (region 993–1197 nt), ORF81 (region 603–783 nt) and ORF82 (region 85–186 nt) genes of CyHV-2, driven by a *Megalobrama amblycephala* β-actin promoter, was constructed. Then, qPCR, Western blotting and immunofluorescence assays showed that the fused gene D4ORFs was successfully delivered and expressed in fish cells or tissues by transduction with BacCarassius-D4ORFs. The fused gene D4ORFs could not be detected by PCR in the *C. auratus gibelio* injected with BacCarassius-D4ORFs after 7 weeks. Specific antibody against ORF72 could be detected in the serum of vaccinated *C. auratus gibelio* by injection with BacCarassius-D4ORFs. Furthermore, when *C. auratus gibelio* were vaccinated with BacCarassius-D4ORFs *via* the oral or injection route, followed by challenge with CyHV-2, the relative survival rate of immunized *C. auratus gibelio* reached 59.3% and 80.01%, respectively. These results suggested that BacCarassius-D4ORFs has the potential to be used as a vector-based vaccine for the prevention and treatment of disease caused by CyHV-2 infection.

1. Introduction

Cyprinid herpesvirus II (CyHV-2), a double-stranded DNA virus, also known as goldfish hematopoietic necrosis virus (GFHNV) or herpesviral hematopoietic necrosis virus (HHNV), is a lethal pathogen of goldfish, crucian carp and the hybrids of goldfish and carp [1,2]. To date, diseases resulting from infection with CyHV-2 have been detected around the world [3–10]. In China, *Carassius auratus gibelio* is one of the most important farmed freshwater fish. An outbreak of hemorrhagic disease of *C. auratus gibelio* gills caused by CyHV-2 infection firstly occurred in Yancheng city of Jiangsu province, China, in 2012 [11]. At present, this hemorrhagic disease of *C. auratus gibelio* gills is spreading to other provinces of China because an effective prevention and control system has not yet been established.

In the aquaculture industry, immunization is widely considered the most effective approach for preventing virus infection of fish. According to their preparation method, vaccines used in aquaculture can be classified into live vaccines, inactivated vaccines, and genetically-engineered vaccines, with genetically-engineered vaccines being further divided into recombinant subunit vaccines, DNA vaccines, gene deletion/mutant vaccines, and live-vector vaccines [12]; however, to date there is still no commercialized vaccine against CyHV-2 in China. Using inactivated virus as a vaccine is the most conventional method of vaccine preparation. It was reported that the β-propiolactone inactivated CyHV-2 vaccine induced both nonspecific and specific antiviral immune responses, and the relative survival rate of immunized

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gibel carp with the inactivated CyHV-2 vaccine reached 71.4% following challenge [13]. Immune protection of inactivated CyHV-2 vaccine with formalin was assessed in goldfish and the results showed that the relative survival rate of the vaccinated fish reached 42.5% and 57.6%, respectively, 4 and 8 weeks after the first vaccination. The fish were CyHV-2-challenged by the immersion route and the relative survival rate of the fish, which were injected with a booster vaccine 4 weeks after the first vaccination, reached 63.6% [14]. The protective immunity elicited by the recombinant subunit vaccines targeting three membrane proteins encoded by genes ORF25, ORF25C, and ORF25D of CyHV-2 was assessed. The relative survival rates for healthy gibel carp immunized with ORF25, ORF25C, and ORF25D recombinant proteins expressed in *Pichia pastoris* were 75%, 63%, and 54%, respectively, when the immunized fish were challenged with CyHV-2 by intraperitoneal injection [15].

Live-vector vaccine, a gene engineering vaccine, which uses non-pathogenic virus or bacteria as a carrier to express protective immune-related antigen genes, can elicit target antigen expression following the cellular entry of the vectors *via* infection, resulting in endogenous antigen processing and MHC class I-restricted antigen presentation [16]. Baculoviruses, double-stranded DNA viruses known to infect invertebrates, are widely used to express vaccine genes in insect cells *in vitro* and *in vivo*, of which *Autographa californica* nucleopolyhedrovirus (AcMNPV) and *Bombyx mori* nucleopolyhedrovirus (BmNPV) are the most widely studied [17]. The influenza vaccines, Provenge™ (www.dendreon.com) and FluBlok™ (https://www.proteinsciences.com), produced by baculovirus, were respectively approved by the Food and Drug Administration in 2010 and 2013. Baculovirus has broad tissue tropism and can deliver foreign genes into mammalian cells *in vitro* and *in vivo* without viral replication [18], and it has been shown that a reporter gene can be delivered into different tissues and organs in mice and chicks by baculovirus vector [19]. Moreover, baculovirus-specific neutralizing serum antibodies are not induced in animals [20–23]. Baculovirus has also been reported to stimulate the host antiviral immune response in mammalian cells [24,25] and to confer protection from lethal influenza virus infection in mice [26]. These studies suggested that baculovirus can be used as a potential vaccine vector for clinical applications [23]; however, investigations into the potential of recombinant baculoviruses as immunizing reagents to mediate protective immunity against viral infections are limited in the aquaculture industry [27].

Baculovirus BmNPV, a pathogen of silkworm (*B. mori*), has been widely studied in terms of its effects on target gene expression in silkworms [17]. Moreover, the application of BmNPV vector was reported to deliver target genes into different tissues and organs in mice and chicks [28]. Our previous studies showed that grass carp reovirus (GCRV) structural protein genes can be delivered into the liver and kidney of grass fish by oral administration of recombinant BmNPV vectors [29,30], and a specific antibody against GCRV could be evoked in the vaccinated fish [29], suggesting that BmNPV can be used as a potential vaccine vector for application in the aquaculture industry. In the present study, a recombinant BmNPV vector vaccine BacCarassius-D4ORFs containing a fused codon-optimized sequence D4ORFs comprising the ORF72 (region 1–186 nt), ORF66 (region 993–1197 nt), ORF81 (region 603–783 nt) and ORF82 (region 85–186 nt) genes of CyHV-2 was constructed. *C. auratus gibelio* were vaccinated with BacCarassius-D4ORFs *via* the oral or injection route, followed by challenge with CyHV-2, and the relative survival rates reached 59.3% and 80.01%, respectively. These results suggested that BacCarassius-D4ORFs could potentially be used as a vector-based vaccine for the prevention and treatment of disease resulting from CyHV-2 infection.

Table 1
Primers used in this study.

primer	Sequence
FA-SM	TCTCCCGGGCTCTTACAGGAAACGGGTC
FA-XH	CTACTCGAGATTGGAGCTCAAAGTGAGG
CyHV-D4-1	ACACTCGAGATGTACGGTCTGAACAACGCTC
CyHV-D4-2	GCAGGTACCTTACAAACCCGGTCTGTTAGC
M13-forward	CCCAGTCACGACGTTGTAAAACG
M13-revers	AGCGGATAACAATTTCACACAGG
ORF72-1	GCTGGATCCATGTACGGCTTAAACAACGCG
ORF72-2	ACAGAATTCAGAGGCCGTTGAATGAATGTATG
CZYM-QF	ACTGCCTTCATCTTGGGTGG
CZYM-QR	CGGCGTATGAATCGTAATCT
Cara-actin-QF	ATGCTCACGGGTCCCATGCTG
Cara-actin-QF	GCTGTAGCCTCTCTCGGTCA

2. Materials and methods

2.1. Expression of CyHV-2 ORF72 in Escherichia coli and preparation of ORF72 polyclonal antibody

The ORF72 coding sequence amplified from CyHV-2 genomic DNA with primer pair ORF72-1 and ORF72-2 (Table 1) was digested with *Eco*RI/*Hin*dIII, and then cloned into pET28a(+) (Novagen, Darmstadt, Germany) to construct pET28a(+)-ORF72. The ORF72 protein fused with a His-tag was expressed in *E. coli* strain BL21. The harvested bacteria were subjected to SDS-PAGE. The proteins in the gel were transferred to PVDF membrane, and Western blotting was carried out with a mouse anti-His6 (Sino Biological Inc., Peking, China) and a HRP-conjugated goat anti-mouse IgG (1:20000) (Sino Biological Inc., Peking, China). The recombinant ORF72 protein with the His-tag was purified using Ni-NTA agarose (Qiagen, Shanghai, China) and was used to immunize ICR mice (Soochow University, Suzhou, China) by subcutaneous and celiac injection to induce antibody.

2.2. Synthesis of the fused DNA sequence from the CyHV-2 ORF66, ORF72, ORF81, and ORF82 partial sequences

Our previous study showed that the complete genome of CyHV-2 strain SY isolated from Sheyang county, Jiangsu province, China, was 290,455 bp in length comprising 154 potential open reading frames (ORFs) (GenBank accession no. KT387800.1), of which, ORF66 and ORF72 code for the putative capsid triplex subunits 1 and 2, respectively, and ORF81 and ORF82 code for membrane proteins ORF81 and ORF82, respectively [19].

To obtain a fused codon-optimized sequence D4ORFs for expressing multi-subunit fusion vaccine, the sequences from regions 1–186 nt of ORF72, 993–1197 nt of ORF66, 603–783 nt of ORF81, and 85–186 nt of ORF82 were linked and synthesized by GenScript Biotech Corp. (Nanjiang, China) based on the preference of the BmNPV codons. The synthesized sequence (Fig. S1) was cloned into vector, pUC57-T (GenScript, Nanjiang, China), to generate pUC57-D4ORFs.

2.3. Construction of a donor vector pFastBac^{TM}Dual-FA-D4ORF

A promoter (0.56 kb) (GenBank accession no. AY170122) of the *Megalobrama amblycephala* β-actin gene, which was amplified from pMD19T-β-actin [29] by PCR using the primer pair FA-SM and FA-XH (Table 1), was cloned into the *Sma*I/*Xho*I sites of pFastBac™ Dual (Invitrogen, Frederick, MD, USA) to generate pFastBac™ Dual-FA. The D4ORFs gene was then amplified from pUC57-D4ORFs by PCR using the primer pair CyHV-D4-1 and CyHV-D4-2 (Table 1), digested with *Xho*I/*Kpn*I, and then cloned into pFastBac™ Dual-FA to generate pFastBac™M Dual-FA-D4ORFs.

2.4. Construction of recombinant baculovirus Bacmid-D4ORFs

The vector pFastBac^{TM}Dual-FA-D4ORFs was transformed into *E. coli* DH10Bac/BmNPV [29] to generate recombinant Bacmid-D4ORFs using the Bac-To-Bac baculovirus expression system (Invitrogen, Frederick, MD, USA) following the manufacturer's instructions. Recombinant Bacmid-D4ORFs (larger than 135 kb in size) was identified by PCR with the M13 forward primer and M13 reverse primer (Table 1). Meanwhile, pFastBac^{TM}Dual was used to generate a control bacmid.

2.5. Generation and identification of recombinant baculovirus vector vaccine BacCarassius-D4ORFs

The confirmed Bacmid-D4ORFs DNA was transfected into cultured BmN cells derived from silkworm ovaries using FuGENE HD Transfection Reagent (Roche Diagnostics, Indianapolis, IN, USA) to generate the recombinant baculovirus vector vaccine BacCarassius-D4ORFs. To further verify the virus, the DNA extracted from the BmN cells infected with BacCarassius-D4ORFs was identified by PCR with the primer pair CyHV-D4-1 and CyHV-D4-2 (Table 1). To amplify the recombinant virus, the harvested virus from the infected BmN cells was used as the P1 virus, and continuously proliferated through further infection into BmN cells until the P3 virus was obtained, and the virus titer was adjusted to 2.2×10^{11} transducing units (Tu)/ml for use as a viral stock. Meanwhile, the control recombinant baculovirus was generated using the control bacmid.

2.6. Multiplication of BacCarassius-D4ORFs in silkworm pupae

To proliferate BacCarassius-D4ORFs in the silkworm, silkworm pupae three days after pupation were inoculated with BacCarassius-D4ORFs by infection. The inoculated pupae were incubated at 25 °C for 120 h, then collected and stored at -20 °C.

2.7. Expression of the fused gene D4ORFs in the cultured Ctenopharyngodon idella kidney (CIK) cell line

To explore whether D4ORF4 could be delivered into fish cells and expressed, BacCarassius-D4ORFs (10 μL of viral stock) was inoculated into cultured *C. idella* kidney (CIK) cells (10^5/mL, 1 mL) provided by Jiangsu Center for Control and Prevention of Aquatic Animal Infectious Disease, Najing, China. After washing twice with 1× PBS, the culture medium was removed, then the cells were cultured in MEM medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum at 28 °C for 72 h, then collected for Western blot analysis. The lysed cells were subjected to SDS-PAGE and then the proteins in the PAGE gel were transferred onto polyvinylidene difluoride (PVDF) membrane and Western blot analysis was carried out with a mouse anti-ORF72 and a HRP-conjugated goat anti-mouse IgG (1:20,000) (Sino Biological Inc.,Peking, China). The un-inoculated cells were used as a negative control.

2.8. Experimental fish

C. auratus gibelio (mean body weight: 50 ± 5 g, body length: 7–13 cm) were purchased from Aquatic Breeding Field of Kunshan city, Jiangsu province, China. The fish were acclimatized in the laboratory at a temperature of 19°C-22 °C for two weeks in a recirculating fresh water system before experimental manipulation. Fish were fed thrice a day with commercial dry pellets (purchased from Tongwei Group, Chengdu, China).

2.9. Vaccination and PCR detection

Intraperitoneal injection immunization: In the vaccination group (Group IIV), the fish were vaccinated by intraperitoneal injection with 100 μL of BacCarassius-D4ORFs (2.2×10^{11} Tu/mL) per fish. In the negative control group (Group IIN), the fish were vaccinated by intraperitoneal injection with 100 μL of the control baculovirus (2.2×10^{11} Tu/mL) per fish. In the blank control group (Group IIC), the fish were injected with 100 μL of normal saline. Thirty fish were treated in each group.

The total RNA extracted from the spleen of the vaccinated fish at different time points post-immunization was subjected to RT-PCR with the primer pair CyHV-D4-1/CyHV-D4-2 to determine the retention time of BacCarassius-D4ORFs in the vaccinated fish.

Oral immunization: The commercial dry pellets (100 mg) were mixed with 100 μL of BacCarassius-D4ORFs (2.2×10^{11} Tu/ml) for preparation of the fodder with BacCarassius-D4ORFs. The control fodder was prepared by replacement of BacCarassius-D4ORFs with normal saline. In the oral vaccination group (Group OV), the fish were fed with the fodder containing BacCarassius-D4ORFs, whereas in the control group (Group OC), the fish were fed with the normal fodder. Thirty fish were treated in each group. Fish were fed thrice a day.

The small fries (about 0.7 cm in length) were fed with the fodder containing BacCarassius-D4ORFs for 8 days. Genomic DNA was extracted from the whole fish at 37 days post-vaccination and was subjected to PCR with primers CZYM-QF and CZYM-QR (Table 1) to determine the retention time of BacCarassius-D4ORFs in the vaccinated fish.

Immersion immunization: Ten pupae infected with BacCarassius-D4ORFs were homogenized and added to water; 5L of water containing BacCarassius-D4ORFs at 500 copies/μL final viral titer was prepared. In the immersion group (Group IMV), six fish were immersed in the water containing BacCarassius-D4ORFs for 5 h. In the control group (Group IMB), six fish were immersed in the normal water for 5 h.

The small fries (about 0.7 cm in length) were soaked in the water containing 1000 copies/μL final viral titer for 5 h. The genomic DNA was extracted from the whole fish at 35 days post-soak and was subjected to PCR with primers CZYM-QF and CZYM-QR to determine the retention time of BacCarassius-D4ORFs in the vaccinated fish.

2.10. Enzyme-linked immunosorbent assay (ELISA)

To determine the antibody titer in the vaccinated fish, blood samples (n = 3) were collected from the caudal vein of fish at 7, 14, 21, and 28 days post-injection immunization and were stored at 4 °C overnight. After centrifugation for 10 min at 2000 × *g*, the supernatants were collected and the antibody titers in the sera were determined by an uncompetitive ELISA. Briefly, different concentrations of recombinant ORF72 with a His-tag were used to coat an ELISA plate. After removing the free recombinant ORF72 with the His-tag, the ELISA plate was blocked with 5% bull serum albumin (BSA), followed by incubation with different dilutions of the sera sampled from the vaccinated fish for 2 h. The serum sampled from the unvaccinated fish was used as a control. After washing with Tris-buffered saline containing Tween (TBST) five times, the prepared ORF72 antibody was added, and incubated for 2 h. After further washing with TBST, HRP-conjugated goat anti-mouse IgG was added and incubated. Finally, the absorbance at 492 nm was measured after coloration.

2.11. qPCR

After digestion with DNaseI to remove genomic DNA contamination, the total RNA extracted from the kidney and spleen was used as a template and cDNA was synthesized using the TransScript First-Strand cDNA Synthesis kit (Transgen Biotech, Beijing, China) according to the manufacturer's manual. To determine the expression of the fused gene D4ORFs in the vaccinated fish, the cDNA was used as template and qPCR was performed with primers CYZM-QF/CYZM-QR (Table 1) using TransStart Tip Green qPCR SuperMix (Transgen Biotech, Beijing, China). The *C. auratus gibelio* actin gene was used as an internal

reference gene. The primers cara-actin-QF/cara-actin-QR (Table 1) were used for determination of actin gene expression. All samples were carried out in triplicate. The relative expression levels to actin were determined using the $2^{-\Delta\Delta Ct}$ method [31].

To explore whether the D4ORFs gene could be delivered into fish by immersion immunization with BacCarassius-D4ORFs, the fish were immersed in water containing BacCarassius-D4ORFs at 500 copies/μL final viral titer for 5 h. Then, total RNA was extracted from 0.05 g of the gill, spleen, and blood of the immersed fish and was dissolved in 20 μL of H_20. Then, 1 μL (2 μg) of total RNA was converted to cDNA (20 μL), and 1 μL of cDNA was subjected to qPCR to determine the level of D4ORFs cDNA.

2.12. Immunofluorescence observations

Immunofluorescence was performed according to our previous study [29]. Briefly, the spleen and kidney dissected from the fish vaccinated by intraperitoneal injection with BacCarassius-D4ORFs at 7 and 14 days post-initiation vaccination were fixed in 4% formalin and embedded with paraffin. Sections were labeled first with mouse anti-ORF72 polyclonal antibody and then with FITC-conjugated goat anti-mouse IgG (H + L) (Bio-world, Dublin, OH, USA). Simultaneously, the sections incubated with pre-immune antiserum were used as a negative control. After the uncombined goat anti-mouse IgG antibody was removed, the cells were stained with 4′, 6-diamidino-2-phenylindole (DAPI) and examined under a fluorescence microscope.

2.13. Challenge trials

Kidney tissue (0.2 g) dissected from the diseased fish infected with CyHV-2 was homogenized and mixed with 5 mL of PBS. After centrifugation at 8000 × *g* for 15 min, the supernatant was collected and used as CyHV-2 stock. The fish at 22 days (injection immunization) and 28 days (oral immunization) post-vaccination were challenged by injection with CyHV-2 stock (100 μl per fish) at the base of the pectoral fin. The challenged fish were raised at 23°C-25 °C. Mortality was recorded over a period of 20 days. The relative survival rate of the immunized fish was determined as follows:

The relative survival rate (%) = [(X-Y)/X] × 100%, in which, X was the mortality rate in the control group and Y was the mortality rate in the vaccination group.

3. Results

3.1. Expression of CyHV-2 ORF72 in E. coli and preparation of ORF72 antibody

E. coli strain BL21 with pET28a(+)-ORF72 was induced with IPTG for 8 h at 27 °C. Following SDS-PAGE and Western blot analysis with a mouse anti-His6 antibody, a specific signal band representing recombinant ORF72-His-Tag could be detected (Fig. S2), suggesting that ORF72 was correctly expressed in the *E. coli*.

The purified recombinant ORF72 protein with a His-tag was used to immunize ICR mice to induce the antibody. To assess the efficacy of the prepared mouse anti-ORF72 antibody, Western blotting was performed. A specific signal band could be detected in the sample *E. coli* strain BL21 with pET28a(+)-ORF72, but no signal band was observed in the *E. coli* strain BL21 with pET28a(+) (data not shown). This result suggested that the prepared ORF72 antibody could be used to detect ORF72.

3.2. Identification of recombinant baculovirus bacmid-D4ORFs

A fused codon-optimized sequence D4ORFs comprising the ORF72 (region 1–186 nt), ORF66 (region 993–1197 nt), ORF81 (region 603–783 nt), and ORF82 (region 85–186 nt) genes of CyHV-2, driven by a *M. amblycephala* β-actin promoter, was cloned into pFastBac^{TM}Dual to generate a recombinant baculovirus transfer vector pFastBac™ Dual-FA-D4ORFs. To generate a recombinant bacmid, the pFastBac™ Dual-FA-D4ORFs DNA was transformed into *E. coli* DH10Bac/BmNPV. To identify the recombinant bacmid, DNAs extracted from seven white colonies were used as templates and PCR was performed with primers M13 forward and M13 reverse. A 3.32 kb product was obtained, which was consistent with the theoretical molecular weight detected in a colony (Fig. 1A), suggesting the D4ORFs expression cassettes had integrated into the bacmid genomic DNA. The generated recombinant bacmid was termed Bacmid-D4ORFs and purified Bacmid-D4ORF DNA was transfected into cultured BmN cells to obtain recombinant baculovirus BacCarassius-D4ORFs. The transfected cells typically increased their cell diameter and nuclei sizes, ceased growing, and exhibited detachment and lysis at 3 days post-transfection (Fig. 1B and C). To verify the recombinant baculovirus BacCarassius-D4ORFs, BacCarassius-D4ORFs DNA was used as a template and PCR was carried out with primers CyHV-D4-1/CyHV-D4-2. A specific product representing the D4ORFs gene could be detected (data not shown), confirming that BacCarassius-D4ORFs was correctly constructed.

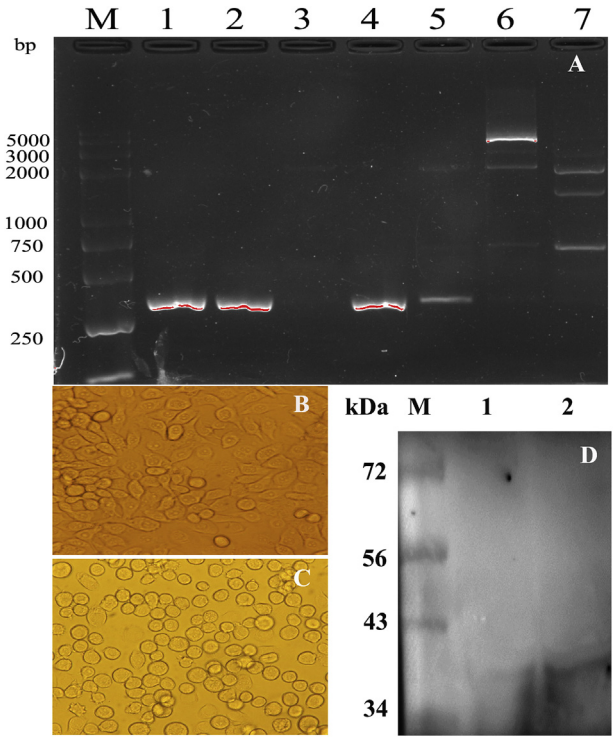

Fig. 1. Identification of Bacmid-D4ORFs and expression of D4ORFs in CIK cells. A, PCR identification of bacmid-D4ORFs. Lane M, DNA marker; Lanes 1–7, the DNA extracted from seven white colonies. B, Normal BmN cells. C, BmN cells transfected with bacmid-D4ORFs. D, Western blot of the expressed ORF72 protein in CIK cells. Lane M, protein marker; lane 1, normal CIK cells; lane 2, CIK cells inoculated with BacCarassius-D4ORFs.

3.3. D4ORFs gene could be delivered into CIK cells and expressed

CIK cells were inoculated with BacCarassius-D4ORFs and 3 days after inoculation, the cells were inspected under a microscope and no pathological changes were found. To explore whether the D4ORFs gene could be delivered into fish cells and expressed, CIK cells inoculated with BacCarassius-D4ORFs were harvested at 72 h post-inoculation and were subjected to SDS-PAGE and Western blot analysis. A specific product representing the D4ORFs protein could be detected suggesting that the D4ORFs gene had been delivered into CIK cells and correctly expressed (Fig. 1D).

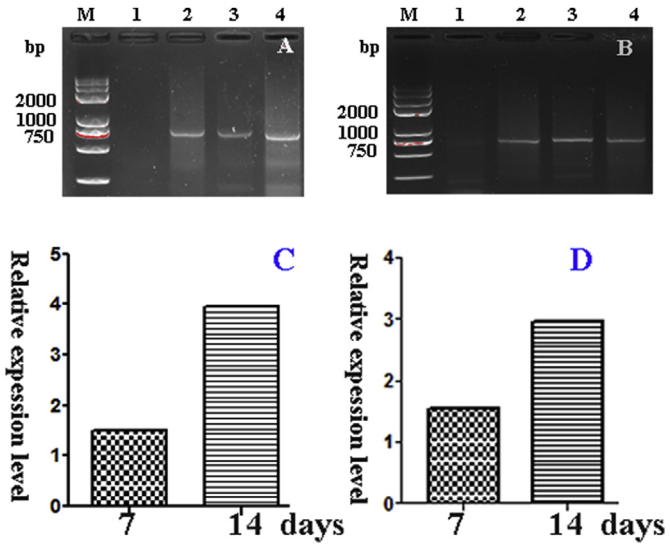

Fig. 2. Detection of BacCarassius-D4ORFs and transcription of the D4ORFs gene in fish injected with BacCarassius-D4ORFs. A, PCR detection of BacCarassius-D4ORFs in the kidneys of fish at different times post-vaccination. Lane M, DNA marker; lane 1, unvaccinated fish; lanes 2, 3, and 4, fish at 7, 14, and 21 days post-vaccination, respectively. B, PCR detection of BacCarassius-D4ORFs in the spleen of fish post-vaccination. Lane M, DNA marker; lane 1, unvaccinated fish; lanes 2, 3, and 4, fish at 7, 14, and 21 days post-vaccination. C, Transcription of the D4ORFs gene in the kidneys of fish at 7 and 14 days post-vaccination with BacCarassius-D4ORFs. D, Transcription of the D4ORFs gene in the fish spleen at 7 and 14 days post-vaccination with BacCarassius-D4ORFs.

3.4. D4ORFs gene could be delivered into fish tissues by injection with BacCarassius-D4ORFs and expressed

The DNAs extracted from the spleen and kidney of *C. auratus gibelio* at 7, 14, and 21 days post-injection with BacCarassius-D4ORFs were subjected to PCR with primers CyHV-D4-1/CyHV-D4-2 and a specific product representing the D4ORFs gene could be detected in the vaccinated fish, but not in the unvaccinated fish (Fig. 2A and B). This suggested that the D4ORF4 gene was delivered into the spleen and kidney of *C. auratus gibelio*. To explore whether the D4ORFs gene was transcribed in the tissues of the vaccinated fish, total RNA was extracted from the tissues of the vaccinated fish. After conversion into cDNA, qPCR was carried out with primers CZYM-QF/CZYM-QR to determine the relative expression level of the D4ORFs gene to the *C. auratus gibelio* actin gene. The transcript could be detected in the kidney (Fig. 2C) and spleen (Fig. 2D) of *C. auratus gibelio* at 7 and 14 days post-injection with BacCarassius-D4ORFs, confirming that the D4ORFs gene driven by the *M. amblycephala* β-actin promoter was transcribed. Moreover, the expression level at 14 days post-vaccination was higher than that at 7 days post-vaccination. Further, immunofluorescence was used to detect the expression of D4ORFs protein in the tissues of the vaccinated fish, and green fluorescence was observed in the kidney (Fig. 3) and spleen (Fig. 4) of the vaccinated fish. These results confirmed that the D4ORFs gene could be delivered into fish tissues by injection with BacCarassius-D4ORFs and expressed.

3.5. D4ORFs gene could be delivered into fish tissues by immersion immunization with BacCarassius-D4ORFs

To explore whether the D4ORFs gene could be delivered into fish by immersion immunization with BacCarassius-D4ORFs, cDNA prepared from the gill, spleen and blood of the immersed fish was subjected to qPCR. The results revealed 1283–1311, 861–1527, and 631–1276 copies of D4ORFs cDNA in 1 μL of cDNA from the gill, spleen, and blood, respectively, suggesting that the D4ORFs gene was delivered into fish by immersion immunization and expressed.

3.6. Specific antibody against ORF72 could be generated in the vaccinated fish by injection of BacCarassius-D4ORFs

To explore whether specific antibody could be generated in the vaccinated fish by injection of BacCarassius-D4ORFs, the antibody titer of the serum sampled from the fish was determined by ELISA. The results showed that no specific ORF72 antibody was detected in the serum of the unvaccinated fish; however, specific antibody (titer ~1:1600) could be detected in the serum of fish at 7 days post-vaccination, with the antibody titer peaking at 21 days post-initial vaccination (titer ~1:6400), then gradually decreasing (titer ~1:3200 at 28 days post-initial vaccination).

3.7. Injection immunization and oral immunization with BacCarassius-D4ORFs confers protection against CyHV-2

To explore whether injection with Baccarassius-D4ORFs can protect fish from infection of CyHV-2, the fish at 22 days post-initial vaccination were challenged by injection with CyHV-2. The fish that had died by 20 days post-challenge were confirmed to be infected with CyHV-2 by PCR (Table 2). The mortality rates resulting from CyHV-2 infection in Group IIC, Group IIN, and Group IIV were 38.46%, 29.17%, and 7.69%, respectively, and the relative survival rates for Group IIN and Group IIV were 24.15% and 80.01%. These results indicated that injection immunization with BacCarassius-D4ORFs conferred protection against CyHV-2. Moreover, when fish were orally administered pellets containing BacCarassius-D4ORFs and challenged with CyHV-2, the mortality rates in Group OC and Group OV were 25.93% and 15.38%, respectively (Table 2), and the relative survival rate in Group OV was 59.31%, indicating that oral immunization with BacCarassius-D4ORFs also conferred protection against CyHV-2.

3.8. BacCarassius-D4ORFs could be eliminated from the vaccinated fish

To determine whether BacCarassius-D4ORFs could be eliminated from the vaccinated fish, the retention time of BacCarassius-D4ORFs in the fish vaccinated by injection immunization was determined by RT-PCR. The results showed that the specific PCR products representing the D4ORFs gene could be detected at 1–7 weeks post-immunization, but could not be amplified from the DNA extracted from the spleens of the vaccinated fish at 8 weeks post-immunization (Fig. 5). These results indicated that the retention time of BacCarassius-D4ORFs in the spleen of vaccinated fish was about 7 weeks and that BacCarassius-D4ORFs could be eliminated from the vaccinated fish.

The retention time of BacCarassius-D4ORFs in the orally immunized and immersion immunized small fries was also determined by RT-PCR and specific PCR product could be amplified at 37 and 35 days post-immunization, respectively (data not shown). This indicated that the retention time of BacCarassius-D4ORFs was more than 35 days in the orally immunized and immersion immunized small fries.

4. Discussion

An outbreak of hemorrhagic disease of *C. auratus gibelio* gills caused by CyHV-2 infection occurred in Yancheng city of Jiangsu province, China, in 2012 [11] and the disease spread widely to almost all of the main culture areas for gibel carp with a 90%–100% mortality rate [29]. Although optimization of the breeding mode, the use of feed containing immunoenhancers, preventing abrupt changes to the feeding environment, and the detection of CyHV-2 in fingerlings were all ensured during *C. auratus gibelio* culture, the occurrence of hemorrhagic disease of *C. auratus gibelio* gills did not decrease significantly, and huge economic losses were still experienced. The mortality rate caused by CyHV-2 infection could be reduced by increasing or decreasing the rearing

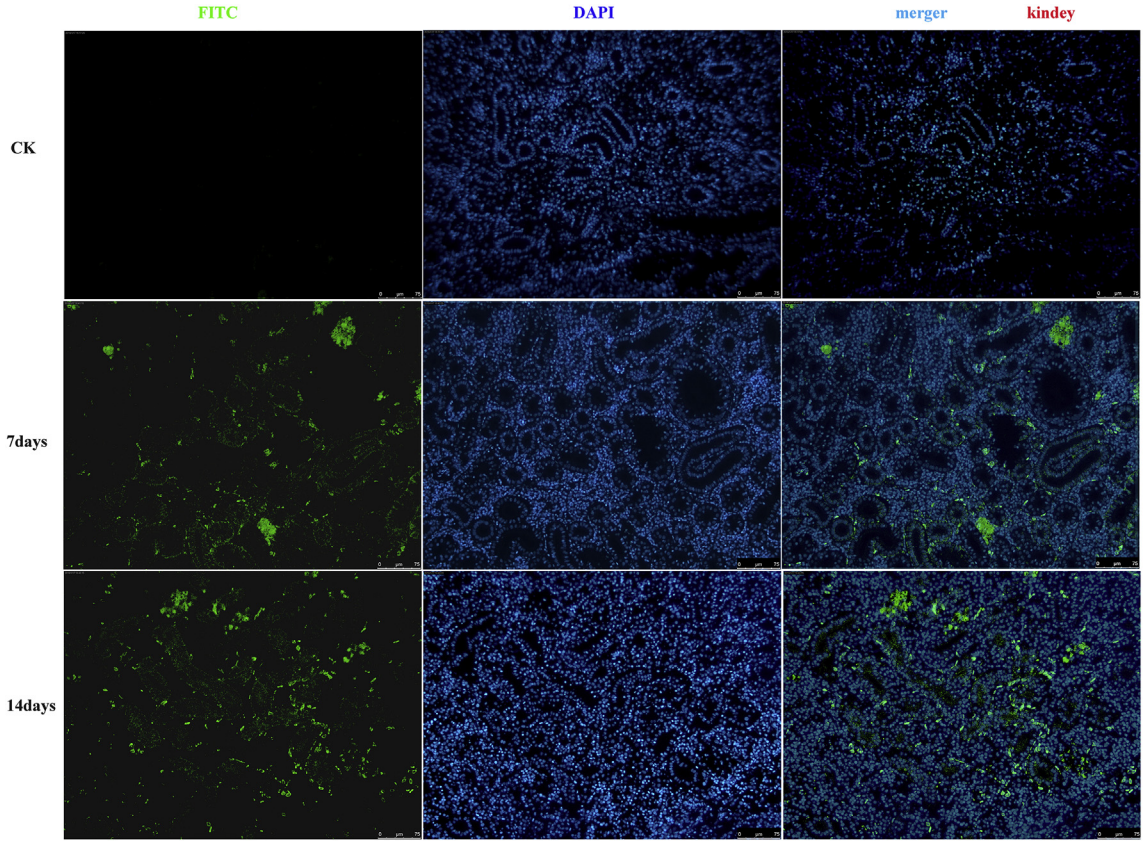

Fig. 3. Detection of D4ORFs protein in the kidneys of fish at 7 and 14 days post-vaccination via injection with BacCarassius-D4ORFs. DAPI was used for nuclear staining and FITC was used for D4ORFs protein staining. FITC, stained with FITC; DAPI, stained with DAPI; merger, the FITC-stained image merged with the DAPI-stained image. CK, the kidneys of the un-injected fish; 7 days, the kidneys of the fish at 7 days post-injection; 14 days, the kidneys of the fish at 14 days post-injection. Immunofluorescence assay was carried out with mouse anti-ORF72 polyclonal antibody and FITC-conjugated goat anti-mouse IgG.

water temperature to 32°C-35 °C [32,33] or 13°C-15 °C [34]. In gibel carp, no mortality was observed after experimental infection with CyHV-2 at 32 °C [35]; however, reducing the mortality rate due to CyHV-2 *via* increasing or decreasing the rearing water temperature is unrealistic at the industry level. The cumulative mortality of gibel carp after CyHV-2 challenge could be reduced significantly by biofloc technology, which involves improving water quality by the addition of an extra carbon source in a limited water exchange system [36]. However, vaccine administration is believed to be the most effective approach in the prevention and control of viral diseases. Immune protection by inactivated CyHV-2 vaccine has been reported in goldfish [14] and gibel carp [13], and recombinant protein ORF25 expressed in *P. pastoris* has been developed as a potential candidate vaccine against CyHV-2 infection in gibel carp [15]. In general, in the case of inactivated vaccine, the immunization dosage is large and the immune protection duration is relatively short and therefore an appropriate adjuvant is needed to enhance the immune effect, whereas in the case of recombinant subunit vaccine, immunogenicity is weaker, the protection duration is shorter, and difficulties are encountered in removing intracellular pathogens [12]. Attenuated viral vaccines typically provoke a strong and sustained immune response against a target disease, but the only attenuated viral vaccine currently available for aquaculture is cyprinid herpesvirus-3 vaccine directed against Koi herpesvirus disease [37], which may result from the limited production of attenuated viral vaccines, their poor biosafety under natural conditions, short shelf-life, and inconvenient storage and transportation requirements. Recombinant DNA vaccines can induce strong and long-lasting humoral and cell-mediated immunity and offer the advantage of easy large-scale production, high stability, no risk of reinfection, and low cost [12]. The immune efficacy of aquatic DNA vaccines for infectious pancreatic necrosis virus [38], atlantic halibut nodavirus [39], infectious hematopoietic necrosis virus [40,41], viral hemorrhagic septicemia virus [42], spring viremia of carp virus (SVCV) [43], snakehead rhabdovirus [44], and GCRV [28,45,46] have been assessed. However, DNA-based vaccines are currently administered by intramuscular injection, which has disadvantages in terms of uncertain immune responses, immune tolerance, and the risk of integration into the genome [47], which hampers their use. In the present study, a live vector vaccine based on recombinant baculovirus BacCarassius-D4ORFs containing a fused codon-optimized sequence D4ORFs driven by a *M. amblycephala* β-actin promoter was constructed. The relative immune protective rate for oral administration and abdominal injection with BacCarassius-D4ORFs reached 59.3% and 80.01%, respectively, suggesting that BacCarassius-D4ORFs is a potential candidate vaccine against CyHV-2 infection.

The selection of vaccine genes plays a critical role in vaccine development. Although the CyHV-2 genomic DNA sequence had been determined, little is known about which genes can be used as vaccine genes [19,48,49]. ORF104, a kinase-like protein encoded by CyHV-2, was considered as a potential vaccine candidate [50], but whether ORF104 vaccine could protect gibel carp from infection by CyHV-2 remains unknown. The ORF25 family of genes encoding membrane proteins were also considered as vaccine genes and the relative percent survival for gibel carp immunized with the truncated ORF25, ORF25C, and ORF25D proteins expressed in *P. pastoris* by intraperitoneal injection reached 75%, 63%, and 54%, respectively [15]. Usually the immune effect is closely related to the expression level of vaccine gene in the vaccinated animal, the truncated ORF25, ORF25C, and ORF25D genes controlled by the alcohol oxidase 1 promoter that was fused to the N-terminus of the α-factor secretion signal could be expressed, but the full length ORF25 family genes were hardly expressed in *P. pastoris*

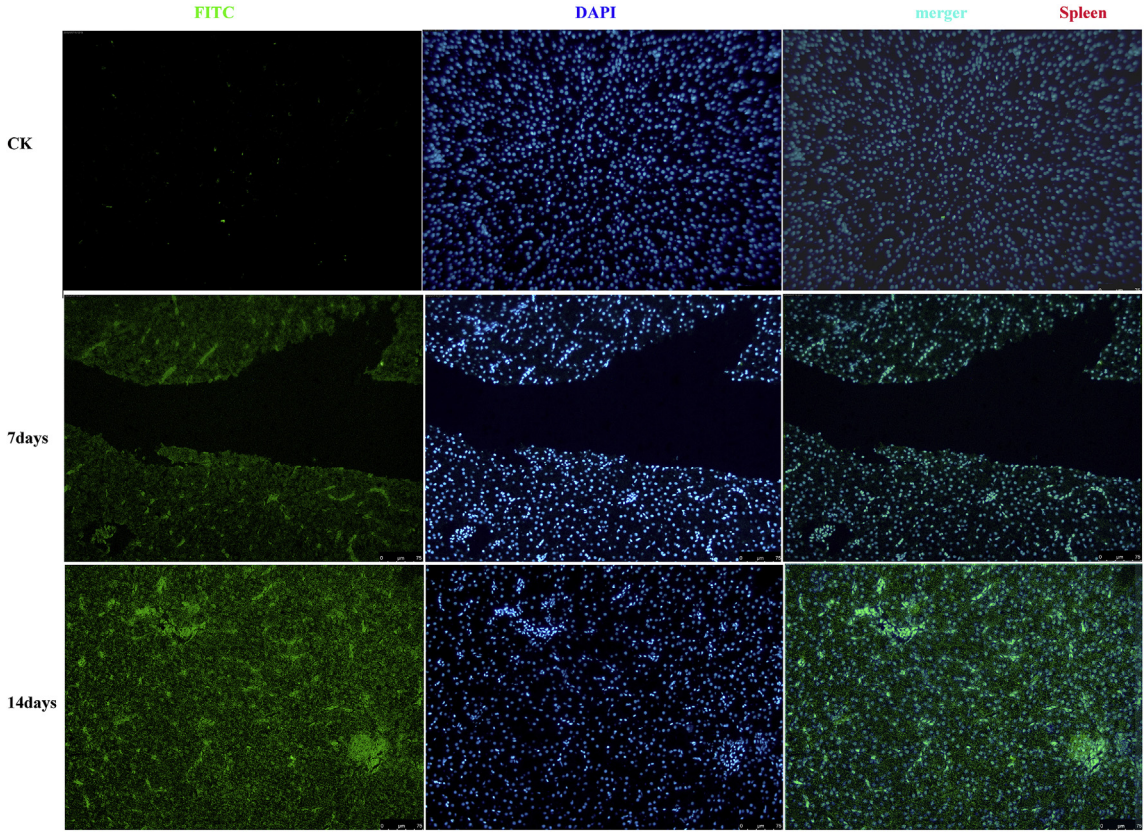

Fig. 4. Detection of D4ORFs protein in the spleen of fish at 7 and 14 days post-vaccination via injection with BacCarassius-D4ORFs. DAPI was used for nuclear staining and FITC was used for D4ORFs protein staining. FITC, stained with FITC; DAPI, stained with DAPI; merger, the FITC-stained image was merged with the DAPI-stained image. CK, the spleen of the un-injected fish; 7 days, the spleen of the fish at 7 days post-injection; 14 days, the spleen of the fish at 14 days post-injection. Immunofluorescence assay was carried out with mouse anti-ORF72 polyclonal antibody and FITC-conjugated goat anti-mouse IgG.

[15]. Therefore, it is difficult to judge whether the ORF25 family genes, which are not fused with signal peptide DNA sequence, can be effectively expressed in fish cells after the target genes were delivered into fish by a recombinant BmNPV vector. Viral membrane proteins and capsid proteins are often considered as candidate vaccine proteins. ORF66 and ORF72 are putative capsid triplex subunit proteins, and ORF81 and ORF82 are membrane proteins encoded by CyHV-2 [19]. In this study, in order to screen and evaluate candidate vaccine genes, a fused codon-optimized sequence D4ORFs that encodes partial amino acid sequences of ORF72, ORF66, ORF81, and ORF82, was used as a vaccine gene to express chimeric vaccine. Our results indicated that D4ORFs protein expression *in vivo* could protect gibel carp from CyHV-2 infection, suggesting that D4ORFs could be used as a vaccine gene against CyHV-2.

In general, a novel vaccine should be safe and cost-effective and elicit an improved immune effect whilst allowing for simple delivery. Live vector vaccine are genetically engineered vaccines that use a non-pathogenic virus, a live-attenuated virus, or bacteria that contains the expression cassettes of the target antigen that can be expressed following cellular entry of the vector *via* infection, resulting in endogenous antigen processing and MHC class I-restricted antigen presentation [16]. In aquaculture, the application of live vector vaccine was reported to prevent infection by *Salmonella* [51], *Streptococcus* [52], *Edwardsiella tarda*, *Aeromonas hydrophila* [53], and Ictalurid herpesvirus 1 [54]. In the present study, BmNPV was used as the vaccine vector and recombinant BmNPV BacCarassius-D4ORFs was constructed. No pathological changes were found in CIK cells transduced with BacCarassius-D4ORFs, but recombinant D4ORFs protein could be detected by

Table 2
Immune protection of BacCarassius-D4ORFs.

Group		Fish	Dead fish at 1–3 days post-challenge	Accumulated dead fish at 4–11 days post-challenge	Accumulated dead fish at 12–20 days post-challenge	Mortality caused by infection of CyHV-2 (%)
Injection immunization	Group IIC	30	2*	10 + 4*	10 + 4*	10/(30–4) × 100 = 38.46
	Group IIN	30	3*	7 + 6*	7 + 6*	7/(30–6) × 100 = 29.17
	Group IIV	30	2*	2 + 4*	2 + 4*	2/(30–4) × 100 = 7.69
Oral immunization	Group OC	30	4	7 + 3*	7 + 3*	7/(30–3) × 100 = 25.93
	Group OV	30	2 + 1*	4 + 4*	4 + 4*	4/(30–4) × 100 = 15.38

*CyHV-2 could be not found in the diseased fish by PCR.
Group IIV, the fish were vaccinated by intraperitoneal injection with 100 μL of BacCarassius-D4ORFs (2.2×10^{11} Tu/ml) per fish.
Group IIN, the fish were vaccinated by intraperitoneal injection with 100 μL of the control baculovirus (2.2×10^{11} Tu/mL) per fish.
Group IIC, the fish were injected with 100 μL of normal saline.
Group OV, the fish were fed with the fodder containing BacCarassius-D4ORFs.
Group OC, the fish were fed with the normal fodder.

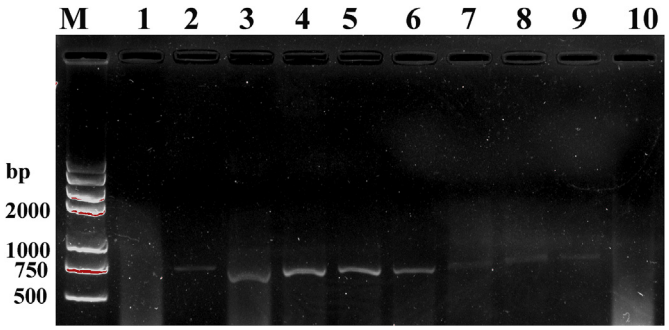

Fig. 5. Retention time of BacCarassius-D4ORFs in the spleen of vaccinated fish by injection. Total DNA was extracted from the spleen of vaccinated fish at 1–9 days post-immunization and subjected to RT-PCR with primer pair CyHV-D4-1/CyHV-D4-2. Lane M, DNA marker; lane 1, unimmunized fish; lanes 2–9, immunized fish at 1, 2, 3, 4, 5, 6, 7, and 8 weeks post-injection.

Western blotting indicating that the target gene was delivered into CIK cells and correctly expressed. PCR, qPCR, Western blotting, and immunofluorescence assays showed that the D4ORFs gene was delivered into the kidney and spleen following injection of BacCarassius into gibel carp, and the target gene was transcribed and expressed suggesting that the *M. amblycephala* β-actin promoter could be used to drive vaccine gene expression in *C. auratus gibelio*. Moreover, specific antibody against ORF72 could be detected in the serum of vaccinated fish at 7 days post-injection with BacCarassius-D4ORFs, and the antibody titer peaked at ~1:6400 at 21 days post-initial vaccination suggesting that expression of recombinant D4ORFs protein *in vivo* could stimulate higher titer antibody responses in gibel carp. The protective level in immunized fish is, in general, related to the antibody response in the serum [55,56]. A previous report showed that the relative survival rate of gibel carp immunized with β-propiolactone inactivated CyHV-2 vaccine reached 71.4%, and the relative survival rates of vaccinated goldfish with formalin-inactivated CyHV-2 vaccine reached 42.5% and 57.6%, 4 and 8 weeks after the first vaccination, respectively [14]. In the present study, the relative survival rate of immunized gibel carp following injection with BacCarassius-D4ORFs was 80.01%, indicating that injection immunization with BacCarassius-D4ORFs conferred protection against CyHV-2, and that BacCarassius-D4ORFs is a potential vaccine candidate that could be administered *via* the injection route.

The efficacy of vaccine protection is dependent on the duration of the protective effect. Baculoviruses have been shown to be replication-defective in animal cells *in vitro* and *in vivo* [18]; however, PCR results indicated that the retention time of BacCarassius-D4ORFs in the spleen of injected fish was about 7 weeks. This indicated that baculovirus-specific neutralizing serum antibodies are not induced in animals [22,23], implying that the duration of BacCarassius-D4ORFs vaccine protection may be longer. Moreover, the relative survival rate of gibel carp injected with BmNPV (Group IIN) was 24.15%, higher than that of the blank control (Group IIC), suggesting that BmNPV can confer protection against CyHV-2 infection in gibel carp. This result was similar to that of a previous report that baculovirus can confer protection against lethal influenza virus infection in mice [26] because baculovirus can stimulate the host antiviral immune response in mammalian cells [24,25].

Fish vaccines can be administered by immersion, injection, or orally, with injection being the main delivery method. Vaccination of fish by injection is labor intensive, causes stress to the fish, and requires the fish to be over a certain size, making vaccination of fry difficult. Oral vaccines are considered to be the most operative immune modes for aquatic vaccines because they are non-stressful, user-friendly, and can be easily administered to large numbers of fish. Much effort has been dedicated to oral vaccine development; however, limited protection has been reported by conventional oral vaccines due to antigen destruction in the gut. Different approaches such as entrapping in liposomes or alginate beads, slow-release microcapsules, yeast carriers, and bacteria carriers have all been used to increase protection. A previous study showed that koi herpesvirus (KHV) liposome oral vaccine can protect common carp from KHV infection [57] and the antigen could be delivered to the hindgut by feeding with Artemia encapsulated with recombinant *E. coli* containing the antigen, thereby conferring protection against direct injection of native *Pseudomonas* exotoxin and bacterial pathogens in zebrafish [58]. The oral immunization of *C. auratus* with modified recombinant A-layer proteins (associated with the fish pathogen atypical *A. salmonicida*) entrapped in alginate beads led to an increase in serum antibody titers in vaccinated fish [59]. In another study, recombinant GCRV VP5 and VP7 vaccines synergistically orally administered to grass carp induced good immunoprotection and less than 10% cumulative mortality [60]. Using *Bacillus subtilis* as the carrier of GCRV VP4, oral immunization of grass carp with recombinant vaccine followed by challenge trials showed that the oral *Bacillus* carrier could protect 50%–60% of grass carp from infection with GCRV [61]. Moreover, a genetically-engineered *Lactobacillus plantarum* surface-displaying G protein of SVCV combined with ORF81 protein of KHV was investigated for protective immunity in carp and koi through oral vaccination. Immunized carp and koi showed effective protection rates of 71% and 53%, respectively [62]. However, a recent study indicated that when carp received paraformaldehyde-fixed whole insect Sf9 cells expressing the recombinant SVCV-G protein by oral gavage of alginate-encapsulated cells, no protection against SVCV was detected [63]. In the present study, challenge experiments showed that the relative survival rate of immunized gibel carp following oral administration with pellets containing BacCarassius-D4ORFs was 59.31%, suggesting that BacCarassius-D4ORFs has the potential to be developed as an oral vaccine against CyHV-2.

Immersion vaccines are effective against a number of bacterial pathogens and are cheap and easy to administer to small fish. *Vibrio* vaccine has achieved success by soaking immunization in salmon [64] and rainbow trout [65]. In the present study, D4ORFs gene expression could be detected in the gills, spleen, and blood of the fish immersed with water containing BacCarassius-D4ORF, suggesting that baculovirus could autonomously enter fish tissues, that the D4ORFs gene was delivered into fish by immersion immunization, and that BacCarassius-D4ORF has the potential to be developed as an immersion vaccine against CyHV-2.

The BmNPV baculovirus expression system has potential not only due to its low cost but also due to its high production capacity (up to 20% of the total cellular protein) [66]. Furthermore, BmNPV vector is more stable against complement inactivation in human serum than the AcMNPV vector [67]. These features make the silkworm, whose pupae are edible, an ideal system for the expression and delivery of oral vaccine. In summary, recombinant BacCarassius-D4ORFs has the potential to be developed as a live vector vaccine against CyHV-2.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.05.065.

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