Journal of Virological Methods 299 (2022) 114342

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Production and application of monoclonal antibodies against ORF66 of cyprinid herpesvirus 2

Baoqin Guo [a,b], Chang Wei [a], Linlin Luan [a,b], Jialin Zhang [a], Qiang Li [a,*]

[a] Department of Ocean Technology, College of Marine and Biology Engineering, Yancheng Institute of Technology, Yancheng, 224051, China
[b] Key Laboratory of Mariculture & Stock Enhancement in North China's Sea, Ministry of Agriculture, Dalian Ocean University, Dalian, 116023, China

ARTICLE INFO

Keywords:
Cyprinid herpesvirus 2
ORF66
Monoclonal antibody
Immunological detection
Carassius auratus gibelio

ABSTRACT

Cyprinid herpesvirus 2(CyHV-2)is the main pathogen causing haematopoietic necrosis disease of goldfish (*Carassius auratus auratus*) and gibel carp (*Carassius auratus gibelio*), which has caused huge economic losses to aquaculture industry of goldfish and gibel carp around the world. Currently, various detection methods based on nucleic acids have been established for the detection of CyHV-2. However, there is still a lack of rapid and effective immunological detection technology. In this study, anti-CyHV-2 ORF66 monoclonal antibodies (MAbs) were prepared to use the recombinant ORF66 protein as the antigen. Firstly, the open reading frame of CyHV-2 ORF66 was cloned into the pET-28a vector and expressed in *Escherichia coli*. Three MAbs (2F11, 2G8, and 3D6) against recombinant ORF66 protein were developed by immunization of Balb/C mice. Among them, MAb-2F11 belonged to the IgG2b isotype, 2G8 and 3D6 belonged to the IgG1 isotype. Western blotting analysis was performed to assess the ability of the MAbs to bind to the ORF66 recombinant protein and CyHV-2 nucleocapsid protein ORF66. In addition, the MAb-2F11 was used to detect the virus particles that infected in cell line and tissues of gibel carp virus infection by immunological methods. These results indicated that the anti-CyHV-2 ORF66 MAb-2F11 prepared in this study could not only detect the presence of the virus but also provide a research tool for further studying the role of ORF66 in the process of CyHV-2 infection.

1. Introduction

Gibel carp (*Carassius auratus gibelio*) is one of the important freshwater species in China (Gui and Zhou, 2010). In recent years, the disease caused by cyprinid herpesvirus 2 (CyHV-2) infection resulted in huge mortality and significant economic losses of gibel carp aquaculture industry (Lu et al., 2018). The CyHV-2 infection has been reported across the world since it was first detected in Japan (Jung and Miyazaki, 1995; STEPHENS et al., 2004; Minamoto et al., 2015; Zhu et al., 2019; Fichi et al., 2013; Daněk et al., 2012).

CyHV-2 known as herpesviral haematopoietic necrosis virus (HVHNV) of goldfish is which member of Cyprinivirus that includes carp pox virus (CyHV-1) and koi herpesvirus (CyHV-3) (Chai et al., 2020). The enveloped viral particles are elliptical with a diameter of 175–200 nm, and the genome consists of a double strand of DNA with a length of about 290,304 bp (Jung and Miyazaki, 1995). The CyHV-2 genomes are predicted to contain 150 unique, functional protein-coding genes, of which four are duplicated in the terminal repeat, which are mainly involved in viral replication, assembly, and capsid formation (Davison et al., 2013). Among them, CyHV-2 has three nucleocapsid proteins, namely ORF66, ORF72, and ORF92 (Thangaraj et al., 2021). Nucleocapsid proteins are the main antigenic proteins of virions. For the infected organism, it is a specific viral antigen that can stimulate the immune system of the organism to produce an immune response (Lang et al., 2020).

At present, there is still a lack of effective prevention and control measures for CyHV-2 infection. Early and rapid detection is still the most effective means to control the spread of the virus. The detection techniques of PCR (Waltzek et al., 2009), real-time quantitative PCR (Goodwin et al., 2006), LMAP (He et al., 2013) have been developed for the detection of CyHV-2. In addition, immunodiagnostic methods based on anti-CyHV-2 antibodies have also been established, such as monoclonal antibodies (MAbs) against the recombinant protein of ORF72, ORF92, and ORF25 (Kong et al., 2017; Shen et al., 2018; Wu et al., 2020).

ORF66 encodes the major capsid protein of CyHV-2 (Thangaraj et al., 2021). In this study, the MAbs against the recombinant protein of CyHV-2 ORF66 were prepared. The specificity of MAbs was analyzed by

* Corresponding author.
E-mail address: fish790608@163.com (Q. Li).

https://doi.org/10.1016/j.jviromet.2021.114342
Received 1 September 2021; Received in revised form 24 October 2021; Accepted 27 October 2021
Available online 30 October 2021

dot blotting, indirect immunofluorescence and western blotting techniques, and the suitability of the antibody in rapid detection of CyHV-2 in infected cells and the infection process of the virus in gibel carp were studied. The results can further enrich the detection methods of CyHV-2 and provide a research tool for further study of the role of ORF66 in the process of CyHV-2 infection.

2. Materials and methods

2.1. Fish, virus, and cells

Healthy gibel carp (*Carassius auratus gibelio*) with an average weight of 50 g ± 0.5 g were purchased from an aquatic farm (Jiangsu province, China) that had no history of CyHV-2 infection. Fish were maintained in tanks (1.5 m × 0.8 m × 1.0 m) containing aerated water at a temperature of 22–25 °C for 1 week before the experiment. The CyHV-2 strain (YC-1) was isolated from diseased gibel carp in Yancheng, Jiangsu province. The cell line of GiCF (*Carassius auratus gibelio* caudal fin cell) was obtained from Shanghai Ocean University as a gift.

2.2. Expression and purification of recombinant protein of ORF66 (rORF66)

Firstly, specific PCR primers for the full-length of CyHV-2 ORF66 gene were designed according to the sequence (No. AKC02013.1) in GenBank. The primer sequences were as followed: Forward 5′-AGGATCCATGACCTCCCTCCAGCAAGCTAC-3′, Revers:5′-TAAGCTTATAAATCATGAGATCGTCTAGGTC-3′ (Underline represents the restriction enzyme sites of BamH I and Hind III respectively). The total DNA extracted from the spleen of gibel carp infected with CyHV-2 was used as the template for PCR amplification. The PCR assay was carried out by denaturation at 95 °C for 5 min, 30 cycles of amplification (95 °C for 1 min, 60 °C for 30 s, 72 °C for 1 min 30 s), and a final extension of 72 °C for 10 min. The product was identified by 1% agarose gel electrophoresis and DNA sequencing. Then, the pET28a- ORF66 plasmid was constructed by inserting the PCR product into the pET-28a expression vector with a His-tag. The plasmids were then transformed into competent *E. coli* BL21 (DH5α) cells, and the transformants were cultured in Luria Bertani (LB) medium supplemented with 50 μg/mL kanamycin. The expression of recombinant ORF66 protein (r ORF66) was induced by the addition of a final concentration of 1 mM of isopropyl-b-D-thiogalactopyranoside (IPTG). Then the bacteria were harvested and analyzed by SDS-PAGE. Subsequently, the ORF66-positive bacteria were induced on a large scale, and purified by magnetic beads (BEAVER, China) followed by the manufacturer's instruction. Finally, the purified proteins were renatured by dialysis against urea concentration gradient and quantified by Bradford protein assay kit (TaKaRa, Japan).

2.3. Preparation of monoclonal antibodies against rORF66

Three female (4 weeks old) Balb/C mice (Experimental Animal Research Center, Dalian Medical University, China) were each inoculated by intraperitoneal injection with 0.1 mL of 1 mg/mL purified rORF66 emulsified with an equivalent volume of Freund's complete adjuvant (Sigma). Two weeks later, mice were boosted with a 1:1 mixture of protein and incomplete Freund adjuvant (Sigma) once a week for three times. Three days after the last injection, the mice were anesthetized with ethyl ether and sacrificed. Spleen cells were collected from the immunized mice and fused with Sp2/0 myeloma cells using polyethylene glycol 1500 (Sigma). The cells were distributed into 96-well culture plates (Costar) in 1640 medium (Solarbio) supplemented with 20 % newborn calf serum (Gibco) and 1% HAT (Sigma), and the culture medium was changed every 3–5 days. The selection of hybridoma cells producing monoclonal antibody specific for rORF66 was accomplished by dot-blotting. Positive hybridomas were cloned three times by limited dilution.

2.4. Dot blotting assay for selection of positive hybridomas

A nitrocellulose (NC) membrane (PALL, America) was cut to the desired size and transferred into the 96-well flat-bottom microplates (Costar). 20 ng rORF66 was spotted on the NC membrane, followed by blocking with 5% BSA in PBST (PBS containing 0.05 % Tween-20) at 37 °C for 1 h, and washed three times with PBST. A 100 μL of hybridoma supernatant was added to each well and incubated at 37 °C for 1 h. Myeloma culture supernatant was used as the negative control. Each well was washed three times with PBST. After washing, 100 μL of AP-labeled goat anti-mouse Ig (Solarbio) diluted 1:2000 in PBST was added to each well and incubated at 37 °C for 1 h,then washed three times in PBST for 5 min each and developed with BCIP/NBT for 3 min. The development reaction was terminated by rinsing with distilled water. Clear purple-blue dot development on NC paper was considered positive. The MAbs were isotyped using a mouse MAb isotyping kit (Beijing Biodragon, China) according to the manufacturer's instruction.

2.5. Western blotting assay for MAbs against rORF66

Recombinant ORF66 protein and CyHV-2 cell culture suspension were separated by 12 % SDS-PAGE and subsequently transferred to a 0.2 μm-pore nitrocellulose membrane at 100 V for 1 h. After blocking at 4 °C overnight with PBST containing 5% BSA, the membrane was incubated with MAbs (2G8, 2F11 3D6) above obtained at 37 °C for 1 h, respectively. Myeloma culture supernatant was used as the negative control. After rinsing the membrane three times in PBST for 5 min each the membrane was treated with AP-labeled goat anti-mouse Ig diluted 1:2000 in PBST at 37 °C for 1 h. Then, the membrane was washed three times in PBST for 5 min each, and developed with BCIP/NBT substrate for 5 min. The development reaction was terminated by rinsing with distilled water.

2.6. Indirect immunofluorescence assay (IIFA) for MAb-2F11 against CyHV-2

GiCF cells were cultured in a 24-well plate with medium 199 (Gibco) supplemented with 10 % FBS (Gibco) and 1% penicillin-streptomycin (Thermos) at 25 °C. GiCF cells were infected with CyHV-2 and immediately washed with PBST after the CPE was observed. The cells were then fixed with acetone solution for 10 min, dried, and washed with PBST 3 times. The cells were incubated with MAb-2F11 against ORF66 for 1 h at 37 °C. After washing three times with PBST, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat-anti-mouse Ig at a dilution ratio of 1:200 in PBST at 37 °C for 1 h. After washing, the cells were mounted in an antifade solution containing DAPI (Solarbio) and examined using fluorescence microscopy. MAbs-1B7 against ORF92 protein of CyHV-2 (Shanghai Ocean University, China) were used to detect GiCF cells infected with CyHV-2 as a positive control. MAbs-2F11 against ORF66 protein of CyHV-2 were used to detect GiCF cells uninfected with CyHV-2 as a blank control.

2.7. The process of CyHV-2 infection in gibel carp

Healthy gibel carp were infected with YC-1 by immersion and maintained at 25 °C. Tissues of spleen, kidney, gill, and fin were sampled at 0 d, 1 d, 3 d, and 5 d after infection and embedded in tissue freezing medium (Leica) and stored at −20 °C. Cryosections of embedded tissues were prepared as follows: 5 mm-thick frozen sections of each sample were cut using a cryostat microtome (CM1900, Leica) at −20 °C, then fixed with pre-cooled acetone for 15 min, and stored at −20 °C for IIFA. The IIFA was performed as described above and the MAb-2F11 was used as a detective antibody.

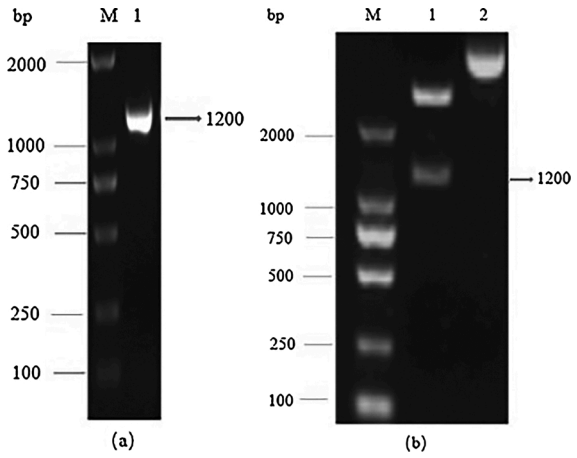

Fig. 1. Amplification of the target gene and the construction of recombinant expression plasmid.
(a) M: Marker 2000; 1: Target gene; (b) M: Marker 2000; 1: Production of pET28a-ORF66 digested with restriction enzyme; 2: Control of pET28a-ORF66 plasmid.

3. Results

3.1. PCR amplification and the construction of pET28a-ORF66 plasmid

After PCR amplification, a size of 1200 bp of the band was observed by agarose gel electrophoresis, which was consistent with the expected size of CyHV-2 ORF66. Then the product was inserted into the expression vector pET-28a. DNA sequencing confirmed that the inserted fragment was correct without any base mismatch or mutation, indicating the vector pET-28a-ORF66 was successfully constructed (As shown in Fig.1).

SDS-PAGE analysis showed that *E. coli* BL21 (DH5a) cells harboring pET28a-ORF66 induced by IPTG expressed a 50-kDa protein, which coincided with the size of predicted molecular weight. However, the bacteria without IPTG induction had no distinct protein band at the corresponding position (Fig.2a). As shown in Fig. 2b, SDS-PAGE analysis showed that the rORF66 was successfully purified, which could apply to animal immunization.

3.2. Screening of the MAbs against the rORF66

Fusion of spleen cells from immunized Balb/C mice with myeloma cells produced several hybridoma clones secreting MAbs against rORF66 of CyHV-2. Positive clones were determined by dot blotting (shown as Fig.3), three positive hybridomas designed as 2F11, 2G8, and 3D6 were cloned and subcloned by limiting dilution. MAb 2F11, 2G8, and 3D6 were isotyped with a mouse monoclonal antibody isotyping kit and the results indicated that 2F11 belongs to IgG2b isotype, 2G8 and 3D6 belong to IgG1 isotype.

3.3. Western blotting assay for MAbs against rORF66

The result of western blotting analysis showed that three MAbs (2F11, 2G8, and 3D6) reacted specifically with the 50 kDa rORF66 (Fig.4.(a)). At the same time, the CyHV-2 cell culture suspension was used to detect MAb 2F11, 2G8, and 3D6. The result is shown in Fig.4.(b) demonstrates that all the MAbs mentioned above can be used as an effective detection tool in western blotting. However, it was found that MAb-2F11 had the strongest reaction with ORF66, therefore, MAb-2F11 was selected for further study.

3.4. MAb-2F11 against CyHV-2 by IIFA

After GiCF cells were infected with CyHV-2 for 48 h, and the cells were fixed. The efficacy and specificity of MAb-2F11 against the protein of ORF66 were determined by IIFA using MAb-2F11 as the first antibody. The results showed that positive signals were clearly detected in virus-infected cells with MAb-2F11 and MAb against protein ORF92 of CyHV-2. There is no signals were observed in the control cells (Fig.5).

3.5. The process of CyHV-2 infection in gibel carp

There is no virus was detected in the tissues of spleen, kidney, and gill at 0 d, 1 d, and 3 d post infection by IIFA. The positive signals were found in fins at 3 days post-infection (Fig.6) and in the tissue of spleen, kidney, fin, and gill at 5 days post-infection (Fig.6).

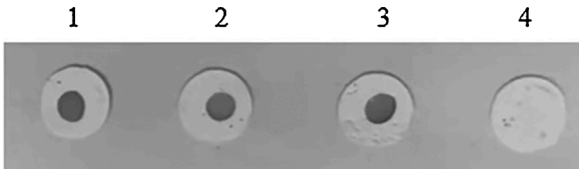

Fig. 3. Dot blotting assay for selection of positive hybridomas.
No.1−4: Immunostaining with MAb 2F11, 2G8, 3D6, and myeloma culture supernatant, respectively.

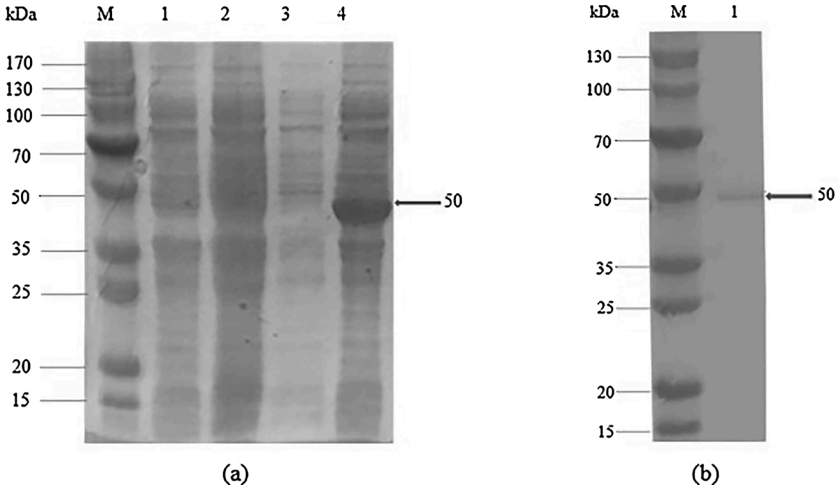

Fig. 2. Expression and purification of rORF66.
(a) Analysis of rORF66 expressed in *E. coli* BL21 by SDS-PAGE. M: Marker; 1−2: The soluble and insoluble protein of pET28a-ORF66 harboring BL21 without IPTG-induction, respectively; 3−4: The soluble and insoluble protein of pET28a-ORF66 harboring BL21 with IPTG-induction, respectively. (b) M: Marker; 1: Target band after purification of rORF66.

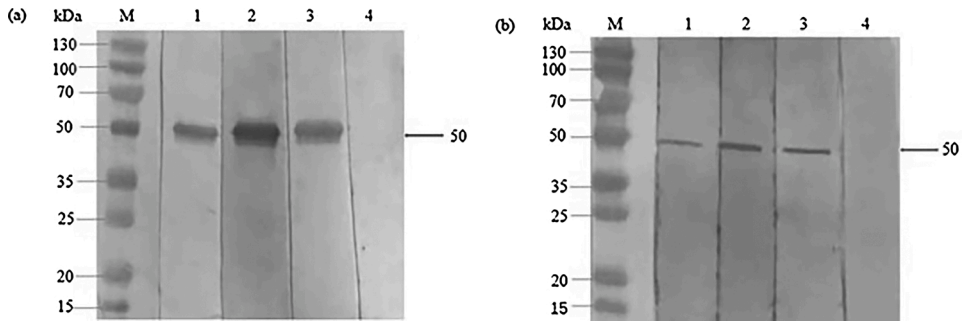

Fig. 4. Western blotting assay for MAbs against rORF66.
(a) MAbs against the rORF66. (b) MAbs against the CyHV-2 suspension. M, Protein marker; Lane 1–4, immunostaining with MAb 2G8, 2F11 3D6, and supernatant of cultured myeloma, respectively.

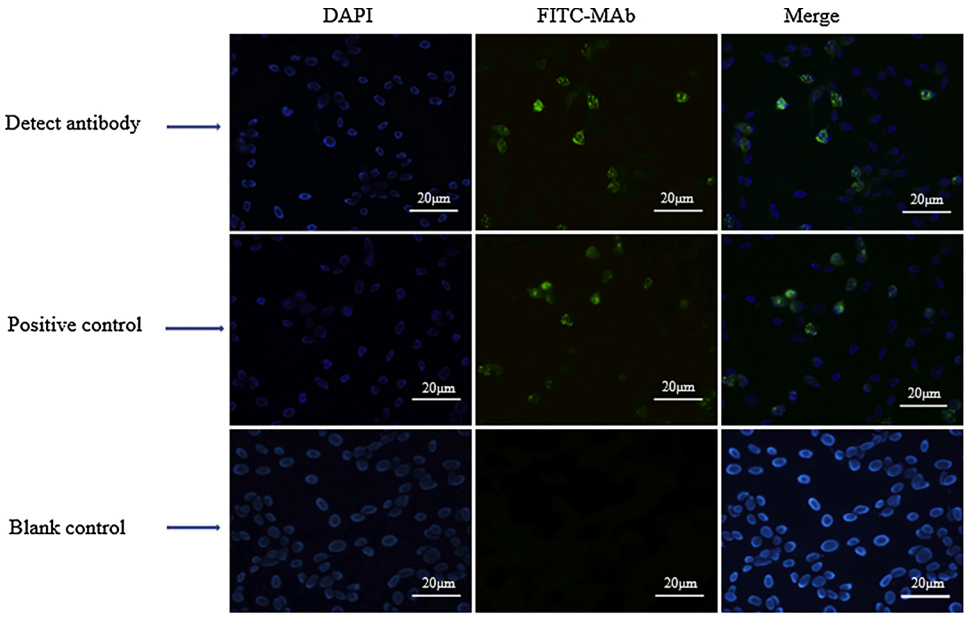

Fig. 5. CyHV-2 detection MAb 2F11 against CyHV-2 by IIFA.
Detect antibody: Detection of CyHV-2 on infected GiCF with MAb-2F11. Positive control: Detection of CyHV-2 on infected GiCF with MAb against ORF92 of CyHV-2. Blank control: Detection of CyHV-2 on uninfected GiCF with MAb-2F11.

4. Discussion

The haematopoietic necrosis of gibel carp caused by CyHV-2 infection is a severe infectious disease that causes huge economic losses to gibel carp aquaculture industry. A rapid, simple, and accurate detection method can contribute to the prevention of the outbreak of the disease.

Monoclonal antibodies (MAbs) are highly homogeneous antibodies that are produced by cloning a single B cell and are specific to a specific epitope. In recent years, MAb as a detection tool has been gradually used to detect viral diseases (Zvirbliene et al., 2006), and the number of antiviral MAb developed for fish virus infection has gradually increased (Hu et al., 2015; Aoki et al., 2011; Jing et al., 2018). Analysis of the protein structure showed that CyHV-2 contained 74 proteins, including 3 capsid proteins, 18 membrane proteins, and 53 unclassified proteins (Thangaraj et al., 2021). The preparation of MAbs against the specified proteins of the virus will provide a powerful tool to study the role of these proteins during virus infection. In addition, the establishment of immunological detection methods using MAbs could contribute to preventing the outbreak of disease. So far, three anti-CyHV-2 MAbs have been prepared by prokaryotic expression and cell fusion, among which ORF72 protein and ORF92 protein are CyHV-2 nucleocapsid protein, and ORF25 protein is virus membrane protein (Kong et al., 2017; Shen et al., 2018; Wu et al., 2020). It has been proved that the MAb against ORF72 can be used as a detection tool for an immunohistochemical protocol with the blood smear method (Kong et al., 2017). The MAb against ORF92 can also specifically recognize CyHV-2 by IFA (Shen et al., 2018). The anti-ORF25 MAb has strong specificity for CyHV-2. In addition, the test for detecting purified CyHV-2 by the immunochromatographic strip (ICS) could be completed in 10 min and the sensitivity was 1 mu g ml(-1) (Wu et al., 2020, 2021).

The goal of this study was to prepare a specific monoclonal antibody against CyHV-2. Dot blotting and western blotting results showed that the anti-ORF66 MAbs could specifically recognize rORF66 and nucleocapsid protein ORF66 of CyHV-2. At the same time, IIFA was carried out with the prepared monoclonal antibody, which proved that MAb-2F11 against ORF66 could not only detect the virus particles in GiCF cells but also could be applied to detect the CyHV-2 in the tissues of gibel carp infected with CyHV-2. The results showed that virus particles were detected in the fins 3 days after infection and in the gill, fin, spleen, and kidney 5 days after infection. These results suggested that the anti-CyHV-2 ORF66 monoclonal antibody is not only helpful for the detection of CyHV-2 infection but also provides a tool for further study of the role of ORF66 in CyHV-2 infection.

Histological analysis in the study by Ding et al. supported that CyHV-

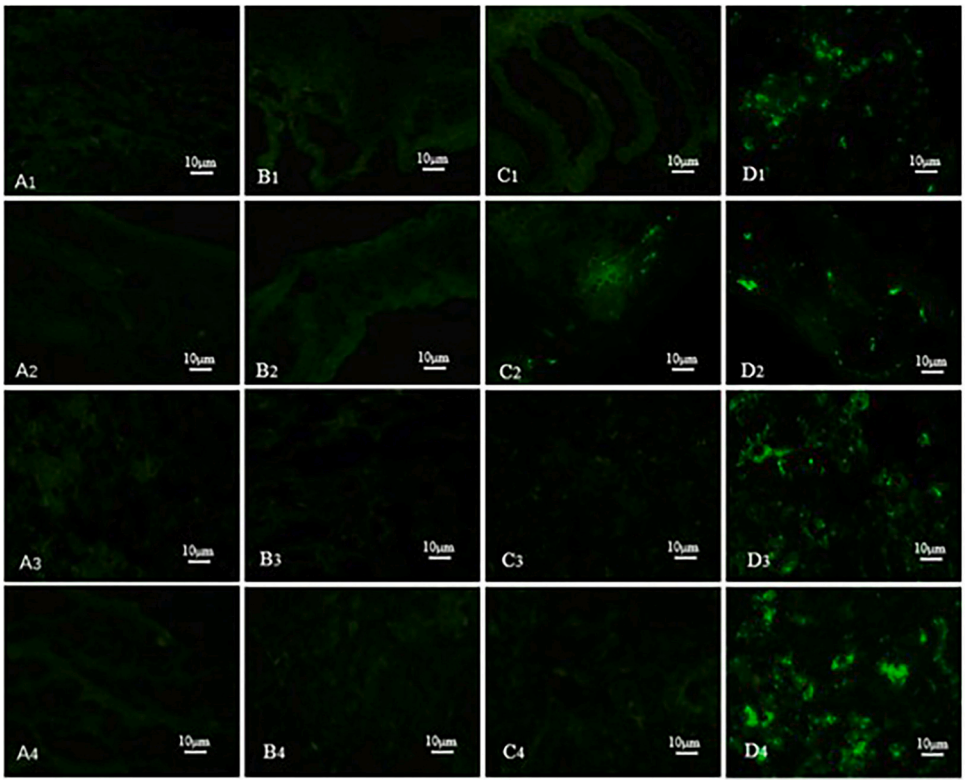

Fig. 6. Virus detection of the tissues of infection gibel carp by IIFA.
A-D: 0,1,3,5d post-infection; 1−4: tissues of gill, fin, spleen, and kidney.

2 may infect the fish through gills and induce mucosal sloughing and necrosis followed by residing in the white blood cells of kidneys and inducing severe interstitial nephritis (Ding et al., 2014; Wu et al., 2013). In addition, the results of histological examination and electron microscope observation of goldfish naturally infected with CyHV-2 also indicated that gills are the portal of CyHV-2 infection (Jiang et al., 2020). In this study, detection of CyHV-2 in the tissues of gibel carp infected with CyHV-2 by immersion found that CyHV-2 was detected in the fins of gibel carp 3 days post virus infection, but no virus particles were found in the gills and other tissues. At 5 days post virus infection, CyHV-2 was detected in the gill, kidney, spleen, and fin. The result of this study is consistent with the study of koi herpesvirus (CyHV-3) infection in *Cyprinus carpio*, which showed that skin and fin are the portal of CyHV-3 infection in Kio (Costes et al., 2009).

5. Conclusion

In this study, the recombinant protein of CyHV-2 ORF66 was successfully prepared by prokaryotic expression, and the MAbs against ORF66 were prepared by cell fusion and limited dilution cloning technique. Dot blotting and western blotting verified that anti-ORF66 MAbs could specifically identify ORF66 recombinant protein and nucleocapsid protein ORF66 of CyHV-2. IIFA results showed that the anti-ORF66 MAb-2F11 could detect CyHV-2 in GiCF cells and gibel carp tissues. Detection of CyHV-2 in different tissues of gibel carp infected with virus indicated that the fins might be the portal of CyHV-2 invasion to fish.

Author statement

Guo Baoqin: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Roles/Writing - original draft.

Wei Chang: Conceptualization, Formal analysis, Methodology, Resources, Supervision, Validation, Writing - review & editing.

Luan Linlin: Data curation, Investigation, Methodology, Software.

Zhang Jialin: Conceptualization, Data curation, Supervision, Validation.

Li Qiang: Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - review & editing.

Ethics in publishing

This study was carried out strictly in line with the guidelines on the care and use of animals for scientific purposes established by the Institutional Animal Care and Use Committee (IACUC) of Yancheng Institute of Technology, China. In this study, the methods used in animal experiments were approved by IACUC (Approval number YCIT (F)-21-010).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the Jiangsu Agricultural Science and Technology Innovation Fund (CX (18)3028). Thanks for the GiCF cell line and anti-CyHV-2 ORF92 monoclonal antibody donated by Shanghai Ocean University.

References

Aoki, T., Takano, T., Unajak, S., Takagi, M., Kim, Y.R., Park, S.B., Kondo, H., Hirono, I., Saito-Taki, T., Hikima, J., Jung, T.S., 2011. Generation of monoclonal antibodies specific for ORF68 of koi herpesvirus. J. Comp. Immunol. Microbiol. Infect. Dis. 34 (3), 209–216. https://doi.org/10.1016/j.cimid.2010.11.004.

Chai, W.J., Qi, L., Zhang, Y.J., Hong, M.M., Jin, L., Li, L.J., Yuan, J.F., 2020. Evaluation of cyprinid herpesvirus 2 latency and reactivation in *Carassius gibel*. J. Microorgan. 8 (3), 1–11. https://doi.org/10.3390/microorganisms8030445.

Costes, B., Raj, V.S., Michel, B., Fournier, G., Thirion, M., Gillet, L., Mast, J., Lieffrig, F., Bremont, M., Vanderplasschen, A., 2009. The major portal of entry of koi herpesvirus in Cyprinus carpio is the skin. J. Virol. 83 (7), 2819–2830. https://doi.org/10.1128/JVI.02305-08.

Daněk, T., Kalous, L., Vesely, T., Krasova, E., Reschova, S., Rylkova, K., Kulich, P., Petrtyl, M., Pokorova, D., Knytl, M., 2012. Massive mortality of Prussian carp *Carassius gibelio* in the upper Elbe basin associated with herpesviral hematopoietic necrosis (CyHV-2). J. Dis. Aquat. Organ. 102 (2), 87–95. https://doi.org/10.3354/dao02535.

Davison, A.J., Kurobe, T., Gatherer, D., Cunningham, C., Korf, I., Fukuda, H., Hedrick, R. P., Waltzek, T.B., 2013. Comparative genomics of carp herpesviruses. J. Virol. 87 (5), 2908–2922. https://doi.org/10.1128/JVI.03206-12.

Ding, Z.F., Xia, S.Y., Zhao, Z.M., Xia, A.J., Shen, M.F., Tang, J.Q., Xue, H., Geng, X.B., Yuan, S., 2014. Histopathological characterization and fluorescence in situ hybridization of Cyprinid herpesvirus 2 in cultured Prussian carp, *Carassius auratus gibelio* in China. J. Virol. Methods 206, 76–83. https://doi.org/10.1016/j.jviromet.2014.05.011.

Fichi, G., Cardeti, G., Cocumelli, C., Vendramin, N., Toffan, A., Eleni, C., Siemoni, N., Fischetti, R., Susini, F., 2013. Detection of Cyprinid herpesvirus 2 in association with an Aeromonas sobria infection of *Carassius carassius* (L), in Italy. J. Fish Dis. 36 (10), 823–830. https://doi.org/10.1111/jfd.12048.

Goodwin, A.E., Merry, G.E., Sadler, J., 2006. Detection of the herpesviral hematopoietic necrosis disease agent (*Cyprinid herpesvirus 2*) in moribund and healthy goldfish: validation of a quantitative PCR diagnostic method. J. Dis. Aquat. Organ. 69 (2/3), 137–143. https://doi.org/10.3354/dao069137.

Gui, J.F., Zhou, L., 2010. Genetic basis and breeding application of clonal diversity and dual reproduction modes in polyploid *Carassius auratus gibelio*. J. Sci. China Life Sci. 53 (4), 409–415. https://doi.org/10.1007/s11427-010-0092-6.

He, J.Q., Shi, X.J., Yu, L., Zheng, X.C., Lan, W.S., Jia, P., Wang, J.J., Liu, H., 2013. Development and evaluation of a loop-mediated isothermal amplification assay for diagnosis of Cyprinid herpesvirus 2. J. Virol. Methods 194 (1/2), 206–210. https://doi.org/10.1016/j.jviromet.2013.08.028.

Hu, S.L., Liou, C.J., Cheng, Y.H., Yiu, J.C., Chiou, P.P., Lai, Y.S., 2015. Development and characterization of two monoclonal antibodies against grouper iridovirus 55L and 97L proteins. J. Fish Dis. 38 (3), 249–258. https://doi.org/10.1111/jfd.12230.

Jiang, N., Yuan, D., Zhang, M., Luo, L., Wang, N., Xing, W., Li, T.L., Huang, X.L., Ma, Z. H., 2020. Diagnostic case report: disease outbreak induced by CyHV-2 in goldfish in China. J. Aquacult. 523, 1–8. https://doi.org/10.1016/j.aquaculture.2020.735156.

Jing, H.L., Zhang, M., Wu, S.Q., Lin, X.M., Gao, L.Y., Wang, N., 2018. Characterization and application of monoclonal antibodies against channel catfish virus (CCV. J. Arch. Virol. 163 (9), 1–4. https://doi.org/10.1007/s00705-018-3884-9.

Jung, S.J., Miyazaki, T., 1995. Herpesviral haematopoietic necrosis of goldfish, *Carassius auratus* (L). J. Fish Dis. 18, 211–220. https://doi.org/10.1111/j.1365-2761.1995.tb00296.x.

Kong, S.Y., Jiang, Y.S., Wang, Q., Lu, J.F., Xu, D., Lu, L.Q., 2017. Detection methods of Cyprinid herpesvirus 2 infection in silver crucian carp (*Carassius auratus gibelio*) via a pORF72 monoclonal antibody. J. Fish Dis. 40 (12), 1–8. https://doi.org/10.1111/jfd.12648.

Lang, Y.K., Chen, K., Li, Z., Li, H.M., 2020. The nucleocapsid protein of zoonotic betacoronaviruses is an attractive target for antiviral drug discovery. J. Life Sci. 282, 1–8. https://doi.org/10.1016/j.lfs.2020.118754.

Lu, J.F., Xu, D., Shen, Z.Y., Lu, L.Q., 2018. Differential expression of miRNA in *Carassius auratus gibelio* in response to cyprinid herpesvirus 2 infection. J. Dev. Comp. Immunol. 82, 1–6. https://doi.org/10.1016/j.dci.2017.12.027.

Minamoto, T., Pu, X.Y., Xie, J., Dong, Y., Wu, D.Y., Kong, H.N., Yang, X.X., Takahara, T., Honjo, M.N., Yamanaka, H., Kawabata, Z., 2015. Monitoring fish pathogenic viruses in natural lakes in Yunnan, China. J. Limnol. 16 (1), 69–77. https://doi.org/10.1007/s10201-014-0440-5.

Shen, Z.Y., Jiang, Y.S., Lu, J.F., Sano, M., Xu, D., Lu, L.Q., 2018. Application of a monoclonal antibody specific for the ORF92 capsid protein of Cyprinid herpesvirus 2. J. Virol. Methods 216, 22–27. https://doi.org/10.1016/j.jviromet.2018.07.012.

STEPHENS, F.J., RAIDAL, S.R., JONES, B., 2004. Haematopoietic necrosis in a goldfish (*Carassius auratus*) associated with an agent morphologically similar to herpesvirus. J. Austr. Vet. J. 82 (3), 167–169. https://doi.org/10.1111/j.1751-0813.2004.tb12650.x.

Thangaraj, R.S., Nithianantham, S.R., Dharmaratnam, A., Kumar, R., Pradhan, P.K., Gopakumar, S.T., Sood, N., 2021. Cyprinid herpesvirus-2 (CyHV-2): a comprehensive review. J. Rev. Aquacult. 13 (2), 796–821. https://doi.org/10.1111/raq.12499.

Waltzek, T.B., Kurobe, T., Goodwin, A.E., Hedrick, R.P., 2009. Development of a polymerase chain reaction assay to detect cyprinid herpesvirus 2 in goldfish. J. Aquat. Anim. Health 21 (1), 60–67. https://doi.org/10.1111/lam.12296.

Wu, T., Ding, Z., Ren, M., An, L., Xiao, Z.Y., Liu, P., Gu, W., Meng, Q.G., Wang, W., 2013. The histo- and ultra-pathological studies on a fatal disease of Prussian carp *(Carassius gibelio*) in mainland China associated with cyprinid herpesvirus 2 (CyHV-2. J. Aquacult. 412 (413), 8–13. https://doi.org/10.1016/j.aquaculture.2013.07.004.

Wu, R.H., Zhang, Q., Li, Y., 2020. Development, characterization of monoclonal antibodies specific for the ORF25 membrane protein of Cyprinid herpesvirus 2 and their applications in immunodiagnosis and neutralization of virus infection. J. Aquacult. 519 https://doi.org/10.1016/j.aquaculture.2019.734904.

Wu, R.H., Xue, Y., Huang, J.A., Ozdemir, E., Li, Y., Ding, S.H., 2021. Development and evaluation of a convenient immunochromatographic strip test for rapid detection of cyprinid herpesvirus 2 (CyHV-2). J. Dis. Aquat. Organ. 143, 195–203. https://doi.org/10.3354/dao03561.

Zhu, M., Li, K., Xuan, Y., Sun, Z.L., Liu, B., Kumar, D., Jiang, M., Pan, Y.S., Zhang, Y.M., Gong, Y.P., Lu, X.P., Yu, D.S., Hu, X.L., Cao, G.L., Xue, R.Y., Gong, C.L., 2019. Host range and vertical transmission of cyprinid herpesvirus 2. Turk. J. Fish. Aquat. Sci. 19 (8), 645–652. https://doi.org/10.4194/1303-2712-v19_8_02.

Zvirbliene, A., Samonskyte, L., Gedvilaite, A., Voronkova, T., Ulrich, R., Sasnauskas, K., 2006. Generation of monoclonal antibodies of desired specificity using chimeric polyomavirus-derived virus-like particles. J. Immunol. Methods 311 (1), 57–70. https://doi.org/10.1016/j.jim.2006.01.007.