Research article

Generation and Application of Monoclonal Antibodies Specific for the Envelope Protein pORF132 of Cyprinid Herpesvirus 3

Zhenxing Liu*, Le Hao, Yanping Ma, Zhiling Liang, Jiangyao Ma and Hao Ke*

Institute of Animal Health, Guangdong Academy of Agricultural Sciences, Guangdong Open Laboratory of Veterinary Public Health, Guangdong Provincial Key Laboratory of Livestock Disease Prevention, Guangzhou 510640, China

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ABSTRACT—Cyprinid herpesvirus 3 (CyHV-3), also named koi herpesvirus (KHV), is a lethal pathogen of koi and common carp *Cyprinus carpio*. In the present study, we described a procedure to generate monoclonal antibodies (mAbs) against the envelope protein pORF132 of CyHV-3 and application of the mAb. Three recombinant plasmids targeting the expression of one complete pORF132 and two truncated pORF132 were constructed, respectively. After induction, a truncated pORF132 was successfully expressed in a soluble form and the purified recombinant protein was used in the preparation of mAbs. Two hybridoma cell clones, 4E11 and 4B11, were obtained and mAb 4E11 reacted to CyHV-3 infected cells and not to CyHV-2 (goldfish hematopoietic necrosis virus) and ICHV-1 (channel catfish virus) infected cells. An antigen-capture ELISA based on the pair of mAb 4E11 and anti-CyHV-3 IgY was primarily constructed. This antigen-capture ELISA could distinguish between the CyHV-3 infected and uninfected fish samples.

Key words: cyprinid herpesvirus 3, KHV, monoclonal antibody, ORF132, ELISA, prokaryotic expression

Cyprinid herpesvirus 3 (CyHV-3), also known as koi herpesvirus (KHV), is a member of genus *Cyprinivirus* belonging to the family *Alloherpesviridae*. CyHV-3 is a lethal pathogen to common and ornamental (koi) carp *Cyprinus carpio*, which leads up to 100% mortality (St-Hilaire *et al.*, 2005). Since its emergence in the late 1990s, CyHV-3 has severely caused financial losses in the carp culture industries worldwide (Michel *et al.*, 2010a; Gotesman *et al.*, 2013).

The diagnostic methods play important roles in the virus control strategies. Targeting the detection of CyHV-3 DNA, a series of methods have been constructed: PCR (Gilad *et al.*, 2002; Gray *et al.*, 2002; Yuasa *et al.*, 2005; Bercovier *et al.*, 2005; Ishioka *et al.*, 2005), nested or semi-nested PCR (Liu *et al.*, 2002; Bergmann *et al.*, 2006; El-Matbouli *et al.*, 2007; Bergmann *et al.*, 2010a), real-time PCR (Gilad *et al.*, 2004; Fan *et al.*, 2007) and LAMP (Gunimaladevi *et al.*, 2004; Soliman and El-Matbouli, 2005; Yoshino *et al.*, 2006, 2009). Immunodiagnostic methods based on the anti-CyHV-3 antibodies have also been established: antigen-capture ELISA relying on polyclonal antiserum

(Dishon *et al.*, 2005), immunofluorescence assay (IFA) and immunohistochemical staining relying on monoclonal antibodies (mAbs) (Aoki *et al.*, 2011; Tu *et al.*, 2014; Fuchs *et al.*, 2014).

MAbs against CyHV-3 pORF68 (Aoki *et al.*, 2011), pORF72 (Tu *et al.*, 2014) and pORF149 (Fuchs *et al.*, 2014) have been generated. CyHV-3 pORF68 and pORF149 are envelope proteins. Envelope proteins might involve the host-virus interactions and become the targets for the establishment of diagnosis method and development of vaccine (Yi *et al.*, 2014). CyHV-3 has a large genome of about 295 kb, which contains 156 different ORFs. To date, 14 ORFs including ORF132 have been identified as genes encoding envelope proteins (Rosenkranz *et al.*, 2008; Michel *et al.*, 2010b; Yi *et al.*, 2014).

In the present study, the recombinant truncated pORF132 was expressed and then used as an antigen for preparation of mAbs. Two mAbs were obtained and one mAb was further subjected to the establishment of IFA and antigen-capture ELISA for the detection of CyHV-3 from fish tissues.

* Corresponding authors

Zhenxing Liu. E-mail: liuzhenxing81@126.com

Hao Ke. E-mail: keha@tom.com

Materials and Methods

Virus and fish

CyHV-3 HZ419 was isolated from a diseased koi carp in a fish farm located in south China in 2012. The virus was propagated in common carp brain (CCB) cells in MEM supplemented with 10% FBS (Gibco). Koi carps weighing 15 g were bought from a fish farm without history of KHVD and were negative for CyHV-3 DNA when tested by PCR (Yuasa *et al.*, 2005).

Construction of the pET32a-ORF132 expression plasmid

Three expression plasmids, pET32a-132A, pET32a-132B and pET32a-132C, were constructed which targeted the expression of complete pORF132 (aa 1–170), truncated pORF132 (aa 26–170) without the signal peptide and truncated pORF132 (aa 97–170) containing the major epitope domain, respectively. Thus, three forward primers (132F1-F3) were designed and paired with the common reverse primer (132R), respectively, for the amplification of corresponding fragments. Primer sequences were as follows: 132F1 (5'-CCGGAATTCATGACGGGTTCCAAGATTAGC-3'), 132F2 (5'-CCGGAATTCCTCAATCCCTCACCGACCTCACG-3'), 132F3 (5'-CCGGAATTCGCGCGTATATCGAGGCACTT-3') and 132R (5'-CCCAAGCTTCTTAGACCTTTTAGACGATTTTGG-3'). Restriction enzyme sites *Eco*R I and *Hind* III (underlined) were incorporated into the forward and reverse primer sequences. CyHV-3 HZ419 genome DNA which was extracted from the mixture of liver and kidney tissue of the diseased fish with a TIANamp Genomic DNA Kit (Tiagen Biotech Co., Ltd.) served as template and other components in the reaction mixture were added according to the manufacturer's protocol of rTaq polymerase kit (TaKaRa). A thermal cycler program was consisted of 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 30 s, followed by a final extension at 72°C for 1 min. The purified PCR products were digested with corresponding restriction enzymes and then inserted into the prokaryotic expression vector pET-32a(+) (Novagen). The *E. coli* Rosetta transformed with recombinant plasmids were screened by PCR as mentioned above, and then the insertion was confirmed by DNA sequencing.

Expression and purification of recombinant pORF132

The recombinant expression plasmids, pET32a-132A, pET32a-132B and pET32a-132C, were transformed into *E. coli* Rosetta, and then were propagated at 37°C in Luria Bertani (LB) broth until the bacterium reached logarithmic growth phase (0.5–0.6 at OD₆₀₀). The recombinant *E. coli* were induced by addition of isopropyl-β-D-thiogalactoside (IPTG) at a final concentration of 1 mM at 25°C for 4 h. Solubility analysis of the recombinant protein was carried out according to

the pET manufacturer's instructions. The expression of recombinant pORF132 was also confirmed by Western blot analysis with the anti-His antibody (Sigma-Aldrich). Purification of the His-tagged recombinant protein was performed using the Ni²⁺-NTA resin (Merck). Concentration of the purified protein was quantified by the BCA Protein Assay Kit (Dingguo), and then was stored at –80°C until used.

Preparation of anti-pORF132 mAbs

BALB/c mice were injected subcutaneously with 60 μg of truncated pORF132 expresses from pET32a-132C emulsified in an equal volume of complete Freund's adjuvant (Sigma-Aldrich) for the primary immunization. Two booster injections were given with 60 μg of the protein in incomplete Freund's adjuvant (Sigma-Aldrich) at 2 wk intervals. Immune response was monitored by indirect ELISA. Briefly, the wells of a 96-well ELISA plate were coated by 100 μL/well of pORF132 at a concentration of 3.125 μg/mL in carbonate-bicarbonate buffer (pH 9.6) at 4°C overnight. After washing with PBS containing 0.05% (v/v) Tween 20 (PBST), the plate was blocked with 200 μL/well of PBS containing 1% (w/v) BSA at 37°C for 2 h. After washing with PBST, two-fold serial dilutions of mouse anti-pORF132 serum (1:3,200–1:204,800) were added in duplicate. The plate was incubated at 37°C for 2 h. After washing with PBST, 100 μL/well of diluted (1:8,000) goat anti-mouse-IgG conjugated with HRP (Tiagen) was added, and then the plate was placed at 37°C for 1 h. The plate was washed again and 100 μL of TMB substrate solution was added into each well. The plate was incubated at 37°C for 10 min, and then the reaction was stopped by 50 μL/well of 2 M H₂SO₄. Absorbance was determined at 450 nm with a plate reader.

Cell fusion (somatic hybridization) was performed when the antibody titers over 1:102,400. Briefly, the spleen cells from immunized BALB/c mice were obtained, which was used in the following polyethylene glycol-mediated cell fusion. The hybridoma cells were screened for antibody production against pORF132 using an indirect ELISA as mentioned above. The primarily screened positive hybridoma cells were further confirmed using the CyHV-3 virion which was prepared as previously described (Liu *et al.*, 2014). The selected hybridoma cells were subcloned three times by limiting dilution. The stability of antibody secretion was determined by *in vitro* passages and anabiosis from the frozen stocks.

Characterization and purification of anti-pORF132 mAbs

The mAb isotypes were determined by the Mouse Monoclonal Antibody Isotyping Reagents (Sigma-Aldrich). In addition, ELISA additivity test was applied to determine whether the mAbs recognized the same

epitopes as previously described (Friguet *et al.*, 1983; Leveux *et al.*, 1995). The hybridoma cell clone 4E11 was inoculated intraperitoneally into pristane-primed BALB/c mice for the production of ascites. The antibody was purified from the ascites using a protein G-coupled sepharose column according to the manufacturer's instructions (Amersham Bioscience). The concentration of purified mAb 4E11 was determined as mentioned above.

Immunofluorescence assay (IFA)

IFA was performed according to the manufacturer's protocol of SABC-Cy3 IFA kit (BOSTER). Briefly, the CCB cells were grown in a 96-well tissue culture plate, and then infected with CyHV-3. When the CPE was observed, the infected cells were fixed with cold methanol (-20°C) for 20 min at RT. Then the plate was washed with PBST three times and blocked with 2% BSA in PBS at 37°C for 1 h. After washing three times, 100 μL /well of purified anti-pORF132 mAb (4E11) was added at a concentration of 2.5 $\mu\text{g}/\text{mL}$ and then incubated at 37°C for 1 h. After washing three times, fixed cells were added with biotin conjugated goat anti-mouse IgG (BOSTER) at a dilution of 1:100, and then were incubated at 37°C for 1 h. After washing three times, the Cy3 conjugated streptavidin-biotin complex (SABC-Cy3) was added at a dilution of 1:100, and then the fixed cells were incubated at 37°C for 1 h. After washing three times, DAPI was added at a final concentration of 5 $\mu\text{g}/\text{mL}$, and then the plate was incubated at 37°C for 1 h. After washing three times, the double-stained cells were visualized under the inverted fluorescence microscope (Axio Observer A1, Zeiss). The Ictalurid herpesvirus 1 (IcHV-1: ATCC VR-665) infected CCO cells (channel catfish ovary cell) and cyprinid herpesvirus 2 (CyHV-2: YC1507) infected crucian carp brain cells served as control to determine the specificity of mAb 4E11.

Antigen-capture ELISA

The koi were infected by inoculating CyHV-3 onto the gills (El-Din, 2011). One week post infection, an outbreak of KHVD was observed, and then confirmed by PCR (Yuasa *et al.*, 2005). The kidney and liver from three dying fish were collected and homogenized in PBS (30%, w/v), and then centrifuged at $3,000 \times g$ at 4°C for 20 min to obtain the supernatant which were served as positive samples. Meanwhile, the homogenate supernatant from three uninfected koi served as negative samples. In addition, the tissue sample of the tilapia *Oreochromis niloticus* was tested by the following antigen-capture ELISA to assess the cross-reactivity. The mean OD_{280} values of koi and tilapia tissue homogenate (1:100 dilution) were 0.829 with a SD of 0.092 and 0.801 with a SD of 0.136, respectively.

The method of square titration was used to confirm the optimum concentration or dilution of capture antibody (mAb), samples (homogenate), detection antibody (IgY) and HRP-IgG (against IgY) in the ELISA. The final procedure was determined as follow: The 96-well ELISA plate was coated with capture antibody, mAb 4E11, at a final concentration of 4 $\mu\text{g}/\text{mL}$ at 4°C overnight. After washing and blocking, the positive and negative samples were added at a dilution of 1:20 in duplicate, respectively. The plate was incubated at 37°C for 1 h and then washed three times with PBST. The anti-CyHV-3 chicken egg yolk immunoglobulin (IgY) which was generated as previously described (Liu *et al.*, 2014) was added as detection antibody at a final concentration of 5 $\mu\text{g}/\text{mL}$, and then the plate was incubated at 37°C for 1 h. After washing three times, rabbit anti-chicken (IgY)-IgG conjugated with HRP (Sigma-Aldrich) was added at a dilution of 1:8,000 and the plate was placed at 37°C for 1 h. After washing three times, TMB substrate solution was added and then the reaction was stopped by 2 M H_2SO_4 . Absorbance was determined at 450 nm with a plate reader.

Fig. 1. The analysis of nucleotide and amino acid sequences of ORF132. The putative cleavage site of the signal peptide is indicated by a downward arrow (↓). The transmembrane helices are shown in grey. The main hydrophobic domains are boxed. The binding sites for primers (132F1, 132F2, 132F3 and 132R) are underlined. The regions of possible epitopes predicted using the DNASTAR software are highlighted with double-headed arrow (↔).

Twenty samples from uninfected koi were used as negative references to determine the cut off value as previously described (Jia *et al.*, 2009).

Results

Expression of recombinant pORF132

CyHV-3 ORF132 was 513 bp in length, which encoded a 170-aa protein with the molecular weight of 18.5 kDa. A possible N-terminal signal peptide was recognized in the pORF132. Based on the analysis of the flexibility in second structure, hydrophilicity, surface probability and antigenic index of pORF132, six possible epitopes were predicted with the DNASTAR software. Two epitopes were located in the N-terminal hydrophobic region and the other four epitopes in the C-terminal hydrophilic region (Fig. 1).

Three fragments were amplified, and then inserted into the pET32-a(+) vector (Fig. 2). The recombinant plasmids, pET32a-132A, pET32a-132B and pET32a-132C, were identified by PCR and DNA sequencing. Theoretically, taking into account the Trx-tag, the molecular weight of fusion proteins expressed by above plasmids were 38.0 kDa, 35.7 kDa and 28.0 kDa, respectively. However, after the induction with 1 mM IPTG at 25°C for 4 h, the plasmids pET32a-132A and pET32a-132B were not successful in producing the detectable pORF132 in *E. coli* Rosetta. A truncated pORF132 was expressed by the plasmid pET32a-132C, which was consistent with the expected molecular weight (Fig. 3). A positive band was also observed in the lysate of *E. coli* Rosetta transformed with pET32a-132C by the analysis of Western blot (Fig. 4A). In addition, the truncated pORF132 was found mainly in the

supernatant of the cell lysate after centrifugation, suggesting that the target protein was in a soluble form (Fig. 4B).

Characterization and purification of mAb

Two hybridoma cell clones, 4E11 and 4B11, were acquired. The corresponding mAbs, 4E11 and 4B11,

Fig. 2. Amplification of ORF132. Lane M: DNA marker DL 1,000; lane 1: the complete CDS of ORF132 (pET32a-132A); lane 2: the partial CDS of ORF132 without signal peptide-encoding sequence (pET32a-132B); lane 3: the partial CDS of ORF132 encoding the major epitope domain (pET32a-132C).

Fig. 3. Expression and purification of the recombinant CyHV-3 pORF132. Lane M: protein marker; lane 1 and 2: lysate from the IPTG-uninduced and induced *E. coli* Rosetta transformed with pET-32a(+); lane 3 and 4: lysate from the IPTG-uninduced and induced *E. coli* Rosetta transformed with pET32a-132A; lane 5 and 6: lysate from the IPTG-uninduced and induced *E. coli* Rosetta transformed with pET32a-132B; lane 7 and 8: the lysate from the IPTG-uninduced and induced *E. coli* Rosetta transformed with pET32a-132C; lane 9: purified recombinant CyHV-3 pORF132.

Fig. 4. Western blot analysis (A) and solubility determination (B) of recombinant pORF132. Lane M: protein marker; lane 1 (A): lysate of IPTG-induced *E. coli* Rosetta transformed with pET32a-132C; lane 2 (A): purified recombinant truncated pORF132; lane 1 (B) and 2 (B): supernatant and the pellet of bacterial lysate from IPTG-induced *E. coli* Rosetta transformed with pET32a-132C.

Fig. 5. Immunofluorescence analysis (IFA) with mAb 4E11. CyHV-3-infected CCB cells (A), ICHV-1 infected CCO cells (B), uninfected CCB cells (C) and CyHV-2-infected crucian carp brain cells (D) were stained with mAb 4E11, then incubated with biotin conjugated goat anti-mouse IgG and Cy3 conjugated streptavidin-biotin complex (SABC-Cy3). The nuclear was visualized using DAPI. The bar represents 20 μ m.

were determined as IgG1 and IgM subclass, respectively. Here the measured additivity index was 5.6%, which implied that the two mAbs targeted the same or similar epitope. In addition, 4E11 showed an apparently higher cell growth rate (data not shown). Thus, the mAb 4E11 purified from the mouse ascites was further applied in the IFA and antigen-capture ELISA.

Application of the mAb in IFA

The red fluorescence signal was detected in the CyHV-3 infected CCB cells, meanwhile, the negative results were observed in the CyHV-2 infected crucian carp brain cells and the ICHV-1 infected CCO cells based on the analysis of IFA, which excluded the cross-reactivity of mAb 4E11 with the above two fish herpesviruses (Fig. 5). The cytoplasm accumulation of the CyHV-3 pORF132 also supported that the pORF132 was an envelope protein, which is consistent with the previous mass spectrometry results (Michel *et al.*, 2010b).

Application of the mAb in the antigen-capture ELISA for infected-fish tissue homogenate

The antigen-capture ELISA was optimized using a checkerboard titration analysis, the optimal capture and detection antibodies were determined as 4 μ g/mL and 5 μ g/mL, respectively. The optimal dilution of HRP conjugated anti-chicken (IgY) rabbit IgG was determined as 1:8,000. The mean OD₄₅₀ value of negative sample was 0.283 with a SD of 0.05. Thus, the positive threshold was 0.433. Results of antigen-capture ELISA showed a significant difference ($p < 0.05$) between the CyHV-3 infected and uninfected sample (Fig. 6).

Fig. 6. Detection of CyHV-3 in fish tissues by ELISA. The homogenate supernatant from the kidney and live pool of CyHV-3 infected koi ($n = 3$), healthy koi ($n = 3$) and tilapia ($n = 3$) were tested by the antigen capture ELISA using mAb 4E11 and anti-CyHV-3 IgY. The bar indicates the means $\pm$ standard errors of the means. Asterisk (*) denotes significant differences from the others ($p < 0.05$).

Discussions

pET system is widely applied in the preparation of recombinant proteins. The Trx fusion tag expressed by the pET32-a(+) vector can contribute to obtain the soluble, active and properly folded target proteins. In this study, pET32-a(+)/Rosetta system failed to express the complete pORF132 as well as fragment without signal peptide. Based on the analysis of the pORF132 structure using the DNASTAR software, a 96-aa N-terminal hydrophobic domain was found in this protein and two transmembrane helices were also predicted in this region by the TMPred program. In addition, the first helix almost overlapped the signal peptide (Fig. 1). According to the result of expression, this N-terminal hydrophobic domain might affect the successful expression of pORF132. The similar phenomenon that the N-terminal hydrophobic domain affects the stability of recombinant CyHV-3-pORF59 has been also observed in our previous study (Ke *et al.*, 2010). The truncated pORF59 could be successfully expressed when the N-terminal hydrophobic domain of pORF59 is deleted. Although without the N-terminal hydrophobic domain, the truncated pORF132 retains the major epitopes. In addition, the 25-aa N-terminal signal peptide has been predicted in previous report by the former version of SignalP program (Michel *et al.*, 2010b). However, the prediction of SignalP 4.1 with the default D-cut off values denies the existence of signal peptide. Taking into account the prediction from PrediSi (<http://www.predisi.de/home.html>) and Signal-3L program (Shen and Chou, 2007), we consider that there is a signal peptide in the pORF132.

The envelope of the mature virion acts as an obstacle to prevent the penetration of antibody into the inner structures of the virion, when the mature virus assembles in the cytoplasm (Yi *et al.*, 2014). The mAb against pORF68 demonstrates a promising prospect in the construction of diagnostic method. The localization of pORF68 in the virion has not been determined. Although a transmembrane (TM) domain is predicted by SOSUI (Aoki *et al.*, 2011), no TM region was detected by TMHMM and pORF68 has not been classified as a member of 30 typical membrane proteins in CyHV-3 virion (Aoki *et al.*, 2007; Michel *et al.*, 2010b), which reduces the possibility of its localization in the envelope. As mentioned above, that the pORF132 located in the envelope makes it easier to react with the antibody.

It is necessary to examine the cross-reactivity of mAb 4E11 between CyHV-3 and other aquatic herpesviruses, especially cyprinid herpesviruses, CyHV-1 and CyHV-2. We confirmed mAb 4E11 reacted to CyHV-3 infected cells and not to CyHV-2 and ICHV-1 infected cells. CyHV-1 which causes viral papilloma in carp is seldom reported in China, and the acquisition of virus from foreign countries is difficult considering govern-

ment regulations. Based on the BLAST search, we find that the pORF132 do not match any other proteins. In other words, it is a CyHV-3-specific protein. In addition, the sequence encoding truncated protein in our study shares 100% identity with that of all the other CyHV-3 isolates, such as CyHV-3-J (AP008984), CyHV-3-U (DQ657948), CyHV-3-I (DQ177346), CyHV-3-GZ11 (KJ627438). Thus, the 4E11 could specifically react with all known CyHV-3 isolates in theory. The actual cross-activity will be evaluated in our further studies.

The genomes of cyprinid herpesviruses (CyHV-1, 2, 3) share 120 orthologous ORFs, which causes the potential problem of cross-reactivity (Davison *et al.*, 2013). For example, the serum antibody from the CyHV-3 infected carp reveals cross reactions with CyHV-1 (Adkison *et al.*, 2005). In addition, goldfish (*Carassius auratus auratus*) has been confirmed as a carrier for CyHV-3, although no typical signs of KHVD could be observed in the infected goldfish. CyHV-3 could be propagated in the goldfish, resulting in transmitting to cohabitated healthy common carp (Bergmann *et al.*, 2010b; El-Matbouli *et al.*, 2011). However, there is a paper which described no virus multiplication occurred in the epidermal cells of goldfish exposed with CyHV-3 (Yuasa *et al.*, 2013). Thus, the goldfish becomes an important target for the detection of CyHV-3. Meanwhile, the goldfish has been suffering from the CyHV-2 infection. It will be reasonable to distinguish the CyHV-3 from CyHV-2 by a specific mAb during the detection from the samples of goldfish.

Dishon *et al.* (2005) has built an antigen-capture ELISA with the rabbit anti-CyHV-3 serum serving as capture antibody, meanwhile, the biotinylated rabbit anti-CyHV-3 IgG serving as detection antibody. That method could detect the viral antigens in the stools of diseased fish. In this study, the mAb 4E11 and specific anti-CyHV-3 IgY have been used for the primary construction of antigen-capture ELISA, which possesses two characteristics: mAb could theoretically improve the specificity and IgY could be largely generated.

In the present study, the truncated CyHV-3 pORF132 which has been expressed in *E. coli* serves as an antigen for the generation of the mAb. In addition, an antigen-capture ELISA for the diagnosis of CyHV-3 has been primarily constructed depending on the mAb. The recombinant pORF132 and its corresponding mAb will also support the further research on the function of this protein.

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