抗コイヘルペスウイルス鶏卵抗体(IgY)の経口投与によるコイの受動免疫

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Research article

Oral Passive Immunization of Carp *Cyprinus carpio* with Anti-CyHV-3 Chicken Egg Yolk Immunoglobulin (IgY)

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ABSTRACT—Cyprinid herpesvirus 3 (CyHV-3), also called as koi herpesvirus, has spread all over the world since its emergence in the late 1990s. CyHV-3 causes a fatal disease, which leads to a mortality rate of more than 80% in common and ornamental (koi) carp *Cyprinus carpio*. The novel anti-CyHV-3 strategies are urgently needed. In this report, CyHV-3 particles were concentrated and then used for the immunization of hens in order to obtain the anti-CyHV-3 egg yolk immunoglobulin (IgY). The proportion of anti-CyHV-3 IgY to total IgY in water-soluble fraction of egg york increased from 0.014% to 23.9% after three times of immunization. The anti-CyHV-3 effects of IgY were then examined by *in vivo* and *in vitro* assays. The results suggested that the anti-CyHV-3 serum of hens and the purified anti-CyHV-3 IgY from eggs could prevent common carp brain (CCB) cells from infection. After oral passive immunization with IgY, the mortality of carp significantly decreased from 85% to 50% ($P < 0.05$) when the fish were challenged with CyHV-3 at a dose of 40 $TCID_{50}$/fish. These results will possibly pave the way for the prevention of CyHV-3 infection.

Key words: Cyprinid herpesvirus 3, KHV, carp, yolk immunoglobulin, IgY, oral passive immunization

Cyprinid herpesvirus 3 (CyHV-3), also called as koi herpesvirus (KHV), represents a member of genus *Cyprinivirus* belonging to the family alloherpesviridae. Since its emergence in the late 1990s, CyHV-3 has spread all over the world, leading to massive economic losses (Michel *et al.*, 2010). Due to its fatal infection, the mortality of common and ornamental (koi) carp *Cyprinus carpio* infected with CyHV-3 is usually over 80% under experimental and natural infection conditions (Bergmann *et al.*, 2010). Vaccination is generally considered to be the most effective method to prevent the virus infection. So far, the attenuated and inactivated vaccines have been developed to control CyHV-3 (Perelberg *et al.*, 2008; Yasumoto *et al.*, 2006). However, the attenuated vaccine seems to retain residual virulence. Moreover, it is hard to distinguish the immunized fish from those infected with wild-type CyHV-3 because of the seropositivity. It might bring a lot of problems in the international trade of such kind of immunized fish. Thus, it is an urgent need to develop the novel and effective strategies against CyHV-3 infection.

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The intestine and periodontal pharyngeal mucosa of the digestive tract have been recently confirmed as portals of entry for CyHV-3, in addition to the skin (Monaghan *et al.*, 2014; Fournier *et al.*, 2012). Moreover, enteritis is one of the clinical signs resulted from the infection (Hedrick *et al.*, 2000), and replication of CyHV-3 is detected in intestine of infected carp (Dishon *et al.*, 2005; Syakuri *et al.*, 2013). These results suggest that alimentary tract might be a potential therapeutic target during CyHV-3 infection. Chicken egg yolk immunoglobulin (IgY) referring to the antibodies transferred from serum to the egg yolk, has been used for the prevention of viral or bacterial pathogens in the digestive tract by oral passive immunization (Rahman *et al.*, 2013). Oral administration of IgY is especially suitable for fish and exhibits favorable effects (Arasteh *et al.*, 2004; Gutierrez *et al.*, 1993; Lee and Mine, 2000). In this study, we have prepared the anti-CyHV-3 IgY and examined its role in the prevention of CyHV-3 infection.

Materials and Methods

Fish, cells and virus

Carp of 10–25 g were bought from Pearl River Fisheries Research Institute, Chinese Academy of Fishery

Sciences and raised in aquaria filled with 40 L water. The water temperature was kept at 25°C with a 1/3 water change once a day. Common Carp Brain (CCB) cells were cultivated at 25°C in Minimum Essential Medium (Invitrogen corp.) containing 10% fetal bovine serum (Invitrogen corp.). The CyHV-3 ZS38 strain was isolated from a diseased carp in a fish farm located in south China in 2009.

Purification of CyHV-3 and immunization of hens

The CyHV-3 ZS38 strain was inoculated to CCB cells. The cultures were harvested until the cytopathic effect (CPE) was observed in 90% of the cells and its titer was determined by $TCID_{50}$ assay (Trkola *et al.*, 1995).

The cell culture was applied to the clarification of CyHV-3 according to the method developed previously (Yasumoto *et al.*, 2006) with slight modifications. Briefly, after 3 freeze-thaw cycles, the debris of CCB cells in the cultures were removed by centrifugation at 3,000 ×*g* at 4°C for 20 min. The supernatant was collected for the clarification of virus by ultracentrifugation at 45,000 ×*g* at 4°C for 60 min. The obtained CyHV-3 particles were suspended in the TEN (0.05 M Tris-HCl, 0.15 M NaCl, 0.001 M EDTA, pH7.2). The concentration of total protein was determined by BCA Protein Assay Kit (Tiangen Biotech Co., Ltd.) and then stored at –80°C until used. The isolated CyHV-3 was negatively stained and examined by transmission electron microscope (Hitachi, Ltd.).

Eighteen laying hens were divided into 3 groups (n = 6). Two groups of them were immunized with concentrated CyHV-3 emulsified in Freund' adjuvant (Sigma-Aldrich, Inc.) and ISA70VG adjuvant (Seppic), respectively, which named as F group and I group. Another group, C group, was injected with PBS without adjuvant and served as the control. A detailed immunization scheme was showed in Table 1.

Serum and IgY

The serum and IgY were collected after 10 days of each immunization and then at 10-day intervals after the third injection. The blood was taken with syringes and clotted at 37°C for 2 h. The serum was then separated by centrifugation at 3,000 ×*g* at 4°C for 20 min. The IgY was obtained in WSF (water-soluble fraction) of the egg yolk according to the method described previously (Lu *et al.*, 2009). The serum and egg yolk IgY were used to detect the antibody response.

Anti-virus ELISA and egg collection

The serum antibody and egg yolk IgY were detected by indirect ELISA. Briefly, the wells of a 96-well ELISA plate were coated by 100 μL/well of concentrated CyHV-3 antigen (1 μg/mL) in carbonate-bicarbonate buffer (pH 9.6) at 4°C overnight. The plate was washed 3 times with PBS (pH 7.4, 0.01 M) containing 0.05% (v/v) Tween 20 (PBST) and then blocked with 200 μL/well of PBS containing 1% (w/v) BSA at 37°C for 2 h. After rinsing with PBST, 100 μL of diluted serum (1:6400) or diluted IgY (1:3200) were added into each well in duplicate. The plate was incubated at 37°C for 2 h. After washing with PBST, 100 μL/well of diluted (1:10000) rabbit anti-chicken (IgY)-IgG conjugated with HRP (Sigma-Aldrich, Inc.) was added and 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 H_2SO_4. Absorbance was determined at 450 nm with a plate reader (Thermo Fisher Scientific Inc.).

When the levels of anti-CyHV-3 reached the peak value (10 days after the third immunization) the eggs from F and I groups were collected, separately. In essence, the specific anti-CyHV-3 IgY in the eggs was not different between the F group and I group. The eggs of F group were used for the next assay.

Purification of IgY

Six eggs were selected randomly from the collection of F group eggs, the yolk was isolated and used as the pooled sample for the purification assay. Five methods were used to purify IgY from the same egg yolk sample. The ethanol precipitation, chloroform extraction, PEG precipitation, Na_2SO_4 precipitation and $(NH_4)_2SO_4$ precipitation were performed according to the procedures described previously (Schade *et al.*, 2001; Lu *et al.*, 2009). The purity of the extracted IgY was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and its anti-CyHV-3 titer was determined by ELISA as described in anti-virus ELISA.

Table 1. Immunization scheme

immunization order	days after first immunization	antigen dosage (μg)	adjuvant	
			F group	I group
1st	–	100	CFA	ISA70VG
2nd	14	200	IFA	ISA70VG
3rd	28	300	IFA	ISA70VG

CFA: complete Freund's adjuvant; IFA: incomplete Freund's adjuvant.

Determination of IgY concentration

The concentration of total IgY was determined by the capture ELISA developed previously (Lee *et al.*, 2002). The concentration of specific anti-CyHV-3 IgY was measured by indirect ELISA. A 96-well ELISA plate was coated with 100 μL of 20 μg/mL concentrated CyHV-3. After blocking, two-fold serial dilutions of WSF and IgY (purified by Na_2SO_4 precipitation) were added in duplicate. Rabbit anti-chicken IgG conjugated with HRP (Sigma-Aldrich, Inc.) was added subsequently. Other ELISA procedures were undertaken according to ELISA described in anti-virus ELISA.

Neutralization assay (in vitro test)

The neutralization activities of specific IgY and serum were calculated in terms of 50% neutralization dose (ND_{50}). CCB cells were inoculated as described above. Two eggs were randomly selected from the collection of F group eggs, then the IgY was purified by Na_2SO_4 precipitation as mentioned above. The anti-CyHV-3 serum was from F group post the third immunization. The non-specific IgY and negative serum from C group were also prepared in the same way. In addition, the anti-CyHV-3 titer of IgY and serum was determined as mentioned above. The IgY and serum were inactivated at 56°C for 1 h and then serially diluted in MEM (1:2–1:256). The CyHV-3 was adjusted to 200 $TCID_{50}$/mL in MEM and mixed with an equal volume of above dilutions. The mixture was incubated at 25°C for 1 h and then added into 8 replicate wells of CCB cells plate (200 μL/well) replacing the original MEM. The plate was kept at 25°C for 14 days. Based on the observation of CPE, ND_{50} was calculated by the formula of Reed and Munch (1938).

Uptake of IgY

The IgY was purified from F group eggs by Na_2SO_4 precipitation as mentioned above, and then adjusted to 8 mg/mL (PBS, pH 7.2). Twenty four carp were used to determine the uptake of IgY. Three unfed fish were severed as the control (0 day). The other 21 carp were intragastrically administrated with the purified IgY at 0.04% body weight. In the following 7 days, 3 fish were euthanized every day and their serum, intestine, liver were collected and pooled, respectively. The serum was diluted in five times volume of PBS. The intestine was dissected and flushed thoroughly with PBS to remove intestinal contents, then the intestine and liver were homogenized in PBS (20%, w/v) and centrifuged at 3,000 ×*g* at 4°C for 20 min to obtain the supernatant. The prepared samples were used to determine the concentration of total IgY by the capture ELISA described above.

Preparation of feed

The IgY was purified from F group or C group through the Na_2SO_4 precipitation, and the concentration of total IgY was determined as mentioned above. Then the carp feed containing specific IgY (from F group) or non-specific IgY (from C group) was prepared according to the method described by Arasteh *et al.* (2004). The IgY was calculated in terms of total IgY and added into the commercial pellets at the weight ratio of 0.05%. Briefly, IgY was sprayed onto commercial pellets (Uni-president Co. Ltd.) to obtain the pellets containing specific or non-specific IgY. The pellets were dried and stored at 4°C until used. One gram samples of each kind of pellets was immersed in ddH_2O at 4°C for 4 h and then centrifuged at 10,000 ×*g* at 4°C for 20 min. The supernatant was collected to determine the anti-CyHV-3 IgY titer by ELISA described in anti-virus ELISA.

Challenge test (in vivo test)

Eighty carp were divided into 4 groups (n = 20) and then were fed above-mentioned pellets for 10 successive days as described below. The fish were fed with the prepared pellets twice daily, at 9:00–10:00 am and 16:00–17:00 pm at 2% body weight throughout the trial. Fish of group 1 and group 3 were orally administered with specific IgY pellets, group 2 and group 4 were fed with non-specific IgY pellets. Carp of group 1, 2 and group 3, 4 were then infected by inoculating CyHV-3 onto the gills (El-Din, 2011) at a dose of 80 and 40 $TCID_{50}$ of CyHV-3, respectively. The mortality of challenged carp was observed daily. The protection effect was evaluated as the relative percent survival: RPS = (1 – passively vaccinated group mortality/control group mortality) × 100% (Yasumoto *et al.*, 2006).

Clinical signs and pathological examination

Six dead carp from the middle of challenge test (7–14 days post infection (d.p.i.)) were dissected for parasitological and microbiological examinations. Todd-Hewitt broth (THB) and nutrient agar (NA) were used to isolate bacteria from the gills, brain, liver and kidney. The isolated bacteria were identified by the PCR amplification and sequence of 16S rRNA gene (Weisburg *et al.*, 1991). The total DNA from the brain tissue of the dead carp was used as the template to amplify the SphI-5 fragment of CyHV-3 (Gray *et al.*, 2002). The PCR products were sequenced. The sera of 3 survived carp were collected at the 28 d.p.i.. Anti-CyHV-3 IgM titer was assessed by the ELISA developed previously (St-Hilaire *et al.*, 2009).

Statistical methods

SPSS13 was used to perform the statistical analysis. The data of IgY concentration was analyzed using the one-way ANOVA followed by the Duncan multi-comparison of means test. The mortalities observed in the challenge test were analyzed using the

Fisher's exact test. The significant level was considered as $P = 0.05$.

Results

Production of the anti-CyHV-3 antibodies

The titer of virus stock reached $10^{4.2}$ $TCID_{50}$/mL and relatively intact virus was obtained (Fig. 1). The antibodies production of F group (immunized with Freund' adjuvant) was slightly faster than that of I group (immunized with ISA70VG adjuvant), and the peak of antibody titer occurred earlier in serum than in egg yolk (Fig. 2). The antibody titer in serum and egg yolk from F group and I group finally rose to the similar level.

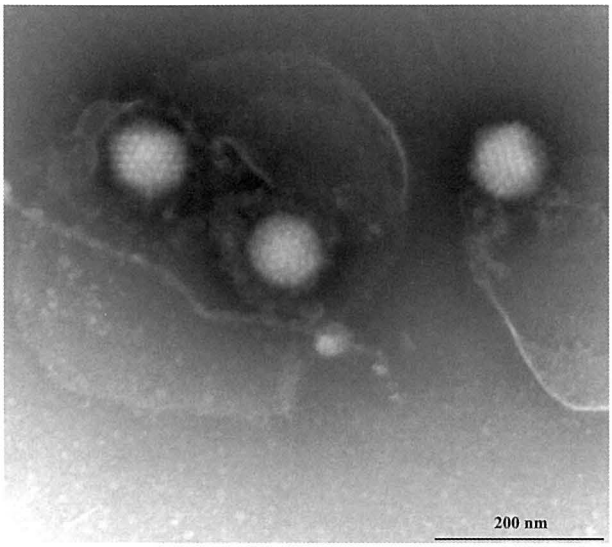

Fig. 1. Transmission electron microscopy. The concentrated CyHV-3 was detected by transmission electron microscopy (negative staining).

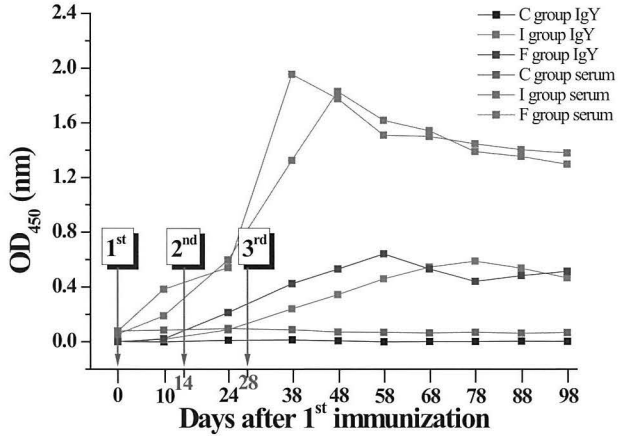

Fig. 2. Production of the anti-CyHV-3 antibodies. Titer of serum and IgY in WSF against CyHV-3 were measured by ELISA. F group: CyHV-3 with Freund' adjuvant, I group: CyHV-3 with ISA70VG adjuvant, C group: PBS without adjuvant. Arrows indicate the immunization time points. Values represent the means of duplicate wells.

Table 2. The anti-CyHV-3 titer of the IgY purified by different methods

Purification methods	chloroform extraction	PEG precipitation	ethanol precipitation	Na_2SO_4 precipitation	$(NH_4)_2SO_4$ precipitation
Antibody titer	> 1:25600	> 1:12800	> 1:6400	> 1:25600	> 1:25600

Values represent the means of duplicate wells.

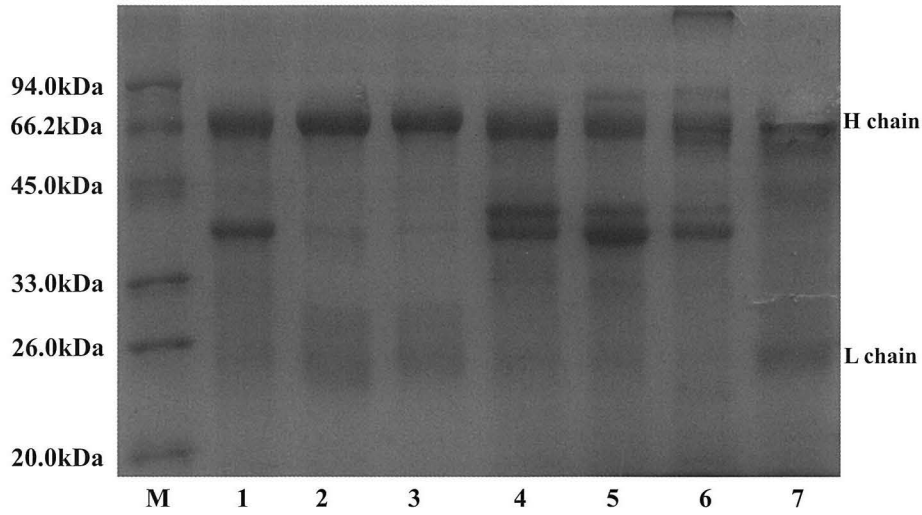

Fig. 3. SDS-PAGE of IgY purified by different methods. The IgY was extracted by $(NH_4)_2SO_4$ precipitation (lane 1), Na_2SO_4 precipitation (lane 2), PEG precipitation (lane 3), ethanol precipitation (lane 4), chloroform extraction (lane 5), WSF (lane 6). The arrows indicate the heavy chain and light chain of the standard IgY (Sigma). Protein molecular weight markers are used to calculate sample molecular weights (lane M).

Purification of egg yolk IgY

IgY purified by chloroform extraction, Na_2SO_4 precipitation and $(NH_4)_2SO_4$ precipitation showed higher antibody titer (Table 2). SDS-PAGE indicated that comparatively pure IgY was purified by the $(NH_4)_2SO_4$ or PEG precipitation (Fig. 3). In this study, Na_2SO_4 precipitation was a preferable method. Therefore, IgY extracted by Na_2SO_4 precipitation was used for the *in vitro* and *in vivo* anti-CyHV-3 tests.

Concentration of egg yolk IgY

The concentration of total IgY and specific anti-CyHV-3 IgY was measured, respectively. The result showed that the concentration of total IgY from F or C group eggs was not significantly different ($P > 0.05$) post the immunization, but the concentration of specific IgY (in the F group eggs) increased remarkably ($P < 0.05$) (Table 3) compared to that in the C group eggs. These data indicated that the immunization of CyHV-3 did not change the total IgY level, but that of specific anti-CyHV-3 IgY. The increased tendency of specific anti-CyHV-3 IgY in F and I group was also observed in Fig.2.

Neutralization activities of IgY in vitro

The neutralization assay revealed that specific IgY or serum could prevent the CCB cells from being infected with CyHV-3. CPE was observed in all control wells (added non-specific IgY and corresponding negative serum) at 5 d.p.i., at the same time, no CPE appeared in the wells added specific IgY or serum. It indicated that the specific antibody could delay the course of CyHV-3 infection. Finally (14 d.p.i.), their values of ND_{50} were 1/18.77 and 1/3.34, respectively. This result was associated with the difference of anti-CyHV-3 titer between serum (> 1:102400) and IgY (> 1:12800).

Uptake of IgY

Three days after the intragastric administration, the relatively constant total concentration of IgY was detected in the liver, intestine and serum, respectively (Fig. 4). The IgY could be transported into serum, suggesting that IgY could be distributed to other tissue by the circulatory system.

Table 3. Concentration of egg yolk IgY

	WSF (C group)	WSF (F group)	IgY (F group) purified by Na_2SO_4 precipitation
total IgY (mg/mL)	1.21 ± 0.11	1.19 ± 0.13	1.89 ± 0.35
specific IgY (mg/mL)	0.00016 ± 0.00059	0.28 ± 0.016*	0.44 ± 0.041
proportion of specific IgY in total IgY (%)	0.014	23.9	23.3

Values of IgY concentration are the means of triple samples ± SD. Asterisk denotes significant differences ($P < 0.05$) of specific IgY concentration in WSF between C group and F group.

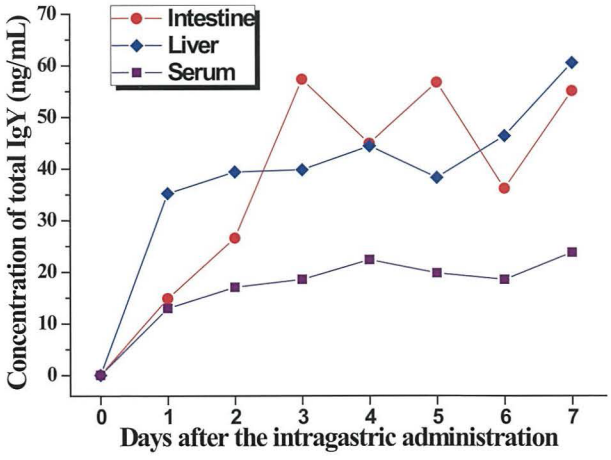

Fig. 4. Detection of total concentration of IgY absorbed in the carp serum, liver and intestine tissue of the fish intragastrically administrated with the specific IgY pellets. Values represent the means of duplicate wells.

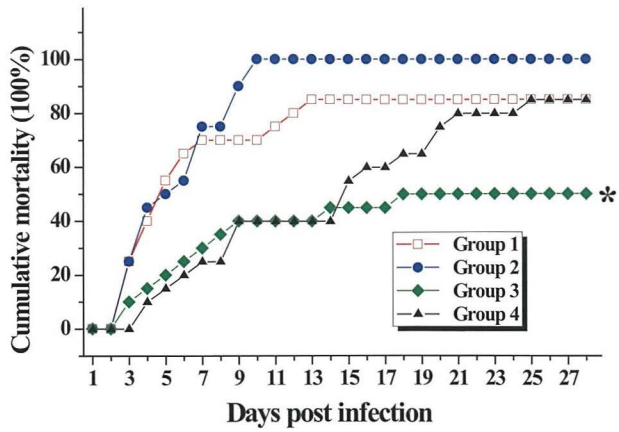

Fig. 5. *In vivo* protection induced by IgY administration. Carp were divided into 4 groups (n = 20 fish/group) and challenged with CyHV-3 (group 1 and 2: 80 $TCID_{50}$/fish, group 3 and 4: 40 $TCID_{50}$/fish) after feeding with IgY pellets (group 1 and 3: 0.05% (w/w) specific IgY, group 2 and 4: 0.05% (w/w) non-specific IgY) for 10 consecutive days.

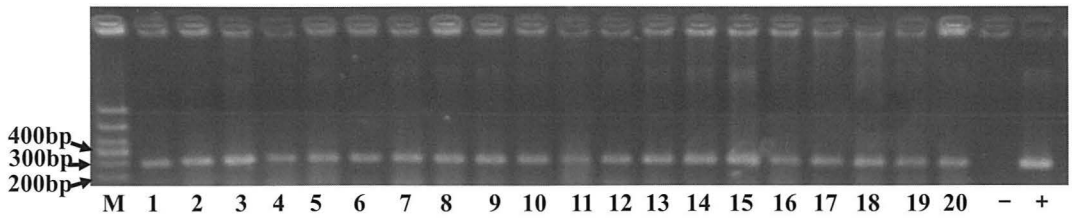

Fig. 6. Detection of CyHV-3 DNA by PCR. The DNA templates were extracted from the brains of the dead carp from the *in vivo* test. One third of the dead carp were randomly examined. M: DNA marker (DL1000); lane 1–5: partial dead carp from group 1, lane 6–10: partial dead carp from group 2, lane 11–15: partial dead carp from group 3, lane 16–20: partial dead carp from group 4; +: positive control; –: negative control.

Anti-CyHV-3 effect of specific IgY in carp

The mortality was associated with the infectious dose of CyHV-3. The infection with 80 $TCID_{50}$ of the virus caused 100% mortality at 10 d.p.i. and the lower mortality (85%) was induced by challenge with 40 $TCID_{50}$ of the virus in the control group fed with non-specific IgY pellets. Passive immunization of carp with specific IgY provided resistance to the CyHV-3 infection. The protective effect was affected by the infectious dose: when fish were challenged with a higher dose of CyHV-3 (80 $TCID_{50}$/fish), the mortality decreased by 30% ($P < 0.05$) at 10 d.p.i.. But the final cumulative mortality (28 d.p.i.) was, however, not significantly different ($P > 0.05$), the survival rate maintained 15% (28 d.p.i.), oral administration of specific IgY could delay the death course of infected fish. Under the condition of a lower infectious dose (40 $TCID_{50}$/ fish), the mortality reduced from 85% to 50% ($P < 0.05$) at 28 d.p.i. (Fig. 5) and RPS was 41.18%.

Pathogeny and anti-CyHV-3 IgM

The typical clinical signs including skin lesion, gill necrosis, kidney enlargement *et al*. were observed in almost all the dead carp. No parasite was found under the microscope and only *Aeromonas veronii* was isolated from the gills of all the 6 diagnosed carp, but nearly no bacteria were cultured from the brain, liver or kidney. On the contrary, CyHV-3 was detected in all dozens of the brain samples (Fig. 6), suggesting that CyHV-3 was the main cause of the acute death. Some carp survived from the challenge, and three of them were chosen for the examination of IgM against CyHV-3. The result of ELISA indicated that the 3 survival fish were seropositive (Table 4).

Table 4. Anti-CyHV-3 serum IgM titer of the survival carp

carp	OD450 (1:400)	OD450 (1:800)
1#	0.521	0.271
2#	0.439	0.268
3#	0.514	0.288
uninfected carp	0.041	0.029

Values represent the means of duplicate wells.

Discussion

Due to the severely financial losses in the common carp and koi carp culture industries worldwide caused by CyHV-3, how to prevent the damage of CyHV-3 becomes a global issue. No effective methods have been applied for the prevention of CyHV-3 except two types of vaccines (Perelberg *et al*., 2008; Yasumoto *et al*., 2006). The attenuated vaccine offers ideal protection against CyHV-3, however, the mortality of the immunized fish could reach 30% when the fish are kept at permissive temperature (24°C). Although the authors assume that a general inferior immune response or CyHV-3 non-related pathogens should be responsible for the mortality, the residual virulence could not be excluded. In addition, there is a risk of reverse to violence for the attenuated virus. The inactivated vaccine is safer and RPS ranging from 65.0% to 74.3% has been obtained. The protection induced by oral passive immunization with specific IgY is inferior (RPS was 41.18%). But the IgY have an advantage that the administration of IgY provides protection by the consumption of little time (10 days in our challenge test). In vaccine researches, a period of 3 to 6 weeks after the initial immunization is generally required for the antibody titer rising to a high level (Ronen *et al*., 2003; Adkison *et al*., 2005), in other words, there is a possible dangerous period of several weeks before the vaccine plays the protective role. In fact, the common or koi carp are usually challenged at 22 days post vaccination (d.p.v.) or 27 d.p.v. (Perelberg *et al*., 2008; Yasumoto *et al*., 2006). By contrast, the oral passive immunization just needs several days. Furthermore, the IgY incorporated into the pellets exhibits excellent stability, the activity does not decrease after storage for 2 months at 4°C (data not shown).

The portals of entry for CyHV-3 are important and ideal drug targets, which largely decide the methods of drug administration. The skin, pharyngeal mucosa, gills and intestine have been indicated as the major portals of entry (Costes *et al*., 2009; Fournier *et al*., 2012; Monaghan *et al*., 2014). They act under different epidemiological conditions: CyHV-3 can enter carp either by skin contact or by digestive tract after ingestion of infec-

tious materials, for example the virus-contaminated freshwater mussels (Kielpinski *et al.*, 2010). There is a hypothesis that the CyHV-3 invades intestinal epithelial cells then spreads to the other tissues via the blood causing a systemic infection (Lee *et al.*, 2012). The first line of defense against CyHV-3 should be located in digestive tract of fish. In our study, the virus has been inoculated onto gills which are connected with the oropharyngeal cavity adjoining intestine. They are the targets of virus entry and replication. On the other hand, the IgY is mainly enriched in the alimentary system including intestine and liver after the oral immunization. So we assume that the IgY plays a preventive role through the neutralization effect in the alimentary system.

Anti-CyHV-3 IgM has been detected in the serum of survival fish, which might result from the uptakes of degenerated CyHV-3 neutralized by IgY or the invasion of virus. Although the challenge dosage is low (40 or 80 $TCID_{50}$ per fish), the virus will replicate in the infectious carp, then the multiplicated virus can be released into the water. In addition, the virus could be also spread through skin contact. The challenge test indicates an intense outbreak of KHVD considering that the mortality of the control fish is no less than 85%. The antigen of virus might be sufficient to induce antibody production in fish. Moreover, fish intestine could absorb large protein molecules even the intact rabbit IgG (Nakamura *et al.*, 2004). So we speculate that the degenerated fragments or intact virus constitute the source of antigen. In fact, the production of anti-CyHV-3 IgM may be more important for carp to survive from the challenge (Perelberg *et al.*, 2008).

In summary, our study suggests the first report of the passive immunization of carp with anti-CyHV-3 IgY, and the administration of specific IgY can reduce the mortality of carp challenged with CyHV-3. These researches supply a possible method to control the infection of CyHV-3.

Acknowledgments

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抗コイヘルペスウイルス鶏卵抗体（IgY）の経口投与によるコイの受動免疫

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世界中でコイヘルペスウイルス（KHV）病による深刻な被害が続いており，本病への新たな防除対策が求められている。そこで，本研究では濃縮したKHVを2週間おきに3回注射して牝鶏を免疫し，その卵から抗KHV鶏卵抗体を精製し，その有効性を*in vivo*および*in vitro*で調べた。なお，試験区には抗KHV IgYを対照区には非免疫鶏卵から精製した等量のIgYを用いた。その結果，抗KHV IgYは，*in vitro*ではCCB細胞へのKHVの感染を阻害し，*in vivo*では攻撃前10日間の経口投与により，40 $TCID_{50}$/fishのKHVで攻撃されたコイの死亡率を85％から50％に有意に低下させた（$P<0.05$）。抗KHV鶏卵抗体は，KHV病の防除対策に新たな道を切り開く可能性を秘めている。

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