



Preparation of rabbit anti-carp IgM and dynamic changes of anti-KHV antibody level in koi serum after vaccination with purified KHV antigen

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Abstract

Koi Herpesvirus Disease (KHVD) is infectious and acute viraemia of common/koi carp caused by Koi herpesvirus (KHV). In this study, common carp IgM was purified and used to immunize rabbits to prepare rabbit anti-common carp IgM. OD of the rabbit anti-common carp IgM (marked as RACIgM) positive serum at 1:10000 was 0.654, while OD of the negative control was 0.192 tested by direct ELISA. Comparison of homemade and kindly presented RACIgM from a French expert showed that the OD values of 1/4000 dilutions of presented RACIgM and 1/5000 dilutions of homemade RACIgM positive serum were similar, all above 1.1, and 19.5 times of negative serum which average OD was 0.0595. KHV isolate F347 from ATCC and 07-1086 from French expert was amplified on the KF and EPC cell lines and collected for virus purification by sucrose density gradient centrifugation. Rabbit anti-KHV antibodies were prepared by immunizing with purified KHV acquired from 30%, 45%, 60% position and pellets of gradient centrifugation (hereinafter, shortened as KHVKF30% ~ 60%, KHVEPC30% ~ 60%, KHVKF, KHVEPC). Titers of rabbit anti-KHV antibody immunized with KHV-KF at 1:1080, while KHVKF30-60% and KHVEPC diluted to 1:120 were similar to that of presented rabbit anti-KHV antibody. They were nearly four times of pre-immune serum. After vaccination with KHV-KF, KHVKF30%, KHVKF45%, KHVKF60%, and KHV EPC into koi carp, The vaccinated koi produced an anti-KHV antibody response except one koi carp inoculated with KHVKF 60% did not change during 88 days of the experiment. Titers Anti-KHV antibody of inoculated koi carp were significantly increased 1 month after vaccination. The time arrived to peak varied with antigen type and individual fish, then the titer slowly decreased, and there was still a high titer of antibody on the day 88. Anti-KHV serum with high titers may serve as positive control for further KHV immunological study.

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Introduction

Koiherpesvirus disease (KHVD) caused by koiherpesvirus (KHV) is contagious and acute viraemia in common carp (*Cyprinus carpio*) and koi carp and other varieties [1]. The disease infects common/koi carp of all ages, causing serious illness and death, with mortality rates as high as 80%-90%. One of the characteristics of Herpesviridae especially for the mammalian herpesvirus is latent infection following virus infection. Studies indicated that survivors following an outbreak of KHVD may become persistent and retain the virus for quite a long time. The virus persisted in experimentally infected common carp subsequently maintained at a lower level than that at permissive temperature [2]. Eide KE et al. [3] reported that no infectious virus or KHV DNA was detected by PCR and no virus isolation in fecal secretion or gill swabs, but KHV DNA other than infectious virus or mRNAs was detected by PCR and Southern blotting in White Blood Cells (WBC) of clinically healthy koi carp from farms with recorded KHV infection. The virus infection in clinically healthy koi carp was reactivated after being subjected to a temperature stress regime, which indicated a latent status of KHV infection, KHV DNA remained at low copy numbers in the latently infected tissues, only small proportion of fish was detected by real-time PCR in WBC preparation. Detection of KHV or viral genomic DNA had been reported difficult beyond 64 days post initial exposure even with highly sensitive quantitative PCR [4].

As difficulties have been encountered for reliable detection of persistent or latent virus in clinically healthy fish already exposed to virus or survivors from KHV infection by PCR-based methods, and very few cell lines are available and quite complicated for the virus isolation, Detection of antibodies may be a valuable method and surveillance tool. Some studies established ELISA tests to detect anti-KHV antibodies in infected common/koi carp serum at 3 weeks after experimental infection and in survivors after 1 year following a natural infection [2,5-8]. All these studies used similar ELISA protocols, i.e., fish serum was added to the plates coated with gradient purified KHV, after wash mouse anti-carp Ig monoclonal antibody (Mab), Horse Radish Peroxidase (HRP) conjugated anti-mouse IgG (or biotin-streptavidin system) and substrate was added to develop color. And also some researchers [9] used rabbit anti-carp Ig to replace the anti-carp Ig Mab.

Studies have shown that carp IgM is the main immunoglobulin in fish serum. Purification of common carp IgM, preparation of anti-carp IgM serum is an essential component for the establishment of indirect ELISA for detection of anti-KHV antibodies in fish serum. Due to lack of easily available anti-carp IgM and other reagents, carp IgM was purified, and rabbit anti-carp IgM was prepared. In this study, the purified KHV was inoculated into rabbits to prepare rabbit anti-KHV serum. Rabbit anti-carp IgM and rabbit anti-KHV serum were compared with those kindly presented by French laboratories. Koi was inoculated with the purified KHV and the Koi anti-KHV serum was prepared. The changes of antibody levels after KHV inoculation were studied, which laid a foundation for the establishment of serological detection system and KHV epidemiological study.

Materials and methods

Experimental materials

Common carp for purification of IgM, were purchased from local Aquatic Products Market, 500~750g. New Zealand rabbits,

2~3kg. Protein A affinity chromatography column (Beijing National Biochemical Engineering Technology Research Center). Freund's complete adjuvant, Freund's incomplete adjuvant (Sigma). Yili skim milk powder (labeled SM), commercial product from Yili Group Company (www.yili.com) in China. KHV isolate F347 from ATCC and 07-1086 from French expert were amplified on the KF and EPC cell lines. Purified KHV antigen described by Hutoran M et al. [10] and Tingqi Liu [9] was diluted to 1:1000 with a carbonate coating solution (pH 9.6), coated with 100 μ L per well, and placed at 4° C overnight or 37° C for 2 hours. Anti-KHV positive serum labeled as JCKh7 and negative serum JCN2, JCN3 from common carp, JCTe from koi carp, Rabbit anti-common carp IgM antibody (2.3 mg/mL, PBS pH 7.4, labeled as JcRACIgM) were kindly presented by Dr. Jennette Castric from the Fish Disease Laboratory, French Food Security and Sanitary Agency (Afssa). Horseradish peroxidase-labeled goat anti-rabbit antibody (labeled as GAR-HRP): Commercially available, Genescript or Boster, 1 mg/mL, PBS (pH 7.4).

The TMB substrate solution was prepared as follows: Liquid A: Urea hydrogen peroxide sodium acetate-citrate buffer. The preparation method is as follows: 1 mol/L pH 5.6 sodium acetate-citric acid buffer: 41.0 g of sodium acetate is dissolved in 500 mL of double distilled water, 4.7 g of citric acid is dissolved in 20 mL of double distilled water. Mixture 500 mL of the above sodium acetate solution and 17 mL of citric acid solution is sodium acetate-citric acid buffer. 140 mg of Urea hydrogen peroxide was dissolved in 100 mL of sodium acetate-citrate buffer. Solution B: TMB-DMSO solution. 60 mg of TMB was dissolved in 10 mL of DMSO, and stored at 4° C in the dark. 250 μ L of TMB and 2 mL of Urea hydrogen peroxide sodium acetate-citrate buffer and 18 mL of water were mixed at the time of use.

Preparation and purification of common carp IgM

20 common carp were purchased from local market, 500~750g, blood was collected from the tail vein, and left at 4° C overnight. After centrifugation at 8000 rpm at 4° C for 6 min, 90 ml serum was collected and frozen at -20°C. The carp serum was purified using Protein A affinity chromatography according to the instruction manual (Beijing National Biochemical Engineering Technology Research Center). The purified IgM was detected by SDS-PAGE described elsewhere, the concentration of the concentrated gel and separated gel was 4% and 12.5%, and the sample to be tested was 15 μ L. The starting voltage was 110V, and changed to 150V after the sample entering the separation gel, and the electrophoresis was stopped when the dye was 0.5 cm from the bottom of the gel.

Preparation of rabbit anti-carp IgM serum

New Zealand rabbits of 2~3kg weight were used for IgM immunization, the immunization protocol was as follows in Table 1:

Blood was taken 16 days after the third immunization, anti-serum was prepared, and serum titer was measured by ELISA. When the requirements were met, blood was taken from the rabbit heart, placed at 4°C overnight at room temperature for 1h, Centrifuged at 2500 rpm for 10 min, serum was taken and dispensed into aliquots.

Table 1: Immunization protocol of New Zealand rabbits

Times Sites	Date	Dose	Concentration	Antigen type	Injection
1	d1	1ml	0.5mg/ml	IgM and complete adjuvant	multi-point subcutaneous injection at the back skin
2	d14	1ml	0.5 mg/ml	IgM and complete adjuvant	as above
3	d28	1ml	0.5 mg/ml	IgM and complete adjuvant	as above

Titer determination of rabbit anti-carp IgM antibody

Rabbit pre-immune serum and post-immune serum were taken, and the serum titer of the immunized rabbit was measured by indirect ELISA. The ELISA was briefly as follows: dilute the carp IgM to 1 µg/ml with a coating solution (8.4 g/L NaHCO₃, 3.56 g/L Na₂CO₃, pH 9.5), add 100 µl to each well of the plate, and place in a refrigerator at 4 ° C overnight. The plate was washed 3 times with 200 µl of washing solution (100 mM Tris-HCl, 0.1% Tween-20, pH 8.0) for 5 min each time. Wash procedures applied after following each step of reagent addition. A 200 µl blocking solution (50 mM Tris-HCl, 5 mg/ml BSA, 5% sucrose, pH 8.0) was placed in a refrigerator at 4 ° C overnight. Each well was added with series double dilution of rabbit antiserum, pre-immune rabbit serum, and PBS 100 µl, and incubated at 37 ° C for 1 h. 100 µl of goat anti-rabbit IgG-HRP was added and incubated at 37 ° C for 1 h. 100 µl of TMB substrate solution was added and incubated at 37 ° C for 10 min. The reaction was terminated by adding 50 µl of 2 mol/L sulfuric acid solution to each well, and the OD (450 nm) was examined.

Comparison of homemade and presented rabbit anti-carp IgM

Direct determination of homemade serum titer

100µl of coated IgM of 1 µg/ml in each well was added to

the following reagents in order (100µl/well except 50µl sulfuric acid) at room temperature for 1 h: the presented rabbit anti-carp IgM (JcRACIgM) (1:4000), and the homemade rabbit anti-carp IgM (ZzRACIgM) was double diluted from 1:5000 to 1:20000, rabbit anti-carp IgM negative serum (1:1000) as a negative control, goat anti-rabbit IgG-HRP(1:5000). The substrate TMB was added, and sulfuric acid was added to stop the color development. The OD value was measured.

Indirect ELISA assay to differentiate standard KHV positive/negative serum

The **Indirect ELISA** protocol was described by Tingqi Liu [9]. Sera JCTE, N2, N3, JCKh7 were diluted with 10% skim milk(SM) in PBST (hereinafter, 10%SM-PBST) to 1:640, 1:1280, and pre-incubated at 25 ° C for 1 h, and then added to the plate coated with purified KHV for 25°C for 1 h, 10%SM-PBST SM diluted JcRACIgM (1: 4000), ZzRACIgM (1:5000) and GAHRRP (1:2000) were added in order and placed at 25° C for 1 h for each step, the substrate was added for color development, and the OD value was measured.

Preparation of rabbit anti-KHV serum

KHV antigen described by Tingqi Liu [9], was purified three times respectively for immunization. The immunization schedule and immunization amount were as follows in Table 2 and Table 3:

Table 2: KHV Immunization to New Zealand White Rabbit

Times Sites	Date	Dose	Concentration	Antigen type	Injection
1	d0	1ml	in table 3	KHV and complete adjuvant	multi-point subcutaneous injection at the back skin
2	d33	1ml	in table 3	KHV and complete adjuvant	as above
3	d70	1ml	in table 3	KHV and complete adjuvant	as above

Table 3: Antigen type and concentration of three vaccination

First vaccination (d0)	Second vaccination (d33)	Third vaccination (d70)
KHV347- EPC: 5.51mg/mL	KHV-KF: 1.456mg/mL	KHV-KF: 3.144 mg/mL
KHV347-EPC30%:1.294mg/mL	KHV-KF	KHV-KF 30%: 0.776mg/mL
KHV347-EPC45%:1.150 mg/mL	30%:0.306mg/mL	KHV-KF 45%: 1.768mg/mL
KHV347-EPC60%: 0.514mg/mL	KHV-KF45%:0.464mg/mL	KHV-KF 60%: 1.68mg/mL
KHV347-KF:1.968mg/mL	KHV and complete adjuvant	KHV07-1086: 1.688mg/mL
	KHV-KF60%:0.922mg/mL	
	KHV and complete adjuvant	
	KHV-EPC:0.52mg/mL	
	EPC-BLANK:0.08mg/mL	
	KF-BLANK:0.321mg/mL	

Note: KHV with no number means KHV347.KHV347-EPC30% means virus purification taken from the place of 30% sucrose of gradient centrifugation. similarly hereinafter. 1/2 dilutions of KHV-KF stock solution, mixture of 330mL each of KHV-KF 30%~60% sucrose purification and 1/2 dilutions of KHV07-1086 KF purification were vaccinated into rabbits for the third vaccination.

Blood was taken and serum was made as 2.3.

Determination of Rabbit anti-KHV antibody titer

Pre-vaccination and post-vaccination rabbit serum was taken, and immunized rabbit serum titer was measured by ELISA and compared with the positive and negative control presented by French expert. ELISA protocol was similar as that used for rabbit anti-carp IgM except 1% BSA –PBST diluted standard positive/negative control and homemade rabbit anti-KHV antibody, goat anti-rabbit conjugate (1:5000) was added and incubated at 37°C for 1 h.

Study on dynamic changes of koi anti-KHV serum after vaccination

The purified KHV antigen KHVKF, KHVKF 30%, 45%, 60% at concentration of 3.144 mg/mL, 0.776mg/mL,1.768mg/mL,1.68mg/mL respectively was inactivated at 50 ° C for 1 min, mixed with an equal amount of incomplete adjuvant, injected into abdominal cavity of koi carp 0.1-0.2 mL per fish, 2 fish for each group. The fish tail was cut and marked for KHV-KF, KHVKF 30%, KHVKF45%, KHV KF60%. Blood was collected periodically and serum was separated for storage. Dynamic changes of koi anti-KHV serum was investigated by ELISA described by Tingqi Liu [9]. Briefly The purified KHVKF, KHVKF 30%, 45%, and 60% antigens above were mixed (final concentration 1.52 mg/mL), coated onto the plates with 1:3000 dilution. Wash three times after each step of reagent addition hereinafter. Homemade and presented serum JCTe, N2, N3, JCKh7 were diluted PBST-10%SM to 1:800 and 1:1600 and pre-incubated at 25°C for 1 h, and then added to the plate coated with purified KHV for 25 h at 1° C. JcRACIgM (1: 4000), GAR-HRP (1:2000) diluted with 10% SM-PBS, each incubated at 25 ° C for 1 h, OD value was measured after addition of substrate TMB solution.

Results

Purification of common carp IgM by Protein A Sepharose affinity column chromatography

SDS-PAGE electrophoresis of effluent and fractions collected from Protein A Sepharose affinity column chromatography showed that purified fractions had bands which molecular weight of about 82 kD and 29 kD which probably represented heavy and light chain of the common carp IgM(data not shown).

Rabbit anti-carp IgM serum titer

Blood was drawn 14 days after the third immunization, and the titer was measured by indirect ELISA (Table 4). IgM coated with 1µg/ml, rabbit anti-carp IgM were diluted to 1:10000 and 1:20000, respectively, negative serum at 1:100 dilution as a control, goat anti-rabbit IgG-HRP diluted to 1:20,000. OD of PBS,negative control,positive serum (1:10000) were 0.046, 0.192, 0.654 respectively.

Table 4: ELISA results of rabbit anti-carp IgM serum

Reagents	OD
PBS	0.046
Negative control	0.192
1#rabbit serum 1:10000	0.654
2# rabbit serum 1:10000	0.593
1# rabbit serum 1:20000	0.511
2# rabbit serum 1:20000	0.504

Comparison of homemade and presented anti-carp IgM

ELISA results of homemade rabbit anti-carp IgM with French counterpart were shown in Fig 1. The effects of block and unblock after antigen coating were not much different. The average OD of rabbit anti-carp IgM negative serum was 0.0595. The OD values of JcRACIgM at 1/4000 and ZzRACIgM at 1/5000 were similar, all were above 1.1, and 19.5 times of negative serum.

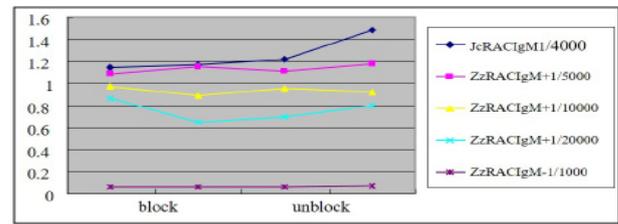


Figure 1: Comparison of homemade and presented rabbit anti-carp IgM

The results of indirect ELISA for the determination of standard fish KHV negative/positive serum were in Fig 2. Whatever French presented or homemade rabbit anti-carp IgM were used in the ELISA the OD values of JCTe, N2 and N3 were 0.2 or less and had not significant difference. For the positive serum JCKh7, the OD value of the homemade rabbit anti-carp IgM was higher than that of the French present serum, and the ratio of the positive to negative OD was more than 2.

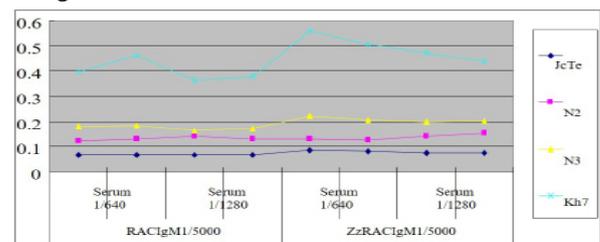


Figure 2: ELISA results of standard negative/positive KHV antibody by using homemade and presented RACIgM

Rabbit anti-KHV antibody titer

Blood was taken 37 days after the second immunization (70 days after first immunization), the titer was measured by ELISA (Table 5). Rabbit anti-KHV antibody (KHVKF1+ and 2+) immunized by KHV-KF antigen at 1:1080, KHVKF30-60%, and KHVEPC+ diluted to 1:120 had similar OD value to that of the present rabbit anti-KHV antibody (rabbit positive KHV+). They were nearly four times of pre-immune serum. OD_{Negative control} was 0.311. the last line of the table was blank control.

Dynamic changes of anti-KHV antibody level in koi serum

After immunization with KHV-KF, KHVKF30%, KHVKF45%, KHVKF60% purified antigen, Anti-KHV serum titers of Koi carps vaccinated with KHVKF30% were not described in this paper because the koi carps died during the early stage of the experiment and the anti-KHV antibody titers did not increase significantly may due to its much lower concentration. Anti-KHV titers in koi serum vaccinated with KHV-KF, KHVKF45%, KHVKF60% except one koi inoculated with KHVKF 60% changed during 88 days of the whole experiment. The inoculated koi produced an anti-KHV antibody response, which was much higher than the three negative controls. The anti-KHV titer increased significantly in one month after immunization, and the peak time was different depending on the antigen type and the individual fish. It then slowly decreased until the 88th day and there was still a higher titer of antibodies (Figure 3,4).

Table 5: Indirect ELISA results of rabbit anti-KHV antibody

dilution	KHV F1+	KHV KF2+	KHV KF 30-60%	KHV EPC+	Pre-immune rabbit serum	Rabbit positive KHV+
1:40	1.854	1.779	1.503	1.586		
1:120	1.741	1.768	1.256	1.280	0.306	1.281
1:120					0.316	1.289
1:360	1.611	1.625	0.903	0.929		
1:1080	1.276	1.285	0.624	0.628		
1:1080 (blank)	0.057	0.054	0.051	0.055		

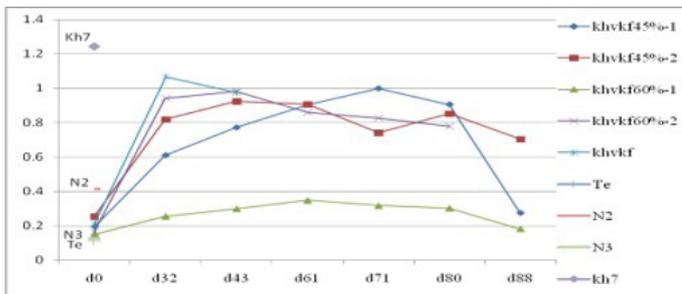


Figure 3: Changes in anti-KHV antibodies in Koi serum and comparison with standard KHV antibodies (1/800 dilution) (1/800 was the antibody dilution, d0 was date of the first vaccination, the others were blood collection dates)

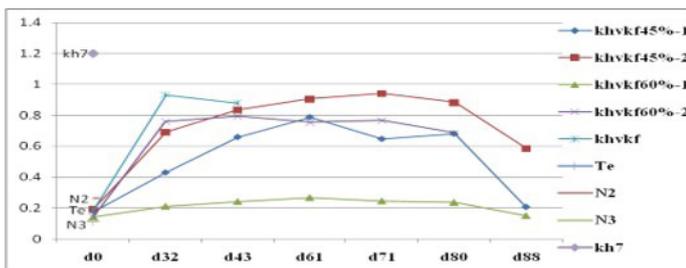


Figure 4: Changes in anti-KHV antibody level in koi serum and comparison with standard KHV antibodies (1/1600 dilution) (1/1600 is the antibody dilution, d0 is date of the first vaccination, the others were blood collection date)

Discussion

Diagnosis of Koi herpesvirus disease (KHVD) mainly includes direct finding or detection of actual virus or parts of virus such as virus isolation and/or PCR etc., and one of the indirect methods is to test the anti-KHV antibody levels for determination whether the fish produced immune response after KHV infection or exposure to the virus. As a non-lethal and valuable tool for surveillance and screen test for large sample size, ELISA were used to detect anti-KHV antibodies after natural/experimental infection, sera screen for the geographic distribution and prevalence of KHV infection [2,5-9]. Due to the lack of reagents and insufficient knowledge of fish serology to KHV infection, the use of ELISA is limited. Here rabbit anti-carp IgM, rabbit anti-KHV antibody were prepared and koi anti-KHV antibody level changes after vaccination of purified KHV antigen were analyzed in this report and results are consistent with that of French presented rabbit anti-carp IgM, rabbit anti-KHV serum and other previous reports.

Molecular weights for most of the teleost Ig H and L chains were generally around 70-81 and 23-28 kDa, respectively. Neeraj Sood et al [11] reported 2 bands of 73.7 kDa and 25.3 kDa were revealed by SDS-PAGE of the affinity purified Ig. Zhong MC

et al. [12] observed H and L chain of 71 and 24-26 kDa for *C. carpio* Ig. Weidong Ding et al. [13] used HiTrap rProteinA Sepharose affinity chromatography to purify grass carp serum IgM. SDS-PAGE and Western-blot results revealed purified IgM had two bands of 78 kD and 28 kD. Rabbit anti-grass carp IgM was prepared by immunized New Zealand rabbits with purified grass carp IgM and the titers of anti-IgM sera were up to 1:25600 by ELISA. In our study the purified common carp IgM had 2 bands of 82kD and 29kD filling in the molecular weight range of reported Ig H and L chains. Slight difference among several studies may due to species or breeds of carp used, different purification methods and measurement for the SDS-PAGE and western blot.

Rabbit anti-carp IgM was prepared in this study by immunization of purified carp IgM in New Zealand rabbits and tested by direct ELISA achieving desired effects. Research showed the titers of rabbit anti- common carp IgM were 0.856 at 1:25600 by ELISA [14]. Weidong Ding et al [13] also reported rabbit anti-grass carp IgM was prepared by immunized New Zealand rabbits with purified grass carp IgM and the titers of anti-IgM sera were up to 1:25600 by ELISA. Comparison of homemade and French rabbit anti-carp IgM showed that the OD values of homemade ZrRACIgM+ (1/5000) and French JcRACIgM (1/4000) were similar, all were above 1.1, 19.5 times of negative serum.

Homemade rabbit anti-carp IgM were used in the ELISA described by Tingqi Liu [9] to test the effectiveness to differentiate the standard fish KHV negative/positive serum. The results showed whatever French presented or homemade rabbit anti-carp IgM were used in the ELISA the OD values of negative sera JCTe, N2 and N3 were 0.2 or less and had not significant difference. For the positive serum JCKH7, the OD value of the homemade rabbit anti-carp IgM was higher than that of the French present serum, and the ratio of the positive to negative OD was more than 2.

Dynamic changes of anti-KHV antibody level in koi serum vaccinated with purified KHV antigen were investigated in this report. Anti-KHV antibody level in koi serum vaccinated with KHV-KF, KHVKF45%, KHVKF60% changed during 88 days of the whole experiment. The inoculated koi produced an anti-KHV antibody response. The anti-KHV titer increased significantly in one month after immunization, which was much higher than the three negative controls. The peak time was different depending on the antigen type and the individual fish. It then slowly decreased until the 88th day and there was still a higher titer of antibodies but exceptions existed. Sera dilution up to 1/800 and 1/1600 were used in the ELISA test. The fact the vaccinated koi produced anti-KHV immune response and maintained for near three months is consistent with previous studies. Adkison Mark A et al. [5] reported anti-KHV antibodies diluted up to 1:62, 500

were detected by ELISA in a group of koi kept in virus-free water for 1 year following a natural KHVD outbreak. Cross reactions with Cyprinid herpesvirus 1 (CyHV-1) were detected in serum less than 1:2, 500 of koi experimentally and naturally exposed to KHV. St-Hilaire S et al. [7] developed an ELISA to reveal that some common carp experimentally infected with KHV produced anti-KHV titers of 1/1600 or greater and continued to the end of the experiments at 65, 46 and 27 weeks post-exposure. Anti-KHV serological survey of the surviving population from episodic outbreaks of KHVD during 2 years following a major outbreak in Japan showed that 54% of the older carp were seropositive and 31% PCR positive. Long-time maintenance of high antibody levels indicated that latent virus may be reactivating periodically and boosting the immune response [15]. TAYLOR N et al. [8] used the ELISA method developed by St-Hilaire S et al. [2,7] to screen carp sera to determinate the geographic distribution and prevalence of KHV infection. The study showed three of four sites affected by clinical outbreaks of KHV a year before and no introductions of fish since then produced 85–93% sero-prevalence in the surviving carp samples [8]. Commercially available mouse anti-carp Ig monoclonal antibody produced by Aquatic Diagnostics Ltd were used in some of the above studies and fish sera were diluted to 1/800 and 1/1600 to achieve good differentiation of positive and negative sera. Tingqi Liu [9] established an indirect ELISA to detect anti-KHV in carp and koi in which the optimal dilution of serum was 1/800 and used rabbit anti-carp IgM.

In this report the mixture of purified KHV and incomplete adjuvant was injected into abdominal cavity on in this study and the experiment started from January to April. Before this experiment the authors tried to inject the purified KHV into koi and found that injected koi died even one day after injection. Possibly purified KHV is pathogenic and lethal to the koi and injection into abdominal cavity is very strong stress. And also due to the small number of experimental koi the experiment was terminated on the 88th day. Changes of koi anti-KHV antibody level in a longer time period and more exposure methods need further investigation.

Conclusion

Rabbit anti-common carp IgM and rabbit anti-KHV serum were prepared by vaccination with purified carp IgM and purified KHV respectively. Rabbit anti-carp IgM and rabbit anti-KHV serum were compared with those kindly presented by French laboratories. Dynamic changes of anti-KHV antibody levels in koi sera after vaccination with purified KHV were studied. The koi serum with high titer may be served as positive control in the following studies. These prepared reagents laid a foundation for the establishment of serological detection system. Finding of dynamic changes of anti-KHV antibody levels after vaccination facilitated the understanding and study of serology and epidemiology of KHV infection.

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References

- Oie. Koi herpesvirus disease. In: Manual of Diagnostic Tests for Aquatic Animals. 2018.
- St-Hilaire S, Beevers N, Way K, Le Deuff R.M, Martrin P. et al. Reactivation of koi herpesvirus infections in common carp *Cyprinus carpio*. *Dis Aquat Org* 2005; 67: 15–23.
- Eide Kathleen E, Tim Miller-Morgan, Jerry R. Heidel, Michael L. Kent, Rob J. Bildfell, Scott LaPatra, Gregory Watson, Ling Jin. Investigation of Koi Herpesvirus Latency in Koi. *J Virol*. 2011; 85: 4954–4962.
- Gilad O, Yun S, Zagmutt-Vergara F, Leutenegger CM, Bercovier H, et al. Concentrations of a herpes-like virus (KHV) in tissues of experimentally infected *Cyprinus carpio* koi as assessed by real-time TaqMan PCR. *Dis Aquat Org*. 2004; 60: 179-187.
- Adkison MA, Gilad O, Hederick RP. An enzyme linked immunosorbent assay (ELISA) for detection of antibodies to the koi herpesvirus (KHV) in the serum of koi *Cyprinus carpio*. *Fish Pathol*. 2005; 40: 53-62.
- Ilouze M, Davidovich M, Diamant A, Kotler M, Dishon A. The outbreak of carp disease caused by CyHV-3 as a model for new emerging viral diseases in aquaculture: a review. *Ecol Res*. 2011; 26: 885-892.
- St-Hilaire S, Beevers N, Joiner C, Hedrick RP, Way K. Antibody response of two populations of common carp, *Cyprinus carpio* L, exposed to koi herpesvirus. *J Fish Dis*. 2009; 32: 311-320.
- Taylor N, Way K, Dixon PF, Peeler EJ, Jeffrey K, et al. Koi herpesvirus (KHV): distribution and prospects for control in England and Wales. *J Fish Dis*. 2010; 33: 221-230.
- Tingqi Liu. Serological Detection Methods of Anti Koi Herpesvirus Antibody [D]. Nanjing Agriculture University. 2012.
- Hutoran M, Ronen A, Perelberg A, Ilouze M, Dishon A, et al. Description of an as yet unclassified DNA virus from diseased *Cyprinus carpio* species. *J Virol*. 2004; 79: 1983-1991.
- Neeraj S, Gaurav R, Raja ST, Rehana A, Mishra BN, et al. Isolation and characterization of serum immunoglobulin of *Cyprinus carpio*. *The Indian journal of animal sciences*. 2007; 77: 642-645.
- Zhong MC, Mor A, Avtalion RR. One-step procedure for the purification of goldfish (*Carrasius auratus*) and carp (*Cyprinus carpio*, L.) serum immunoglobulin by precipitation with 9% polyethylene glycol 6000. *Israeli Journal of Aquaculture-Bamidgeh*. 1999; 51: 3-9.
- WeiDong, Ding, Cao LiPing, Cao ZheMing. Purification of Serum IgM from Grass Carp (*Ctenopharyngodon* Dellus) and Preparation of Rabbit Sera Anti-IgM. *Acta Hydrobiologica Sinica*. 2010; 34: 164-169.
- Shu-zhu L, Wan-bo L, Xian-ju L, Fan Y, Chuan Q, et al. Production and HRP Labeling of Rabbit Anti-fish Immunoglobulins Antibodies *Chin J Comp Med*. 2008; 18: 45-49.
- Uchii K, Matsui K, Iida T, Kawabata Z. Distribution of the introduced cyprinid herpesvirus-3 in a wild population of common carp, *Cyprinus carpio* L. *J Fish Dis*. 2009; 32: 857-864.