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# A developed subunit vaccine based on fiber protein VP56 of grass carp reovirus providing immune protection against grass carp hemorrhagic disease

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ABSTRACT

Grass carp reovirus (GCRV) is the main viral pathogen that endangers grass carp seriously. Application of vaccine has been considered to be the most effective way to prevent virus infection. VP56 is a protein encoded by gene segment 7 of grass carp reovirus, and is predicted to share homology with fiber protein of mammalian reovirus (MRV). In our study, the immunogenicity of VP56 was evaluated by neutralization test. GCRV was incubated with mouse anti-VP56 antibody, and then was injected into grass carp. Results showed that disease progress and death occurrence was hindered in the experimental group compared with the control group. For further study, the recombinant VP56 protein (rVP56) expressed by pET-32a (+) vector was purified, and was used as subunit vaccine to immunize grass carp. After each fish (15 ± 1.5 g) was injected with 30 μg purified rVP56 intraperitoneally, the immune protective efficacy of recombinant VP56 protein was assessed by a series of immune parameters. The population of red blood cells in immunized fish increased significantly after 5 d post injection (dpi), and reached a peak with (2.98 ± 0.17) × $10^9$/ml at 7 dpi ($p < 0.05$). The numbers of white blood cells peaked with (8.42 ± 1.01) × $10^7$/ml at 7 dpi ($p < 0.05$). Additionally, the percentage of monocytes and neutrophils rose to a peak with (9.05 ± 0.92)% and (25.93 ± 2.60)% respectively at 5 dpi ($p < 0.05$ or $p < 0.01$), whereas lymphocytes reached the highest value of (85.81 ± 2.73) % at 14 dpi ($p < 0.01$). Serum antibody titer in the vaccinated fish increased significantly and reached a peak at 21 dpi ($p < 0.01$). The mRNA expression levels of type I interferon (*IFN1*), major histocompatibility complex class I (*MHC I*), Toll-like receptor 22 (*TLR22*), and immunoglobulin M (*IgM*) were significantly up-regulated in head kidney and spleen ($p < 0.05$ or $p < 0.01$). The GCRV challenge test showed that the relative survival rate in immunized group was 71%–75%. Collectively, the results indicated that rVP56 protein can induce immune protection in grass carp, and can be consider as a candidate vaccine against GCRV infection.

## 1. Introduction

Grass carp (*Ctenopharyngodon idella*) is widely farmed in China and represents one of the most important commercial fish species. The production of grass carp exceeded 5.3 million tons in 2017, accounting for approximately 10% of total aquaculture production in the country, according to the China Fishery Statistical Yearbook. Moreover, grass carp is also widely cultured in other Asian countries [1]. However, grass carp farming is threatened by several viral and bacterial diseases, in particularly, the hemorrhagic disease caused by grass carp reovirus (GCRV), is the most serious disease hampering juvenile grass carp [2]. The mortality rate of GCRV infection is up to 85% or even higher. Outbreaks of epidemic grass carp hemorrhagic disease occur every year and lead to huge economic loss [3].

GCRV, a member of the genus *Aquareovirus* in the family *Reoviridae*, was initially isolated from grass carp in China in 1983. In recent decades, more GCRV strains were isolated and their whole genome information was obtained. The bioinformatics analysis revealed that all of the virus strains can be divided into three distinct subtypes, type I (the initial strain), type II (such as GCRV-HZ08, GCRV-GD108, GCReV-109 strain), and type III (HGDRV strain) [4–8]. All types of GCRV contain 11 double-stranded RNA and two-layer capsid. The inner core of capsid is composed by VP1, VP2, VP3, VP4, VP6 protein. Comparing different subtypes of GCRV, their inner core proteins share similarities, but their outer capsid proteins are very different. Although VP5 protein is found in the outer shell of all types GCRV, another outer capsid protein composing the outer shell in type I GCRV is VP7, and it was predicted to be VP35 and VP38 in type II and type III GCRV respectively. The

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similarities between VP7 and VP35 amino sequences were below 20%. Besides, type II GCRV and type III GCRV have a fiber-like protein (VP56 and VP55 respectively) that resemble mammalian reovirus σ1 protein, which cannot be found in type I GCRV [6].

Currently, most of the vaccines against GCRV were developed based on the outer capsid protein of type I GCRV, such as subunit and DNA vaccines made by VP5 and VP7 [9–13]. However, epidemiological investigations showed that type II GCRV was the dominant virus type in China [8]. But the vaccines against type II GCRV were very few. Previous studies showed that subunit vaccine prepared by VP5 could induce strong immune response, and subunit or DNA vaccine produced by VP35 provided protective immunity against GCRV [14–16]. There is only one commercial vaccine available developed by attenuating the GCRV-892 strain through serial passages in cell culture, which has the risk of reverse mutation [17,18]. Thus, study on the immunogenicity of GCRV proteins and searching for novel candidate vaccines is very essential. VP56 protein is encoded by S7 segment of type II GCRV, which shares 27% sequence identity with adenovirus fiber protein in the region of amino acid sequence 100–300, and VP56 has a predicted coiled-coil region at the N-terminus, followed by a β-spiral motif, that is similar to other known fiber proteins [6,19]. Therefore, VP56 is predicted to be a fiber protein that exists on the surface of outer capsid, which may have immunogenicity.

Compared with inactivated and attenuated virus vaccines, subunit vaccine was safer, which can stimulate immune response without introducing pathology. Our present study aims to evaluate the immunogenicity of VP56 protein and the potential of VP56 as subunit vaccine against GCRV infection, providing foundation for the development of commercial vaccines. Meanwhile, this research could help to understand the mechanism of antiviral immunity in grass carp.

## 2. Materials and methods

### 2.1. Fish and virus

Healthy grass carp with average weight of 15 ± 1.5 g were obtained from local aquaculture farm in Xinxiang city, China. The fish were randomly divided and cultured in the aerated freshwater in plastic aquaria. Water temperature was maintained at 25 ± 2 °C. The fish were fed with commercial pellets twice a day. Fish were acclimatized for two weeks prior to the treatment, and GCRV infection was detected from randomly selected individuals by RT-PCR to eliminate the possibility that these fish were virus carriers. The GCRV-HN14 strain, which is a type II GCRV, was propagated in grass carp liver cell line L8824 and applied in this study [16]. The virus titer was determined by half lethal dose $LD_{50}$ in grass carp ($10^{4.3}$ $LD_{50}$/ml). The genomic sequences of GCRV-HN14 strain were submitted into GenBank of National Center for Biotechnology Information (NCBI). GenBank accession numbers were MK675075-MK675085.

### 2.2. Cloning of VP56 gene and analysis

GCRV genomic dsRNA was extracted using RNAiso plus (TaKaRa, Japan) according to the protocol of manufacture. The *VP56* gene was obtained by RT-PCR with the forward primer *vp*56-F1 (5′-TTCGGTACCAGTGGAAAATTAGACA-3′) and reverse primer *vp*56-R1 (5′-GCACTCGAGGTACTTACAGCAAACTACC-3′) targeting the C-terminal of open reading fragment (from 547 bp to 1539 bp) Restriction enzyme sites of *Kpn* I and *Xho* I were indicated by underlined letters respectively. The targeting fragment was inserted into pMD-19T vector (Takara, Japan) for sequencing. The homology analysis of *VP*56 gene was conducted using BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) from NCBI database. The molecular weight (MW) and isoelectronic point (pI) were calculated using online software (http://web.expasy.org/compute_pi/). Antigenic epitopes were predicted by Protean in DNAStar software.

### 2.3. Construction of recombinant plasmid and expression of recombinant VP56

The *VP56* DNA sequence and pET-32a (+) vector were digested by restriction enzyme *Kpn* I and *Xho* I (New England Biolabs, USA). The enzyme-digested fragments were purified and ligated together by and T4 ligation enzyme (TakaRa, Japan). The recombinant pET32a-VP56 plasmid was transferred into *E. coli* strain BL21 (DE3). Positive clone was inoculated into LB medium that containing 100 μg/ml ampicillin, and was cultured overnight at 37 °C until the optical density at 600 nm ($OD_{600}$) reached 0.6. The recombinant VP56 (rVP56) protein was induced to express by adding isopropyl-β-*d*-thiogalactoside (IPTG, Sigma, USA) into the medium with a final concentration of 1 mM. The induction of rVP56 protein lasted for 6 h at 37 °C. The cells were harvested by centrifugation at 8,000 *g* for 30 min at 4 °C.

### 2.4. Purification of recombinant protein

The bacteria induced by IPTG were collected and re-suspended in 15 ml binding buffer (0.1 M Tris, 5 mM imidazole, pH 8.0), and then was broken by ultrasonic in ice bath for 45 min. The cell mixture was centrifuged at 10,000 g for 10 min to collect the precipitate. The rVP56 protein in the precipitate was purified according to the method described previously [20]. The purified protein was identified by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration was detected by measuring the absorption at 562 nm by BCA protein assay kit (Solarbio, China) with BSA as a standard protein.

### 2.5. Preparation of antibody against VP56 and neutralization test

The purified rVP56 protein was mixed with an equal volume of Freund's adjuvant (Sigma, USA), and was used to immunize mice by intraperitoneal injection. Each mouse was immunized with 100 μg recombinant protein every 7 days and anti-VP56 serum was collected after four times immunization. Antibody titer was detected by ELISA. Normal mice serum was used as control. Anti-VP56 serum and normal serum were heat-inactivated at 56 °C for 30 min and serially 4-fold diluted (1:4, 1:16, 1:64) in PBS. The diluted serum was gently mixed with 200 $LD_{50}$ of virus in a 1:1 vol ratio and then was incubated at 25 °C for 1 h, referring to previous study [21]. The mixture was injected into grass carp intraperitoneally. Fish injected with an equal volume of PBS was set as blank control. The water supply was kept at 28 °C and aerated continuously. Dead fish were collected and spleens were sampled for GCRV detection by RT-PCR. Specific primers (S9−F: GCATATCGTACGTTGGACCTTGC, *S*9-R: AGATCCTCGAATTTCCGTTTCTT) targeting the S9 gene segment of GCRV-HN14 with predicted amplified fragment length 389 bp were used for detection.

### 2.6. Vaccination and sample collection

Fish were divided into experimental and control groups. 180 fish in each treatment group were divided into three parallel subgroups, with 60 fish in each subgroup. In the immunized group, each fish was intraperitoneally injected with 100 μl (0.3 mg/L) rVP56, referring to previous study [14,15]. In the control group, each fish was intraperitoneally injected with an equal volume of PBS. Blood, head kidney, liver, spleen and kidney were sampled from three fish in each subgroup at 1, 3, 5, 7, 14, 21, 28, and 35 d post injection (dpi). Subsequently, 50 mg tissues (head kidney and spleen) were respectively used for RNA extraction. Whole blood sample was diluted at 1:200 with Dacie's reagent, and the blood cells were counted with Neubauer hemocytometer. The number of red blood cells (RBCs) or white blood cells (WBCs) in each sample was the average of triplicate values. Blood smears were prepared from each sample and air-dried, methanol fixed, stained with Giemsa for 15 min, and then rinsed and dried. A total of 100 white blood cells composed of lymphocytes, neutrophils, and

monocytes in each slice were classified and counted in randomly selected visual fields under oil immersion microscopy.

### 2.7. Detection of serum antibody titer by ELISA assay

The whole blood from each sample was allowed to clot at 37 °C for 1 h, and then was centrifuged at 1,500 g for 5 min. The serum was collected for the detection of serum antibody titer. The serum antibody titer in grass carp was determined by the enzyme-linked immunosorbent assay (ELISA). Briefly, each well of 96-well plate was coated with 5 μg rVP56. After the plate was incubated overnight at 4 °C, it was washed with PBS that containing 0.1% Tween-20 (PBS-T). Then it was blocked with 1% skimmed milk powder and 0.5% bovine serum albumin blocking buffer at 37 °C for 2 h. Plate was washed three times with PBS-T, and fish serum from immunized group and control group were added at dilution of 1:200. The plate was incubated with test sera at 37 °C for 1 h. The plate was washed again and incubated with 1:1000 diluted horseradish peroxidase (HRP) conjugated rabbit anti-grass carp IgM antibody (Zoonbio Tech Co, China) at 37 °C for 0.5 h. Finally, 0.1 ml 3, 3′, 5, 5′-tetramethylbenzidine (TMB) (Solarbio, China) was added in each well and developed for 15 min. The reaction was stopped by adding 50 μl of 2 M sulfuric acid. After the calibration with blank control, the OD value of samples at 450 nm was read with an automated microtiter plate reader.

### 2.8. mRNA expressions of immune-related genes detected by quantitative real-time PCR

The mRNA expressions level of type I interferon (*IFN I*), immunoglobulin M (*IgM*), Toll-like receptor 22 (*TLR22*) in head kidney and spleen of grass carp were determined by quantitative real time PCR (qRT-PCR). The first strand cDNA was synthesized by using 500 ng of total RNA and PrimeScript™ RT Master Mix (Takara, Japan) and stored at −80 °C. The primers used for quantitative real-time PCR were referenced from the previous studies [22–24]. The qRT-PCR was carried out in the Applied Biosystems 7500 Real Time PCR System (ABI, USA) using AceQ® qPCR SYBR® Green Master Mix (Takara, Japan). The amplification conditions were as follows: 95 °C for 30s, 40 cycles of 95 °C for 5s and 60 °C for 30s. The relative mRNA expression levels of immune-related genes were calculated as the relative folds of expression of *β-actin* by the $2^{-\triangle\triangle Ct}$ method [25].

### 2.9. Challenge test

Each fish in immunized group and control group (about 35 fish in each group) was intraperitoneally injected with 0.1 ml GCRV (contain 10 $LD_{50}$) on 21 days post immunization. The experiments were performed in triplicate. Subsequently, the mortality was recorded daily and the dead fish were promptly removed from the tank. Dead fish were collected and RNA from the spleen was extracted to detect the presence of the virus by PCR amplification. Relative percentage survival (RPS) was calculated as: RPS = (1 - the ratio of mortality percent in the immunized group to in the control) × 100%.

### 2.10. Statistical analysis

Data were presented as mean ± standard deviation (SD). The statistical significance between the experimental group and the control group was analyzed by using ANOVA and Tukey's tests in SPSS software (version 20.0). The significant level was set as $P < 0.01$ or $P < 0.05$.

## 3. Results

### 3.1. Construction of prokaryotic expression plasmid pET32a-VP56

The *VP56* DNA sequence with the predicted length of 1011 bp was cloned using specific primers targeting the C-terminal of GCRV gene segment 7. The obtained gene fragment was verified by sequencing. Afterwards, it was inserted into the pET-32a (+) plasmid by restriction enzymes *Kpn* I and *Xho* I. Recombinant plasmid pET32a-VP56 was further verified via sequencing. The result confirmed that *VP56* gene was connected to the vector correctly. Furthermore, pET32a-VP56 was transferred into *E. coli* BL21 (DE3).

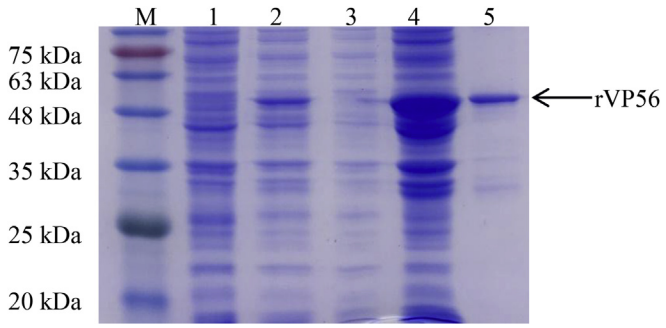


**Fig. 1.** Expression and purification product of pET32-VP56 in *E.coli* on SDS-PAGE: Lane M, protein markers; lane 1, un-induced BL21 with pET32a-VP56; lane2, IPTG induced BL21 with pET32a-VP56; lane 3, supernatant of induced cells after ultra-sonication; lane 4, precipitate of induced cells after ultra-sonication; lane 5, purified rVP56 protein.

### 3.2. Expression and purification of recombinant protein

*E. coli* BL21 (DE3) with recombinant plasmid pET32a-VP56 was induced to express recombinant protein by 1 mM IPTG for 6 h. The expression level of rVP56 was tested by SDS-PAGE. A distinct band with molecular mass of approximately 55 kDa was exhibited in IPTG induced cells (Fig. 1, lane 2), which was equal to the total molecular mass of the predicted rVP56 (~37 kDa) and his-tag protein (~18 kDa). In addition, the recombinant protein was mainly presented in the inclusion bodies of induced cells (Fig. 1, lane 4). Then rVP56 was successfully purified (Fig. 1, lane 5), and was used as subunit vaccine to immunize fish.

### 3.3. Neutralization capacity of the anti-VP56 antiserum

Mouse anti-VP56 serum was prepared and diluted in the ratio of 1:4, 1:16, 1:64, and mixed with equal volume of GCRV suspension (200 $LD_{50}$). The mixture was incubated at 25 °C for 1 h. Normal mouse serum with the same treatment was used as negative control. PBS without virus was used as blank control. Grass carp in the negative control group showed symptoms of hemorrhage, and death occurred at 4 dpi of the mixture. While, death occurrence was delayed to 6 dpi in the experimental group injected with mixture of virus and anti-VP56 serum (1:64), and fish began to die until 7 dpi in the experimental group with antiserum 1:4 and 1:16 dilution (Table .1). The mass mortality occurred earlier in the control group (6 and 7 dpi) than in the experimental group (8 dpi). No more fish died after 10 dpi in all groups (Fig. 2A). A survival rate of 10% in the experimental group with antiserum 1:4, 1:16 dilution and control group with 1:16 dilution was observed. The mortality rate was 100% in other dilutions. Dead fish during the experiment were collected and five of them were randomly selected for RT-PCR detection by specific primers targeting GCRV-HN14 gene segment 9. A band of about 400 bp was amplified in each fish (Fig. 2B). The fragments obtained were sequenced and GCRV infection in these fish was confirmed.

### 3.4. Blood cell counts

Healthy grass carp was injected intraperitoneally with rVP56 protein or PBS, and immune response was monitored in each group. The changes in peripheral blood cell counts were observed and the result

**Table 1**
Virus neutralization by anti-VP56 antiserum in grass carp.

| Group(a) | Dilution | LD50 | Number of survivors | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 3(c) | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Anti-VP56 serum(n = 10) | 1:4 | 200 | 10 | 10 | 10 | 10 | 9 | 3 | 1 | 1 |
| | 1:16 | 200 | 10 | 10 | 10 | 10 | 8 | 3 | 3 | 1 |
| | 1:64 | 200 | 10 | 10 | 10 | 7 | 5 | 2 | 0 | 0 |
| Normal serum (n = 10) | 1:4 | 200 | 10 | 9 | 8 | 6 | 1 | 1 | 0 | 0 |
| | 1:16 | 200 | 10 | 10 | 8 | 7 | 3 | 2 | 1 | 1 |
| | 1:64 | 200 | 10 | 10 | 9 | 4 | 2 | 0 | 0 | 0 |
| PBS (n = 10) | | N(b) | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |

(a) Groups were divided into fish infected with 50 μl containing 200 $LD_{50}$ of virus mixed with 50 μl of anti-VP56 antiserum or 50 μl normal serum, or with 100 μl PBS without virus.
(b) N, none: fish injected with PBS without virus.
(c) In the first 3 dpi, fish did not die.

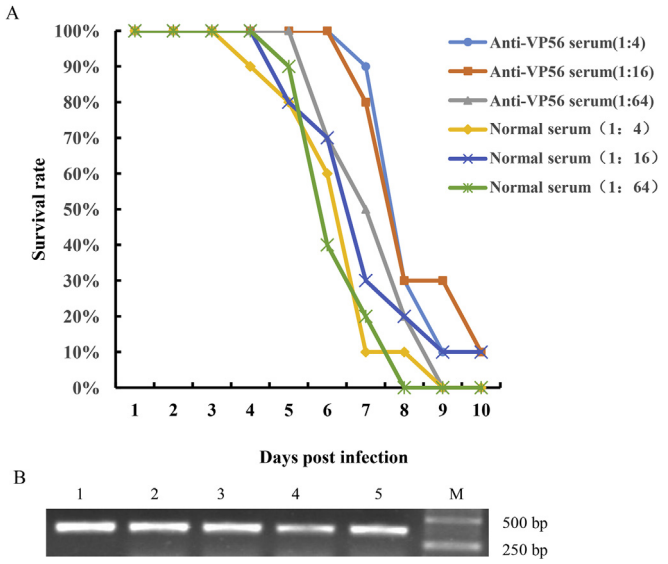


**Fig. 2.** The neutralization assay of anti-VP56 antiserum to GCRV in vivo. (A) Grass carp in the experimental groups were infected intraperitoneally with a lethal dose of 200 $LD_{50}$ mixed with increasing dilutions (1:4, 1:16, 1:64) of anti-VP56 antiserum. Fish in control groups were given viruses with normal serum (1:4, 1:16, 1:64), or PBS without viruses. Death was monitored daily until no more fish died. (B) RT-PCR detection of GCRV infection. 1–5: five dead fish randomly selected for detection, M: marker.

showed that the number of RBCs increased significantly at 5 and 7 dpi, and reached a peak with (2.98 ± 0.17) × $10^9$/mL at 7 dpi in the immunized group ($p < 0.05$) (Fig. 3A). The number of WBCs were significantly higher in immunized fish than in the control group at 5, 7 and 14 dpi ($p < 0.05$ or $p < 0.01$) and peaked with (8.42 ± 1.01) × $10^7$/mL at 7 dpi ($p < 0.05$) (Fig. 3B).

### 3.5. Counts of differential leukocytes

Changes in the ratio of neutrophils, monocytes, and lymphocytes were observed at different time points post immunization. The percentage of neutrophils among the leukocytes in the immunized group increased significantly and rose to a peak with (25.93 ± 2.60)% at 5 dpi, which was significantly higher (approximately 1.7-fold) than that of the control ($p < 0.01$) (Fig. 4A). The percentage of monocytes increased and reached the highest value of (9.05 ± 0.92)% on day 5, which was approximately 1.4 times higher than that of the control ($p < 0.05$) (Fig. 4B). However, as a result of remarkable increase in the number of neutrophils and monocytes, the percentage of lymphocytes

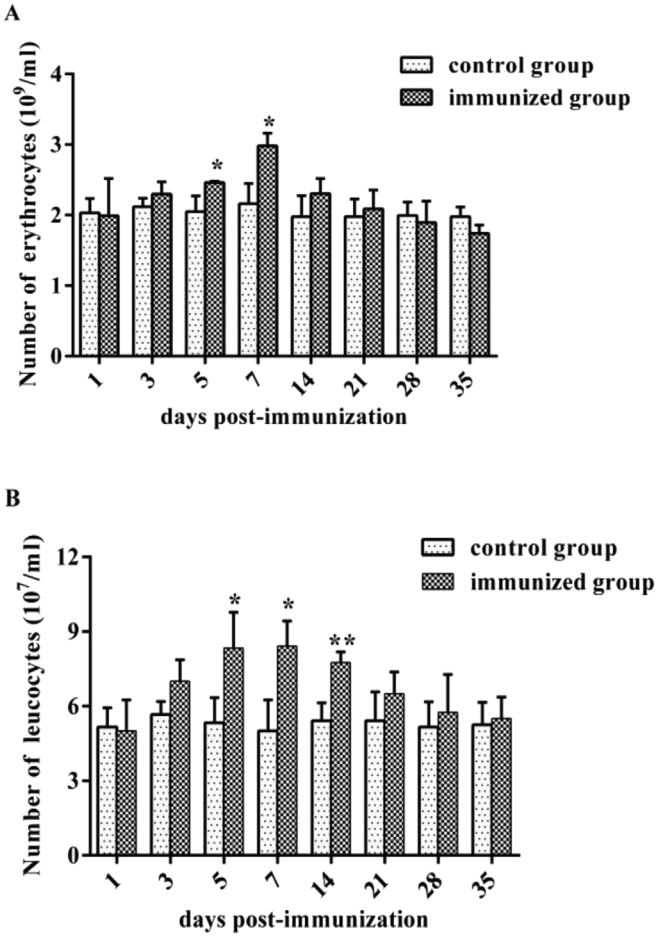


**Fig. 3.** Changes in the number of erythrocytes and leucocytes in the peripheral blood samples from grass carp immunized with rVP56 or PBS. (A) RBCs; (B) WBCs. Data are the means of three assays and are presented as mean ± SD (n = 3). * represents $p < 0.05$ and ** represents $p < 0.01$.

declined significantly at 5 dpi. The percentage of lymphocytes reached the highest point with (85.81 ± 2.73)% in the immunized group at 14 dpi ($p < 0.01$) subsequently (Fig. 4C).

### 3.6. Detection of serum antibody titer by ELISA assay

The serum antibody titer was detected via ELISA assay, and changes in antibody levels against rVP56 in grass carp was shown in terms of $OD_{450}$ (Fig. 5). The results showed that the antibody level in rVP56-immunized fish began to increase significantly from 3 dpi and kept on rising to a peak with $OD_{450}$ of (0.34 ± 0.01) at 21 dpi ($p < 0.01$). Then the antibody level in rVP56-immunized group decreased steadily, but was still significantly higher than that in the control group at 35 dpi ($p < 0.05$).

### 3.7. mRNA expression levels of immune-related genes

The mRNA expression levels of immune-related genes *IgM*, *IFN1*, *MHC I*, and *TLR22* were detected in the head kidney and spleen by qPCR. The relative expression levels of these genes were initially up-regulated and then returned to a normal level in the immunized group (Fig. 6). The relative expression levels of *IFN1*、*MHC I* and *TLR22* in vaccinated fish were significantly increased within a week. Specifically, the highest expression levels of *IFN1* in head kidney and spleen were approximately 20 and 7.5 fold higher than that in the control at 5 dpi ($p < 0.01$). The mRNA expression levels of *TLR22* in head kidney began to rise significantly at 3 dpi and reached a peak at 5 dpi, being

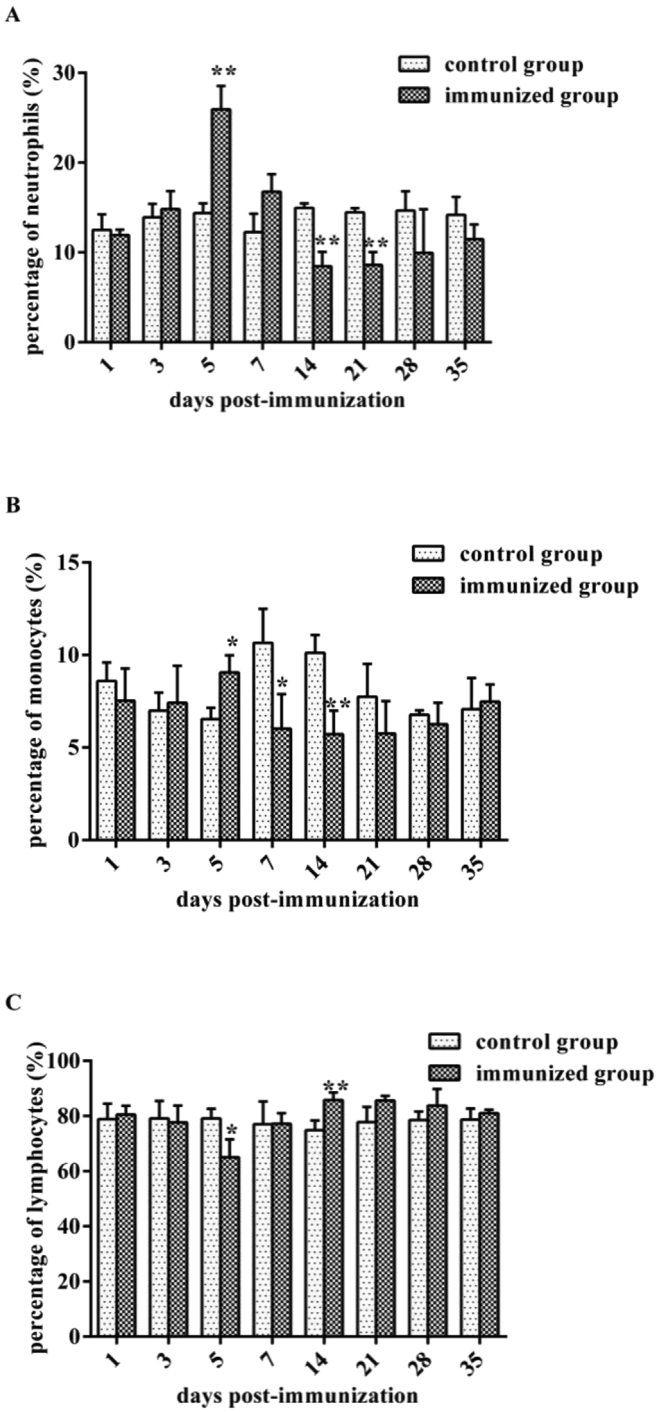


**Fig. 4.** Changes in differential leukocyte counts of neutrophils, monocytes and lymphocytes in the peripheral blood of grass carp after immunization with rVP56 or PBS. (A) neutrophils; (B) monocytes; (C) lymphocytes. Data are the means of three assays and are presented as mean ± SD (n = 3). * represents $p < 0.05$; ** represents $p < 0.01$.

approximately 3.2 fold of values obtained from the control ($p < 0.05$). It increased more significantly in spleen and peaked at 3 dpi with approximately 10 fold of control ($p < 0.01$). The expression of *MHC I* in head kidney and spleen both reached the highest value at 5 dpi, were approximately 40 and 7.5 fold of the control, respectively ($p < 0.05$ or $p < 0.01$). Moreover, the relative mRNA expression levels of *IgM* in immunized fish started to increase significantly at 5 dpi in head kidney ($p < 0.01$) and 7 dpi in spleen ($p < 0.05$). The expression level of *IgM* reached the peak with 48 fold of control in head kidney and 10 fold of control in spleen at 21 dpi ($p < 0.05$ or $p < 0.01$).

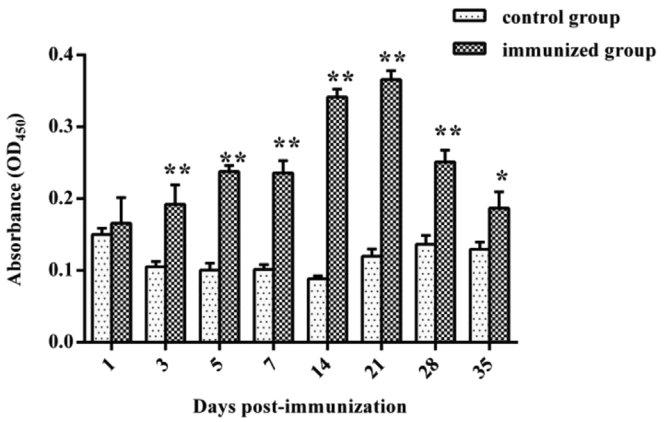


**Fig. 5.** Detection of serum antibody levels in grass carp. Results are expressed as the value of $OD_{450}$. Blood samples $OD_{450} > 0.20$ were recorded to have a positive antibody response. Data are presented as mean ± SD (n = 3). * represents $p < 0.05$ and ** represents $p < 0.01$.

### 3.8. GCRV challenge test

Grass carp was observed after injected with rVP56 or PBS. No fish died in each group during the 21 days post injection, indicating rVP56 protein was safe to use as a subunit vaccine. Grass carp in each treatment group were challenged with 10 $LD_{50}$ GCRV at 21 dpi to evaluate the protective efficacy. The mortality and clinical signs of GCRV-challenged fish were recorded daily for 15 days. The results showed that the survival percentage in the immunized group was 73.3%–76.7%, which was significantly higher than that in the control (6.67%–16.7%) ($p < 0.01$) (Fig. 7). The relative percentage survival was approximately 71%–75%. In the trials of GCRV challenge, the dead fish showed typical clinical symptoms of haemorrhagic disease, such as haemorrhage at the base of fins, orbit and muscles. GCRV infection was confirmed in the dead individuals by RT-PCR (data not shown).

## 4. Discussion

The production of cultured grass carp is steadily growing in China, but the development of vaccines against GCRV is relatively slow, and commercial vaccine is not widely used. The first vaccine for grass carp hemorrhagic disease is “inactivated tissue vaccine” that was developed in 1960s [26]. Nowadays, the most commonly applied method for prevention of GCRV is immunization by inactivated virus vaccines prepared by scientific institutions or universities [22]. But preparation of inactivated vaccines of type II GCRV was not easy. Because of the low virus copies in fish cell lines [27], it is very time consuming to collect large amount of virus. In contrast, subunit vaccines developed based on genetic engineering technology can be produced faster and more efficiently, thus subunit vaccines were more suitable for large-scale production and application. In this study, rVP56 protein used as subunit vaccine was evaluated, and it showed that rVP56 could elicit immune reaction effectively in grass carp, which could be a candidate vaccine against GCRV.

VP56 protein of type II GCRV is unique, which has no equivalent protein with type I GCRV and share only 15% amino sequence identity with VP55 protein of type III GCRV [6]. VP56 is predicted to be a fiber protein, and the fiber protein in mammalian reovirus is σ1 protein that involved in the cell-attachment and invasion [28,29]. Recent studies showed that recombinant VP56 could bind to fish cells, demonstrating that VP56 protein may also function as a cell attachment protein [30,31]. In our study, the disease progress was significantly delayed in the group of fish injected with anti-VP56 serum incubated virus,

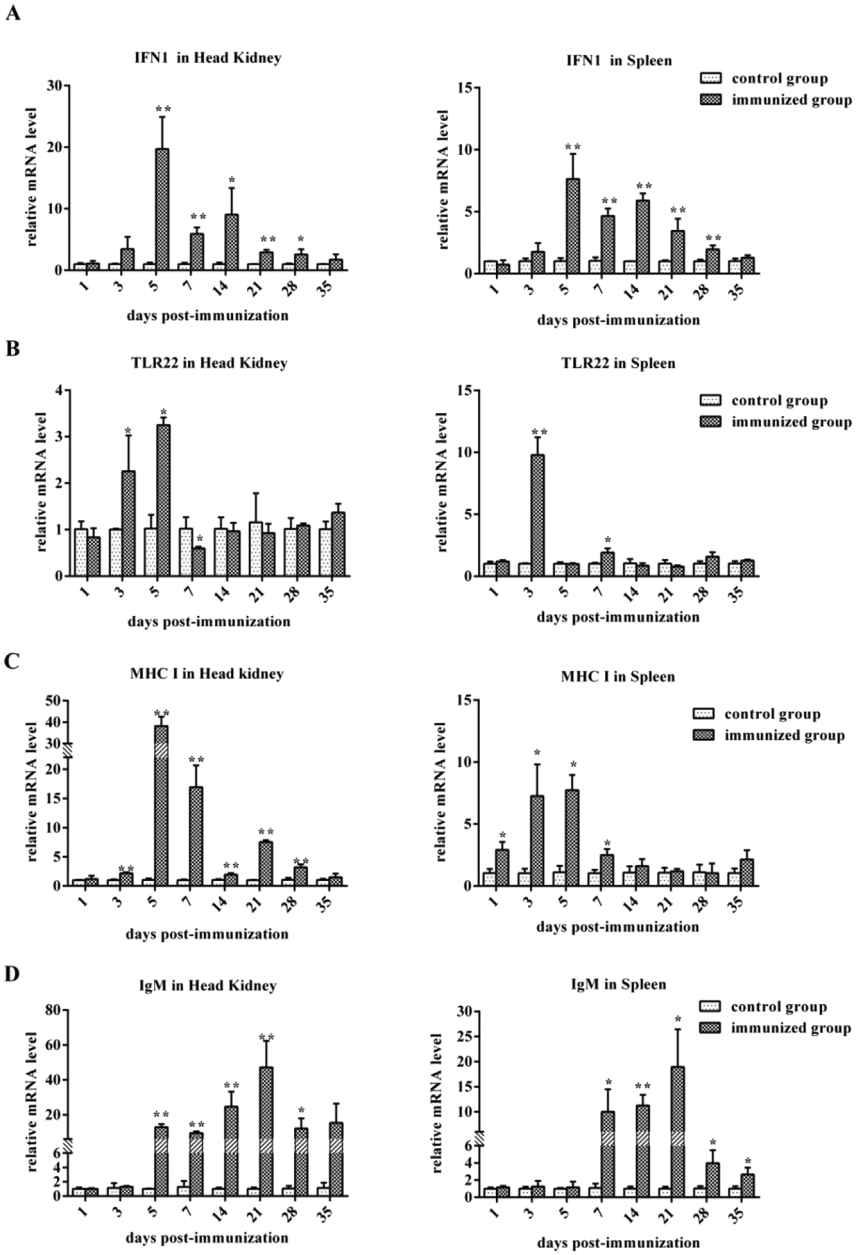


**Fig. 6.** The mRNA expression levels of immune-related genes *IFN1*, *TLR22*, *MHCI* and *IgM* in head kidney and spleen of grass carp. The mRNA level of each gene was normalized based on *β-actin* gene expression. Data are average of triplicate values and are presented as mean ± SD (n = 3). * represents $p < 0.05$ and ** represents $p < 0.01$.

indicating that anti-VP56 serum had neutralizing efficacy to GCRV, although the survival rate in the experimental group was not significantly increased. This result was in accordance to the previous studies in GCRV, MRV, and ARV [30,32,33]. Fiber proteins of MRV and ARV applied as subunit vaccine can trigger robust immune response [34,35].

The intensity of immune reactions could be reflected by the number changes of blood cells, including RBCs and WBCs. The number of RBCs in rVP56 immunized fish was significantly higher at 5 and 7 dpi than that in the control, indicating that RBCs might also take part in immunity. This phenomenon could also be observed in other fishes injected with vaccine [36,37]. It seemed that the RBCs in fish also played an important role in immunity, like the RBCs in mammals [38,39]. In addition, fiber proteins in MRV and adenovirus also act as a viral hemagglutinin [40,41], but there was no evidence to prove that fiber proteins in GCRV could induce agglutination of RBCs [30]. The population of WBCs in vaccinated fish also increased at 5, 7, and 14 dpi, among which the percentage of monocytes and neutrophils increased firstly at 5 dpi, and then the ratio of lymphocytes rose at 14 dpi. These changes reflected the process of immune response to infection. Non-specific immune response conducted by monocytes and neutrophils played a major role in the early stage of infection, followed by specific immunity mediated by lymphocytes. According to the results in previous study, pET-32a plasmid or rTrx tag alone cannot elicit immune response effectively [42,43], indicating the changes in immune parameters in the immunized were caused by rVP56.

Apart from indicators of innate immunity, the antibody expression level was vital for individual to prevent infection after vaccination. The major immunoglobulin isotype of teleost fish was IgM [44]. In the present study, production of specific IgM against rVP56 in immunized fish started uprising sustainably from 3 dpi to 21 dpi. The changes in protein level of specific IgM was in consistent with the change in total

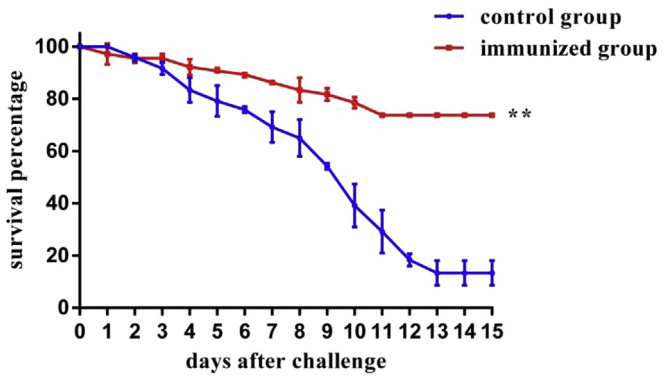


**Fig. 7.** Cumulative survival percentage of experimental fish challenged with GCRV. Grass carp were challenged with GCRV at 10 $LD_{50}$ after 21 dpi. ** represents $p < 0.01$.

mRNA level of *IgM* gene, indicating that the up-regulation of *IgM* gene expression post immunization was mainly applied to produce specific IgM. Moreover, the IgM level was still higher in vaccinated group compared with the control group at 35 dpi, which means that the antibody could continuously provide protection for the fish against GCRV infection.

The expression changes in immune-related genes, especially the genes of antiviral immunity, can reflect the effect of vaccination. *IFN* is a key gene in innate immunity, which induced antiviral proteins in cells to inhibit virus propagation [45]. *TLRs* are pattern recognition receptor that recognize pathogens and initiate highly expression of *IFN* or cytokines. *TLR22* is responsible for recognizing dsRNA and initiating antiviral response against GCRV [46,47]. *MHC* I can present viral peptide antigens to $CD8^+$ T cells to initiate adaptive immune response [48,49]. Our results showed that the expression levels of *TLR22* and *MHC* I were up-regulated firstly in head kidney and spleen of immunized grass carp at 3 dpi, followed by *IFN* at 5 dpi, indicating that the immune system made a quick response to extrinsic antigens. The total results indicated that the antiviral immunity was successfully activated by vaccination. These changes in immune indexes in rVP56 protein immunized fish strongly supported the result of high relative percentage survival after GCRV challenge.

In conclusion, the present study demonstrated that the subunit vaccine prepared by rVP56 protein could induce strong innate and adaptive immune responses and provide effective protection against GCRV infection. The antiserum of rVP56 protein showed neutralizing effect to GCRV, indicating that VP56 was on the outer surface of virus particles, which could be a target for neutralizing antibodies. The results of changes in RBCs and WBCs, expression levels of immune related genes, IgM antibody titer, and relative percentage survival, all proved the effectiveness of rVP56 in activating antiviral immunity of grass carp. Therefore, VP56 protein could be considered as a candidate for the development of commercial vaccine against GCRV.

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