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Plasmid pcDNA3.1-*s11* constructed based on the S11 segment of grass carp reovirus as DNA vaccine provides immune protection

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ABSTRACT

Although some commercial vaccines against grass carp reovirus (GCRV) are available, given the many varieties of GCRV and limited types of vaccines, the disease caused by GCRV remains a major problem, which leads to economic losses in grass carp aquaculture. A reovirus strain (GCRV-HN14) was recently isolated from local diseased fish in our laboratory. The S11 segment of GCRV-HN14 was speculated to encode the virus capsid protein VP35. In our study, the S11 segment was cloned into the eukaryotic expression vector pcDNA3.1(+) to construct the recombinant plasmid pcDNA3.1-*s11*, which was then transfected into CIK cells, and the VP35 protein was successfully expressed. Grass carp was immunized with pcDNA3.1-*s11*, and the *in vivo* distribution and expression of the pcDNA3.1-*s11* plasmids were analyzed by PCR and Western blot. Recombinant plasmids were detected in the blood, liver, spleen, kidney, and muscle. However, protein expression could only be detected in the muscle. The immune protection of the pcDNA3.1-*s11* plasmid in grass carp was evaluated using a series of experiments. Results showed that the population of white blood cells significantly increased at 1, 7, and 14 days post-immunization (dpi) and reached a peak with $(9.58 \pm 0.72) \times 10^7$/ml at 7 dpi ($P < 0.01$ or $P < 0.05$). The percentage of neutrophils reached a peak with (24.13 ± 2.38)% at 7 dpi ($P < 0.01$), whereas the lymphocytes peaked with (93.30 ± 4.71)% at 14 dpi ($P < 0.05$). Serum antibody levels were significantly enhanced in immunized fish at 14, 21, and 28 dpi ($P < 0.01$). The mRNA expression levels of type I interferon, immunoglobulin M, Toll-like receptor 22, and major histocompatibility complex class I were significantly up-regulated in the head kidney and spleen of immunized fish ($P < 0.05$). Grass carp immunized with pcDNA3.1-*s11* exhibited a higher survival percentage (70.4%–73.3%) than the controls (5%–13%). Overall, as a DNA vaccine, the pcDNA3.1-*s11* plasmid could induce immune protection against GCRV.

1. Introduction

In recent years, remarkable progress has been made in aquaculture [1]. For instance, the total aquaculture production in China exceeded 69.01 million tons in 2016, according to the China Fishery Statistical Yearbook, and is currently undergoing rapid, stable, and sustainable development. Grass carp (*Ctenopharyngodon idella*) is widely distributed in China and represents one of the most important aquaculture species; it accounts for approximately 18% of global freshwater aquaculture production and has great commercial value and worldwide distribution [2]. However, outbreaks of severe epidemic grass carp hemorrhagic disease can cause the annual yield to reduce by 30% and lead to a huge loss to aquaculture in China [3,4].

Grass carp hemorrhagic disease, which is caused by the grass carp reovirus (GCRV), is the most serious infectious disease that causes significant loss of fingerlings during rearing [3]. GCRV, a member of the genus *Aquareovirus* in the family Reoviridae, was the first viral pathogen identified from aquatic animals in China in 1983 [5]. A comparative study of gene sequences revealed that GCRV could be divided into three distinct subtypes: type I GCRV with GCRV-873 as the representative strain, type II GCRV with GCRV-HZ08 as the representative strain, and type III GCRV with GCRV-104 being the only strain found in China [6]. Type I GCRV is less virulent and has a longer latent period prior to the induction of a mild host immune response, whereas type II GCRV is more virulent and has a shorter latent period before stimulating a strong and extensive host immune response [7]. Recent studies showed that most GCRV strains isolated in China are type II GCRVs, such as GCRV-HZ08, GCRV-GD108, and GCReV-109, which can cause outbreaks of hemorrhagic disease in grass carp [7,8]. Therefore,

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searching for a novel vaccine is very important for preventing diseases caused by type II GCRV in grass carp aquaculture.

Currently, various types of GCRV vaccines have been developed, including inactivated vaccines of GCRV 854, 836-w, and GCRV HuNan1307; a commercial attenuated GCRV 892 vaccine; and recombinant subunit and DNA vaccines [4,9,10]. These vaccines have been widely applied in China to improve immune protection in grass carp and are effective against GCRV [4]. GCRV variations have led to the need of subunit and DNA vaccines, which can be developed via biological techniques. For example, some vaccines were developed in laboratories based on structural (VP4, VP5, VP6, and VP7) and non-structural (NS38) proteins of GCRV [11–14]. In addition, these vaccines provide a relative percentage of survival (RPS) of 23%–60% [14]. However, a major disadvantage of inactivated vaccines is the limited duration of immunity protection, which only lasts for 2–5 weeks [9]. The attenuated vaccine needs longer time to prepare compared with other vaccines. Immunization with naked subunit vaccines generally induces a weak immune protection in fish as they require more effective carriers to improve host protection [11,15]. Compared with these vaccines, DNA vaccines are more advantageous due to their ease in production, genetic stability, absence of cold chain requirement, and activation of humoral and cellular immunities [16,17].

The S11 segment of type I and type III GCRVs (e.g., GCRV-873 and GCRV-104) encodes non-structural (NS26 or NS20) and undefined proteins, whereas the S11 segment in type II GCRV encodes the VP35 protein that has a conserved putative zinc-binding motif CxxC-n16-HxC sequence [18], which is also present in the VP7 protein [19]. In a previous study, anti-VP35 serum could effectively neutralize GCRV infection [18]. Therefore, the protein encoded by the S11 segment in type II GCRV is predicted to be an outer-clamp protein and has antigenicity. However, to the best of our knowledge, a DNA vaccine based on the S11 segment of GCRV has not been reported previously.

In this study, a strain of GCRV (GCRV-HN14) was isolated in our laboratory. It belongs to the type II GCRV, where the S11 segment of GCRV could encode the VP35 protein. The S11 segment of GCRV was integrated into the expression vector pcDNA3.1(+) to construct the recombinant plasmid pcDNA3.1-*s11*, which was used as the DNA vaccine to immunize grass carp. The aims of this study were to (1) verify the effective expression of the VP35 protein for the constructed pcDNA3.1-*s11* plasmid *in vitro* and *in vivo* and (2) evaluate the protective effect of the recombinant pcDNA3.1-*s11* plasmid, as a DNA vaccine in fish, following a type II GCRV challenge.

2. Materials and methods

2.1. Virus, cells, and plasmids

GCRV-HN14 was isolated from a grass carp with hemorrhagic disease in Henan, China, in 2014. It was stored in our laboratory and used in the preparation of a DNA vaccine. Grass carp liver cell line L8824, obtained from the China Center for Type Culture Collection (Wuhan, China), was used for the propagation of GCRV. The grass carp kidney cell line (CIK) was kindly offered by the Institute of Hydrobiology (Wuhan, China). The cells were maintained at 25 °C in an M199 medium that contained 10% (v/v) FBS (HyClone, USA). Competent *Escherichia coli* DH5α cells were purchased from Biomed (Beijing, China), and the pMD^{TM}19-T vector was obtained from Takara (Dalian, China).

2.2. Cloning of S11 into the pcDNA3.1(+) vector

GCRV-HN14 was cultured in the L8824 cell line, and subsequently, the GCRV-cultured cells were collected and used for total RNA extraction, which was conducted using a TRIzol reagent kit (Invitrogen, USA), according to the manufacturer's instructions. Extracted GCRV RNA was stored at −80 °C. Afterward, reverse transcription was performed to produce cDNA, which was used to clone the GCRV-HN14 S11 segment.

The S11 segment of GCRV-HN14 was amplified via PCR with the following primers: forward primer 5′-TGTGGATCCACCAATTATCGGTAAGTATGGAA-3′ and reverse primer 5′-CTGCTCGAGTGGTATGGAATCAGTCATTACTG-3′ (the underlined letters in the forward and reverse primers indicate *BamH* I and *Xho* I restriction enzyme sites, respectively). The PCR product was examined via electrophoresis using a 1.5% agarose gel. The target DNA was purified using the E.Z.N.A.™ gel extraction kit (Omega, USA). Subsequently, the target DNA and pcDNA3.1(+) (Invitrogen, USA) were respectively digested by restriction enzymes of *BamH* I and *Xhol* I. The enzyme-digested fragments were purified and ligated together using T4 DNA ligase. The ligation mixture was transferred into *Escherichia coli* DH5α. The constructed pcDNA3.1(+) plasmids containing the S11 segment of GCRV (designated as pcDNA3.1-*s11*) were extracted from positive clones and confirmed by sequencing and enzymatic digestion with the *BamH* I and *Xho* I restriction enzymes.

2.3. Detection of recombinant plasmid expression products in cells by Western blot

To confirm that the VP35 protein can be expressed by pcDNA3.1-*s11* in cells, the recombinant plasmid pcDNA3.1-*s11* was extracted using a plasmid extraction kit (Omega, USA). In total, 4 μg of plasmid was transfected into CIK cells cultured in 6-well cell culture plate with Lipofectamine 2000 reagent (Invitrogen, USA), according to the manufacturer's instructions. At 48 h post transfection, CIK cells with pcDNA3.1-*s11* were collected, split, and used for Western blot. Untransfected and pcDNA3.1(+)-transfected CIK cells were used as negative controls.

Following SDS-PAGE with 12% separating gel, the recombinant protein was transferred onto the surface of an NC membrane (Merck, USA). The membrane was blocked via PBS (containing 1% skimmed milk powder and 0.5% bovine serum albumin) and nurtured in mouse anti-VP35 serum (developed in our laboratory with a 1:200 dilution) for 2 h. After rinsing with PBST (containing 0.1% Tween-20), alkaline phosphatase conjugated rabbit anti-mouse IgG was added for nurturing as a second antibody for 1 h at room temperature. Then, the protein was fixed onto the membrane, and Western Blue® Stabilized Substrate for Alkaline Phosphatase (Promega, USA) was used for development for 10 min until the bands could be seen clearly. The development was stopped by rinsing the membrane with distilled water. The relative molecular weight of the protein was compared and evaluated using the PageRuler Prestained protein molecular weight standard (Solarbio, Beijing).

2.4. Immunization with plasmid pcDNA3.1-s11 for grass carp

The grass carps with a body length of (13.0 ± 1.5) cm and body weight of (12.0 ± 1.7) g were obtained from a grass carp farm in Xinxiang City, Henan, China. They were acclimatized for 2 weeks prior to the experiments and did not exhibit GCRV infection as validated by PCR. The grass carps were divided randomly into three groups (180 fish/group). Each fish received a dose of 10 μg recombinant pcDNA3.1-*s11* in a volume of 50 μl PBS via an intramuscular injection in the epaxial muscular tissue to the side, 5 mm away to the right middle of the dorsal fin. The negative control group was intramuscularly injected with 10 μg of pcDNA3.1(+) diluted in 50 μl of PBS, and the control group was intramuscularly injected with 50 μl of PBS. Then, 180 fish in each treatment group were randomly divided into three parallel groups with 60 fish/group.