Fish & Shellfish Immunology 54 (2016) 473–480

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Immunogenicity of a cell culture-derived inactivated vaccine against a common virulent isolate of grass carp reovirus

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ARTICLE INFO

Article history:
Received 4 March 2016
Received in revised form
22 April 2016
Accepted 29 April 2016
Available online 30 April 2016

Keywords:
Inactivated vaccine
Grass carp reovirus
Humoral immune response

ABSTRACT

Grass carp (*Ctenopharyngodon idella*) hemorrhagic disease, caused by grass carp reovirus (GCRV), is emerging as a serious problem in grass carp aquaculture. There is no available antiviral therapy and vaccination is the primary method of disease control. In the present study, the immunological effects and protective efficacy of an inactivated HuNan1307 vaccine in grass carp were evaluated. The GCRV isolate HuNan1307 was produced by replication onto the grass carp PSF cell line, and inactivated with 1% β-propiolactone for 60 h at 4 °C. Grass carp were injected with inactivated GCRV vaccine, followed by challenge with the isolate HuNan1307. The results showed that the minimum dosage of the inactivated vaccine was $10^{5.5}$ $TCID_{50}$/0.2 mL to induce immune protection. All grass carp immunized with the inactivated vaccine produced a high titer of serum antibodies and GCRV-specific neutralizing antibody. Moreover, the inactivated vaccine injection increased the expression of 6 immune-related genes in the spleen and head kidney, which indicated that a immune response was induced by the HuNan1307 vaccine. In addition, grass carp immunized with the inactivated vaccine showed a survival rate above 80% after the viral challenge, equal to that of grass carp immunized with a commercial attenuated vaccine, and the protection lasted at least for one year. The data in this study suggested that the inactivated HuNan1307 vaccine may represent an efficient method to induce immunity against GCRV infection and the induced disease in grass carp.

1. Introduction

Grass carp (*Ctenopharyngodon idella*) is an economically important freshwater fish in China and its production accounted for 18.10% of the output of all freshwater fisheries in 2013 [1]. It is also the most commonly cultivated fish in the world and is cultured extensively in more than 40 countries [2]. However, hemorrhagic disease caused by grass carp reovirus (GCRV) has seriously hampered the development of grass carp aquaculture [3]. GCRV, a member of genus *Aquareovirus* in the family *Reoviridae*, was the first viral pathogen to be identified from aquatic animals in China in 1983 [4]. This pathogen can provoke severe hemorrhagic disease in fingerling and yearling populations of grass carp, and causes a mortality rate of 60–100% during outbreaks [5]. GCRV was recognized to be the most virulent among *Aquareovirus* species so far [6]. In recent years, with the rapid development of aquaculture industry, diseases caused by GCRV have become increasingly significant and resulted in huge financial losses [7].

Attempts to control GCRV infection are hindered by a lack of thorough knowledge of the pathogenesis of the virus, the existence of diverse genetic make-ups, and the lack of anti-viral therapeutics [8,9]. Vaccination is considered the most effective way of protecting grass carp from this disease. To date, four vaccine types have been investigated for the control of GCRV: (i) inactivated vaccine; (ii) attenuated vaccine; (iii) recombinant subunit vaccine, and (iv) DNA vaccine. Inactivated vaccines are the most commonly applied method for the prevention of GCRV. In China, the first vaccine for grass carp hemorrhagic disease “inactivated tissue vaccine” was developed in the 1960s [10]. Subsequently, significant

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http://dx.doi.org/10.1016/j.fsi.2016.04.133
1050-4648/

achievements have been obtained in the development of inactivated vaccines for GCRV through cell culture [11]. Currently, there is only 1 licensed GCRV vaccine available in the international market, which is an attenuated vaccine developed by attenuating the GCRV-892 strain through serial passages in tissue culture [1]. With attenuated vaccines, the difference between vaccine virus isolates and wild-type viruses often results in a failure to produce effective immunity against the wild-type viruses. In some cases, fish populations immunized with attenuated vaccines have shown outbreaks; in these cases, safety considerations stopped further work [13]. The development and manufacture of subunit vaccine and DNA vaccine are expensive and time consuming, and difficulties in applying them in the aquatic environment have also resulted in many limitations [14]. Especially, the subunit vaccine is easily degraded during processing, delivery, and in the animals. So far, studies of recombinant subunit vaccine and DNA vaccine are still at an experimental stage. At present, it became necessary to develop a new GCRV vaccine due to the occurrence of rare serious adverse events following treatment with existing vaccines. The development of a new vaccine that reduces or eliminates the disadvantages of existing vaccines would be highly beneficial for grass carp aquaculture.

In this study, aiming to produce a vaccine with an improved efficacy and safety profile, an inactivated vaccine was prepared by inactivating GCRV isolate HuNan1307, which is commonly known as a virulent isolate. The aim of this work was to evaluate the immunological effects and protective efficacy of this inactivated GCRV vaccine in grass carp.

2. Materials and methods

2.1. Experimental fish and virus

Grass carp were kindly provided by a fish farm located in Foshan (Guangdong, China) with a mean total length and body weight of 12.0 ± 0.5 cm and 25.0 ± 0.5 g, respectively (data are presented as mean values ± SD). Fish were acclimatized at 28 °C under laboratory conditions for 2 weeks before experimental manipulation, then were maintained in aerated water and fed daily with commercial dry feed pellets (Hello Fish Dry Pellets; CVM Products, Beijing, China). Possible viral contamination in fish and feed was checked by reverse transcription quantitative real-time PCR (RT-qPCR) to verify that they were free from pathogens [7]. Care of animals was performed in compliance with the guidelines of the Animal Experiment Committee, South China Agricultural University. The protocol was approved by China Guangdong Province Science and Technology Department (permit number: SYXK(Yue) 2014–0136). The GCRV HuNan1307 that was used to prepare the inactivated vaccines in this study was isolated from infected grass carp in a fish farm located in Zhuzhou (Hunan, China). The complete genome sequences of HuNan1307 are publicly available (GenBank accession nos. KU254566 ~ KU254576).

2.2. Cell lines

The proboscis snout into fibers (PSF) cell line, which is derived from grass carp, was used for the propagation of GCRV [15]. The cells were maintained at 28 °C in M199 medium (Gibco, USA) containing 10% (v/v) fetal bovine serum (FBS; HyClone™, GE Healthcare Life Sciences, Logan, UT, USA). The concentration of FBS in the cell culture medium was reduced to 3% for virus propagation.

2.3. Virus replication

Infectious GCRV was replicated onto PSF cells seeded in 150 cm^2 flasks (Corning Inc., Corning, NY, USA) and infected using a multiplicity of infection (MOI) of 0.1. After 1 h of viral adsorption at 28 °C, the cells were washed with sterile phosphate-buffered saline (PBS, pH 7.5) and maintained in M199 medium supplemented with 3% FBS at an incubation temperature of 28 °C. At 5 days after infection, the GCRV-infected cell monolayers were harvested by scraping the sterile PBS-washed monolayer with 1 mL M199 followed by centrifugation of the cell suspension at 1500*g* and 4 °C for 10 min. The titer of the virus was determined according to the 50% tissue culture infectious dose ($TCID_{50}$) assay, which was performed as described previously with some modifications [16]. Ten-fold serial dilutions of the viral samples were prepared, and 100 μL aliquots of each dilution were inoculated in duplicate onto PSF cell monolayers in 24-well cluster plates. Virus adsorption was allowed to proceed for 3 days at 28 °C in a 5% CO_2 incubator. Then, the virus presents was assayed using indirect immunofluorescence assay (IFA) as described previously [17].

2.4. Virus inactivation

The GCRV preparations were inactivated with formaldehyde or β-propiolactone (BPL; Sigma-Aldrich, St. Louis, MO, USA) and samples were removed at specific time intervals to determine the infectivity titer [18]. 0.05% or 1% (w/v) formaldehyde was added to live GCRV and the mixture was agitated at different temperatures. BPL was mixed with live GCRV to a final concentration of 0.1% (v/v) and the mixture was agitated at 4 °C (Table 1). Virus inactivation by BPL was neutralized by the addition of 1 M sodium thiosulfate to a final concentration of 20 mM.

2.5. Testing for remaining live virus

Confirmation of virus inactivation in the vaccine preparations was performed by cell culture of PSF cells inoculated with the inactivated GCRV preparations. The titer of virus preparation after inactivation was determined using qPCR [7]. In addition, 1 mL of the inactivated viral suspension was initially inoculated into a 25 cm^2 cell culture flask and incubated at 28 °C/5% CO_2. The cell monolayer was assessed daily using IFA. Every 7 days, 1 mL of the viral supernatant was removed from the prior flask and inoculated in a new flask, and the suspensions were evaluated for a total period of 21 days. The absence of live GCRV during this period confirmed that the viral inactivation had been successful. The sample was considered to be inactivated only when confirmed as inactive by two separate trials. The inactivated virus preparations were stored at −80 °C.

2.6. Grass carp immunization

Healthy grass carp (n = 1800) were randomly divided into 3 groups (600 fish per group). The immunized group was administered with 5×10^4 $TCID_{50}$ of the inactivated virus preparation in a volume of 200 μL by intraperitoneal injection. The negative control group was injected with 200 μL of PBS. The positive control group was immunized with a single dose of 1×10^3 $TCID_{50}$/200 μL of the commercial attenuated GCRV vaccine (PuLin Biological Products Co., Ltd., Guangzhou, China). Subsequently, the fish in the control and treatment groups were transferred to different tanks and maintained as described above.

2.7. Measurement of antibody response by antibody ELISA

An anti-IgM monoclonal antibodies (MAbs) preparation was made according to the methods described in our previous study [19]. For antibody determination, the immunized and control fish