Article @ Virology

Research Progress of Grass Carp Reovirus

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ABSTRACT

Grass Carp Reovirus(GCRV), also called Grass Carp Hemorrhagic Disease Virus, is the first fish virus isolated in China. Grass carp hemorrhagic disease caused by GCRV is a highly infectious and pathogenic fish disease. It has the characteristics of wide epidemic range, long onset season, high morbidity and mortality. However, its frequent outbreak has affected the development of aquaculture industry in China and caused huge economic losses to freshwater fisheries. Therefore, it is imperative to study the theory of grass carp reovirus.

This paper mainly reviews the biological characteristics, pathogenic mechanism and vaccine of GCRV, aiming at summarizing the relevant theoretical basis and understanding the new technologies and methods related to GCRV vaccine. To provide a strong and powerful theoretical reference for the prevention of the virus disease and development of green fisheries.

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Abbreviation: GCRV,Grass Carp Reovirus; VP;Viral Protein; NS; Nonstructural roteins

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1. Biological characteristics

1.1 Physical and chemical characteristics

The Grass carp reovirus (GCRV) belongs to the family Reoviridae, aquatic animals belonging to the genus Aquareovirus. GCRV is a spherical particle of icosahedron with a diameter of 60~85 nm, no envelope, having double capsids. It mainly composed of proteins and nucleic acids, and also contains a small amount of carbohydrate in the form of glycoproteins, without lipids. The viral particles are arranged in a lattice in the cytoplasm. The optimum temperature of the virus polymerase is 28 ℃, and it is easy to break out at 25~30℃, and some viruses lose their infectivity below 20℃[1]. It is Stable in the range of pH 3~10, stable to heat (56℃, 1 h), insensitive to lipid solvents, Repeated freezing and thawing have a great effect on the virus. The buoyancy density is 1.30~1.31 g/mL in sucrose, 1.36m/l in cesium chloride, and the sedimentation coefficient is 550s

1.2 Biological characteristics

There is no definite standard for GCRV genotyping, most of which are based on nucleotide sequences or deduced amino acid sequence and phylogenetic tree construction. According to the current study, there are at least three GCRV genotypes[2]. According to the different gene sequences, it can be divided into genotype I (representing GCRV 873 and GCRV-873-JX09-01), genotype II (representing GCRV-HZ08 and GCRV-GD10) and genotype III (representing GCRV-104 and GCRV-HB1007)[1]. According to Li Yonggang et.al, the main prevalent grass carp reovirus in China is type II[3].

GCRV is a double stranded RNA (dsRNA), which consists of 11 gene fragments. According to the gel electrophoresis mobility, the 11 fragments can be divided into 3 groups, large segment L1、L2、L3, medium segment M4、M5、M6, smaller segments S7、S8、S9、S10、S11. From the whole genome sequencing, the large fragments are between 3600bp and 4200bp, the medium fragments are between 2000 bp ~2400 bp, the small segments are between 700 bp ~1700 bp. There is no poly (a) tail at the 3 'end of GCRV gene, and a short repetitive conserved sequence at the 5' and 3 'ends.

GCRV can proliferate in CIK、GCO、GCF、GSB cell lines. Yuan Xuemei er al[4]. infected GCRV-873 CIK cells, found that the virus titer began to rise at 12h, increased rapidly from 24 to 48h, and then stabilized at 48h, the virus titer reached the maximum at 72 h and reached the peak at 36 h after CIK infection. After 72 hours, the cells almost fell off[5].

1.2.1 Structural protein

At present, it is believed that the proteins encoded by the three subtypes of GCRV include seven structural proteins (VP1, VP2,VP3,VP4,VP5,VP6 and VP7) and five nonstructural proteins(NS80,NS38, NS16, NS26 and NS31)[6]. Among them, VP1, VP2 and VP3 proteins have similar molecular weights, while VP6 and VP7 components have smaller molecular weights. The content

of VP5 component accounts for the largest proportion of capsid protein[7], while VP4 band is the weakest, which is the trace protein component[8]. The inner core of capsid is composed by VP1, VP2, VP3, VP4 and VP6 protein. The outer proteins are different, although VP5 protein is found in the outer shell of all types GCRV, VP7 protein is in GCRV type I, VP35 is in GCRV type II and VP38 is in GCRV type III. There is no fibrous protein in GCRV type I, VP56 protein is in type Ⅱ, which is encoded by S7, and VP55 is in GCRV type Ⅲ[9].

VP1 proteins form an open channel across the inner and outer layers, With the methyltransferase activity, that can cap the transcribed RNA[10]. VP2 and VP4 are microstructural proteins and do not form viral capsid component of the virus[11]. Respectively, They have RNA polymerase and NTPase activities. VP3 forms an inner nucleocapsid skeleton protein, which first assembles the inner nucleocapsid and then enters the inclusion body for outer capsid assembly[12]. It has helicase and NTPase activities. VP5 and VP7 complex form the outer protein layer, Wang Hangjun et.al found that VP5 had NTPase activity[9], which could induce the body to produce immunogenicity, VP7 is mainly participates in the process of virus adsorption and infection, which has good antigenicity[12]. VP6 is used to connect the inner protein layer and the outer protein layer, and has the function of stabilizing the structure. The VP33 protein encoded by the S11 segment of GCReV-109 gene has good immunogenicity, and its antibody can inhibit the infection of GCRV[13].

1.2.2 unstructured proteins

Unstructured proteins play an important role in viral replication. NS80 contains a coiled coil fragment, which plays a role in the early morphology of the virus and form a polymer. NS38 is the initiator of inclusion body formation. NS26 is a specific protein of GCRV, which has ATP dependent RNA helicase functional site. NS31 is related to the release, diffusion, cell fusion of viral particles[7]. Shen believes that NS31 can increase the virus titer, which could be the viral proteins that inhibit host RNAi[14].

2. Pathological features

The main pathological symptoms of GCRV infected fish are as follows: there is no obvious apparent phenomenon in the incubation period, and the activity and feeding are normal. In the early stage of the disease, the appetite of the sick fish decreased, the body color become black, the epidermis tissue at the edge of the fin become necrotic, and the color become lighter. Then the congestion and bleeding appear in different parts of the diseased fish. When bleeding is serious, the diseased fish appears anemia, at this time the gills are grayish white, the blood color becomes lighter, the blood volume decreases. The color of liver, kidney, spleen often turns pale, and there are also bleeding spots.

According to the bleeding symptoms and location, it can be roughly divided into the following three situations[13,15]:

(1) Red muscle type: It usually appears in small grass carp species (7~10cm), the disease fish is mainly muscle bleeding, but there is no obvious bleeding symptoms or only slight bleeding on the surface;

(2) Red fin and red gill cover type: generally appears in larger fish species (more than 13cm), the diseased fish is mainly the body surface hemorrhage, with obvious bleeding or congestion in the mouth, jaw, gill cap, eye socket and fin base;

(3) Enteritis type: It can be seen in all size fish species, diseased fish appear intestinal congestion, bleeding.

GCRV pathogenicity is mainly reflected in the destruction of blood circulation system and parenchymal organs[16]. Through histopathological examination, the pathological features of diseased fish are systemic capillary endothelial cell damage, increased vascular wall permeability, degeneration and necrosis of tissues and organs. Red blood cells, white blood cells and hemoglobin are significantly lower than normal fish, the activity of serum alanine aminotransferase, serum lactate dehydrogen--ase and serum isocitrate dehydrogenase are increased, and the level of plasma total protein, serum albumin, urea nitrogen, cholesterol are decreased[17].

3. Pathogenic mechanism

There are four stages of virus infection[6]: incubation period, prodromal stage, obvious stage and outcome stage(host death or potential source of infection). Viral particles enter the fish body through the gills and directly invade the kidneys and intestines[3]. GCRV proliferates in cytoplasm and induces apoptosis[18,19]. Therefore, the endothelial cells that destroy capillaries or microvessels cause diffuse vascular coagulation to form microthrombus, consume a large number of coagulation factors, cause bleeding, and reduce circulating blood volumn[3]. And then cause physiological disorders and organ necrosis[6].

Virus entry: GCRV adsorbs onto the cell surface. There are two main ways for the virus to enter the cell, one is to make the virus enter the cell through the endocytosis, the other is to make virus particles directly through the membrane[20,21]. Cell lysosomes enzymatic hydrolysis of virus capsid. VP5 mediates viral entry, and VP7 is associated with the viral adsorption.

Synthesis of nucleic acids and proteins: VP1 and VP2 are the main participants in this process. All the 11 mRNA of GCRV are synthesized by VP2 with RNA polymerase activity, Five VP1 molecules form a cylindrical pentamer, which across the inner and outer protein layers. Synthetic templates are the dsRNA negative chains.

Assembly of viral particles: after the viral protein is synthesized in the cytoplasm, it

may first form a spherical inclusion bodies from the NS80, which can bind VP3 and NS38. firstly VP3 formed the inner nucleocapsid skeleton protein, and then entered the inclusion body for outer protein assembly 30 minutes later. VP4 and VP6 play an auxiliary role, VP4 can fix the inclusion body on the microtubules, VP6 can be stabilize the inner protein layer.

Virus release: the release of virus is related to NS31. The transmembrane protein composed of NS31 has the function of membrane permeability and membrane fusion, which releases virus particles out of the cell.

On the one hand, apoptosis is the mechanism of resisting virus infection, on the other hand, it can promote the transmission of virus between tissues. Li Bo experiment confirmed that CIK cells infected with GCRV induced apoptosis through caspase pathway, resulting in the activation of caspase-9 and downstream Caspase-3, and the expression of upstream Bax Pro apoptotic protein and Bcl-2 antiapoptotic protein were up-regulated and down regulated respectively[19]. Indicating that GCRV activated the mitochondrial apoptosis pathway and lead to apoptosis.

4. Vaccine development

Grass carp hemorrhagic disease is an infectious fish disease caused by GCRV. With strong pathogenicity, transmissibility and high mortality, which seriously threatens the development of fish breeding industry in China. But since GCRV proliferation process is only located in the cytoplasm[22], so it is difficult for general chemicals to inhibit the virus. At present, drug control and immunotherapy are the main methods of fish disease prevention and treatment. With the development of modern molecular biology and genetic engineering, the vaccines that can be used for immunization and epidemic prevention mainly include inactivated vaccine, live attenuated vaccine, subunit vaccine and DNA vaccine[23,24].

4.1 inactivated vaccines

Inactivated vaccine refers to the use of chemical or physical methods to make the pathogen lose its pathogenicity and retain the immunogenicity of antigen. Inactivated vaccine has the advantages of short development period, safe use and easy to preserve, but its inoculation dose is large and the immunization duration is short. In the 1960s, the earliest “indigenous vaccine” used in China was tissue homogenate inactivated vaccine. In the 1980s, yang xianle et al. carried out research on cell culture inactivated vaccine for grass carp hemorrhagic disease. The results showed that the immune protection rate of the cell inactivated vaccine was high (about 85%), which induced immunity of fish in 4~5d. And the protection could last for more than 60d. Inactivated vaccine may enhance the body's ability to present antigen and activate the complement system in the specific immune response of grass carp. At present,

the most widely used vaccine in production is inactivated vaccine.

4.2 Live attenuated vaccines

The live attenuated vaccine is a kind of vaccine which can reproduce and infect the body normally after the structure of the toxic subunit of the pathogen is changed by physical or chemical methods, and its virulence is weakened or disappeared, but it still retains good immunogenicity. The live attenuated vaccine has the advantages of less vaccine dosage, longer immunization duration and no need to add adjuvant, but the screening cycle is long and difficult to preserve, sometimes even appears the phenomenon of virulence reversion, which increases pathogenicity.

The lyophilized cell attenuated vaccine for grass carp hemorrhagic disease developed by Pearl River Fisheries Research Institute has the advantages of strong immunogenicity, good immune effect and long immune protection period, with the immune protection rate of over 90%. After observing the immune protective effect, Xu shuying et al[25]. have found that eucalyptus is a good attenuated agent, successfully attenuated grass carp hemorrhagic virus (GCRV-892 strain) after continuous transmission to 19 generations, and maintained the attenuated virulence without regurgitant, the immunogenicity of antigen did not change, and the immune protection rate was 100%, but the live attenuated vaccine has the risk of reversing mutations[9,26]. With the develop-ment of gene recombination technology, attenuated vaccines are prepared by using gene deletion technology to make pathogenic virulence genes missing or key genes of pathogen metabolism missing. However, because of the large number of viral mutation genotypes the viral vaccines are seldom used. Mainly used in bacterial disease vaccine production. Min et al[27]. constructed recombinant viral hemorrhagic septicemia virus with G gene deletion. The vaccine could not replicate and proliferate due to the lack of G gene in natural state, and the risk of disease is low, so it could be used as a preventive vaccine[13].

4.3 Recombinant subunit vaccine

Recombinant subunit vaccine is a kind of protein preparation which contains only pathogen antigen which is expressed in vitro and purified by gene recombination technology[28]. It is mainly divided into virus vaccine, bacterial vaccine and hormone vaccine, which has the characteristics of high purity, high yield and good stability, but the poor immune effect and need adjuvant assistance. Subunit vaccines stimulate immunogenicity without causing pathological reactions. To date, several GCRV structural proteins, such as VP35,VP4,VP6 and VP56, have been studied as potential subunit vaccines against GCRV infection[13]. As GCRV II coat shell protein,VP35 has antigenicity. Studies have shown that anti-VP35 serum can effectively neutralize GCRV infection. The recombinant

VP35 proteins can induce immunity and protect grass carp from GCRV infection and can be used as the subunit vaccine[29]. Chao Pei et al. studies on fibrin VP56 have neutralizing effect on GCRV after injection of antiserum, indicating that VP56 is a target of neutralizing antibody on the outer surface of virus. The activation effect of anti-VP56 on grass carp antiviral immunity was verified, so VP56 protein can be used as a candidate protein for the development of GCRV II type commercial vaccine[30].

4.4 gene vaccine

Gene vaccine refers to the DNA vaccine, which inserts the gene encoding exogenous antigen into the plasmid containing eukaryotic expression system, and then directly introduces the plasmid into human or animal body to express antigen protein in host cells and induces the body to produce the immune response. The advantages of DNA vaccine are low production cost, mass production, simple preparation process, no need to add other adjuvants, good stability, but there is a risk of integration into the host genome, which sometimes induces the body to develop immune tolerance. Li Zhen constructed a protein-nucleic acid vaccine expression vector controlled by double promoter pFastTMDua-VP7-VP6-β-actin, and obtained recombinant baculovirus Bacmid-VP7-VP6-β-actin. The recombinant baculovirus Bacmid-VP7-VP6-β-actin can enter the fish body by RT-PCR and immunohistochemistry and express the antigenic gene VP6, This study showed that the recombinant virus could be used as a DNA vaccine[31]. Bin zhu et al. used carbon nanotube as the carrier to investigate the immune effect of grass carp reovirus VP7 gene DNA vaccine on grass carp, After injecting SWCNTs-pcDNA-VP7 vaccine into the fish, With the increase of plasmid DNA released by SWCNTs, More antigen expression and stronger immune response were induced[32]. It can stimulate persistent innate and adaptive immunity in the fish. On the 28th day after injection, The plasmid was still stable in the fish, and had a protective effect on GCRV, indicating that the stability of the plasmid was good[33], However there's no result in the paper on its long-term protection mechanism, which requires further investigation. But the duration of plasmid expression in fish, Whether long-term expression has other side effects on the body, Whether the plasmid foreign gene can be integrated into the host cell are still controversial, which still limit the development of DNA vaccine. At present, most of them are in the stage of laboratory research and development, But it has very broad development prospect and application value for the development of DNA vaccine.

5. Outlook

GCRV belongs to the reovirus family, which is the first fish virus isolated in China and the most virulent strain in aquatic reovirus genus. China began to study grass carp hemorrhagic disease in 1950s. In the

late 1970s, the pathogen was isolated and identified as virus particles. In 1991, the International Commission on virus Classification named it grass carp reovirus[34]. Although many aspects of grass carp reovirus have been studied, the prevention and control situation of grass carp hemorrhagic disease is still grim, The annual outbreak of the disease has caused huge economic losses to fishery production[35].

Currently, Vaccine immunization is the most effective way to prevent grass carp hemorrhagic disease, But development of GCRV vaccine is slow, and the commercial vaccines are not widely used. Most of the vaccines in the market for GCRV are based on the outer capsid protein of type I GCRV[36], such as subunit vaccines and DNA vaccines made by VP5 and VP7[22]. Epidemiological investigations showed that the dominant virus type in China was II GCRV type, but there were few vaccines against GCRV[37].

At present, Inactivated vaccine and live attenuated vaccine are used more frequently in production. the highest research level is grass carp hemorrhagic disease live vaccine (GCHV-892 strain)[38]. Because of its high production technology, high cost and complex preparation process, subunit vaccine and DNA vaccine have not been widely used in production, and still remain at the level of laboratory research. However, with the development of modern molecular biology and gene recombination technology, through the understanding of GCRV pathogenesis and the analysis of functional structure, It is the most urgent task to develop multivalent or unit price inactivated vaccine and a new generation of attenuated live vaccine for the current epidemic type of grass carp hemorrhage. The DNA vaccine with carbon nanotubes as a carrier has good stability, can prolong the degradation time of plasmid DNA, and has remarkable effects on non-characteristic immunity and adaptive immunity of fish[39]. We know that non-specific immunity is more important in fish and is the first line of defense against fish immunity, and adaptive immunity is limited because of the limited antibody library of fish, the slow proliferation and growth of lymphocytes. Therefore, the DNA vaccine with carbon nanotubes can be used as an effective DNA vaccine carrier against fish virus pathogens. Development of new genetic engineering vaccines such as subunit vaccine and DNA vaccine is the future development direction of grass carp hemorrhage vaccine. Moreover, oral vaccine can also play a good immune effect. Mu Changyong et al. study have shown that the new recombinant baculovirus BmNPV--VP35-VP4 can induce immunity and resist GCRV infection, the combination of oral administration of the two can provide the best effect[39].

Compared with other reovirus genera, there are relatively few studies on grass carp reovirus at home and abroad, and the

pathogenic mechanism of GCRV has not been fully understood, which has caused limitations to the effective antiviral treatment against GCRV infection. In the next step, the research on GCRV is necessary to analyze the pathogenic mechanism and functional structure of the virus with GCRV as the model, which will have a profound significance in the theory and practice of grass carp reovirus.

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