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Prokaryotic expression and immunoassay of grass carp reovirus capsid VP6 protein

Y. ZHOU[#], Y. D. FAN[#], L. B. ZENG[*], J. MA

Division of Fish Disease, Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Wuhan, Hubei 430223, P. R. China

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Summary. – Grass carp reovirus (GCRV) of the genus *Aquareovirus* and the family *Reoviridae* causes a severe hemorrhagic disease in grass carp fingerlings in China. GCRV genome is composed of 11 double-stranded RNA segments, of which segment 8 encodes the major core capsid protein VP6. In this study, the VP6 gene following an RT-PCR-amplification from the GCRV 104 strain was cloned into an expression vector pET-32a to obtain pET-32(a)-VP6. The VP6 protein was expressed in *Escherichia coli* BL21 as a fusion protein of 64 kDa. After purification with the HisLink Spin Protein Purification System the VP6 protein was used to raise a specific polyclonal antibody in Balb/c mice. Presence of VP6 protein was proved in bacterial lysates containing VP6 fusion protein by Western blot analysis and in GCRV-infected CIK cells by immunofluorescent staining using polyclonal antibody. These results may be helpful in further studies of interactions between GCRV and cells and in preparation of an engineered vaccine against GCRV.

Keywords: grass carp reovirus; VP6 gene/protein; polyclonal antibody; Western blot analysis; immunofluorescence staining

Introduction

Grass carp hemorrhage caused by GCRV (Cheng *et al*., 2008) is a highly infectious, fatal viral disease affecting grass carp fingerlings in China. GCRV is a member of the genus *Aquareovirus* of the family *Reoviridae,* which belongs to the species *Aquareovirus C* (Fang *et al*., 2005; Lupiani *et al*., 1993; Regenmortel *et al*., 2000; Attoui *et al*., 2002). The genome of GCRV is composed of 11 double-stranded RNA segments.

It has been reported that the GCRV could be detected by staphylococcus protein A coagglutination (Yang *et al*., 1991), ELISA, fluorescent antibody technique (Li *et al*., 1999), Dot-ELISA (Shao *et al*., 1996) and RT-PCR (Wang *et al*., 1997). Since GCRV VP6 protein has high immunogenicity, it will be suitable for raising the polyclonal antibody (Wang *et al*., 1995). In this study, we amplified, cloned and expressed the GCRV VP6 gene in *E. coli*. Using a purified VP6 protein, we raised in Balb/c mice a polyclonal antibody which was able to specifically detect the VP6 protein in bacterial lysates by Western blot analysis and in virus-infected cells by immunofluorescence staining. These results contribute to the knowledge of the interaction between GCRV and cells and preparation of an engineered vaccine against GCRV.

Materials and Methods

Virus and cells. The GCRV104 (CCTCC NO: V201217) was originally isolated from the diseased grass carp with hemorrhage symptoms in 2009 in China and was passaged in the CIK cell line. The CIK cells were cultured in MEM supplemented with 10% bovine fetal serum with pH 6.8 at 28°C (Zuo *et al*., 1983).

Cloning of VP6 gene. GCRV genomic dsRNA was extracted from the infected CIK cell cultures with Trizol Reagent (Invitrogen, USA) following manufacturer′s recommendations. A pair of specific primers P1/ P2 for amplifying GCRV VP6 gene was

[*]Corresponding author. E-mail:zenglingbing@gmail.com; phone: +86-27-81780158. [#]These authors equally contributed to this project.

Abbreviation: CIK = *Ctenopharyngoden idellus* kidney; GCRV = grass carp reovirus; MRV = mammalian orthoreovirus

designed by using Primer Premier 5 software based on the sequence information of GCRV VP6 gene and the expression vector pET-32a(+) (Novagen, USA). The sequence of P1 is CGGGATCC ATGGCACGTGTGGTTTATG (forward) containing the *Bam*HI and P2 is CCAAGCTT ATGTGGTTACGCGGGTCAG (reverse) containing the *Hind*III restriction site (restriction sites are underlined). A full length GCRV VP6 gene was produced by RT-PCR amplification, and the amplified fragment was cloned into prokaryotic expression vector pET-32a(+). The recombinant plasmid, which was designated as pET-32(a)-VP6, was then transformed into *E. coli* BL21(DE3) host cells. The recombinant plasmid was extracted using the Plasmid Miniprep kit (Omega Bio-Tek, USA) and analyzed by PCR, restriction enzyme digestion, and sequencing (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd).

Expression of his-tagged VP6 protein. The VP6 protein expression was induced by addition of 0.5 or 1 mmol/l IPTG. At 1, 2, 4 or 8 hrs after the addition of IPTG, 1ml of the culture was collected and centrifuged. The bacterial pellet was resuspended in Laemmli sample buffer (Bio-rad, USA) and boiled for 5 min before SDS-PAGE analysis.

Preparation of polyclonal VP6 antisera. Recombinant proteins were purified using HisLink Soin Protein Purification System kit (Promega, USA) and analyzed by SDS-PAGE. Concentration of the purified proteins was determined by using the Bradford assay. To prepare antisera, 20 μg of the recombinant proteins were used to immunize Balb/c mice intraperitoneally with Quick Antibody immunoadjuvant (Kbqbio, BeiJing) at day 1 and 21.

Titration of VP6 antisera by ELISA. The 96-well plate (Corning, USA) was coated with recombinant protein at concentration of 5 μg/ml at 4°C, overnight. Polyclonal VP6 mouse antiserum and unimmunized mouse serum (negative control) were added to the plate separately and were incubated 1 hr at 37°C. The plates were washed with buffer (PBS containing 0.05% Tween 20) and HRP-conjugated rabbit anti-mouse antibody (Kbqbio, BeiJing) was added to each well and incubated 1 hr at 37°C. After washing, O-phenyldiamine (OPD) was added to each well and the reaction was stopped with 2 mol/l sulfuric acid. ELISA titers were measured at A490 and expressed as the reciprocal of the last dilution of sera.

Western blot analysis. CIK cells infected with GCRV were collected and total proteins were extracted by boiling and thawing, separated by 12% SDS-PAGE and then transferred to a nitrocellulose membrane by a semi-dry transfer cell (Bio Rad, USA) at 15 V for 30 min. The membrane was blocked with 5% BSA in the TBS buffer (50 mmol/l Tris-HCl, 200 mmol/l NaCl, pH 7.5) at 37°C for 2 hrs. Subsequently, the membrane was probed with ONE-HOUR Western kit (Genscript, NanJing) following the manufacturer′s instructions.

Immunofluorescent staining. CIK cell monolayer was infected by GCRV (MOI = 0.5) at 28°C for 24 hrs and fixed with 4% paraformaldehyde. After fixation, cells were incubated with polyclonal VP6-specific antiserum for 1 hr, followed by washing three times with PBS. Goat anti mouse IgG Cy3 (1:50) was added and incubated at 37°C for 45 min. Images were taken with fluorescence microscope (Leica, germany).

Results

Cloning and expression of VP6 gene

The full-length GCRV VP6 gene was composed of 1,319 bp (Acc. No. JN967636) and an open reading frame (ORF) of 1,257 bp encoding an 418 aa protein VP6. Primer pair P1/P2 was designed to amplify the full length ORF of GCRV VP6 gene. The PCR amplified product was analyzed in 1.2% agarose gel (Fig. 1) and cloned into the vector pMD18-T and confirmed by sequencing.

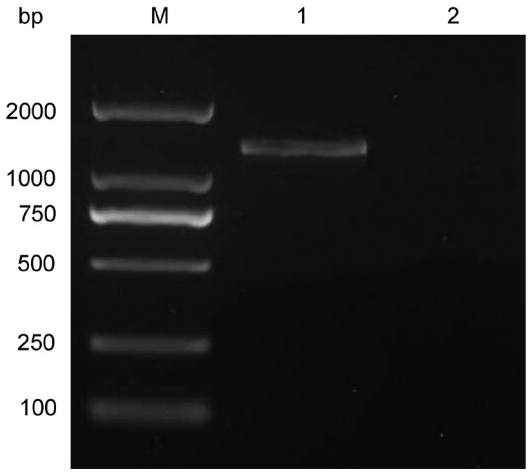

Fig. 1

RT-PCR amplification of GCRV VP6 gene

Agarose gel electrophoresis. DNA size markers (lane M), GCRV VP6 gene RT-PCR product (lane 1), negative control (lane 2).

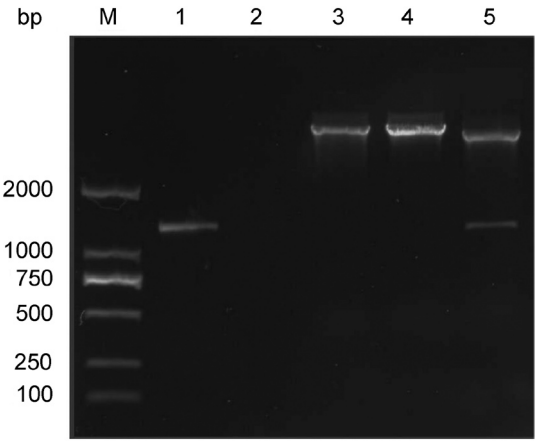

Fig. 2

Restriction analysis of recombinant plasmid pET-32a-VP6

The legend: Agarose gel electrophoresis. DNA size markers (lane M), GCRV VP6 gene (lane 1), negative control (lane 2), plasmid pET-32a-VP6 digested with *Bam*HI (lane 3), *Hind*III (lane 4), *Bam*HI + *Hin* III (lane 5).

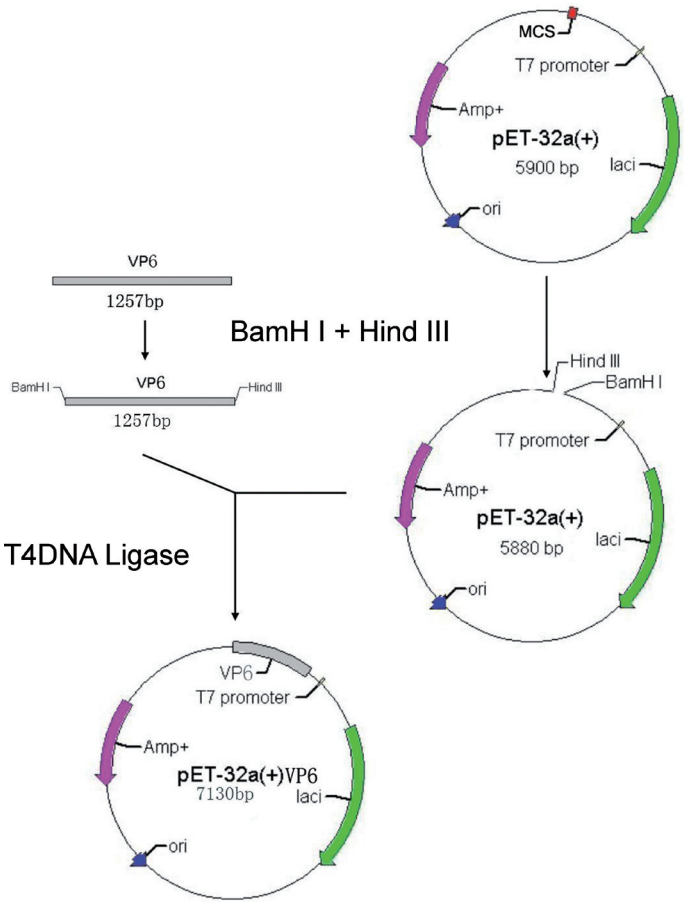

Fig. 3

Construction of recombinant plasmid pET-32a-VP6

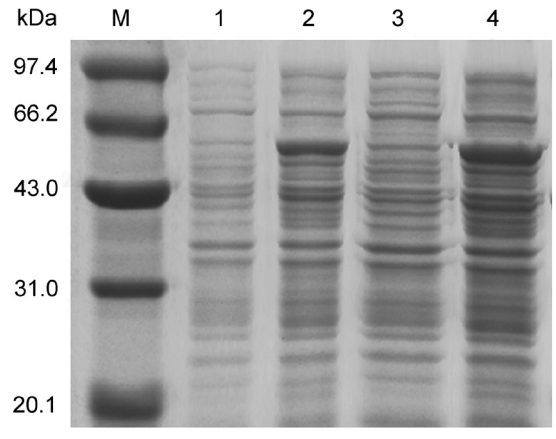

Fig. 4

SDS-PAGE analysis of the production of recombinant plasmid pET-32a-VP6 in transformed *E. coli*

Induction with 1 mmol/l IPTG for 4 hrs. DNA size markers (lane M), negative control (lane 1), total bacterial lysate (lane 2), supernatant (lane 3), pellet (lane 4).

Amplified GCRV VP6 gene was digested with *Bam*HI and *Hind*III and cloned into expression vector pET-32a(+) digested with the same enzymes to construct the recombinant plasmid pET-32(a)-VP6. The obtained recombinant clones were verified by *Bam*HI and *Hind*III digestion (Fig. 2), which generated two bands at 1,257 bp and 5,900 bp, respectively. Both restriction enzyme digestion and DNA sequencing were used for plasmid pET-32(a)-VP6 verification (Fig. 3).

SDS-PAGE analysis showed a band with molecular mass of 64 kDa, which was equal to the molecular mass sum of the VP6 protein (46 kDa) and the expression vector pET-32a(+) (18 kDa) (Fig. 4). In addition, SDS-PAGE analysis showed that target protein was mainly present in the inclusion bodies.

Optimization of induction

To determine the optimal conditions for VP6 protein expression, two major parameters were examined including the IPTG concentration and incubation duration. The results from different induction times are shown in Fig. 5. After

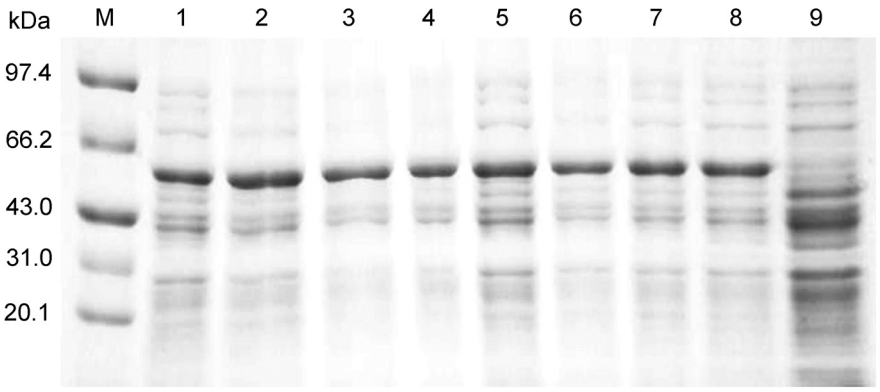

Fig. 5

SDS-PAGE analysis of the production of recombinant plasmid pET-32a-VP6 under different induction conditions

Induction with 0.5 mmol/l (lanes 1–4) and 1 mmol/l IPTG (lanes 5–8). The products were harvested after 1, 2, 4, and 8 hrs induction (lanes 1–4 and 5–8, respectively). DNA size markers (lane M), negative control (lane 9).

induction for 1–8 hrs, a steady expression level of VP6 was observed, and the highest production of VP6 was after 8 hrs. There was no significant difference in the different IPTG concentration conditions (0.5 mmol/l or 1 mmol/l).

Characterization of polyclonal VP6 antisera

The recombinant proteins with the 3' terminal fused his-tagged proteins were purified using HisLink Soin Protein Purification System kit and analyzed by SDS-PAGE (Fig. 6). The SDS-PAGE result showed the purity of purified fusion protein. The concentration of the fusion protein was 140 μg/ml as determined by Bradford assay.

Serum samples were taken at day 26 post-immunization and analyzed by ELISA and Western blot analysis. Antibody titers (A492) were detected as high as 20,000 (Fig. 7). The VP6 protein was detected by SDS-PAGE followed by Western blot analysis with the polyclonal antibodies against the VP6 protein. The Western blot analysis demonstrated a protein with a molecular mass of 64 kDa (Fig. 8).

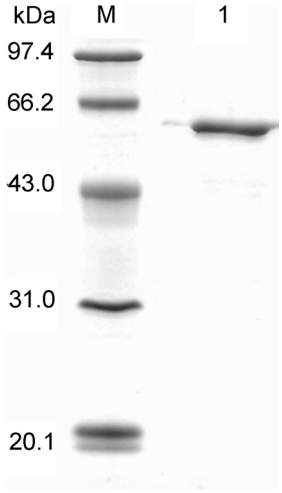

Fig. 6

SDS-PAGE analysis of the purified recombinant GCRV VP6 protein

DNA size markers (lane M), GCRV VP6 protein (lane 1).

Immunofluorescent staining of virus-infected cells for VP6 protein

The CIK cells infected by GCRV were positive in immunofluorescence already at day 1 post-infection. Increased fluorescence, in comparison to uninfected CIK cells, could be seen also after 3 days (Fig. 9).

Discussion

The GCRV VP6 protein encoded by the genomic segment 8 is similar to the σ2 protein of MRV and occupies a similar structural position in the virus (Fang *et al*., 2005). Early studies have also demonstrated that the σ2 protein of MRV or the VP6 protein is essential for core shell assembly (Leslie *et al.*, 2007; Kim *et al*., 2002). In recent years, Hill and Zhou found that some reoviruses in the genus of *Cypovirus*, are similar to the GCRV core and have nodule proteins at the same positions as those of other aquareoviruses which serve as a stabilizing clamp to enhance VP3 (λ1 analogue) core frame intensity (Hill *et al*., 1999; Zhou *et al*., 2003). Some researchers have investigated immunogenicity of rotavirus VP6. This protein is highly immunogenic but does not induce neutralizing antibodies. Since antibodies against VP6 are highly cross reactive among group A of rotaviruses,

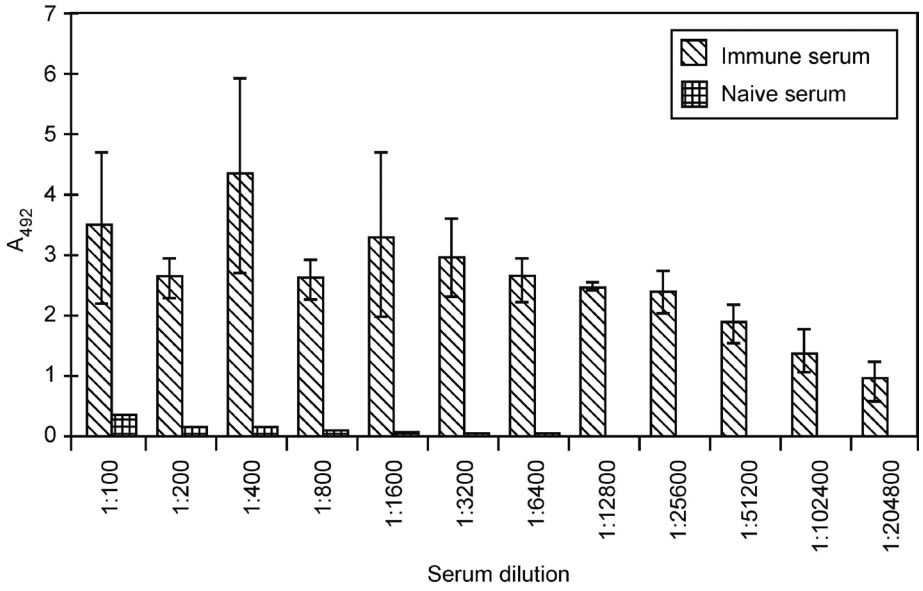

Fig. 7

Detection of antibody titers by ELISA

The antibody titers (A_{492}) against the VP6 protein were detected by ELISA in serum from mice immunized after 26 days. Antibody titers were as high as 1:20,000.

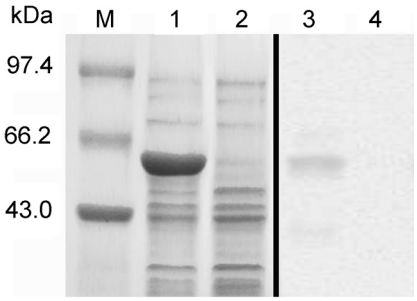

Fig. 8

SDS-PAGE and Western blot analysis of lysate of transformed *E. coli*

Induction with 1 mmol/l IPTG and harvest after 4 hrs. SDS-PAGE (lanes 1–2), Western blot analysis for VP6 using the antiserum (lanes 3–4). Induced GCRV VP6 product (lanes 1, 3), non-induced control (lanes 2, 4). Protein size markers (lane M).

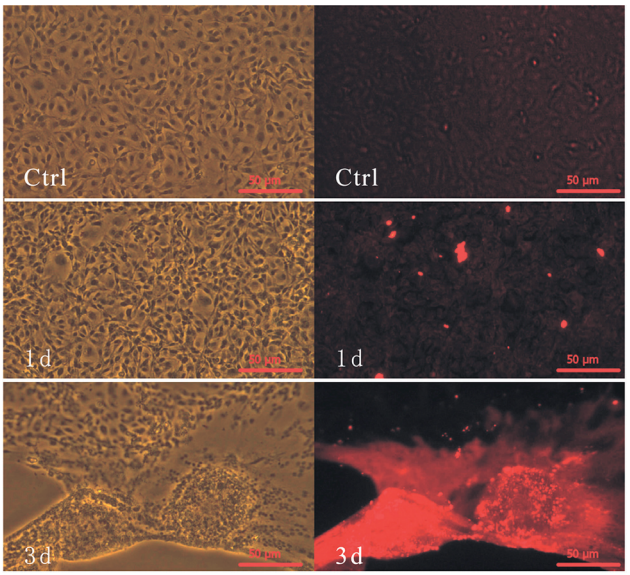

Fig. 9

Immunofluorescent staining of GCRV-infected CIK cells for VP6 protein

Images were taken with white light (left) and fluorescent light (right). The CIK cells infected by GCRV were firstly detected to be positive by immunofluorescence at day 1 post-infection and increased in fluorescent intensity after 3 days, no fluorescent in uninfected CIK cells (Ctrl).

VP6 immunization could potentially provide heterotypic protection (Tang *et al.*, 1997). Vaccine development studies have shown a high level of protection when VP6 was used as an immunogen in experimental models (Choi *et al.*, 2002; Dong *et al.*, 2005; Yu and Langridge, 2003), so VP6 protein is a good target protein for immunological assays (Wang *et al.*, 1995).

In this study, the recombinant protein VP6 present in inclusion bodies formed a compact particle composed of aggregate proteins due to the hydrophobic interactions and high expression level of recombinant protein. Inclusion bodies can prevent protein from degradation and also protect host cells from incorrectly folded recombinant proteins. Compared to antibodies against GCRV, recombinant protein

VP6 antibodies could be a better tool for the study of GCRV VP6 (Zhang *et al*., 2008).

In conclusion, we expressed the VP6 protein in the host strain *E. coli* BL21 (DE3). Fusion protein was used to raise specific polyclonal antibodies in Balb/c mice. Western blot and immunofluorescence analysis indicated high specificity of VP6 polyclonal antibody. These results provide a solid foundation for further research on aspects of immunity and preparation of immunoreagents.

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References

Attoui H, Fang Q, Mohd Jaafar F, Cantaloube JF, Biagini P, de Micco P, de Lamballerie X (2002): Common evolutionary origin of aquareoviruses and orthoreoviruses revealed by genome characterization of Golden shiner reovirus, Grass carp reovirus, Striped bass reovirus and golden ide reovirus (genus Aquareovirus, family Reoviridae). J. Gen. Virol. 83, 1941–1951.

Cheng L, Fang Q, Shah S, Atanasov IC, Zhou ZH (2008): Subnanometer-resolution structures of the grass carp reovirus core and virion. J. Mol. Biol. 382, 213–222. http://dx.doi.org/10.1016/j.jmb.2008.06.075

Choi AH, McNeal MM, Basu M, Flint JA, Stone SC, Clements JD, Bean JA, Poe SA, VanCott JL, and Ward RL (2002): Intranasal or oral immunization of inbred and outbred mice with murine or human rotavirus VP6 proteins protects against viral shedding after challenge with murine rotaviruses. Vaccine 20, 3310–3321. http://dx.doi.org/10.1016/S0264-410X(02)00315-8

Dong JL, Liang BG, Jin YS, Zhang WJ, Wang T (2005): Oral immunization with pBsVP6-transgenic alfalfa protects mice against rotavirus infection. Virology 339, 153–163. http://dx.doi.org/10.1016/j.virol.2005.06.004

Leslie AS, Max LN, Kenneth LT (2007): Orthoreoviruses and Their Replication. In David MK, Peter PH (Eds): Fields Virology. 5th edition. Lippincott Williams & Wilkins, USA, Philadelphia. pp.1854–1915.

Fang Q, Shah S, Liang Y, Zhou ZH (2005): 3D reconstruction and capsid protein characterization of grass carp reovirus. Sci. China C Life Sci. 48, 593–600. http://dx.doi.org/10.1360/062004-105

Hill CL, Booth TF, Prasad BV, Grimes JM, Mertens PP, Sutton GC, Stuart DI (1999): The structure of a cypovirus and the functional organization of dsRNA viruses. Nat. Struct. Biol. 6, 565–568. http://dx.doi.org/10.1038/9347

Kim J, Zhang X, Centonze VE, Bowman VD, Noble S, Baker TS, and Nibert ML (2002): The hydrophilic amino-terminal arm of reovirus core shell protein lambda1 is dispensable for particle assembly. J. Virol. 76, 12211–12222. http://dx.doi.org/10.1128/JVI.76.23.12211-12222.2002

Li J, Wang TH, Lu RH, Chen HX. (1999): Advances in research of hemorrhagic virus of grass carp. Oceanologia Et Limnologia Sinica 30, 445–453.

Lupiani B, Hetrick FM, Samal SK (1993): Genetic analysis of Aquareoviruses using RNA-RNA blot hybridization. Virology 197, 475–479. http://dx.doi.org/10.1006/viro.1993.1616

Regenmortel MHV, Fauquet CM, Bishop DHL (2000): Virus Taxonomy-Seventh Report of the International Committee on Taxonomy of Viruses. Academic Press, USA, San Diego.

Shao JZ, Xiang LX, Li YN (1996): Studies on the detection of grass carp haemorrhagic virus by enzyme linked immunosorbent assay on nitrocellulose membrance (DOT-ELISA). J. Fisheries China 20, 6–12.

Tang B, Gilbert JM, Matsui SM, Greenberg HB (1997): Comparison of the rotavirus gene 6 from different species by sequence analysis and localization of subgroup-specific epitopes using site-directed mutagenesis. Virology 237, 89–96. http://dx.doi.org/10.1006/viro.1997.8762

Wang TH, Li J, Yi YL (1997): Detection of hemorrhagic of grass carp by reverse transcription-polymerase chain reaction. Oceanologia Et Limnologia Sinica 28, 1–6.

Wang W, Zhao TY, Fang Q (1995): Immunogenicity of grass carp hemorrhage virus polypeptides. Virologica Sinica 10, 66–68.

Yang GZ, Luo YZ, Ye XP (1991): Rapid serological diagnosis of grass carp haemorrhagic virus by coagglutination test using antibody-sensitized staphylococci protein A. J. Fisheries China 15, 27–33.

Yu J, Langridge W (2003): Expression of rotavirus capsid protein VP6 in transgenic potato and its oral immunogenicity in mice. Transgenic Res. 12, 163–169. http://dx.doi.org/10.1023/A:1022912130286

Zhang LL, Shen JY, Lei CF, Li XM, Fang Q (2008): High level expression of grass carp reovirus VP7 protein in prokaryotic cells. Virologica Sinica 23, 51–56. http://dx.doi.org/10.1007/s12250-008-2921-3

Zhou ZH, Zhang H, Jakana J, Lu XY, Zhang JQ (2003): Cytoplasmic polyhedrosis virus structure at 8 A by electron cryomicroscopy: structural basis of capsid stability and mRNA processing regulation. Structure 11, 651–663. http://dx.doi.org/10.1016/S0969-2126(03)00091-1

Zuo WG, Qian HX, Xu YF (1983): A cell line derived from the kidney of grass carp (ctenopharyngodon idellus). J. Fisheries China 10, 11–17.