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ISSN 0792 - 156X

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PUBLISHER:
Israeli Journal of Aquaculture - BAMIGDEH -
Kibbutz Ein Hamifratz, Mobile Post 25210,
ISRAEL

Phone: + 972 52 3965809

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Hemorrhagic Disease of Grass Carp: Status of Outbreaks, Diagnosis, Surveillance, and Research

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Key words: hemorrhagic disease of grass carp, fish disease, viral disease, aquareovirus, grass carp reovirus (GCRV)

Abstract

Hemorrhagic disease of grass carp is the most serious infectious disease of grass carp and causes significant losses of fingerlings. The main clinical signs are external and internal hemorrhage. The disease is caused by aquareovirus and has several serotypes. The optimal epidemic temperature of this disease is 25-28°C. The disease can be transmitted by water or parasite bite. Susceptible hosts are grass and black carp. Other cyprinids are only carriers. Vaccination can control hemorrhagic disease of grass carp. An inactivated vaccine prepared from organs of sick fish is simple and easy to produce with good efficacy. In China, hemorrhagic disease of grass carp was prevalent but has been controlled by wide application of the vaccine. In other Southeast Asian countries, it is a new disease. For surveillance and diagnosis of hemorrhagic disease of grass carp, isolation of the virus using carp kidney (CK) cells and reverse transcriptase polymerase chain reaction (RT-PCR) are the main methods of detection.

Introduction

Grass carp (*Ctenopharyngodon idellus*) is an important species of freshwater fish in China. It accounts for about 20% of the total freshwater fishery production. Hemorrhagic disease of grass carp is the most serious infectious disease of grass carp and causes significant losses of fingerlings during rearing. Survival to market size is less than 20% if this disease occurs.

Hemorrhagic disease of grass carp was first discovered in a fish farm in Hubei Province in 1972. It was identified as of viral etiology in 1978 and became the first fish viral disease studied in China (Section of Virus Study, 1978). In 1980, virus particles in kidney sections of infected grass carp were observed by electron microscopy and the virus was tentatively designated a herpesvirus (Section of Virus Study, 1980). However, further study revealed that these were icosahedral particles with double capsids, no envelope, containing 11 segments of double-stranded RNA (Chen and Jiang, 1984). It was similar to some reoviruses isolated from aquatic animals, such as chum salmon reovirus (CSV), catfish reovirus (CRV), and golden shiner virus (GSV). Hence, it was classified as an aquareovirus and renamed grass carp reovirus (GCRV) in 1984.

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Clinical Signs

The clinical signs of this infection are hemorrhages of organs showing spots or plate forms, in combination of some or all of the following signs: exophthalmia, body darkening, hemorrhage of the mouth cavity, hemorrhagic or pale gills, and hemorrhage at the base of fins and gill covers (Fig. 1). Internal hemorrhage may occur throughout the musculature, liver, spleen, kidney, and intestines (Jiang, 1995).

Some farmers tried to classify this disease into three types according to clinical signs: "red muscles", "red intestine", or "red fin and gill cover". In fact, these are only some of the signs; sick fingerlings show one or all (Fig. 2).

It is easy to confuse these clinical signs with bacterial enteritis and viral enteritis of fish. In both infections, the intestines are reddish, but the intestine of fish infected by bacteria develops ulcers, while the intestine of fish infected only by virus is smooth and elastic. Histopathological changes consist of degeneration and necrosis in liver cells, hyperemia, and hemorrhage in liver and spleen vessels (Guo and Jiang, 1993). Infected fish can also be infected with bacteria or a secondary bacterial infection in warm seasons (Fig. 3). It is important to distinguish between bacterial and viral infections because clinical signs of bacterial infection may conceal the viral infection.

Causative Agent

The pathogen of hemorrhagic disease of grass carp is the grass carp reovirus (GCRV). Virus particles, orderly arranged in lattice form in the cytoplasm, were observed in a kidney tissue section

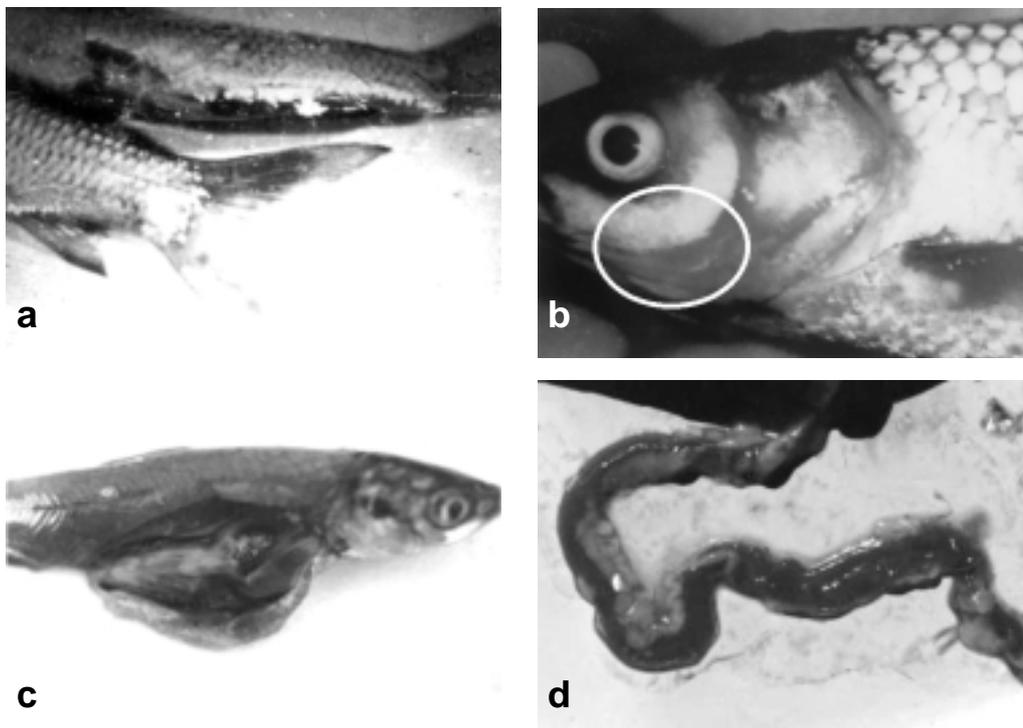


Fig. 1. Hemorrhage of (a) fins, (b) gill cover, (c) musculature and organs, and (d) intestine of grass carp infected with grass carp reovirus (GCRV).

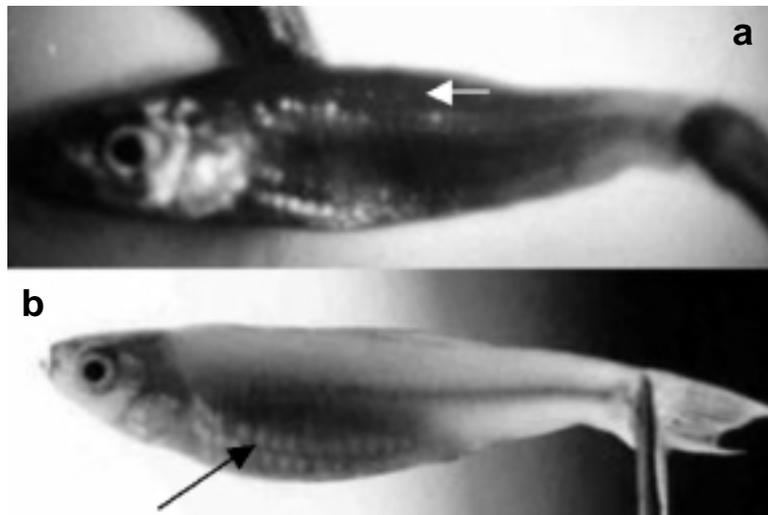


Fig. 2. Hemorrhage of the (a) musculature and (b) organs of grass carp infected with grass carp reovirus (GCRV).



Fig. 3. Grass carp fingerlings infected by bacteria as well as grass carp reovirus (GCRV).

of artificially infected grass carp (Fig. 4). By negative staining specimens of purified virus particles, the virus showed icosahedral particles with a diameter of 60-70 nm, a double capsid, and no envelope (Fig. 5). Twenty peripheral capsomers were seen on the outer capsid (Fig. 6). When purified virus was mixed with serum of grass carp that survived infection and incubated, negatively stained specimens revealed the presence of complex agglomerates, formed by a combination of antibodies and corresponding virus particles. In the agglomerates, a number of intact virus particles and a few hollow capsids could be seen, indicating that the antigen-antibody agglutination of the virus was specific (Jiang and Chen, 1983). The virus was stable to chloroform treat-

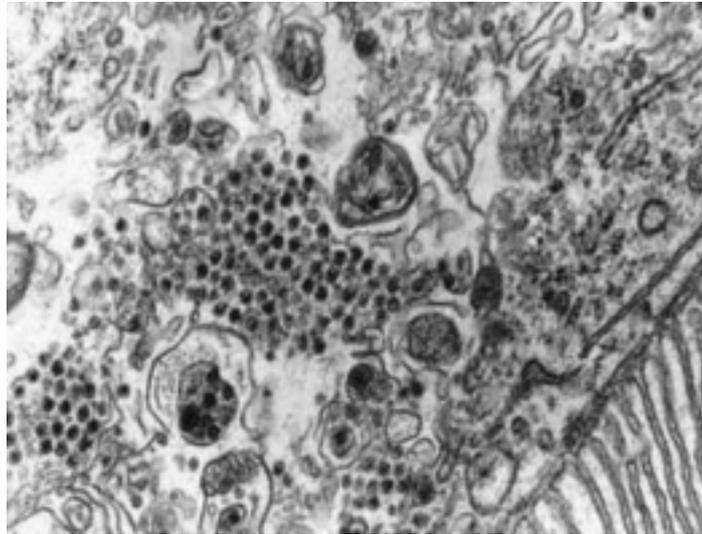


Fig. 4. Electron micrograph of the kidney section of grass carp artificially infected with grass carp reovirus (GCRV). Note the virus particles, arranged in lattice form in the cells.

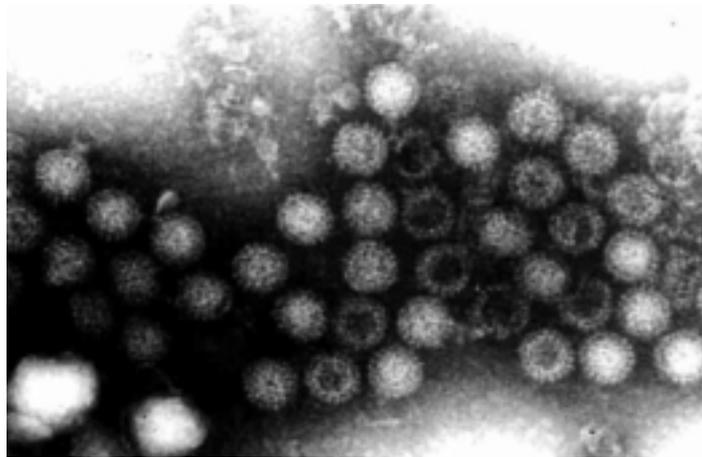


Fig. 5. Electron micrograph of negatively stained grass carp reovirus (GCRV).

ment, to acid (pH 3) and alkaline (pH 10) conditions, and to heating at 56°C for 30 min. Virus replication was not inhibited by 5-iodo-2'-deoxyuridine (IUDR), a DNA inhibitor, indicating its RNA virus nature. Particles viewed along the five-fold axis of symmetry exhibited twenty capsomers.

SDS-PAGE analysis of the viral nucleic acid exhibited eleven segments of double-stranded RNA, divided into large, medium, and small segments. The total molecular weight of the RNA is approximately 15×10^6 d.

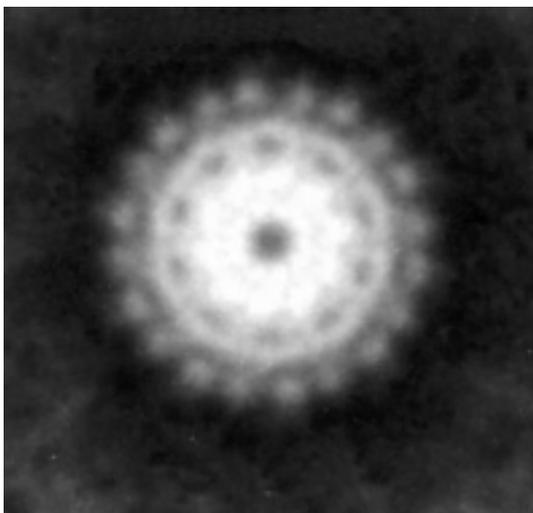


Fig. 6. Electron micrograph of GCRV particles. Note the twenty peripheral spherical capsomers with a diameter of 7-9 nm.

Morphologically, GCRV is similar to members of the *Reovirus* genus. However, viruses in this genus contain only ten segments of dsRNA, while the GCRV has eleven. Nor does GCRV belong to the *Rotavirus* genus, as the shape of the peripheral capsomer is different. Capsomers of GCRV are spherical while capsomers of rotavirus are long and baculoform. In addition, the patterns of RNA segments detected by electrophoresis are quite different from each other (Jiang and Ahne, 1989). GCRV and other reoviruses isolated from aquatic animals (e.g., CSV, CRV, and GSV) have many characteristics in common but can be differentiated from one another. Hence, in the sixth report of International Committee for the Taxonomy of Viruses (ICTV), these viruses were placed into a new genus: *Aquareovirus* (Chen and Jiang, 1984).

Virulence and Serotypes

Formerly, hemorrhagic disease was observed in grass carp and black carp and considered two separate diseases: hemorrhagic disease of grass carp and hemorrhagic disease of black carp (Fig. 7). Further investigation and comparison indicated that there were no differences in morphological, physico-chemical, or biological characteristics between the two pathogens from a single region. In cross infection tests, the pathogens induced similar clinical signs and mortality in both grass carp and black carp. When both viruses were used to prepare vaccines, cross-protection was observed. Hence, the same virus affects both hosts.

On the other hand, serotypes of the virus exist. When collected from different regions and compared, the serotypes differ in virulence, antigenicity, and profile of RNA segments in electrophoresis (Jiang and Li, 1995). So far, two serotypes of GCRV have been confirmed in China: GV-87/3 from Hunan province and GV-90/14 from Hubei province (Fig. 8). Artificial infection tests showed that although both serotypes are pathogens of hemorrhagic disease, GV-90/14 possesses stronger virulence and causes higher mortality in grass carp fingerlings (Table 1; Guo and Jiang, 1993).

Fish infected with serotype GV-90/14 died within 3-5 days and histopathological changes appeared mainly in external organs (hyperemia and hemorrhage in muscles and mouth cavity). Fish infected with serotype GV-87/3 died in 5-10 days and histopathological changes appeared mainly in internal organs (degeneration and necrosis of liver cells, hyperemia, and hemorrhage in liver and spleen vessels). Inactivated vaccine prepared from organs of sick fish from one region had very good effects in the same region but not as



Fig. 7. GCRV-infected black carp showing hemorrhage of the musculature and other visceral organs.



Fig. 8. SDS-PAGE profile of two serotypes of GCRV RNA: left lane = GV-90/14, right lane = GV-87/3.

good effects in the other region, confirming the existence of different GCRV serotypes (Yang et al., 1989). Enzyme-linked immunosorbent assay (ELISA) also shows that the virus strains have different antigenicity (Table 2).

Susceptible hosts of GCRV are grass carp (*Ctenopharyngodon idella*), black carp (*Mylopharyngodon piceus*), and topmouth gudgeon (*Pseudorasbora parva*). Other cyprinids, such as bighead carp (*Aristichthys nobilis*), silver carp (*Hypophthalmichthys molitrix*), golden carp (*Carassius auratus*), and common carp (*Cyprinus carpio*), can carry the virus, but exhibit neither clinical signs nor mortality. Fry and one-year fingerlings are most susceptible, resulting in death. The disease occasionally occurs in 2 or 3-year old fish. Usually adults do not exhibit clinical signs but are reservoirs of the virus.

Hemorrhagic disease of grass carp is transmitted horizontally from sick fish or apparently healthy carriers by the water or a parasite bite. Fingerlings can be infected by immersion in water containing GCRV. The optimum temperature of the disease is 22-30°C, with mortality peaking at 25-28°C. During summer, infections run an acute course, resulting in high mortality. In experimental infections, fish showed clinical signs and mortality within 1-2 weeks at 25°C or higher. The target organ of GCRV is the head kidney. Hemorrhagic disease leads to reduced immunity of infected fish; as the optimal temperature of this disease is about 25°C, bacteria in the fish body multiply quickly, leading to secondary infection, septicemia, and even higher mortality.

Control and Vaccination

There is no medicine to cure this disease. However, disinfecting ponds, reducing density, and killing parasites might decrease mortality and prevent the spread of the disease.

Prevention is the most effective and economic control measure against hemorrhagic disease. This includes preventing the introduction of virus carriers, use of virus-free water, and disinfection of water and tools when outbreaks occur on farms. The disease can also be controlled by vaccine. A simply-prepared inactivated vaccine produced from organs of infected fish has been used in China since 1970 to reduce losses of grass carp fingerlings (Chen et al., 1985). To prepare the inactivated vaccine, organs such as the liver, spleen, and kidney from infected grass carp are collected and homogenized, diluted with 0.85% NaCl to a concentration of 1:100, and

Table 1. Mortality (%) of grass carp fingerlings challenged by different dilutions of two grass carp reovirus (GCRV) serotypes at 25°C for three weeks (n = 60 fish/group).

GCRV serotype	Dilution of virus suspension					Control
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	
GV-87/3	38	20	5	0	0	0
GV-90/14	100	90	50	30	0	0

Table 2. Cross reaction in ELISA test of two serotypes of GCRV (OD value).

Serotype	Antiserum	
	As-GV-87/3	As-GV-90/14
GV-87/3	3.06	1.06
GV-90/14	1.06	2.70

development of fish immunity is related to water temperature. Usually immunity is induced in 90% of the fingerlings about 1 week after vaccination at 20°C or above (Jiang et al., 1991). Safety and efficacy tests were carried out on healthy grass carp fingerlings. Depending on fish size, 0.2-0.5 ml GCRV vaccine was injected into each fish. The fish were reared at 25-28°C for 15 days. The vaccine was considered safe if no onset of clinical signs appeared during this period. Afterwards, the immunized fish were challenged with a suspension of GCRV. Control (not immunized) fish were also challenged. The fish were observed for another 15 days. The vaccine was considered effective if no deaths occurred in the immunized group while mortality reached 70% or above in the control group. The protection rate can usually reach 70% or above and is calculated as follows: protection rate = [(mortality of control group - mortality of immunized group)/mortality of control group] x 100%.

Vaccines and immunization methods have been improving. Vaccination by injection is time-consuming, labor intensive, and stressful to the fish. However, the protection rate of vaccination by immersion is usually very low. Hence, researchers are trying to improve the efficacy of immersion methods. The hyperosmotic method was tested on fingerlings immersed in 3% NaCl for 2-3 min and a bath in vaccine for 10 min. This method raised the protection rate to 60-70% (Zhang et al., 1990). Other researchers used large-scale culture of carp kidney (CK) cells inoculated with GCRV to prepare the vaccine and, thus, improve its quality (Ye et al., 1992). An attenuated virus vaccine was developed in 1989. GCRV passaged over 50 times in CK cells at 20°C became attenuated. Fish inoculated with the attenuated virus by injection or bath resisted GCRV attacks. The virus did not revert to virulent after 10 passages *in vivo*. However, the protection rate was still not high enough (Jiang, 1995). Lyophilized vaccine is applied in some provinces in China.

In summary, the main vaccination method in China against GCRV is injection of an inactivated vaccine prepared from fish organs which is easy to produce, inexpensive, and has good efficacy. Now that vaccination has been applied widely in China, the mortality of fingerlings has been reduced from over 80% to less than 30%. In China, at least 300,000 kg of the vaccine are produced annually and several billion grass carp fingerlings are vaccinated (Fig. 9).

Diagnosis and Detection

Isolation of the virus from infected fish by cell culture and identification by RT-PCR are the main methods of surveillance and diagnosis of the hemorrhagic disease of grass carp,

To isolate the virus, the kidney, spleen, and liver of infected fish are collected. The virus is isolated using CK cells at 25°C for 7 days, blind passaged twice if required. Cytopathogenic effects (CPE) usually appear 3-7 days after inoculation (Deng et al., 1985). However, only some strains of GCRV can replicate and induce CPE in CK cells (such as GV-87/3). In contrast, other strains replicate in CK cells but do not induce CPE (such as GV-90/14). This difference has been demonstrated by electron microscopy, gel electrophoresis, and experimental infection tests. It was easy to observe the 11 bands of viral RNA in SDS-PAGE using only 2-10 ml of infected CK cell suspension (Jiang and Li, 1995).

Primary diagnosis in fish farms is based on typical external and internal clinical signs, espe-

centrifuged at 3,000 rpm for 30 min. Then 0.1% formalin is added to the supernatant and the well-mixed solution is inactivated at 32°C for 72 h. Safety and efficacy tests are conducted before the vaccine can be used. The vaccine can be preserved at 4-8°C for 14 months (Yang and Zuo, 1990).

During vaccination, 0.2-0.5 ml vaccine (depending on fish size) is injected intraperitoneally or intramuscularly to each fingerling when the water temperature is above 20°C (Yang and Zuo, 1994). The



Fig. 9. Vaccination of grass carp against GCRV in farms (inset shows the vaccine).

cially the “red muscles” that appear in some sick fish. However, some clinical signs may be caused by a bacteria in a mixed infection and conceal clinical signs caused by the virus. If mortality is high and cannot be controlled by antibiotics, the possibility of viral infection should be confirmed by virus isolation or RT-PCR detection.

Epidemiological investigations are usually carried out in summer when it is easy to detect the virus from a carrier or sick fish. Because some strains of GCRV cannot induce CPE, virus isolation using CK cells should be done first. If CPE appears, the virus can be identified by a neutralization test, ELISA, or RT-PCR. However, neutralization tests can only identify a virus that can produce CPE in cells. Otherwise, gel electrophoresis or RT-PCR are used to detect viral RNA.

RT-PCR assay can be used to identify GCRV in CK cell culture or in fish organs using two pairs of specific primers (Li et al, 1997). The set for the GCRV-87/3 strain amplifies the 697 bp fragment in the S10 gene and consists of GV873S10R: 5' ccc-cga-tca-tca-cca-cga-t 3' and GV873S10F: 5' cgc-gtt-cgc-tga-tgt-aag-g 3'. The set for the GCRV-90/14 strain amplifies the 320 bp segment in the M6 gene and consists of GV14S6R: 5' agt-tct-caa-agc-tga-gac-ag 3' and GV14S6F: 5' acg-tgc-gat-tgg-aag-agc-tt 3'.

Prevalence of the Disease

Hemorrhagic disease was widely prevalent in central, southern, and eastern China, especially along the Yangzhi River, and caused serious losses every year. Since 1990, the disease has been controlled, basically because of extensive vaccination, and has not occurred in epidemic proportions in southern China, although it is sporadic in some farms.

Red spot disease (RSD), a most serious disease, was discovered in Vietnam in 1996, causing significant economic losses. RSD occurred in March-April and October-November at 20-25°C (Fig. 10). Later, researchers found that RSD is not an appropriate name for the disease, but a description of one clinical sign. There might be two diseases: one similar to the hemorrhagic disease of grass carp in China and a second caused by bacteria. It was believed that grass carp with the virus were introduced from China to Vietnam.

It is possible that the geographic range of GCRV is wider than known. In some regions, the



Fig. 10. Red spot disease (RSD) in grass carp in Vietnam.

water temperature is too low for infected fish to show clinical signs and these fish may be carriers. Transnational fish trades cross boundaries without quarantine. Such activity can provide an opportunity to spread pathogens from one country to another and should be seriously considered.

References

- Chen Y.X. and Y. Jiang**, 1984. Morphological and physico-chemical characterization of the hemorrhagic virus of grass carp. *Chinese Sci. Bull.*, 29:832-835.
- Chen Y.X., Gao H.J., Jiang Y. and Y.X. Wang**, 1985. A study of pathogenesis of hemorrhage of grass carp and its immunization with inactivated vaccine. pp. 332-342. In: *Proc. Asian Symp. Freshwater Fish Culture*. Beijing, China.
- Deng C.X., Yang X.Q. and H.X. Chen**, 1985. Susceptibility to grass carp reovirus (GCRV) of several fish cell lines. *Acta Hydrobiol. Sin.*, 9: 351-358.
- Guo Q.L. and Y. Jiang**, 1993. Histopathological studies of the hemorrhage disease of grass carp infected by two types of grass carp reovirus. *Trans. Res. Fish Dis.*, 1:7-11.
- Jiang Y.**, 1995. Advances in fish virology research in China. pp. 211-226. In: M. Shariff, J.R. Arthur, R.P. Subasinghe (eds.). *2nd Symp. Diseases in Asian Aquaculture*. Phuket, Thailand.
- Jiang Y. and W. Ahne**, 1989. Some properties of the etiological agent of the hemorrhagic disease of grass carp and black carp. pp. 227-240. In: W. Ahne, E. Kurstak (eds.). *Viruses of Lower Vertebrates*. Springer-Verlag Publ., Berlin, New York.
- Jiang Y. and Y.X. Chen**, 1983. Purification and characterization of hemorrhage virus of grass carp. p. 21. In: *Abstract Chinese Symp. Diseases of Grass Carp*. Wuhan, China.
- Jiang Y. and Z.Q. Li**, 1995. Comparison of the characteristics of four aquareoviruses. *Trans. Res. Fish Dis.*, 2:3-8.
- Jiang Y., Li Y. and P. Yu**, 1991. A preliminary study on immune response of grass carp. *Acta Hydrobiol. Sin.*, 15(4):321-326.
- Li J., Wang T.H., Yi Y.G., Liu H.Q., Lu R.H. and H.X. Chen**, 1997. A detection method for grass carp hemorrhagic virus (GCHV) based on a reverse transcription-polymerase chain reaction. *Dis. Aquat. Org.*, 29:7-12.
- Section of Virus Study, 3rd Laboratory, Institute of Hydrobiology, Academia Sinica**, 1978.

Studies on the causative agent of hemorrhage of the grass carp (*Ctenopharyngodon idellus*). *Acta Hydrobiol. Sin.*, 6:321-330.

Section of Virus Study, 3rd Laboratory, Institute of Hydrobiology, Academia Sinica, 1980. Studies on the causative agent of hemorrhage of the grass carp (*Ctenopharyngodon idellus*): electron microscopic observation. *Acta Hydrobiol. Sin.*, 7: 75-79.

Yang X.L. and W.G. Zuo, 1990. Inactivated vaccine for hemorrhage of grass carp: storage of vaccine. *Freshwater Fish.*, 4:1-3.

Yang X.L. and W.G. Zuo, 1994. Inactivated vaccine for hemorrhage of grass carp (*Ctenopharyngodon idellus*): the stability of vaccine and influence of adjuvants and booster on immune response. *Acta Hydrobiol. Sin.*, 18(3):222-226.

Yang X.L., Xia C. and W.G. Zuo, 1989. Inactivated vaccine for hemorrhage of grass carp: comparison of immunogenicity and immunizing dose between two strains. *J. Fish. China*, 13:138-144.

Ye X.P., Yang G.Z., Luo Y.Z., Chen Y.L. and Z.H. Chen, 1992. Large-scale culture of grass carp cell and virus by using bioreactor. *J. Fish. China*, 16:1-6.

Zhang N.C., Cao J., Wu L.D., Yin W.L. and J.J. Shi, 1990. Supplementary effect of henbane on the bathing immunization of summer fingerlings of grass carp. *J. Zhejiang College Fish.*, 9:79-84.