

The pathogenicity and biological features of Santee-Cooper Ranaviruses isolated from Chinese perch and snakehead fish

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ABSTRACT

Ranavirus has become a noticeable threat to both farmed and natural populations of fish and amphibians. Herein, we reported that 3 strains of novel viruses, designated as ScRIV-GM-20150902, CmRIV-XT-20150917 and ScRIV-ZS-20151201, were isolated from diseased Chinese perch and snakehead fish in China. Efficient propagation of these isolates were determined in Chinese perch brain (CPB) cell line by the means of cytopathic effect observation, PCR amplification and electron microscopy observation. And their viral titers in CPB cells reached $10^{8.13}$ TCID₅₀ ml⁻¹, $10^{7.71}$ TCID₅₀ ml⁻¹ and $10^{7.94}$ TCID₅₀ ml⁻¹, respectively. While the challenge experiment results showed that 3 isolates resulted in 100% mortality of Chinese perch after virus infection. Electron microscopy analysis showed that two kinds of viral inclusion bodies (intracytoplasmic and intranuclear inclusion body) were observed in infected CPB cells. Sequence alignment and phylogenetic analysis of major capsid protein gene sequences of isolates revealed that these isolates belonged to the species Santee-Cooper Ranavirus.

1. Introduction

China is the biggest aquaculture country in the world and aquaculture production grew dramatically in recent decades [1]. With intensive farming operations developing, the more and more infectious diseases were emerging. Among them, Iridovirus has become a noticeable threat to Chinese aquaculture because they infected invertebrates and poikilothermic vertebrates [2]. Iridoviruses are large cytoplasmic double-stranded DNA viruses and divided into five genera: *Iridovirus*, *Lymphocystivirus*, *Chloriridovirus*, *Ranavirus* and *Megalocytivirus*. Ranaviruses caused severe systemic disease and infected fish, amphibians and reptiles [3]. The genus *Ranavirus* comprises seven virus species, including frog virus 3 (FV-3), *Epizootic haematopoietic necrosis virus* (EHNV), *Bohle irido virus* (BIV), *Ambystoma tigrinum virus* (ATV), *European catfish virus* (ECV), *Santee-Cooper Ranavirus* (SCRV) and *Singapore grouper iridovirus* (SGIV) (ICTV 2016).

The first case of largemouth bass infected with *Santee-Cooper Ranavirus* was found in Santee-Cooper Reservoir, South Carolina, during 1995 [4]. Now three viruses have been combined under the *Santee-Cooper Ranavirus*, including largemouth bass virus (LMBV), doctor fish virus (DFV) and guppy virus 6 (GV-6) [2]. In 2008, LMBV was detected and isolated from cultured largemouth bass with ulcerative syndrome disease in China [5].

In this study, we detected and recovered three Ranavirus isolates from diseased Chinese perch *Siniperca chuatsi* and snakehead fish *Channa maculata* in Guangdong provinces, China. The virus characterization and pathogenicity were determined. Comparison of the nucleoid sequence of major capsid protein (MCP) indicated that the three isolates were closely related to *Santee-Cooper Ranavirus*.

2. Materials and methods

2.1. Cells

The Chinese perch brain cell line (CPB) was established from the brain tissues of Chinese perch in our laboratory [6]. CPB cells were cultured in Leibovitz's L-15 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) at 28 °C.

2.2. Virus isolation

Diseased Chinese perch and snakehead fish were collected from fish farms in Guangdong, China. Each fish was dissected to allow observation of pathological changes in the viscera. The tissues of liver, spleen and kidney were thawed and homogenized with 10 folds volume of sterile PBS (pH 7.4). The suspension was centrifuged at $7500 \times g$ for

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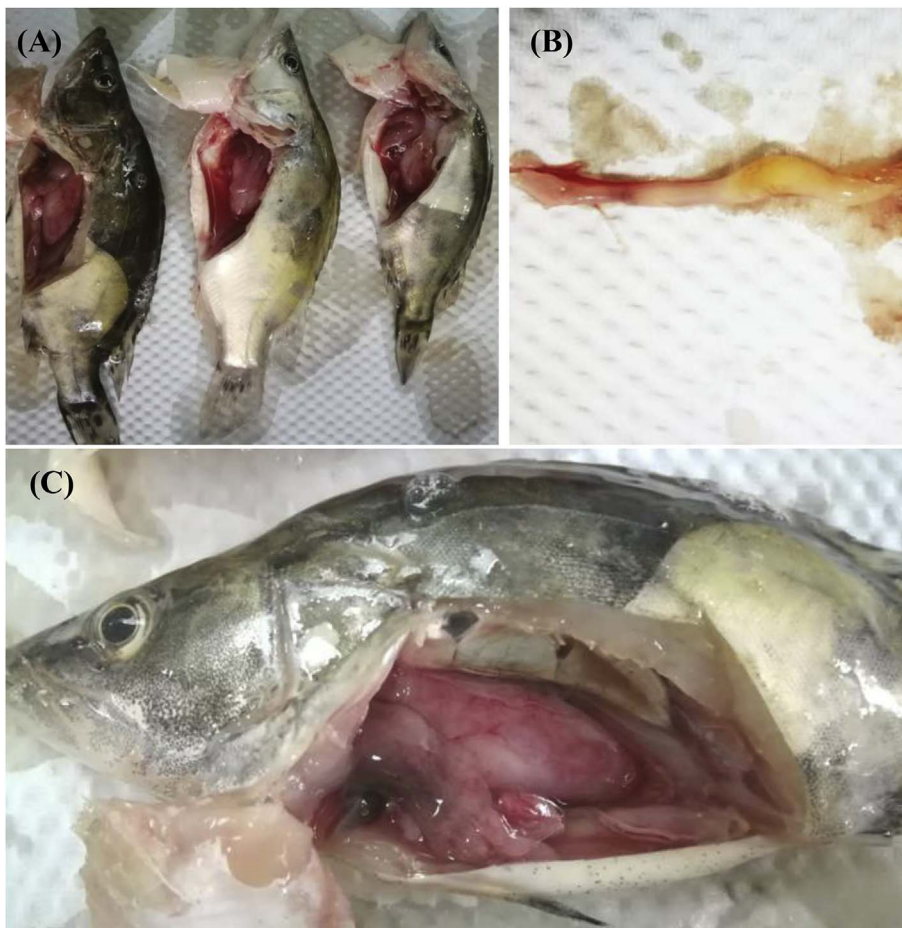


Fig. 1. Clinical signs of Chinese perch infected with CmRIV and ScRIV. Diseased fish had no specific external signs, but internally had ascites (A, C), mesentery hemorrhages (A, C), as well as pus in the intestine (B).

20 min at 4 °C and then filtered through 0.22 µm filter membranes. The 1000-fold diluted virus filtrate was added to a monolayer CPB cells and incubated for 60 min at 28 °C. After absorption, unattached viruses were removed and 5 ml maintaining L-15 medium (containing 2% FBS, 100 IU ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin and 0.25 µg ml⁻¹ amphotericin B) were added to flasks until the cytopathic effect (CPE) was observed.

2.3. Viral titration determination

Until the CPE was stable after serial passage, viral titers were determined. When CPB cells grew in monolayers in 96-well plate, the medium was discarded, and serial 10-fold dilutions (10⁻¹ to 10⁻¹¹) of viral suspension in L-15 medium with 2% FBS were inoculated into cell monolayers and incubated at 28 °C for 1 h. Each dilution was repeated in eight wells. After 10 days of incubation, the CPE was observed and the viral titer was determined using the 50% tissue culture infective dose (TCID₅₀) method (method based on Reed and Muench, 1938) [7].

2.4. Transmission electron microscopy observation

The infected CPB cells at 48 h post incubation were collected and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 h at 4 °C and then post-fixed in 0.1 M phosphate buffer containing 1% osmium tetroxide for 1 h. Ultrathin sections were stained with uranyl acetate-lead citrate and examined by a Philips CM10 electron microscope (Eindhoven, Netherlands).

2.5. Challenge experiments

Healthy Chinese perch with an average body weight of 100 g were used for the challenge test. Each group consisted of 10 individuals in a 50-L glass tank with an air-pumped circulating water system and water temperature was kept 28 ± 0.5 °C. Cells infected with CmRIV-XT-20150917 or ScRIV-GM-20150902 or ScRIV-ZS-20151201 were collected and centrifuged at 4500 × g for 10 min at 4 °C, and filtered (0.22 µm membranes). For the challenge experiments, viral suspensions at different dose were injected intraperitoneally. Sterile PBS was used as control. Fish were observed daily. Dead fish were collected and their liver, spleen and kidney tissues were obtained for PCR analysis. The PCR reactions were performed as part 1.6.

2.6. MCP gene sequencing and phylogenetic analysis

Total genomic DNA was extracted from infected spleen with E.Z.N.A.™ Tissue DNA Kit (OMEGA, USA) according to the manufacturer's instructions and stored at -20 °C until used. The PCR primer sets, Rana-F (5'- TATGTGCTCAACTCTTG GCTGGTC -3') and Rana-R (5'- CCACGATGGGCTTGACTTCTCC -3'), were designed and used for PCR detection basing on the *Santee-Cooper Ranavirus MCP* gene sequence. PCR amplification was carried out in a 25 µl reaction mix containing 12.5 µl of 2 × Taq PCR Master Mix (TIANGEN, China), 0.5 µl of each primer (20 µM each), and 2 µl of the extracted genomic DNA. The optimum conditions for PCR were as follows: 94 °C for 3 min, 35 cycles at 94 °C for 30 s, 58 °C for 45 s and 72 °C for 30 s, and a final elongation at 72 °C for 10 min. The PCR products were analyzed in 1% agarose and sequenced by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China). The DNA sequences

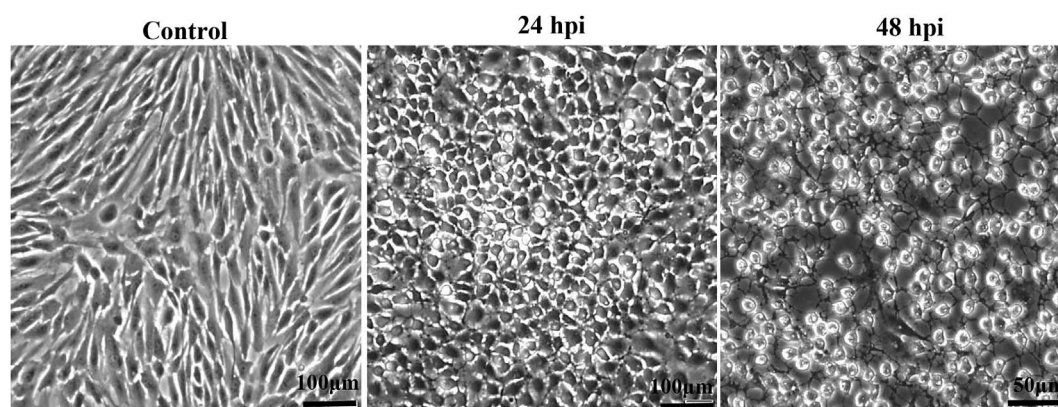


Fig. 2. Cytopathic effect (CPE) induced by the CmRIV-XT-20150917 isolate in CPB cell monolayer.

were aligned using CLUSTAL X 1.81 and the final phylogenetic tree was drawn with the MEGA 6.0 program using maximum likelihood algorithm. The bootstrap values were determined from 1000 replicates of the original data.

3. Result

3.1. Clinical signs of diseased fish

In 2015, Ranavirus diseases were emerging in Chinese perch and snakehead fish farm in Guangdong province, China. These diseased fish had no specific external signs. Internally, diseased fish had ascites, mesentery hemorrhages, as well as pus in the intestine (Fig. 1).

3.2. Virus isolation and titers

The liver, kidney and spleen of diseased fish were sampled and tissue filtrates diluted 10^4 times were inoculated onto the CPB cell monolayer and cultured with L-15 medium containing 2% FBS at 28 °C. As shown in Fig. 2, initial CPE consisted of numerous pyknotic cells at 24 h post-inoculation (hpi). Then pyknotic cells became rounding and intercellular filaments at 48 hpi. Finally, all of cells were detached at 96 hpi. The viral titers were measured on the basis of TCID₅₀ ml⁻¹ described by Reed and Muench (1938) [7]. The results showed that viral titers of ScRIV-GM-20150902, CmRIV-XT-20150917, and ScRIV-ZS-20151201 were $10^{8.13}$ TCID₅₀ ml⁻¹, $10^{7.71}$ TCID₅₀ ml⁻¹ and $10^{7.94}$ TCID₅₀ ml⁻¹, respectively (Table 1).

3.3. Pathogenicity of the virus

The pathogenicity of ScRIV-GM-20150902, CmRIV-XT-20150917 and ScRIV-ZS-20151201 to Chinese perch was determined by challenge experiments. Challenged fish died as early as 5 days post-infection. When the dose of intraperitoneal injection was higher than $10^{4.71}$ TCID₅₀ per fish, the cumulative mortality was 100%. In contrast, control fish exhibited no mortality and no clinical signs of infection. The detailed data are shown in Table 2. Ranaviruses were detected in tissues of dead fish by PCR, but no amplification in control fish.

Table 1
Different isolates used in this study.

Virus taxonomy	Isolates	Titers (TCID ₅₀ ml ⁻¹)	Origin	Isolation region	Isolation year
Ranavirus	ScRIV-GM-20150902	$10^{8.13}$	Chinese perch	Gaoming, Guangdong, China	2015
	CmRIV-XT-20150917	$10^{7.71}$	Snakehead fish	Shunde, Guangdong, China	2015
	ScRIV-ZS-20151201	$10^{7.94}$	Chinese perch	Zhongshan, Guangdong, China	2015

Table 2
The mortality of chinese perch infected with the 3 ranavirus.

Virus	Titers (TCID ₅₀ ml ⁻¹)	Injection volume (ml)	Deaths	Mortality (%)
ScRIV-GM-20150902	$10^{5.13}$	0.1	10/10	100
CmRIV-XT-20150917	$10^{4.71}$	0.1	10/10	100
ScRIV-ZS- 20151201	$10^{4.94}$	0.1	10/10	100

3.4. Transmission electron microscopy analysis of viruses

Electron microscopy analysis showed that large amounts of intracellular viral particles in the infected CPB cells (Fig. 3). The infected cells appeared the nucleus displaced to the periphery of the cell, karyopyknosis, and marginated chromatin (Fig. 3B). Two kinds of viral inclusion bodies were observed, including the intracytoplasmic inclusion body (Cib) and intranuclear inclusion body (Nib) (Fig. 3B and C). Numerous viral cores were observed in Nib, and numerous nucleocapsids or capsids in Cib. Nib could export from nucleus to cytoplasm and arrive at Cib (Fig. 3D, E, F). Lysosomes were observed in infected cells (Fig. 3G). Virus endocytosis and virus budding were also observed (Fig. 3H and I).

3.5. Phylogenetic analysis

Specific DNA fragments of the MCP were successfully amplified by PCR from DNA extracted from the EPC cells infected with ScRIV-GM-20150902, CmRIV-XT-20150917 and ScRIV-ZS-20151201, and the negative controls had no PCR products (Fig. 4A). The PCR products of MCP were purified and sequenced. To determine the taxonomy of the virus, the nucleoid sequences of MCP were aligned with published sequences of other Iridoviruses. The phylogenetic tree showed that three isolates in this study formed one monophyletic lineage with LMBV, DFV and GV-6, indicating that these three viruses belonged to the species *Santee-Cooper Ranavirus*, genus *Ranavirus* (Fig. 4B).

4. Discussion

In 2015, we detected and isolated three Ranavirus isolates from

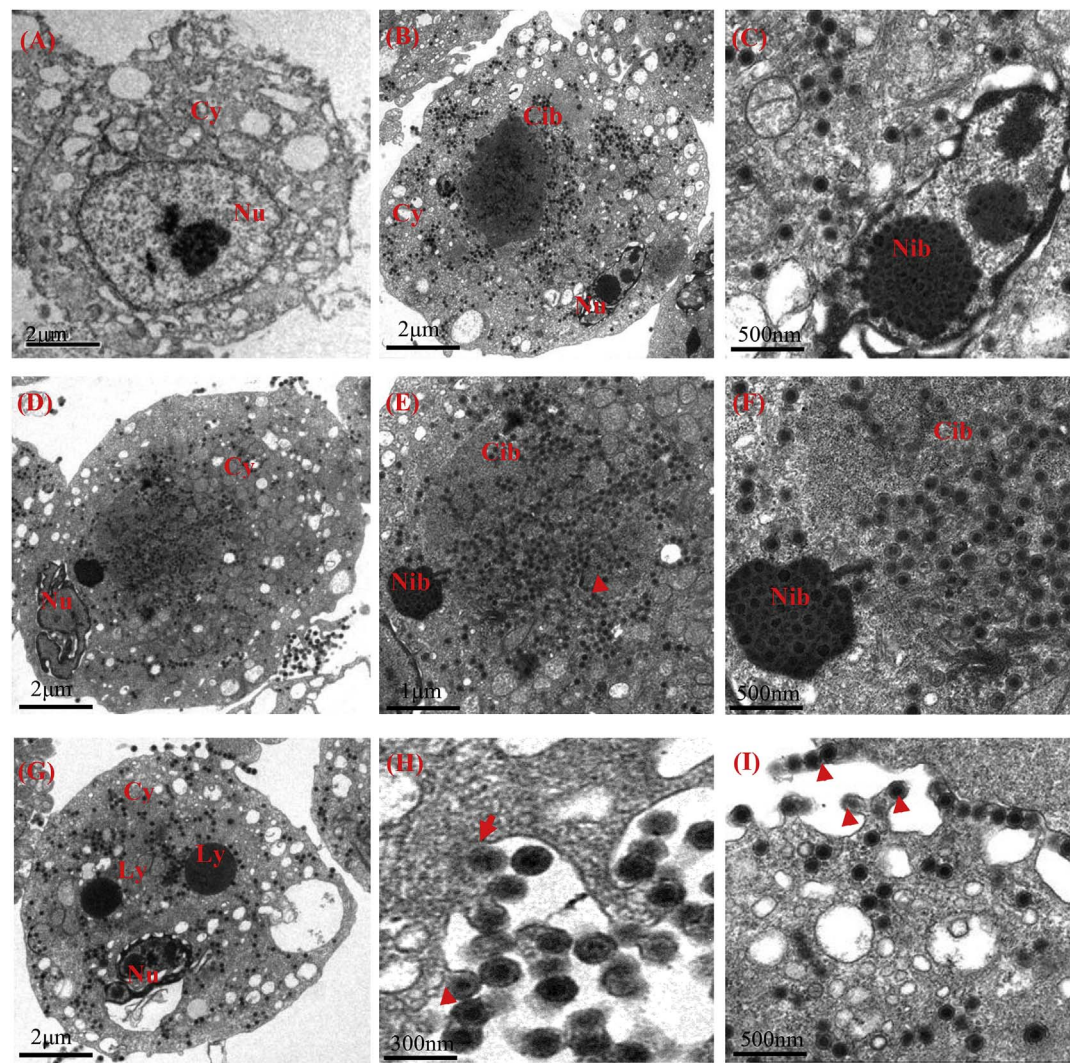


Fig. 3. Ultrathin section micrographs of CPB cells (A) or CPB cells infected with CmRIV-XT-20150917 or ScRIV-GM-20150902 (B–I). Nu: cell nucleus; Cy: cell cytoplasm; L: lysosomes. Cib: intracytoplasmic inclusion body; Nib: intranuclear inclusion body; Arrow: virus endocytosis; Arrow head: virus budding.

diseased Chinese perch and snakehead fish in Guangdong provinces, China. These diseased fish had no specific external signs, which was similar with the Plumb's reports but different to Deng's report [5,8]. While internally diseased fish appeared ascites, mesentery

hemorrhages, as well as pus in the intestine, which was different to the largemouth bass reported previously [5,8]. The different clinical symptom may be related to the different fish species.

The MCP gene is one of the most important gene for analysis of

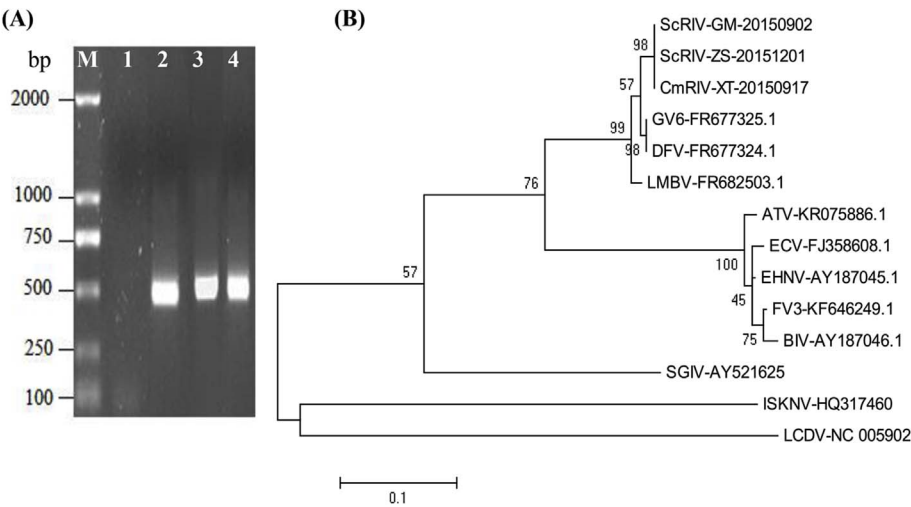


Fig. 4. Ranavirus determination of different virus isolates. (A) PCR amplification of 3 ranaviruses. M: DNA marker; 1: negative control; 2–3: ScRIV-GM-20150902, CmRIV-XT-20150917, ScRIV-ZS-20151201 (B) Molecular phylogenetic tree of the genetic relationship among different iridoviruse strains based on MCP gene sequence. Bootstrap values at 1000 times construction are shown at major nodes in the tree. The scale bar is for a genetic distance marker (number of replacement nucleotides per site).

genetic relationships among iridoviruses, because it is relatively conserved among viruses belonging to the family *Iridoviridae* [9]. To determine the taxonomy of the three viruses, the sequences of MCP genes were aligned with published sequences of other Iridoviruses. The result verified that three isolates, ScRIV-GM-20150902, CmRIV-XT-20150917 and ScRIV-ZS-20151201, belonged to the species *Santee-Cooper Ranavirus*, genus *Ranavirus*. Deng et al. [5] also reported that the virus isolated from largemouth bass in China was identical to doctor fish virus (DFV) and closely related to largemouth bass virus (LMBV), which are the represented species *Santee-Cooper Ranavirus*. Thus we speculated the Ranavirus isolates from different fish in Guangdong maybe belong to the same species. Recent two years we surveilled the Ranavirus in the Chinese perch, largemouth bass, snakehead fish and marbled sand goby cultured in Guangdong province, and we found that Ranaviruses disease had epidemic trend (data not shown).

Electron microscopy analysis showed that the infected cells appeared the nucleus displaced to the periphery of the cell, karyopyknosis and marginated chromatin, which was similar with the previous reports [5,10]. Two kinds of virus inclusion bodies including Cib and Nib were observed, and interestingly we found that Nib could export from nucleus to cytoplasm and arrive at Cib. We speculated that Nib transportation was used for viral particles assemble. Lysosomes observed in infected cells may help virus replicate or digest viral particles through autolysosome [11]. Goorha et al. reported that the nucleus was a site of Frog Virus 3 DNA and RNA synthesis [12]. However, in the previous studies on different *Ranaviruses* including LMBV, FV3, EHN, BIV and SGIV Nib was not observed [3,5,13–16]. Thus it is necessary to further investigate the functions of two kinds of inclusion bodies in the future. According to above results, we speculated the CmRIV or ScRIV multiplication process as following: (1) the virus infection was mediated by adsorptive endocytosis, (2) DNA and mRNA were synthesized in the nucleus, then with certain proteins organized the dense viral core in the Nib, and the icosahedrally symmetric capsids were assembled in the Cib, (3) Nib penetrated the nuclear envelope and transported near the Cib, viral cores were released into Cib and nucleocapsids were assembled, (4) at last mature viruses were released by budding.

In conclusion, three novel Ranaviruses belonged to the species *Santee-Cooper Ranavirus* were isolated in this study and they propagated efficiently in Chinese perch brain cell line. All of three isolates were pathogenic to Chinese perch and mortalities were 100% post virus infection. Interestingly, two kinds of viral inclusion bodies were observed, and their function would be investigated in the future.

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