

Identification and Characterization of a Nervous Necrosis Virus (NNV) Isolated from Tilapia (*Oreochromis niloticus*) in China

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Short Report

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Abstract

Nervous necrosis virus (NNV) is a viral pathogen that infected variety of cultured and farmed fish around the world. In this study, we isolated a novel NNV from diseased cultured tilapia (*Oreochromis niloticus*). The typical vacuolates were discovered in the brain of diseased tilapia through histological analysis. The virus was identified as NNV with RT-PCR tested of brain tissues and named as ONNNV-ZS. In addition, the virus was purified by incubating GS cells with tissue suspension of brain from diseased *O. niloticus*. The obvious CPE (cytopathic effect) was observed in infected cells and the virus was confirmed by RT-PCR. Electron microscopy of the infected cells revealed the virus particles with non-enveloped spherical shape. The pathogenicity of ONNNV-ZS was explored in challenge experiments using *O. niloticus* juveniles. The cumulative mortalities reached 100% at day 10 post-infection. Together, this is the first report for isolation and identification of a NNV isolate from *O. niloticus* in China.

1. INTRODUCTION

The Nile tilapia (*Oreochromis niloticus*) is a major and crucial economic freshwater fish species in global aquaculture; and its aquaculture and exported abroad have witnessed rapid development in China[1]. In recent years, water quality has declined sharply across many *O. niloticus* farms, while stocking densities continue to rise. Together, these pressures have fueled frequent disease outbreaks.[2]. To date, so many pathogens are identified in cultured or wild *O. niloticus* around the world, including parasite(*Prohemistomum vivax*)[3], bacteria(*Vibrio*, *Aeromonas*, *Pseudomonas* and *Streptococcus spp.*)[4, 5]and virus(Tilapia lake virus (TiLV) and Nervous Necrosis Virus(NNV)[6, 7].

NNV (Nervous Necrosis Virus) was initially documented in parrotfish cultured at the Nagasaki Aquaculture Research Institute in Japan and in Australian lungfish raised at an hatchery in northern Queensland, Australia, classified within the genus Betanodavirus in Nodaviridae[8]. Its genome consists of two positive-sense single-stranded RNA segments, and the virion encapsidates a single coat protein (CP) subunit.[9]. To date, more than 120 fish species have found infected by NVV around the world and serious threat to aquaculture industry. According to nucleotide sequences of CP gene, NNV is classified into four genotypes:SJNNV (Striped jack nervous necrosis virus),TPNNV (Tiger puffer nervous necrosis virus),BFNNV (Berfin flounder nervous necrosis virus), JFNNV (Japanese flounder nervous necrosis virus), and RGNNV (Red-spotted grouper nervous necrosis virus)[10]. Although NNV infections have been widely reported in marine fish, evidence from freshwater systems is still limited. This study describes the identification of an NNV strain (ONNNV-ZS) from diseased farmed *Nile tilapia* (*O. niloticus*). In addition to isolation, we examined the biological properties and virulence of ONNNV-ZS. These results contribute to a better understanding of how NNV interacts with *O. niloticus* and may inform future research directions.

2. MATERIALS AND METHODS

2.1 Fish and cell line

Fish showing not swim or move around alone were collected from a tilapia farm in Zhongshan, Guangdong Province. As a control group, apparently healthy *O. niloticus* from the same farm were also sampled, after testing negative for NNV. Brain tissue from both groups was then subjected to histopathological analysis and RT-PCR test.

Prof. Qin kindly provided the grouper spleen (GS) cell line used in this study. Cells were cultured at 25°C in L15 medium containing 10% fetal bovine serum (FBS, Excell), 1% streptomycin (100 µg/mL), and 1% penicillin (100 µg/mL).

2.2 RNA extraction and RT-PCR detection

Total RNA was extracted and cDNA was reverse-transcribed according to the kit instruction manual (Trans, China). A pair of primers targeting NNV was designed with reference to an earlier study[11]. The forward primer (5'-CGT GTC AGT CAT GTG TCG CT-3') and the reverse primer (5'-CGA GTC AAC ACG GGT GAA GA-3') were chemically designed for use in RT-PCR detection. And the PCR was performed using the following steps: 97°C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 30 s, followed by a final extenuation step at 72°C for 10 min. PCR products were cloned into pSWE-Topo Zero Vector (G3022, Servicebio) and transformed into competent DH5a and sequenced.

2.3 Sequence analysis

Nucleotide sequences were compared with reference sequences deposited in GenBank using the BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>). To clarify the genetic relationship among the isolates, we downloaded partial CP gene sequences of NNV from NCBI and aligned them using MEGA 6.0. The resulting phylogenetic tree was constructed on the basis of these aligned sequences.

2.3 Virus isolation and titer determination

According previous research that GS cell is commonly used to detected NNV infection[12]. At first, the GS cells (1×10^4 per well) were grow in a 96-well plates at 25°C for 18 h. Then, the brain of diseased fish was frozen by liquid nitrogen and grinded. Afterwards, tissue suspension was filtered by a 0.22 µm sterile filter and incubated GS cells with 2 hours. After the incubation period, the spent medium was carefully aspirated from each well, and an equal volume of fresh L15 medium was added back. Observing the GS cells under the light microscopy every 12 hours to check for cytopathic effect (CPE). The GS cells, which incubated diseased fish brain homogenate, exhibiting CPE lesions were subjected to three cycles of freeze-thaw cycles in an -80°C freezer, followed by collection of the supernatant and re-infection of the cells. After two generations of blind infection, the CPE was also appeared. Subsequently, RT-PCR was employed for further confirmation. Following sequencing, it was confirmed that the NNV virus had been successfully isolated.

To further characterize the infection, GS cells were collected at 48 h post-inoculation. A portion of the infected cells was prepared for ultrastructural observation by transmission electron microscopy (TEM), whereas the remaining cells were subjected to viral titration. The titer was calculated and expressed as

median tissue culture infective doses (TCID₅₀) per milliliter, using the method described by Reed and Muench.[12]. In short, the supernatant and GS cells were collected at 48h post infection. GS cells were inoculated in a 96-plater with the 10 times ratio diluted NNV supernatant for 7 days. At designated time points post-infection, the wells in each plate were examined under an inverted microscope.

2.4 Challenge experiment

To verify the virulence and pathogenicity of ONNNV-ZS, the juvenile *O. niloticus* were infected via intraperitoneal. A total of 180 juvenile *O. niloticus* (6-8g) were randomly divided into two groups, such as control groups and experimental group. To evaluate pathogenicity, fish assigned to the experimental group were challenged by intraperitoneal injection with 100 μ L of a viral suspension containing ONNNV-ZS at a dose of $10^{3.12}$ TCID₅₀/mL. Fish in the control group were mock-inoculated with an equal volume of sterile L15 medium. Throughout the experimental period, all fish were examined twice per day and the number of mortalities was documented on each observation day. Brains were harvested from fish that died during the experiment. The collected tissues were then processed for RT-PCR detection to verify the presence of ONNNV-ZS.

3. RESULTS AND DISCUSSION

As a serious pathogen, NNV affects both cultured and wild fish populations. Its widespread impact has become a considerable constraint on the sustainable growth of global aquaculture.[13]. The occurrence of NNV in freshwater aquaculture systems is far less well characterized than in marine settings. To explore this knowledge gap, we focused on the brain as the organ most consistently associated with NNV pathogenesis. Brain tissue was aseptically harvested from *O. niloticus* specimens exhibiting disease signs and processed for parallel analysis by histopathology and molecular detection. Histological sections stained with hematoxylin and eosin revealed extensive vacuolar degeneration within the brain parenchyma—a pathological feature typical of betanodavirus infection. Complementing these findings, RT-PCR amplification generated a fragment of roughly 420 bp, which was resolved on a 1.5% agarose gel, excised, and sequenced to confirm its identity.(Fig. 1B). Nucleotide BLAST analysis against the GenBank database revealed that the PCR fragment shared 99.00% nucleotide identity with an SGNNV strain (AB373029.1). Phylogenetic relationships were evaluated by constructing a neighbor-joining tree with 1000 bootstrap replicates, using aligned partial CP sequences. The newly isolated strain grouped consistently with SGNNV reference sequences and was therefore named ONNNV-ZS (Fig. 2). Notably, this is the first report to confirm NNV infection in farmed *O. niloticus* in China.

It has been documented that the grouper spleen (GS) cell line supports efficient NNV replication [14]. Leveraging this susceptibility, we used GS cells as the isolation host. Cells were plated at a density of 1×10^5 per well in 96-well plates and, after attachment, were inoculated with a 10% (w/v) brain homogenate. The homogenate supernatant was clarified by centrifugation and rendered bacteria-free by passage through a 0.22 μ m pore-size membrane before being added to the cell cultures. After 48 hpi (hours post infected), the CPE was clearly existed under the microscope(Fig. 3A). TEM observation confirmed the presence of ONNNV-ZS virions in infected cells. High-magnification images revealed

abundant non-enveloped particles clustered in the cytoplasm (Fig. 3B). These particles measured 20–30 nm in diameter, a size range that matches the typical dimensions reported for other NNV isolates.[7].

To further investigate the pathogenic potential of ONNNV-ZS, juvenile *O. niloticus* were used for detect the viral virulence strength of isolated NNV and checked with viral isolates by intraperitoneal injection. The fish of challenged group began to die at 2 dpi (day post-injection) and death rate rise to 100% at 10 dpi. But the fish in control group were all survival during the whole challenged experiment(Fig. 4A). Typical disease signs, including dark body coloration and abnormal swimming behavior, were recorded in infected fish, in agreement with the clinical description provided by previous reported [15]. Histological examination of brain sections from challenged fish also revealed pronounced vacuolation (Fig. 4B). To verify that the observed mortality was caused by the isolated strain, we performed RT-PCR on brain tissue collected from challenged fish, which yielded a positive signal (Fig. 4C). Taken together, the evidence obtained here establishes ONNNV-ZS as a serious pathogen of cultured *O. niloticus*. Subsequent studies will be directed at elucidating the pathogenic mechanism and likely source of this virus, with the ultimate goal of preventing NNV epizootics in farmed fish populations.

Declarations

Acknowledgements

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CONFLICT OF INTEREST

There were no conflicts of interest.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

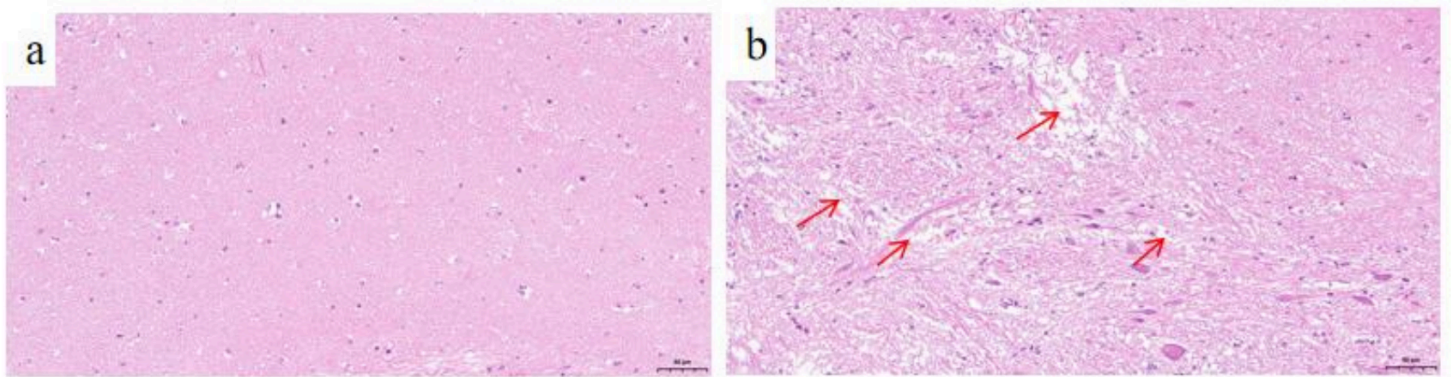
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Figures

A



B

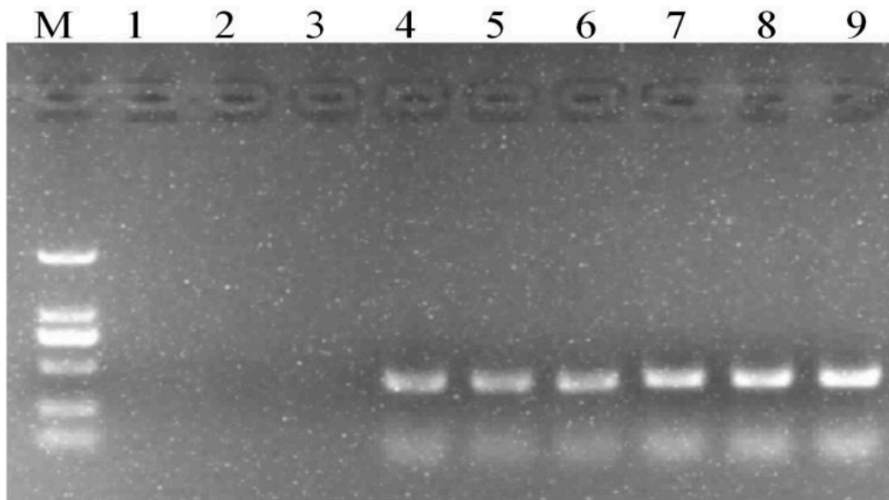


Figure 1

(A) a: the brain of healthy tilapia; b: the brain of diseased tilapia; Note: the red arrow indicated vacuolation. (B) PCR detection of NNV from the brain tissue of diseased tilapia M = DL2000 DNA Marker; Lane 1-3: negative control; Lane 4-6: positive control; Lane 7-9: the brain tissues from three diseased tilapia.

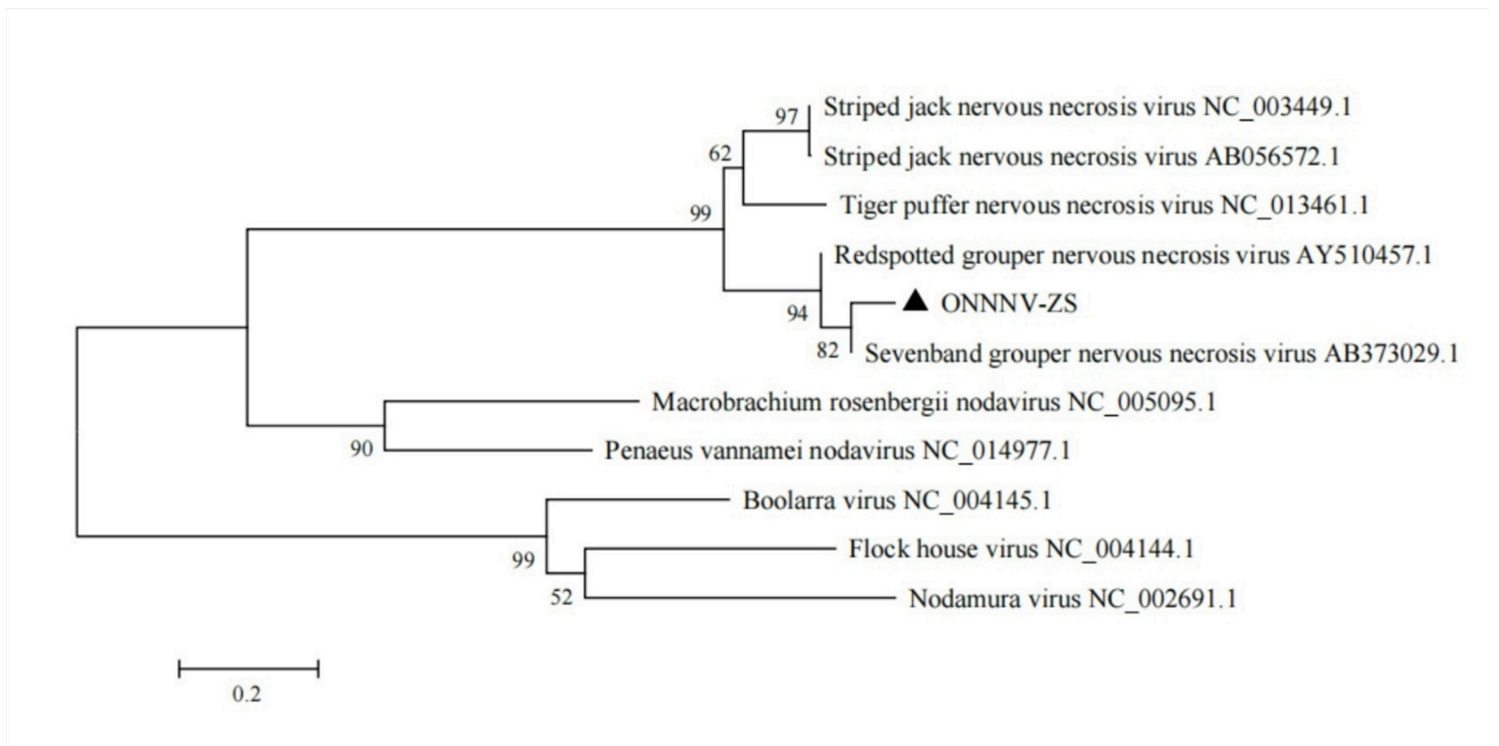
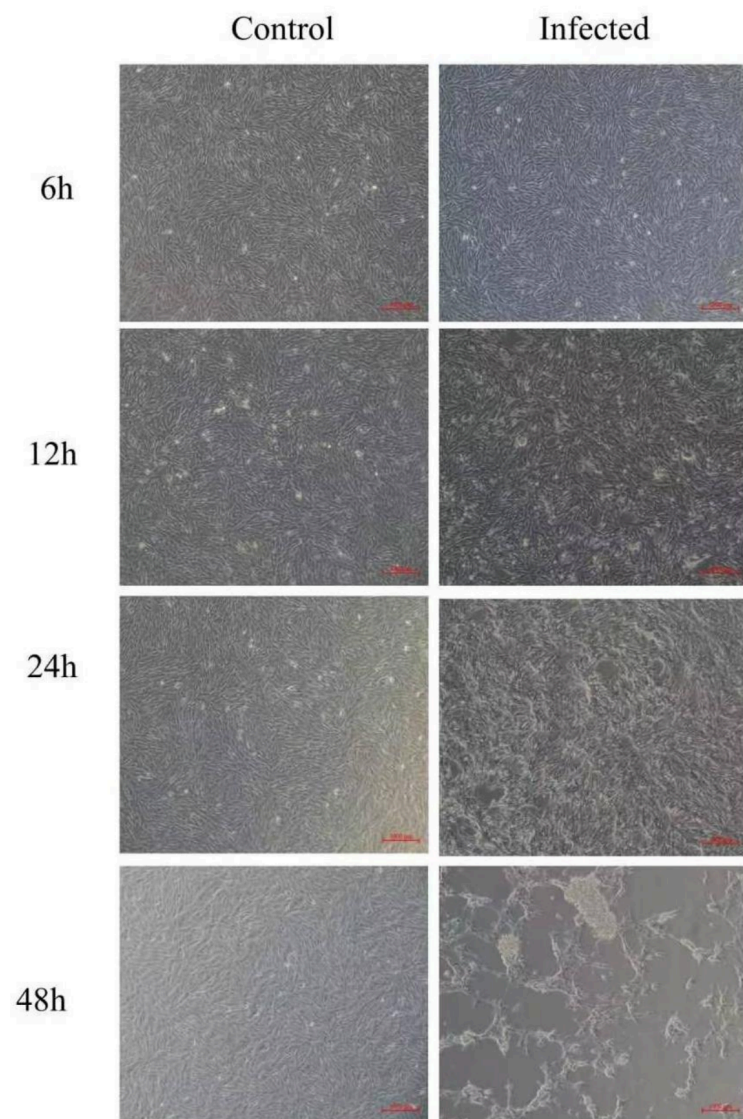


Figure 2

Phylogenetic tree based on partial nucleotide sequences showing the virus isolate was clustered closely with Sevenband grouper nervous necrosis virus AB373029.1.

A



B

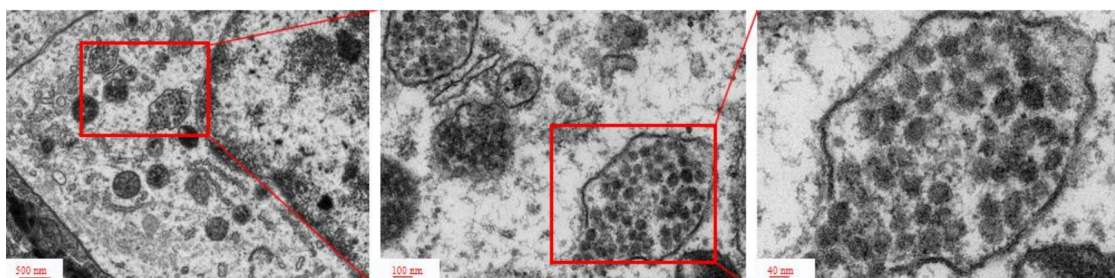
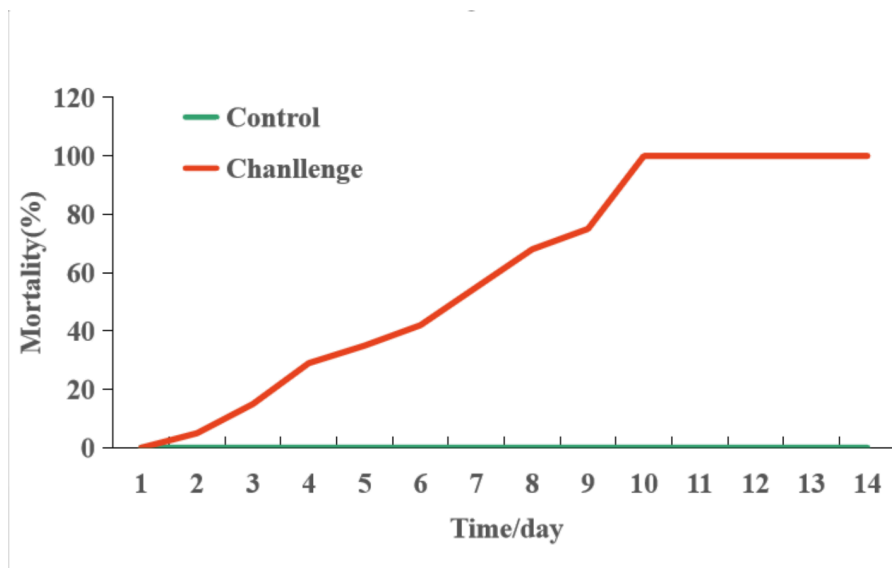
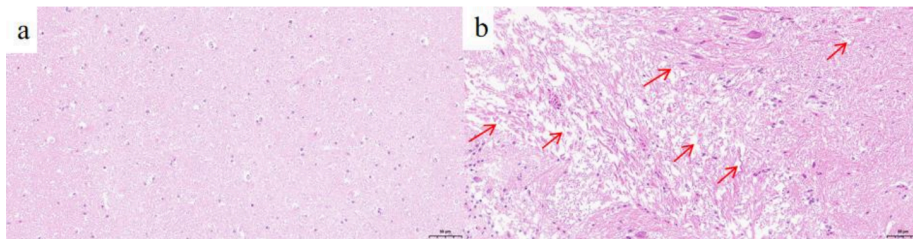
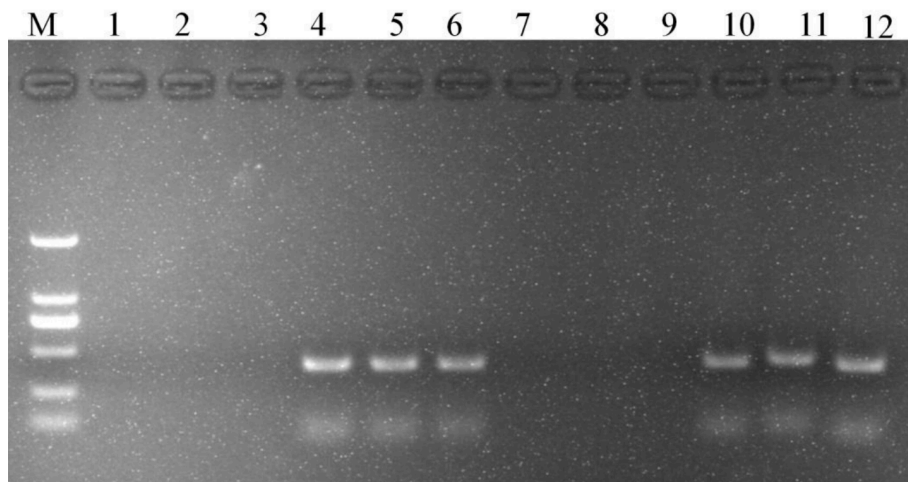


Figure 3

Morphology changes (A) and Electron micrograph in GS cells after infected with ONNV-ZS for 48h (B).

A**B****C****Figure 4**

(A) Cumulative mortality of juveniles *O. niloticus* experimentally infected by ONNV-ZS. The fish were intraperitoneally injected with $1 \times 10^{3.12}$ TCID₅₀/ml ONNV-ZS. The control group was injected with medium. The cumulative mortality was determined from 2 days to 14 days follow infection. (B) a: the brain of control tilapia; b: the brain of infected tilapia; (C) RT-PCR detection of ONNV-ZS in the brain tissues from dead fish after infection. M: DL2000 DNA marker; Lane 1-3: negative control; Lane 4-6:

positive control; Lanes 7-9: the brain tissues from control group; Lane 10-12: the brain tissues from dead fish.