Fish and Shellfish Immunology 95 (2019) 328–335

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Deletion of the Infectious spleen and kidney necrosis virus *ORF069L* reduces virulence to mandarin fish *Siniperca chuatsi*

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ARTICLE INFO

Keywords:
ISKNV
Iridovirus
Virulence gene
Mandarin fish
Vaccine

ABSTRACT

Mandarin fish (*Siniperca chuatsi*) is a significant cultured species with high added value in China. With the expansion of farming, diseases of mandarin fish such as Infectious spleen and kidney necrosis virus (ISKNV) diseases are becoming more and more serious. Human endogenous retrovirus subfamily H long terminal repeat associating protein 2 (HHLA2) is a type 1 transmembrane molecule with three extracellular Ig domains (IgV-IgC-IgV) and plays important roles in the T cell proliferation and tumorigenesis. The HHLA2-homologues have not been found in virus. In this study, a viral HHLA2 protein encoded by ISKNV *ORF069L* was identified and the virulence of the deleted *ORF069L* reconstruction ISKNV strain (Δ*ORF069L*) was investigated. ISKNV *ORF069L* gene was predicted to encode a 222-amino acids peptide. The bioinformation analysis revealed that ISKNV *ORF069L* contained an Ig HHLA2 domain and was homologous to vertebrate B7-CD28 family proteins. The recombinant virus strain of Δ*ORF069L* was constructed by homologous recombination technology. The virus titer and growth curves between ISKNV wild type (WT) and Δ*ORF069L* on cellular level showed no significant differences indicating that the *ORF069L* did not influence the ISKNV replication. The expression levels of immune-related genes (*Mx1, IL-1β, IL-8, TNF-α* and *IgM*) were increased in fish infected with Δ*ORF069L*, compared to those in fish infected with ISKNV WT. Furthermore, the lethality caused by Δ*ORF069L* declined by 40% compared with ISKNV WT, indicating that *ORF069L* was a virulence gene of ISKNV. Most importantly, the protection rate was nearly 100% for fish immunized with Δ*ORF069L* strain. Those results suggested that Δ*ORF069L* could be developed as a potential attenuated vaccine against ISKNV. Our work will be beneficial to promote the development of gene deletion attenuated vaccines for ISKNV disease.

1. Introduction

Mandarin fish (*Siniperca chuatsi*) is a significant cultured species with high added value in China [1]. With the expansion of farming, diseases of mandarin fish such as iridovirus diseases are becoming more and more serious. Iridovirus is regular icosahedral, enveloped double strands DNA macromolecular virus with radius range from 125 to 300 nm. Iridovirus infects insects, fishes, amphibians and reptiles [2]. According to the tenth virus classification report of International Committee on Taxonomy of Viruses, *Iridoviridae* is divided into five genera: *Iridovirus*, *Chloriridovirus*, *Ranavirus*, *Lymphocystivirus* and *Megalocytivirus*. Infectious spleen and kidney necrosis virus (ISKNV) is the type species of *Megalocytivirus* genus [3]. ISKNV disease has caused huge economic losses to the mandarin fish farming industry since the 1990s [4]. Mandarin fish infected with ISKNV is characterized lesions in the spleen, kidney, brain and heart, as well as cells enlargement of spleen and kidney [4]. The mortality rate of infected mandarin fish is nearly 100% in the following 15 days at 28 °C.

Researches on the viral gene functions of ISKNV have made a lot of progresses [5]. ISKNV is the first megalocytivirus to be fully sequenced [6]. The length of ISKNV genome is 111,362 bp with 124 hypothetical open reading frames (ORFs) [7]. These ORFs encode a large number of

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https://doi.org/10.1016/j.fsi.2019.10.039
Received 21 July 2019; Received in revised form 13 October 2019; Accepted 22 October 2019
Available online 23 October 2019
1050-4648/

host range genes and virulence related genes, for example, ISKNV-encoded VP48R protein could cause abnormal phenotype of zebrafish embryos [8]; ISKNV-encoded VP103R protein interacted with JAK1 protein and inhibited its tyrosine kinase activity to escape the IFN-α response [9]; ISKNV-encoded VP111L protein induced apoptosis through the extrinsic pathway [10]; ISKNV-encoded VP119L protein disrupted the integrin linked kinase, parvin, and PINCH proteins, termed the IPP complex, resulting in a pericardial enlargement of the fish, cardiac malformation, sparse cardiac sarcomere, and ventricular muscle [11]; ISKNV-encoded VP124L protein interacted with IKKβ to inhibit the NF-κB signaling pathway [12]. However, several ISKNV virulence factors, for example, ISKNV *ORF069L* that is likely a viral human endogenous retrovirus subfamily H long terminal repeat associating protein 2 (HHLA2), have not been identified.

HHLA2 is a type 1 transmembrane molecule with three extracellular Ig domains (IgV-IgC-IgV) which belongs to the B7 family. As a negative co-stimulatory molecule of T cells, HHLA2 regulates the proliferation of $CD4^+$ and $CD8^+$ T cells by binding to the CD28 family receptors [13,14]. This molecule is widely expressed in antigen-presenting cells and T cells and can also be found in dendritic cells, macrophages, and B cells under the stimulation of inflammatory factors [15]. The specific receptor of HHLA2 is transmembrane and immunoglobulin domain containing (TMIGD2) (also called CD28H). HHLA2 binds to TMIGD2 at the T-cell surface, and then activates the PI3K/AKT signaling pathway and inhibits the expression of cyclin-related proteins and T cell proliferation [16]. These changes are believed to be closely related to the occurrence of some tumors [17]. HHLA2 expressed by tumor cells can promote the occurrence and growth of tumors by interacting with activated T cells and unknown receptors on other immune cells. Under the stimulation of inflammatory factors, HHLA2 in resting state of dendritic cells and macrophages induces T cell apoptosis, which can reduce T cell immune effect in tumor micro-environment [18]. Current studies on HHLA2 mainly focus on the areas related to T cell proliferation and tumorigenesis. HHLA2-homologues have not been found in virus, and the role of viral HHLA2 in virus virulence is unknown.

In this study, ISKNV *ORF069L* encoding a viral HHLA2 protein was identified, and the virulence of the deleted *ORF069L* reconstruction ISKNV strain (Δ*ORF069L*) was investigated in mandarin fish.

2. Materials and methods

2.1. Animal, cells and virus

Healthy mandarin fish (*S. chuatsi*) weighing 150 ± 30 g were provided by farmers from Nanhai district of Foshan, Guangdong province, China. Mandarin fish fry (MFF-1) cells were separated and saved in our laboratory [19]. The ISKNV wild type (WT) strain was separated and obtained by the Nanhai mandarin fish farm in 2017 and stored in our laboratory. ISKNV was grown in MFF-1 cells. Purified virus particles were infected at a ratio of multiplicity of infection (MOI) = 0.1. About 7 days after MFF-1 cells lesions, swelling round and falling off, the cell suspension was collected. The cell suspension became virus suspension as following procedures: being frozen and being thawed for 3 times in turns in a −80 °C refrigerator, and then being sterilized through a 0.22 μm filter.

2.2. Construction of ISKNV ORF069L recombinant transfer vector

The pDsRed-Monomer-N1 fluorescent reporter vector (Takara, Japan) and pLVX-Puro vector (Youbio, China) as reporter gene sources, the PCR was used to generate a 2181 bp fragment including the whole expression frame of red fluorescent protein (*RFP*) and puromycin resistance (*Puro*) genes. This fragment included the original vector's multiple cloning site (MCS) between the promoter and the *RFP* gene. Therefore, we removed the 82 bp fragment of MCS with the overlap PCR method. Then the *Puro* gene was linked with overlap PCR to form a fusion protein. The above fragment was amplified by PCR and then was inserted into MCS of pUC19 expression vector with the restriction enzyme Kpn I and BamH I. Finally, the upper and lower arm segments of ISKNV *ORF069L* were attached to both sides of the reporter gene with the restriction enzyme EcoR I/BamH I and Kpn I/Hind III, forming the ISKNV *ORF069L* recombinant transfer vector, named pUC19-Δ*ORF069L*. The primers used in this section are listed in Table 1.

Table 1
Primers for obtaining target genes.

Primers	Sequence(5′-3′)
RFP-F	ATAGTAATCAATTACGGGGT
RFP-R	TGATGAGTTTGGACAAACCA
RFP-ol-F	CAGATCCGCTAGCGCTCGCCACCATGGACAACACCG
RFP-ol-R	GTTGTCCATGGTGGCGAGCGCTAGCGGATCTGACGG
Puro-ol-F	AAATGTGGTATGGCTGATTATCAGGCACCGGGCTTGCGGG
Puro-ol-R	CCCGCAAGCCCGGTGCCTGATAATCAGCCATACCACATTT
RFP-K-F	GGGGTACCATAGTAATCAATTACGGGGT
RFP-B-R	CGGGATCCTGATGAGTTTGGACAAACCA
ORF069L-upper-F	CGGAATTCGCCATGGCTACAGGCACGTC
ORF069L-upper-R	GGGGTACCCTTCCTGGATATCAAACATT
ORF069L-lower-F	CGGGATCCTTGCAGCGCTCTACAAGCAT
ORF069L-lower-R	CCCAAGCTTACTTCTGTATGTTGTACATG
ORF069L-F	CTGGATATCAAACATTTTCAA
ORF069L-R	TCACAAGTACAAGCCACATCA

2.3. Purification and identification of recombinant virus strain

The MFF-1 cells were inoculated into six-well cell culture plates and then were transfected with the pUC19-Δ*ORF069L* vector by using the FuGENE® HD transfection reagent (Promega, USA). After 24 h post transfection, cells were infected with ISKNV WT strain (MOI = 1) to generate recombinant virus. These above recombinant virus-infected healthy cells and puromycin are used to kill cells not infected by recombinant viruses. Then red fluorescent plaques formed by recombinant viruses were picked out under an inverted fluorescence microscope. This process was repeated five times to obtain the purified recombinant virus strain, named Δ*ORF069L*. Then, the Δ*ORF069L* strain was further confirmed by PCR.

2.4. Determinations of virus titer and growth curve

The virus infection titers and growth curves of the Δ*ORF069L* strain were detected in MFF-1 cells. Cells were infected with ISKNV WT or Δ*ORF069L* strain (MOI = 1). The fluids were removed after 2 h. To determine the virus titer, the infection titration tissue culture infective dose 50% ($TCID_{50}$) was measured using a limiting dilution method. After 7 days, the incidence of each well was observed, and the $TCID_{50}$ value was calculated. For growth curve assay, total DNAs from cells were extracted at 2, 4, 8, 12, 24, 48, 72, and 96 h after infection to measure the numbers of virions by absolute quantitative PCR by using the LightCycler® 480 System (Roche Diagnostics, Switzerland) with the primers for ISKNV major capsid protein (*MCP*) gene. The plasmid with ISKNV *MCP* gene is considered as the standard substance [20]. The copy number of viral genome DNA was determined by the copy number of ISKNV *MCP* gene in genome by using SYBR premix Ex Taq™ (Takara, Japan), as described previously [20].

2.5. The expression levels of immune-related genes induced by ΔORF069L in mandarin fish

To determine the expression levels of immune-related genes after infection with Δ*ORF069L*, mandarin fish were infected with ISKNV WT or Δ*ORF069L*. Blood samples were collected at 0, 3, 6, 9, 12, 15 and 18 d post infection (each group 3 fish per day). RNA samples were isolated using an SV Total RNA Isolation System (Promega, USA) and

Table 2
Primers used for qRT-PCR.

Genes	Primers	Sequence(5′-3′)
MCP	Forward	TTACAGGATAGGGAAGCCTG
	Reverse	ATGTCTGCAATCTAGGTGC
Mx1	Forward	GCTTTGACTTTCCGTTTGTCTTCC
	Reverse	TGAATCCACGCTGAGAACCCA
IL-1β	Forward	CAAGGATGGTCCCGAAGCAA
	Reverse	ACGGAAGGCGACAGACACGA
IL-8	Forward	CAAAGTGGAGATTGTTGTCACCCTAA
	Reverse	GAGGTTTTCTGTTTGATGATGTTAGCC
TNF-a	Forward	TCTCGTTGTGGCCCTTTGTTT
	Reverse	GAGGCTTCCTGCTGTTTGCTTG
IgM	Forward	ACAGAAGGGCTCAACGTGAC
	Reverse	ACAGGTGTATTCATCTCCTC

then reverse transcribed into cDNAs using Takara's ExScript reverse transcription (RT) reagent kit (Takara, Japan) following the manufacturer's protocol. The expression levels of the immune-related genes (*Mx1, IL-1β, IL-8, TNF-a* and *IgM* genes) were determined by real-time quantitative RT-PCR (qRT-PCR) using the corresponding forward and reverse primers (Table 2).

2.6. qRT-PCR

All qRT-PCR reactions were performed in triplicate. PCRs were performed on a total reaction volume of 10 μL containing 0.2 μM primers, 1 μL of cDNA, 5 μL of 2 × SYBR premix ExTaq™, and 3.6 μL of ultrapure water using the following setting: 40 cycles of amplification for 5 s at 95 °C, 40 s at 60 °C, and 1 s at 70 °C. The expression level of each transcript was normalized to the expression of the *β-actin* gene, which was used as an internal housekeeping control. The qRT-PCR data of the target genes were analyzed using the Q-gene statistics add-in, followed by unpaired sample *t*-test. Statistical significance was accepted at $p < 0.05$, and high significance was accepted at $p < 0.01$. All data were expressed as the mean ± standard deviation (SD).

2.7. The virulence and immunoprotection of ΔORF069L

The virulence and immunoprotection experiments were independently conducted twice, using 50 mandarin fishes respectively as the test groups. For virulence assay, in the *ΔORF069L* group, fish were injected with 100 μL of the *ΔORF069L* suspension at 10^5 $TCID_{50}$/mL; in the WT group, fish were injected with 100 μL of the ISKNV WT suspension at 10^5 $TCID_{50}$/mL; in the blank control group, fish were injected with 100 μL of DMEM solution. After 20 days of feeding, the mortality rates of mandarin fish were determined. For immunoprotection assay, above survived fish were re-infected with ISKNV WT suspension at 10-folds concentration. And another 20 days later, protection rates were calculated. After the dead mandarin fish were dissected, the total DNAs from spleen were extracted and detected for the presence of virus to rule out other accidental factors of deaths.

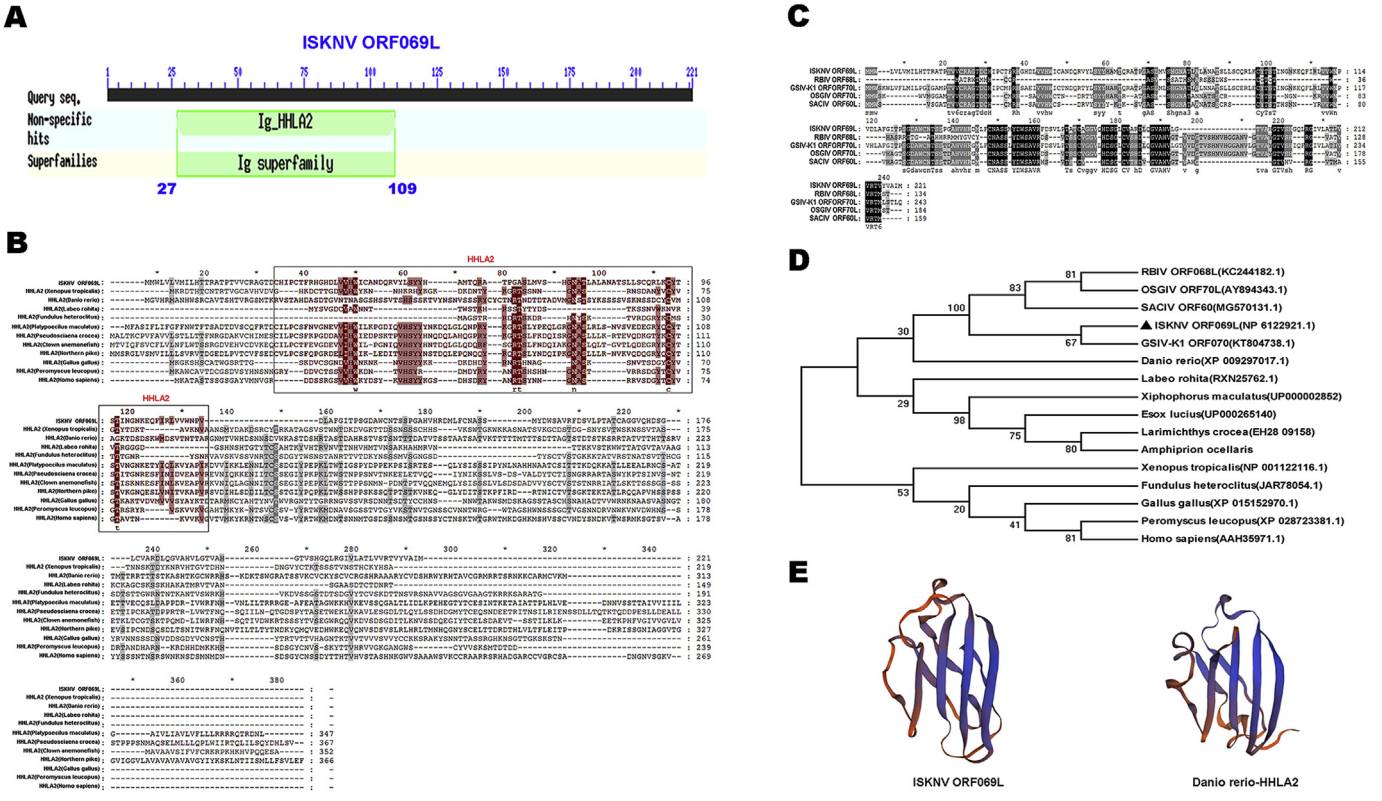

Fig. 1. Bioinformatics analysis of ISKNV *ORF069L*. (A) Domain analysis of ISKNV *ORF069L* by using the BlastP. (B) Amino acid sequences are compared to show the relationship between the ISKNV *ORF069L* and the vertebrate HHLA2 domains. The sequences of HHLA2 proteins are as follows: ISKNV (NP 61229.1), *Xenopus tropicalis* (NP_001122116.1), *Danio rerio* (XP_009297017.1), *Labeo rohita* (RXN25762.1), *Fundulus heteroclitus* (JAR78054.1), *Platypoecilus maculatus* (UP000002852), *Pseudosciaena crocea* (EH28_09158), *Amphiprion ocellaris* (XP_022074839.1), *Northern pike* (UP000265140), *Gallus gallus* (XP_015152970.1), *Peromyscus leucopus* (XP_028723381.1), and *Homo sapiens* (AAH35971.1). (C) Amino acid sequences are compared to show the relationship between the ISKNV *ORF069L* and other viral proteins. The sequences of viral HHLA2 proteins are as follows: ISKNV *ORF ORF069L* (NP 61229.1), RBIV *ORF 068L* (KC244182.1), GSIV-K1 *ORF070L* (KT804738.1), OSGIV *ORF070L* (GenBank: AY894343.1), and SACIV *ORF060L* (MG570131.1). (D) Phylogenetic tree analysis of HHLA2 domains in megalocytivirus and fish. The phylogenetic tree was constructed according to the alignment of amino acid sequences by using the neighbor-joining method in MEGA v6.0 with 1000 bootstrap replications. (E) Results of 3D structure prediction of zebrafish HHLA2 domain and ISKNV *ORF069L*. They have similar size and barrel structures made of β-folds.

2.8. Histopathological section of hematoxylin-eosin (HE) staining

During the experiment, the spleen and opisthonephros from dying or healthy mandarin fish were cut into squares of approximately 3 mm square, fixed with formalin and acetic acid fixative for 24 h, then washed with phosphate-buffered saline twice, and preserved with 70% ethanol. Dehydration was sequentially performed using 70%, 80%, 95%, and anhydrous ethanol. Then, these squares were embedded by paraffin and sectioned. The slices were stained with HE staining and made into permanent slides after they were dried, dewaxed, and hydrated. The slices were then observed under the microscope.

3. Results

3.1. Bioinformatics analysis of ISKNV ORF069L

The coding region of ISKNV *ORF069L* (Gene ID: 935332) is 669 bp in length and was predicted to encode a 222 amino acid peptide (*ORF069R*) [7]. NCBI-Blast Conserved Domain search analysis revealed that ISKNV *ORF069L* contained an HHLA2 domain and was homologous to the vertebrate B7-CD28 family proteins (Fig. 1A). The multiple-sequence alignment analysis showed that ISKNV *ORF069L* was homologous with vertebrate HHLA2 proteins and contained some conserved amino acids sites, such as V48, W50, N95, T97, C113 and T118 of ISKNV *ORF069L* (Fig. 1B). Interestingly, the viral HHLA2 domain-containing proteins were only found in megalocytiviruses, such as orange-spotted grouper iridovirus (OSGIV), turbot reddish body iridovirus, and red sea bream iridovirus (Fig. 1C). The phylogenetic trees results showed that ISKNV ORF069L was clustered to viral proteins and was closed with zebrafish HHLA2 domain protein (Fig. 1D). Furthermore, the 3D protein structures displayed that the ISKNV *ORF069L* was similar to the zebrafish HHLA2 protein (Fig. 1E). These results indicated that ISKNV *ORF069L* was a viral HHLA2 protein.

3.2. Screening and identification of ΔORF069L

ISKNV *ORF069L* left and right flanking gene fragments of approximately 1000 bp were cloned into a previously constructed pUC19-RFP-Puro vector upstream and downstream of the *RFP-Puro* gene, respectively, to construct the ISKNV *ORF069L* transfer vector (pUC19-Δ*ORF069L*) (Fig. 2A). Then, MFF-1 cells were transfected with pUC19-Δ*ORF069L*. After 24 h transfection, cells were infected with ISKNV WT strain. The proportion of the recombinant virus became increasingly high in the collected virus mixture after several generations of purification. In the fifth generation of purification, the cultured cells were almost all infected with the recombinant virus. The swollen lesion cells almost all exhibited red fluorescence under the fluorescence microscope (Fig. 2B). Test primers were designed to identify the purity of the recombinant virus strains. The purity of the recombinant virus strains, named Δ*ORF069L*, was tested by PCR. The replacement of *ORF069L* gene by the *RFP-Puro* genes in Δ*ORF069L* was demonstrated by agarose gel electrophoresis analysis and sequencing of PCR products amplified using primers 100 bp upstream and downstream of the *ORF069L* in the ISKNV genome. As shown in Fig. 2C, the full length of ISKNV *ORF069L* with 30 bp both upstream and downstream sequences (726 bp) was amplified from ISKNV WT genome (positive control), but these were not detected in the negative control and recombinant virus genome. The full length of *RFP-Puro* gene of 2106 bp, including upstream and downstream fragments, was amplified from recombinant virus genome, but these were not detected in the negative control and WT virus genome (positive control). Above results suggested that Δ*ORF069L* strain was successfully obtained. The Δ*ORF069L* has been applied for China national invention patent (No. 2017104653566).

3.3. The characteristics of the recombinant virus strain

The proliferation ability of Δ*ORF069L* was evaluated in MFF-1 cells by calculating the copy number of ISKNV *MCP* gene in genome by using absolute quantification PCR analysis. As shown in Fig. 2D, Δ*ORF069L* proliferation has a similar growth curve with ISKNV WT in MFF-1 cells. Furthermore, the virus titers of both ISKNV WT and Δ*ORF069L* strains were evaluated. As shown in Fig. 2E, no significant differences were observed on the virus titers between the two virus strains. These results indicated that ISKNV *ORF069L* was a non-essential gene for virus growth and replication in MFF-1 cells.

3.4. ΔORF069L induced expression of immune-related genes in Mandarin fish

The expression of immune-related genes in mandarin fish infected with Δ*ORF069L* or ISKNV WT were detected by qRT-PCR. Mandarin fish infected with ISKNV WT died within 15 d, so, the samples were collected within 12 d. As shown in Fig. 3, the expression levels of *Mx1* (3A), *TNF-α* (3B), *IL-1β* (3C) and *IL-8* (3D) genes in fish infected with Δ*ORF069L* were all up-regulated compared with those in fish infected with ISKNV WT. Among them, the expression levels of *Mx1* and *IL-8* genes in fish infected with Δ*ORF069L* were significantly higher than those in fish infected with ISKNV WT after 3 d immunization; the expression levels of *TNF-α* and *IL-1β* genes in fish infected with Δ*ORF069L* were significantly higher than the those in fish infected with ISKNV WT after 12 d immunization. Furthermore, the expression level of total IgM in blood of Δ*ORF069L*-infected mandarin fish was also detected. The results showed that the expression level of *IgM* was significantly up-regulated in fish infected with Δ*ORF069L* compared with those in fish infected with ISKNV WT (Fig. 4F). These results suggested that Δ*ORF069L* could better activate host immune response compared with WT group.

3.5. Lethality and protection rate of ΔORF069L strain

Mandarin fish were divided into three groups to investigate the Δ*ORF069L* virulence *in vivo*. The fish in each group were infected *via* intraperitoneal injection with one of the following: Δ*ORF069L* (66 fish), WT (33 fish), or sterile DMEM as control (33 fish). The survival ratio was assessed in a 20 days period. As shown in Fig. 4A, the fish infected with ISKNV WT began to die on 6 days post-infection, and none of them survived after 11 days post-infection. Ultimately, the survival rate was 0%. By contrast, fish infected with Δ*ORF069L* began to die on 11 days post-infection, and 29 of them survived after 20 days post-infection. Ultimately, the survival rate was 44%. For histopathological analysis, kidney and spleen samples were obtained from ISKNV WT or Δ*ORF069L* infected fish at 8 days post-infection. The samples were treated by tissue slice and HE staining. The results showed that the swelling cells were widespread in the spleens (4C) and kidney (4E) from fish infected with ISKNV WT. By contrast, swelling cells were not observed in the spleens (4D) or kidneys (4F) from fish infected with Δ*ORF069L*. These results suggested that *ORF069L* was a virulence gene of ISKNV.

Furthermore, the immune protective effect of Δ*ORF069L* was evaluated. Healthy mandarin fish (as a control) or above survived mandarin fish (immunized with Δ*ORF069L*) were challenged by 10-fold dose of ISKNV WT. As shown in Fig. 4B, all healthy mandarin fish died after being infected with ISKNV WT. Meanwhile, all the Δ*ORF069L* immunized and survived fish did not die, and the survival rate was 100%. These results suggested that Δ*ORF069L* provided high immune protection effect for mandarin fish against ISKNV.

4. Discussion

A viral HHLA2 protein encoded by ISKNV *ORF069L* was first identified in the present study. The virulence of the deleted *ORF069L*

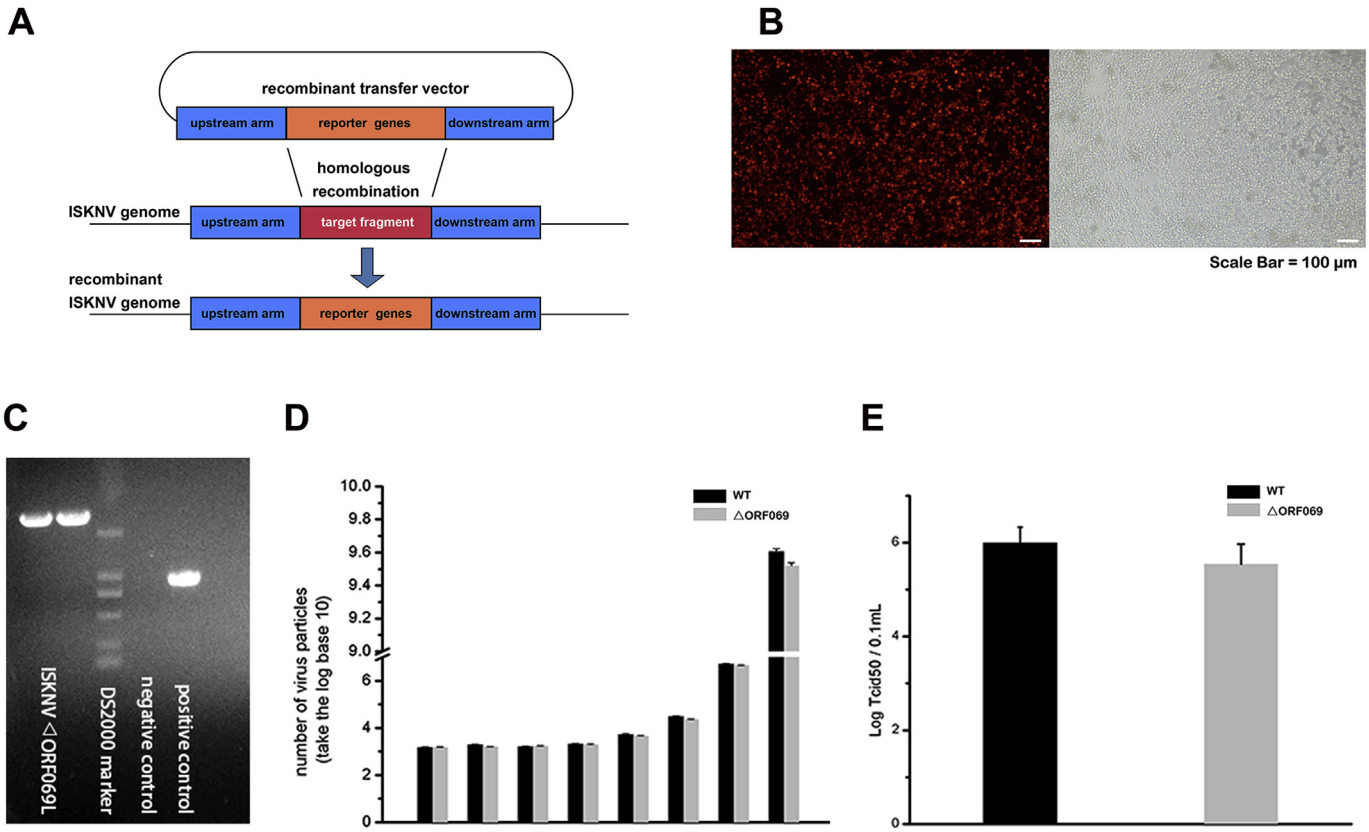

Fig. 2. Screening and identification of Δ*ORF069L*. (A) Whole construction process. We constructed upstream and downstream recombinant arms and transferred the marker genes in the recombinant transfer vector to the ISKNV genome by using the principle of homologous recombination. The tag carries a red fluorescent protein, and by fluorescence, we can obtain recombinant virus strains. (B) Left: after purification, Δ*ORF069L*-infected cells presented red light under an inverted fluorescence microscope. Right: Bright field under the same visual field of Fig. 1B left. (C) Purity of the recombinant virus strain was detected using PCR, and lanes 1 and 2 are Δ*ORF069L* samples to be tested. Lane 3 is standard DL2000 marker, and lanes 4 and 5 are the negative and positive control, respectively. (D) Growth curve of ISKNV WT and Δ*ORF069L* strain. The growth curve of the ISKNV WT and Δ*ORF069L* strain was measured on MFF-1 cells for 72 h from the beginning of infection to adoption until the cells were completely shed and died. (E) Infection titers of both ISKNV WT and Δ*ORF069L* strain. The titer of virus infection was measured on a 96-well cell culture plate by finite dilution method. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

reconstruction ISKNV strain (Δ*ORF069L*) declined by 40% compared with ISKNV WT. The immune-protection ratio was nearly 100% in the survived fish against ISKNV after being immunized with Δ*ORF069L*. These results suggested that ISKNV *ORF069L* was a virulence gene of ISKNV and Δ*ORF069L* could be developed as a potential attenuated vaccine against ISKNV.

Gene knockout is an important method to study the function of viral genes. Homologous recombination technique is a common way for viral gene knockout, which mainly depends on matching of the same homology sequence between fragment/vector and target genome. For example, both poxviruses and iridovirus have been knockout and insertion of exogenous genes [21,22]. In the present study, a recombinant virus strain of the ISKNV *ORF069L* deletion was successfully constructed. On one hand, the replication and transmission abilities of virus are not highly vulnerable after *ORF069L* has been knocked out. On the other hand, the lethality that caused by Δ*ORF069L* declines by 40%. The sequence analysis showed that ISKNV *ORF069L* includes a HHLA2 domain. HHLA2 is the most recently discovered ligand of the B7 family and was first demonstrated as a T cell co-inhibitory molecule [23,24]. The known functions of HHLA2 proved that it is a negative regulation factor of the immunity. As a viral HHA2 protein, ISKNV *ORF069L* may play a similar role as cellular HHLA2 to suppress the host immunity. The concrete function needs further study.

To control the iridovirus disease, vaccine development is urgent. To date, the ISKNV inactivated vaccine has obtained a certain effect, but no reports of ISKNV attenuated vaccine are available [25]. The DNA recombinant techniques have been used to produce protective antigens that are cheap and high-quality, including live vector vaccines and genetic deletion-attenuated vaccine, which is stable in natural conditions, hard to back mutation, and has good immunogenicity. In the present study, the protection rate of Δ*ORF069L* was nearly 100% in laboratory study. This result proved that Δ*ORF069L* could be as a potential attenuated vaccine against ISKNV. However, the Δ*ORF069L* still has certain virulence. The probable solution lies in knocking out other virulence genes from Δ*ORF069L* recombinant viral strains to construct recombinant viral strains of double-gene deletion to further weaken the virulence.

Acknowledgement

This work was supported by the Science and Technology Planning Project of Guangzhou (Nos. 201904020043 and 201607020014), the National Key Research and Development Program of China (Nos. 2018YFD0900504 and 2018YFD0900501), the National Natural Science Foundation of China (No. 31702381), the Guangdong Key Research and Development Program (No. 2019B020217001), the China Agriculture Research System (No. CARS-46), the Guangdong Natural Science Foundation (Nos. 2016B020202001), and Guangdong Provincial Special Fund for Modern Agriculture Industry Technology Innovation Teams.

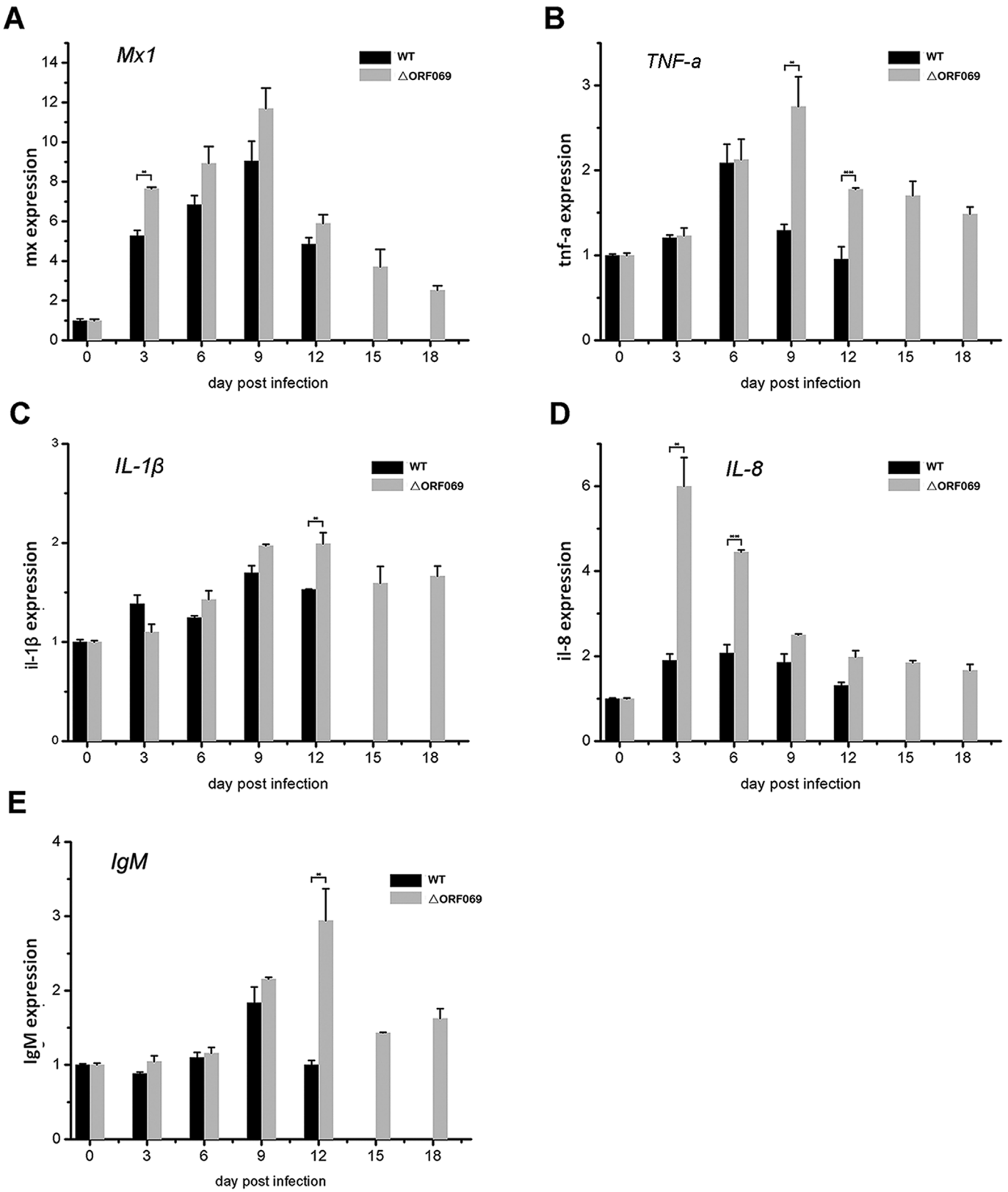

Fig. 3. Expression levels of innate immune related genes after mandarin fish infected with Δ*ORF069L*. After fish infected with ISKNV wild-type or the ΔORF069L strain, the blood of 3 mandarin fishes each group every 3 days were drawn and then RNAs were extracted to test the expression of relevant immune genes (*Mx1*, *IL-1β*, *IL-8*, *TNF-α* and *IgM*). The *y*-axis represents the relative mRNA expression. Line points indicate the mean ± SD of 3 technical replicates. The asterisks above the bars represent statistically significant differences of the control samples. "*" at $p < 0.05$ and "**" at $p < 0.01$.

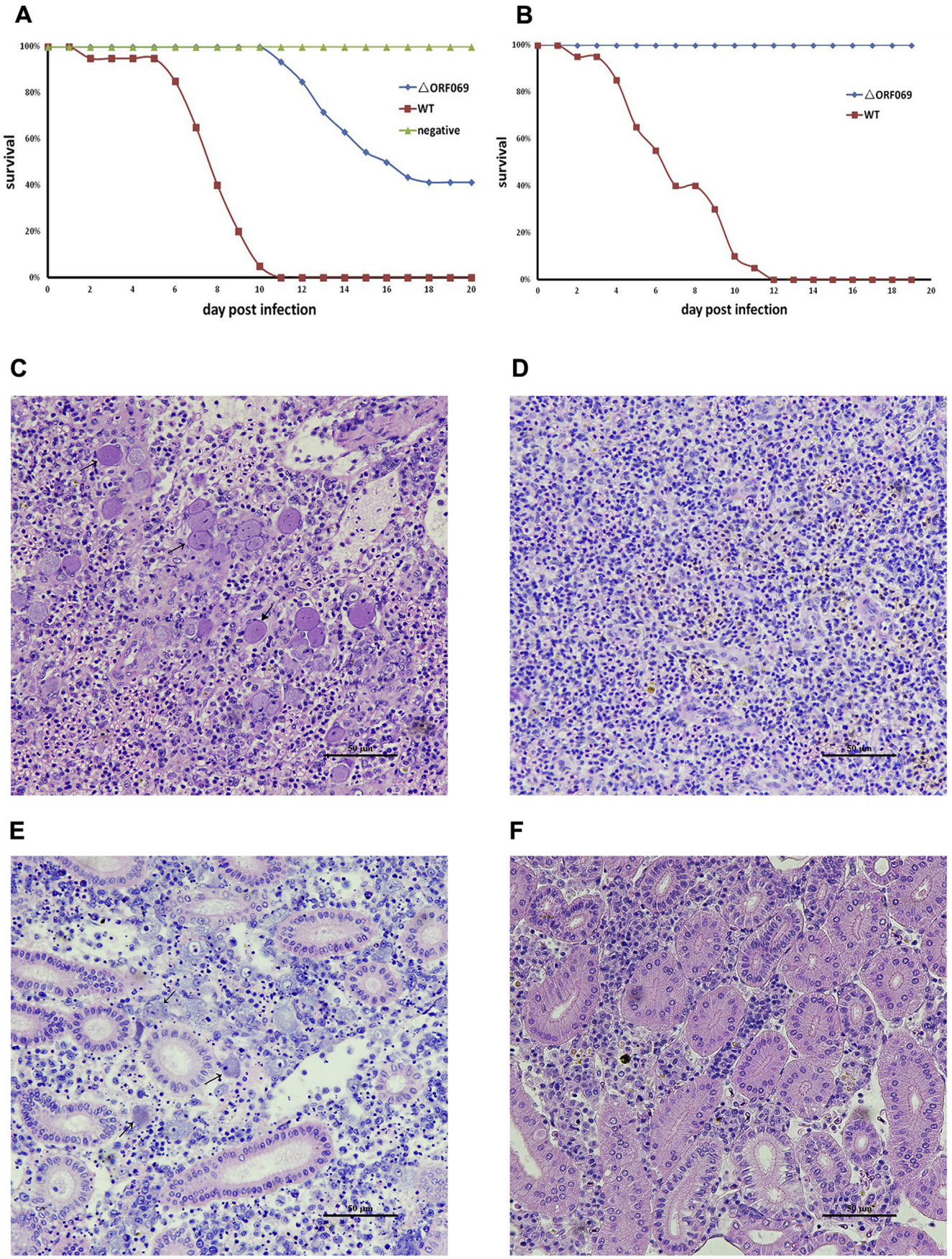

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Fig. 4. Lethality and protection rates of ΔORF069L infection with mandarin fish. (A) Survival rate of mandarin fish after fish were infected with ISKNV WT or Δ*ORF069L*. The WT group all died within 11 days, and 60% of the Δ*ORF069L* group nearly died and then remained stable after 17 days. Meanwhile, the control group did not die unexpectedly. (B) Protection rates were determined. Above survival mandarin fish infected with Δ*ORF069L* were further injected with 10 times of the dose of ISKNV WT (named Δ*ORF069L* group). Healthy mandarin fish were injected with the same dose of ISKNV WT (named WT group) as a positive control. All of the mandarin fish of the Δ*ORF069L* group survived, whereas the mandarin fish of the WT group died within 13 days. (C–F) Tissue sections of the spleens and kidneys from ISKNV WT or Δ*ORF069L*-infected mandarin fish. The spleen (C) and kidney (E) from the mandarin fish infected with ISKNV WT. The spleen (D) and kidney (F) from the mandarin fish infected with Δ*ORF069L*. Arrows indicate the swollen cells. 40× magnification.

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