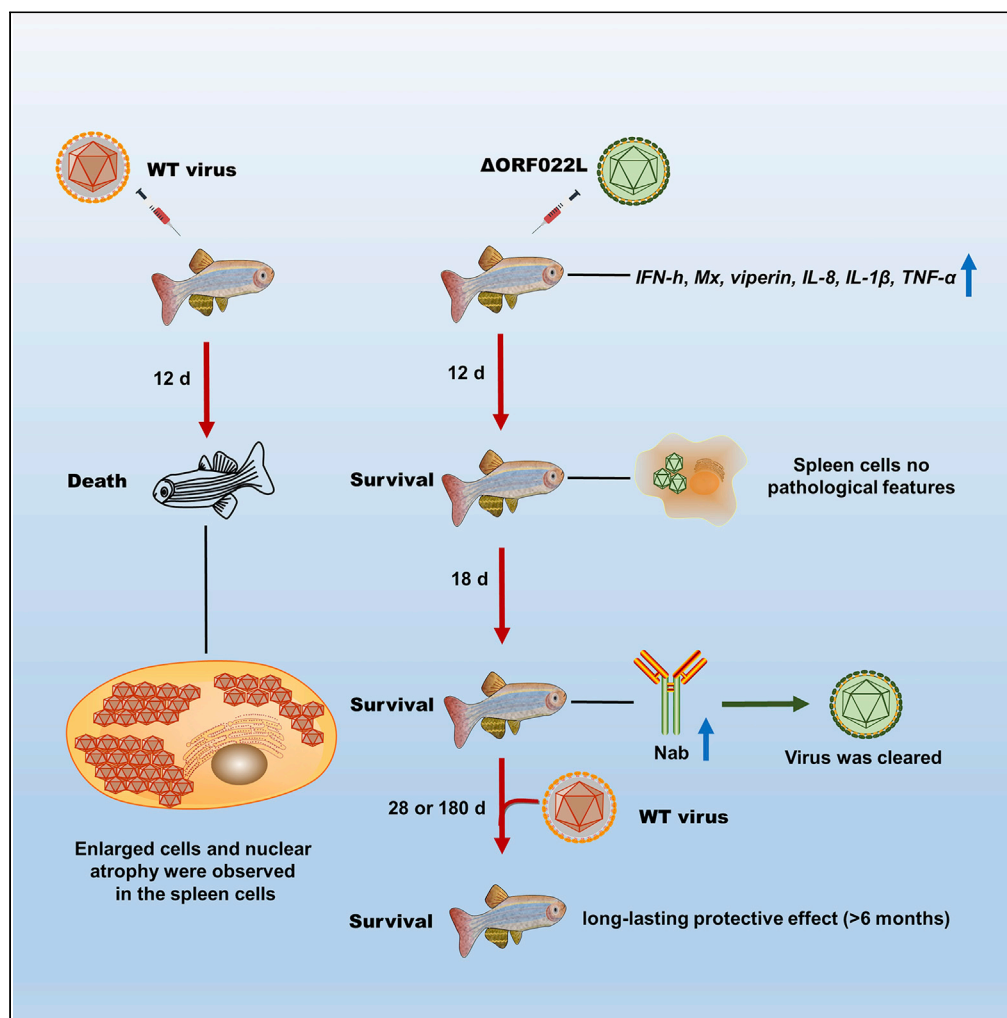# iScience

Article

Development of a gene-deleted live attenuated candidate vaccine against fish virus (ISKNV) with low pathogenicity and high protection

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Highlights

ISKNV ORF022L deletion
recombinant virus
(ΔORF022L) was
constructed

ΔORF022L was greatly
attenuated and caused
only 2% mortality in
mandarin fish

ΔORF022L-immunized
mandarin fish were 100%
protected from ISKNV
challenge

ORF022L induced
immune-related genes
and ISKNV-specific
antibody responses

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Article

Development of a gene-deleted live attenuated candidate vaccine against fish virus (ISKNV) with low pathogenicity and high protection

Ruoyun Zeng,[1,2,3] Weiqiang Pan,[1,3] Yifan Lin,[2] Jian He,[1] Zhiyong Luo,[1] Zhimin Li,[1] Shaoping Weng,[2] Jianguo He,[1,2,*] and Changjun Guo[1,2,4,*]

SUMMARY

Aquaculture provides important food, nutrition, and income sources for humans. However, aquaculture industry is seriously threatened by viral diseases. Infectious spleen and kidney necrosis virus (ISKNV) disease causes high mortality and economic losses to the fish culture industry in Asia and has been listed as a certifiable disease by the International Epizootic Office. Vaccine development is urgent to control this disease. Here, a gene-deleted live attenuated candidate vaccine (ΔORF022L) against ISKNV with low pathogenicity and high protection was developed. ΔORF022L replicated well in mandarin fish fry-1 cells and showed similar structure with wild-type ISKNV. However, the pathogenicity was significantly lower as 98% of the mandarin fish infected with ΔORF022L survived, whereas all those infected with wild-type ISKNV died. Of importance, 100% of the ΔORF022L-infected fish survived the ISKNV challenge. ΔORF022L induced anti-ISKNV specific antibody response and upregulation of immune-related genes. This work could be beneficial to the control of fish diseases.

INTRODUCTION

Fish and fish products have a crucial role in nutrition and global food security as they represent a valuable source of nutrients and micronutrients of fundamental importance to diversified and healthy diets (FAO, 2018). Between 1961 and 2016, the average annual growth rate of the global consumption of fish food (3.2%) has exceeded the growth rate of all terrestrial animal meat consumption (2.8%) (FAO, 2018). Aquaculture production has supported the rapid and continuous growth of human consumption of fish supplies (FAO, 2016). Unfortunately, fish diseases, especially infectious viral diseases, are becoming increasingly serious, causing major economic losses to the fish aquaculture industry (FAO, 2016). For example, in China, disease-related losses were approximately 5.3 billion USD in 2017, and these losses involved 62 cultured species and 96 diseases (FAO, 2019). Therefore, disease prevention is crucial to the sustainability of fish aquaculture.

Vaccine is an effective way to control fish viral diseases. Inactivated vaccines possessing advantages of good safety, high protection rate, and short research and development cycle have become the most commonly used vaccines to control fish viral diseases (Hu et al., 2017). However, inactivated vaccines usually require multiple administrations with the help of appropriate adjuvants to elicit sufficient serum antibody responses (Jang and Seong, 2012). Meanwhile, gene-deleted live attenuated vaccines, which are produced by the knockout of virulent gene(s) in the viral genome (Sun et al., 2014), could induce a long-lasting and broad immune response (humoral and cellular), which closely resembles natural immunity after infection (Sridhar et al., 2015). However, they have seldom been developed for fish virus. Therefore, the development of genetic engineering vaccine for fish virus needs to be promoted.

Infectious spleen and kidney necrosis virus (ISKNV) is a large icosahedral, double-stranded DNA-containing virus that is the type species of *Megalocytivirus* genus from Iridoviridae family (Chinchar et al., 2017a); it causes spleen and kidney necrosis with lethality (Wu et al., 1997). ISKNV infection has occurred in more than 50 marine and fresh species, and mandarin fish is one of the most typical host (Guo et al., 2012). Owing to the strong infectiousness and high lethality of ISKNV, billions of economic losses are being incurred on the aquaculture every year (He JG et al., 2000). Thus, ISKNV disease has been a high concern of researchers, and it has been listed as a certifiable disease by the International Epizootic Office (Hu et al., 2017). In

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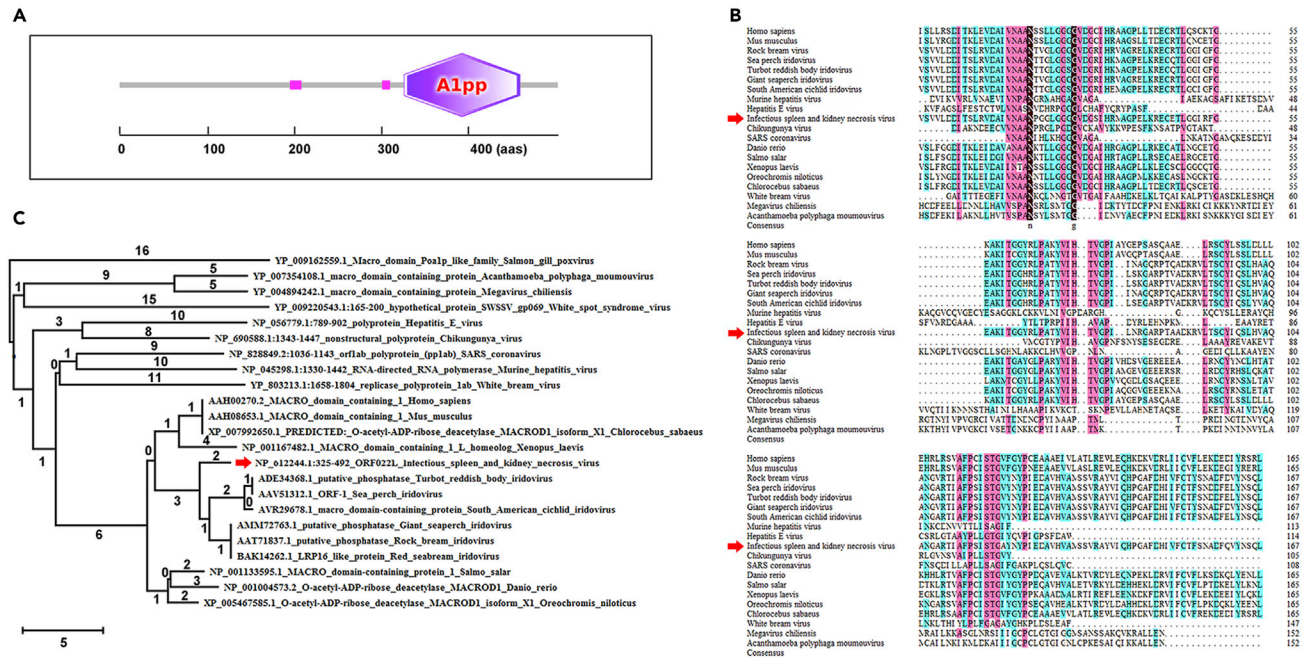https://doi.org/10.1016/j.isci.2021.102750

Figure 1. ORF022L amino acid sequence analysis and alignment with macro domains derived from other species

(A) SMART analysis of ISKNV ORF022L showed an A1pp domain (also named macro domain) at the C terminus of ORF022L amino acid sequence.

(B) Amino acid sequence alignment between the macro domains from ISKNV and Murine hepatitis virus, Chikungunya virus, Hepatitis E virus, White bream virus, Megavirus chiliensis, *Acanthamoeba polyphaga* moumouvirus, human, house mouse, zebrafish, salmon, African clawed frog, tilapia, and green monkey. Identities of 100%, 75%–100%, and 50%–75% were indicated in black, pink, and blue, respectively.

(C) Phylogenetic tree of macro domains from ISKNV and Murine hepatitis virus, Chikungunya virus, Hepatitis E virus, White bream virus, White spot syndrome virus, Salmon gill poxvirus, Megavirus chiliensis, *Acanthamoeba polyphaga* moumouvirus, human, house mouse, zebrafish, salmon, African clawed frog, tilapia, and green monkey.

the present study, a candidate ISKNV gene-deletion attenuated vaccine (ΔORF022L) was constructed and its pathogenicity and immunogenicity were determined for safety and effectiveness evaluation.

RESULTS

Sequence analysis of ISKNV ORF022L

Searching for virulence genes is the first step in the construction of the ISKNV gene-deletion attenuated vaccine. The amino acid sequences of ISKNV open reading frames (ORFs) were analyzed to screen the potential virulence genes. Simple modular architecture research tool (SMART) results showed an A1PP domain, which was also named macro domain, at the C terminus of ORF022L (Allen et al., 2003) (Figure 1A). Macro domain family proteins widely exist in all life forms, including virus, bacteria, archaea, and eukaryotes. Multiple sequence alignments and phylogenetic analysis were performed to compare the evolutionary relationships of the macro domains of ISKNV with those of other viruses and vertebrates. As shown in Figure 1B, the ISKNV macro domain shared a high protein sequence similarity with the macro domains from Rock bream iridovirus (92.26% identity), Giant sea perch iridovirus (91.07% identity), and Sea perch iridovirus (90.48% identity), indicating that the macro domain was conserved among different iridoviruses. Moreover, the macro domain of ISKNV showed higher similarity with macro domains from vertebrates (64.00% identity) than those from viruses of other families (28.35% identity). A similar result was obtained from phylogenetic analysis (Figure 1C). Previous studies have shown that virus-encoded macro domain-containing proteins are important for virulence (Nan et al., 2014; Eriksson et al., 2008). Therefore, ORF022L may be a virulence gene of ISKNV, and it could be selected as the target gene when constructing the gene deletion vaccine.

Construction and purification of ΔORF022L

Mandarin fish fry-1 (MFF-1) cells were transfected with recombinant transfer vector and subsequently infected with wild-type (WT) ISKNV to obtain the recombinant ORF022L deletion viruses. After several passages of puromycin selection, the plaques that emitted red fluorescent were selected to obtain the purified
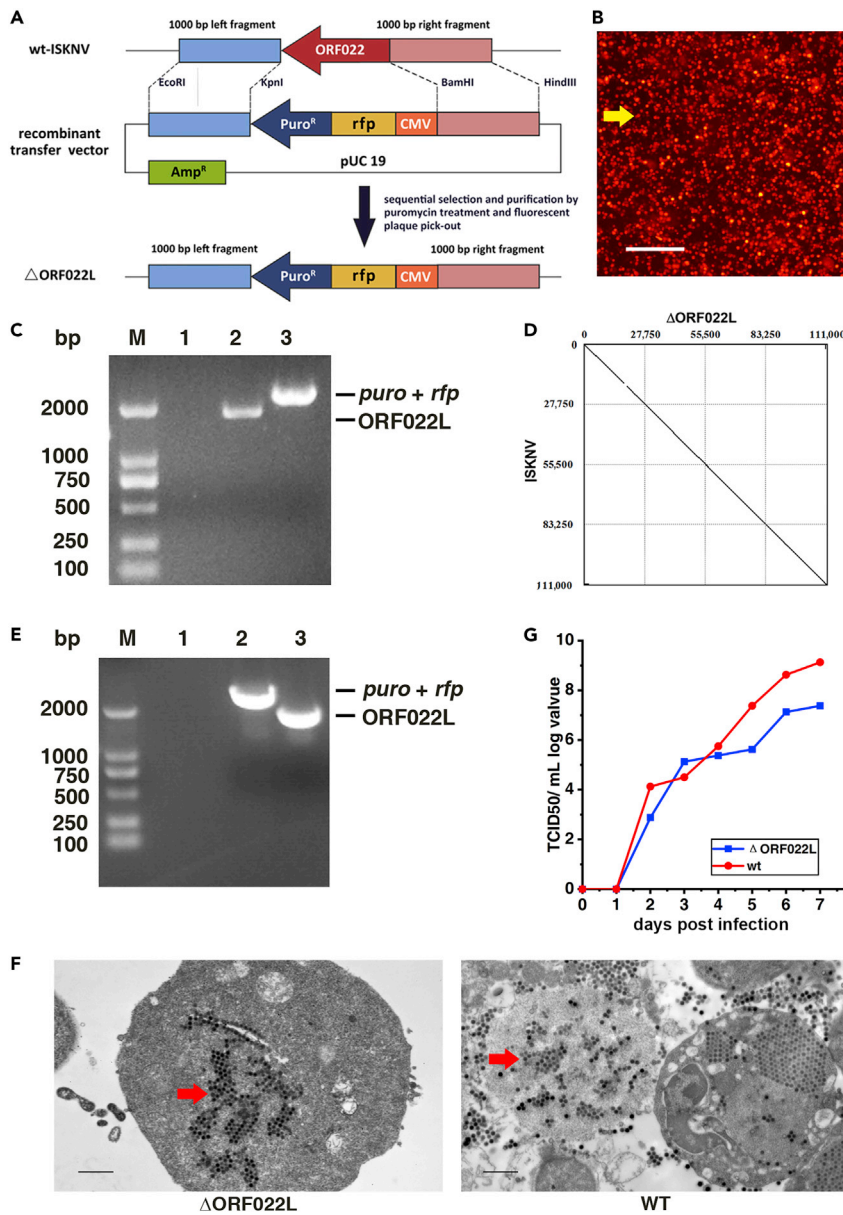
Figure 2. Construction and characteristics of ΔORF022L

(A) The ORF022L of ISKNV genome was deleted by homologous recombination with a transfer vector containing puro and rfp tags.

(B) MFF-1 cells infected with ΔORF022L were observed under the inverse fluorescence microscope. The arrow indicates the cells infected by the ΔORF022L that emitted red fluorescence. Scale bar, 50 μm.

(C) Purity of ΔORF022L was confirmed by PCR and agarose gel electrophoresis analysis. M: DS2000 marker; lane 1: negative control; lane 2: WT DNA amplified by ORF022L outer primers; lane 3: ΔORF022L DNA amplified by ORF022L outer primers.

(D) Purity of ΔORF022L was further confirmed by resequencing, and the result was shown by a dot matrix graph that compared the genome of ΔORF022L with that of WT ISKNV. The gap represents the replacement of ORF022L by the tag genes.

(E) ΔORF022L purity (10th passage) was confirmed by PCR and agarose gel electrophoresis analysis. M: DS2000 marker; lane 1: negative control; lane 2: ORF022L (10th passage) DNA amplified by ORF022L outer primers; lane 3: WT DNA amplified by ORF022L outer primers.

Figure 2. *Continued*

(F) ΔORF022L- or WT-infected MFF-1 cells were fixed and sectioned for transmission electron microscopic observation to determine the structure of the viral particles. Scale bar, 1 μm. The viral particles are indicated by arrows. See also Figure S1.

(G) Growth kinetics of ΔORF022L and WT ISKNV were analyzed in MFF-1 cells after infection. At the indicated time points, cells were collected and viral titers in the cells were measured *via* $TCID_{50}$ assays.

recombinant virus strain, which was named ΔORF022L. A schematic of the construction process is shown in Figure 2A. Red fluorescence in cells infected with ΔORF022L was observed under the inversed fluorescent microscope (Figure 2B). Replacement of *ORF022L* by the red fluorescence protein gene (*rfp*)- puromycin-resistant gene (*puro*) in the ISKNV genome was detected via PCR (Figure 2C). Furthermore, resequencing of the whole genome confirmed the recombinant virus strain (Figure 2D). The purity of genome from the 10th passage of ΔORF022L was determined via PCR to confirm the stability of ΔORF022L, and the result showed that no WT contamination was present in the 10th passage. Therefore, ΔORF022L was stable enough for vaccine development (Figure 2E). These results confirmed that the ISKNV ORF022L gene-deletion recombinant virus was constructed.

Characterization of ΔORF022L

The sections of WT ISKNV- or ΔORF022L-infected cells were observed via transmission electron microscopy (TEM) to investigate the structure of ΔORF022L. As shown in Figures 2F and S1, ΔORF022L virus particles showed similar size (diameters of approximately 150 nm) and arrangement to those of the WT viral particles, indicating that the deletion of ORF022L did not influence the structure of ISKNV. Furthermore, the proliferation ability of ΔORF022L was evaluated in MFF-1 cells. The cells were infected with ΔORF022L or WT, and then viral titers at the indicated time points after infection were measured. As shown in Figure 2G, ΔORF022L replicated well in MFF-1 cells, showing similar titers with WT at 3 and 4 days post infection. Although the final titer of ΔORF022L at 7 days post infection was 56 times lower than that of WT, it reached 2.4×10^7 $TCID_{50}$/mL. Therefore, the deletion of ORF022L did not influence the normal proliferation of the virus, which was an essential feature for large-scale production of the candidate vaccine.

Characterization of ΔORF022L as a vaccine candidate

Given that ΔORF022L grew well in MFF-1 cells, its growth phenotype and pathogenesis in an animal model were interesting because high pathogenicity is not suitable for vaccine development. Mandarin fish were inoculated with ΔORF022L or WT ISKNV via intraperitoneal injection, and then the mortality ratio was assessed during a 28-day period. As shown in Figure 3A, the fish infected with WT ISKNV died out within 18 days post infection. By contrast, all dosages of ΔORF022L showed only 2% residual virulence. Mandarin fish were infected with ΔORF022L or WT ISKNV to determine parameters of the ΔORF022L pathogenicity *in vivo*, and the viral titers in the spleens were determined at indicated time points. As expected, WT ISKNV replicated well, reaching a peak at 9 days post infection, but ΔORF022L replicated noticeably in spleens of the vaccinated animals. Although ~100-fold difference retained in the titers between the two viruses during 3–9 days post infection, the variant achieved a maximum titer in the same order of magnitude as WT at 12 days post infection. WT ISKNV killed all fish within 12 days, whereas ΔORF022L was eventually cleared at 15–18 days post infection (Figure 3B). For pathological analysis, spleen samples from the fish of WT ISKNV and ΔORF022L groups were collected at 12 days post infection and observed using TEM. As shown in Figure 3C, enlarged cells were widespread in the spleen of fish infected with WT virus. On the contrary, almost no enlarged cells were observed in the spleen of mandarin fish infected with ΔORF022L, which was similar with that of the uninfected fish. The enlarged cells in three random sights of each sample were quantified under the microscope with 200× magnification. The average number of enlarged cells in the WT samples was 34.67, whereas that of ΔORF022L was only 0.33, showing a highly significant difference (Figure 3D). These results suggested that the pathogenicity of ΔORF022L was considerably attenuated, and it could be eliminated by mandarin fish. Therefore, ΔORF022L was safe enough for vaccine development. The expression levels of flanking genes by WT and ΔORF022L were compared at 7 days post infection to exclude the possibility that the attenuation observed is the consequence of a polar effect of the ORF022L deletion. Figure 3E showed that the ORF019R, ORF020L, ORF021L, ORF023R, and ORF024R expressed by ΔORF022L were comparable with those expressed by WT, whereas ORF022L was not expressed by ΔORF022L. This
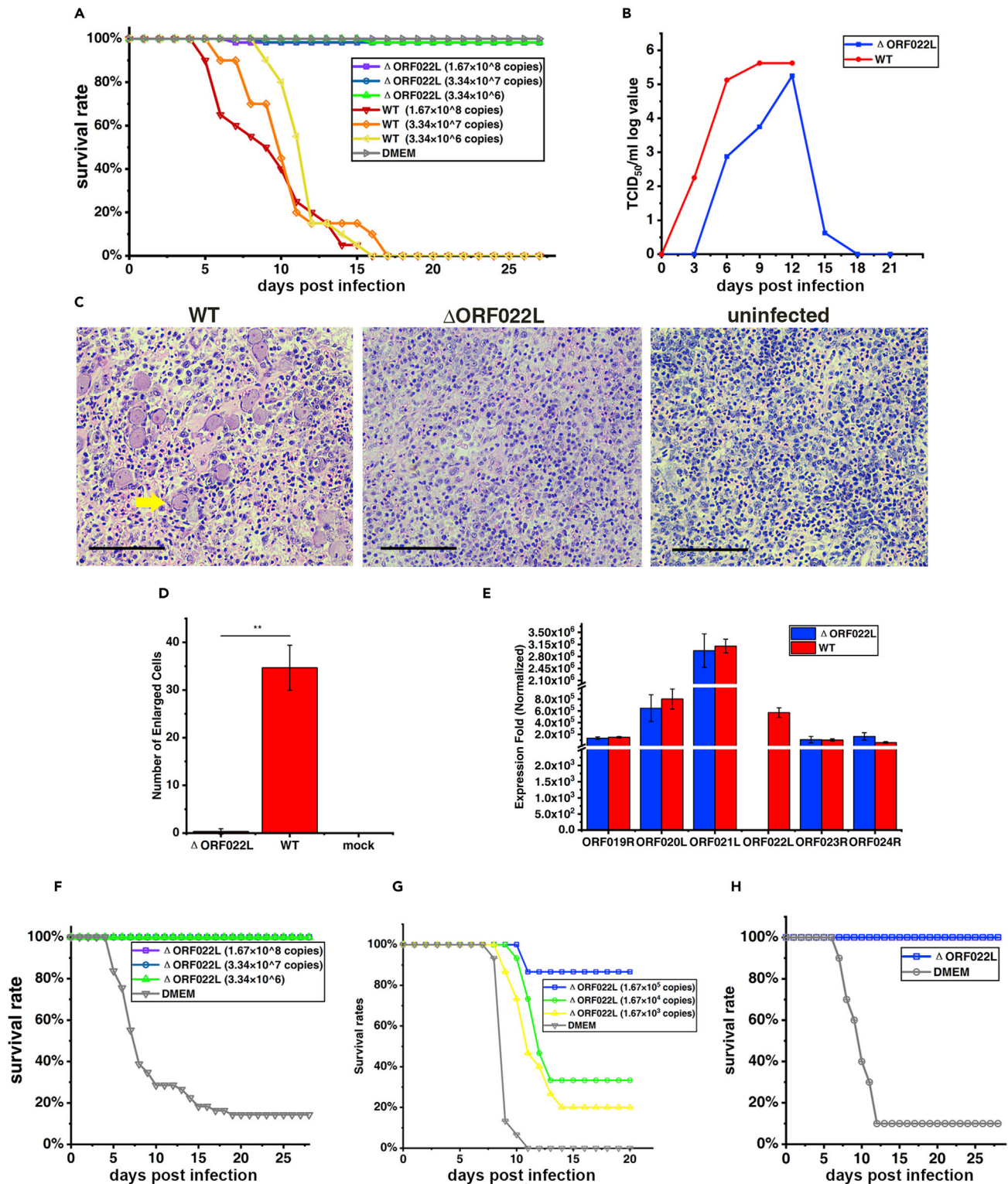
Figure 3. Characterization of ΔORF022L as a vaccine candidate in mandarin fish

(A) Groups of 60 mandarin fish were intraperitoneally injected with ΔORF022L or WT at indicated doses, and their survival rates were monitored for 28 days post infection.

Figure 3. *Continued*

(B) Mandarin fish were intraperitoneally injected with ΔORF022L or WT. On the indicated days, viral titers in the spleens were determined by $TCID_{50}$ methods in MFF-1 cells.

(C) Mandarin fish were injected with ΔORF022L or WT. At 12 days post infection, the spleens of fish were transformed into H&E-stained tissue sections for microscopic observation. Scale bar, 60 μm. Arrow indicates enlarged cells.

(D) Enlarged cells were quantified in three random sights of each sample under the microscope with 200× magnification. The results are presented as means of n=3 independent experiments, and error bars represent SD. Statistical analysis of differences was performed via one-way ANOVA by using the SPSS 20.0 software. The asterisks above the bars represent statistically significant differences of the control samples, $**p < 0.01$.

(E) Expression levels of ORF019R, ORF020L, ORF021L, ORF022L, ORF023R, and ORF024R by ΔORF022L or WT were quantified. The results are presented as means of n=3 independent experiments, and error bars represent SD.

(F) At 28 days post infection, mandarin fish from the ΔORF022L or mock-inoculated groups were challenged with WT ISKNV, and their survival rates were monitored for 28 days post infection.

(G) Fish were immunized with ΔORF022L at a dose of $1.67×10^5$, $1.67 × 10^4$, or $1.67 × 10^3$ copies and then challenged with WT ISKNV at 21 days post infection, and their survival rates were monitored for 28 days.

(H) Six months post immunization of ΔORF022L, mandarin fish were challenged with WT, and the mortality ratios were calculated over a 28-day period.

result suggested that the attenuation observed was caused by the deletion of ORF022L rather than the silence of the expression levels of the flanking genes.

At 28 days post inoculation, the mandarin fish from the ΔORF022L or mock inoculated groups were challenged with WT ISKNV to further evaluate the protective effect of ΔORF022L. Despite the great attenuation in mandarin fish, ΔORF022L was remarkably proficient at protecting against WT ISKNV. Only 14% of the fish from the mock group survived the challenge, whereas those from the ΔORF022L groups were completely protected from the challenge (Figure 3F). To clarify the lowest effective dose for ΔORF022L to protect the fish from ISKNV, we further diluted ΔORF022L when inoculating. And in the WT ISKNV challenge experiment, protection rate of the $1.67×10^5$ copies group was 86.6%, whereas those of the $1.67 × 10^4$ and $1.67 × 10^3$ copies groups were 33.3% and 20%, respectively (Figure 3G). Therefore, the minimum effective dose of ΔORF022L was about $1.67×10^5$ copies. To determine whether the protective effect was long lasting, we challenged the ΔORF022L immunized fish at 6 months post immunization. As shown in Figure 3H, all the ΔORF022L survived, proving the long-lasting protective effect of ΔORF022L. These results proved that ΔORF022L had strong and long-lasting protective effects and could be developed into an ISKNV candidate vaccine.

ΔORF022L induced antibody response in mandarin fish

The protective effects of ΔORF022L suggested a strong host response, including adaptive immunity. The production of neutralizing antibodies (NAbs) in the serum of ΔORF022L-immunized fish at 15 days post immunization was determined using an antibody neutralization assay. In brief, WT ISKNV suspension was incubated with the serum samples overnight to infect MFF-1 cells. At 72 h post infection, the cells infected with ΔORF022L serum-incubated virus showed the least enlarged cells compared with those infected with unincubated or mock-serum-incubated virus, and it showed a dose-dependent effect (Figure 4A). Quantification of this observation is shown in Figure 4B. The levels of viral mRNAs and proteins were detected to further quantify this result. Real-time quantitative reverse transcription-PCR (qRT-PCR) results showed that the expression levels of viral *major capsid protein* (*MCP*) gene in the cells infected with ΔORF022L serum-incubated virus were remarkably lower than those infected with unincubated or mock serum-incubated virus, showing dose dependence (Figure 4C). Western blot results also proved the dose-dependent reduction in viral protein (ORF101L) by ΔORF022L serum (Figures 4D and 4E). Therefore, the serum from ΔORF022L-treated fish more strongly inhibited ISKNV infection than the serum from mock-treated fish, thereby proving the induction of anti-ISKNV specific NAbs by ΔORF022L in mandarin fish. NAb titers against ISKNV were also measured through microneutralization assay at 15 days post immunization. As shown in Figures 4F and S2, the MN_{50} NAb titer of ΔORF022L serum was 143.51 ($lgMN_{50}$=2.2), whereas that of mock serum was not detectable (<10, $lgMN_{50}$<1). These results indicated a strong neutralizing antibody response induced by ΔORF022L at 15 days post immunization. As IgM constitutes the main systemic immunoglobulin (Magadan et al., 2015), the levels of anti-ISKNV specific IgM in fish infected with ΔORF022L or WT were also determined. The results were shown in Figure 4G. Comparing with the mock-injected fish, the level of ISKNV specific IgM in the ΔORF022L-immunized fish reached a peak at 12 days post immunization, lasting to 21 days post immunization. On the contrary, the fish that were infected with WT died out within 5 days, and they did not show a production of ISKNV-specific IgM. Together,
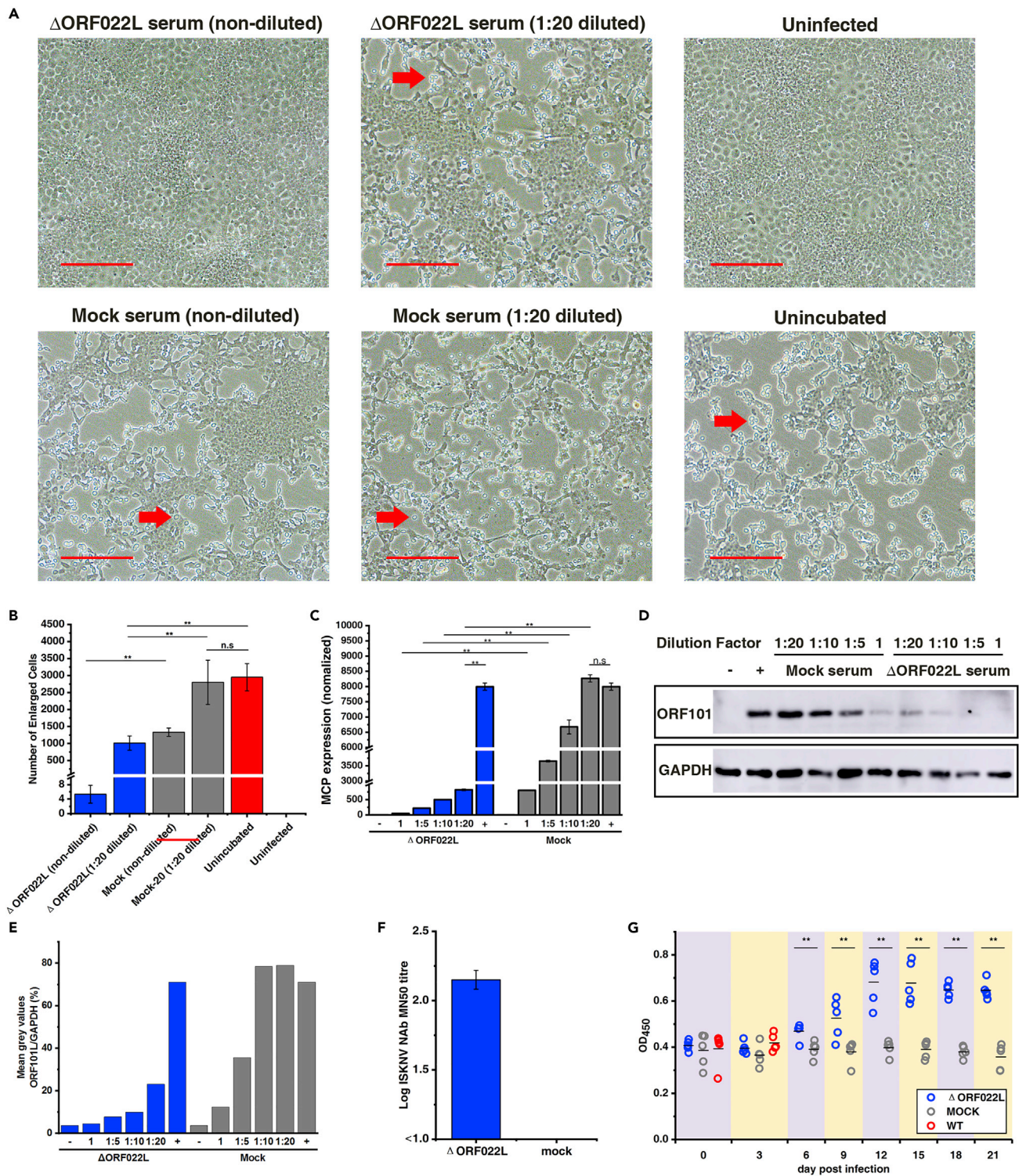
A

B

C

D

E

F

G

Figure 4. Determination of antibody response aroused by ΔORF022L in mandarin fish

(A–E) Serum samples were collected from mandarin fish treated with ΔORF022L at 15 days post infection or mock treated with DMEM. WT ISKNV suspension was incubated with the serum samples to infect MFF-1 cells. At 72 h post infection, MFF-1 cells were observed under the microscope (A). Scale bar, 50 μm. Arrows indicate enlarged cells. The numbers of enlarged cells were quantified in three random sights under the microscope with 200× magnification. The results are presented as means of n = 3 independent experiments, and error bars represent SD. The statistics were analyzed by one-way ANOVA. The asterisks above the bars represent statistically significant differences of the control samples n.s $p>0.05$, **$p<0.01$ (B). ISKNV *MCP* expression was determined

Figure 4. *Continued*

by qRT-PCR at 72 h post infection, representing the viral load in MFF-1 cells infected with ISKNV suspension, which were incubated with serum samples (C). The results are presented as means of $n=3$ independent experiments, and error bars represent SD. The statistics were analyzed by one-way ANOVA. The asterisks above the bars represent statistically significant differences of the control samples n.s $p>0.05$, **$p<0.01$. ISKNV ORF101L protein abundance was determined through Western blot at 72 h post infection, representing the viral load in MFF-1 cells infected with ISKNV suspension, which were incubated with serum samples (D). Mandarin fish GAPDH protein was used as reference. The ΔORF022L and mock serum samples derived from the same experiment and the blots were processed in parallel. The blots were cropped. Western blot analysis result was quantified using ImageJ software (E).

(F) MN_{50} titers of the ΔORF022L and mock serum samples were measured. The results were presented as the means of $n=3$ independent experiments, and error bars represent SD.

(G) Serum samples were collected from mandarin fish infected with ΔORF022L, WT or mock treated on the indicated days, and the levels of ISKNV-specific IgM were measured using ELISA. The statistics were analyzed by one-way ANOVA. The asterisks above the bars represent statistically significant differences of the control samples **$p<0.01$.

these results proved that, as an ISKNV candidate vaccine, ΔORF022L could induce antibody response in mandarin fish.

ΔORF022L induced the expressions of immune-related genes

With the above results that indicated ΔORF022L-induced adaptive immunity of the host to resist viral infection, whether ΔORF022L could affect the expressions of the immune genes associated with antiviral defense was explored. The available gene database of mandarin fish and selected genes (i.e., *IFN-h*, *viperin*, *mx*, *IL-1β*, *IL-8*, *TNF-α*, *Iκb*, and *IKK-β*) known to be involved in innate immune defense against viral infection was searched (Chinchilla et al., 2020). The potential effect of ΔORF022L on the expression levels of these genes in the MFF-1 cells and in the spleen of mandarin fish was examined at indicated time points after ΔORF022L infection. The results of the cell experiments (Figure 5) showed that following infection of ΔORF022L, the expression levels of *IFN-h*, *IL-1β*, *TNF-α*, and *viperin* were upregulated in high degrees, reaching 120-, 80-, 58- and 45-fold, respectively. The upregulation levels of *IL-8*, *IKKβ*, and *Iκb* were 10- to 20-fold, whereas that of *mx* was only 4-fold. ΔORF022L was capable of upregulating higher levels of *IFN-h*, *viperin*, *mx*, *IL-8*, and *TNF-α*; similar levels of *Iκb* and *IKK-β*; and lower levels of *IL-1β* than WT. The above-mentioned results preliminarily confirmed that ΔORF022L induced the host antiviral immune defense in MFF-1 cells. The *in vivo* experiments (Figure 6) showed that, in the spleen of fish infected with ΔORF022L or WT ISKNV, the expression levels of all the examined genes reached a peak at 6 days post infection, except the *IKK-β* in the ΔORF022L group, which peaked at 3 days post infection, whereas the *TNF-α* in the WT group was not upregulated. For the ΔORF022L inoculated fish, the expression of *IFN-h* (600-fold) was the most upregulated among the observed genes, followed by *viperin* (112-fold), *IL-8* (88-fold), and *mx* (38-fold), whereas those of other immune genes, including *IL-1β*, *TNF-α*, *Iκb*, and *IKK-β* were slightly upregulated by 2- to 6-fold. Moreover, the expression levels of *IFN-h*, *IL-8*, *TNF-α*, *Iκb*, and *IKK-β* could be more upregulated by ΔORF022L than by WT ISKNV. These results suggested that, as an ISKNV candidate vaccine, ΔORF022L could upregulate the expressions of immune genes associated with antiviral defense.

DISCUSSION

In this study, an ISKNV gene-deletion viral strain ΔORF022L was constructed, and proliferation ability, pathogenicity, immunogenicity, and immune protective effect were evaluated. The results suggested that ΔORF022L with low pathogenicity and high protective effects could be developed as a gene-deleted attenuated candidate vaccine to control ISKNV disease.

Few gene deletion strains of fish viruses have been reported in recent years. However, in many occasions, the reported gene deletion fish viral strains were not suitable for vaccine development because of high virulence, low protective effect, or poor replicative capability. Some gene-deleted fish viruses remained to be highly virulent. For example, the knockout of non-virion gene in Snakehead rhabdovirus did not influence viral replication in cultured fish cells, and it had a similar lethality with the parental virus in zebrafish (Alonso et al., 2004). On the contrary, deletion of some genes, such as ORF057R of Ambystoma tigrinum virus (Jancovich and Jacobs, 2011), and VP51(Yu et al., 2017) and VP88 (Yuan et al., 2016) of Singapore grouper iridovirus, resulted in the attenuation of virulence. However, the protective effects of these mutant strains were not mentioned in the articles. Only a few gene deletion strains of fish virus have been reported to protect the host from the parental viruses, but most of their effects were unsatisfactory because the genes knocked out were important for immunogenicity. The protection rate of G gene-deleted Viral hemorrhagic
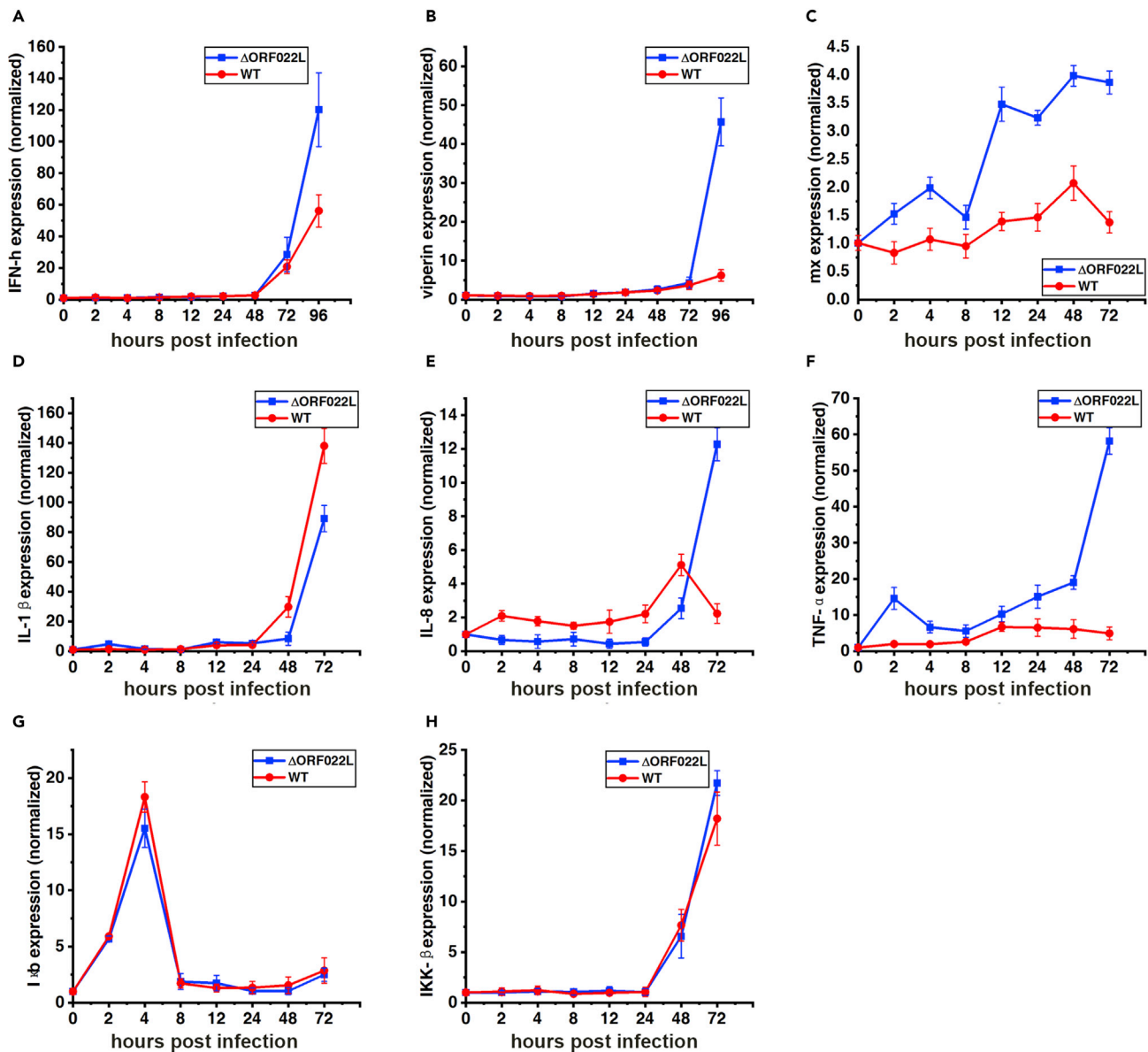
Figure 5. Transcriptional levels of immune-related genes in MFF-1 cells infected with ΔORF022L

(A–H) MFF-1 cells were infected with ΔORF022L or WT. At indicated time points, cell samples were collected, and the expression levels of *IFN-h* (A), *viperin* (B), *mx* (C), *IL-1β* (D), *IL-8* (E), *TNF-α* (F), *Iκb* (G), and *IKK-β* (H) were quantified using qRT-PCR. *β-actin* served as the reference gene. The results are presented as means of *n*=3 independent experiments, and error bars represent SD.

septicemia virus (VHSV) for olive flounder (*Paralichthys olivaceus*) fingerlings was 80% (Kim et al., 2015). Another problem of G gene-lacking recombinant VHSV (rVHSV-ΔG) is its inability to produce infective viral particles and the need to replicate in G gene-expressing cells. Moreover, the G protein expression efficiency could be a limiting factor in viral replication, thereby leading to low vaccine production efficiency. Some mutations resulted in slower replication, and elicited strong, protective immune response in the host, but the vaccine strains had residual virulence. For example, death rates of zebrafish post immunization of $10^{2.5}$, $10^{3.5}$, $10^{4.5}$, and $10^{5.5}$ TCID$_{50}$/ml of the 3′-UTR A4G-G5A variant of VHSV were 5%–13.3%. When challenged with WT VHSV, the relative percent survival in immunized groups ranged from 81.6% to 100%, correlating with vaccination dose (Kim et al., 2016). Therefore, a virus strain with low virulence, high protection, and efficient replication is the key to the development of gene-deleted attenuated vaccine. The Δ56-
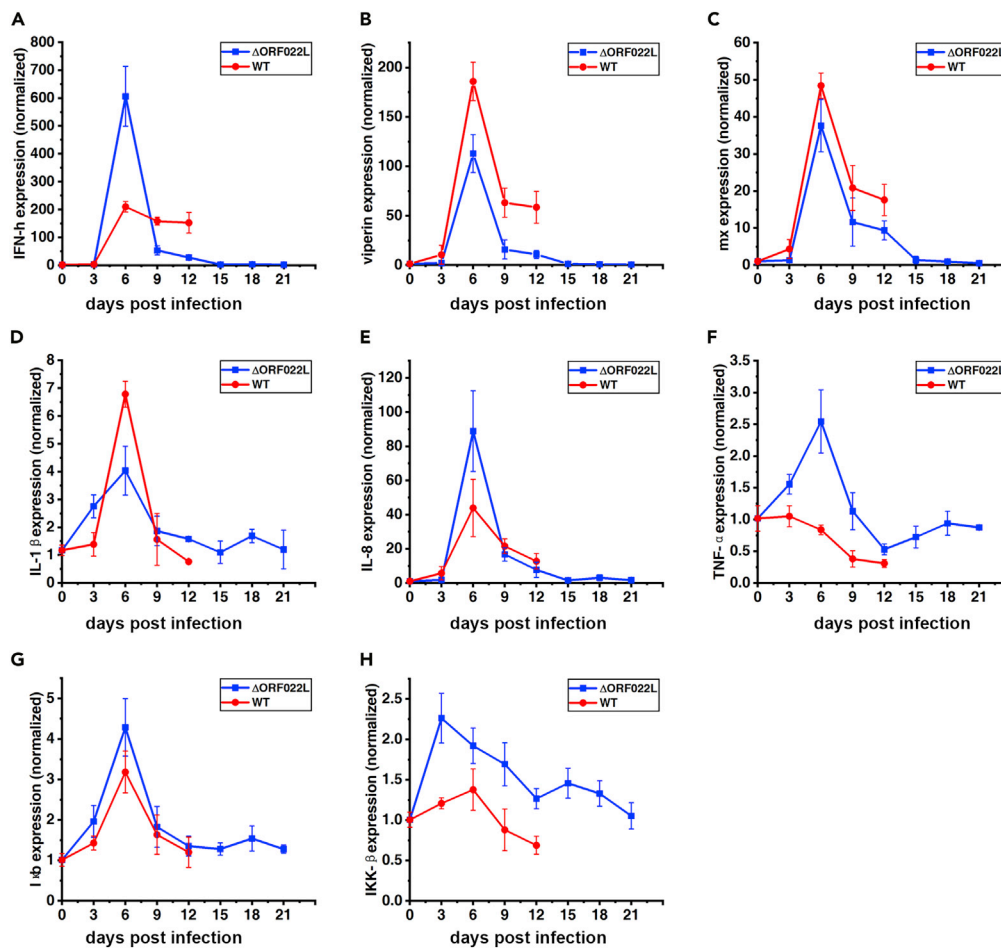
Figure 6. Transcriptional levels of immune-related genes in mandarin fish infected with ΔORF022L

(A–H) Mandarin fish were infected with 1.67 × 10⁸ copies of ΔORF022L or WT. At indicated time points, spleen samples were collected, and the expression levels of *IFN-h* (A), *viperin* (B), *mx* (C), *IL-1β* (D), *IL-8* (E), *TNF-α* (F), *Iκb* (G), and *IKK-β* (H) in the spleen samples were quantified using qRT-PCR. *β-actin* served as the reference gene. The results are presented as means of *n*=3 independent experiments, and error bars represent SD.

57 strain of Cyprinid herpesvirus 3 (CyHV-3) provided a successful example for the development of gene-deleted attenuated vaccine against fish virus; it did not cause any death when infecting carps at a dose of 400 pfu/mL and completely protected the fish from lethal challenge (Boutier et al., 2015). However, no such effective gene-deleted attenuated vaccines were reported for iridoviruses. In the present study, ΔORF022L had the potential to become a candidate vaccine with good safety, protection efficiency, and proliferation ability.

Although ΔORF022L was attenuated and only caused 2% mortality *in vivo*, its immunogenicity was retained. The upregulation of some mandarin fish immune-related genes and the induction of ISKNV-specific antibody by ΔORF022L in mandarin fish were proven. Of importance, ΔORF022L provided complete protection to mandarin fish. At present, the ISKNV vaccine with the best protective effect is the cell-inactivated vaccine developed by Dong et al. (2013), with a protection rate of higher than 90% in the laboratory and the field. Although ISKNV DNA vaccines and subunit vaccines are not commercially applied, their research and development have also progressed well. Fu et al. (2012) developed an *MCP* DNA vaccine with a protection rate of 80%. The subunit vaccine developed by He et al. (2016) contains ORF024R and ORF050L recombinant proteins, with a protection rate of 70%. In comparison with these data, the performance of ΔORF022L was one of the best in protecting against ISKNV, thereby showing high potential as a gene-deleted live attenuated candidate ISKNV vaccine.

Safety is another important problem for gene-deleted live attenuated vaccines, which remained the ability to infect and replicate in the host. Therefore, they have the possibility to spread in the environment and injure the cohabitant fish. Boutier et al. (2015) showed that the double ORF56-57 deletion reduced the ability of CyHV-3 to spread within infected fish and impaired virus transmission from infected fish to naive sentinel fish. In our research, the vaccinated fish cleared the ΔORF022L by 15–18 days post immunization. During this period, ΔORF022L might be released into the environment. To clarify this concern, we monitored the existence of ΔORF022L DNA in the feces as well as the naive cohabitant mandarin fish every 3 days for 30 days post immunization, and no viral DNA could be determined from the above samples (data not shown). Therefore, ΔORF022L did not show transmissibility and was safe enough to be an ISKNV candidate vaccine.

Furthermore, the macro domain exists in many iridoviruses (Figure 1B). Among them, megalocytiviruses have the potential to cause mortalities of nearly 100% in infected fish, and it had a markedly negative effect on commercial aquiculture operations in Asia (Chinchar et al., 2017b). For example, Red sea bream virus has caused mass mortalities in more than 30 species of cultured marine fish in the Western part of Japan since 1991 (Kurita and Nakajima, 2012). Turbot reddish body iridovirus has been identified as the primary cause of viral reddish body syndrome in turbot aquaculture, and it is associated with high transmissibility and mortality (Fan et al., 2010). Considering that the macro domain is highly conserved among different iridoviruses, it has a potential to be a universal target of gene deletion vaccines for iridovirus diseases. The present work could be beneficial to the promotion of the development of gene-deleted attenuated vaccines for viral diseases in fish.

Limitations of the study

An important advantage of gene-deleted live attenuated vaccine is the ability to elicit adaptive cellular immunity of the host. Clarifying whether protection effect of ΔORF022L is dependent on cellular responses could provide an evidence-based guide to ISKNV vaccine development. However, owing to lack of monoclonal antibodies such as anti-CD8$^+$ antibody, which were required for mandarin fish T cell responses determination, our studies did not address this question. Moreover, function of ISKNV ORF022L and the mechanism how ΔORF022L was attenuated have not yet been clarified, remaining an uncertainty for macro domain to be a universal target of gene-deleted live attenuated vaccines against other iridoviruses. In addition, live attenuated vaccines have potential to be delivered through a natural route of infection, thereby providing a vaccination approach compatible with mass vaccination, but further experiments are still required to establish an optimal immune process of the immersion vaccine to acquire satisfied protection effects.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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AUTHOR CONTRIBUTIONS

C.-J.G and J.-G.H. conceived the study. R.-Y.Z. and Y.-F.L. performed the construction and purification of recombinant virus. R.-Y.Z. and W.-Q.P. performed the measurement of characteristics, pathogenicity, and protection effects of ΔORF022L. R.-Y.Z. and Z.-M.L. performed the picture drawing of this article. W.-Q.P., Z.-Y.L., J.H., and S.-P.W. performed fish breeding and sampling. R.-Y.Z. and C.-J.G. wrote the manuscript. All authors discussed the results and commented on the manuscript.

DECLARATION OF INTERESTS

ΔORF022L has applied for the national invention patent of the PR China (No. 2017104662300).

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-mandarin fish IgM	Laboratory of Lin Tianlong	N/A
Mouse monoclonal anti-ISKNV ORF101L	Jia, et al., 2013b	N/A
Bacterial and virus strains		
Infectious spleen and kidney necrosis virus (2006)	Isolated from the mandarin fish (Guo et al., 2011)	N/A
Infectious spleen and kidney necrosis virus (2017)	Isolated from the mandarin fish (Lin et al., 2019)	N/A
ISKNV ΔORF022L	This paper	N/A
Chemicals, peptides, and recombinant proteins		
Puromycin	Gibco™	A1113803
Critical commercial assays		
FuGENE® HD transfection reagent	Promega	E2311
SV total RNA isolation system	Promega	Z3101
GoScript reverse transcription system	Promega	A5001
Experimental models: cell lines		
Mandarin fish fry cells (MFF-1)	Dong et al., 2008	N/A
Experimental models: organisms/strains		
Mandarin fish (*Siniperca chuatsi*)	Jinji Fish Farm, Foshan, Guangdong, China	N/A
Oligonucleotides		
See Table S1 for primers used for ISKNV genes amplification	This paper	N/A
See Table S2 for primers used for real-time quantitative RT-PCR	This paper	N/A
Recombinant DNA		
pUC19-*rfp-puro* vector	This paper	N/A
ISKNV ORF022L transfer vector	This paper	N/A
Software and algorithms		
ImageJ	ImageJ	https://imagej.nih.gov/ij/download.html, RRID:SCR_003070
DNAman version 5.0	Lynnon Biosoft	https://www.lynnon.com/dnaman.html
Molecular Evolutionary Genetics Analysis software version 5.0	MEGA	https://www.megasoftware.net/, RRID:SCR_000667
SPSS version 10.0	IBM	https://www.ibm.com/cn-zh/analytics/spss-statistics-software, RRID:SCR_019096

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Guo Changjun (gchangj@mail.sysu.edu.cn).

Materials availability

All unique/stable reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

Data and code availability

All data reported in this paper will be shared by the lead contact upon request.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines

MFF-1 cells (Dong et al., 2008) were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (Hyclone, USA) under humidified atmosphere containing 5% CO_2 (Jia et al., 2013a).

Virus strains

The WT ISKNV strain used for the construction of ΔORF022L was separated from mandarin fish in 2006 (Guo et al., 2011) and stored in the laboratory, while that used in the challenge experiment was obtained in 2017 (Lin et al., 2019).

Animals

Mandarin fish (2 months old, premature, and weigh ∼100 g) were obtained from the fish farms in Foshan, Guangdong, China. Both male and female fish were included in the experiments, and sex had no influence on this study. All animal experiments were performed in accordance with the regulation for animal experimentation of Guangdong Province, China, and were permitted by the Ethics Committee of Sun Yat-sen University.

METHOD DETAILS

Sequence analysis

Sequence homology was obtained using SMART (http://smart.embl-heidelberg.de/). Sequence alignments were performed using the DNAman software. The phylogenetic tree was constructed using the Bootstrap Neighbor-Joining method of the Molecular Evolutionary Genetics Analysis software version 5.0 program. Bootstrap sampling was reiterated by 1000 times.

Construction of ISKNV ΔORF022L transfer vector

A pUC19-*rfp*-puro vector was constructed by successively cloning *rfp* and *puro* under the control of the CMV promoter into the pUC19 vector. The left and right genome fragments of approximately 1000 bp that flank the ISKNV ORF022L gene were amplified through PCR by using the primers listed in Table S1 and cloned into the pUC19-*rfp*-puro vector upstream and downstream of *rfp* and *puro* to construct the ISKNV ORF022L transfer vector.

Construction of ISKNV ΔORF022L deletion recombinant viral strain

Cells were inoculated into six-well cell culture plates and transfected with ISKNV ORF022L transfer vector by using the FuGENE HD transfection reagent (Promega, USA). At 24 h post-transfection, the cells were infected with WT ISKNV at a multiplicity of infection (MOI) of 0.02 to generate recombinant viruses. After five blind passages of puromycin (at a concentration of 2 μg/mL) selection, the red fluorescent plaques formed by recombinant viruses were picked out under an inverted fluorescence microscope, and these processes were repeated three times to obtain the purified recombinant viral strain, which was named ΔORF022L. The thorough replacement of ORF022L by the tag genes was confirmed *via* PCR by using ORF022L outer primers (listed in Table S1), and resequencing was performed to further prove the purification of ΔORF022L. For stability analysis, ΔORF022L was passaged in MFF-1 cells for 10 generations, and the genome of ΔORF022L was extracted for PCR determination by using ORF022L outer primers.

Structure of ΔORF022L

The cells were fixed with glutaraldehyde fixing solution for electron microscopic section at 3 days post-infection with WT ISKNV or ΔORF022L strain at an MOI of 0.02. The sections were observed *via* negative staining TEM measurements (Huang et al., 2018).

Viral growth kinetics in MFF-1 cells

Cells were cultured in six-well plates and infected with WT ISKNV or ΔORF022L at an MOI of 0.01. At 1–7 days post infection, samples were collected by repeated freezing and thawing and analyzed for virus titers *via* TCID$_{50}$ assays by using Reed–Muench methods.

Immunization experiment of ΔORF022L

Groups of mandarin fish (*n*=60) received ΔORF022L or WT at various doses (1.67×10^8, 3.34×10^7, or 3.34×10^6 copies) by intraperitoneal injection. The fish (*n*=60) injected with sterile DMEM were used as the mock-treated group. The mortality ratios were calculated in a 28-day period. Then, 28 days post-infection, the fish from ΔORF022L and mock-treated groups (*n*=50) were challenged with the virulent ISKNV at a dose of 1.67×10^8 copies, and the mortality ratios were calculated over a 28-day period. For examination of long-lasting protective effects, 20 fish were intraperitoneally injected with ΔORF022L at a dose of 1.67×10^8 copies and challenged with 1.67×10^8 copies of WT ISKNV at 6 months post immunization. The mortality ratios were calculated over a 28-day period. For evaluation of the minimum effective dosage, 15 fish were immunized with ΔORF022L at a dose of 1.67×10^5, 1.67×10^4 or 1.67×10^3 copies and then challenged with WT ISKNV on 21 days post-infection at a dose of 1.67×10^8 copies, and the mortality ratios were calculated over a 28-day period.

Histopathological section of hematoxylin-eosin (H&E) staining

Mandarin fish were infected with ΔORF022L, WT, or DMEM *via* intraperitoneal injection at a dose of 1.67×10^8 copies. 12 days post infection, spleen samples were collected and cut into fragments of approximately 3 mm^3. After fixing with formalin and acetic acid fixative for 24 h, the tissue fragments were washed with phosphate-buffered saline two times and preserved using 70% ethanol. Ethanol solutions (70%, 80%, 95%, and anhydrous) were sequentially applied for dehydration, and the tissue fragments were embedded by paraffin before sectioning. The slices were stained with H&E and became permanently reservable after drying, dewaxing, and hydration. The slices were then observed under the microscope. The numbers of enlarged cells were quantified in three random sights of each sample under the microscope with 200× magnification.

Determination of viral replication in Mandarin fish

Mandarin fish were infected with ΔORF022L or WT ISKNV *via* intraperitoneal injection at a dose of 1.67×10^8 copies. Prior to viral infection and every 3 days post-infection, spleen samples with similar weights were collected from the fish and homogenized. The viral titer was measured using TCID$_{50}$ assay to determine the viral replication in the spleen samples (Reed and Muench, 1938).

Expression levels of genes flanking ORF022L

MFF-1 cells were infected with 0.02 MOI of ΔORF022L or WT. At 7 days post infection, RNA samples were extracted from the cells and reverse transcribed into cDNA. The expression levels of ORF019R, ORF20L, ORF021L, ORF022L, ORF023R, and ORF024R were quantified *via* qRT-PCR methods (He et al., 2019), as described previously. The primers used are listed in Table S2.

Expression levels of immune-related genes induced by ΔORF022L in MFF-1 cells and mandarin fish

To determine the expression levels of the immune-related genes (*IFN-h, viperin, mx, IL-1b, IL-8, TNF-a, Iκb* and *IKK-b*) post-infection, cells were cultured in six-well plates and then infected with WT ISKNV or ΔORF022L at an MOI of 0.02. Cell samples (*n*=3) were collected at 0, 2, 4, 8, 12, 24, 48, and 72 h post-infection. RNAs were extracted from the samples by using an SV total RNA isolation system (Promega, USA) and then reverse-transcribed into cDNAs by using the GoScript reverse transcription system (Promega, USA). The levels of immune-related genes in the cDNA samples were quantified *via* qRT-PCR methods, with mandarin fish *β-actin* as the reference gene. Mandarin fish were treated with WT ISKNV or ΔORF022L (20 fish per group) at a dose of 1.67×10^8 copies to determine the expression levels of the above immune-related

genes after infection with ΔORF022L. Spleen samples (*n*=3) were collected at 0, 3, 6, 9, 12, 15, and 18 days post-infection, with three fish samples per day from each group. The levels of the immune-related genes (*IFN-c*, *IFN-h*, *IFN-d*, *IL-18*, *IgM*, *TNF-a*, and *IL-1*) were quantified *via* qRT-PCR methods, as described previously (He et al., 2019). The primers used are listed in Table S2.

Neutralization assay

Serum samples (*n*=5) were collected from fish that received ΔORF022L at a dose of 1.67×10^8 copies or DMEM in the immunization experiment on 15 days post immunization. 10 μL of ISKNV suspension (at a concentration of 1.67×10^9 copies/mL) was incubated with 10 μL of non-diluted or 1:5, 1:10, and 1:20 diluted (in DMEM) serum samples for 14 h at 4°C. Subsequently, the mixture was added to the MFF-1 cells cultured in a six-well plate for 72 h. The numbers of enlarged cells were quantified in three random sights of each sample under the microscope with 200× magnification. For qRT-PCR assay, RNA samples were extracted from the cell and then reverse transcribed to cDNA at 72 h post-infection. The ISKNV *MCP* in the cDNA samples was quantified using qRT-PCR. At 72 h post-infection, cell samples were collected using sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer and boiled for Western blot analysis to determine the viral protein in MFF-1 cells (Reed and Muench, 1938). ISKNV ORF101L, which is a structural protein and one of the most abundant proteins in ISKNV, was used to represent the viral load (Jia et al., 2013b), and GAPDH was used as the reference protein (Liu et al., 2014). For microneutralization assay, serum samples were serially diluted with DMEM in two-fold steps. The diluted serums were mixed with ISKNV suspension of 100 $TCID_{50}$ in 96-well plates at a ratio of 1:1, followed by overnight incubation at 4°C. Then, 2×10^4 MFF-1 cells were added to the serum–virus mixture, and the plates were incubated for 5 days at 27°C in a 5% CO_2 incubator. The wells with enlarged cells were recorded under the microscopes, and the neutralization titers were expressed as MN_{50}, which is the reciprocal of the serum dilution capable of neutralizing 50% of the test virus dose (Stephenson et al., 2020). Statistical analysis was performed using SPSS version 10.0. ANOVA was performed by comparing the data from ΔORF022L-infected fish with those from the mock-infected ones. A *p*-value of <0.05 was considered statistically significant.

ISKNV-specific IgM determined *via* ELISA

Serum samples (*n*=6) were collected from fish on 0, 3, 6, 9, 12, 15, 18, and 21 days post-injection of ΔORF022L, WT or DMEM at a dose of 1.67×10^8 copies. The ISKNV-specific antibody response was determined using ELISA. In brief, ELISA plates (Nunc Maxisorp) were coated with purified ISKNV in carbonate-bicarbonate buffer under pH 9.8 at 4°C overnight. The ISKNV-coated plates were then blocked by 5% goat serum in PBST overnight at 4°C. Subsequently, the plates were loaded with serum samples diluted at 1:100 in 5% PBST for 2 h at room temperature. The plates were washed, and ISKNV-specific antibodies were detected by successive hybridization with mandarin fish IgM-specific antibodies and horseradish peroxidase-labeled goat anti-mouse antibodies. Absorbance (optical density) was measured at 405 nm using BIOTEK ELx808 ELISA reader.

QUANTIFICATION AND STATISTICAL ANALYSIS

Sequence homology was obtained using SMART (http://smart.embl-heidelberg.de/). Sequence alignments were performed using the DNAman software. The phylogenetic tree was constructed using the Bootstrap Neighbor-Joining method of the Molecular Evolutionary Genetics Analysis software version 5.0 program. Bootstrap sampling was reiterated by 1000 times. The numbers of enlarged cells were quantified in three random sights of each sample under the microscope with 200× magnification. Western blot analysis result was quantified using ImageJ software. Statistical analysis was performed using SPSS version 10.0. ANOVA was performed by comparing the data from ΔORF022L-infected fish with those from the mock-infected ones. A *p*-value of <0.05 was considered statistically significant.

ADDITIONAL RESOURCES

This study has not generated or contributed to a new website/forum.