**Tetraodon nigroviridis** as a nonlethal model of infectious spleen and kidney necrosis virus (ISKNV) infection

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**A B S T R A C T**

Infectious spleen and kidney necrosis virus (ISKNV) is the type species of the genus Megalocytivirus, family Iridoviridae. We have previously established a high mortality ISKNV infection model of zebrafish (*Danio rerio*). In this study, a nonlethal Tetraodon nigroviridis model of ISKNV infection was established. ISKNV infection did not cause lethal disease in Tetraodon but could infect almost all the organs of this species. Electron microscopy showed ISKNV particles were present in infected tissues. Immunofluorescence and quantitative real-time PCR analysis showed that nearly all the virions and infected cells were cleared at 14 d postinfection. The expression profiles of interferon-γ and tumor necrosis factor-α gene in response to ISKNV infection were significantly different in Tetraodon and zebrafish. The establishment of the nonlethal Tetraodon model of ISKNV infection can offer a valuable tool complementary to the zebrafish infection model for studying megalocytivirus disease, fish immune systems, and viral tropism.

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**Introduction**

Iridoviruses are a group of icosahedral cytoplasmic DNA viruses with circularly permuted and terminally redundant DNA genomes that can infect invertebrates and poikilothermic vertebrates, including insects, fish, amphibians, and reptiles (Williams, 1996; Darai et al., 1983, 1985; Delius et al., 1984; Tidona and Darai, 1997). The family Iridoviridae is divided into five genera: Iridovirus, Chloriridovirus, Ranavirus, Lymphocystivirus, and Megalocytivirus (Chinchar et al., 2005). Megalocytivirus infection is characterized by marked hypertrophy of infected cells and is one of the most harmful viruses to cultured fish (Chinchar et al., 2005; Inouye et al., 1992; Jung and Oh, 2000). Infectious spleen and kidney necrosis virus (ISKNV), the causative agent of a disease causing high mortality in mandarin fish, *Siniperca chuatsi*, is regarded as the type species of the Megalocytivirus genus (Chinchar et al., 2005). ISKNV-like viruses can also infect large-mouth bass, *Micropterus salmoides*, Murray cod, *Maccullochella peeli peeli*, and more than 60 other species of marine and freshwater fish (Go et al., 2006; Wang et al., 2007; Jeong et al., 2008). Cell hypertrophy in spleen, kidney, cranial connective tissues, and endocardia is the typical sign of ISKNV infection in mandarin fish (He et al., 2000, 2002). The genome of ISKNV (GenBank accession no. AF371960) is highly methylated and was sequenced in 2001 (He et al., 2001). We have previously reported a zebrafish (*Danio rerio*) model of ISKNV infection, which is the first established DNA virus-zebrafish infection model (Xu et al., 2008). ISKNV infection of zebrafish results in high mortality and causes similar symptoms, such as organ hemorrhage and cell hypertrophy, to those of mandarin fish. Both the zebrafish and ISKNV genomes have been sequenced; thus, the ISKNV-infected zebrafish model is likely to prove very useful in the study of the interactions between ISKNV and its host.

The green-spotted pufferfish, *Tetraodon nigroviridis*, has the smallest known vertebrate genome (~350 Mb) (Roest Crollius et al., 2000b; Volff et al., 2003). The genome of *Tetraodon* was sequenced in 2004 (Jallon et al., 2004). The genome catalogue and gene structures of *Tetraodon* are very similar to those of humans and zebrafish. With short introns and small intergenic regions, the *Tetraodon* genome is highly compact, being eight times smaller than that of humans and four times smaller than that of zebrafish (Volff, 2005; Roest Crollius et al., 2000a). These advantages make *Tetraodon* ideal for genomic studies, which may help in the annotation and functional studies of genes in other species (Lutfalla et al., 2003). Furthermore, *Tetraodon* is potentially a novel model fish, in addition to zebrafish, for further use in various research areas, such as developmental biology, immunology, physiology, biochemistry, genetics, and evolutionary biology (Bui et al., 2010; Clelland et al., 2010; Sepulcre et al., 2009).

In this study, we established an ISKNV-*Tetraodon* infection model. ISKNV can successfully infect *Tetraodon* cells. This is the first report of virus infection in *Tetraodon*. The infected cells were present in almost all organs, especially in the spleen and kidney. However, ISKNV infection...
did not cause death in *Tetraodon*, and nearly all the ISKNV-infected cells were cleared at 14 d postinfection (dpi), suggesting that *Tetraodon* can resist the lethality of ISKNV infection. The expression of interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) was significantly different in the ISKNV-infected *Tetraodon* and zebrafish, which may help to explain why *Tetraodon* can survive ISKNV infection while zebrafish cannot. Complementary to zebrafish, the *Tetraodon* model of ISKNV infection may serve as an important platform for the studies of megalocytivirus disease, fish immune systems, and viral tropism.

**Result**

**Clinical signs**

We found that *Tetraodon* was hard to be infected by challenge using immersion method. The experiments to challenge *Tetraodon* using intraperitoneal injection method have been repeated many times using different viral doses. No *Tetraodon* died after challenging with all these low or high doses. We found that challenging with low dose of virus, the cell infection rates in *Tetraodon* tissues were also low (data not shown). In order to obtain a high tissue infection rate, the viral dose was normalized to 25 μL/g body weight of ISKNV at 4×10^{11} genome equivalents (GE)/mL. Following inoculation with this dose, a concentration that was approximately double that used in the establishment of zebrafish infection (Xu et al., 2008), no *Tetraodon* died from ISKNV infection in the 6 weeks period postinoculation, whereas all the infected zebrafish were dead by 9 dpi (Fig. 1). Compared with the control fish, the spleen and intestine of the challenged *Tetraodon* showed obvious signs of enlargement at 8 dpi, and return to normal after 14 dpi.

**Histopathology and electron microscopy**

Histopathology studies by comparing the spleens and kidneys of the control and ISKNV-infected fish at 8 dpi revealed that the spleens of control fish contained normal white and red pulp regions and brown granules present in the areas involved in erythrocyte phagocytosis (Fig. 2A). In contrast, the spleens of ISKNV-infected *Tetraodon* showed signs of hyperemia and reduction of the areas containing brown granules (Fig. 2B). In the kidneys of infected fish (Fig. 2C), the glomeruli showed slight shrinkage, and the areas between renal tubules and glomeruli appeared less tightly arranged compared to those of control fish (Fig. 2D). Unlike in mandarin fish (He et al., 2000, 2002), only a few hypertrophic cells can be stained by hematoxylin in the spleens and kidneys of ISKNV-infected *Tetraodon* (Figs. 2B and D, blue arrows). Other organs showed no significant signs of pathological changes. Histological changes in infected *Tetraodon* tissues were milder than those observed in mandarin fish and zebrafish (He et al., 2000, 2002; Xu et al., 2008).

The spleen sections of ISKNV-infected *Tetraodon* at 8 dpi were examined by electron microscopy (EM). Discrete areas containing abundant virus particles were observed in infected cells (Fig. 2E). The ISKNV particles were approximately 150–200 nm, the same size as described in the infected mandarin fish cells (He et al., 2000, 2002). The mature virions had cores and were located along side immature virus particles that appeared to be undergoing assembly (Fig. 2F).

**Tissues distribution of the infected cells**

The paraformaldehyde-fixed, paraffin-embedded sections of organs from *Tetraodon* at 8 dpi were stained by immunohistochemistry using the polyclonal mouse antiserum against viral protein VP23R, as previously described (Xu et al., 2008). Like in zebrafish, the ISKNV-infected cells were present in almost all organs of the infected *Tetraodon* (Fig. 3) with an exception of the skin tissues where the infected cells were difficult to be found (Fig. 3L). Interestingly, the infected cells showed different locations in the kidney at 8 and 12 dpi. At 8 dpi, the infected cells existed in the gap regions between renal tubules and inside some of the renal glomeruli but not in the epithelia region of renal tubules (Fig. 3B). At 12 dpi, more infected cells were found inside the renal glomeruli than in the gap regions, and the infected cells were also found inside the lumen of the renal tubules (Fig. 3C).

**ISKNV infection kinetics in Tetraodon tissues**

To study features of ISKNV infection and replication in *Tetraodon* at different stages of infection, double-stain immunofluorescence analysis was performed with VP23R protein visualized in green fluorescence under stimulation of 488 nm light and major capsid protein (MCP) visualized in red fluorescence under 633 nm light stimulation. Infected spleens were sampled from 3 to 14 dpi (Fig. 4A). The number of the infected cells increased from 3 dpi to 8 dpi. After peaking at 8 dpi, the number of the infected cells began to decrease until almost all the infected cells were cleared at 14 dpi. The red fluorescence signals representing MCP antigen began to appear at 6 dpi, and the release of virus particles from infected cells (Fig. 4A, white arrows) was observed at 8 dpi, peaked at 10 dpi, and nearly all the MCP signals disappeared after 12 dpi. As controls, the infected spleens were detected using mock-immunized sera and the mock-infected spleens were detected using anti-VP23R and anti-MCP specific antibodies. No fluorescent signals were observed (data not shown). As a comparison, immunofluorescence analyses demonstrated that the number of the infection cells in zebrafish spleens also reached a peak at 8 dpi (Fig. 4A). All the infected zebrafish were dead by 9 dpi and did not undergo a recovery period. MCP antigen signals were less abundant in zebrafish spleens than in *Tetraodon*.

Consistent with the results of immunofluorescence analyses, the quantitative analysis of ISKNV GE using quantitative real-time PCR revealed variations in the quantities of virus DNA present in the infected spleens of *Tetraodon* and zebrafish at various time points postinfection (Fig. 4B). In *Tetraodon* spleens at 1–3 dpi, the quantity of viral DNA was extremely low (<8.55×10^{5} GE/μg DNA). Viral DNA subsequently increased in concentration up to a maximum of 3.17×10^{7} GE/μg DNA at 10 dpi. The quantity of viral DNA then decreased rapidly to 3.94×10^{5} GE/μg DNA at 14 dpi (Fig. 4B). In zebrafish spleens, the concentrations of ISKNV DNA also showed an increase during infection and reached a maximum of 7.52×10^{6} GE/μg DNA at 8 dpi. The transcription of the VP23R and MCP genes of ISKNV was also examined in infected spleen cells at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, and 14 dpi by quantitative real-time reverse transcription–PCR (RT–PCR) (Fig. 4C). Periodic expression of the VP23R gene was detected with
peaks at 2 and 6 dpi, but the expression level of the second peak at 6 dpi was only 29.5% of that of the first peak at 2 dpi. MCP transcription was barely detectable before 3 dpi. At 3–4 dpi, MCP transcription increased sharply and reached a peak value at 5 dpi and then declined to the basal level after 10 dpi.

Expression profile analysis of immune-related genes

Expression of antiviral immune-related genes in the ISKNV-infected zebrafish and Tetraodon was determined by quantitative real-time RT–PCR (Fig. 5). Following inoculation, the expression of Tetraodon type I IFN gene was upregulated by 9–10 folds, with two peaks at 16 h postinfection (hpi) and 4 dpi. The expression of zebrafish type I IFN gene increased 7–14 folds, with three peaks at 4 and 24 hpi, and 3 dpi. The magnitude of the changes in IFN-α expression in the two fishes was similar, although the temporal patterns were slightly different. Compared with the controls, the expression of zebrafish IFN-γ gene showed no apparent change, whereas Tetraodon IFN-γ transcription was significantly upregulated periodically, with the first peak of 18-fold increase at 16 hpi and the second peak of 52– to 54-fold increase at 3 to 4 dpi. The TNF-α expression in Tetraodon exhibited a 3-fold increase at 4 hpi and then returned to the basal level at 24 hpi where it remained unchanged. In contrast, the expression of zebrafish TNF-α changed markedly after infection, showing a sharp increase at 24 hpi which was maintained at 67– to 43-fold levels of up-regulation up to 2 dpi, followed by a sharp decline at 3 dpi.

Discussion

Virus infection has not yet been reported in Tetraodon. The establishment of a nonlethal Tetraodon model of ISKNV infection filled this gap. As an ideal genomic study model organism, Tetraodon has such a compact and sequenced genome that makes it easy to identify the immune-related genes and characterize their modulation roles in the process of antiviral immunity. ISKNV can cause high mortality in zebrafish but does not cause death in Tetraodon, so these two infection models may be complementary in the study of ISKNV infectious disease, greatly facilitating studies of the fish immune system, ISKNV gene function, lethality mechanisms of megalocytiviruses, and virus–host interactions. Tetraodon survived ISKNV infection despite being challenged with a high titer of virus particles. The pathological changes in infected Tetraodon tissues were milder than those of mandarin fish and zebrafish, although cell infection rates in the Tetraodon tissues were similar to
those observed in mandarin fish and zebrafish hosts (He et al., 2000, 2002; Xu et al., 2008). The prevalence of infection in cells of Tetraodon tissues reached a peak at 8 dpi, when the tissues exhibited obvious pathological changes, and then decreased rapidly to a very low level at 14 dpi, when the clinical signs of infection also disappeared. This indicates that Tetraodon was able to clear ISKNV infection. Changes in the quantities of ISKNV genomic DNA in spleen tissues during the infection also support this conclusion. ISKNV genomic DNA concentration peaked at 10 dpi and then almost disappeared at 14 dpi, suggesting that the infection had been cleared.

It has been speculated that ISKNV can spread through the host circulation system (Xu et al., 2008). ISKNV-infected hypertrophic cells were present in almost all zebrafish organs, including spleen, kidneys, liver, gills, esophagus, gut, muscles, and eyes (Xu et al., 2008). In Tetraodon, we also observed ISKNV infecting almost all organs, suggesting that ISKNV-susceptible cells may be common in Tetraodon and zebrafish. The infected cells in the kidney of Tetraodon were mainly present in renal glomeruli at 12 dpi when most infected cells had disappeared in the gap areas between renal tubules. Renal glomeruli are blood capillary-rich areas; therefore, this observation supports the idea that ISKNV spreads through the circulation system. The infected cells localized in the renal tubule lumens may be derived from renal glomeruli.

ISKNV infection of Tetraodon is a nonlethal model and of zebrafish is a high-mortality model; therefore, the clinical signs and pathological

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Fig. 3. Immunohistochemical examination of Tetraodon infected with ISKNV by i.p. injection at 8 dpi (magnification, ×400). The Tetraodon sections were incubated with polyclonal mouse antisera against viral protein VP23R and developed using diaminobenzidine (DAB) mixture. The ISKNV-infected cells were specifically stained brown (black arrows). (A) spleen, (B) kidney (at 8 dpi), (C) kidney (at 12 dpi), (D) liver, (E) heart, (F) digestive tract, (G) eyes, (H) brain, (I) sex gland, (J) gill, (K) muscle, and (L) skin.

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Fig. 4. (A) Double-stain immunofluorescence analysis of spleens from ISKNV-infected Tetraodon and zebrafish. Infected cells were labeled with Alexa Fluor 488 (Invitrogen, USA) for VP23R (green fluoresence) and the ISKNV particles were labeled with Alexa Fluor 633 (Invitrogen, USA) for MCP (red fluorescence). White arrows indicate the released virions. (B) Quantitative real-time PCR analysis of ISKNV GE in the spleen tissues of infected Tetraodon and zebrafish at various time points postinfection. (C) Quantitative real-time RT-PCR analysis of ISKNV VP23R and MCP mRNA expression in ISKNV-infected Tetraodon at 12, 24, and 36 hpi and 2, 3, 4, 5, 6, 8, 10, 12 and 14 dpi.

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changes in tissues and the behavior of virus inside the cells of the two species differ significantly. In zebrafish, MCP synthesis, viral particle assembly, and release were blocked to a certain extent due to still unknown mechanisms (Xu et al., 2008). MCP antigen signals were more abundant in Tetraodon tissues than in zebrafish, and the released virions were readily detected in Tetraodon at 10 dpi, suggesting that Tetraodon cells may be more suitable for replication and maturation of ISKNV particles than zebrafish cells. This is probably because Tetraodon is phylogenetically closer to mandarin fish than zebrafish. However, Tetraodon experienced no mortality following ISKNV infection, whereas

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mortality of the infected zebrafish was high (Xu et al., 2008), indicating that replication and release of virions are unlikely to be main reasons for host death. Infection rates of Tetraodon cells were much higher and peaked at 8 dpi, when the infected organs showed slight signs of disease. However, no death of the host occurred, suggesting that cell infection rate is also unlikely to be directly related to host mortality. Similarly, for the neuroviroient Sindbis virus (SINV), viral replication and infection rate also have little to do with host death. In this case, the failure of the host immune response, but not the proliferation of the SINV infection itself, is the cause of host death (Rowell and Griffin, 2002). Further studies are needed to elucidate the mechanisms that modulate the lethality of ISKNV infection.

The antiviral immune responses against ISKNV of zebrafish and Tetraodon were subjected to preliminary comparison by quantitative real-time RT–PCR. The expression profiles of three antiviral immune-related genes, including type I and II IFN and TNF-α, in response to ISKNV infection were examined and compared. The response profiles of type I IFN genes, which showed periodic up-regulation that may relate to the virus replication cycle, were similar in both hosts, whereas those of type II IFN (IFN-γ) showed a significant difference. ISKNV induced IFN-γ expression at a much higher level in Tetraodon than in zebrafish. IFN-γ has antiviral activity and is also an immunomodulatory protein that can activate Th1 cells to initiate cellular immunity (Novelli and Casanova, 2004; Dardalhon et al., 2008; Templeton and Perlman, 2008). It has been reported that cellular immunity plays a crucial role in protecting fish against red seabream iridovirus (RSIV), another important member of the Megalocytivirus genus (Caipang et al., 2006).

Thus, dramatically upregulated expression of IFN-γ following infection can probably explain why infected cells in Tetraodon can be completely cleared without causing serious adverse effects on the host.

ISKNV infection can induce much higher transcription levels of the TNF-α gene in zebrafish than in Tetraodon. It is well known that TNF-α plays a critical role in proinflammation and immunoregulation and can partially mediate cellular immunity against infections of bacteria, parasites, and viruses (Bradley, 2008; Cawthorn and Sethi, 2008; Wilhelm et al., 2005). However, overexpression of TNF-α has a toxic effect on normal tissues and can cause severe pathological effects (Katagiri et al., 2008; Kudo et al., 2009; Song et al., 2008). In dengue virus-infected patients, the elevation of TNF-α expression results in endothelial cell apoptosis, vascular leakage, and hemorrhage (Atrasheuskaya et al., 2003; Chen et al., 2007; Martina et al., 2009). Interestingly, organ hemorrhage is one of the typical signs of megalocytivirus infection (Chinchar et al., 2005). A possible explanation for this is that overexpression of TNF-α in zebrafish and suppression of it in Tetraodon resulted in more severe tissue damage in zebrafish than in Tetraodon. This may also be related to the high mortalities of infected zebrafish. This hypothesis requires further study. Moreover, the expression profiles of TNF-α in infected zebrafish showed sharp changes from 16 to 48 hpi. Further studies are also required to determine if the 32-h period of high expression of TNF-α causes adverse effects on the infected host.

The difference in the expression of the IFN-γ and TNF-α in the two fish may help to explain why Tetraodon can survive ISKNV infection while zebrafish cannot. These results support the idea that cytokines play an important role in viral tropism (McFadden et al., 2009). Then, the ISKNV infection models of Tetraodon and zebrafish may also provide a useful platform for studies of viral tropism. Moreover, further studies on the comparison of other immune-related genes and signal pathways in the two fish should be performed to identify possible prevention and cure measures for megalocytivirus infection.

Materials and methods

Fish and virus

Wild-type Tetraodon, weighing about 10 g each, were bought from a pet market in Guangzhou, China. Fish were maintained in clean water containing 1.5% sea salt in a 40-L tank with recirculating and filtering units and a 12-h light/12-h dark cycle at 28 °C and fed with chironomid larvae. Each tank contains 30 fish. Wild-type zebrafish were maintained as previously described (Xu et al., 2008). Fish were maintained more than 10 days before the experiment. Before infection experiments, fish were confirmed free of ISKNV infection by PCR as previously described (Deng et al., 2000; Wang et al., 2007). Briefly, 10% cultured fish were randomly sacrificed and the spleens were sampled. DNA from each sample was extracted as a template. The first-step PCR was performed using ISKNV MCP-specific primers (mF1, 5′-AGACCACTGTGAGCGC-3′; mR1, 5′-CCATGTCGAACTGACGGC-3′). Nested PCR was performed using primer mF2 (5′-CGTGAGACCGTGCGTACG-3′) and mR2 (5′-AGGGTGACGGTCGA-3′). Each sample was detected in triplicates to confirm the result.

ISKNV was purified from diseased mandarin fish identified in our laboratory (He et al., 2002) and propagated in a cultured mandarin fish fry cell line (MFF-1) (Dong et al., 2008). The supernatants of ISKNV infected cells at 6 dpi were collected and ISKNV particles were purified by sucrose gradient centrifugation. The residual sucrose was eliminated by ultrafiltration using a 100-kDa cutoff membrane (Millipore, USA). Purified ISKNV particles were quantitated by quantitative real-time PCR as previously described (Xu et al., 2008) and diluted in phosphate-buffered saline (PBS, pH 7.4) to 4×10^11 ISKNV GE/mL. The titer of the virus in diluted PBS was determined in MFF-1 cells as previously described (Dong et al., 2008) as 10^{-2}-2% tissue culture infective dose (TCID_{50})/0.1 mL.

To determine the mortality of Tetraodon caused by ISKNV infection, adult Tetraodon were injected intraperitoneally (i.p.) with 25 μg/g body weight of the purified ISKNV particles (infection group, n = 60) or PBS (control group, n = 60) supplemented with 50 IU/mL penicillin and 50 μg/mL streptomycin. As comparisons, 3-month-old zebrafish weighing about 1 g were challenged with 25 μL of the purified ISKNV (infection group, n = 60) or PBS (control group, n = 60) in the same way.

Histology analysis

Spleens and kidneys of 20 ISKNV-infected Tetraodon at 8 dpi were sampled for histology and placed in 10 mL of buffered 10% formalin. The samples were dehydrated by passage through a gradient of ethanol solutions, embedded in paraffin, and cut into 4-μm-thick sections. The sections were stained with hematoxylin and eosin for microscopic examination.

EM analysis

Spleens of 20 ISKNV-infected Tetraodon at 8 dpi were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The specimens were rinsed with 0.1 M phosphate buffer four times and postfixed in 0.1 M phosphate buffer containing 2.0% osmium tetroxide for 1 h at 4 °C and embedded in Epon’s 812 following dehydration. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined on a Philips CM10 EM for the presence of virus particles in hypertrophied cells.

Immunohistochemistry and immunofluorescence analyses

Spleens of ISKNV-infected Tetraodon at 3, 6, 8, 10, 12, and 14 dpi, and zebrafish at 3, 6, and 8 dpi were sampled, fixed with 4% paraformaldehyde, paraffin-embedded, and sectioned. Immunohistochemistry and immunofluorescence analyses of the VP23R and MCP of ISKNV were performed as previously described (Xu et al., 2008). As controls, the ISKNV-infected spleens of Tetraodon at 8 dpi were detected using sera from PBS-mock immunized mice and rabbits, and the PBS mock-infected spleens were also detected using mice anti-VP23R polyclonal antibodies and rabbit anti-MCP polyclonal antibodies.
### Table 1
Analyzed genes and their specific primers.

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TN, Tetraodon nigroviridis; ZF, zebrafish.

*Identified by comparing its encoding product with the IFN-γ protein sequence of zebrafish (GenBank accession no. AB158361) and Takifugu rubripes (GenBank accession no. CAEB2301; identified by Zou et al., 2004).

**Identified by comparing its encoding product with the TNF-α protein sequence of T. rubripes (GenBank accession no. NP_001036974; identified by Savan et al., 2005).**

### Quantitative real-time PCR

A total of 400 Tetraodon and 600 zebrafish were challenged with purified ISKNV in the same way as described in the Fish and virus section. To determine the quantities of ISKNV GE in the infected tissues, the spleens of 15 Tetraodon per sample time were collected at 1, 2, 3, 4, 5, 6, 8, 10, 12, and 14 dpi. As a comparison, spleens of ISKNV-infected zebrafish were sampled at 1, 2, 3, 4, 5, 6, and 8 dpi in the same way. ISKNV in each sample were quantified by quantitative real-time PCR as previously described (Xu et al., 2008). The levels of ISKNV GE per microgram (μg) of tissue DNA were calculated.

For quantitative real-time RT–PCR analysis of levels of gene transcription, Tetraodon spleens at 0, 4, 8, 12, and 16 hpi and 1 and 2, 3, 4, 5, 6, 8, 10, 12, and 14 dpi were sampled. Each sample was collected from 15 fish. As a comparison, spleens of zebrafish were sampled at 0, 4, 8, 12, and 16 hpi and at 1, 2, 3, 4, 5, 6, and 8 dpi. Each sample was collected from 15 fish. As controls, spleens of healthy Tetraodon and zebrafish injected with PBS were sampled in an identical manner to that of the challenged groups. Total RNA of each sample was purified and reverse transcribed to cDNA by reverse transcriptase. Quantitative real-time RT–PCR was performed as previously described (Xu et al., 2008). The genes analyzed and their specific primers are listed in Table 1. Expression levels were normalized to glyceraldehyde phosphate dehydrogenase (GAPDH) gene transcription in both Tetraodon and zebrafish. For types I and II interferon genes and TNF-α gene, the expression levels at 0 hpi were defined as basal levels, and the transcription levels in other samples were calculated based on basal transcription levels. For the VP23R and MCP genes of ISKNV, the expression ratio values of samples were calculated over the highest observed values, which were defined as 100%.

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