Betanodavirus infection in the freshwater model fish medaka (*Oryzias latipes*)

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SUMMARY

Betanodaviruses, the causal agents of viral nervous necrosis in marine fish, have bipartite positive-sense RNA genomes. Because the genomes are the smallest and simplest among viruses, betanodaviruses are well studied using a genetic engineering system as model viruses, like the cases with the insect viruses, alphanodaviruses, the other members of the family *Nodaviridae*. However, studies of virus–host interactions have been limited because betanodaviruses basically infect marine fish at early developmental stages (larval and juvenile). These fish are only available for a few months of the year and are not suitable for the construction of a reversed genetics system. To overcome these problems, several freshwater fish species were tested for their susceptibility to betanodaviruses. We have demonstrated that adult medaka (*Oryzias latipes*), a well-known model fish, is susceptible to both *Striped jack nervous necrosis virus* (the type species of the betanodaviruses) and *Redspotted grouper nervous necrosis virus* which have different host specificity in marine fish species. Infected medaka exhibited erratic swimming and the viruses were specifically localized to the brain, spinal cord, and retina of the infected fish, similar to the pattern of infection in naturally infected marine fish. Moreover, medaka were susceptible to the virus at the larval stage. This is the first report of a model virus–model host infection system in fish. This system should facilitate the elucidation of the mechanisms underlying RNA virus infections in fish.

INTRODUCTION

Medaka (*Oryzias latipes*) is a well-recognized vertebrate model used in various studies of development, genetics, environmental research, and human diseases (Ishikawa, 2000; Wittbrodt *et al.*, 2002). Compared with mammalian models, such as mice and rats, the medaka is small, cost effective, easy to breed in large numbers, prolific, and easily confined. Furthermore, most experimental tools for the analysis of gene function can be applied to medaka. These characteristics also define another model fish, zebrafish (*Danio rerio*).

The development of viral infection systems using model fish has been limited. Experimental infections of zebrafish with infectious hematopoietic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV), spring viremia of carp virus (SVCV), and snakehead rhabdovirus (SHRV) have been reported (Alonso *et al.*, 2004; LaParta *et al.*, 2000; Phelan *et al.*, 2005; Sanders *et al.*, 2003). No experimental viral infection has yet been demonstrated in medaka. In contrast to the genomes of the betanodaviruses, the genomes of these other viruses are relatively complex, and complicated interactions between viral factors and host factors are likely. A model virus with a small and simple viral genome should more easily facilitate the study of virus–host interactions.

Betanodaviruses, members of the family *Nodaviridae*, are the causal agents of a highly destructive disease of hatchery-reared larvae and juveniles of a variety of marine fish. The disease, designated viral nervous necrosis (VNN) when it was first described in 1990 (Yoshikoshi & Inoue, 1990), is also known as viral encephalopathy and retinopathy (Office International des Epizooties, 2003). VNN disease has spread to more than 30 marine fish species from 14 families in the Indo-Pacific and

Mediterranean regions, Scandinavia, and North America. Recently, adult and mature fish have also been reported to suffer from the disease (Munday *et al.*, 2002). The virus localizes in the brain, spinal cord, and retina of the affected fish. Affected fish exhibit erratic swimming patterns and a range of neurological abnormalities, including vacuolization and cellular necrosis in the central nervous system and retina.

Betanodaviruses are nonenveloped, spherical viruses with a bipartite positive-sense RNA genome. The larger genomic segment RNA1 (3.1 kb) encodes an RNA-dependent RNA polymerase (protein A). The smaller genomic segment RNA2 (1.4 kb) encodes the coat protein (CP). Recently, we characterized a subgenomic RNA3 (0.4 kb), which encodes protein B2 having a suppressor function for post-transcriptional gene silencing (Iwamoto *et al.*, 2005). The viruses can be classified into four types, designated *Striped jack nervous necrosis virus* (SJNNV), *Barfin flounder nervous necrosis virus* (BFNNV), *Tiger puffer nervous necrosis virus* (TPNNV), and *Redspotted grouper nervous necrosis virus* (RGNNV), based on similarities in the partial RNA2 sequences encoding the C-terminal halves of the CPs (Nishizawa *et al.*, 1997). The host ranges of SJNNV and TPNNV are limited to striped jack (*Pseudocaranx dentex*) and tiger puffer (*Takifugu rubripes*), respectively, whereas BFNNV has been isolated from some coldwater species, such as barfin flounder (*Verasper moseri*) and Pacific cod (*Gadus macrocephalus*). RGNNV has a broad host range and causes disease among a variety of warm water fish species, particularly groupers and sea bass.

Betanodavirus has key features as a model virus, as is the case with the insect virus, *Alphanodavirus* (Ball & Johnson, 1998), the other genus of the family *Nodaviridae*. *Betanodavirus* has one of the smallest genomes (4.5 kb in total) among the known viruses, which encodes only three viral proteins (protein A, CP, and protein B2).

Furthermore, a genetic engineering system has already been established based on a cDNA-mediated infectious RNA transcription strategy (Iwamoto *et al.*, 2001, 2004). Qualitative and quantitative analysis of virus multiplication is possible with betanodaviruses, using cultured cells. However, studies of virus–host interactions have progressed slowly because of the limited availability of host fish. Betanodaviruses basically infect marine fish larvae and juveniles, which are only available for a few months of the year. Other problems involving marine fish species are their long life cycles, large bodies, and the difficult rearing conditions required, which preclude easy experimentation, and especially the use of genetic engineering strategies.

Recently, we have tested several ornamental freshwater fish for their susceptibility to betanodaviruses. We observed that medaka exhibit disease symptoms following inoculation with the viruses. In this study, we evaluated the susceptibility of medaka to betanodaviruses in detail and discuss the potential of medaka as a viral infection model. This is the first report of the experimental infection of freshwater fish with betanodaviruses.

MATERIALS AND METHODS

Viruses and cells. SJNNV (SJNag93 strain; Iwamoto *et al.*, 2001) and RGNNV (SGWak97 strain; Iwamoto *et al.*, 1999) were used in this study. The E-11 cell line (Iwamoto *et al.*, 2000) was grown at 25 °C in Leibovitz's L-15 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 5 % fetal bovine serum.

Purification of SJNNV and RGNNV virions. The viruses were inoculated into E-11 cells and the cells were incubated at 25 °C for 3–5 days. The culture supernatants of the

infected cells were harvested and mixed with three volumes of 40 % (w/v) polyethylene glycol (PEG 8000; Nacalai Tesque, Kyoto, Japan). The mixtures were incubated on ice for 1 h and centrifuged at 12000 × g for 15 min at 4 °C to sediment the progeny viruses. The sedimented viruses were suspended in extraction buffer (0.1 M Tris-HCl, 1 mM EDTA, pH 7.2) and stored at –80 °C until use. Viral titers were quantified by determining the 50 % tissue culture infective dose ($TCID_{50}$)/ml (Nguyen *et al.*, 1996).

Intramuscular injection of adult medaka. The himedaka, a variety of medaka (*Oryzias latipes*), was used in this study. Adult medaka, weighing 150–300 mg, were purchased from a local ornamental fish market, maintained at 25 °C throughout the experimental period, and fed daily with powdered dry flakes (TetraMin, TetraWelke, Germany). For intramuscular inoculation, the fish were injected with 3 µl of RGNNV ($10^{5.0}$, $10^{7.0}$, or $10^{9.0}$ $TCID_{50}$/ml) or SJNNV ($10^{4.7}$, $10^{6.7}$, or $10^{8.7}$ $TCID_{50}$/ml) suspensions using microsyringes (Hamilton, Reno, NV, USA). Control groups were similarly injected with 3 µl of extraction buffer. Inoculations were performed in quadruplicate with 20 fish per treatment and the inoculated fish were monitored daily for 14 days. Cumulative morbidity was calculated based on the numbers of fish showing abnormal swimming, and the cumulative mortality was recorded.

Bath challenge of adult medaka. For viral exposure by immersion, two groups of 10 adult medaka were maintained at 25 °C in 1 l glass beakers containing 500 ml of aerated water with 10^{10} $TCID_{50}$/l of RGNNV in 500 µl of extraction buffer. Two groups of control fish were exposed to no virus but only extraction buffer. After exposure for 5 h, the fish were transferred to aquaria and were kept at 25 °C for further 14 days to monitor them for abnormal swimming and mortality.

Bath challenge of medaka larvae. Medaka eggs were purchased from a local ornamental fish market and maintained at 25 °C to hatch. Two groups of 50 hatched one-day-old medaka larvae were maintained at 25 °C in 200 ml glass beakers containing 100 ml of water with 10^{10} $TCID_{50}$/l RGNNV and 5 μg/ml kanamycin sulfate. Two equally sized control groups were maintained under the same conditions but with no added virus. The fish were fed daily with powdered dry flakes. The fish were observed for morbidity and mortality for nine days because mortality increased substantially with further maintenance even in the control groups.

Titration of viruses in fish brains. Adult medaka were injected intramuscularly with 3 μl of RGNNV suspension ($10^{7.0}$ $TCID_{50}$/ml) using microsyringes. The brains were recovered from the inoculated fish at 0, 2, 4, 6, 8, 10, and 14 days after inoculation (n = 3 per day) and stored at –80 °C until use. The brain samples were thoroughly homogenized with 500 μl of Hanks' balanced salt solution (Nissui, Tokyo, Japan) and the homogenates were passed through a 0.45 μm membrane filter (Advantec, Tokyo, Japan). Viral titers of the homogenates were quantified as described above.

Histopathology. Fish were immersed in Bouin's solution for one week at 4 °C and then soaked in 15 % trichloroacetic acid for one week at 4 °C. The fish samples were then embedded in paraffin and sectioned to 8 μm. One of two consecutive sections was stained with hematoxylin and eosin (H&E), and the other was subjected to immunofluorescence staining with anti-SJNNV rabbit polyclonal antibody and fluorescein isothiocyanate-conjugated swine immunoglobulin raised against rabbit immunoglobulin (Dako, Copenhagen, Denmark), as described previously (Nguyen *et al.*, 1996). This anti-SJNNV polyclonal antibody has been shown to detect both RGNNV and SJNNV (Iwamoto *et al.*, 2004).

RESULTS

Susceptibility of adult medaka to viruses. Because experimental infection by *Betanodavirus* has never been successfully achieved in freshwater fish, we tested several adult freshwater fish for their susceptibility to betanodaviruses. A few freshwater fish, including medaka, exhibited erratic swimming after inoculation with RGNNV that resembled the erratic swimming of saltwater fish affected with VNN disease reported previously (Munday *et al.*, 2002). To establish a betanodavirus infection system in medaka, a well-studied model fish, the susceptibility of medaka to betanodaviruses was further investigated in detail.

The susceptibility of adult medaka to viruses was tested using RGNNV and SJNNV. When the fish were injected intramuscularly with $10^{6.5}$, $10^{4.5}$, or $10^{2.5}$ $TCID_{50}$ of RGNNV, erratically swimming fish were observed in all treatment groups (Figs. 1A and 2). In the intramuscular injection experiments, viral susceptibility was evaluated on the basis of the cumulative morbidity rate, not by cumulative mortality, because about 15 % of mock-inoculated fish died during the experimental period without displaying erratic swimming. Therefore, the evaluation of cumulative morbidity eliminates the nonspecific responses of inoculated fish. Cumulative morbidity reached 85 % after eight days and 100 % after 12 days in fish inoculated with $10^{6.5}$ $TCID_{50}$ of RGNNV. Cumulative morbidity reached 52 % and 21 % in 11 days after inoculation with $10^{4.5}$ and $10^{2.5}$ $TCID_{50}$ of RGNNV, respectively. In contrast, SJNNV inoculation produced a maximum of 73 % cumulative morbidity, even though $10^{6.2}$ $TCID_{50}$ of viruses were inoculated (Fig. 1B). Delayed and low levels of morbidity were observed after

inoculation with $10^{4.2}$ and $10^{2.2}$ $TCID_{50}$ of SJNNV, respectively. No SJNNV dose higher than $10^{6.2}$ $TCID_{50}$ was tested in the intramuscular injection experiments. Overall, medaka were more sensitive to RGNNV than to SJNNV. All fish that displayed erratic swimming died within 3–5 days throughout the experiments. Curves drawn from the cumulative mortality data were similar to the curves drawn from the cumulative morbidity data (Fig. 1) with a delay of 3–5 days (data not shown). No differences were observed in the modes of erratic swimming between fish inoculated with RGNNV and those inoculated with SJNNV. Bath challenge of adult medaka with RGNNV did not result in erratic swimming or evidence of viral multiplication, even though the fish were exposed to viruses at 10^{10} $TCID_{50}$/l (data not shown).

Localization of viral antigens in inoculated adult medaka. To determine the viral tropism and multiplicity in medaka, fish showing erratic swimming after viral inoculation (Fig. 1) were sectioned and subjected to immunofluorescence staining with anti-SJNNV antibody to detect viral antigens. Strong fluorescent signals were detected in the brains, spinal cords, and retinas of both the RGNNV- and SJNNV-infected medaka (Fig. 3), as observed with betanodavirus infections of marine fish species. Viral antigens were detected in all the inoculated fish that displayed erratic swimming. No fluorescent signal was observed in the mock-inoculated fish (Fig. 3).

Histopathology of infected adult medaka. Fish exhibiting erratic swimming (Fig. 1) were histopathologically investigated by sectioning and staining with H&E. No vacuolation was observed, even in the brain, spinal cord, or retina, in either the RGNNV- or SJNNV-infected medaka, although high levels of viral antigens were present in these tissues (Fig. 3). However, when the same RGNNV samples were injected intramuscularly into adult sevenband groupers, from which RGNNV originated,

vacuolation of the nerve tissues was observed in all the infected fish tested (data not shown).

RGNNV multiplication in medaka brain. Virus multiplication in the brains of adult medaka injected intramuscularly with RGNNV ($10^{6.5}$ $TCID_{50}$/fish) was measured every two days (Fig. 4). Viral multiplication was detected two days after inoculation and reached near maximal at four days. Viral titers then increased gradually to $10^{9.2}$ $TCID_{50}$/g brain tissue during the experimental period. Interestingly, there was no significant difference in viral titer between the normal and the erratically swimming fish at the same sampling times (data not shown). These results suggest that the appearance of erratic swimming is not controlled simply by the amount of virus in the brain, although the accumulation of virus would be a prerequisite for symptom development.

Susceptibility of medaka larvae to RGNNV. To determine the viral susceptibility of medaka larvae, one-day-old larvae were bath challenged with RGNNV and monitored for the appearance of moribund or dead fish. In this case, susceptibility was evaluated on the basis of cumulative mortality, not on cumulative morbidity, of the inoculated fish (Fig. 5) because most inoculated larvae died without showing distinctly erratic swimming, although these fish had high levels of virus in the brain, eye, and spinal cord (Fig. 6A). Medaka larvae exposed to RGNNV showed 76 % cumulative mortality over nine days, and cumulative mortality increased rapidly six days after inoculation (Fig. 5). Mock inoculation produced 16 % cumulative mortality, most of which occurred eight and nine days after inoculation, but no viral antigen was detected in the dead fish (Fig. 6B). No histopathological abnormalities were observed in the brain, spinal cord, or retina of the virus-infected larvae, despite the high levels of viral antigen found in those tissues (Fig. 6A).

DISCUSSION

Adult and larval medaka were successfully infected with two different types of *Betanodavirus*. Evidence of viral infection included abnormal swimming, the presence of viral antigens, and viruses in the target tissues of exposed medaka. Our immersion experiments indicated that medaka in the larval stage are more sensitive to betanodaviruses than medaka in the adult stage. These experimental infections of medaka are similar to the natural and experimental infections described in saltwater fish (Munday *et al.*, 2002). Our study demonstrates that betanodavirus infections of medaka provide a unique model fish system for the further study of virus–host interactions among RNA viruses.

In virus-infected medaka, no obvious lesions were observed in the central nervous system or retina, although high levels of viral accumulation were observed in these tissues (Figs. 3 and 4). Therefore, we conclude that the occurrence of abnormal swimming in medaka does not necessarily require obvious lesion formation, such as vacuolation. Some histopathological abnormalities may have occurred in the target tissues of infected medaka that were undetectable by H&E staining and light microscopy. This hypothesis is supported by the fact that viral multiplication in the brains of RGNNV-inoculated fish preceded erratic swimming by 6–7 days (Figs. 1 and 4). This suggests the occurrence of a pathological event(s) after viral multiplication in the target tissues. Generally, histopathological features, characterized by vacuolation of the central nervous system and retina, have been observed in VNN-affected marine fish (Munday *et al.*, 2002). One possible explanation for the lack of vacuolation in the virus-

infected medaka is that the histopathology differs from that observed in affected marine fish. A similar situation is observed in poliovirus infections of mice. The histopathology of wild-type mice infected with mouse-adapted polioviruses differs from that of human poliovirus receptor-expressing mice, although the poliomyelitic symptoms were the same (Gromeier *et al.*, 1995). Alternatively, infected medaka may die before any vacuolation becomes apparent in the target tissues. VNN-affected striped jack larvae seldom showed vacuolation in the central nervous system or retina, although those tissues accumulated significant levels of virus (unpublished data).

The levels of viral multiplication in the brain were similar in the normal and erratically swimming adult medaka when the fish were injected intramuscularly with RGNNV (data not shown). These results suggest that the appearance of erratic swimming is not controlled simply by the amount of virus in the brain, even though viral accumulation is a prerequisite for symptom development. This apparent discrepancy may arise from the nonuniform viral accumulation in the brain. This hypothesis is supported by the fact that the patterns of viral antigens localized within the brain varied among the infected fish (unpublished data). Thus, infected fish would not display erratic swimming when the brain areas controlling motility and balance escaped significant viral accumulation by chance. With greater viral accumulation, the likelihood that important brain areas will be damaged increases. Precise mapping of the areas of viral accumulation within the brains of normal and erratically swimming medaka would test this hypothesis.

We have demonstrated that both adult and larval medaka are susceptible to *Betanodavirus*. Only a limited number of fish species have been reported to be betanodavirus hosts in the adult stage (e.g., groupers, European sea bass, Atlantic

halibut; Munday *et al.*, 2002). Surprisingly, we have shown that adult medaka, which are taxonomically distant from the original saltwater fish host, are susceptible to betanodaviruses when they are injected intramuscularly. Intramuscular injection of SJNNV or RGNNV into adult striped jacks and kelp groupers, respectively, induced no betanodavirus-specific symptoms or death (unpublished data), although these fish are sensitive to the respective viruses in their larval stages. Therefore, medaka appear to be highly sensitive to the viruses compared with the native hosts (Munday *et al.*, 2002).

Many vertebrate model organisms (e.g., chicken, mouse, and rat) are used for virus–host interaction studies. Mice and rats are especially important because they are taxonomically close to humans. However, these animals are relatively large, are difficult to raise in large populations, are relatively expensive, and are subject to complex bioethical considerations. Consequently, mammals are not always advantageous hosts for the expeditious study of the mechanisms underlying virus–host interactions. The medaka has been used as a vertebrate model for various studies involving embryology, neuroembryology, pharmacology, toxicology, etc. (Ishikawa, 2000; Wittbrodt *et al.*, 2002). The genome size of the medaka is 800 Mb, which is much smaller than that of zebrafish (1700 Mb), human (3.0 Gb), or mouse (3.3 Gb). A medaka whole-genome sequencing project has been undertaken (Naruse *et al.*, 2004), and substantial sequence data are already available (http://medaka.dsp.jst.go.jp/MGI/). The medaka genomic sequence data will be useful in identifying and characterizing host factors that play important roles in viral infection and multiplication. Furthermore, fundamental experimental techniques, reverse genetics, classical genetics, and breeding systems are already available for medaka, which will greatly facilitate the study of host factors. Recently, IPNV, IHNV, SVCV, and SHRV were shown to infect zebrafish (LaPatra *et*

al., 2000; Phelan *et al.*, 2005; Sanders *et al.*, 2003), a well-studied model fish, as is medaka (Pradel & Ewbank, 2004). However, these birnaviruses and rhabdoviruses have large and complex genomes compared with those of the betanodaviruses, implying that the interactions between their viral factors and between the viral and host factors are complicated. Therefore, to determine the complete viral infection mechanisms of these virus–host systems would be highly labor intensive, even if zebrafish were used as the host. Thus, our betanodavirus–medaka system appears suitable for extensive studies of virus–vertebrate interactions because the model virus–model fish combination has several important advantages.

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FIGURE LEGENDS

Fig. 1. Cumulative morbidity of adult medaka injected intramuscularly with RGNNV or SJNNV. Adult medaka were injected intramuscularly with RGNNV (A) (◆, $10^{6.5}$ $TCID_{50}$/fish; ■, $10^{4.5}$ $TCID_{50}$/fish; ▲, $10^{2.5}$ $TCID_{50}$/fish) or SJNNV (B) (◆, $10^{6.2}$ $TCID_{50}$/fish; ■, $10^{4.2}$ $TCID_{50}$/fish; ▲, $10^{2.2}$ $TCID_{50}$/fish). Inoculated fish were maintained at 25 °C and fish exhibiting erratic swimming were counted. Data are representative of four independent experiments. Mock inoculation (●).

Fig. 2. Typical erratic swimming of an adult medaka infected with RGNNV. Adult medaka were injected intramuscularly with RGNNV at $10^{6.5}$ $TCID_{50}$/fish and were maintained at 25 °C. A medaka showing typical erratic swimming is indicated with the arrow.

Fig. 3. Immunofluorescence staining and histopathology of adult medaka infected with RGNNV or SJNNV. Adult medaka showing erratic swimming after intramuscular injection with RGNNV or SJNNV were harvested. The fish were sectioned and subjected to immunofluorescence staining with anti-SJNNV antibody or H&E staining. After mock inoculation, healthy fish were used for sectioning. Each scale bar is 1 mm for the head samples (A) or 0.5 mm for the eye samples (B). Representative data for each treatment are shown.

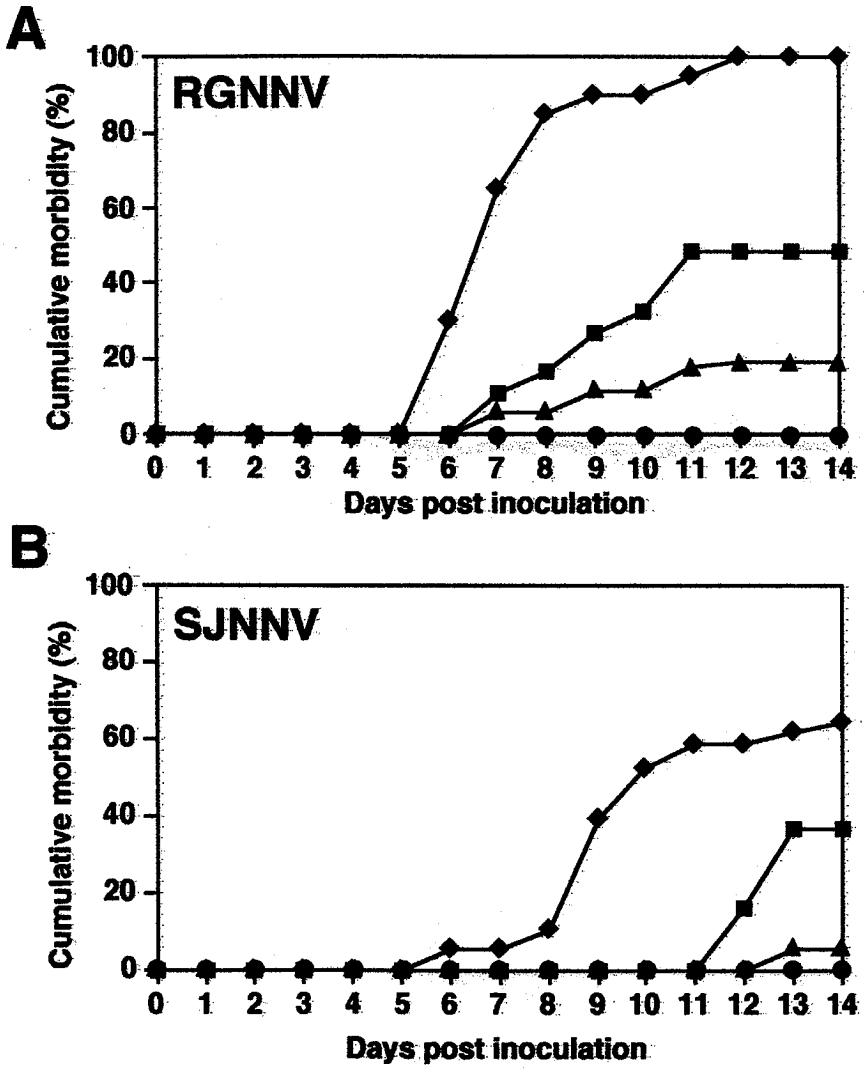
Fig. 4. Viral titers in the brains of RGNNV-inoculated adult medaka. Adult medaka were injected intramuscularly with RGNNV at $10^{6.5}$ $TCID_{50}$/fish. Fish (n = 3) were collected at the indicated times and viral titers in the brains were measured. Data are the means and standard errors of two independent experiments.

Fig. 5. Cumulative mortality of medaka larvae bath challenged with RGNNV. One-day-old medaka larvae were maintained at 25 °C in a glass beaker containing 100 ml of

water with 10^{10} (◆) or 0 (●) $TCID_{50}$/l RGNNV. Fish were exposed in duplicate, with 50 fish per experiment. Data are representative of four independent experiments.

Fig. 6. Immunofluorescence staining of RGNNV-infected medaka larvae. One-day-old medaka larvae were bath challenged with RGNNV and maintained at 25 °C in a glass beaker. After immersion, dead fish (A) were collected at seven days after inoculation, sectioned, and subjected to immunofluorescence staining with anti-SJNNV antibody. Mock-inoculated larvae (B) were similarly subjected to immunofluorescence staining. Representative data for each treatment are shown. Scale bar = 1 mm.

Fig. 1


A
RGNNV
Cumulative morbidity (%)
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Days post inoculation
B
SJNNV
Cumulative morbidity (%)
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60
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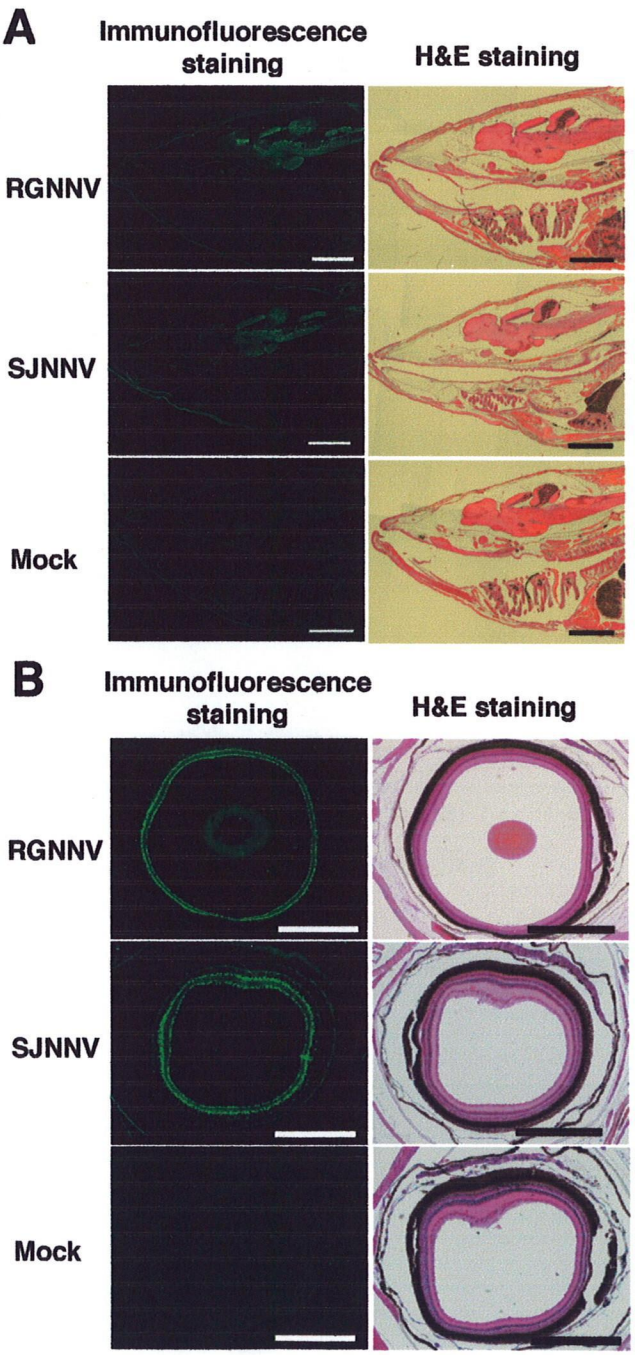
Days post inoculation

Fig. 2

Fig. 3


A
Immunofluorescence staining
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B
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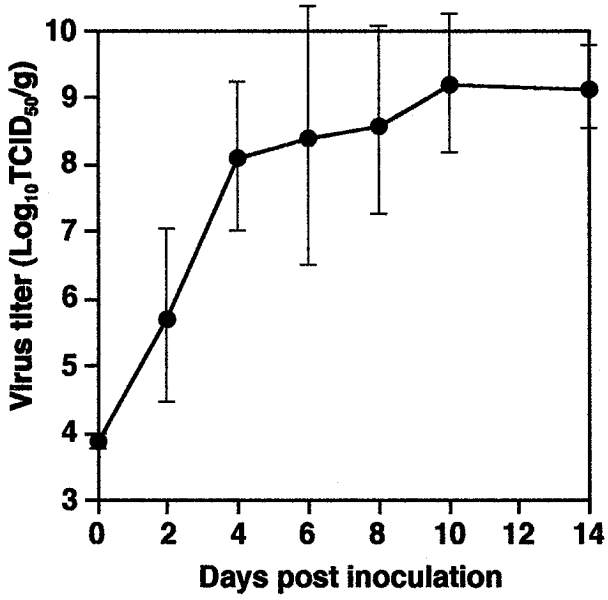
H&E staining
RGNNV
SJNNV
Mock

Fig. 4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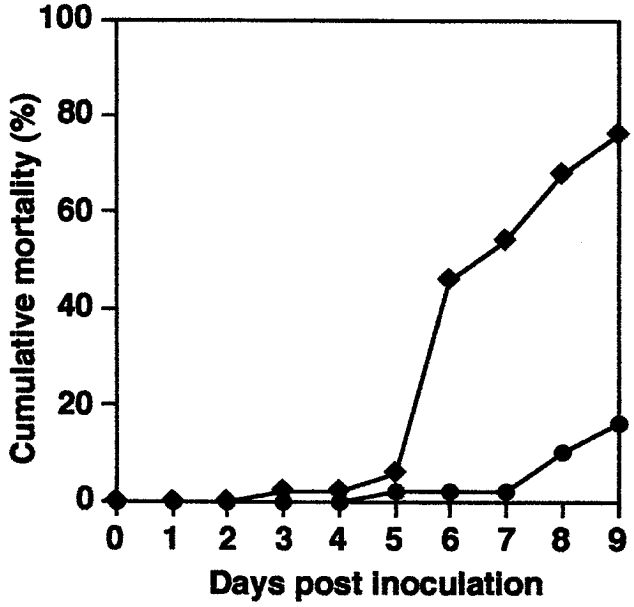
12
14
Days post inoculation

Fig. 5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60
40
20
0
Cumulative mortality (%)
0 1 2 3 4 5 6 7 8 9
Days post inoculation