



Short communication

Detection of nervous necrosis virus RGNNV genotype in pearl gentian grouper (*Epinephelus lanceolatus* ♂ × *E. fuscoguttatus* ♀) fry imported to Thailand



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ABSTRACT

Nervous necrosis virus (NNV) is a deadly virus that affects >120 fish species worldwide, but there is little information about it in Thailand. In August 2019, a population of pearl gentian grouper fry imported to Thailand experienced mass mortality. The diseased fish exhibited darkening, floating on the water surface, 'sleepy behaviour', and erratic swimming. PCR analysis revealed that the diseased samples were positive for NNV but negative for *Megalocytivirus* ISKNV and *Ranavirus*. Sequencing the virus's genome and phylogenetic analysis revealed that it is a member of the red-spotted grouper nervous necrosis virus (RGNNV) genotype. *In situ* hybridization using an NNV-specific probe revealed localization of the virus in the vacuolation lesions of the central nervous system (brain and spinal cord) and the retina of infected fish. The virus was successfully isolated from tissue homogenate from diseased fish using the E-11 cell line. This study identified NNV (RGNNV genotype) as a virus associated with massive die-offs in imported pearl grouper fry in Thailand. Considering the infectious nature of the virus and its broad host range, appropriate biosecurity measures are needed to prevent any loss to the marine aquaculture industry.

1. Introduction

Nervous necrosis virus (NNV) is the causative agent of viral nervous necrosis (VNN), also known as viral encephalopathy and retinopathy (VER). This virus is a member of the genus *Betanodavirus*, family *Nodaviridae* and has been responsible for disease outbreaks in both wild and farmed fishes (OIE, 2019a; Bandín and Souto, 2020). Since the first incidence of VER or VNN reported over 30 years ago, >120 freshwater and marine fish species, including grouper (*Epinephelus spp.*), have been affected by NNV (OIE, 2019a; Bandín and Souto, 2020; Xing et al., 2020).

NNV is a non-enveloped RNA virus with an icosahedral shape and a mean diameter of 25 to 30 nm. The NNV genome comprises two positively stranded RNAs (RNA1 and RNA2) (Breuil et al., 1991; Doan et al., 2017; Tan et al., 2015; Zorriehzahra, 2020). The RNA1 gene is 3.1 kb long and encodes the RNA-dependent RNA polymerase (RdRp) enzyme required for virus replication. RNA2 (1.4 kb) encodes the capsid protein of NNV. In virus-infected cells, RNA1 transcribes sub-genomic RNA3, which encodes proteins B1 and B2. Different genotypes of NNV have been identified based on the homology of RNA2, and their geographical distribution patterns were affected by water temperature (Bandín and Souto, 2020). Red-spotted grouper NNV (RGNNV), Tiger puffer NNV

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(TPNNV), Striped Jack NNV (SJNNV), and Barfin flounder NNV (BFNNV) were the first four genotypes identified (Nishizawa et al., 1995). New genotypes were discovered later, including the Turbot betanodavirus strain (TNV) and Korean shellfish NNV (KSNNV) (Johansen et al., 2004; Kim et al., 2019). RGNNV is widely distributed among these genotypes, affecting the greatest number of fish (Bandín and Souto, 2020).

Pearl gentian grouper is cross-hybrid between giant grouper (*Epinephelus lanceolatus* ♂) and tiger grouper (*E. fuscoguttatus* ♀) (Jiang et al., 2015). This species inherits favourable characteristics from both parents, including disease resistance, rapid growth, and high-quality flesh; as a result, it is gaining popularity in China and Southeast Asian countries (Xing et al., 2020). In 2019, a disease outbreak killed 99% of imported pearl gentian grouper fry in a marine hatchery in Thailand. We employed various diagnostic methods, including PCR, histopathology, *in situ* hybridization, genome sequencing and phylogenetic analysis, as well as cell culture to identify and characterize the imported causative virus associated with this unusual disease event.

2. Materials and methods

2.1. Fish sample collection

The samples used in this study were obtained from the imported pearl gentian grouper (*E. fuscoguttatus* × *E. lanceolatus*) fry from a marine hatchery in Thailand that experienced massive mortality of up to 99% in the quarantine tanks in August 2019 due to an unknown disease event. The hatchery had twelve tanks with a stocking density of one million fry per five-metric-ton tank. Water parameters were 30–32 °C, 28–30 ppt salinity, DO 5–6 ppm, pH 7.8–8.3, alkalinity ~160 ppm, ammonia <1.0 ppm, and nitrite <0.5 ppm. The onset of mortality started on day 21 with clinical signs including lethargy, darkening of the skin, floating on the water surface, ‘sleepy behaviour’, and erratic swimming. We received a collection of samples (35 day-old) to identify the causative agent, including whole-body fry preserved in 10% neutral buffered formalin for histological examination and freshly dead fish for PCR diagnosis and cell culture.

2.2. Nucleic extraction and viral pathogen detection by PCR

Considering small size of fry, whole fish bodies were used for extraction of nucleic acids. Three pooled samples of diseased fry (3 individuals/pool) were subjected to RNA extraction using Trizol reagent (Invitrogen) according to manufacturer’s protocol and DNA extraction using conventional phenol/chloroform extraction and ethanol precipitation as previously described (Meemetta et al., 2020). The quality and quantity of the obtained DNA and RNA were measured by spectrophotometry at OD 260 and 280 nm. Single PCR or RT-qPCR assays were performed based on established protocols to diagnose NNV (OIE, 2019a), *Megalocytivirus* ISKNV (Gias et al., 2011), and *Ranavirus* (OIE, 2019b) from the obtained nucleic acid samples. 200 ng of either DNA or RNA template was used in a reaction. Quantification of NNV was later conducted using probe-based qPCR according to the published method (Hick and Whittington, 2010). Table 1 shows a list of the PCR primers and probes used in this study. The pGEM T-easy (Promega) based positive control plasmid of NNV was constructed from a 605 bp RNA2 fragment amplified from NNV-infected orange-spotted grouper (laboratory sample). It was also used for building a standard curve for viral quantification in the RT-qPCR assay. Similarly, pGEM T-easy containing a 214 bp fragment of the *Megalocytivirus* ISKNV major capsid protein (MCP) gene (Dong et al., 2017) was used as a positive control for ISKNV detection. Note that this study did not have a positive control for *Ranavirus* detection.

Table 1
Primers used in this study.

Virus	Primer names/ Sequence (5'-> 3')	Size (bp)	Reference	Usage
NNV	VNNV1/ ACA CTG GAG TTT GAA ATT CA VNNV2/ GTC TTG TTG AAG TTG TCC CA NNVR1-F/ TAA CAT CAC CTT CTT GCT CTG TTG AGT NNVR1-R/ CCG AAG CGT AGG ACA GCA TAA AGC T NNVR2-F/ TAA TCC ATC ACC GCT TTG CAA TCA CAA TG NNVR2-R/ CGA GTT GAG AAG CGA TCA GCG GCC AT NNVR1-F1/ TGT GCT CCC GAC CTC GTG GT NNVR1-R1/ TGT CGT CAA TCT CTG ACT GG qR2TF/ CTT CCT GCC TGA TCC AAC TG qR2TR/ GTT CTG CTT TCC CAC CAT TTG R2probe2/FAM CAA CGA CTG CAC CAC GAG TTG - BHQ1 M153/ ATG ACC GTC GCC CTC ATC AC M154/ CCA TCG AGC CGT TCA TGA TG	605 3102 1431 – 93	OIE, 2019a This study This study This study Hick and Whittington, 2010	Viral detection & probe preparation RNA1 genome amplification RNA2 genome amplification RNA1 sequencing qPCR
<i>Ranavirus</i>	M153/ ATG ACC GTC GCC CTC ATC AC M154/ CCA TCG AGC CGT TCA TGA TG	625	OIE, 2019b	Viral detection
<i>Megalocytivirus</i> ISKNV	Mega I/ GTG TGG CTG CGT GTT AAG MegaII/ TGC CAA TCA TCT TGT TGT AGC	214	Gias et al., 2011	Viral detection
IMNV	IMNV13-FN/ TGT TTA TGC TTG GGA TGG AA IMNV13-RN/ TCG AAA GTT GTT GGC TGA TG	282	Senapin et al., 2007	Probe preparation

2.3. Nervous necrosis virus genome amplification

Primer sets (Table 1) used for amplification of genomic RNAs of NNV were designed based on genome segments of RGNNV (accession numbers NC_008040 and NC_008041) and sevenband grouper nervous necrosis virus (SGNNV, accession numbers AB373028 and AB373029). RNA extracted from diseased grouper was used as a template for viral genomic segment amplification. RT-PCR reaction was carried out in a final volume of 25 µL containing 500 ng RNA template, 200 nM of each primer, 1 µL of SuperScript III RT/Platinum Taq Mix (Invitrogen), and

1× supplied buffer. The amplification conditions were reverse transcription at 50 °C for 30 min and denaturation at 94 °C for 2 min followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 3 min (for RNA1) or 1.5 min (for RNA2), and final extension at 72 °C for 5 min. RT-PCR amplicons were electrophoresed on agarose gel, purified, and cloned into a pGEM-T easy vector (Promega). Recombinant clones were then sent to Macrogen (South Korea) for DNA sequencing using T7 and SP6 promoter primers. A recombinant clone of RNA1 was additionally read to fill the gap with NNVR1-F1 and NNVR1-R1 primers designed in this study (Table 1).

2.4. Phylogenetic tree construction

The amplified sequences of NNV from the diseased fish were subjected to homology searches using the NCBI blast tool. For phylogenetic analysis, publicly available genomes of NNV isolates whose bi-segmented genome belonged to the same genotype (not reassortment) were selected (Table S1) and aligned with the NNV sequences from this study. Following multiple sequence alignments and trimming, the phylogenetic trees were constructed based on the consensus sequences using Maximum Likelihood with the best model TN93 + G suggested by MEGA10 software. Bootstraps was performed at 1000 replicates.

2.5. Histological examination and in situ hybridization (ISH)

The preserved specimens ($n = 5$) were dehydrated and embedded in paraffin prior to consecutive sectioning for haematoxylin and eosin (H&E) staining and *in situ* hybridization (ISH) assay. An ISH assay using an NNV-specific probe was performed to determine the presence of the NNV viral genome in diseased fish tissues. Digoxigenin (DIG)-labelled probes were prepared using a commercial DIG-labelling mix (Roche, Germany). The recombinant plasmid containing a 605 bp insert derived from the NNV RNA2 fragment mentioned above was used as a template in labelling reaction, whereas a 282 bp fragment of infectious myonecrosis virus (IMNV) was used as an unrelated negative control probe (Senapin et al., 2007). The ISH procedure was carried out as previously described (Dinh-Hung et al., 2021) and a digital microscope was used to analyze and photograph the section.

2.6. NNV isolation using E-11 cell line

The tissue sample from NNV positive fish was homogenized in Leibovitz 15 (L-15) medium. The homogenized tissue was centrifuged at 10,000g for 20 min at 4 °C, and the suspension was filtered through a 0.22 µm membrane filter. The collected filtrate was used for infection of the E-11 cell line, which was cloned from the SSN-1 cell line derived from snakehead fish (Iwamoto et al., 2000). The procedure for cell culture was performed as previously described (Yamashita et al., 2005) with minor modifications. Briefly, E-11 cell lines were grown in L-15

medium supplemented with 5% fetal bovine serum. The monolayer of the E-11 cell line was inoculated with 500 µL of filtrate at 27 °C for 1 h and then replaced by a new culture medium. The control E-11 cell line was inoculated with L-15 medium only. The cytopathic effect (CPE) was observed daily using an inverted microscope. For confirmation, three continuous passages were performed on the E-11 cell line for CPE observation, and culture supernatant was subjected to RNA extraction and RT-qPCR (Hick and Whittington, 2010).

3. Results

3.1. Clinically sick fish were tested positive for NNV

Three pooled samples of the diseased fish were tested positive for NNV by a single RT-PCR assay, indicating severe infection (Fig. 1A). On the contrary, these samples were tested negative for *Megalocytivirus* ISKNV and *Ranavirus* as no amplification was detected (Fig. 1B, C). The subsequent result obtained from RT-qPCR showed that NNV positive fish samples had high virus loads ranging from 8.08×10^6 to 2.64×10^7 viral copies/200 ng RNA template (Fig. S1).

3.2. The genome sequence revealed RGNNV infection

The whole genome of NNV was obtained by amplifying RNA1 and RNA2 using their specific primers. Two expected bands of 3102 bp (RNA1) and 1431 bp (RNA2) were successfully amplified from RNA extracted from diseased fish and sequenced (Fig. 1D). The RNA1 and RNA2 nucleotide sequences were deposited to the NCBI GenBank database under accession numbers MW265973 and MW265974, respectively. The viral isolate in this study was named NNV G-2019. The RNA1 of NNV G-2019 predictably contained two ORFs encoding an RdRp protein (982 amino acids) and a protein B (75 amino acids). The RNA2 putatively encoded a capsid protein, which has 338 amino acids.

The blast results showed that nucleotide sequences of NNV G-2019 had significantly high percent identity to the represented viruses belonging to RGNNV genotype (95.24–98.36% for RNA1 and 98.18–98.74% for RNA2, Table S1) while lower percent identity to other genotypes (77.09–83.41%, Table S1). Moreover, the phylogenetic trees generated using representative genome sequences of five genotypes of NNV revealed that the genome sequences of NNV G-2019 were consistently clustered in the group of RGNNV genotype based on both RNA1 and RNA2 sequences (Fig. 2A-B). Notably, the genome identities of the viral isolates isolated from pearl gentian grouper in this study and a previous study (Xing et al., 2020) were 95.94% for RNA1 and 98.60% for RNA2 (Table S1).

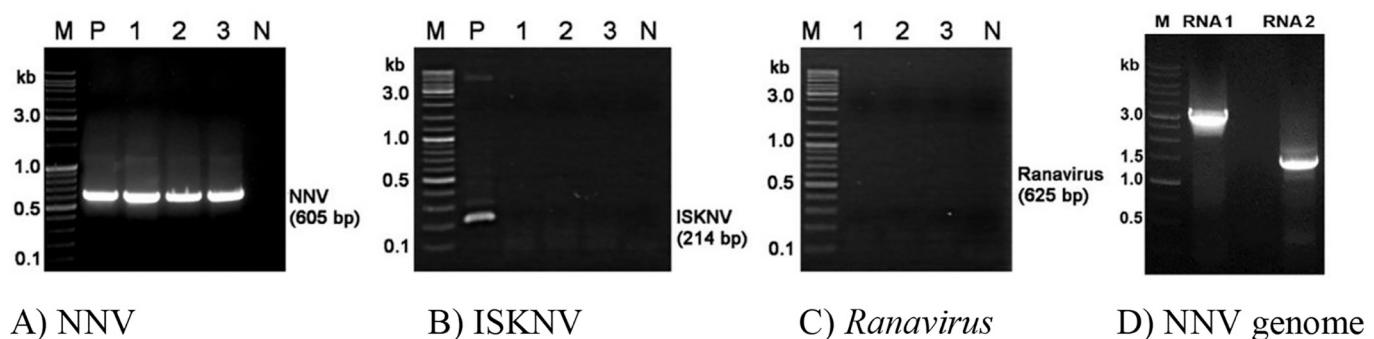


Fig. 1. The photomicrographs of agarose gel electrophoresis after PCR amplification of NNV (A), *Megalocytivirus* ISKNV(B), and *Ranavirus* (C), NNV RNA1 (3102 bp) and NNV RNA2 (1431 bp) (D). M, DNA marker; P, positive control; N, no template control; 1–3, tested pooled samples.

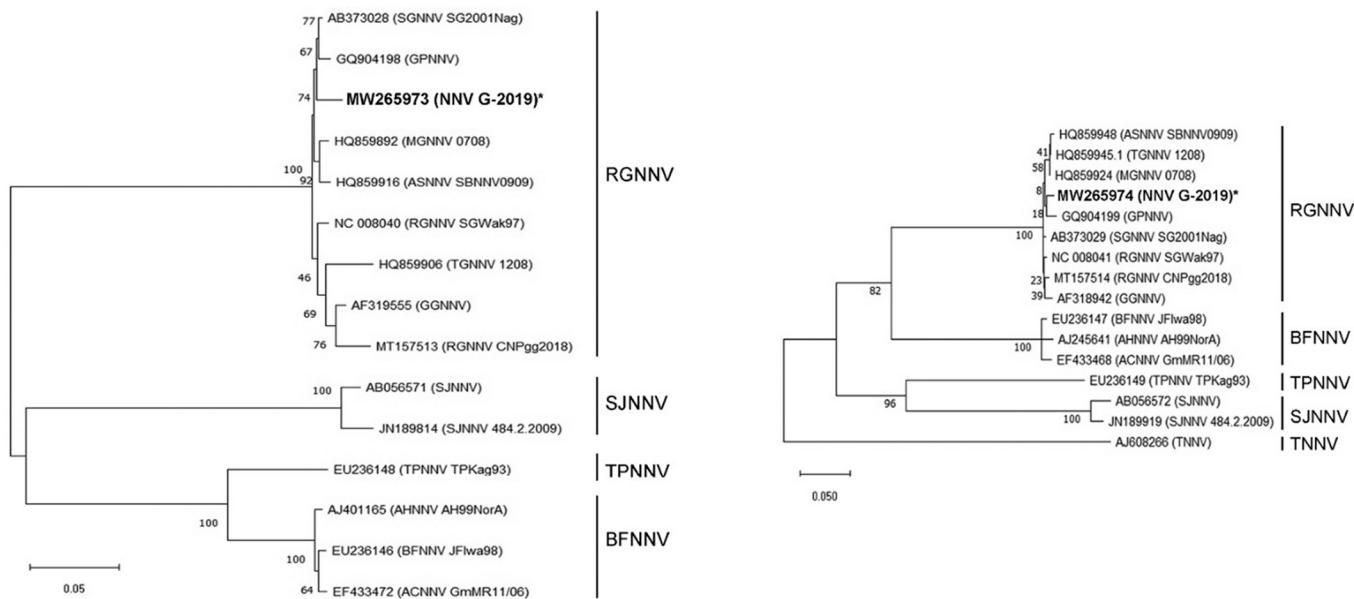


Fig. 2. The maximum likelihood phylogenetic trees were constructed using NNV RNA1 (A) and RNA2 sequence (B) from this study and publicly available sequences obtained from the NCBI database. Consensus lengths for RNA1 and RNA2 were 2841 and 981 nucleotides, respectively. Bootstraps of 1000 replicates were performed. * Denotes NNV G-2019 isolate obtained from this study.

3.3. Pathognomonic lesion and NNV-positive ISH were found in the diseased fry

Neuronal vacuolation, a pathognomonic lesion of NNV infection, was obviously observed in the outer nuclear layer (onl), inner nuclear layer (inl), and ganglion layer (gl) of the retina of clinically sick fish (Fig. 3A, arrows). The vacuoles were not clearly observed in the brain (Fig. 3D) but occasionally observed the spinal cord (Fig. 3G, arrows). ISH using an NNV-specific probe, detected dense positive signals (dark color) in the retina and central nervous system (brain and spinal cord) of diseased fish (Fig. 3, middle panel). The intensity of the signal seems to be strongest in the midbrain and along the cellular zone of the spinal cord. The ISH assay performed on the same samples using an unrelated probe (IMNV) revealed no hybridization signals (Fig. 3, right panel).

3.4. RGNNV infection revealed by cell culture method

Under the light microscope, cytopathic effect (CPE) was observed in the cells transfected with filtrate of diseased fish at 3 days post-inoculation (dpi). The main characteristic of CPE was intracellular vacuolation. At 5 dpi, clearer CPE was observed, including increased vacuoles, rounded cells and even detachment of cells from the flask's bottom (Fig. S2). No CPE was observed from control cells. In the RT-qPCR quantification of NNV from cell culture, supernatant from the third passage revealed high copy numbers of the virus, ranging from 1.66×10^8 to 1.98×10^9 copies/mL (Fig. S1). No viral copies were detected from control cells.

4. Discussion

The NNV is a highly contagious virus that has spread throughout the world. This virus has caused disease outbreaks in grouper hatcheries in Taiwan, Japan, China and other countries (Chi et al., 1997; Fukuda et al., 1996; Xing et al., 2020). Earlier in Thailand, there were some case reports of disease outbreaks caused by NNV in red-spotted grouper (*E. coioides*), and Nile tilapia (*Oreochromis niloticus*) larvae (Keawcharoen et al., 2015; Roongkammertwongsa et al., 2005). The detection of NNV in pearl gentian grouper fry imported to Thailand highlights the critical role of improved biosecurity measures, particularly diagnostic

testing and quarantine inspection, in mitigating the risk of disease spread from infected imported stock.

In this investigation, a variety of disease diagnostic techniques, including histopathology, molecular detection, and cell culture, were utilized to confirm NNV infection in pearl gentian grouper fry. This study did not employ other classic approaches, such as electron microscopy and experimental infection studies, which are also useful for pathogen identification. The phylogenetic analysis based on RNA1 and RNA2 consistently indicated that the imported NNV to Thailand belonged to the RGNNV genotype and that no reassortment event occurred in the genome of this strain (called NNV G-2019). Previous reports of RGNNV in fish from Asian countries included the infections detected in Asian seabass from India (Parameswaran et al., 2008) and Malaysia (Ransangan and Manin, 2010), red-spotted grouper from Japan (Mori et al., 1992), grouper from Singapore (Hegde et al., 2002), and pearl gentian grouper from China (Xing et al., 2020). Although the sources of imported contamination were not specified, the viral genotype likely implied their possible origin from the same region.

As previously reported, an NNV outbreak in pearl gentian grouper juveniles in China resulted in 98% mortality and typical vacuolation of the retina and CNS (Xing et al., 2020). Neuronal vacuolation in the CNS, particularly the medulla oblongata, would result in abnormal swimming and neurological disorders (Xing et al., 2020). Our study further revealed the widespread presence of NNV throughout the brain, as indicated by dense ISH signals, supporting the described abnormal clinical symptoms of the NNV infected fish. NNV appears to have a significant impact on larvae and juveniles (Zorriehzahra, 2020; Xing et al., 2020), whereas adults remain an asymptomatic carrier of the disease (Gomez et al., 2004). Vertical transmission of NNV was reported in various fish species such as European seabass, Seven-band grouper, Atlantic halibut (Breuil et al., 2002; Grotmol et al., 1999; Watanabe et al., 2000; Bandín and Souto, 2020) and was suspected in the outbreak case of pearl gentian grouper in China (Xing et al., 2020). Understanding the NNV transmission route may provide insight into the development of more effective control measures. While NNV could be isolated in various cell lines, E-11 was found to be the most permissive cell line for *in vitro* propagation of NNV, including RGNNV, SJNNV, and reassortant RGNNV/SJNNV genotypes (Valero et al., 2021).

In conclusion, this study reported a case of disease outbreak caused

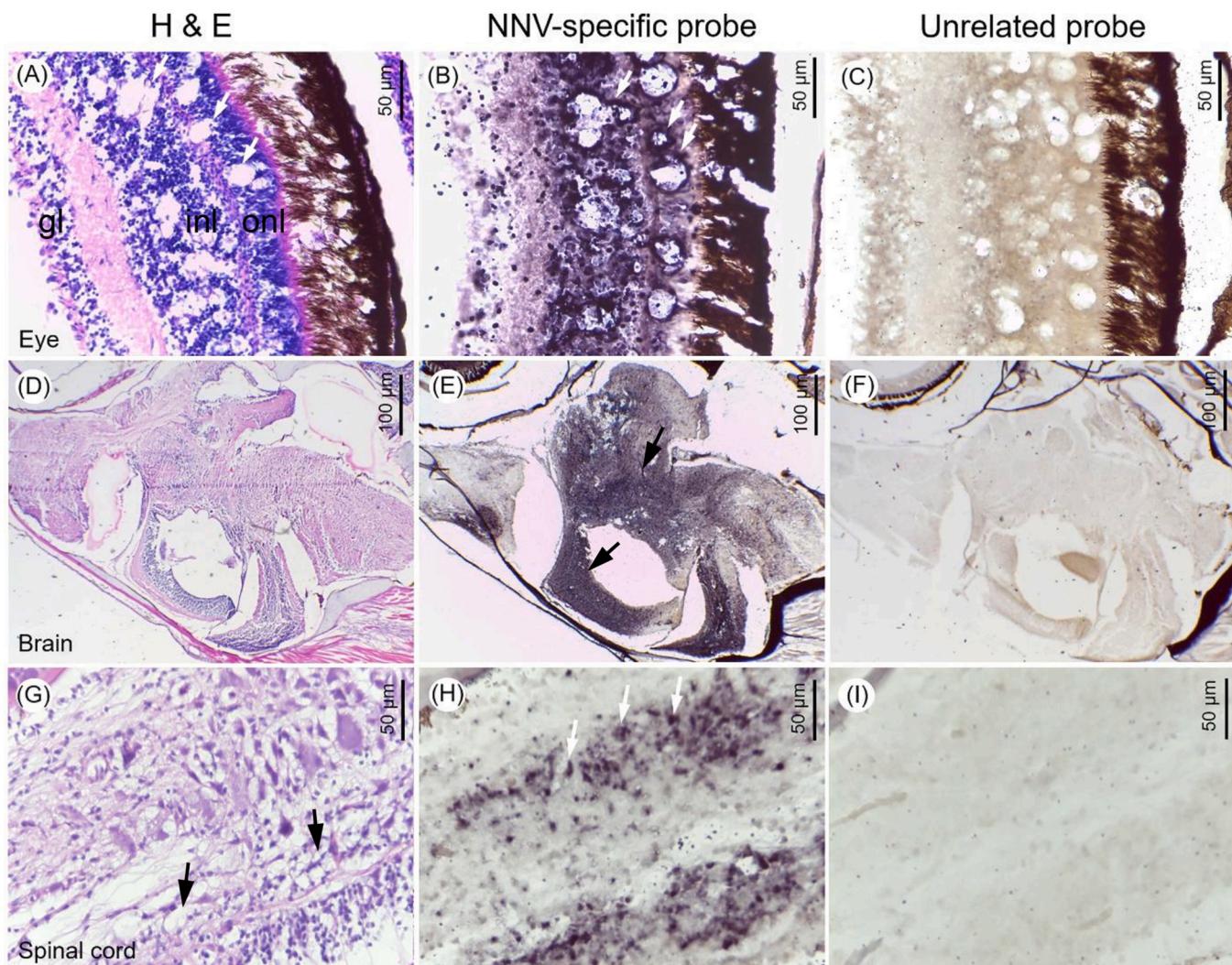


Fig. 3. Consecutive sections of the eye (A-C), brain (D-F) and spinal cord (G-I) of grouper infected with NNV subjected to H&E-staining (left panel), ISH using NNV-specific probe (middle panel) and unrelated probe (right panel), respectively. Arrows in H&E-stained section indicate vacuoles. Arrows in ISH using NNV probe demonstrate positive signals (dark color in B, E and H). No signals were detected using an unrelated probe (C, F and I).

by NNV RGNNV genotype in a population of pearl gentian grouper fry imported to Thailand in 2019. The virus was successfully isolated and bio-banked for further investigation. This is particularly useful for future research into its virulence through experimental infection, genetic diversity, and evolution, as well as for developing control measures. Given the NNV's diverse host specificity, which includes tilapia and Asian seabass, on which Thailand's aquaculture industry thrives, adequate precaution and biosecurity practices are essential to avoid any losses.

Credit authorship contribution statement

S.S. and H.T.D., conceptualization, methodology, data curation, project administration and supervision; S.V.J., S.T., P.K., K.P., investigation, methodology and data curation; S.S., H.T.D and S.V.J., Writing - original draft; M.S., B.N., and K.R.S., resources, writing - review-editing. All authors have read and agreed to the current version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aquaculture.2022.738659>.

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