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ORIGINAL ARTICLE

Resistance of pearlspot larvae, *Etroplus suratensis*, to redspotted grouper nervous necrosis virus by immersion challenge

Makesh Marappan | Aathithya Rengarajan | Venkata Satyanarayana Nallala | Krishna Sukumaran | Aritra Bera | Thirugnamurty Sivaramakrishnan | Govindarajan Thiagarajan | Muniyandi Kailasam | Vijayan Koyadan Kizhakedath

ICAR- Central Institute of Brackishwater Aquaculture, Chennai, India

Correspondence
Makesh Marappan, ICAR- Central Institute of Brackishwater Aquaculture, Chennai, India.
Email: mmakesh@ciba.res.in

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Abstract

Viral nervous necrosis (VNN) affects more than 120 species mostly belonging to the order Perciformes. However, none of the brackishwater species belonging to the family Cichlidae under the order Perciformes are reported to be susceptible. Hence, the present experiment was undertaken to study the susceptibility of the brackishwater cichlid, pearlspot, *Etroplus suratensis* to NNV. Thirty-day-old pearlspot larvae were infected with NNV by immersion. Mortality was recorded till 14 days post-infection, and the infected larvae were subjected to nested RT-PCR and histology. The virus was isolated from infected larvae using SSN-1 cells. To study the replication of the virus in vitro, primary cultured brain cells of *E. suratensis* and IEK cells were infected with NNV. No mortality was observed in any of the control or experimentally infected larvae. However, the experimentally infected larvae were positive for NNV by nested RT-PCR and the virus was isolated using SSN-1 cells. Further, the infected pearlspot brain cells and IEK cells showed cytopathic effect at second and third passage of the virus and they were positive for NNV by nested RT-PCR. Pearlspot is relatively resistant to VNN although the virus could replicate in the larvae and in cell culture.

KEYWORDS
Etroplus suratensis, nervous necrosis virus, pearlspot, resistance, viral nervous necrosis

1 | INTRODUCTION

Nervous necrosis virus (NNV), a betanodavirus, is the causative agent of viral encephalopathy and retinopathy (VER) or viral nervous necrosis (VNN) in fish. VNN is characterized by dark body coloration, uncoordinated swimming, hyper-inflation of swim bladder, emaciation, necrosis and vacuolation of the brain and retina, and high mortality, particularly during the larval and juvenile stages (Bovo et al., 1999; Chi, Lin, Su, & Hu, 1999; Mori et al., 1992). It is one of the most devastating viruses infecting a large number of species causing up to 100% mortality, threatening the marine and brackishwater aquaculture. NNV is an icosahedral, non-enveloped RNA virus measuring about 25 nm in diameter. The genome of the virus consists of two single-stranded positive-sense RNA molecules, RNA1 and RNA2 (Mori et al., 1992).

The NNV belongs to the family Nodaviridae and genus Betanodavirus. Officially, four species of betanodavirus has been identified based on the T4 variable region of RNA2 viz. Barfin flounder nervous necrosis virus (BFNNV), Redspotted grouper nervous necrosis virus (RGNNV), Striped jack nervous necrosis virus (SJNNV) (type species) and Tiger puffer nervous necrosis virus (TPNNV) (Nishizawa, Furuhashi, Nagai, Nakai, & Muroga, 1997). NNV genome

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being segmented, natural genetic reassortment happens and new reassortant strains such as SJNNV/RGNNV and RGNNV/SJNNV have been reported in several species (Olveira et al., 2009; Panzarin et al., 2012; Toffan et al., 2017; Toffolo et al., 2007). VER is mostly caused by RGNNV in tropical countries where the water temperature ranges from 25–30°C which is optimal for the growth of the virus (Doan, Vandeputte, Chatain, Morin, & Allal, 2017; Iwamoto, Nakai, Mori, Arimoto, & Furusawa, 2000), and all the reported outbreaks in India are caused by RGNNV (Rajan, Ezhil Praveena, Bhuvaneswari, & Jithendran, 2016). NNV is transmitted horizontally through water and vertically through eggs (Azad, Jithendran, Shekhar, Thirunavukkarasu, & de la Pena, 2006; Mori, Mushiake, & Arimoto, 1998; Munday, Kwang, & Moody, 2002). The virus can be transmitted experimentally by immersing the susceptible species in purified virus suspension or infected homogenates (Arimoto, Mori, Nakai, Muroga, & Furusawa, 1993).

The virus affects more than 120 species belonging to 30 families from 11 different orders (Costa & Thompson, 2016). At least 70 species have been described as carrier of the virus making it difficult to control the disease. Although the virus infects mostly fishes of marine and brackishwater origin, many freshwater fishes were also found to be susceptible (Doan et al., 2017). Most of the affected species are from the order Perciformes. However, none of the brackishwater species belonging to the family Cichlidae under the order Perciformes are reported to be susceptible. The only Cichlid species reported to be susceptible is the freshwater species *Oreochromis niloticus* (Keawcharoen et al., 2015). Hence, the present experiment was undertaken to study the susceptibility of a brackishwater cichlid, pearlspot, *Etroplus suratensis*, widely cultured in India and Southeast Asian countries for food and ornamental purposes, to NNV.

2 | MATERIALS AND METHODS

2.1 | Experimental fish

Thirty-day-old pearlspot, *E. suratensis* larvae of average length 17 mm were obtained from fish hatchery of Central Institute of Brackishwater Aquaculture, Chennai, India, acclimatized for seven days in 30 ppt sea water in FRP tanks with continuous aeration and fed with commercial weaning feed of 200–400 μm size. Optimum water quality was maintained by 20% water exchange daily. The water temperature during the experimental period ranged from 26–28°C. Before the experimental infection, the larvae were ensured that they were free of NNV by subjecting two groups of six larvae each to nested PCR for NNV. The experimental fish were handled as per the ethical guidelines of the institute.

2.2 | Cell culture

Etroplus suratensis fingerlings weighing about 10 g were used to prepare primary culture of brain cells. The brain of *E. suratensis* was collected aseptically in L-15 cell culture medium (Gibco, USA) containing 1 × antibiotic–antimycotic solution (Gibco, USA). The brain tissue was triturated gently in a hand-held homogeniser. The cell suspension was washed thrice in L-15 medium by centrifuging at 200 × *g* for 10 min and resuspending in L-15 medium. Finally, the cell pellet was resuspended in L-15 medium containing antibiotics and 20% foetal bovine serum (FBS) (Gibco, USA), and cells were counted using a haemocytometer. The cell concentration was adjusted to 1×10^6 cells ml^{-1}, seeded in 25 cm^2 tissue culture flasks (TPP, Switzerland) and incubated at 27°C. The next day, the medium was decanted and fresh growth medium was added. The cells were subcultured after confluency at a split ratio of 1:2. The cells were also cultured in chamber slides (SPL, Korea) for immunofluorescence assay.

Indian Etroplus kidney (IEK) cell line (RRID:CVCL_W363) derived from kidney of *E. suratensis*, obtained from National bureau of fish genetic resources, Lucknow, India, was subcultured in L-15 medium supplemented with 10% FBS in 25 cm^2 flasks.

2.3 | Experimental Infection

The pearlspot larvae were divided into four groups of 30 numbers each. NNV isolated from Asian seabass (*Lates calcarifer*), proven to cause 100% mortality in 15-day-old seabass larvae, was used for experimental infection. Two of the groups were infected with NNV by immersing the larvae in sea water containing a virus titre of 1×10^5 $TCID_{50}$ ml^{-1} for one hour with vigorous aeration. This dose was previously reported (Jaramillo, Hick, & Whittington, 2017) and also tested in our laboratory and found to cause mortality in *L. calcarifer* larval stages by immersion challenge. The two groups of control larvae were treated similarly without the viral inoculum. Mortality was recorded till 14 days post-infection (dpi). At 7 dpi, six larvae from each group were pooled for nested reverse transcription PCR (RT-PCR) and three larvae from each group were collected in neutral buffered formalin for histology.

The primary cultured brain cells of pearlspot at about 90% confluency were infected with NNV. 0.5 ml of virus suspension having a virus titre of 1×10^7 $TCID_{50}$ ml^{-1} was added to the flask and incubated at 27°C for 1 hr. Subsequently, the virus suspension was removed and the cells were washed twice with L-15 medium. L-15 maintenance medium containing 2% FBS was added to the flask and incubated at 27°C. Seven days after the experimental infection, the cells were freeze-thawed three times and culture medium containing the virus was used to infect another flask of brain cells. The virus was passaged once more in pearlspot brain cells. Control cells were also treated similarly without the viral inoculum. The chamber slides were also infected similarly with NNV. The control and the infected slides at 7 dpi were fixed with 95% methanol and 5% glacial acetic acid for 10 min at −20°C. Similarly, IEK cells at about 90% confluency were also infected with NNV and the virus was passaged twice in IEK cells.

2.4 | Isolation of virus

Nervous necrosis virus was isolated from infected pearlspot using striped snakehead cell line (SSN-1 cells) (RRID:CVCL_4306). Briefly,

five infected pearlspot seeds were pooled and homogenized in TN buffer (50 mM Tris-Cl, pH 7.6; 150 mM NaCl). The homogenate was clarified by centrifuging at 12,000 × *g* for 20 min at 4°C. The clarified homogenate was filtered through a 0.22 µm syringe filter. One ml of the filtrate was added to about 90% confluent monolayer of SSN-1 cells in a 25 cm^2 flask. The cells were incubated at 27°C for 1 hr. Subsequently, the cells were rinsed three times with L-15 medium and incubated with L-15 medium supplemented with 2% FBS at 27°C. The cells were observed daily under inverted microscope for the presence of CPE. After the appearance of the CPE, the cells were freeze-thawed thrice and were subjected to nested RT-PCR to confirm the presence of the virus.

2.5 | Nested Reverse Transcription PCR (RT-PCR)

Total RNA was extracted from NNV infected and control *E. suratensis* larvae, infected and uninfected cultured *E. suratensis* brain cells, and infected SSN-1 cells aseptically using TRIzol™ Reagent (Thermo fisher Scientific, Mumbai, India.) as per manufacturer's protocol. One µg of RNA was reverse transcribed into cDNA using iScript™ cDNA synthesis Kit (Bio-Rad, Lucknow, India). Nested RT-PCR was performed according to the method described by Dalla Valle et al., (2000) using NNV capsid protein gene-specific primers. RT-PCR with a product size of 605 bp was performed using forward (5'-ACACTGGAGTTTGAAATTCA-3') and reverse (5'-GTCTTGTTGAAGTTGTCCCA-3') primers. The PCR cycle conditions were as follows: initial denaturation at 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 45 s, annealing at 58°C for 45 s, and extension at 72°C for 45 s. A final extension at 72°C for 7 min was performed at the end of 30 cycles. Nested PCR with product size of 255 bp was performed using forward (5'-ATTGTGCCCCGCAAACAC-3') and reverse (5'-GACACGTTGACCACATCAGT-3') primers. The PCR cycle conditions were as follows: initial denaturation at 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 45 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. A final extension was carried out at 72°C for 5 min. The PCR amplification products were analysed on 1.5% agarose gel, stained with ethidium bromide under UV light using gel documentation system (Gel Doc ™ XR, Bio-Rad, Lucknow, India).

2.6 | Production of recombinant NNV capsid protein and polyclonal antibodies

Total RNA was isolated from NNV infected SSN-1 cells aseptically using TRIzol™ Reagent (Thermo fisher Scientific, Mumbai, India.) according to the manufacturer's protocol. After cDNA synthesis using iScript™ cDNA synthesis kit (Bio-Rad, Lucknow, India), PCR was performed to amplify NNV capsid protein gene. NNV capsid protein gene (1.1 kb) was PCR amplified using custom-designed primers NNV-*F* (5'-GCAC<u>AAGCTT</u>ATGGTACGCAAAGGTGAGAAG-3') and NNV-R (5'-CAGC<u>CTCGAG</u>TTAGTTTTCCGAGTCAACCCTAG-3', underlined sequences indicating sites for restriction enzymes). The PCR product was cloned into pET-28b(+) vector and transformed into BL21 (DE3) host cells. The clone was confirmed by digesting the recombinant plasmid with *Hind* III and *Xho* I restriction enzymes and sequencing the insert. The recombinant protein was expressed after induction with 1 mM IPTG for 3 hr at 37˚C. Cells were harvested by centrifugation at 8,000 × *g*, 4°C for 10 min and cell pellet was washed and then resuspended in 50 mM Tris, pH 8.0. The cells were lysed by sonication using 30% amplitude for 10 min and the insoluble cell fraction was pelleted by centrifugation at 12,000 × g for 20 min. The supernatant and pellet were subjected to SDS-PAGE, and the target protein was found in the pellet as inclusion bodies. The pellet was resuspended in 10 ml of

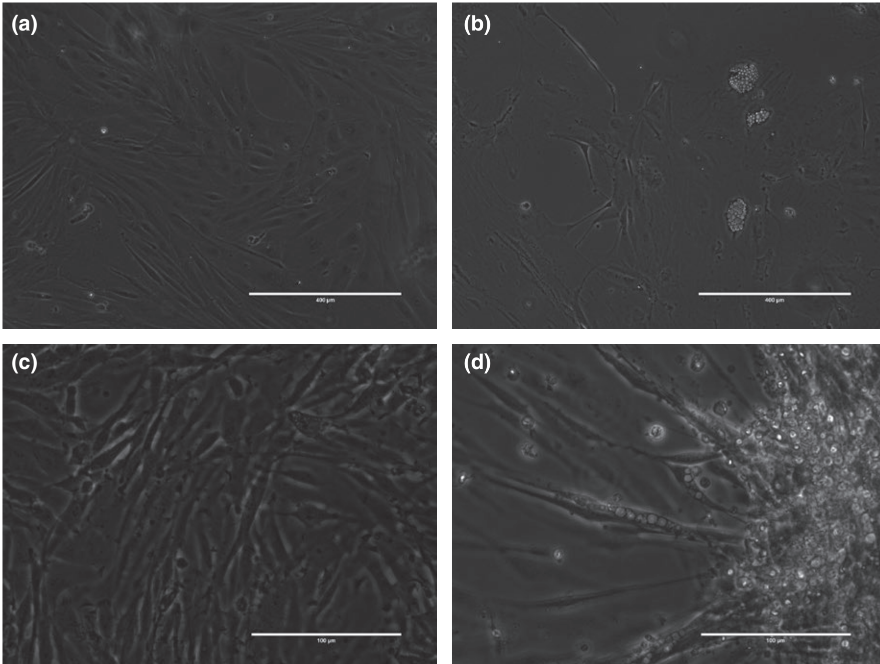


FIGURE 1 Primary cultured pearlspot brain cells; control (a) and nervous necrosis virus (NNV) infected (3rd passage) (b) showing vacuolations and IEK cell line derived from *Etroplus suratensis* kidney; control (c) and nervous necrosis virus (NNV) infected (3rd passage) (d) showing vacuolations, rounding and granulation

FIGURE 2 Nested RT-PCR PCR for control and infected pearlspot larvae (a), in vitro cultured pearlspot brain cells (b) and IEK cells (c). (a): lanes 1—Pearlspot larvae infected with nervous necrosis virus (NNV); 2—uninfected larvae; 3—positive control; 4—Negative control; 5–100 bp DNA ladder. (b): lanes 1—Negative control; 2—positive control; 3– 100 bp DNA ladder; 4—uninfected pearlspot brain cells; 5—NNV infected Pearlspot brain Passage 1; 6—Uninfected pearlspot brain cells; 7—NNV infected pearlspot brain passage 2; 8—Uninfected Pearlspot brain cells; 9—NNV infected pearlspot brain passage 3. (c): lanes 1—NNV infected IEK passage-1; 2—NNV infected IEK passage-2; 3—NNV infected IEK passage-3; 4—Uninfected IEK; 5—Negative control; 6—Positive control; 7—1 kb DNA ladder

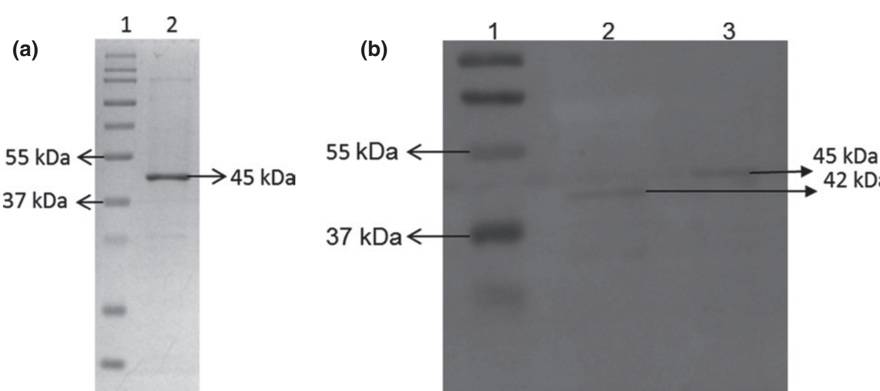


FIGURE 4 (a) SDS-PAGE for purified recombinant nervous necrosis virus (NNV) capsid protein (Lane 1: protein marker; Lane 2: Purified recombinant NNV capsid protein). (b) Western blot for NNV using polyclonal antibodies against recombinant capsid protein and NNV (Lane 1—protein Ladder; 2—NNV infected SSN-1 cell culture supernatant; 3—purified recombinant NNV capsid protein)

50 mM Tris and 8 M urea (pH 8.0) to solubilize the recombinant protein. The pellet was sonicated using 30% amplitude for 5 min at 4°C. The recombinant protein was purified using nickel affinity chromatography according to the manufacturer's protocol (GE Healthcare Life Sciences, Chennai, India). The recombinant protein was eluted with buffer containing 150 mM imidazole and 8 M urea. The eluted protein was dialysed against buffer containing 50 mM sodium phosphate, 1.5 M urea, 400 mM arginine and 20% glycerol for 12 hr to renature recombinant protein. The purified recombinant protein was subjected to SDS-PAGE to check the purity and molecular weight.

Polyclonal antibody was produced against the recombinant capsid protein of NNV in New Zealand white rabbit using the commercial services of M/s Aura Biotechnologies Private Limited, Chennai, India. The antibody titre was estimated by ELISA, and the specificity of the antibodies was tested by western blot against the recombinant capsid protein and NNV.

2.7 | Histology

The formalin-fixed samples were dehydrated and embedded in paraffin blocks following standard histology procedure. Five-µm-thick sections were made from paraffin blocks and transferred to 3-Aminopropyltriethoxysilane-coated microscope slides. The sections were deparaffinised in xylene and rehydrated using descending grades of ethanol. The sections were stained with Harris haematoxylin and dehydrated using ascending grades of ethanol. The sections were counter stained with 2% eosin and mounted using DPX mountant. The sections were visualized under microscope (Nikon, Eclipse N*i*-U, USA). Tissue sections were also processed for immunofluorescence.

2.8 | Immunofluorescence staining

Immunofluorescence staining was performed on sections of control and infected pearlspot larvae as well as control and infected cultured pearlspot brain cells as described by Azad et al., (2006) with minor modifications. Briefly, the slides were blocked with 3% BSA for 30 min. Then the slides were washed and incubated with polyclonal anti-NNV primary antibody raised in rabbit (1:2,000 dilution) for 1 hr at room temperature. The slides were washed with Tris-buffered saline (TBS) (pH 7.6) to remove unbound antibody. For immunofluorescence staining, goat anti-rabbit IgG FITC secondary antibody (1:500 dilution) (Sigma, USA) was added and incubated for 30 min at room temperature. The slides were washed again with TBS (pH 7.6) to remove unbound secondary antibody. The slides were mounted using glycerol and visualized under EVOS™ FL Auto Imaging System (Thermo Fisher Scientific Inc., CA, USA).

3 | RESULTS

3.1 | Experimental infection of Pearlspot

No clinical signs of VNN or mortality were observed in the experimentally infected pearlspot larvae till 14 dpi. No CPE was observed in pearlspot brain cells at the first passage. However, vacuolation was observed sporadically in the second and third passages of the virus in pearlspot brain cells at 4 dpi (Figure 1a,b). Similarly, infection in IEK cells produced extensive vacuolation, rounding and granulation of cells after two passages (Figure 1c,d).

3.2 | Nested RT-PCR

The experimentally infected pearlspot larvae were positive for NNV by nested PCR (Figure 2a) as also the infected brain cells cultured in vitro at all the three passage levels (Figure 2b). Second and third passages of NNV in pearlspot brain cells were also positive by first step RT-PCR. NNV infected IEK cells at first passage were negative by nested PCR while the second and third passages tested positive (Figure 2c).

3.3 | Isolation of the virus

The experimentally infected larval homogenate when inoculated to SSN-1 cells, produced characteristic CPE such as vacuolation, granulation, rounding off and detachment by 72 hr post-infection (Figure 3a,b). The culture supernatant, when subjected to nested RT-PCR, was positive for NNV by first step RT-PCR (Figure 3c).

3.4 | Production of recombinant NNV capsid protein and polyclonal antibody

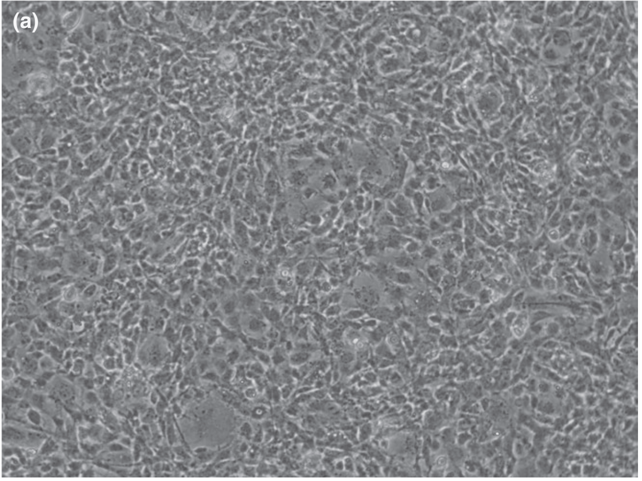
NNV capsid protein was expressed as inclusion bodies in the BL21 (DE3) cells and hence dissolved in 8 M urea buffer to solubilize the inclusion bodies after which the protein was dissolved and obtained in the soluble fraction. The recombinant protein purified using nickel affinity column produced protein of more than 90% purity (Figure 4a). Western blot of whole virus and recombinant protein with anti-NNV antibody resulted in specific reaction at 42 and 45 kDa, respectively as expected (Figure 4b).

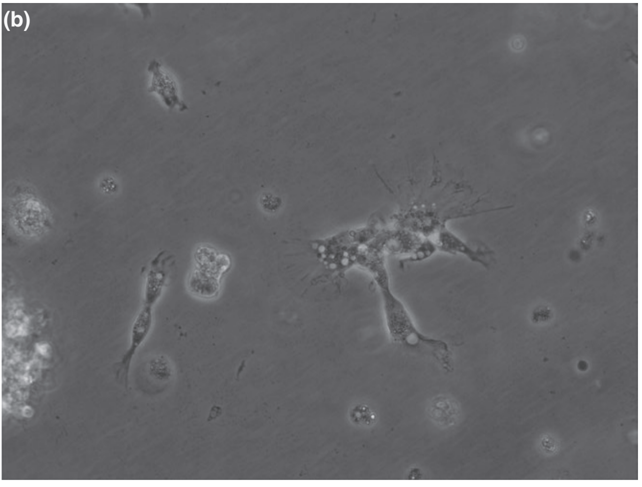
3.5 | Histology

Histology of experimentally infected pearlspot larvae did not show any significant pathological changes (Figure 5a).

3.6 | Immunofluorescence assay

The NNV infected cultured pearlspot brain cells showed specific immunofluorescence in the cytoplasm upon immunofluorescence staining while the control cells did not reveal any specific reaction (Figure 5b,c). The control and infected larval sections also did not show any specific fluorescence (Figure 5d) while the NNV infected SSN-1 cells showed specific fluorescence (Figure 5e).





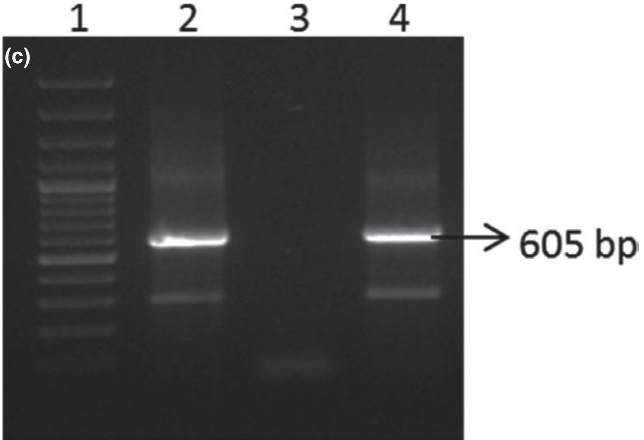


FIGURE 3 Control (a) and SSN1 cells infected with NNV infected pearlspot homogenate, 72 hr post-infection (b), RT-PCR for NNV infected SSN-1 cells (c). (c): Lanes 1—100 bp DNA ladder; 2—Positive control; 3—Negative control; 4—NNV infected SSN-1 cells

4 | DISCUSSION

NNV is known to infect a large number of species of marine, brackishwater and freshwater fishes. However, it was observed that pearlspot are not affected by the virus although other susceptible species such as Asian seabass stocked in the same culture facility

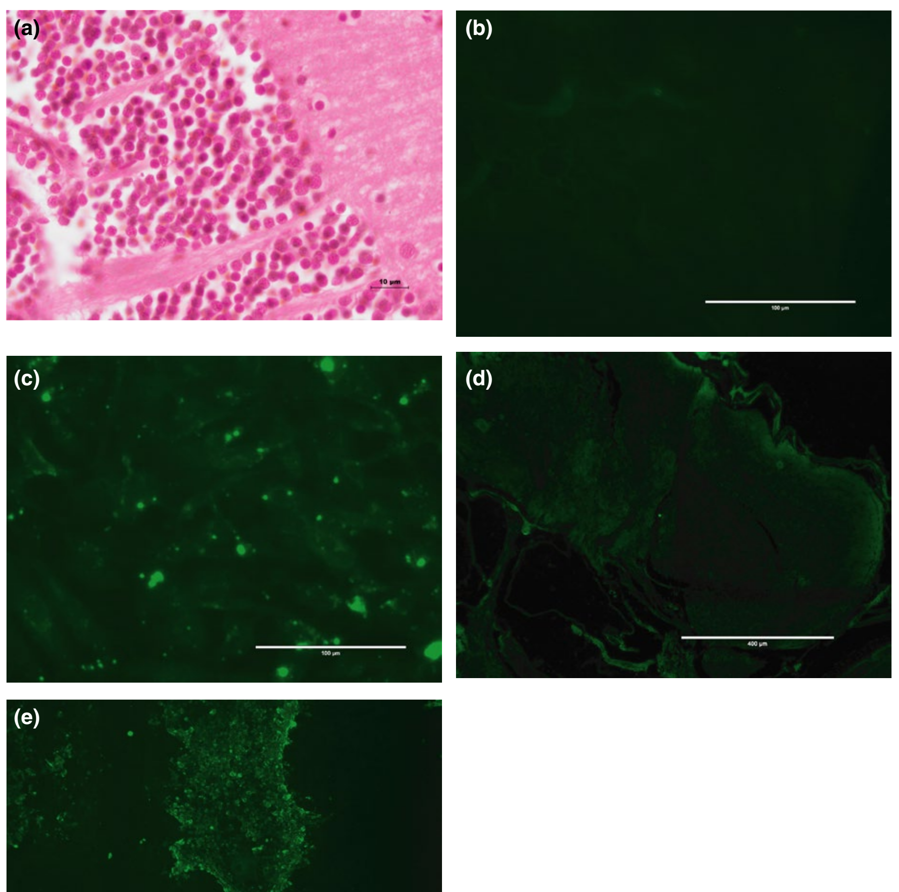


FIGURE 5 Histology of NNV infected pearlspot brain showing normal architecture (a), Immunofluorescence assay for control (b) and nervous necrosis virus (NNV) infected (c) cultured pearlspot brain cells, and immunofluorescence assay for NNV infected pearlspot brain section (d) and NNV infected SSN-1 cells (e) [Colour figure can be viewed at wileyonlinelibrary.com]

suffered acute mortality. To investigate the susceptibility of pearlspot to NNV, the experimental infection trial was carried out. NNV is more pathogenic to larval stages than juveniles or adult fish. Hence, 30-day-old pearlspot larvae were chosen for experimental infection by immersion, simulating natural infection. Although the virus did not cause any clinical sign or mortality in pearlspot, the virus was detected by nested RT-PCR at 7 dpi. The NNV used for experimental infection is a virulent strain which produced 100% mortality in 15-day-old Asian seabass larvae (data not shown). It is apparent from the experimental infection that pearlspot could resist natural infection with NNV although the virus could replicate in the host. However, the viral load observed in the infected pearlspot larvae was quite low since PCR amplification could be observed only in the nested PCR indicating that pearlspot is relatively resistant to the disease. Histology of the infected fish brain did not show any pathological changes indicative of VER, and no specific fluorescence was observed in the brain of the experimentally infected larvae by immunofluorescence assay suggesting that the viral load is too low to cause pathological changes or immunofluorescence. However, the virus could be isolated from the infected larvae. The virus is quite stable in the environment and survives up to six months in sea water at 15°C and up to 4 weeks in cell culture media at 25°C (Frerichs, Tweedie, Starkey, & Richards, 2000). Hence, infected pearlspot may act as a carrier for the virus and aid in the transmission of the virus to other susceptible host especially when cultured together. Similarly, although gilthead sea bream is generally considered resistant to VNN, the virus could be isolated from apparently healthy adults and larvae (Toffan et al., 2017).

In spotted wolffish (*Anarhichas minor*), mortality started 4 weeks post-bath challenge and peaked after 6–8 weeks post-challenge with SJNNV (Johansen, Amundsen, Dannevig, & Sommer, 2003). The present experiment was terminated 14 days post-infection as the incubation period for NNV in warm water fishes are much shorter compared to cold water fishes. RGNNV produced CPE within 48 hr of infection in SSN-1 cells and resulted in complete cell detachment by 72 hr when incubated at 27°C (present study) while BFNNV produced CPE only after 5 days in SSN-1 cells when incubated at 20°C (Dannevig et al., 2000) demonstrating that the virus replicates faster at higher temperature and thus a shorter incubation time in warm water fish. In sevenband grouper, *Hyporthodus septemfasciatus*, mortality was observed 3 dpi by intramuscular and immersion challenge and the mortality lasted till 6 and 12 dpi for intramuscular and immersion challenge, respectively when the animals were maintained at 27°C (Kim, Kim, Kim, Kim, & Oh, 2018).

The virus could replicate in cultured brain cells of pearlspot as observed by nested RT-PCR although the reaction was positive only by the second step of nested PCR. On subsequent passages, the PCR gave a positive reaction in the first step of the nested RT-PCR indicating that the virus could get adapted to multiply in the brain cells of pearlspot. The virus could also be adapted to IEK cell line after two passages. The ability of the virus to adapt to the primary cultured cells and cell lines of pearlspot gives us an opportunity to passage the virus in these cells to reduce its virulence resulting in a potential live attenuated vaccine against VER. The mechanism of resistance of pearlspot to RGNNV is not known. Receptor-transporting protein 3 (Rtp3) belonging to the RTP family has been reported to be associated with resistance to VNN in Asian seabass (Liu et al., 2017). However, the function of Rtp3 in pearlspot with respect to viral disease resistance has not been studied. The resistance to viral infections may involve multiple mechanisms including genetic background and capacity to mount an early interferon response (Verrier et al., 2018, 2012). It will be imperative to study the resistance mechanism in pearlspot to NNV which will help in formulating control measures for this lethal virus.

In conclusion, pearlspot is relatively resistant to NNV although the virus could be isolated from the experimentally infected larvae. However, pearlspot could act as a carrier of the virus for other susceptible population. Study of the relative disease resistance mechanism could help in developing control strategies for VER. Adaptation and passaging of NNV in cultured pearlspot cells could lead to the development of a potential live attenuated vaccine.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ORCID

Makesh Marappan https://orcid.org/0000-0001-8494-3820

Venkata Satyanarayana Nallala https://orcid.org/0000-0002-9879-486X

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