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Experimental infection of *Betanodavirus* in freshwater fish *Gambusia affinis* (Baird and Girard, 1853)—a potential infection model for viral encephalopathy and retinopathy

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Abstract Betanodaviruses are the causative agents of the disease known as viral nervous necrosis (VNN) or viral encephalopathy and retinopathy (VER) in a variety of marine and freshwater fish species. The aim of this study was to demonstrate experimental infection of an isolate of betanodavirus (RGNNV genotype) in freshwater fish, *Gambusia affinis*, for elucidation of transmission mechanism and potential use as a laboratory model. Morbidity and mortality rate was significantly higher by injection route of infection as compared to immersion by bath and resembled the natural infection of juvenile marine fish. The fish in disease affected group showed severe neurological disorders accompanied by extensive vacuolar degeneration and mild to moderate neuronal necrosis of the brain in comparison to control. Amplification of ~427 bp product in the variable region of the coat protein gene of betanodavirus was achieved by RT-PCR with 100% sequence homology to RGNNV genotype.

Keywords Viral nervous necrosis · *Betanodavirus* · *Gambusia affinis* · Experimental transmission · RT-PCR · Histopathology · Electron microscopy

Introduction

Betanodaviruses are the causative agents of the disease known as viral nervous necrosis (VNN) or viral encephalopathy and retinopathy (VER) in a variety of cultured marine and

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freshwater fishes (Munday et al., 2002; Maltese and Bovo, 2007; Doan et al. 2017). However, development of virus-fish infection model has been limited, though zebrafish (*Danio rerio*) has been used to study many infectious viruses including betanodavirus (Lu et al. 2008). Medaka (*Oryzias latipes*) has been reported as model fish to study VNN (Furuzawa et al., 2006). However, most of these fish did not exhibit either clinical manifestations of the disease or specific cellular changes in target organs in spite of the presence of virus confirmed in these studies by cell culture, immunofluorescence antibody technique (IFAT), or by polymerase chain reaction (PCR). *Gambusia affinis*, a small "livebearer" fish, commonly known as mosquito fish, is a hardy and easy to breed in large numbers in wide variety of environment. Further, the ovoviviparous nature could allow the virus transmission pattern more clearly, upon experimental infections. Hypothesis that this fish could be important as a model fish to study mechanism of disease transmission as against high cost and input required for maintenance of marine fish. The present paper pertains to study the experimental transmission of betanodavirus by injection and bath exposure to test the virulence of the virus, clinical signs of infection or pathogenesis in target organs clinic-pathology, histology, and RT-PCR in *G. affinis*.

Materials and methods

Fish

For experimental infection, freshwater fish *G. affinis* of mixed sex ($n = 30$) weighing 0.85 ± 0.45 g and a length of 4.23 ± 0.56 cm reared at 26 °C were procured from a private fish farm at Tambaram (Chennai, Tamil Nadu) and transported back to aquatic health testing laboratory of Central Institute of Brackishwater Aquaculture, Chennai, in oxygen pack. Fishes were acclimatized and maintained at 28–29 °C in rectangular glass tanks (120 L capacity) filled with filtered and aerated freshwater for 2 days. Fishes were fed two times using 0.5-mm sinking commercial fish feed pellets (New Life spectrum, USA) at 2% of their body weight and daily water exchange throughout the experimental period. Prior to challenge experiments, a couple of additional fish from the same lot from the farm were randomly selected and checked for parasite examination using skin and gill scrapings, bacterial isolation on tryptic soy agar (TSA) plates, and betanodavirus using brain samples by RT-PCR to ensure disease-free status of the experimental fish.

Viral strain and propagation in cell culture

An isolate of betanodavirus (RGNNV) designated as BARL-LC02 originally isolated from an acute disease outbreak of VNN in Asian seabass (*Lates calcarifer*) during September 2014 and characterized earlier was used in the experiment (Rajan et al. 2016). This virus isolate was propagated on SISS cell line from Asian seabass spleen (provided by Dr. Sahul Hameed, C. Abdul Hakeem College, Melvisharam, India) as per Bandín et al. (2006) and used in this study to induce experimental infection in fish. Larval samples harvested from an outbreak of viral nervous necrosis in seabass were homogenized in PBS (1:10 *w*/*v*) and clarified by centrifugation, and subsequent filtration (0.22 μm) was used for inoculation. Briefly, cell monolayers were grown in L-15 medium containing 5% fetal bovine serum, penicillin (100 IU ml^{-1}), and streptomycin (100 mg ml^{-1}) in 25 cm^2 tissue culture flasks. The inoculated flask was incubated at 28 °C for 10 days. When the cytopathic effect (CPE) became extensive (Fig. 1), culture

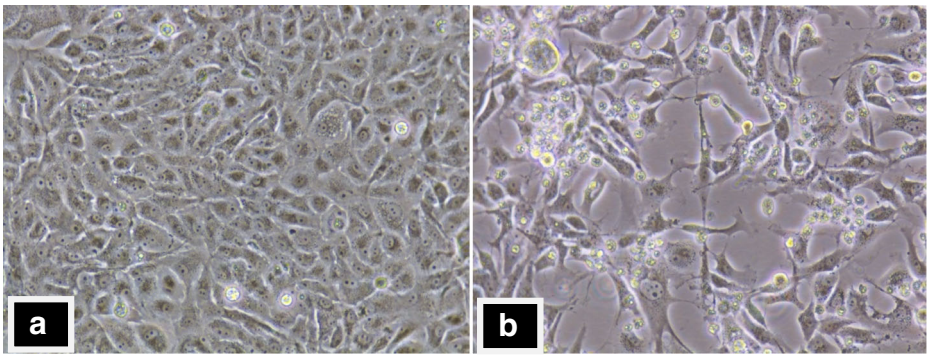

Fig. 1 SISS cell line monolayers after 96 h of incubation; **a** uninfected control, **b** infected with betanodavirus showing cytopathic effect (CPE), × 20

supernatant was harvested and after freeze-thawing of infected cells for three times followed by centrifugation (3000 *g*) for 15 min at 4 °C. The supernatant was syringe-filtered (0.22 μm) and stored at − 80 °C until use. Virus titration was conducted on monolayers of cells in 96 well plates using tenfold serial dilutions in triplicate. Plates incubated for 14 days at 28 °C. The 50% of the tissue culture infective dose ($TCID_{50}$ mL^{-1}) was calculated as described by Reed and Muench (1938). The titer of the culture supernatant was 10^3 $TCID_{50}$ mL^{-1} at 4 days post infection (DPI) and 10^8 $TCID_{50}$ mL^{-1} at 7 DPI. The culture supernatants were subjected to RNA extraction and further analyses by RT-PCR. The inoculum of betanodavirus for experimental infection consists of an aliquot in PBS, retrieved from infected culture supernatants which was maintained and preserved at − 196 °C in liquid nitrogen, was used with the simultaneous infection in cell lines for the presence of cytopathic effect within 76 h.

Experimental design

The experimental fishes were divided into three groups of 10 each; group 1, 2, and 3 (Gr. 1, 2, and 3) in 20 L plastic tanks. Gr. 1 fish were exposed to rearing fresh water (500 ml) spiked with viral inoculum (1:10,000 *w*/*v*) by immersion for 1 h under aeration. Gr. 2 fish received 10 μl of viral inoculum (1:10 dilution, *w*/*v*) as i/m injection using Hamilton syringe. Gr. 3 served as negative control fish without any infection, but half of the fish under this group received 10 μl of sterile PBS as injection control. The course of infection including clinical symptoms, morbidity, and/or mortality of the experimental fish was monitored thrice a day for 2 weeks.

Collection of samples

Moribund fish with characteristic symptoms of VNN were removed from the tank and two fish samples were collected from each of the infected and control groups at an interval of 2, 3, 5, 7, and 14th DPI or during the morbid state of the fish in each of the experimental group. However, the first mortality was observed in Gr. 1 after 28 h post immersion and 48 h in Gr. 2 with clinical signs of spiraling movement, lethargy, and fluid accumulation in the abdomen. This was followed by slow mortality in both immersion and injection group with clinical symptoms of typical cork screw spiraling movement, darkening of the body, black peppering on the flanks, orange colored head region, loss of appetite, and death. On each

sampling, one fish was used for RT-PCR and another fish for histopathology. For RNA extraction and RT-PCR, the head portion of the morbid fish consisting brain and retina was collected in Trizol (Invitrogen, USA) and stored at − 80 °C until use. Samples of the brain, eyes, and other visceral organs like intestine, liver, kidney, gonad, and heart were excised and fixed in 10% neutral buffered formalin (NBF) for histopathological examination under light microscopy. A portion of the brain from one fish from each of the infection group was fixed in 4% glutaraldehyde for transmission electron microscopic studies at Cancer Institute (WIA), Chennai.

RNA extraction and RT-PCR

RT-PCR analysis of the target organ was carried out to confirm the betanodavirus infection in fish samples collected at different time intervals by the following standard protocol. Total RNA was extracted as per manufacturer's instruction from the head portion consisting (brain, eye) of adult fish or whole larvae using Trizol method (Invitrogen, USA). Similarly, RNA extracted from seabass larvae with previous history of VNN outbreak, healthy larvae (negative control) stored at − 80 °C, and rearing water (spiked with betanodavirus) were also included in each assay. The RNA quality and purity (A_{260}/A_{280} ratio) was estimated using a Nano Spectrophotometer (Implen, Germany). A non-infectious sample of ethanol precipitated (80% *v*/*v*) tissue culture supernatants from active virus culture of betanodavirus (RGNNV) provided by CSIRO, Australia, was used as positive control.

All PCR reactions were conducted in Pro Flex PCR system (Applied Biosystems). First strand (cDNA) synthesis was carried out using ProtoScript® First Strand cDNA Synthesis Kit (New England Biolabs) in 10 μl reaction mixture (Oligo d (T) 23 VN 1 μl, M-MuLV Reaction Mix 5 μl, M-MuLV Enzyme Mix 1 μl, Nuclease-free Water 2.5 μl, and template RNA of 0.5 μl) using PCR cycling conditions of priming at 25 °C for 5 min, reverse transcription at 42 °C for 30 min, and reverse transcriptase inactivation at 80 °C for 5 min. The cDNA was quantified using a nanodrop at 260 nm. For PCR amplification, a single step 25 μl reaction mix, containing 1 μl of cDNA product, 12.5 μl master mix (Ampliqon Taq DNA polymerase, × 2.0 Master mix red, $MgCl_2$ 2.0 mM), 1 μl of each primers, and 9.5 μl distilled water was used. RT-PCR amplification was done using primers; F2 - 5' CGT GTC AGT CAT GTG TCG CT 3' and R3 - 5' CGA GTC AAC ACG GGT GAA GA 3' as per Nishizawa et al. (1994) by following cycling conditions: 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 40 s, annealing at 55 °C for 40 s, and elongation at 72 °C for 40 s, and a final extension at 72 °C for 5 min. The PCR products were analyzed for purity and size by electrophoresis in agarose gel (1.2%) stained with ethidium bromide (0.5 μg ml^{-1}) and visualized using a GelDoc XR^+ Imaging system (BioRad, USA). The identity of the amplified products representing the target size was purified from the gel using Power Gel Extraction kit (Sigma) following the manufacturer's protocol and sequenced by outsourcing (First BASE, Malaysia).

Histopathology

For histological studies, the head portion consisting brain, eye, and visceral organs were fixed separately in 10% neutral buffered formalin and processed by an automatic tissue processor (Microme, Germany) and histoembedder (Leica, Germany) as per standard protocol. Sections of tissues were cut (4–5 μm) using a rotary microtome (Leica RM 2245, Germany) and stained with hematoxylin and eosin (H&E). Stained sections were analyzed by light microscopy

(Nikon Eclipse E200, Japan) and digital images were taken using a Nikon DS-Fi2 digital camera using Nikon NIS-Elements Imaging software suite (Version F 4.30.01).

Transmission electron microscopy

Transmission electron microscopy of the samples fixed in 4% glutaraldehyde, washed in 0.1 M cacodylate buffer at 4 °C, post fixing in 1% osmium tetroxide, and washing in buffer were processed for electron microscopy. Ultra-thin sections were cut using Ultracut microtome (Leica, Germany) and stained with uranyl acetate and toluidine blue. The sections were examined and photographed using a transmission electron microscope (JEOL TEM-1400) and Olympus iTEM imaging platform.

Results

In the present study, we attempted to transmit betanodavirus (RGNNV) experimentally to freshwater fish *G. affinis* by injection and bath exposure to test the virulence of the virus and cause clinical signs of infection or pathogenesis in target organs. The VNN-free status of the experimental fish prior to infection was tested by RT-PCR and histology of target organs from representative samples of fish. Histopathology and RT-PCR of the coat protein gene were used for viral detection. Primers specifically designed to amplify coat protein gene of betanodavirus proved negative for the batch test of experimental animals, while the infective viral inoculum used for injection and the water samples drawn from bath exposure showed presence of virus by first step PCR (Fig. 2).

Clinical symptoms

Clinical manifestation started as early as 2 DPI in Gr. 2. The main clinical signs include darkening of the body, reduced feed intake, isolation, and descaling. Pigmentation with peppered appearance

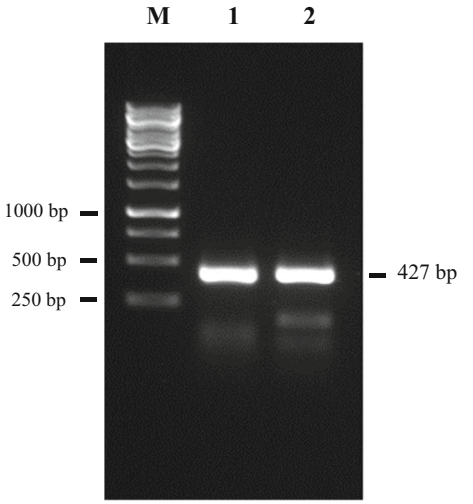

Fig. 2 PCR testing of experimental virus inoculum and rearing seawater (spiked with viral inoculum). Lanes: M 100 bp DNA marker, 1 betanodavirus affected cell culture supernatant, 2 rearing water (spiked with betanodavirus)

beginning at caudal end and gradual progression towards anterior end was also noted. In advanced stage, redness in the head region and loss of coordination were noted. The clinical course before mortality includes erratic swimming, typical cork screw swimming behavior, and settling at the bottom in a belly up position with ascites. Fish in Gr. 1 showed mild darkening and pigmentation and typical symptom only in few animals as compared to normal behavior and health status of control animals under Gr. 3. The fish in Gr. 2 showed maximum morbidity from 3 DPI with mortality commencing 6 DPI, while immersion group showed a slow pace of manifestation of symptoms marked by darkening of the body, twitching of tails, restlessness, and reduced feed intake from 4 DPI onwards lasting for a period of 14 days. The progression of the infection was manifested by dark pigmentation beginning from caudal end and sometimes only one side of the body. Some of the affected fish exhibited pigmentation with peppered appearance all over the body and caudal end in particular. The affected fish swim in isolation and in advanced stage, spiraling and cork screw swimming pattern in upside down position, inflated abdomen, and ascites followed by death within a day.

Laboratory investigations on experimental infections

Reverse transcriptase–polymerase chain reaction

RT-PCR is the most rapid, precise, and convenient method of diagnosing clinically affected fish. In the present study, RT-PCR showed positive results in injected group by 1 DPI, while in case of immersion group, it was on 3 DPI indicating the presence of the virus in the body and manifestation of clinical signs leading to mortality. Results of the laboratory analysis of the experimental animals at regular interval are shown in Table 1 and Table 2. All animals (except 3 DPI) in Gr. 1 were PCR positive upto 14 DPI and four fishes showed positive clinical signs of betanodavirus. All animals in injection group (Gr. 2) were found positive by PCR from 3 to 14 DPI with nine fishes exhibiting typical clinical signs (mortality of 3), while tissues collected from Gr. 3 (non-infected control) were found negative for betanodavirus throughout the course of the study (Fig. 3). The identity of the PCR product (427 bp) representing the target size was confirmed by sequencing results and NCBI BLAST analysis. The 423 bp nucleotide sequence generated in the present study has been deposited in GenBank (Accession No. KY040104). Two female fish used in this experiment with clinical symptoms belonging to injected group gave birth to larvae (7–9 larvae) on 9 DPI. These larvae released by the bearer fish suffered 50% mortality and also showed positive for betanodavirus by RT-PCR.

Histopathology

Histopathological examination of fish specimens sampled on 3, 5, 7, and 14 DPI showed VNN specific lesions characterized by vacuolations in the brain and eye tissue of animals under Gr. 1

Table 1 Experimental infection of betanodavirus in *Gambusia affinis* and the results of the laboratory analysis

Method of exposure	No. of fish	Morbidity	No. positive (RT-PCR)	Histopathology
Immersion (bath, 1 h)	10	4 (10)	4 (5)	+
Injection (i/m)	10	9 (10)	5 (5)	++
Control	10	0 (10)	0 (10)	−

Data in parenthesis indicate total number of animals

Table 2 Summary of the RT-PCR results on experimental infection of betanodavirus infection in *Gambusia affinis* at different days post infection (DPI)

Sample	PCR	Days post infection (DPI)						
		0	2	3	5	7	10	14
Group 1 (immersion)	I step	−	+	−	+	+	ND	+
Group 2 (injection)	I step	−	ND	+	+	+	+	+
Group 3 (control)	I step	−	−	−	−	ND	−	−

ND not done, + positive, − negative

and 2 (Fig. 4), while no lesions of any significance were seen in Gr. 3 animals. In Gr. 1, the prominent lesion noticed includes mild vacuolation of brain during 3 and 5 DPI and severe vacuolation on 7 and 14 DPI. Neuronal degeneration, spongiosis, engorged blood vessels, and gliosis were seen to be mild during early stage and found to be severe on 7 and 14 DPI. Kidney tubular necrosis with erythrocytic infiltration was evident. In Gr. 2, mild vacuolation and neuronal necrosis were observed on 3 DPI. Subsequently, these vacuolations assumed different sizes and shapes and found dispersed throughout the brain. Neuronal degeneration with neuropil accumulation was noticed in almost all days of sampling. Spongiosis was evident in almost all cases. Mild vacuolations were also observed in the eye from 3 to 5 DPI onwards. Fusion of the gill filaments was noticed on the 10 DPI. Myocardial muscle degeneration was observed on 6 DPI. Kidney tubular degeneration and necrosis was observed in almost all the DPI. Vacuolar degeneration was noticed in the eye tissue on 3 and 5 DPI. All major organs including brain, eye, liver, kidney, heart, spleen, and gills of uninfected control group appeared normal. The larval brain also had vacuolations and mild gliosis.

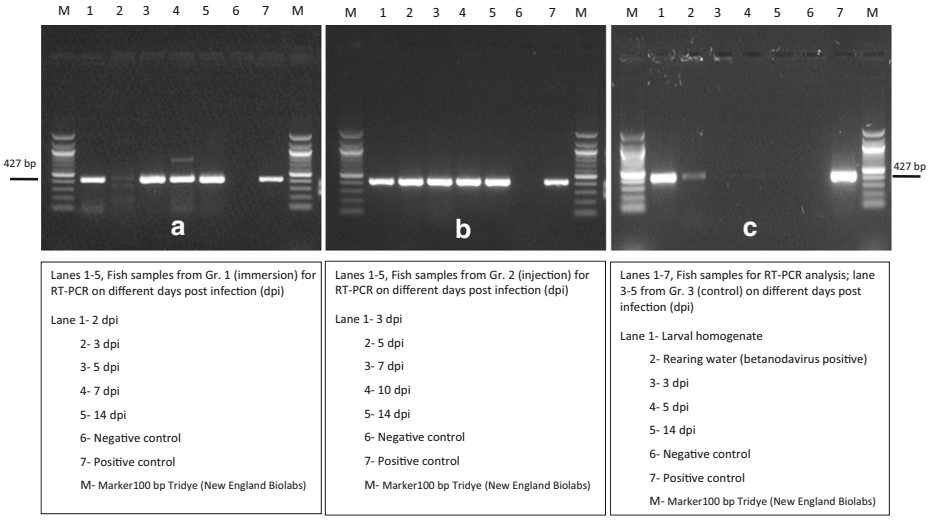

Fig. 3 RT-PCR (I step) analysis of experimental fish *Gambusia affinis* for betanodavirus on different days of post infection; **a** immersion (Gr.1), **b** injection (Gr.2), and **c** uninfected control (Gr.3)

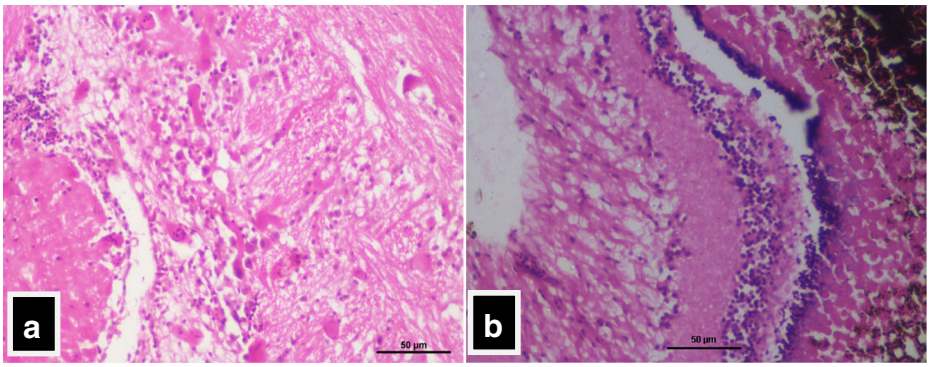

Fig. 4 Histological section (brain) of the experimental fish *Gambusia affinis* showing vacuolations in the brain and eye tissue of different groups on 4 DPI; **a**-immersion (Gr. 1) and **b** injection (Gr. 2)

Transmission electron microscopy

Ultra-thin sections showed numerous damaged myelinated nerve fibers with vacuolations were observed in the infected brain tissue. The cytoplasm of virus infected cells had membrane bound virion particles in neurons in close association with intracytoplasmic cell membrane (Fig. 5). Large vacuoles were always seen in heavily infected cells. Necrosed nerve cells were seen discretely in the brain tissue.

Discussion

Betanodavirus infection has been reported in at least 70 fish species across habitat barriers from freshwater to the seawater environments throughout the world, with the exception of South America (Munday et al., 2002; Maltese and Bovo, 2007; Doan et al. 2017). In India, the disease is increasingly becoming important in wild and culture conditions (Azad et al., 2005; Parameswaran et al., 2008; Jithendran et al. 2011; Binesh and Jithendran, 2013; Jithendran and Binesh, 2013; John et al., 2014; Banerjee et al., 2014). Infection experiment often revealed lack of clinical symptoms and/or histopathological lesions in target organs in spite of positive

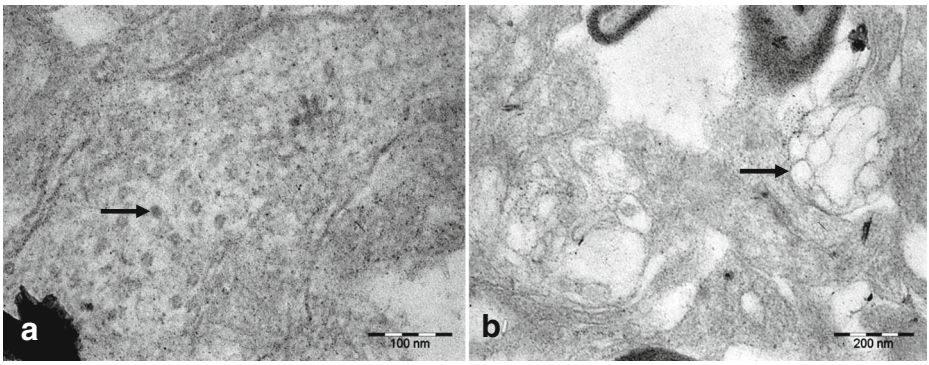

Fig. 5 Transmission electron photomicrograph of the brain of the experimental fish *Gambusia affinis* showing betanodavirus virion particle (arrow) affecting the neuron (**a**) and brain cells with large vacuoles (arrow) in the cytoplasm (**b**); lead citrate and uranyl acetate stain

analysis by PCR or cell culture isolation. Freshwater model has been reported earlier and many species of aquarium fishes were found susceptible to infection without much apparent clinical signs (Hegde et al., 2003; Furusawa et al., 2006, 2007; Gomez et al., 2006; Hasoon et al., 2011; Jithendran and Binesh, 2013). Contrary to this, guppy fish (*Poecilia reticulata*) has been shown to have developed clinical signs, typical cytopathic effect (CPE) in cell lines, and histological changes in target organs by challenge experiments (Nazari et al., 2014).

In this work, *Gambusia affinis* is a "livebearer" fish that give birth to well-developed larvae or juveniles. In these species, males typically bear an intromittent organ for inseminating the female, and the eggs are fertilized and developed within the ovary. In so-called "ovoviviparous" species, the developing embryos and larvae obtain nutrients only from the egg (lecithotrophy) as compared to viviparous species, including many poeciliids; the contribution of the yolk mass is less and nutrients are transferred directly from maternal circulation or secretions to the developing offspring (matritrophy) (Wootton and Smith, 2015). *Gambusia* spp. has shown susceptibility to epizootic hematopoietic necrosis virus (EHNV), an Australian strain of iridovirus in the genus *Ranavirus* by laboratory experiments (CFSPH, 2007). Epizootic hematopoietic necrosis is a systemic iridoviral disease of fish characterized by necrosis of the liver, spleen, and hematopoietic tissues within the kidney.

The present study confirmed the horizontal transmission of betanodavirus through water by immersion exposure and by intramuscular injection with manifestations of characteristic clinical signs and conspicuous histopathological lesions, although the natural susceptibility to this viral infection has not been recorded in literature till date. Further, this finding has extended the list of known susceptible hosts of the betanodaviruses in freshwater. Histologically, severe vacuolation in the brain, spinal cord, and retinal tissues of the eye is the most important and reliable diagnostic feature of VNN in fish. The brain of the clinically affected fish shows conspicuous vacuolation that varied from fish to fish in infected groups. No lesions of any major significance were seen in other organs. However, experimentally induced infections need not necessarily show these features especially in freshwater model fishes (Furusawa et al., 2007; Binesh, 2013). Histopathological examination of fish infected by immersion and injection route showed degenerative lesions in both brain as well as eye as compared to uninfected animals in a manner similar to that of other susceptible marine fish species. TEM study also confirmed the presence of intracytoplasmic vacuolation and presence of viral particles.

RT-PCR detection of betanodavirus was possible on 3 DPI in case of injection mode of infection and by 5 DPI in case of bath exposure of the virus. Sequencing of the PCR product also showed complete homogeneity with the RGNNV strain used as reference sample. Admittedly, one of the limitations of the present study is the lack of quantification of the viral load during the course of infection, though the initial viral titer ($TCID_{50}$) was 10^8 mL^{-1}.

Interestingly, a couple of female fish ($n = 2$) used in this experiment with clinical symptoms belonging to injected group gave birth to well-developed larvae on 9 DPI. Spontaneous release of larvae from bearer fish suffered 50% mortality and showed positive for betanodavirus by RT-PCR. This observation in freshly hatched larvae from female fish under Gr. 2 (injection) indicates the possible vertical route, in addition to the confirmation on presence betanodavirus by immersion and injection route of infection irrespective of the sex of the fish. More controlled studies are needed to verify this observation and further validation. However, this virus infection model should facilitate the elucidation of the transmission mechanism of the disease and potential use as a laboratory model fish. Moreover, the ovoviviparous reproduction should facilitate the study on vertical transmission of betanodavirus in this fish species as a lab

model as the fish can be bred very easily within a short span of time and minimum laboratory facilities.

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