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Characterisation of betanodavirus of finfishes from India and its purification using CsCl density gradient ultracentrifugation

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Abstract

Betanodavirus is a pathogen of many tropical and temperate marine and brackishwater fish species. It is an etiological agent of viral nervous necrosis (VNN) in fish larvae and juveniles, leading up to 100% mortality. One among the best and effective approaches to control viral diseases in aquaculture is vaccination. Purified viral particles are useful for the development of immunodiagnostics and vaccines. In this study, purification of betanodavirus was attempted through ultracentrifugation using CsCl density gradient. SSN-1 cells were infected with betanodavirus and after recording cytopathic effect (CPE), cells were harvested. Virus infection was confirmed by PCR assay using reported primers and sequencing. Transmission electron microscopy (TEM) was carried out for confirmation of infection. Virus from cell culture supernatants was purified using CsCl density gradient ultracentrifugation. Purified virus was further confirmed by PCR assay, SDS-PAGE analysis and TEM. The purified particles were tested for infectivity by incubating with SSN-1 cell line for its biological activity.

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Introduction

Many types of viruses infect finfishes and shellfishes (Crane and Hyatt, 2011). Betanodavirus among them is a major threat to aquaculture industry as about 40 marine and freshwater finfish species have been reported to be prone to betanodavirus infection leading to 100% mortality in juveniles (Furusawa *et al.,* 2006; Gomez *et al.,* 2006; Furusawa *et al.,* 2007; Nishi *et al.,* 2016). Further, more and more species are being added to the list of susceptible host species (Bandin and Souto, 2020). However, survived adult fishes become potential carriers. Infection can spread from wild fish to cultured ones and *vice versa* (Bitchava, *et al.,* 2019). Nodavirus of finfishes is a non-enveloped virus with bipartite genome comprising of two units of positive sense, single-stranded RNA molecules. RNA1 is responsible for RNA Dependent RNA Polymerase, which is necessary to duplicate viral RNA genome without the formation of intermediate DNA. It is about 3.1 to 3.2 kb long. RNA2 encodes for capsid protein which is about 1.2 to 1.4 kb. A subgenomic transcript of RNA1 and designated as RNA3 is also reported in betanodavirus and it codes for non-structural protein B2 (Mori *et al.,* 1992; Hayakijkosol, 2012). The concentration of nodavirus with polyethylene glycol (PEG) combined with density gradient ultracentrifugation is a method of choice for virus purification (Comps *et al.,* 1994). Cell culture isolation and observation of specific cytopathic effects (CPEs) (Frerichs *et al.,* 1996; Krishnan *et al.,* 2010; Benkaroun *et al.,* 2021), transmission electron microscopy (TEM) (Comps *et al.,* 1994; Frerichs *et al.,* 1996), SDS-PAGE analysis of capsid proteins (Comps *et al.,* 1994; Frerichs *et al.,* 1996; Krondiris and Sideris, 2002) and Sanger sequencing or Next Generation Sequencing (NGS) of partial sequences or whole genome are effective methods for characterisation of viruses (Ransangan and Manin, 2012). Vertical transmission is the main mode of nodavirus spreading (Murwantoko *et al.,* 2016) and hence screening of potential brooders gets significance. RT-PCR is the

method of choice for detection of different isotypes (Gomez *et al.,* 2004; Furusawa *et al.,* 2007; Nakai *et al.,* 2009). Specific antibody based non-lethal methods of nodavirus detection have been found to be suitable for early detection (Ferreira *et al.,* 2019). Purification and characterisation of nodavirus isolated from a particular source is important for development of a suitable diagnostic method. Therefore, this study was undertaken with the aim of characterising betanodavirus isolated from VNN outbreak in *Lates calcarifer* hatchery in India (Parameswaran *et al.,* 2006).

Materials and methods

Virus propagation

SSN-1 cells (Sigma-Aldrich, USA) developed from striped snakehead *Channa striata* at a passage level of 248 were used for virus propagation. The betanodavirus inoculum was provided by C. Abdul Hakeem College, Vellore, Tamil Nadu, India. The cells were grown at 28ºC in Leibovit's L-15 medium supplemented with 10% FBS and 1X antibiotic antimycotic solution (Sigma-Aldrich, USA). Growth medium was decanted from flasks having more than 90% confluency. Virus inoculum was mixed with L-15 medium without FBS and without antibiotic-antimycotic solution. Thirteen to fifteen millilitre inoculum was added to each 75 cm^2 cell culture flask and incubated for two hours after which inoculum was replaced with growth medium with 5% FBS and antibiotic-antimycotic solution. The cells were examined regularly for the presence of CPE for fifteen days. After appearance of CPE, flasks were harvested by repeated freezing and thawing cycles to detach and lyse the cells. The virus particles were separated from cell debris using low-speed centrifugation (3000 *g* at 4ºC for 10 min). The virus was confirmed with PCR assay. The centrifuged fraction was used for re-infection studies, cloning, SDS-PAGE and electron-microscopy.

Purification of virus using CsCl density gradient

For virus purification, SSN-1 cells were cultured on large scale and inoculated with virus. Cell cultures exhibiting CPE were collected and preserved at 4ºC until further use. Cells from one flask with CPE were harvested and pelleted by centrifugation at 15000 g for 10 min. Pellet was preserved in 3% glutaraldehyde for TEM study. Cells from remaining flasks were subjected to three cycles of freezing at -80ºC followed by thawing at 37ºC. Cell lysate was clarified by centrifugation at 3000 *g* for 15 min followed by centrifugation at 10,000 *g* for 30 min.

Method of Comps *et al.* (1994) was followed for virus purification from cell culture supernatant, with slight modifications. Briefly, cell culture supernatant was transferred to a 500 ml sterile container. Five percent of Polyethylene glycol (molecular weight-20,000) and 2.2% NaCl were added to the mixture and stirred well for 6 h at 4ºC. Fused virus particles were further sedimented and pelleted by centrifugation at 10,000 *g* for 1 h at 4ºC. Pellet was resuspended in sufficient quantity of TNE buffer (pH-7.3). Virus suspension was layered on preformed CsCl density gradient (20-35%) and topped with TNE buffer. Ultracentrifugation was carried out at 2,68,000 *g* for 8 h in SW 55Ti swinging bucket rotor using Beckman Coulter Ultracentrifuge (USA). Bands observed at interface of 20/25 density was aspirated and collected in a separate tube. An aliquot was preserved for PCR assay, SDS-PAGE analysis and TEM with negative staining for confirmation. Purified viral suspension was used for re-infection to study retention of infectivity after purification process.

SDS-PAGE analysis of purified virus

Structural proteins of purified virus particles were ascertained by SDS-PAGE analysis method of Laemmli (1970) with modifications. Briefly, viral proteins in purified form were disrupted by boiling at 95ºC for ten min in SDS reducing buffer and protein polypeptides were separated by polyacrylamide gel electrophoresis (PAGE) in a vertical gel apparatus (Genei, India) using 5% stacking gel and 10% resolving gel. Protein molecular mass standard (Bio-Rad) 6.5-200 kDa range was used as standard. Protein in gel was visualised by staining with 0.1% Coomassie Blue R-250 followed by destaining.

Transmission electron microscopy

Infected SSN-1 cells pelleted for TEM were initially fixed in 3% glutaraldehyde in 0.2M sodium cocodylate buffer (pH 7.4) at room temperature and shifted to 4ºC after 30 min. Later pellet was washed with sodium cocodylate buffer (2 times x 10 min) and post-fixed in 1% osmium tetroxide in sodium cocodylate buffer for one hour at 4ºC, followed by washing with distilled water 2 times x 10 min each. The pellet was further dehydrated in a series of increasing ethanol grade *viz.,* 50, 70, 90 and 100%. This was followed by two changes of propylene oxide, one change each of propylene oxide: epon (1:1) and (1:3) for 30 min and 100% epon for one hour. Ultrathin sections (90 nm) were prepared using ultracut microtome and placed on 300 mesh copper grids, stained with uranyl acetate and lead acetate and observed under JEOL 1010 transmission electron microscope.

A drop of purified virus suspension was placed on copper grid, stained with negative stain (phosphotungstic acid) dried under electric lamp and directly observed under JEOL 1010 electron microscope.

Partial cDNA sequencing

Total RNA isolation and cDNA synthesis

Total RNA was isolated from infected SSN-1 cell pellet using commercial reagent TRIzol® (Invitrogen, USA) following manufacturer's instructions. Trizol™ reagent was used for tissue disruption and RNA fraction was separated by using 150 µl of chloroform (0.2 ml per ml of Trizol™ reagent). RNA was precipitated by adding 400 µl of ultrapure isopropanol (0.5 volume per ml Trizol™ reagent). The precipitated RNA was washed twice with 1 ml of 70% ethanol. The RNA pellet was dissolved in 30 µl of nuclease-free water and stored at -80°C until further use. The concentration and purity of the isolated RNA was measured using Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). A purity ratio of 1.85-2.0 was considered acceptable for further analysis.

Complementary DNA (cDNA) synthesis

Total RNA was treated with 'RNase free' Dnase-I (MBI Fermentas, USA) before cDNA synthesis following manufacturers' protocol.

Total RNA was reverse transcribed to its complementary DNA (cDNA) using the M-MuLV reverse transcriptase First Strand cDNA Synthesis kit (Thermo Fisher scientific, USA) as per the manufacturer's instructions. The synthesised first strand cDNA was directly used in PCR to amplify the gene using specific primers.

PCR amplification

PCR amplification for coat protein genes for cloning was carried out using specific PCR primers (forward primer NNVRNA2 F2-5'CCCTCGAGGAACCCCGCCGACGTGCTAACA 3' and reverse primer NNVRNA2 R2 - 5' GGAATTCACGCCGTCAAGGGTACCAACAATA3'). Primers and cycling conditions as reported by Nishizawa *et al.* (1994) were used. The PCR conditions consisted of initial denaturation at 94ºC for 3 min followed by 30 cycles of denaturation at 94ºC for 30 s, annealing at 58ºC for 45 s and elongation at 72ºC for 1 min. Final elongation was for 10 min at 72ºC. cDNA (2.5 µg) reverse transcribed from total RNA was used as template. PCR product was visualised on 1.5% agarose gel. PCR was performed by using 25 µl of a PCR mixture containing cDNA (2.5 µg) template, 0.5 µl containing 10 pmol of each specific primer, 0.5 µl of 10 mM dNTP, 0.25 µl of Taq DNA polymerase and 2.5 µl of 10X Taq polymerase buffer containing 1.5 mM $MgCl_2$.

Cloning and characterisation of coat protein gene

The amplified PCR product was eluted using Gel Extraction kit (Qiagen, USA) following manufacturer's instructions. The eluted PCR product was cloned into pTZ57R/T vector using InsTAclone™ PCR Cloning Kit (Thermo Fisher Scientific, USA) following manufacturer's protocol. The vector used for the transformation of recombinant plasmid was DH5α strain of *E. coli*. Competent cells (DH5α) were prepared according to the supplier's protocol described in the InsTAclone™ PCR Cloning Kit (Thermo Fisher Scientific manual, USA). Colony PCR was performed with gene-specific primers for rapid confirmation of recombinant clones. Plasmid DNA from the positive clone was isolated using the Gene JET plasmid miniprep kit (Thermo Fisher Scientific, USA) as per the manufacturer's protocol. The integrity of the isolated plasmid was checked on 1% agarose gel.

Gene sequencing

The confirmed recombinant plasmid was sequenced using ABI Big DYE terminator method (Eurofins, Bangalore, India) and using M13 (-20) forward (5'-GTAAAACGACGGCCAGT-3') and M13 (-24) reverse (5'- GGAAACAGCTATGACCATG-3') primers. The data obtained in chromatogram was analysed using Chromas LITE 2.1.1. software. Phylogenetic analyses were conducted using MEGA version 7 (Kumar *et al.*, 2016).

Results

Virus isolation and propagation

Cells inoculated with virus started showing gradually increasing CPEs, on third day post-infection. Control SNN-1 cells had uniform monolayer with full confluency (Fig. 1a, b). At initial stage, infected cells were exhibiting vacuole formation followed by loss of cell-to-cell contact and multiple vacuolisation (Fig. 1c, d). Furthermore, elongated cells, formation of multinucleated syncytial masses of cells at multiple locations, grouping and rounding of cells were observed (Fig. 1e, f) and finally detachment of cells was noticed (Fig.1g, h). Infection of betanodavirus in SSN-1 cells was confirmed by PCR assay (Fig. 5).

Transmission electron microscopy

The TEM micrographs of infected cells harvested at different time intervals also indicated progressive changes such as formation of autophagic vacuoles (Fig. 2a, b) which are characteristic of NNV

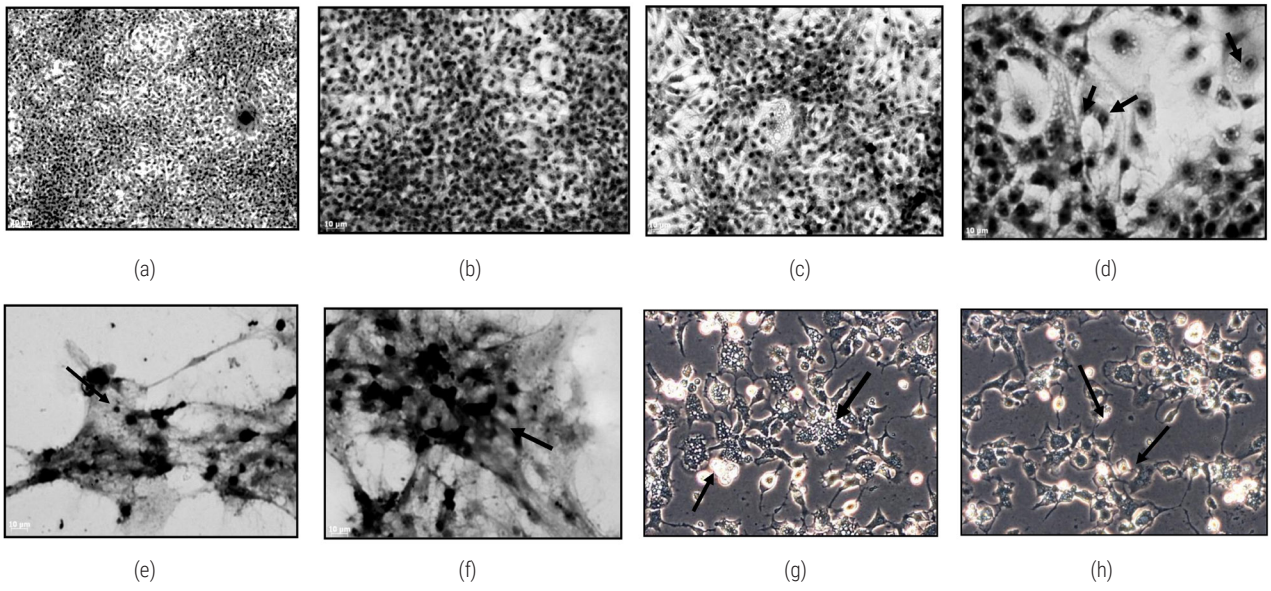


Fig 1. Cytopathic effects observed in SSN-1 cells. (a) and (b) Uninfected cells (x100); (c) (x100) and (d) (x200) : Infected cells showing vacuole formation; (e) (x100) and f (x200): Infected cells showing grouping and syncytial mass formation (Bold arrows); (g) and (h) (x100): Detachment of cells (Bold arrows)

infection along with chromatin condensation (Fig. 2c), membrane blebbing (Fig. 2d) and irregular shaped mitochondria (Fig. 2e, f). At higher magnification, membrane bound aggregations of viral (Fig. 2g) particles and virus particles released from autophagic vacuoles (Fig. 2h) were clearly seen.

The prominent changes noted in electron micrographs were changes in cytoplasmic organelles. Cytoplasmic autophagic vacuoles were a constant feature in many cells (Fig. 2a, b). Nuclear shrinkage and chromatin condensation were also noticed. Marked mitochondrial swelling, probably due to disturbed osmotic gradient balance and increased mitochondrial number, and irregular shape was also observed (Fig. 2c, d). The cell membrane blebbing was probably related to active membrane changes relevant to stages of infection and virus budding from the infected cells. Blebbing of cell membrane, which is an approved feature of virus infection was also noticed (Fig. 2e). Certain electron micrographs of ultrathin sections of infected SSN-1 cells showed intra-cytoplasmic, membrane bound as well as free aggregates of virus particles ranging in size from 24-29 nm (Fig. 2f, g).

Purification of virus using CsCl density gradient

In CsCl density gradient, two distinct virus bands were observed at the interface between 25 and 30%, the other one at 35% and 40% (Fig. 4b). PCR analysis of the band at 25 and 30% interfaces confirmed the presence of virus (Fig. 5). Transmission electron microscopy of CsCl density gradient purified virus particles by negative staining confirmed the presence of betanodavirus. The size range varied from 24-29 nm (Fig. 3).

SDS-PAGE analysis of purified virus

SDS-PAGE analysis of CsCl density gradient purified virus confirmed the presence of protein bands at approximately 42 KDa corresponding to major coat protein of the betanodavirus (Fig. 4a).

Phylogenetic analysis

Phylogenetic analysis of partial mRNA coat protein sequence elucidated in this study was found close similarity to Red spotted grouper nervous necrosis virus (NNV) and Guppy NNV coat protein sequences as shown in Fig. 6.

Discussion

Concentration and purification of virus particles is essential for the development of rapid and specific diagnostics through production of monoclonal antibodies. SSN-1 cells are an ideal cell line for isolation and propagation of betanodavirus (Frerichs *et al.,* 1991; Frerichs *et al.,* 1996; Lai *et al.,* 2003; Liu *et al.,* 2005; Lu *et al.,* 2012; Benkaroun *et al.,* 2021). Vacuolisation and pyknosis leading to necrosis are the prominent features of cells or tissues infected with betanodavirus (Yoshikoshi and KInoue, 1990; Lu *et al.,* 2003; Qin *et al.,* 2006; Crane and Hyatt, 2011). CPE specific to fish nodavirus in different cell lines was recorded as localised areas of rounded and refractile cells that later spread to cover a wider area of monolayer. This progressed to degenerated cells with multiple vacuolisation (Krishnan *et al.,* 2010; Wang *et al.,* 2022; Zhang *et al.,* 2022). CPEs such as localised areas of rounded, granular, refractile cells which later on formed a network of degenerating cells was recorded in SSN-1 cell monolayer in our study.

Macropinocytosis due to membrane ruffling was quoted as one of the probable mechanisms of entry of virus into cells. Area of localisation and arrangement of viral pathogens inside cells can be specific to the pathogen as reported by Qin *et al.* (2006). Arrangement of virus particles in paracrystalline arrays in cytoplasm and also in membrane-bound aggregates noted in this study, has been reported to be specific to *Epinephelus tauvina* nervous necrosis virus (ETNNV). Similar arrangements have been reported

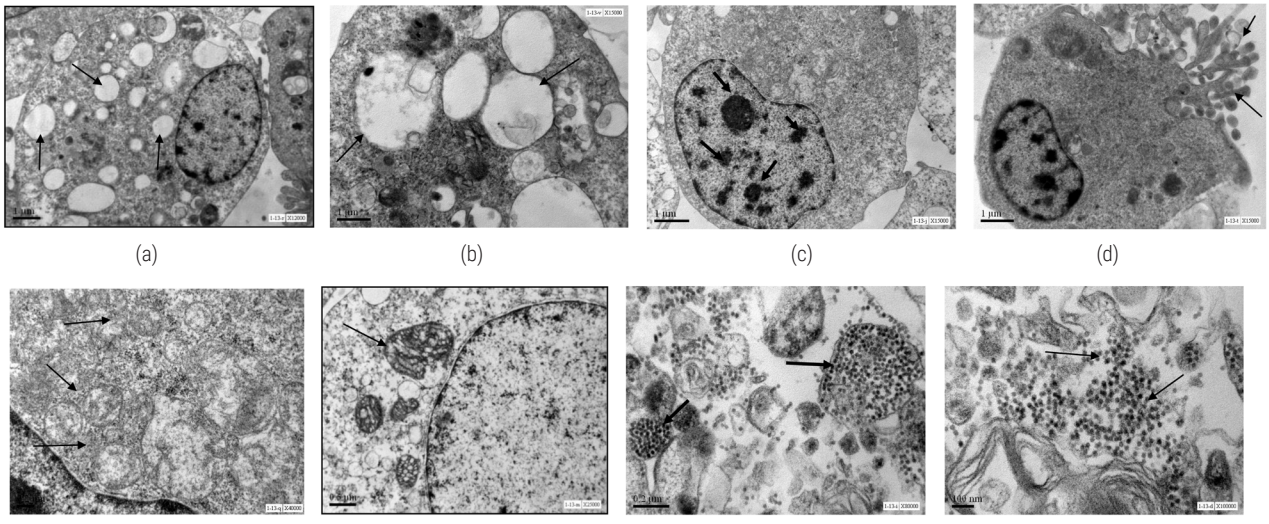
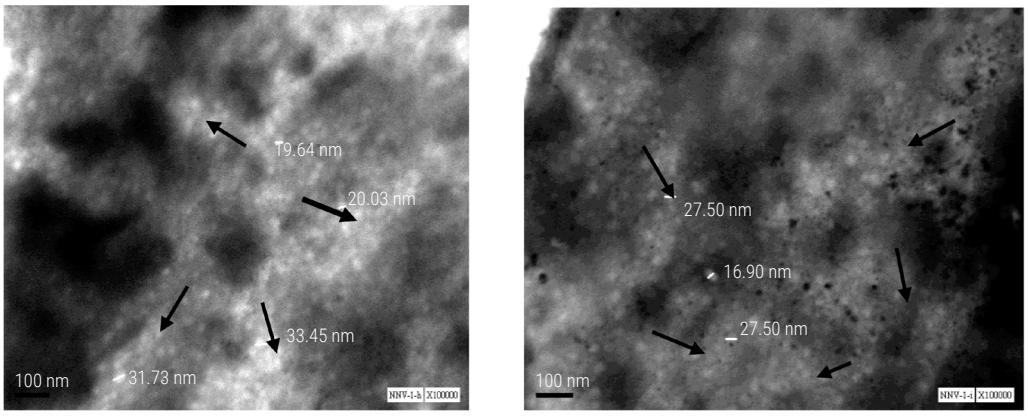
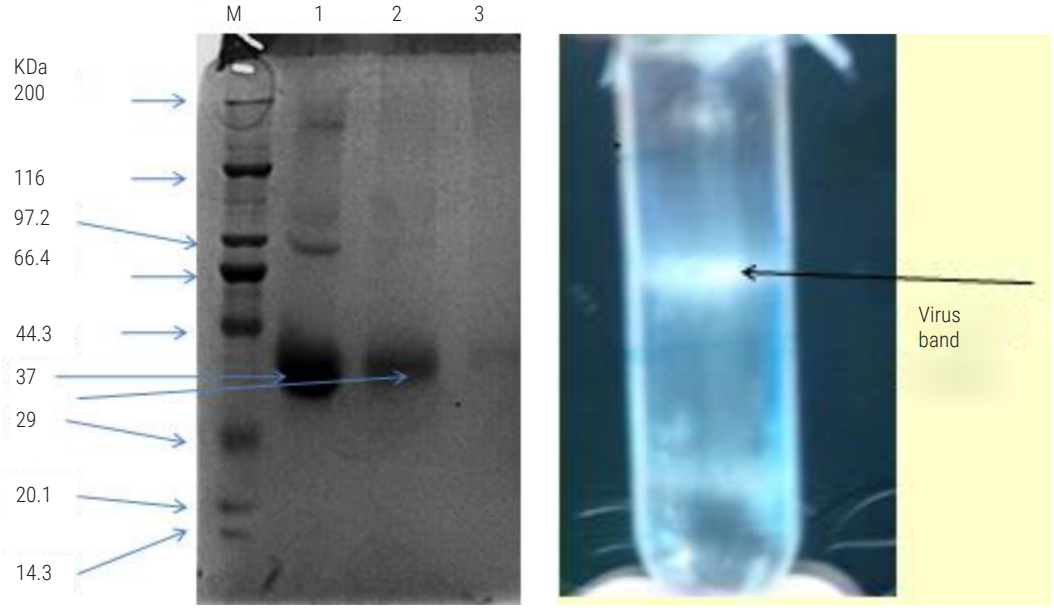


Fig. 2. Transmission electron micrographs exhibiting intracellular changes in infected SSN-1 cells. (a) x12000 and (b) x15000: Cytoplasmic autophagic vacuoles (Line arrows); (c) x15000: Chromatin condensation (Bold arrows); (d) x15000: Membrane blebbing (Line arrows); (e) x40000 and (f) x250000: Irregular shaped mitochondria (Line arrows) (M); (g) x80000: Membrane bound aggregations of virus particles (Bold arrows) (VPs); (h) x100000: Release of virus particles from autophagic vacuoule. Scale bar: 100 nm



(a) (b)

Fig. 3. TEM image of negatively stained purified virus particles (Bold arrows) (x100000). Scale bar – 100 nm



(a) (b)

Fig.4. SDS- PAGE showing (a). Viral coat protein at 42 KDa in sample lanes; M-200 KDa protein molecular marker, L1, L2 and L3- Samples of purified virus; (b) Band of virus particles (Arrow)

in other studies (Lai *et al.,* 2003; Liu *et al.,* 2005; Lu *et al.,* 2012). TEM examination of infected SSN-1 cells in this study showed intracytoplasmic, membrane-bound aggregates of virus particles.

One of the key steps in characterisation of virus particles is to verify the size and shape of the virus. Fission, fusion and mitophagy, lysis of affected mitochondria, are means of achieving homeostasis in mitochondrial as well as cellular functioning. These changes have the potential to decide the outcome of viral infections (Khan *et al.,* 2015). In another study, native dragon grouper nervous necrosis virus (DGNNV) and VLPs are found to be localised in endocytic vesicles (Lu *et al.,* 2003; Liu *et al.,* 2005). Further viral infection efficiency depends upon number of viral particles attached to cell surface (Lee *et al.,* 2023).

Virus infections can cause aggregation of mitochondria around nucleus leading to fission or fragmentation as observed in this study. Same observation was also reported in virus infected human neurons by Teodorof-Diedrich and Spector (2018). In negative staining, the betanodavirus particles are reported to range in size from 24 to 34 nm (Yoshikoshi and Inoue, 1990; Nguyen *et al.,* 1996; Muroga, 2001). The size reported in this study also found to fall within this range.

The purified fraction of virus revealed two closely associated virus polypeptides at 40 and 42 KDa structural proteins under reduced conditions on discontinuous SDS-PAGE gradient (Frerichs *et al.,* 1996). However, coat protein was found to migrate at 40 KDa due to breaking of disulfide bonds under a totally reduced state.

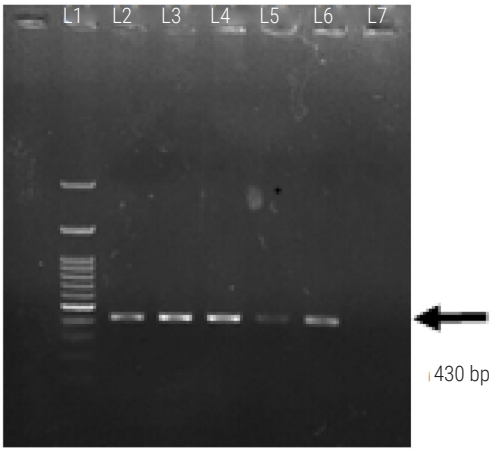


Fig. 5. Betanodavirus confirmation by PCR. L1 - 100 bps DNA molecular marker; L2: Virus band collected at interface of 20% and 25% CsCl density gradient; L3: Ultra purified virus particles; L4: Cell culture supernatant from (CCSN) SSN-1 cells infected with purified virus; L5: CCSN from SSN-1 cells infected with normal virus inoculum; L6: Positive control; L7: Negative control

The protein reoxidised at 38 KDa when electrophoresed under partially reduced or non-reduced condition, which was very close to the expected value derived from deduced amino acid sequence (Krondiris and Sideris, 2002). Virus like particles (VLPs) and native DGNN virus particles both were found to possess a 37 KDa coat protein under partially or non-reducing conditions (Lu *et al.,* 2003). Liu *et al.* (2005) also estimated molecular weight of NNV VLPs at 37 KDa. Thus, based on conditions of electrophoresis variations in the molecular weight of coat protein can be observed. Molecular weight of coat protein reported in our study falls within the reported range. Thus, CPE in cell culture, transmission electron microscopic study, molecular characterisation, coat protein analysis, particle size of betanodavirus as recorded in this study is in tandem with characteristics of betanodavirus as mentioned in previous studies. Phylogenetic analysis done in this study also placed partial coat protein sequence close to other coat protein sequences of betanodavirus.

Phylogenetic analysis with closely related and distant species has also been used for identification and characterisation of different strains of betanodaviruses (Thiery *et al.,* 2004; Toffolo *et al.,* 2007). Betanodavirus poses a great risk to the aquaculture industry due to its wide host range. A number of studies have been conducted to address the multiple aspects of nodavirus infection. However, a very few studies have focused on cell culture isolation and purification. Studies on infectivity of purified virus particles are also scarce. The betanodavirus can be successfully purified by methods used in this study while maintaining its infectivity. The outcome of the study is highly suitable for development of monoclonal antibody-based immunodiagnostics for betanodavirus using hybridoma technology and also for the development of viral vaccine.

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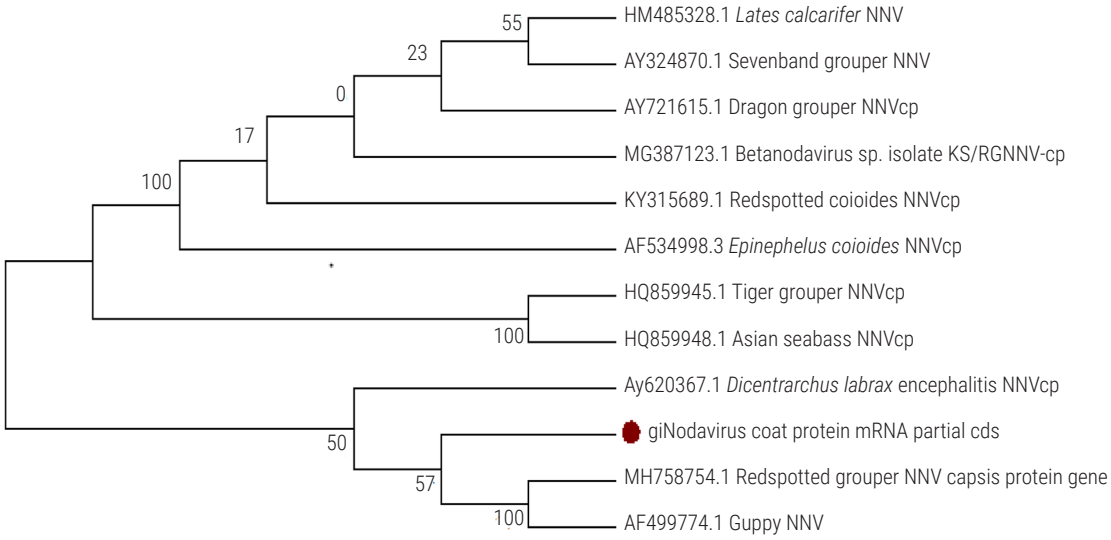

Fig. 6. Phylogenetic analysis using MEGA 7.0.25

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