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BRIEF REPORT

Comparison of betanodavirus replication efficiency in ten Indian fish cell lines

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Abstract Ten cell lines established from Indian marine, brackishwater and freshwater fish were tested for their susceptibility to fish nodavirus. In addition, the efficiency of betanodavirus replication was tested in these cell lines. Multiple vacuolation, a typical cytopathic effect for virus infection, was observed in infected SISK, SISS, SIGE and ICF cells. Infection of the different fish cell lines was confirmed by RT-PCR, immunodot blot assay and indirect ELISA. The virus concentration in culture supernatant collected from infected sea bass and grouper cell lines increased progressively from 10^3 at day 1 postinfection to 10^8 $TCID_{50}$ ml^{-1} at day 9. The amount of virus in different cell lines was also quantified by real-time PCR. These results indicate the suitability of the SISK, SISS, and SIGE cell lines for fish nodavirus propagation for developing viral diagnostics and vaccines.

Keywords Nodavirus · Susceptibility · Fish cell line · RT-PCR · $TCID_{50}$

Nodavirus infection has been one of the major limiting factors in the culture of marine fish species all over the world during the past decade. Nodaviruses have been reported to affect 40 different fish species worldwide. The occurrence of nodaviral infection in India was reported for the first time by Azad et al. [4] using RT-PCR analysis. Nodaviruses (betanodaviruses) that affect fish belong to the family *Nodaviridae*. They are non-enveloped, icosahedral, single-stranded RNA viruses that cause a disease called viral nervous necrosis (VNN) or viral encephalopathy and retinopathy (VER) in larvae and juveniles of marine finfish. The main target organ for nodavirus infection in fish is the central nervous system (CNS), including the brain, spinal cord and retina, where it causes extensive cellular vacuolation and neuronal degeneration [29]. The disease is therefore referred to as either VER or VNN and usually affects the larval or juvenile stages of fish, in which 80 to 100 % mortality has been recorded [4]. However, in recent years, significant mortality has been reported in older fish up to harvesting size [3, 20].

A nodavirus that is responsible for high mortality in sea bass larvae and brooders in the hatcheries has been isolated for the first time in India using a sea bass kidney cell line, and this has been confirmed by RT-PCR [37]. Susceptibility of cell lines to viral pathogens is the basis for isolating and characterizing fish viruses and is also very important for developing diagnostics and vaccines for prophylactic measures in the culture systems. Cell lines from many organs of fish of different species have been developed and tested for their susceptibility to fish nodaviruses for the purpose of their isolation and replication [5, 6, 8, 27, 35]. Some attempts to isolate nodavirus in a variety of cell lines have been largely unsuccessful [8, 28]. However, successful isolation of nodaviruses has been reported in other cell lines, such as the SB cell line [15], the SSN-1 snakehead cell line [19], the GF-1 grouper cell line [13], the SF sea bass cell line [11], the TF turbot cell line [1], the BB cell line from sea bass [14], sea bass kidney and spleen cell lines [35, 41], and a grouper eye cell line [36]. In most of the above cases, the information on nodavirus replication is lacking [12, 13]. In the present study, ten cell lines established from Indian fishes have been tested for

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their susceptibility to nodaviruses. The viral replication efficiency in these cell lines was also reported so that they can be used for large-scale propagation for the development of diagnostics and whole-virus vaccines.

Ten cell lines developed from Indian fishes were used to test the replication efficiency of a nodavirus for large-scale propagation. These ten cell lines were SISK (kidney cell line of *Lates calcarifer*), SISS (spleen cell line of *L. calcarifer*), SIGE (eye cell line of *Epinephelus coioides*), IGK (kidney cell line of *E. coioides*), ICF (fin cell line of *Clarias batrachus*), IEE (eye cell line of *Etroplus suratensis*), IEG (gill cell line of *E. suratensis*), IEK (kidney cell line of *E. suratensis*), SICH (heart cell line of *Catla catla*) and SICE (eye cell line of *C. catla*). Two control cell lines, SSN-1 (whole fry cell line of *Ophicephalus striatus*) and E-11 (clone of SSN-1 cell line), were used for comparison. All of the cell lines were cultured at 28 °C in Leibovitz's L-15 medium (L-15) (Invitrogen, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum (FBS).

The susceptibility of fish cell lines to a nodavirus was evaluated according to the method described by Fan et al. [18]. The fish nodavirus used in the present study was isolated from infected Asian sea bass (*L. calcarifer*) larvae during a massive outbreak in sea bass hatcheries located in Chennai and Nagapattinam of Tamilnadu, India [37]. This virus was confirmed by RT-PCR using nodavirus-specific primers designed by Nishizawa et al. [31]. The nodavirus was propagated in the SISK cell line for infectivity studies. Viral inoculum was prepared by the method described by Kang et al. [25]. For infection, the cells of different fish cell lines, including SSN-1 and E-11, were inoculated in a 24-well plate and incubated for 12–24 h at 28 °C to 60 % to 70 % confluence. After removing the medium, 0.1 ml of virus suspension with a titre of 10^3 $TCID_{50}$/ml was inoculated onto the cell culture in the 24-well plate and allowed to adsorb for 1 h. Then, 0.5 ml of maintenance medium containing 5 % FBS was added. The cells were incubated at 25 °C and examined daily for the appearance of a cytopathic effect (CPE) for up to 2 weeks. The virus was harvested from the infected cultures and processed by three cycles of freezing and thawing. The virus harvest was clarified by low-speed centrifugation (3000 × g) at 4 °C, and the viral titre was then determined in a 50 % tissue culture infective dose ($TCID_{50}$) assay in 96-well tissue culture plates based on the procedure described by Reed and Muench [40].

RT-PCR analysis was carried out to confirm the nodaviral infection in fish cell lines following standard RT-PCR protocol. Primers specific for nodaviruses designed by Nishizawa et al. [31] were used. The sequences of the primers were 5′-CGT GTC AGT CAT GTG TCG CT -3′ (forward) and 5′-CGA GTC AAC ACG GGT GAA GA -3′ (reverse) (GenBank accession no. AJ698093). The 426-bp amplicon was then analyzed by electrophoresis on a 1 % agarose gel.

A dot blot assay was carried out using the procedure described by Hawkes et al. [21] to confirm the nodaviral infection in cell lines using antiserum raised against a whole nodavirus. Indirect ELISA was performed to quantify the nodavirus in infected fish cell lines at different time points after inoculation according to the method of Schurrs and van Weemen [42].

All of the fish cell lines used were tested further for their ability of to support nodavirus replication. The nodavirus inoculum was prepared and the viral titre was determined as described above. Fish cell lines were exposed to nodavirus as described above, and samples were collected at 1, 3, 5, 7 and 9 days postinfection (d p.i.). The samples collected at different time intervals were then assayed to determine virus titres ($TCID_{50}$ ml^{-1}) using the SISK cell line. For determining $TCID_{50}$, 1 day before the day of titration, microtiter plates (96-well, flat bottom) were seeded with the SISK cell line. After 24 h, serial tenfold dilutions of nodavirus harvested from different cell lines were made in maintenance medium; 3 to 9 wells were used for the dilution range of 10^{-1} to 10^{-9}, and three wells were kept as controls. After an adsorption time of 1 h, all of the wells were emptied carefully, and 100 µl of maintenance medium was added to all the test and control wells. The plate was then incubated at 25 °C for 9 days. The plate was examined daily, and the observations were recorded. The $TCID_{50}$ was calculated by the method described by Reed and Muench [40].

Quantitative real-time PCR was carried out to determine the C_T (cycle threshold) value of the nodavirus in the different fish cell lines exposed to nodavirus at different time intervals (d.p.i.) [17, 38]. Total RNA was extracted from nodavirus-infected cell samples collected at different times, and cDNA was made by RT-PCR as described above. The cDNA was quantified using a NanoDrop 2000C spectrophotometer (Thermo Scientific, Waltham, USA) at 260 nm. Primers specific for the RNA-2 coat protein gene (GenBank accession no. AJ698093) of fish nodaviruses published previously by Nishizawa et al. [32] were used with a modification, namely the addition of restriction sites. The sequences were 5′-CCG GAA TTC GAT TTG GAC GTG CGA CCA A-3′ (forward) and 5′-CCC AAG CTT CAA CAG CGT ATC GCT GGA AG-3′ (reverse). The size of the amplicon was 175 bp. The viral load was estimated using the StepOnePlus™ system (Applied Biosystems, Singapore) with a DyNAmoTMSYBR® Green qPCR Kit (Finnzymes, Espoo, Finland).

Ten fish cell lines established recently in India were tested for their susceptibility to nodavirus. Except for the eye and heart cell lines of *C. catla*, all of the cell lines were found to be susceptible to nodavirus infection. The typical

CPE for nodaviral infection is multiple vacuolations in the cytoplasm of cells. The CPE with multiple vacuolations was prominent in the infected cells of SISK, SISS, SIGE and ICF cells (Fig. 1B, D, F and H). Vacuoles were not prominent in IEE, IEG, IEK and IGK cells (Fig. 1J, L, N and P), although morphological changes such as shrunken cells and degeneration of the monolayer were observed. A progressive CPE was observed in SISS, SISK, SIGE and ICF cells at 3 to 5 d.p.i. The CPE developed in the infected cells was characterized by vacuole formation in the cytoplasm of cells in some areas, after which the CPE spread to other areas of the monolayer. Finally, complete degeneration of the monolayer of cells was observed from 9 d.p.i. (Table 1). A similar CPE was observed in all subsequent passages in the cell lines SISS, SISK, SIGE and ICF. However, CPE formation and degeneration of monolayer appeared earlier in the subsequent passages when compared to the first passage (Table 1). The CPE observed in SISS, SISK and SIGE cells was found to be more prominent when compared to the CPE observed in SSN-1 and E-11 cells (Fig. 1R and T). No CPE was observed in uninfected cell lines (Fig. 1A, C, E, G, I, K, M, O, Q, and S) or in cells of catla exposed to nodavirus.

Nodaviral infection in different fish cell lines used in the study was confirmed by RT-PCR analysis using primers specific for nodaviruses. All of these cell lines except the catla cell lines were found to be positive for fish nodavirus by RT-PCR. The results of RT-PCR were further confirmed by dot blot analysis. The results of dot blot analysis revealed that SISK, SISS, SIGE, ICF, SSN-1 and E-11 were found to be positive at 3 d.p.i. while other cell lines were positive at 5 d.p.i. The catla cell lines were found to be negative. The intensity of nodaviral infection in different fish cell lines was quantified by indirect ELISA. The OD value was found to be greater than 2 in nodavirus-infected SISK, SISS, SIGE, SSN-1 and E-11 cells at 9 d p.i, whereas the OD value in ICF, IEE, IEK, IEG and IGK cells infected with nodavirus ranged from 1.5 to 2.0 at 9 d.p.i. The OD value for the cell lines from catla exposed to nodavirus was found to be similar to that of the uninfected cell lines.

The virus titre was determined by $TCID_{50}$ assay to study the replication efficiency of nodavirus in the ten fish cell lines used in this study, and the results are shown in Fig. 2. The $TCID_{50}$ of the culture supernatant collected from infected SISS, SISK and SIGE cells increased progressively from 10^3 at 1 d p.i. to 10^8 $TCID_{50}$ ml^{-1} as observed in the E-11 cell line at 9 d.p.i. Similarly, the maximum viral titer of approximately 10^7 $TCID_{50}$ ml^{-1} was observed in IEE, IEG and ICF cells and the control cell line SSN-1 at 9 d.p.i. In IEK and IGK cells, the viral titre was calculated as 10^6 $TCID_{50}$ ml^{-1} at 9 d.p.i.

The nodavirus was quantified in the fish cell lines exposed to nodavirus on different days by real-time PCR. The viral load increased during the course of infection in these cell lines after exposure to nodavirus. Lower C_T values for nodavirus were observed in different the fish cell lines infected with nodavirus at 9 d.p.i. when compared to the cell lines exposed for 5 days. The C_T value was found to be higher in nodavirus-infected SISK, SISS, SIGE, ICF, SSN-1, and E-11 cell lines when compared to the IGK, IEE, IEG and IEK cell lines. As a positive control, with a C_T value of 21.53 and low production of virus at a C_T of 32-35, the mean C_T values for the SISK, SISS, and SIGE cell lines were 29-35 at 5 dpi and faster to 20-25 at 9 dpi. At 5 dpi, the C_T value for the SSN-1 and E11 control cells was 32-35, while SISK and SISS outperformed them, reaching a C_T value of 28-29 of C_T at 5 dpi. The coefficient of variation (CV) for the C_T values for pFNV-RT was found to be less than 5 %, and this indicated that the assay was highly reproducible.

Cell lines are important for propagating viruses and for characterizing and studying mechanisms of infection [19]. Frerichs et al. [19] successfully used the SSN-1 cell line, derived from striped snakehead (*Ophicephalus striatus*), to isolate nodaviruses from diseased sea bass juveniles. The cloned E-11 cell line derived from SSN-1 [24] was subsequently used in a number of studies to isolate nodaviruses. Other cell lines used for propagating nodaviruses include simian COS-1 cells [16], GF-1 cells derived from grouper (*Epinephelus coioides*) [13], a tropical marine fish cell line (SF) from Asian sea bass (*Lates calcarifer*) [11], and the TF line from turbot (*Scophthalmus maximus*) [1]. The SAF-1 cell line proved to be a suitable alternative to the SSN-1 type. However, the widespread distribution of nodaviruses has led to the development of several other susceptible cell lines that have proven to be effective for propagating a number of different nodavirus strains [7, 14, 27, 39, 41].

In the present study, the cell lines developed from sea bass, grouper, pearl spot, catla and catfish were tested for their ability to propagate a nodavirus strain and used to determine the efficiency of these cell lines for viral replication. The cells are potentially useful for propagating nodaviruses on a large scale for developing diagnostics and vaccines in the near future. Each cell line was tested for its susceptibility to infection by CPE, RT-PCR and immunodot blot analysis. In addition, the replication efficiency was determined by $TCID_{50}$, real-time PCR and ELISA.

Establishment of specific and sensitive diagnostic methods for nodaviruses is important for early detection [23]. The nodavirus-infected cells were characterized by typical vacuole formation in the cytoplasm of cells followed by complete degeneration of the cell monolayer. The CPE observed in SISS, SISK, SIGE and ICF cells was

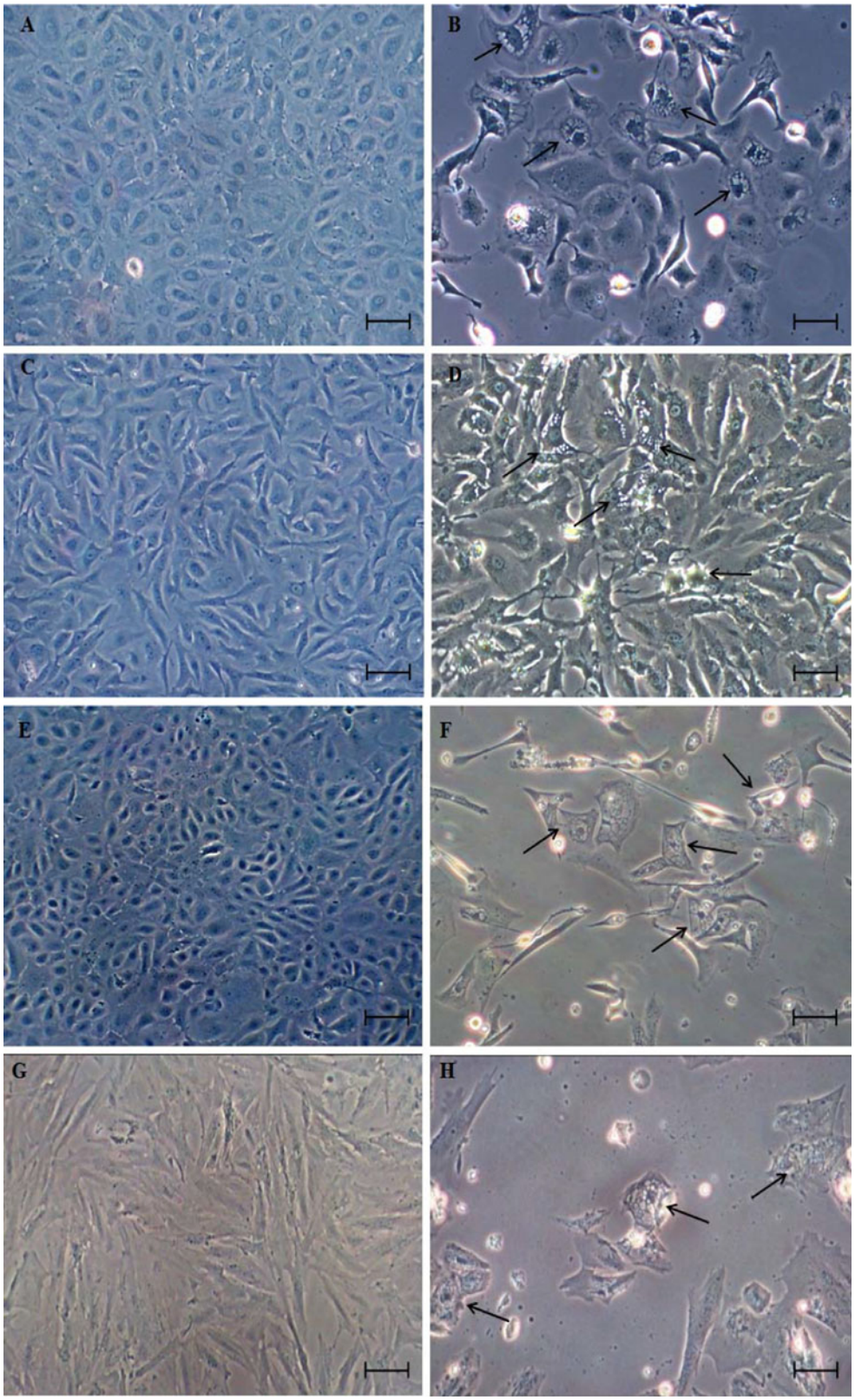
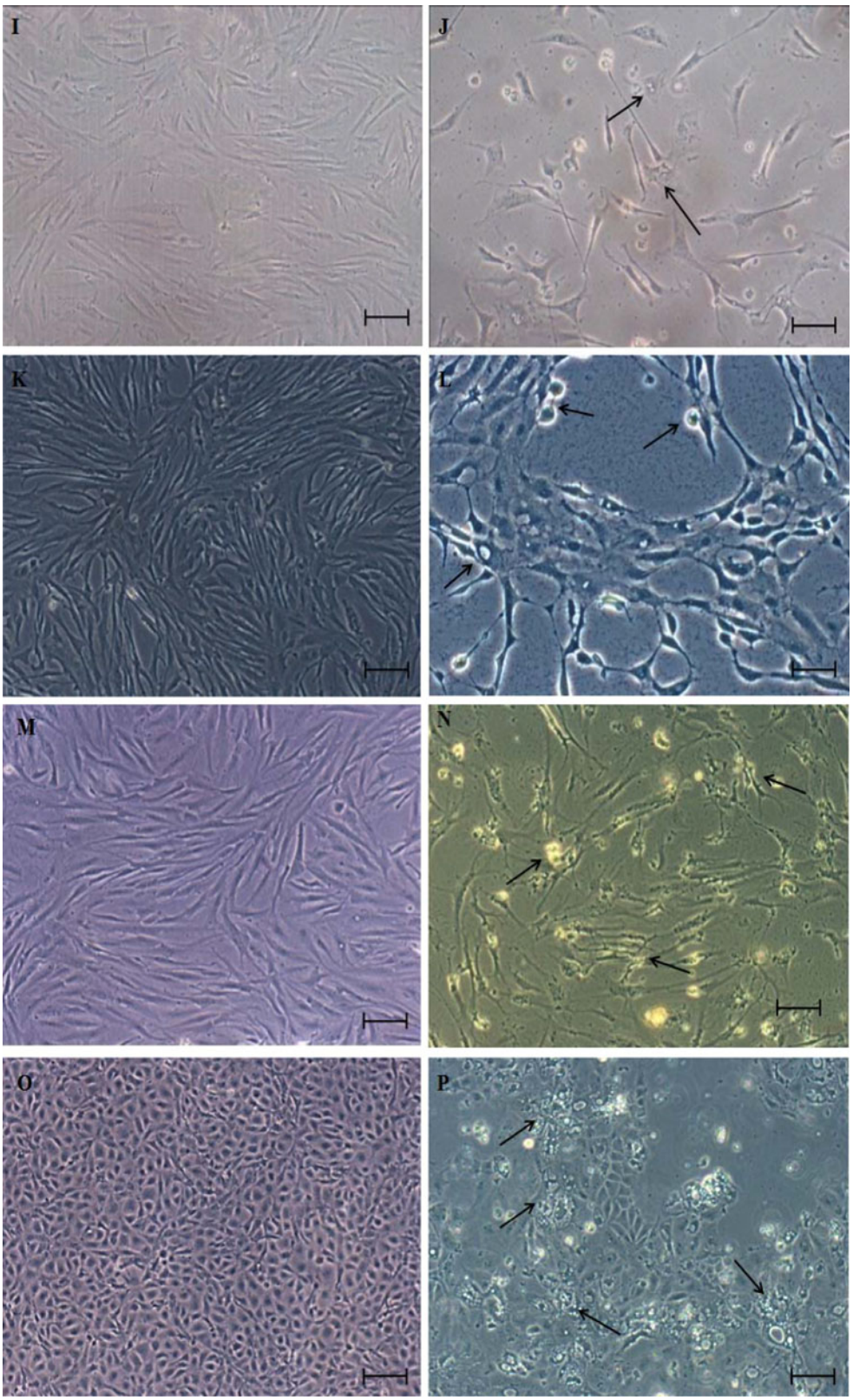


Fig. 1 Susceptibility of Indian fish cell lines to nodavirus infection. Cytopathic effect (CPE) with multiple vacuolations in infected cells (arrow). SISK cell line (**A** and **B**), SISS cell line (**C** and **D**), SIGE cell line (**E** and **F**), ICF cell line (**G** and **H**), IEE cell line (**I** and **J**), IEG cell line (**K** and **L**), IEK cell line (**M** and **N**), IGK cell line (**O** and **P**), SSN-1 cell line (**Q** and **R**) and E-11 cell line (**S** and **T**). Uninfected cell lines (**A**, **C**, **E**, **G**, **I**, **K**, **M**, **O**, **Q** & **S**); infected cell lines (**B**, **D**, **F**, **H**, **J**, **L**, **N**, **P**, **R** & **T**). Scale bar, 50 μm



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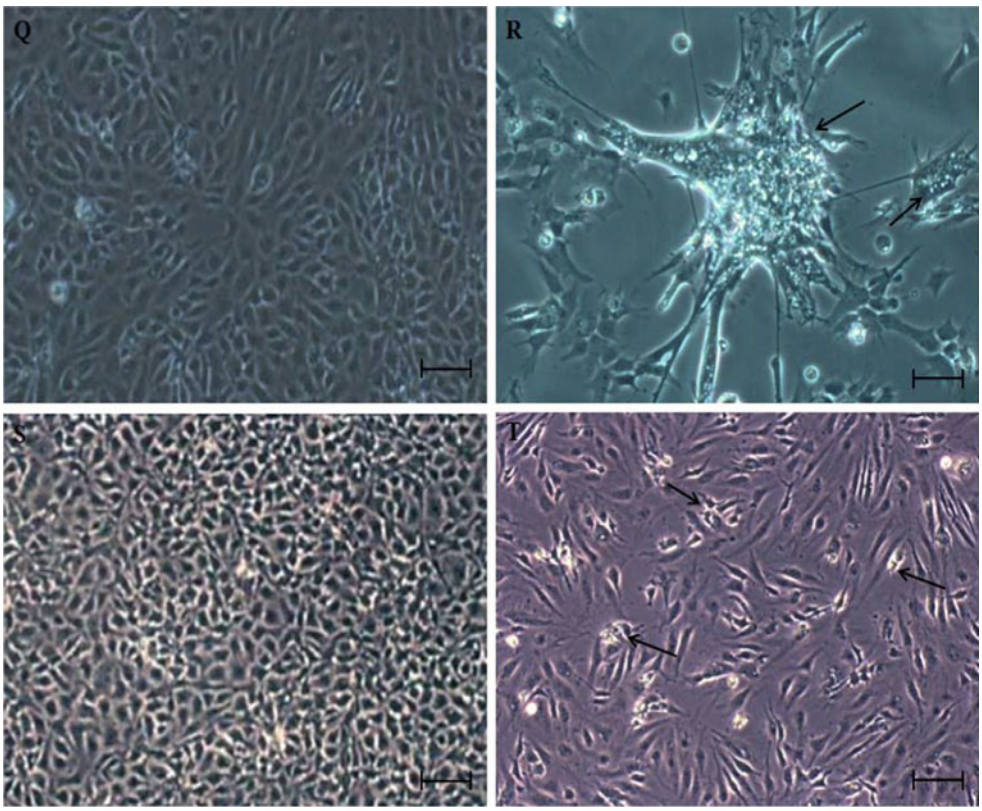

Fig. 1 continued

Table 1 Comparative infectivity of a fish nodavirus in different fish cell lines at 25 °C

Cell line (fish species)	Organ	First passage		Second passage	
		CPE	CD	CPE	CD
SISK (*Lates calcarifer*)	Kidney	3[a]	9	2	7
SISS (*Lates calcarifer*)	Spleen	3	9	2	7
SIGE (*Epinephelus coioides*)	Eye	4	10	3	8
ICF (*Clarias batrachus*)	Fin	5	11	3	9
IEE (*Etroplus suratensis*)	Eye	4	11	3	10
IEG (*Etroplus suratensis*)	Gill	5	12	3	10
IEK (*Etroplus suratensis*)	Kidney	5	12	4	11
IGK (*Epinephelus coioides*)	Kidney	5	12	4	11
SSN-1 (*Ophicephalus striatus*)	Fry	5	11	4	9
E-11 (*Ophicephalus striatus*)	Clone of SSN-1	6	12	4	10

CPE, cytopathic effect; CD, complete destruction

[a] Day postinfection when CPE or CD was observed

compared with the CPE observed in SSN-1 and E-11 cells. This CPE was similar to that reported by Frerichs et al. [19], Chua et al. [15], Chang et al. [11] and Qin et al. [39] in the SSN-1, SB, SF and GS cell line, respectively. The same CPE was observed in all subsequent passages of cells infected with fresh inoculum. The observation of CPE is also considered to be one of the simple detection methods, but it may not be confirmatory, and infection should therefore be confirmed by other diagnostic methods such as ELISA, electron microscopy studies or RT-PCR [34]. The CPE observed in nodavirus-infected SISS, SISK and SIGE cells was found to be prominent, and infection could easily be detected when CPE was compared to that observed in SSN-1 and E-11 cell lines. This is one of the advantages of using the SISS, SISK and SIGE cell lines for nodaviral isolation and propagation.

The nodavirus infection in the cell lines was confirmed by RT-PCR using primers designed by Nishizawa et al. [31]. In addition to RT-PCR, antibody-based diagnostic methods such as ELISA, western blot and indirect fluorescent antibody test (IFAT) have also been developed by different workers worldwide for detection of nodaviruses [30, 33]. Arimoto et al. [2] applied an ELISA technique to detect SJNNV using a rabbit polyclonal antiserum. Later, Shieh and Chi [43] used an antigen capture ELISA for diagnosis of nodaviral infection. ELISA was also used to detect nodavirus in different fish species to monitor their health status [9, 10, 23]. In the present study, polyclonal antibodies were successfully raised against heat-inactivated whole nodavirus in a rabbit and used to detect nodaviral

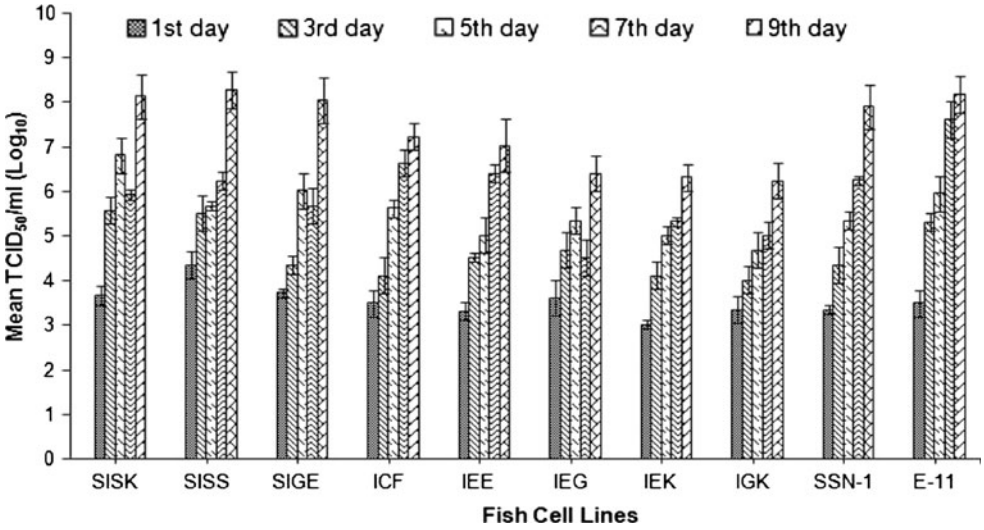

Fig. 2 Nodavirus titre in different fish cell lines at different times after infection (*days*). Titre is expressed as $TCID_{50}$ ml^{-1} (Mean ± SE) of the cell suspension

infection in different cell lines by ELISA and immunoblot assay.

The efficiency of nodavirus replication in different Indian fish cell lines was determined by $TCID_{50}$ assay, and the results were compared with those obtained using SSN-1 and E-11 cell lines. The cell lines from sea bass, grouper and Indian catfish supported the propagation of nodavirus and yielded significant titres ranging from 10^6 to 10^8 $TCID_{50}$ ml^{-1}. A virus titre of 10^8 $TCID_{50}$ ml^{-1} was achieved in the cell lines from sea bass and grouper, as observed in the SSN-1 and E-11 cell lines. Qin et al. [39] reported the susceptibility of a grouper spleen (GS) cell line to nodavirus infection and reported a viral titre of 10^7 $TCID_{50}$ ml^{-1}, whereas the eye cell line of grouper in the present study supported the propagation of nodavirus, and the virus titre increased to 10^8 $TCID_{50}$ ml^{-1}. The kidney and spleen cell lines of Asian sea bass supported the propagation of nodavirus and the viral titre increased to 10^8 $TCID_{50}$ ml^{-1} as observed by Hegde et al. [22] in an Asian sea bass cell line. Lai et al. [27] have established four cell lines from *Epinephelus awoara* and studied their susceptibility to nodaviruses. The grouper cell lines established by Lai et al. [26, 27] support the replication of nodaviruses, as evidenced by an increase in the viral titre to $10^{8.5}$ $TCID_{50}$ ml^{-1}. Wen et al. [44] observed a high nodaviral titre of about 10^{10} $TCID_{50}$ ml^{-1} in a brain cell line of orange spotted grouper. In the present study, the cell lines established from sea bass, grouper, catfish and pearl spot were tested for their ability to propagate a nodavirus. Their replication efficiency was assessed by $TCID_{50}$ assay. The results revealed that all of these cell lines support the propagation of nodaviruses and yielded viral titres ranging from 10^6 to 10^8 $TCID_{50}$ ml^{-1}, as has been observed by different workers using sea bass and grouper cell lines [22, 26, 27, 39, 44]. The load of fish nodavirus in different Indian fish cell lines was determined by real-time PCR at different time intervals, and the results revealed a decrease in C_T value of nodavirus in the cell lines exposed for 9 days. The C_T value was found to be lower in the cell lines from sea bass, grouper and catfish, and higher in the case of the *Etroplus* cell lines.

In conclusion, ten Indian fish cell lines derived from marine, brackishwater and freshwater fish species were applied for studying susceptibility to nodavirus infection. These cell lines were also tested for viral replication efficiency. Their susceptibility to nodavirus infection was confirmed by CPE, RT-PCR and immunodot blot assays. The replication efficiency of a nodavirus in these cell lines was tested by virus titration, indirect ELISA and real-time PCR. The SISK and SISS cell lines from sea bass and the SIGE cell line from grouper were found to be highly susceptible and allowed nodavirus replication at high efficiency. Furthermore, these three cell lines should be very useful for virus propagation and also for the large-scale production of viral diagnostics and vaccines for the benefit of the aquaculture industry.

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