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ORIGINAL ARTICLE

Characterization of a virulent ranavirus isolated from marine ornamental fish in India

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Abstract A viral agent implicated in the mortality of marine ornamental "Similar Damselfish" (*Pomacentrus similis* Allen, 1991) was isolated and characterized. The virus grew well in marine and freshwater fish cell lines from seabass and snakehead. The virus was sensitive to chloroform, acidic pH (3.0) and heat treatment at 56 °C. Biochemical characterisation indicated that the virus had double stranded DNA genome. Transmission electron microscopic analysis of ultrathin sections of infected cell pellets showed iridovirus-like icosahedral virus particles of 120–130 nm. Purified virus had six structural protein bands that ranged from of 44 to 132 kDa. PCR analysis confirmed the presence of viral DNA in infected cell cultures and sequence analysis of the major capsid protein gene showed an identity of 99.82% to that of largemouth bass virus. Serum neutralization studies involving the viral agent and koi ranavirus (KIRV) indicated partial homogeneity between the two isolates. Experimental infection of seabass (*Lates calcarifer*) and similar damselfish (*P. similis*) fingerlings with the similar damselfish virus showed cumulative mortalities of 68.75 and 93.33%. The biophysical and biochemical properties of the viral agent isolated, serological characteristics, size of major capsid proteins and the sequence similarity of the MCP gene proved that the virus belongs to the genus *Ranavirus* of the family *Iridoviridae*. Ability of the virus to grow in marine and freshwater fish cell lines and its pathogenicity to one of the cultivable marine fish shows the wide host range of the virus. This is the first report of ranavirus induced mortality in marine fish in India.

Keywords Aquaculture · *Iridoviridae* · *Ranavirus* · Seabass · Ornamental fish · India

Introduction

Culture and trade of ornamental fish are growing rapidly in India and worldwide. Ornamental fish contributed to a world export value of US$ 174 million, with imports of US$ 257 million in nineties. At present, the global ornamental fish sector is reported to be worth around US$ 15 billion (http://www.safea.org/facts-and-figures.html). Ornamental fish export from India has increased from Rs. 3.2 crores (US$ 0.6 million) in 2001–2002 to Rs. 10 crores (US$ 2.17 million) in 2010. Bright and vibrant colours of the marine ornamental fish in the tropics have caught the attention of many fish farmers and entrepreneurs in the South and Southeast Asian countries. Endowed with large areas of coral reefs and protected marine biosphere, India was successful in breeding and seed production of several marine ornamental fish in captivity. Damselfish are among the most popular marine ornamental fish due to their dazzling colours and ease of maintenance under controlled conditions. Several species inhabit the Indo Pacific region and more than 30 species belonging to various genera are commonly found in Indian coral seas [12].

Captive breeding and hatchery production of marine fish have always been hindered by infectious diseases caused by bacterial and viral pathogens. Due to the enormity of

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infection and lack of ideal control measures, viral diseases pose serious threat to the rearing of juveniles and maintenance of brood stock of marine ornamental fish [16, 41, 42, 44].

Viruses of the family *Iridoviridae,* which cause large scale mortalities in marine fish are divided into five genera viz. *Iridovirus, Chloriridovirus, Ranavirus*, *Lymphocystivirus* and *Megalocytivirus* [21]. Both wild and freshwater fish including ornamental fish have been threatened by viruses of *Iridoviridae* family [4, 7, 8, 19, 35, 47]. Iridoviruses found infecting fish have been accommodated in three genera viz. *Ranavirus*, *Lymphocystivirus* and *Megalocytivirus.* The genus *Ranavirus* (type virus, FV3) causes systemic infection in many fish and reptiles, where as *Lymphocystivirus* (type species, fish lymphocystis disease virus, LCDV) is associated with hyperplasia of connective tissue [45] and Megalocytiviruses (type species, infectious spleen and kidney necrosis virus ISKNV) infect multiple internal organs of marine fish species [46]. In 1990, red seabream (*Pagrus major*) iridovirus (RSIV) was first detected in Japan [19]. Several viruses belonging to *Iridoviridae* family have been isolated from different fish including infectious spleen and kidney necrosis virus (ISKNV), largemouth bass (*Micropterus salmoides*) virus (LMBV), African lampeye *Aplocheilichthys normani* iridovirus (ALIV), dwarf gourami *Colisa lalia* iridovirus (DGIV), rock bream *Oplegnathus fasciatus* iridovirus (RBIV), grouper *Cromileptes altivelis* sleepy disease virus (GSDIV) and RBIV IVS-1 [14, 15, 42]. Cultured ornamental fish in India has also been reported to suffer from virus induced mortalities due to nervous necrosis virus, ranavirus and cyprinus herpesvirus-2 [11, 22, 40].

The current investigation was aimed at finding out the aetiology of the continuous mortality observed in farm reared similar damselfish maintained in the culture tanks of an ornamental fish farm in South India. The fish that exhibited clinical signs such as abnormal swimming behaviour, anorexia, skin discoloration, surface ulcerations finally succumbed to the infection without any improvement after conventional treatments. We, therefore, carried out virological analysis of the infected fish and describe here the first time isolation and characterization of a virulent ranavirus in India based on biophysical, biochemical, molecular and serological characteristics and transmission studies. In order to ascertain the infectivity of the virus, we experimentally infected similar damselfish and seabass fingerlings with cell culture grown virus and observed that the virus was virulent to these two species of fish and could cause significant mortality in both the species.

Materials and methods

Fish

Frequent mortalities of farm reared similar damselfish (*Pomacentrus similis* Allen, 1991) were reported from local marine ornamental fish farms of South India. Several fish stocked in different tanks were dead following the infection leaving very few surviving fish in the tanks. Moribund fish were collected from the tanks and transported to the laboratory in ice. Infected fish brought to the laboratory had erythemic lesions on the dorsal head region and caudal peduncle indicating onset of ulcers. The fish upon arrival were aseptically dissected and tissues such as spleen and kidney were processed for virus isolation.

Cell lines

Seabass (*Lates calcarifer*) cell lines, SBCP2 and SBCP2a developed from seabass caudal peduncle and snakehead (*Channa striata*) cell line SNKD2a, derived from snakehead kidney, in the Department of Fish Pathology and Health Management of FCRI [24] were used for isolation, propagation and infectivity assays of the viral agent isolated. Other viruses such as blotched snakehead virus (BSNV, [25]) and koi ranavirus (KIRV, [11]) used in the study were grown in striped snakehead (*C. striata*) SSN-1 cells [10] and in bluegill (*Lepomis macrochirus*) fry BF-2 [48] cells respectively. The cell cultures were maintained in Leibovitz-15 (L-15) medium (Gibco, USA) supplemented with 10% foetal bovine serum (Gibco, USA) and 1× antibiotic antimycotic solution (Gibco, USA) in 25 cm^2 cell culture flasks (Thermo, Korea) at 27 °C.

Virus isolation and propagation

Kidney and spleen tissues collected aseptically from infected damselfish were homogenised in L15 medium containing 2% FBS and 1× antibiotic–antimycotic mix at 1/10 w/v dilution. The homogenate was centrifuged at 3000×*g* for 10 min at 10 °C and clarified supernatant was filter sterilised by 0.22-µm syringe membrane filter (Millipore). The filtered homogenate (0.7 mL) was inoculated to freshly prepared snakehead kidney cells (SNKD2a) in a 25-cm^2-flask. The inoculated cells were incubated at 27 °C along with control and observed for the development of cytopathic effect. Once the CPE was complete, the supernatant was removed and clarified by centrifugation at 3000×*g* for 10 min and filtered through 0.22-µm syringe membrane filter and passaged once for confirmation of virus isolation. Following full scale CPE of the cell monolayer, the virus stock (hereinafter referred to as

similar damselfish virus, SRDV) was prepared as before and stored at − 80 °C in 1 mL aliquots for further use.

Biophysical and biochemical characterization of the virus

Similar damselfish virus was tested for its heat stability, sensitivity to organic solvent and resistance to pH treatment (pH 3, 7 and 11) as described earlier [9, 11]. The type and nature of nucleic acid of the virus were analysed using a DNA inhibitor, 5-ido-2 deoxyuridine (IUDR) and acridine orange staining as described by Rovozzo and Burke [39].

Purification of virus

Similar damselfish virus was concentrated by ultracentrifugation of the virus preparation in SNKD2a cells. Confluent monolayers of SNKD2a cells in 175 cm^2 flasks inoculated with SRDV were harvested and clarified by centrifugation at 3000×*g* for 15 min at 4 °C after the cell layer was damaged by extensive CPE. The culture supernatant and cell pellet were processed separately. Pellet was resuspended in 2 mL of TNE buffer (50 mM Tris HCL, 140 mM NaCl, 5 mM EDTA, pH 7.5), transferred into a cryovial and subjected to 3 cycles of rapid freezing and thawing (35 °C) using liquid nitrogen. The suspension was clarified by centrifugation at 3000×*g* for 15 min at 4 °C and mixed with the culture supernatant. The virus was purified by ultracentrifugation of clarified cell culture supernatant at 100,000×*g* for 1 h in a SW-41 Ti rotor (Beckman, USA) using 20% sucrose cushion. The supernatant was discarded and the purified virus was diluted in 200 µl TNE buffer and stored at − 70 °C until use.

Transmission electron microscopy

The stock virus preparation of the isolate was inoculated on to SBCP2 cells and the cell pellet was harvested after 24–36 h and used for electron microscopy as per the general protocols described earlier [23]. Briefly, the pellet was fixed in 1 mL Karnovsky's fixative and held at 4 °C for 15–20 min. It was then washed in 0.1 M cacodylate buffer and post fixed in 1% osmium tetroxide. The cell pellets after dehydration were embedded in araldite resin and ultrathin sections (80 nm) cut using an Ultracut microtome (Leica Ultracut UCT). The sections were stained with uranyl acetate and lead citrate and examined using a Tecnai T12 Spirit transmission electron microscope (The Netherlands) at 60 kV.

Analysis of structural proteins by SDS-PAGE

Structural proteins of purified SRDV were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using the 0–300 V/0–100 mA vertical gel electrophoresis system (GenNei™, India) as described earlier [11].

PCR detection and sequencing of viral DNA

DNA from SRDV infected cell pellet preparation and fish tissues from pathogenicity study was extracted using 500 µl of DNA extraction solution (GeNei™, India) as per the manufacturer's instructions. The extracted DNA was allowed to air dry for 5 min and dissolved in 100 µl of deionised water and stored at 4 °C before use. The DNA extracted from SRDV infected SBCP2 cells and uninfected SBCP2 cells were used for PCR in 25 µl reaction mixture with Smart Prime Mix (Ampliqon, Denmark). Primers and cycling conditions used for amplification of different genes are given in the Table 1. The amplification products along with molecular markers stained by ethidium bromide were visualized in 1.5% agarose gels (GeNei, Merck Millipore, India) by a gel documentation system (UVI Tec, UK). The PCR products generated by MCP gene primers were sequenced by outsourcing to MWG, Eurofins Genomics India Pvt Ltd Bangalore, India. The sequences were analysed using CLC Main Workbench (CLC Bio) for multiple alignments and phylogenetic analysis with MCP gene of largemouth bass virus, LMBV (FR682503), koi ranavirus, KIRV (KJ939444), epizootic haematopoietic necrosis virus, EHNV (FJ433873) and frog virus 3, FV3 (AY548484).

Serum neutralization test

Polyclonal rabbit antiserum against the virus was prepared in New Zealand white rabbits as described by Lilley and Frerichs [27]. Cross-neutralising activity of the antisera of SRDV was determined by calculating neutralisation index (NI) after titrating the antiserum against both viruses by the beta (β) procedure [25]. Neutralisation Index (N.I.) is expressed as logarithmic difference in neutralizing antibody titres of the two viruses.

Pathogenicity studies

Experimental fish

Pathogenicity of the virus to the fish was investigated in cultured and ornamental fish. Marine cultured fish seabass (*L. calcarifer*) fingerlings obtained from a nearby hatchery were used for the pathogenicity study. Fish were

Table 1 Primers and cycling conditions used for amplification of MCP gene

Name	Sequence	Target size (bp)	Cycling conditions (35 cycles, final extension 72 °C 5 min)
M151 (EHNMCP1FW)	AACCCGGCTTTCGGGCAGCA	321	94 °C/30 s, 60 °C/30 s, 72 °C/40 s
M152 (EHNMCP1RE)	CGGGGCGGGGTTGATGAGAT		
M153 (EHNMCP2FW)	ATGACCGTCGCCCTCATCAC	625	94 °C/40 s, 55 °C/30 s, 72 °C/60 s
M154 (EHNMCP2RE)	CCATCGAGCCGTTCATGATG		
M151 (EHNMCP1FW)	AACCCGGCTTTCGGGCAGCA	1201	94 °C/40 s, 58 °C/30 s, 72 °C/60 s
M1542 (EHNMCP2RE)	CCATCGAGCCGTTCATGATG		

maintained in the laboratory until non-specific mortalities had ceased and acclimatisation achieved to the tank conditions. Fish were held in glass aquarium tanks of 100-L capacity maintained with 40-L seawater at a temperature of 30 ± 1 °C, salinity 25–28 ppt and pH 8 ± 0.2 and fed with commercial pelleted feed twice daily. Tanks were provided with continuous aeration and cleaned daily by siphoning out waste material along with partial water exchange (10–20%).

Pathogenicity study was also conducted for the marine ornamental similar damselfish (*P. similis*) fingerlings. Fish were procured locally and maintained in the laboratory until non-specific mortalities had subsided and acclimatised to the tank conditions. Fish were held in glass aquarium tanks maintained at a water temperature of 32 ± 1 °C, salinity 33–35 ppt and pH 8 ± 0.2 with closed recirculatory system having carbon filters but without additional aeration. Fish were fed with commercial pelleted feed twice daily. The tanks were cleaned daily to remove the wastes along with partial exchange (10%) of water. Seabass and damsel fish were tested for the presence of koi ranavirus and *L. calcarifer* nervous necrosis virus using standard protocols and were confirmed negative before the study.

Experimental infection

Seabass fingerlings (5.02 cm, 1.72 g) in three groups of eight fish each in duplicate were used for inoculating freshly grown SRDV in SBCP2a cells. First group was injected with neat undiluted virus suspension at 50 µl per fish and second group with 50 µl of 10^{-2} diluted virus suspension per fish intraperitoneally. Third group was administered with 50 µl SBCP2a cell culture supernatant diluted in the same manner as the virus isolates intraperitoneally and served as control. Fish were maintained as before and examined daily for any behavioural and morphological changes. Dead and moribund fish were assayed for the presence or absence of SRDV.

Experimental similar damselfish (6.74 cm, 6.48 g) were divided into two groups. First group containing eight fish each in duplicate was used for injecting SRDV intraperitoneally at 50 µl per fish. The second group containing seven fish each in duplicate was injected intraperitoneally with 50 µl cell culture supernatant diluted in the same manner served as control. The fish were examined daily for any behavioural and morphological changes. Dead and moribund fish were assayed for the presence or absence of SRDV.

Virological examination

Tissue samples of liver, spleen and kidney from of both seabass and similar damselfish were aseptically removed, pooled separately for each fish and examined for virus infection by cell culture isolation and PCR detection of MCP gene as described earlier. Cells without tissue extract served as control. Supernatant from all the flasks was again passaged once after CPE was observed.

Results

Susceptibility of cell lines to virus infection

Similar damselfish virus (SRDV) isolated in SNKD2a cell line developed from snakehead fish grew well in caudal peduncle cell lines (SBCP2 and SBCP2a) of seabass at 27 °C. The cytopathic effects in all the cells started with the formation of focal areas of cell destruction in the monolayer. After appearance of small foci with cell lysis, the CPE progressed by formation of granulated cells, rounding of cells at the edge of foci, localized cell death and complete damage to the monolayer of cells within 3–5 days. Subsequent passage of the above supernatant to the uninfected cell lines showed CPE as early as 2–3 days post-inoculation. Complete destruction of monolayer of cells occurred by 7-days post infection. Control SBCP2, SBCP2a and SNKD2a cells remained healthy throughout the 7 days of incubation.

Transmission electron microscopy

Transmission electron microscopic observation of SRDV-infected SNKD2a cells at 27 °C for 1 day showed virus particles in cytoplasm. Ultrathin sections of the virus-infected SNKD2a cells showed that the virus replicated in the cytoplasm. Viral nucleocapsids in different stages of assembly were observed in the cytoplasm, ranging from incomplete particles containing empty or partially formed electron-dense cores to complete mature icosahedral particles containing full electron-dense cores. Virions in the SNKD2a cells were seen empty or complete icosahedral particles of 120–130 nm diameter size (n = 7) (Fig. 1a, b). Virions were also seen budding from the plasma membrane of infected cells acquiring envelope (Fig. 1c).

Biophysical and biochemical characteristics

The replication of virus was found to be inhibited by IUDR compared to control with a reduction of infectivity by about two logs. The known RNA virus BSNV on the other hand, showed no reduction in the infectivity on treatment with IUDR. SNKD2a cells grown on glass cover slips infected with SRDV and stained with acridine orange showed fluorescent yellow cytoplasmic inclusion bodies. No such structures were observed in control uninfected SNKD2a cells (Fig. 2). SRDV titrated in SBCP2 cells for heat sensitivity showed that the virus lost infectivity within 1 h of exposure to heat at 56 °C. The similar damselfish virus on treatment with chloroform reduced the infectivity from $10^{5.8}$ to $10^{1.5}$ TCID 50 mL-1. Stability of SRDV at acid (3.0) and alkaline (11.0) pH determined following incubation of the virus suspension at room temperature for 30 min showed that SRDV infectivity was reduced by 2.5 logs after exposure to acidic conditions at pH 3.0 but not at pH 11.

Analysis of structural proteins

Purified virus preparation analysed using 12% SDS-PAGE showed six distinct bands having molecular mass ranging from 44 to 132 kDa (Fig. 3). The molecular weights of the proteins were estimated to be 132, 101, 63, 51, 46 and 44 kDa. Two of the six resolved as major (63 and 51 kDa) proteins and remaining four as minor proteins.

PCR amplification of SRDV DNA and sequencing analysis of major capsid protein

PCR analysis of the infected SBCP2 cells showed different results. Primer set I (M151 and M152) targeting the major capsid protein of the ranavirus amplified the characteristic product of 321 bp. The primer set II M153 and M154 did not amplify the expected 625-bp amplicon. Combination of M151 forward and M154 reverse amplified a larger amplicon of the expected size of 1201 bp. Similar set of reaction using uninfected control cells did not produce any amplification (Fig. 4). Similar results were also obtained in the analysis of fish tissues of experimental infection analysed for MCP genes.

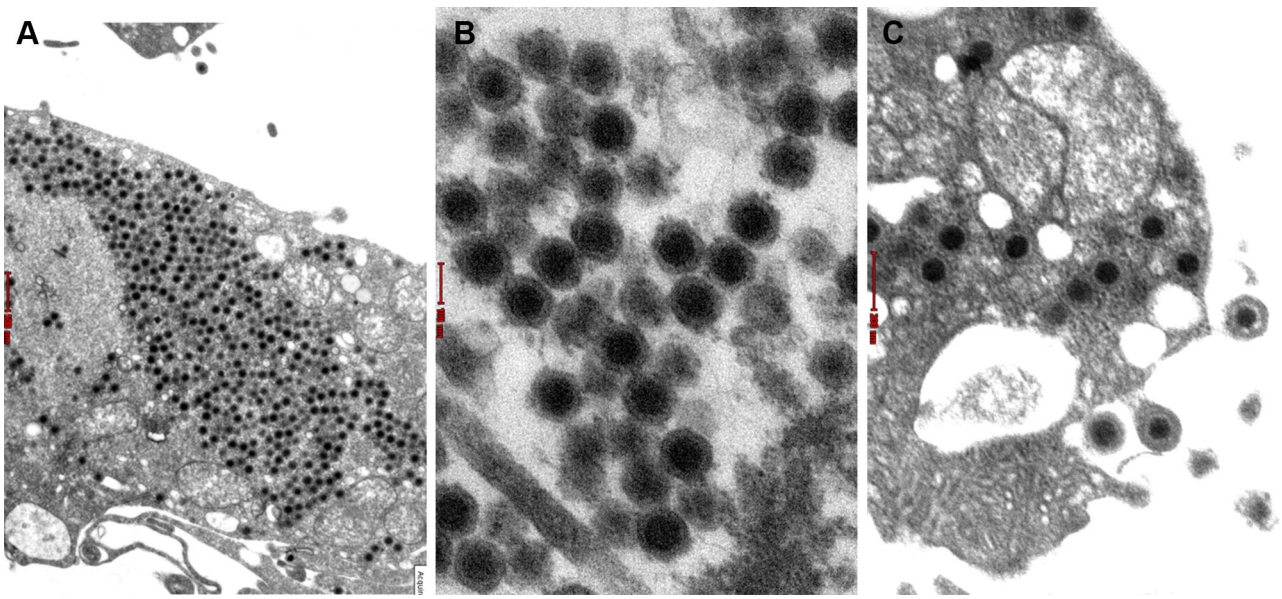

Fig. 1 Transmission electron micrographs of SRDV. **a** Electron micrograph of SRDV-infected SNKD2a cells showing virus particles in the assembly site in the cytoplasm (scale bar 500 nm) and **b** higher magnification of the virus particles in the cytoplasm with sizes ranging from 120 to 130 nm (scale bar 100 nm). **c** Virus particles budding from the plasma membrane of an infected cell acquiring the envelope (scale bar 200 nm)

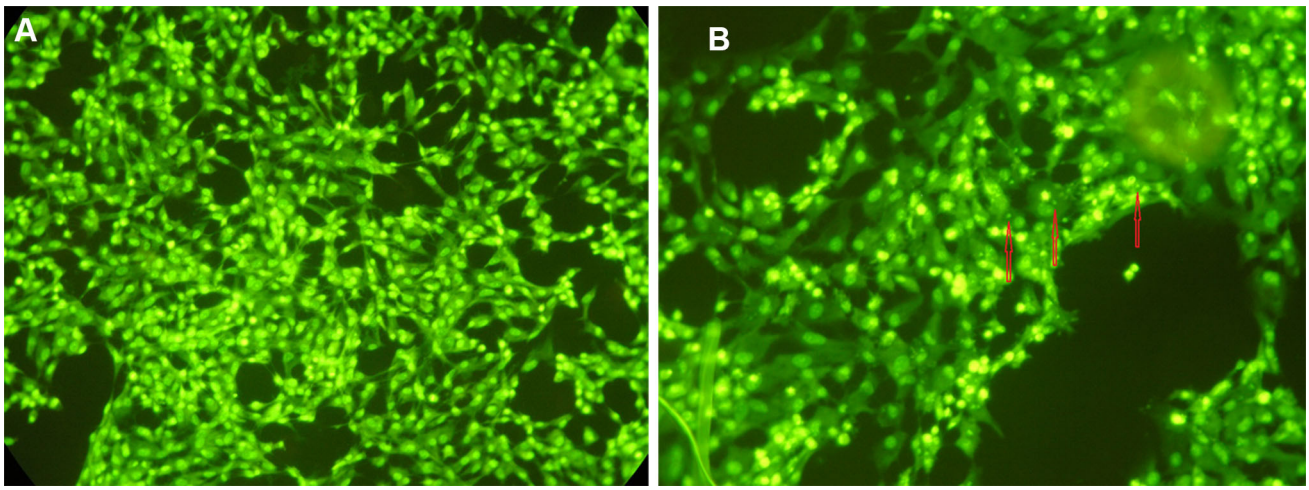

Fig. 2 Acridine orange staining of SRDV infection in SNKD2a cells. **a** Uninfected control SNKD2a cells. **b** SRDV infected SNKD2a cells. Yellow coloured extrachromosomal intracytoplasmic inclusion bodies indicated by arrow

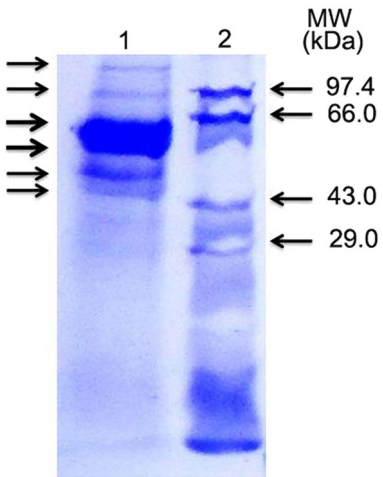

Fig. 3 SDS-PAGE analysis of structural proteins of SRDV in 12% acrylamide gel stained with 0.1% Coomassie brilliant blue. Lane 1: purified SRDV; Lane 2: medium range molecular mass markers (GeNei). Two major structural proteins of 63 and 51 kDa (thick arrow) and 4 minor proteins of 132, 101, 46 and 44 kDa (thin arrow) are indicated

Consensus sequence of the MCP gene obtained from the forward and reverse reads for the 1201 bp fragment showed 99.82% identity across 1130 bp fragment with the LMBV MCP gene sequence FR 682503; 99.29% with KIRV and 77.7% with EHNV and FV3. One amino acid was found different in the MCP sequence of SRDV from that of LMBV owing to a change in a single nucleotide at position 291 of the sequence. While it was a glycine in SRDV, it was aspartic acid in LMBV. A phylogenetic tree constructed with MCP sequences of SRDV, LMBV, KIRV, EHNV and FV3 is given in the Fig. 5. It clustered SRDV, LMBV and KIRV in one group and EHNV and FV3 in a distinctly different group.

Serum neutralization test

Rabbit antisera raised against the purified similar damselfish virus was used to test antigenic relationship between SRDV and KIRV. The serum had a homologous neutralisation dose (ND50) of 3.65 and heterologous ND50 of 3.19 with cross neutralisation index of 0.46.

Pathogenicity studies of SRDV

Seabass fingerlings injected intraperitoneally with undiluted and diluted SRDV preparation developed clinical signs 1 day after injection. Clinical signs such as sudden jerky movement, circling around the central axis and settling at the bottom of the tank were observed in all the virus inoculated fish before mortality. Clinical signs also included, lethargy, anorexia, followed by abnormal swimming before mortality. By day 35, the cumulative mortality of the neat virus infected seabass fingerlings had reached 62.5% and the 10^{-2} diluted virus infected seabass fingerlings interestingly reached 68.75% (Fig. 6a). As compared to the infected fish, no clinical signs or mortality were noticed in control fish injected with uninfected cell culture supernatant. Virus was re-isolated from the pooled homogenates of spleen and kidney of each of the dead and moribund SRDV injected seabass fingerlings. No CPE was noticed in the cell cultures inoculated with tissue extracts of control seabass fingerlings.

In the second experiment, SRDV injected damselfish developed clinical signs 2-day post injection. Clinical signs such as discolouration of skin, imbalanced swimming, lack of appetite indicated by no feed intake, settling at the bottom of the tank and finally mortality were observed in all the virus injected fish. Ulcer was also found on the dorsal region behind the head of the injected fish. By day

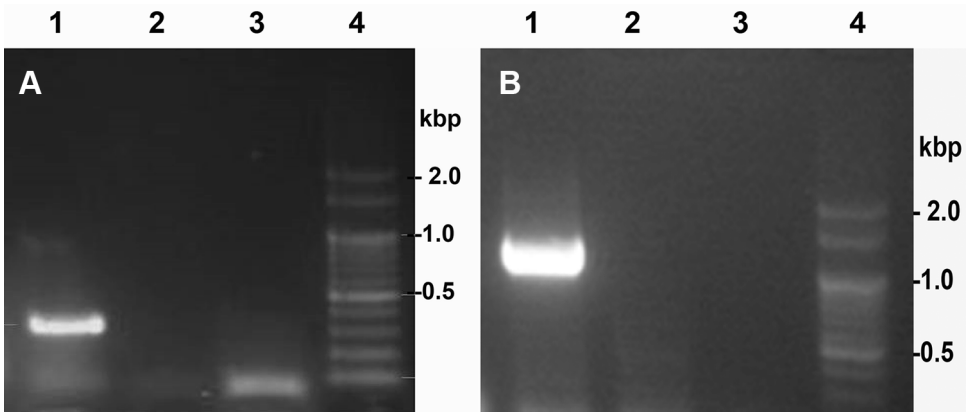

Fig. 4 Agarose gel electrophoretic analysis of the PCR products obtained from SRDV infected cell culture pellet preparation with primers targeting major capsid protein gene of EHNV. **a** with M151 (EHNMCP1FW) and M152 (EHNMCP1RE) primers. Lane 1: SRDV infected SBCP2 cell culture pellet; Lane 2: uninfected SBCP2 cell culture pellet; Lane 3: PCR negative control; Lane 4: 100 bp molecular marker (GeneRuler, Fermentas, Thermo Scientific). **b** with M151 (EHNMCP1FW) and M154 (EHNMCP2RE) primers. Lane 1: SRDV infected SBCP2 cell culture pellet; Lane 2: uninfected SBCP2 cell culture pellet; Lane 3: PCR negative control; Lane 4: 100 bp molecular marker

Fig. 5 Phylogenetic tree constructed using partial MCP gene sequences of five similar ranaviruses LMBV (FR682503), KIRV (KJ939444), EHNV (FJ433873) and FV3 (AY548484) by neighbour-joining method using CLC Main Workbench indicating the relationship of SRDV with other similar ranaviruses including the type virus of the genus, FV3

21, the cumulative mortality of the injected damselfish fingerlings reached 93.33% (Fig. 6b). As compared to the injected fish, no clinical signs or mortality were noticed in control fish. Virus was re-isolated from the pooled homogenates of spleen and kidney from each of the SRDV-injected dead fish. No CPE was noticed in the SNKD2a cells inoculated with tissue extracts of the control similar damselfish.

Discussion

Frequent mortalities were observed in similar damselfish maintained in the culture tanks of an ornamental fish farm in South Indian coast. We examined the infected fish for viral aetiology as the farm reported unabated mortality despite conventional therapeutics. The fish received in the laboratory had external clinical signs such as haemorrhagic lesions, surface ulcerations and damaged caudal fin. Post mortem analysis revealed the presence of ascitic fluid in the peritoneal cavity. Extensive ulcerations of the skin and muscles due to ranavirus infection were observed in largemouth bass in China [8]. Largemouth bass virus (LMBV) had been isolated from many fish species since its

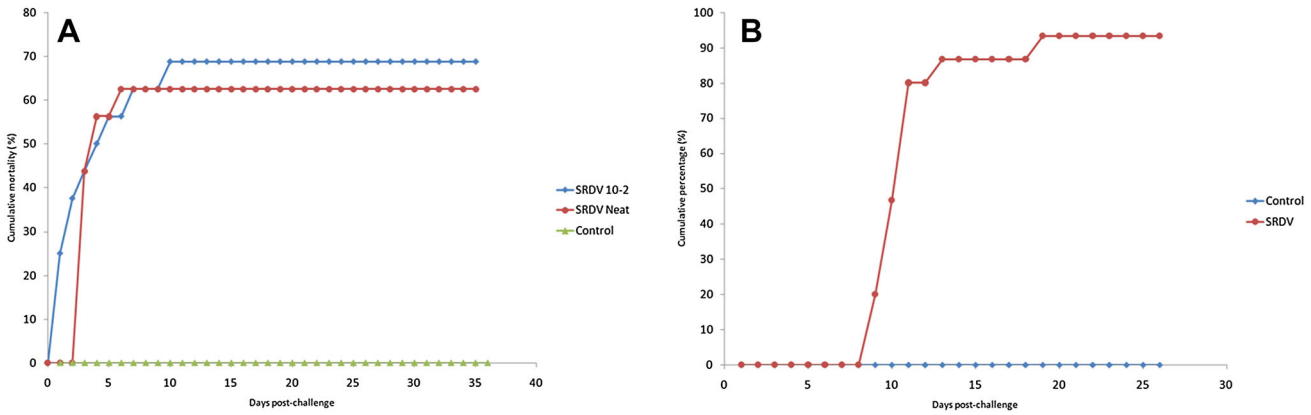

Fig. 6 **a** Cumulative mortality of seabass fingerlings experimentally challenged with SRDV. **b** Cumulative mortality of similar damselfish fingerlings experimentally challenged with SRDV

first isolation from diseased largemouth bass in Santee-Cooper reservoir [37]. Unlike the ulcerations observed in largemouth bass in China, LMBV infection has not been reported to cause any serious clinical signs in some of the fish species from which it was isolated [45]. Cell cultures inoculated with clarified and membrane filtered supernatant of tissue homogenates derived from characteristically infected similar damselfish exhibited cytopathic effect upon sustained passaging, which indicated the presence of a viral agent. The virus was found capable of growing in both marine and freshwater fish cell lines developed from seabass (SBCP2 and SBCP2a) and snakehead (SNKD2a) pointing to a wider host range for the virus. Most iridoviruses have been reported to cause cytopathic effects in cell cultures developed from different fish [1] and ranaviruses exhibit a wide host range among freshwater and marine fish [45]. Some of the ranaviruses induce severe mortality in fish with epizootic haematopoietic necrosis virus (EHNV) listed as a typical example coming under the OIE list of notifiable diseases [34]. The nature of the cytopathological changes induced by SRDV was similar to the CPE induced by SGIV and koi ranavirus [11, 38]. The CPE started with vacuolation of the cells, formation of granulated cells, rounding up of cells followed by localized cell death and complete damage to the monolayer of cells within 3–4 days. Similar CPE was noticed in SBCP2a and SNKD2a cells inoculated with the viral agent. Infectivity of the similar damselfish virus (SRDV) was comparable in both marine and freshwater cell lines (SBCP2a and SNKD2a respectively) with the virus yield ranging between 10^5–10^6 TCID50 mL-1 indicating the infectivity potential in both the fish species.

Characteristics of the virus particles such as the size ranging between 120–130 nm and acquiring an envelope by budding from the cell membrane observed in the transmission electron micrographs of SRDV revealed that virions are similar to that of the iridoviruses [3, 17, 26], which have icosahedral capsid with a size range of about 120–200 nm and a genome size ranging from 102 to 210 kbp [21]. Ranaviruses display icosahedral symmetry with size range of 120–200 nm in diameter and a genome size ranging between 105 and 140 kbp [5, 6]. Biophysical and biochemical characteristics of the virus were similar to that of the ranaviruses including intracytoplasmic inclusions, sensitivity to heat and low pH and presence of a double stranded DNA genome and an envelope [21, 38, 43]. The Chinese giant salamander virus (CGSV-L) characterised as a ranavirus was also sensitive to heat treatment at 56 °C for 30 min [49]. SRDV conforms with CGSV-L by its sensitivity to acidic pH and differs by its stability at alkaline pH (pH 11), where the CGSV-L was reported to lose its infectivity at pH 10. Ultrastructural, biochemical and biophysical characteristics of SRDV demonstrated its relatedness to the genus *Ranavirus* of the family *Iridoviridae* [36, 38, 43].

The SDS-PAGE analysis resolved the structural proteins of the virus into six polypeptides of 132, 101, 63, 51, 46 and 44 kDa molecular mass. Two of these six viz. 63 and 51 kDa formed the major structural proteins of the virus similar to other reported ranaviruses. Compared to six structural viral proteins of similar damselfish virus, koi ranavirus (KIRV) was reported to have two major and six minor structural proteins [11]. The major capsid proteins of the iridoviruses are in the range of 48–55 kDa, which forms almost 40% of the proteins [21]. MCP of EHNV, FV3 and GIV has a molecular weight of about 50 kDa [20, 30, 32].

Presence of the LMBV MCP gene sequences was detected in the infected cell culture pellet and infected fish tissues by only one set of the EHNV primers. However, the forward primer of the first set and reverse primer of the second set amplified the expected larger fragment of 1201 bp. Similar results were obtained for the KIRV isolate making both these viruses similar to LMBV than to EHNV [11]. Based on major capsid protein (MCP) gene sequences, three groups of ranaviruses could be recognised [33]. Many of the published ranavirus detection methods are based on amplification followed by sequencing analysis of the MCP gene. The MCP is the main structural protein of iridoviruses and is used as a marker for the identification, differentiation and classification of ranaviruses [28]. The first group contains viruses originating from fish, reptiles and amphibians, such as EHNV, FV3, BIV, ECV/ESV, ATV and SSTIV. The second group consists of Santee Cooper ranaviruses LBMV and DFV/GV6 and the third group comprises of the grouper iridoviruses GIV and SGIV. Sequence analysis of SRDV MCP gene fragment of 1130 bp showed 99.82% homology to LMBV (Santee-Cooper virus) MCP sequences (Genbank No. FR 682503). Similar findings were also reported in the MCP gene of the koi ranavirus, where the sequence homology was 99.91% with LMBV MCP. However, the SRDV MCP differed from EHNV and FV3 MCP sequences substantially with only 78.32 and 78.23% homology respectively. Although the cluster of three viruses SRDV, LMBV, and KIRV differed from EHNV and FV3 group by several changes in the nucleotides, all had a glycine at 291 bp except LMBV, which had an aspartic acid substitution at this location. Sequence similarity of the MCP gene fragment combined with the structural protein characteristics of the viral agent confirms the SRDV to belong to the genus *Ranavirus.* [13, 18, 29–31].

Serological studies undertaken with rabbit SRDV antisera against homologous virus and KIRV showed that both these viruses share similar immunogenic epitopes on their capsid proteins and has antigenic similarity. Further

studies through cross neutralisation studies and Western blot analysis would be required to delineate the two isolates.

Experimental infection study of SRDV caused mortality in seabass and similar damselfish during the 35-day observation period. SRDV caused cumulative mortality of 62.5 and 68.75% in seabass fingerlings infected with undiluted and 10^{-2} diluted virus preparations. In similar damselfish fingerlings, SRDV caused 93.33% cumulative mortality. This variation in the susceptibility of two different species is a key phenotypic factor of ranaviruses and LMBV in particular [45]. Challenge trials with ESV in sheatfish cultured at 24 °C resulted in 100% mortality 8 days after bath challenge and 11 days after exposure via co-habitation [2]. Juvenile koi, injected with tissue extracts prepared from brain, gill and pooled samples of spleen and kidney of infected koi from which the ranavirus was isolated, showed clinical signs such as uncoordinated swimming, rolling over and vertical hanging before death [11]. Similarly, SRDV injected fish also displayed clinical signs after 1 day of injection, such as sudden jerky movement, circling around the central axis and settling at the bottom of the tank. Clinical signs also included, lethargy, followed by abnormal swimming before mortality. Ulcer was noticed on the skin of dorsal region of virus infected fish in the present study. The EHNV infected fish shows clinical signs like distended abdomen, darkened skin, petechial haemorrhages at base of fin and in gill [45]. The SRDV infected experimental fish after exhibiting clinical signs, died in 2–3 days. The virus was recovered from liver, spleen and kidney homogenates of the moribund and dead fish in cell culture. In contrast, cell culture grown KIRV could not cause mortality in koi when the fish was experimentally infected with virus [11]. Though the two isolates shared similar biochemical and molecular properties, they showed variation in virulence indicating the differences in their biological characteristics. Santee Cooper viruses are reported to show high virulence variations in different species of fishes [45]. Further investigations involving more cultured and ornamental fishes are required to delineate the virulence variations of these two isolates to other fishes and also to establish causes of these biological variations.

Thus in the present study, we successfully characterised a virulent viral agent isolated from "Similar damselfish" through investigations on morphological, biophysical and biochemical, molecular, serological and pathogenic characteristics. The virus has high potential to infect both brackish water and marine fish apart from its ability to infect freshwater fish as indicated through cell line susceptibility study in snakehead cell line. Since this ranaviral agent is isolated for the first time from a marine fish in India, the name 'similar damselfish virus' (SRDV) is proposed for the agent.

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