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Isolation and characterization of a ranavirus from koi, *Cyprinus carpio* L., experiencing mass mortalities in India

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Abstract

We investigated mass mortalities of koi, *Cyprinus carpio* Linnaeus, 1758, experienced in South Indian fish farms by virus isolation, electron microscopy, PCR detection, sequencing of capsid protein gene and transmission studies. Samples of moribund koi brought to the laboratory suffered continuous mortality exhibiting swimming abnormalities, intermittent surfacing and skin darkening. Irido-like virus was isolated from the infected fish in the indigenous snakehead kidney cell line (SNKD2a). Icosahedral virus particles of 100 to 120 nm were observed in the infected cell cultures, budding from the cell membrane. Virus transmission and pathogenicity studies revealed that horizontal transmission occurred associated with mortality. PCR analysis of infected fish and cell cultures confirmed the presence of *Ranavirus* capsid protein sequences. Sequence analysis of the major capsid protein gene showed an identity of 99.9% to that of largemouth bass virus isolated from North America. Detection and successful isolation of this viral agent becomes the first record of isolation of a virus resembling Santee–Cooper Ranavirus from a koi and from India. We propose the name koi ranavirus to this agent.

Keywords: fish cell line, India, koi, ranavirus, snakehead.

Introduction

Common carp, *Cyprinus carpio* Linnaeus, 1758, enjoys worldwide distribution and is a widely cultivated fish species with a current aquaculture production of 3.44 million tonnes (FAO year book 2010). The koi is a coloured variety of common carp and is extensively used as an ornamental fish in large display aquaria and backyard ponds as personal hobby. The hobby has now been transformed into an art and science in Japan, and then subsequently spread worldwide (Balon 1995). Presently, koi is one of the most transcontinentally traded fishes among the ornamental fishes. Both common carp and koi culture suffered mass mortalities as last two decades in many countries, and the causative agent was identified to be koi herpesvirus. However, mortalities due to iridoviruses have not been reported from koi. Iridovirus infections can cause varied clinical signs ranging from death to no observable signs depending on the species infected (Langdon *et al.* 1986; Ahne *et al.* 1989a; Hedrick *et al.* 1992; Pozet *et al.* 1992; Hedrick & McDowell 1995).

Iridoviruses are double-stranded DNA viruses having icosahedral capsid with a size range of about 120–200 nm and a genome size ranging from 102 to 210 kbp (Jancovich *et al.* 2012). Iridoviruses have been found to infect both vertebrate and invertebrate hosts including fish, amphibians, reptiles, crustaceans, molluscs and insects (Williams 1996; Chinchar *et al.* 2009). The family Iridoviridae is subdivided into five genera, the *Iridovirus* and *Chloriridovirus* genera, which infect insects; the *Lymphocystivirus* and *Megalocytivirus* genera, which infect fish species; and *Ranavirus*, which contains viruses that are more genetically diverse and associated with mortality in amphibians, fish and reptiles (Chinchar 2002). Genome sizes of ranaviruses vary from 105 to 140 kbp with number of putative ORFs ranging between 92 and 139 (Chinchar, Yu &

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Jancovich 2011). Ranaviruses can cause acute, systemic disease in fish with increasing severity resulting from necrosis of kidney and spleen and haemorrhages on the skin and internal organs (Chinchar 2002; Williams, Barbosa-Solomieu & Chinchar 2005). Viruses of the genera *Ranavirus* are of growing concern to aquaculture owing to their ability to cause large-scale mortality in a wide variety of host species (Ahne *et al.* 1997; Mao, Hedrick & Chinchar 1997; Qin *et al.* 2003; Williams *et al.* 2005).

Within the genus *Ranavirus*, there are viruses that appear to be different in several respects from the type species FV3. Two tropical ranavirus isolates, guppy virus 6 (GV6) and doctor fish virus (DFV), were found to be different from European and Australian ranavirus isolates based on the nucleotide sequences of major capsid protein (MCP), DNA polymerase and neurofilament triplet H1-like (NF-H1) protein gene (Holopainen *et al.* 2009). However, these two viruses are found to be very similar but not identical with the North American Santee–Cooper ranavirus isolated from largemouth bass (Mao *et al.* 1999). Some authors have suggested that the Santee–Cooper ranavirus and related viruses such as doctor fish virus and guppy virus may not belong to the genus (Hyatt *et al.* 2000; Whittington, Becker & Dennis 2010).

Ranaviruses infect multiple coldblooded vertebrates and have been found undergone several host shifts suggesting the possibility of these viruses crossing the poikilothermic species barriers leading eventually to potentially devastating diseases in new hosts (Jancovich *et al.* 2010). Some strains of iridoviruses such as EHNV have also been isolated from fishes not showing clinical disease indicating their likely role as the carriers of the virus. Experimental inoculation resulting in sero-conversion but without clinical signs has been reported from EHNV in Australian frogs or the cane toad *Bufo marinus* (Zupanovic *et al.* 1998). In the present study, we have investigated infected juvenile koi suffering from mass mortality in an ornamental fish farm with apparently no effect up on treatment with antibiotics.

Materials and methods

Cell cultures

Snakehead kidney cell line (SNKD2a) derived from striped snakehead *Channa striata* (Bloch, 1793; John & George 2006) was used for isolation, multiplication and infectivity assays of this virus. Cell lines developed in the laboratory from seabass, *Lates calcarifer* Bloch, 1790, caudal peduncle (SBCP2), seabass kidney (SBKD; John & George 2006) and different tissues from clownfish, *Amphiprion sebae* Bleeker, 1853, such as fin (CFFN), brain (CFBR), spleen (CFSP2; John & George 2011), bluegill, *Lepomis macrochirus* Rafinesque, 1819, fry (BF2; Provided by Dr. Milind Patole, National Centre for Cell Sciences, Pune), *Epithelioma papulosum cyprini* (EPC; Provided by Dr. Espen Rimstad, Norwegian School of Veterinary Science, Oslo) and Brown bullhead, *Ictalurus nebulosis* (Lesueur, 1819) cells (BB; Provided by Dr. Somkiat Kanchanakhan, AAHRI, Bangkok) were also used for testing the susceptibility of the viral agent. Cell lines were grown and maintained in Lebovitz (L-15) medium (Gibco Invitrogen) supplemented with 10% foetal bovine serum (FBS; Gibco Invitrogen) and 1% antibiotic–antimycotic mix (Gibco Life Technologies) in 25 or 75 cm^2 tissue culture flasks (Greiner Bio-One) at 28 °C.

Fish

Mass mortalities were observed in ornamental fish farms of South India where the koi were bred and reared. The koi after breeding in cement cisterns were transferred to open freshwater earthen ponds for nursery rearing. The ponds received tube well water with water hardness ranging between 400 and 600 ppm as $CaCO_3$. The juvenile koi stock was later transferred to a set of cement cisterns when the mortalities were observed following clinical signs such as skin darkening, loss of scales, vertical hanging, uncoordinated swimming, turning upside down, lateral rotation, intermittent surfacing, settling at the bottom laterally and death. The fish, which showed clinical signs, always succumbed to death despite antibiotic treatment by the farmers. In many cisterns, the mortality often reached 100%. A sample of 25 juvenile koi of about 11.7 g average weight from one of the cement cisterns experiencing such large-scale mortalities in an ornamental fish farm was brought to the laboratory in live condition for investigation. The fish were maintained in freshwater aquarium of 100 L capacity with 60 L water where they exhibited clinical signs such as uncoordinated swimming, rolling over, intermittent surfacing and skin darkening and continued to suffer from

mortality. The fish were sampled for virus isolation, detection and used for virus transmission studies.

Virus isolation and propagation

Pooled tissue extracts from kidney and spleen of the infected koicarp were aseptically prepared in a tissue homogenizer at 1/10 dilution in L15 medium containing 2% FBS and 1× antibiotic–antimycotic mix. The homogenate was centrifuged at 3000 ***g*** for 10 min at 10 °C, and clarified supernatant was filtered through 0.22-μm syringe membrane filter (Millipore). The filtered homogenate (0.7 mL) was added to freshly prepared snakehead kidney cells (SNKD2a) in a 25-cm^{2-}flask. The inoculated cells along with control were incubated at 28 °C and observed daily for the development of CPE. Once the CPE was complete, the supernatant was collected, clarified and filtered through 0.22-μm syringe membrane filter, and 0.7 mL was inoculated to new SNKD2a cells in a 25-cm^2 flask for confirmation of viral agent, and CPE was observed. The cell culture supernatant with full blown CPE at the end of first passage of virus was clarified at 3000 ***g*** for 15 min and stored in aliquots at −50 °C for further use. The isolated viral agent was investigated for the presence of an envelope by treating the virus suspension with chloroform and checking for the retention of infectivity (Feldman & Wang 1961). The virus was also tested for its ability to withstand heat treatment at 56 °C for 2 h and pH treatment at pH 3 and 9 for 30 min. Acidic (pH 3.0) and alkaline (pH 9.0) solutions were prepared by adding 0.1 N HCl/NaOH to the cell culture medium without FBS. After adding 1 mL virus to 9.0 mL acidic or alkaline medium at the respective pH for ½ h, an aliquot 100 μL each was used for titration by serial dilution to find out the tissue culture infective dose (TCID 50 mL^{-1}) along with control.

Virus titration and cell line susceptibility studies

To find out the TCID 50 mL^{-1} of the virus preparation, the virus suspension was titrated in SNKD2a cells in a 96-well-microtitre plate. Actively growing cells were trypsinized, and the cell suspension diluted using L-15 medium supplemented with 10% FBS and antibiotics. The cells were added in simultaneous mode to each well having tenfold serially diluted virus suspension in quadruplicate. The plate was incubated at 28 °C, and development of CPE was observed for a period of 10 days. TCID 50 mL^{-1} was calculated using Spearman and Karber formula (Karber 1931). Cell line susceptibility studies were carried out using cell lines such as BB, BF2 and EPC and other indigenous cell lines developed in the laboratory such as SBKD, SBCP2a, CFFN, CFBR and CFSP2. All the cell lines were inoculated with the virus by simultaneous inoculation method. The cell lines were subcultured for simultaneous inoculation at a rate already standardized for formation of monolayer at the end of 24-h incubation for each cell line. The cell lines after subculturing were simultaneously inoculated with 0.7 mL of the virus preparation of $10^{8.5}$ TCID 50 mL^{-1} in 25-cm^2 flasks and incubated at 28 °C along with control. The flasks were observed daily for the onset and development of CPE.

Transmission electron microscopy

The virus was grown on SNKD2a cells (24–48 h), fixed in 3% glutaraldehyde, washed in 0.1 M cacodylate buffer, and the cell pellets were held overnight at 4 °C. Following post-fixing in 1% osmium tetroxide and washing in buffer, the cell pellets were processed for electron microscopy (John *et al.* 2001). Ultrathin sections (80 nm) of cell pellets were cut using an Ultracut microtome (Leica ultracut UCT) and stained with uranyl acetate and Reynold's solution. The sections were examined and photographed using a Philips 201C transmission electron microscope (the Netherlands) at 80 kV.

Virus purification and DNA extraction

The virus was concentrated by ultracentrifugation following propagation of the virus in EPC cells grown in 175-cm^2 flasks (Greiner Bio-one). When CPE was extensive, culture fluid was harvested and clarified by centrifugation at 2000 ***g*** for 15 min to remove the cellular debris. Supernatant and pelleted cells were then processed separately. Pelleted cells were resuspended in 2-mL TNE buffer and subjected 3 times to freeze-thawing in liquid nitrogen. After centrifugation at 2000 ***g*** for 10 min, the supernatant was pooled with the cell culture clarified supernatant. Approximately 100 mL of collected supernatant was pelleted in a

Beckman L 80 ultracentrifuge (Beckman) at 100 000 ***g*** for 90 min in an SW-41 Ti rotor (Beckman) over a 50% sucrose cushion in TNE buffer (0.01 M Tris–HCl, 0.1 M-NaCl, 0.001 M-EDTA, pH 7.5). The virus pellets were pooled and resuspended in 1-mL TNE buffer. DNA from purified virus preparation was extracted using DNA Extraction Solution (Merck Millipore) as per the manufacturer's instructions. DNA quality was assessed by electrophoresis using 0.4% agarose gel and ethidium bromide staining.

Analysis of structural proteins

Structural proteins of the purified virus preparation were analysed by SDS–polyacrylamide gel electrophoresis (PAGE) using a Genei Mini Gel System (GeNei, Merck). The proteins of the virus were resolved by 12% discontinuous polyacrylamide–SDS slab gels (Laemmli 1970) by electrophoresis at 90 V for 45–55 min along with medium and low range molecular weight markers (GeNei, Merck), and the gels were stained in 0.1% Coomassie brilliant blue. Molecular weights of the virion proteins were determined by UVIDOC software in a gel documentation system (UVI Tec).

PCR detection of viral agent

The dead and moribund fish from the pathogenicity study, infected cell culture (EPC) pellet and purified virus preparation were used for detection of ranavirus DNA by PCR. DNA from kidney of the fish was extracted using DNA Extraction Solution (GeNei, Merck Millipore) as per the manufacturer's instructions. The DNA at the end of extraction was dissolved in sterile deionized water (Biocel Millipore) and subjected to amplification using several primer sets including the primers (Table 1) and slightly modified protocol of Marsh *et al.* (2002) for the detection of EHNV described in the Manual of Diagnostic Tests for Aquatic Animals (OIE 2012) in a Master Cycler Gradient (Eppendorf). PCRs were conducted in 50-μL reaction mixture with Smart Prime Master mix of Ampliqon, Denmark. The amplification conditions were as follows for the first set of primers (M151 and M152): 94 °C/4 min, one cycle; 94 °C/30 s, 60 °C/30 s, 72 °C/40 s, 35 cycles with a final extension of 72 °C/5 min. Slight change in cycling conditions was used for the second set of primers (M153 and M154) as follows: 94 °C/4 min, one cycle; 94 °C/30 s, 55 °C/30 s, 72 °C/60 s, 35 cycles with a final extension of 72 °C/5 min. A third set of amplification was carried out with M151 and M154 primers with cycling conditions of 94 °C/4 min, one cycle; 94 °C/30 s, 58 °C/30 s, 72 °C/60 s, 35 cycles with a final extension of 72 °C/5 min. The amplification products along with molecular markers were visualized in 1.2% agarose gels (GeNei, Merck Millipore), stained by ethidium bromide and recorded in the gel documentation system. DNA from uninfected cell cultures and fish were used as negative controls.

Sequencing of the major capsid protein gene of viral agent

The PCR products obtained by two primer sets directed for the EHNV major capsid protein (MCP) gene were purified by gel extraction kits (Qiagen) and sequenced by outsourcing. The sequences obtained were analysed using CLC Main Workbench (CLC Bio) for multiple alignments and phylogenetic analysis with MCP gene of largemouth bass virus, LMBV (FR682503), guppy virus, GV6 (FR677325), doctor fish virus, DFV (FR677324), epizootic haematopoietic necrosis virus, EHNV (FJ433873) and frog virus 3, FV3 (AY548484).

Virus transmission and pathogenicity

Virus transmission and pathogenicity study were conducted using healthy juvenile koi (average

Table 1 Primers used for the successful amplification of *Ranavirus* DNA from the infected tissues and cell culture supernatants

Primer	Sequence	Target gene	Product size, bp
M151 (EHNMCP1FW)	AACCCGGCTTTCGGGCAGCA	EHNV Major capsid protein gene	321
M152 (EHNMCP1RE)	CGGGGCGGGGTTGATGAGAT		
M153 (EHNMCP2FW)	ATGACCGTCGCCCTCATCAC	EHNV Major capsid protein gene	625
M154 (EHNMCP2RE)	CCATCGAGCCGTTCATGATG		
M151 (EHNMCP1FW)	AACCCGGCTTTCGGGCAGCA	EHNV Major capsid protein gene	1201
M154 (EHNMCP2RE)	CCATCGAGCCGTTCATGATG		

weight – 9.3 g) obtained from a local fish farm. The fish were acclimatized to laboratory conditions for 7 days in well-aerated glass aquarium tanks of 100-L capacity and fed with commercial pelleted feed twice daily. Following acclimatization, four fish each was assigned to five groups for four different treatments. Three groups received intraperitoneal injection with 50-μL tissue extracts prepared from brain, gill and pooled samples of spleen and kidney from infected koi, respectively. Tissue extracts were prepared by homogenizing pooled individual tissues from infected fish, and 0.5 g tissues each was mixed with 4.5 mL of L15 medium. Tissue extracts were clarified by low-speed centrifugation and membrane filtration using 0.22-μm syringe membrane filter. The fourth group was maintained in aquarium tank with 40-L freshwater mixed with 1/10th water from the infected fish tank. A group of four fishes intraperitoneally injected with 50 μL L15 medium served as control. The fishes were maintained for 35 days for observation in well-aerated 100-L glass tanks with 40 L water and fed *ad libitum* with commercial pelleted feed. Uneaten food and faecal matter were removed daily and disinfected before discharge all through the duration of the experiment. Recovery of the virus was also attempted from dead and live fish by inoculating tissue homogenates of pooled samples ($n = 3$) of kidney and spleen on to SNKD2a cells.

A similar study was conducted in koi juveniles using cell culture grown virus by intraperitoneal injection. Koi juveniles had an average weight of 7.4 g, and five fish each were maintained in duplicate in 100-L glass tanks as above. Virus grown in EPC cells was diluted using cell culture medium, and 50 μL virus preparation containing 10^6 TCID50 was injected to each fish intraperitoneally. Two sets of five fish each were used as control and were injected with clarified cell culture supernatant without virus. Fish were maintained for a period of 4 weeks for clinical signs and mortality.

Results

Clinical pathology

The koi in the fish farm were experiencing continuous mortalities leading to large-scale loss of fishes. The live fish samples brought to the laboratory also suffered from progressive mortality, and the entire sample of 25 fishes except 3 were dead in 14 days. The fishes had clinical signs such as erratic and uncoordinated swimming, loss of balance, vertical hanging, turning upside down, lateral rotation, intermittent surfacing, settling at the bottom and death. Externally, fishes had skin darkening, loss of scales, discolouration, swollen and pale gills and emaciation.

Isolation of virus in cell culture and susceptibility of cell lines to the virus

Virus isolation studies conducted with pooled tissues of kidney and spleen from infected koi in SNKD2a cells showed that the viral agent was capable of growing in snakehead kidney cell line at 28 °C. The CPE was characterized by focal destruction of cells discernible on day 1 and further progression over 3-day duration till the complete destruction of the monolayer (Fig. 1). CPE started with several small foci of rounded cells appearing in the cell monolayer followed by aggregation of the round cells at the periphery of the foci, which became more and more enlarged in size. The rounded cells got detached from the monolayer, underwent lysis and CPE-progressed till the monolayer is completely destroyed. The virus on first passage through the same cell line was found to induce complete CPE in 2 day post-infection (dpi). Cell line susceptibility studies demonstrated that the virus could grow on a variety of cell lines tested *viz.* CFFN, CFBR, CFSP2, SBKD, SBCP2, BF2, EPC and BB cells (Table 2). KIRV however grew very slowly on BB cells. Quantitation of virus by titration experiments in SNKD2a cell line revealed that the stock virus had a titre of $10^{8.5\ +/-\ 0.94}$ TCID 50 mL^{-1}. Chloroform treatment of the virus suspension resulted in the loss of infectivity of the virus indicating the likely presence of an envelope. While treatment at pH 9 did not reduce the infectivity of the virus, treatment of the virus at pH 3 reduced the virus infectivity by 3.5 logs and heat treatment at 56 °C by 2.33 logs.

Electron microscopy of infected cell cultures

Transmission electron microscopic analysis of the infected SNKD2a cell cultures revealed the presence of several icosahedral virus particles of 100–120 nm size ($n = 14$) scattered in the cytoplasm

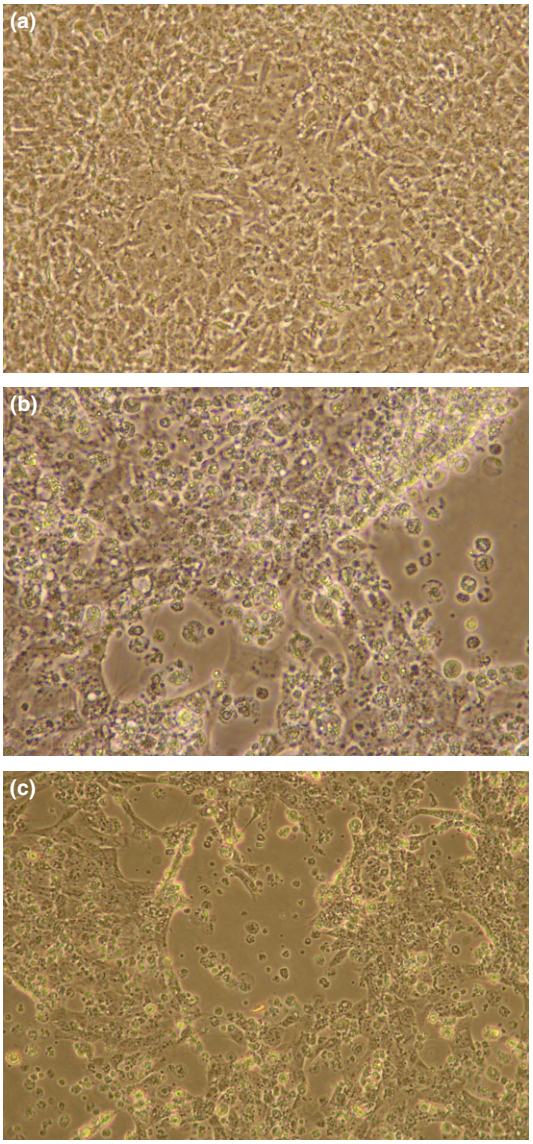

Figure 1 Cytopathic effect caused by KIRV in SNKD2a cell line (a) Control uninfected cells (200×) and (b and c) infected cells showing the induced CPE (200 and 100×, respectively).

of the cells (Fig. 2). Large numbers of virus particles were found in the periphery of the cytoplasm of the infected cells. Budding of the virus particles could also be noticed from the surface of the membranes (Fig. 3).

Analysis of structural proteins

Structural proteins of the virus resolved into two major and six minor proteins in the SDS–PAGE analysis (Fig. 4). Molecular mass of the 8 proteins ranged from 18 to 151 kDa. Two major proteins of the virus had molecular weight of 50 and 63 kDa.

Table 2 Details of cell lines investigated for the susceptibility to KIRV indicating onset of CPE and the complete destruction of the monolayer

Cell line	Onset of CPE (days)	Completion of CPE (days)
SNKD2a	1	3
CFFN	1	3
CFBR	2	3
CFSP2	1	3
SBKD	3	5
SBCP2	2	3
BF2	2	3
EPC	1	3
BB	3	10[a]

[a]Incomplete CPE.

PCR detection of the *Ranavirus*

PCR analysis of the infected fish tissues showed that the viral DNA is present in the infected tissues such as spleen and kidney of the koi. While the first primer set targeting the MCP amplified a characteristic product of 321 bp, second set of primers did not amplify the expected 625-bp amplicon. However, when the forward primer of the set I and reverse primer of the set II were used, an amplicon of about 1200 bp was obtained indicating the expected target sequence amplification. Similar results were also obtained when purified virus preparation was subjected to PCR amplification (Fig. 5a–c). No amplicons were obtained from uninfected healthy koi tissues and control cell cultures used in PCR.

Sequence analysis of major capsid protein gene

Sequences of the two gel purified amplified products from the PCR were multiple-aligned with MCP genes of LMBV, GV6, DFV, FV-3 and EHNV. The analysis showed 99.91% similarity with LMBV with single change of one nucleotide at position 291 of the sequence generated, which resulted in the change of one amino acid G (Glycine) to D (Aspartic acid; Fig. 6). The ranavirus isolated in this study showed 78.46% identity with MCP gene of EHNV and 78.38% with FV3, the type species of *Ranavirus*. Although, KIRV carried several nucleotide changes with EHNV and FV3 in the sequenced fragment, it had same nucleotide at position 291 (relative to

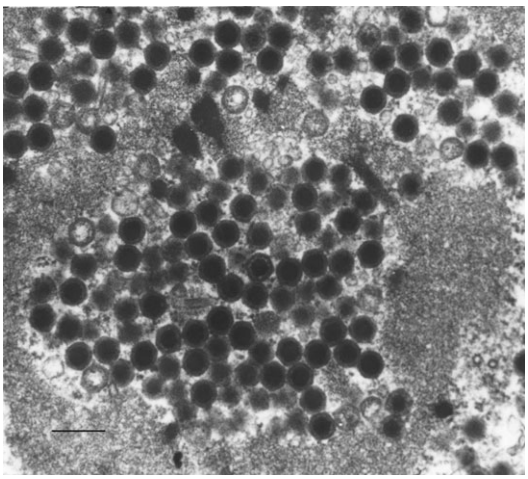

Figure 2 Transmission electron micrograph of KIRV grown in SNKD2a cells showing icosahedral particle of 100–120 nm size. Virus particles at the end of virus morphogenesis (bar = 200 nm).

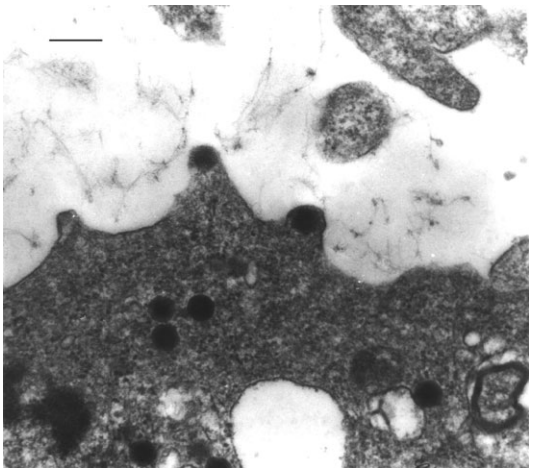

Figure 3 Transmission electron micrographs of KIRV particles distributed in the infected cell and virions seen budding from the cell membrane (bar = 200 nm).

start in Fig. 6) and the amino acid remained as glycine similar to that of GV6, DFV, EHNV and FV3 unlike that of LMBV. A phylogenetic tree constructed with the six sequences by neighbour-joining method is in Fig. 7.

Pathogenicity of KIRV to koi juveniles

All the fish injected with pooled extracts of kidney and spleen were dead by the end of the experiment. These fish had similar clinical signs such as

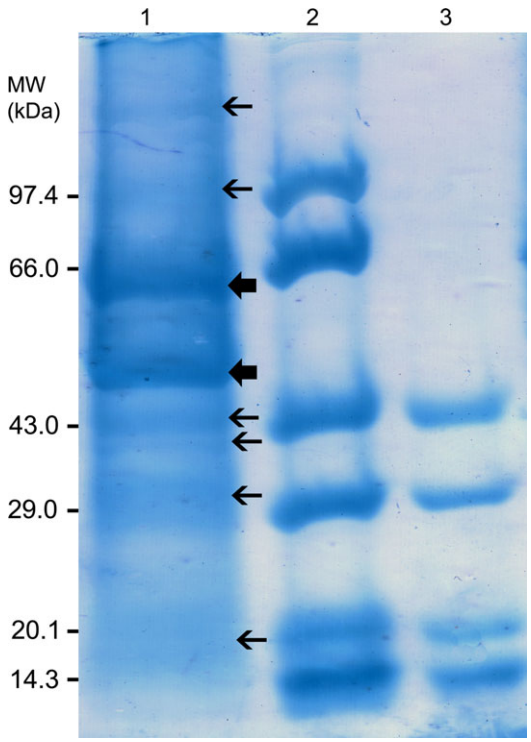

Figure 4 SDS–PAGE analysis of structural proteins of KIRV in 12% acrylamide gel stained with 0.1% Coomassie brilliant blue. Lane 1: KIRV. Two major structural proteins of 60 and 53 kDa (thick arrow) and 6 minor proteins (thin arrow) are indicated; Lane 2: Medium range molecular mass markers (GeNei); lane 3: Low range molecular mass markers (GeNei).

uncoordinated swimming, rolling over and vertical hanging before death. Half of the fish in the tank having 1/10th water from the infected tank also died during the experiment. However, the fishes, which received brain and gill extracts by intraperitoneal injection, did not show any mortality (Fig. 8). Fish also had no clinical signs in these two tanks. No mortality was observed in the control fishes, which received only cell culture media by intraperitoneal injection. The virus was recovered from the spleen and kidney of the dead fishes from all the treatment tanks using SNKD2a cell line but not from live fish samples collected from control tank. No mortality was observed in second set of fishes injected with virus grown in EPC cell line.

Discussion

Koi mortalities were found to be causing havoc in an ornamental fish farm of Southeast coast of

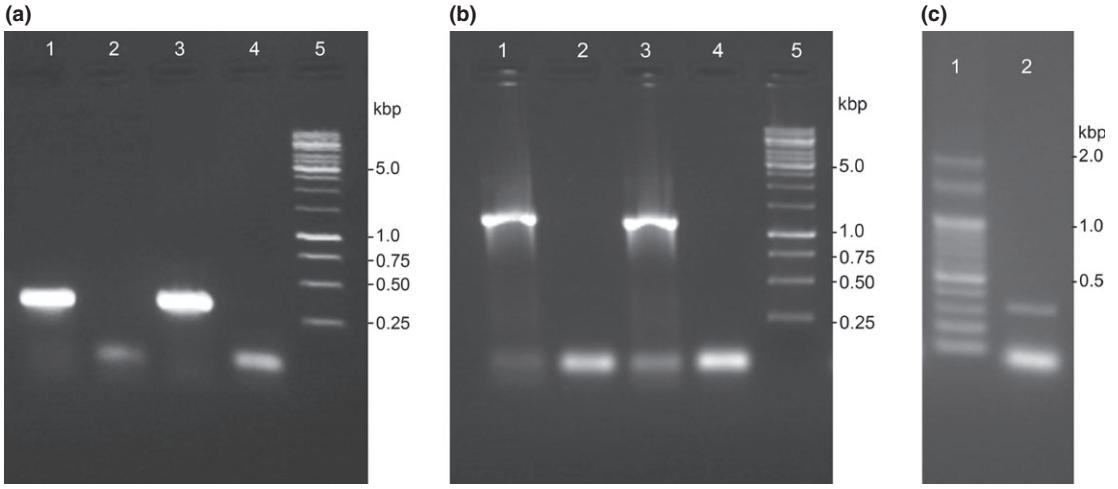

Figure 5 Agarose gel electrophoretic analysis of the PCR products obtained from KIRV infected cell culture pellet and purified virus preparation with primers targeting major capsid protein gene of EHNV (a) with M151 & M152 primers. Lane 1: KIRV infected EPC cell culture pellet, Lane 2: uninfected EPC cell culture pellet, lane 3: Purified preparation of KIRV, Lane 4: PCR negative control, lane 5: 1 kb molecular marker (GeneRuler, Fermentas, Thermo Scientific). (b) with M151 & M154 primers. Lane 1: KIRV infected EPC cell culture pellet, Lane 2: uninfected EPC cell culture pellet, lane 3: Purified preparation of KIRV, Lane 4: PCR negative control, lane 5: 1 kb molecular marker. (c) PCR amplification of infected koi kidney using M151 and M152 primers. Lane 1: 1 kb Molecular marker, Lane 2: Infected koi kidney.

India. To ascertain the aetiology of the large-scale mortality in the koi farm, we investigated the incidence through virological analysis of infected fishes. The fish samples brought to the laboratory were a collection of healthy and moribund animals, which showed varying clinical signs such as loss of scales, uncoordinated swimming, vertical hanging, lateral rotation turning upside down and intermittent surfacing before settling at the bottom and death. All but three fishes died within 14 days of the arrival of the fish to the laboratory. Similar clinical manifestations such as erratic swimming or hyperbuoyancy associated with swim bladder overinflation were found associated with infections of wild largemouth bass with Santee–Cooper ranavirus (Grizzle & Brunner 2003). Natural wild infections with variable clinical manifestations ranging from inapparent infection to sporadic epizootics of mortality also reported in largemouth bass (Goldberg *et al.* 2003).

The virus isolate resembling Santee–Cooper ranavirus obtained in the present investigation was found to grow on a variety of cell lines developed in the laboratory from marine (clown fish), brackish water (seabass) and freshwater (snakehead) fishes and also on EPC, BF2 and BB cell lines. In BB cell line however, KIRV did not cause full scale CPE unlike other cell lines. Many of the ranaviruses have wide host range with EHNV infecting as many as 13 species of fishes (Whittington *et al.* 2010). The susceptibility of a panel of freshwater and marine fish cell lines to the KIRV obtained in this study indicates the potential of the virus to multiply in different fish species leading to clinical or subclinical infection. Further investigation may be required to prove the susceptibility of these fish species by *in vivo* pathogenicity studies. The virus in SNKD2a cells had grown to a titre of $10^{8.5\ +/-\ 0.9428}$ TCID 50 mL^{-1} similar to the titres obtained for Australian EHNV isolates grown in BF2 cells (Gould *et al.* 1995; Ariel *et al.* 2009) and largemouth bass virus in EPC cells (Deng *et al.* 2011). Like ranaviruses in general, the KIRV also lost infectivity by low pH (3.0) and heat treatment at 56 °C (Jancovich *et al.* 2012).

The virus grown in cell culture examined under the transmission electron microscope had icosahedral particles of 100–120 nm size. The virions of vertebrate iridoviruses are reported to range in size from 100 to 300 nm (Hyatt & Chinchar 2008). The virus particles were seen at the periphery of the cell membrane and were also found budding out of the cell membrane. Virions of ranaviruses are reported to accumulate in the cytoplasm

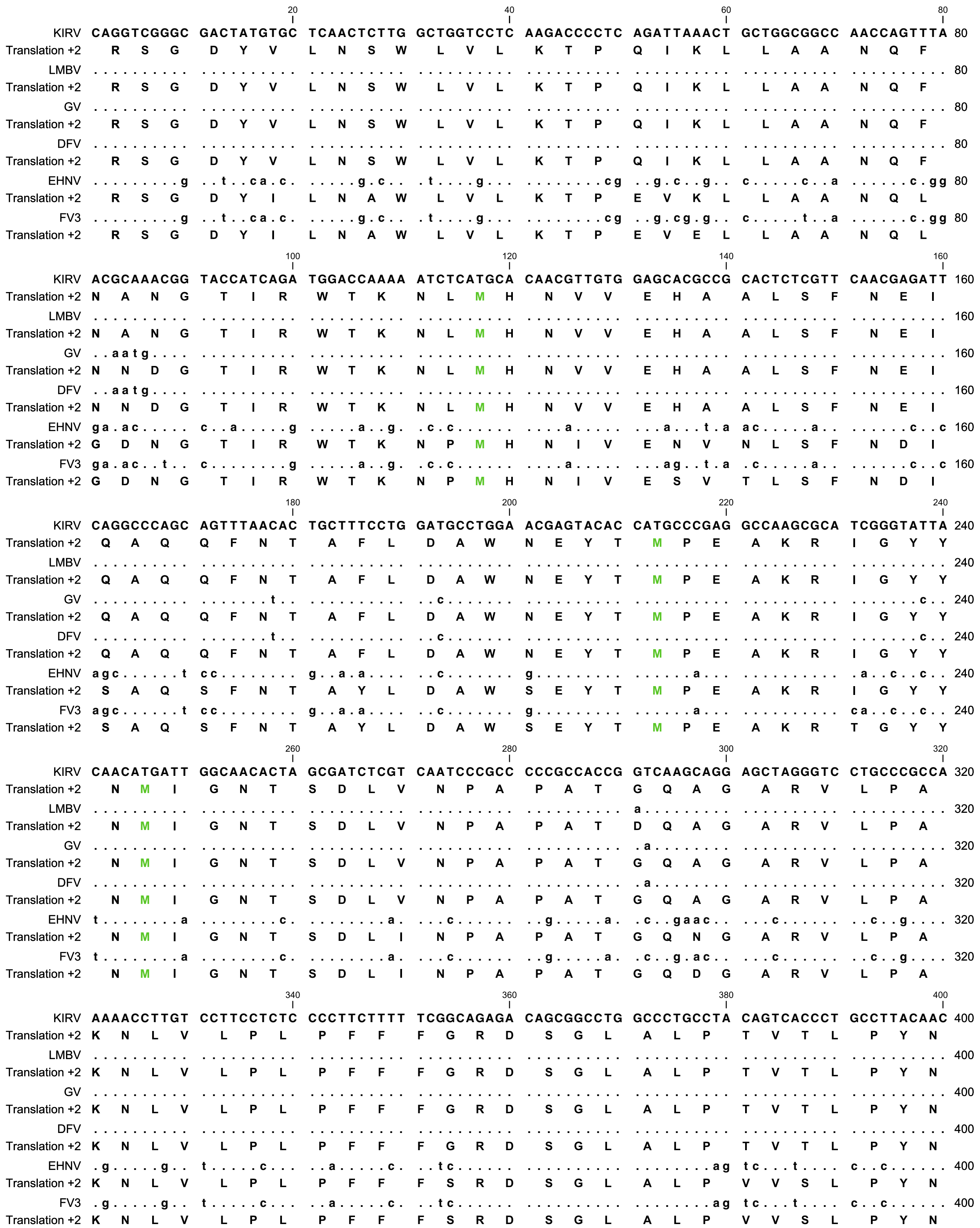

Figure 6 Partial MCP sequence alignment of KIRV, LMBV (GenBank Accession No FR682503, nt 403–1525), GV6 (FR677325), DFV (FR677324), EHNV (FJ433873, nt 21008–19886) and FV3 (AY548484, nt 97557–98679) were compared. Amino acid translation with reference to full coding sequence of the MCP gene is indicated. Identical nucleotides are represented by dots.

within large paracrystalline arrays and are released by budding from the plasma membrane acquiring an envelope (Chinchar 2002). The presence of the envelope for KIRV was also confirmed by the loss of the viral infectivity up on treatment with the organic solvent.

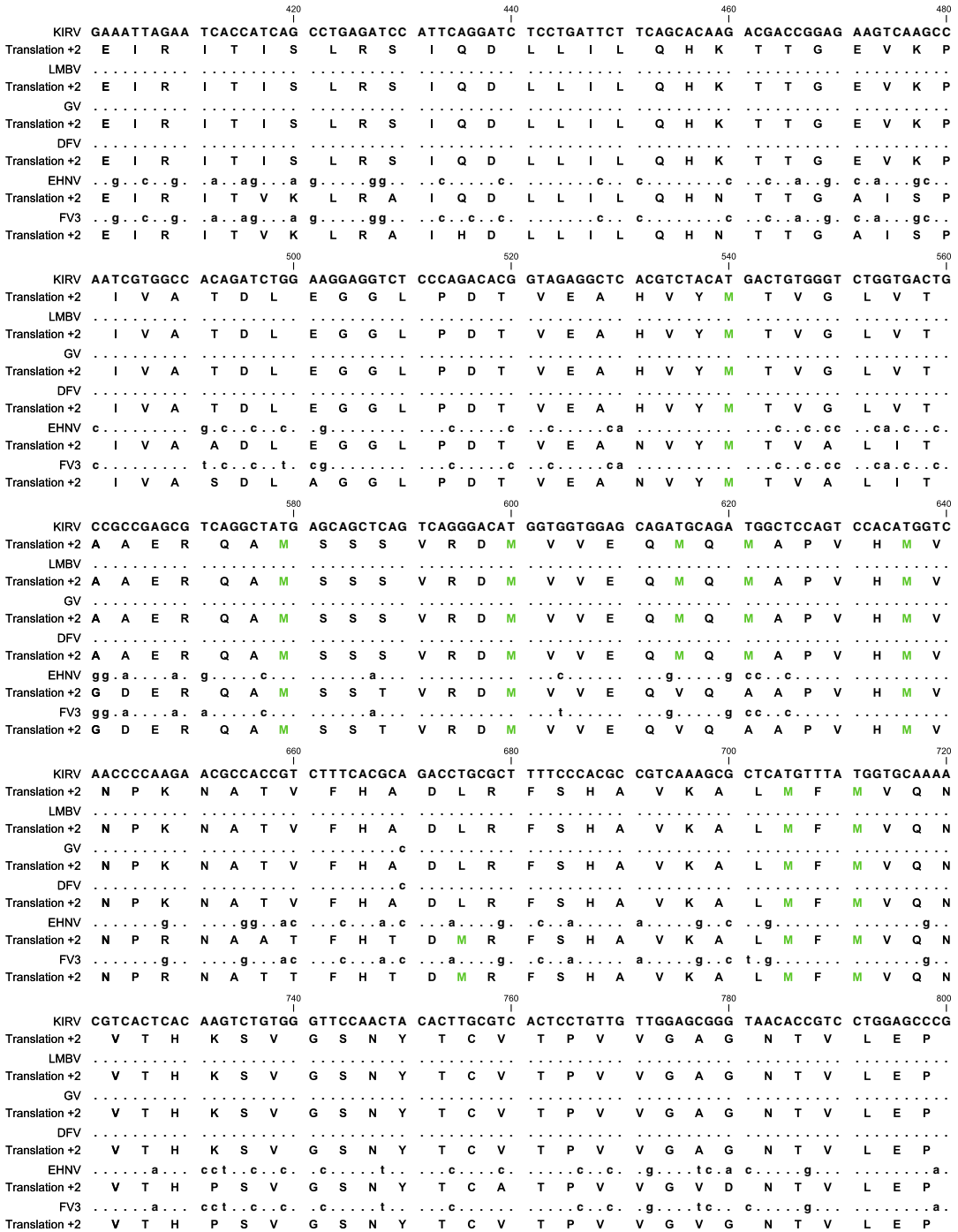

Figure 6 Continued.

The SDS–PAGE analysis of the structural proteins of the virus indicated the presence of major and minor viral proteins in the purified virus preparation having molecular weights ranging from 18 to 151 kDa. Two proteins of the size 50 and 63 kDa formed the major proteins of the virus. The major capsid protein of the EHNV (Jancovich *et al.* 2010), FV3 (Mao *et al.* 1996) and GIV (Murali *et al.* 2002) also has similar molecular weight of about 50 kDa. The major capsid proteins of the size

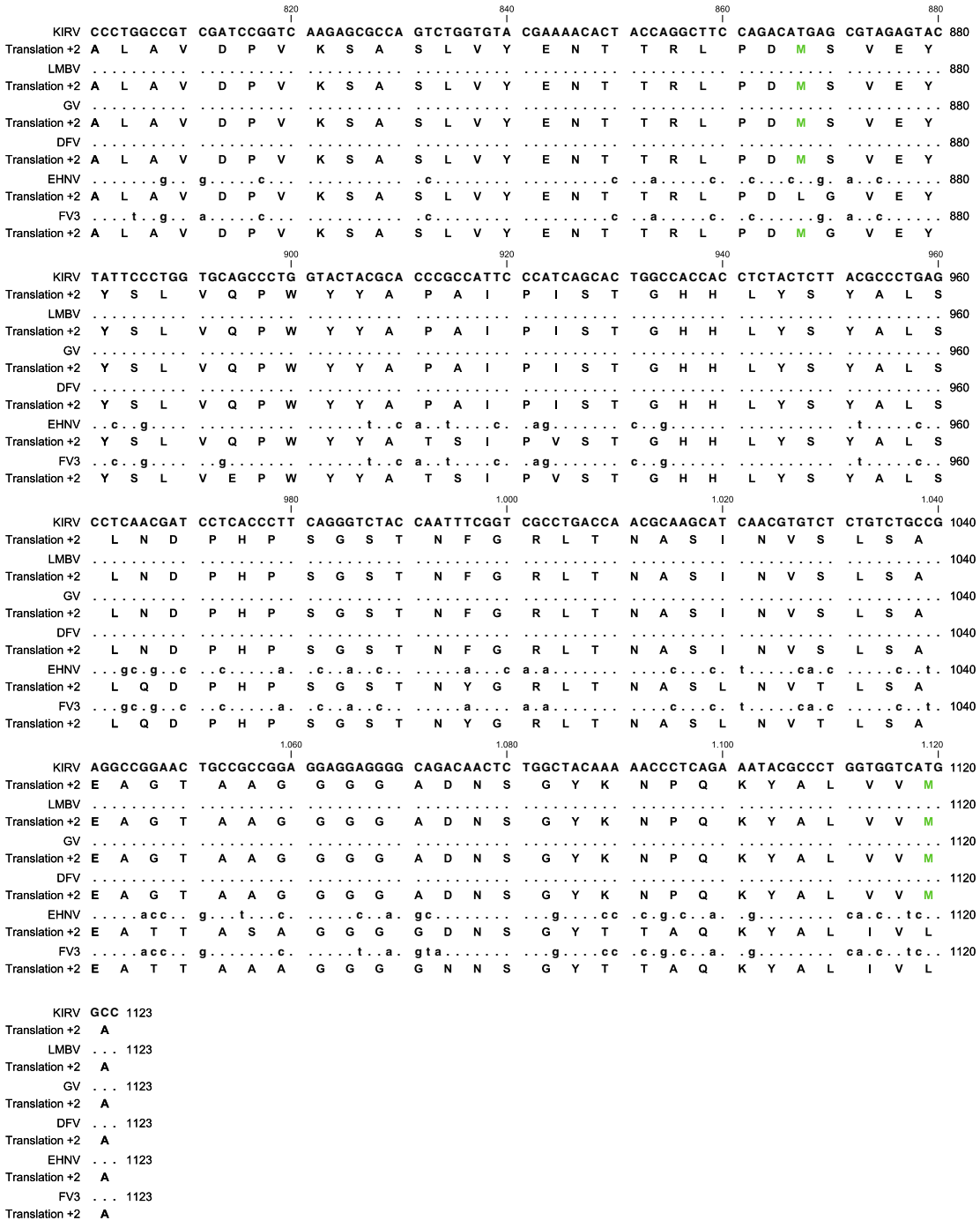

Figure 6 Continued.

range 48–55 kDa form about 40% of the proteins of the family Iridoviridae (Jancovich *et al.* 2012).

Two sets of primers targeting the MCP of EHNV were used to amplify the MCP gene of the KIRV isolate. While the first set amplified the expected 321 fragment, the second set of primers did not amplify the expected 625 fragment indicating a sequence difference from EHNV. However, an approximately 1200-bp fragment was amplified when the forward primer of the first set

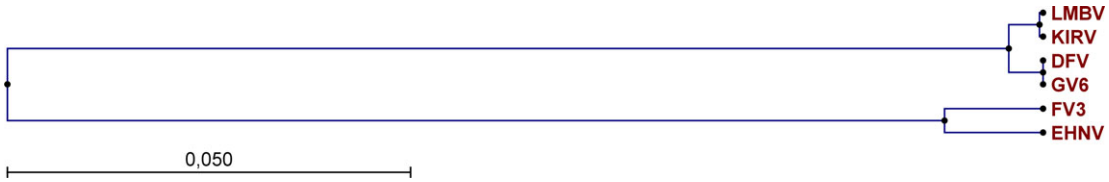

Figure 7 Phylogenetic tree constructed using partial MCP gene sequences of six similar ranaviruses LMBV (FR682503), GV6 (FR677325), DFV (FR677324), EHNV (FJ433873) and FV3 (AY548484) by neighbour-joining method using CLC Main Workbench indicating the relationship of KIRV with other similar ranaviruses including the type virus of the genus, FV3.

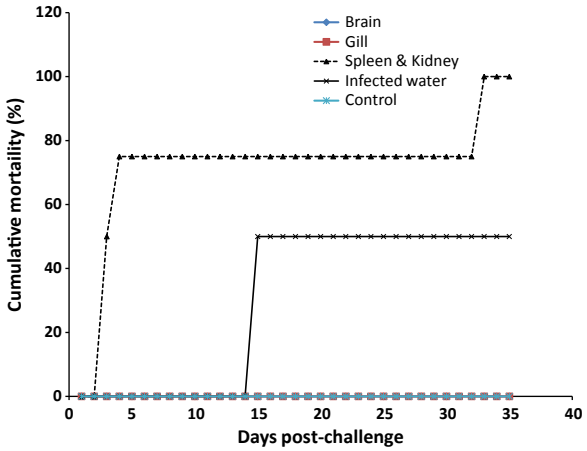

Figure 8 Cumulative mortality of koi juveniles experimentally challenged by intraperitoneal injection with 0.22-μm membrane filtered pooled tissue extracts of infected koi along with control and koi challenged with 1/10th infected tank water.

and reverse primer of the second set were used. Although the OIE primer sequences used were based on EHNV MCP sequence, they were not present in LMBV sequence. However, the analysis of sequences showed that the primers shared some identity with LMBV MCP sequence FR682503. As the EHNV primer sets could amplify both 321- and 1200-bp fragments, we proceeded through sequencing of the amplicons for identifying the pathogen instead of further redesigning primers based on LMBV sequences.

The sequence analysis of the partial coding region of the MCP gene amplified in the present virus spanning 1123 bp demonstrated that the isolate is similar to the Santee–Cooper ranavirus (LMBV) with 99.91% sequence homogeneity. LMBV MCP sequence shows 99.21% identity with DFV and GV6 MCP sequences, which within themselves are identical (Ohlemeyer *et al.* 2011). MCP gene sequences have been used as main identifying character of the ranaviruses (Whittington *et al.* 2010), which shows over 84% percentage similarity between the ranavirus groups by blast searches of the full MCP gene. With other major group of ranaviruses comprising EHNV and FV3, which shows 97.7% homogeneity of MCP gene between them, the ranavirus isolated in the present study showed an identity of only 78.46% with EHNV and 78.38% with FV3 for the fragment of 1123 bp. The present analysis, however, showed an interesting change in the amino acid sequence in the LMBV and FV3, the type species of the *Ranavirus* group. While the amino acid at position 168 of the FV3 and EHNV MCP gene is a glycine, it is an aspartic acid for LMBV. However, the present ranavirus isolate from India compares favourably with the type species FV3 in this regard by retaining the amino acid glycine at this position.

Early epizootic ranavirus EHNV was isolated in 1985 from Australia (Langdon *et al.* 1986), later European sheatfish virus (ESV) and European catfish virus (ECV) from Europe (Ahne, Schlotfeldt & Thomsen 1989b; Pozet *et al.* 1992). The vertebrate ranavirus in North America was isolated in 1995 from an epizootic affecting largemouth bass

Micropterus salmoides (Lacepe′de) in Santee–Cooper reservoir in SC, USA (Plumb *et al.* 1996). Two virus isolates similar to Santee–Cooper virus were reported later from ornamental fishes imported from South-East Asia to the United States, the guppy virus (GV6) and doctor fish virus (DFV), which had the partial MCP nucleotide sequence almost identical to that the MCP gene of Santee–Cooper ranavirus (Mao *et al.* 1999). The present isolate KIRV is more homologous to Santee–Cooper ranavirus at 99.91% than to GV6 and DFV, which is showing only 99.2% homology for the same region of 1123 bp, thus forming a separate isolate from these two Asian ranaviruses. Singapore grouper iridovirus (SGIV), another Asian ranavirus isolate, shows only 71.2% identity with Santee–Cooper ranavirus isolate over the full MCP gene sequence and hence has substantial difference between the Indian ranavirus isolate obtained in the present study as well.

Pathogenicity study was conducted for the virus isolate using filtered infected tissue homogenate administration and by cell culture grown virus with two groups of koi. While all the fish injected with tissue homogenates of spleen and kidney developed similar clinical signs seen in the farm-infected fishes and died at the end of the experiment, there was no mortality in the fish injected with brain or gill homogenates indicating the importance of kidney as an ideal organ for isolation of ranaviruses from infected fishes (Ariel *et al.* 2009). Present study also indicated that the virus could be transmitted through contaminated water. However, the cell culture grown virus did not induce mortality in koi injected with virus intraperitoneally. Similar instances were also reported in experimental infections of largemouth bass, where different populations have demonstrated profound variability in susceptibility to Santee–Cooper ranavirus (Goldberg *et al.* 2005). Experimental infections with no clinical signs or mortality was also observed in 1-year-old wild largemouth bass injected with Santee–Cooper ranavirus although viraemia was established (Plumb *et al.* 1996). At the same time, mortality up to 100% in 5 days was observed in largemouth bass infected via injection of Santee–Cooper ranavirus (Plumb & Zilberg 1999). Although it is reported that there are only lower levels of environmental virus shedding through cutaneous mucus (Plumb *et al.* 1996; Woodland *et al.* 2002), in the present study, the infection was transmitted through water taken from the infected tank when used as a source of the virus to induce experimental infection by causing 50% mortality in koi. EHNV has been reported to be an indiscriminate pathogen of several fish with high variability in susceptibility of host species. While redfin perch are highly susceptible, rainbow trout were resistant to bath exposure in $10^{2.2}$ TCID 50 mL^{-1} and succumbed only after intraperitoneal infection (Whittington & Reddacliff 1995).

The experimental fish after exhibiting clinical signs, died in 2–3 days. Samples of moribund and dead fish were processed for virus isolation, and the results indicated the presence of a viral agent capable of multiplying in snakehead kidney cells. The virus caused cytopathic effect in snakehead cell cultures in 2 days characterized by the presence refractile detached cells causing damaged areas in the cell monolayer. The confirmatory diagnosis for the presence of ranavirus DNA in the infected fish tissues was obtained by the PCR amplification of DNA in the infected fish tissues. The recovery of the virus from the dead fishes in the laboratory experiment inoculated with the virus isolate indicated that the virus isolated from the dying fishes was associated with the mortality of koi in the farm. The role of koi herpesvirus (KHV) in inducing the mortality was not investigated as the virus isolate obtained was a ranavirus like agent. As the cell culture grown ranavirus could not induce mortality, the possibility of original infection carrying other viral agents such as KHV could not be ruled out.

We have thus isolated and characterized the viral agent from infected koi experiencing large-scale mortality in the koi farms of South India. The physicochemical properties of the viral agent isolated, size of major capsid protein, sequence identify of the MCP gene with the Santee–Cooper virus (LMBV) coupled with the clinical pathology of infection prove that virus belongs to the genus *Ranavirus* of the family Iridoviridae. The disease caused by *Ranavirus* in fish is a problem in many countries but was hither to unreported from India. The detection of a ranavirus isolate from infected koi in India in the present investigation becomes the first record of isolation of a virus resembling Santee–Cooper ranavirus infection of koi and in the country. The name koi ranavirus (KIRV) is proposed for this agent.

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