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Research Article

Detection and genetic analysis of infectious spleen and kidney necrosis virus (ISKNV) in ornamental fish from non-clinical cases from India

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Abstract

Infectious spleen and kidney necrosis virus (ISKNV), a type species of the genus *Megalocytivirus*, poses a threat to ornamental fish trade as most cases shown on specific symptoms, thus making timely diagnosis challenging. Apparently healthy molly (*Poecilia sphenops*) and angel fish (*Pterophyllum scalare*) collected from two distinct geographic localities of India were screened for four genera under *Iridoviridae*, *Megalocytivirus* i.e, ISKNV, turbot reddish body iridovirus (TRBIV) and red sea bream iridovirus (RSIV); rana viruses and Singapore grouper iridovirus; and Lymphocystis virus through molecular approach. In total seven numbers out of 35 samples (20%), ISKNV genome fragments were detected. A PCR assay using major capsid protein (MCP) gene was standardised to detect and differentiate infections within the *Megalocytivirus* genus, even without aid of sequencing. This forms the first report of ISKNV infection in ornamental fish from eastern part of India. Moreover, the ISKNV infection was confirmed by PCR and sequence analysis of MCP and ATPase genes. The sequence of these genes showed that Indian isolate being 99-100% similar to the complete genome or reference strain of ISKNV. Phylogenetic reconstruction demonstrated the present strain belonging to ISKNV genotype I. Furthermore, structural stability of the MCP revealed this strain was more stable than ISKNV genotype II, RSIV and TRBIV at 25°C and pH 7.0. Thus, strong pan-India surveillance is recommended to reduce trade risk.

Keywords: ISKNV, Major capsid protein, Ornamental fish, PCR, Phylogeny

Highlights

- ISKNV is reported from non-clinical samples of ornamental fishes
- The sequence of MCP and ATPase genes showed that Indian isolate being 99-100% with reference strains.
- A PCR assay using MCP gene was standardised to detect ISKNV from other *Megalocytivirus*

INTRODUCTION

Ornamental fish culture and trade is widely accepted as an industry showing enormous potential and aggressive growth worldwide. In India, its popularity has boomed in recent times owing to its economic benefits due to a large internal market and the substantial unexplored potential for exports. The major groups of farm-bred ornamental fish are goldfish, barbs, tetras, swordtails, mollies, gourami, guppies, angelfish, fighting fish and platy fish in India. The molly (*Poecilia latipinna*) and angelfish (*Pterophyllum scalare*) are two crucial ornamental fish species, equally attractive and vibrantly coloured species, both of which can be kept as pets in confined spaces like aquaria or garden pools (Kumari *et al*., 2017). The total export of ornamental fish from India stood at around 42 tonnes, grossly estimated to have a value of approximately 8.4 crores (US $1.2 million) in 2017-18 alone (Singh, 2019).

On the flip side, the global ornamental fish trade poses a potential source for the spread of many exotic pathogens, particularly viruses causing both morbidity and mortality in fishes (Oyamatsu *et al*. 1997; George *et al*., 2015; Pragyan *et al*., 2019; Sahoo *et al*., 2016, 2020a,b). Similarly, Sahoo *et al*. (2020b) investigated the infectious diseases in freshwater aquaculture farms of eastern India and found that the incidences of viral infections are more in ornamental fish. To-date, viral infections viz., viral nervous necrosis (VNN) in freshwater aquarium fishes 'goldfish and rainbow shark' (Jithendran *et al*., 2011), Cyprinid herpes virus-2 (CyHV-2) in goldfish (Sahoo *et al*., 2016), carp edema virus (CEV) in koi carp (Swaminathan *et al*., 2016; Sahoo *et al*., 2020a), ISKNV in ornamental fishes (Girisha *et al*.,, 2021), ranavirus in koi carp (George *et al*., 2015) and marine ornamental similar damselfish (Sivasankar *et al*., 2017) have been reported from Indian ornamental fishes.

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The family *Iridoviridae* consists of a large group of enveloped viruses possessing double-stranded DNA (Jancovich, 2012), which are known to cause large-scale mortalities in fish. Taxonomists have divided this family into five genera viz. *Megalocytivirus*, *Iridovirus*, *Ranavirus*, *Lymphocystivirus* and *Chloriridovirus* (Kurita and Nakajima, 2012). Viruses belonging to the *Iridoviridae* family are typically vulnerable to both wild as well as freshwater fish, including ornamental fish. Iridoviruses capable of infecting fish have been accommodated in three major groups or genera viz., *Ranavirus*, *Lymphocystivirus* and *Megalocytivirus* (Chinchar *et al*., 2009). Since their discovery in the early 1990s, the group of *Megalocytiviruses*, in particular, have caused significant morbidity as well as financial losses in marine and freshwater fish culture, predominantly in Asia. *Megalocytiviruses* have been broadly distributed into three clusters, i.e., red seabream iridovirus (RSIV), infectious spleen and kidney necrosis virus (ISKNV) and turbot reddish body iridovirus (TRBIV), based on their major capsid protein (MCP) and ATPase gene phylogenetic analysis (Kurita and Nakajima 2012), and recently another strain, three spine stickleback iridovirus (TSIV) have also been described (Murwantoko *et al*., 2018). He *et al*., (2000) first isolated ISKNV, a species of the genus *Megalocytivirus*, from mandarin fish in China. Subsequently ISKNV has also been reported from Japan (Tanaka *et al*., 2014), Malaysia (Subramaniam *et al*., 2014), Australia (Mohr *et al*., 2015), USA (Subramaniam *et al*., 2016), Germany (Jung-Schroers *et al*., 2016), Vietnam (Dong *et al*., 2017), Africa (Ramires *et al*., 2019) and Thailand (Thanasaksiri *et al*., 2019) in both freshwater and marine ornamental fishes. Phylogenetic analysis of the *Megalocytivirus* MCP gene sequence revealed the existence of two genotypes I and II of ISKNV (Kurita and Nakajima, 2012; Dong *et al*., 2017; Murwantoko *et al*., 2018). Recently Girisha *et al*. (2021) reported ISKNV infecting ornamental fishes in India.

Ornamental fish viruses have inherent inability to exhibit growth in commonly used fish cell lines, thus making diagnosis difficult. ISKNV like isolates, mostly those from freshwater ornamental fish, are challenging to culture *in vitro* (Kurita and Nakajima, 2012, Rimmer *et al*., 2017) and CEV also being reported not to grow in any studied cell lines (Swaminathan *et al*.,2016). On multiple occasions, viral infection may remain latent until adverse environmental conditions like poor quality of water, rough handling of fish, or overcrowding, activate the disease (Sahoo *et al*., 2016, 2020a). ISKNV poses a threat to ornamental fish trade because *Megalocytiviral* infections have non-specific symptoms, thus making timely diagnosis challenging (Jeong *et al*., 2006, 2008; Subramaniam *et al*., 2014; Zainathan *et al*., 2017) and persistence of sub-clinical detected level of virus at the retailer site (Rimmer *et al*., 2015). Viral diagnosis based on candidate gene PCR-sequencing seems to be more accurate, reliable and faster than traditional phenotypic methods. Despite different molecular techniques like PCR, nested PCR, real-time PCR (Xu *et al*.,, 2008), and LAMP assay (Suebsing *et al*., 2016) being used for detection of ISKNV, the specificity for ISKNV has usually been low (Razak *et al*., 2014; Shiu *et al*., 2018) thereby failing to reliably differentiate ISKNV from other subgroups of *Megalocytiviruses*, i.e. RSIV and TRBIV. Hence, the current investigation was aimed at detecting the presence of *Megalocytiviruses* in two species of ornamental fishes, i.e., molly (*Poecilia sphenops*) and angel fish (*Pterophyllum scalare*) collected from two geographically distinct Indian ornamental fish markets from apparently healthy fish with a PCR based method even without aid of sequence confirmation. We standardised a precise and reliable nested PCR-based approach to detect ISKNV and segregate it from other species of *Megalocytiviruses*. Further this virus was also confirmed by molecular approach as described by OIE (OIE, 2019).

MATERIALS AND METHODS

Case history and sample collection: Thirty-five numbers of apparently healthy fish (Molly, *Poecilia sphenops* and angelfish, *Pterophyllum scalare*) were collected from seven different ornamental retailer shops of Kurla, Maharashtra (25 no's) and Bhubaneswar, Odisha (10 no's) states of India, for routine screening of viruses in the National Referral Laboratory of Freshwater Fish Diseases at ICAR-Central Institute of Freshwater Aquaculture, Bhubaneswar, India. The fish were euthanized with overdose of MS222 (Sigma, USA), and liver, kidney, gills, brain and eye samples of individual fish were collected in 100% ethanol. The experiments were performed following the approval of the Institute Animal Ethics Committee.

DNA isolation: Tissue samples (~ 10 mg) were treated with proteinase K in lysis buffer (50 mM Tris/HCl, 100 mM NaCl, 100 mM EDTA, 1% [w/v] SDS, pH 8.0) and subjected to extraction with phenol/chloroform/isoamyl alcohol, followed by ethanol precipitation. The isolated DNA was diluted in TE (50 mM Tris/HCl, one mM EDTA, pH 7.5) buffer. Concentration and purity of the extracted DNA was determined by measuring OD at 260 and 280 nm using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies Inc., USA). The samples were stored at --20ºC for further analysis. Before processing

Table 1. Details of the primers used in this study with expected size of amplicons

Sl No	Virus/target gene	Primer	Nucleotide base sequence (5'-3')	Amplicon size (bp)	Reference
1	Megalocytivirusn PCR MCP gene	C1105	GGGTTCATCGACATCTCCGCG	430	Rimmer *et al*., 2012
		C1106	AGGTCGCTGCGCATGCCAATC		
		C1173	AATGCCGTGACCTACTTTGCG	167	Rimmer *et al*., 2012
		C1174	ATCTTAACACGCAGCCACA		
2	ISKNV nPCR MCP gene	C1105	GGGTTCATCGACATCTCCGCG	1075	Current Study
		MCP-uni1108-R8	TCTCAGGCATGCTGGGCGCAAAG		
		MCP-specI465-F3	GCTGTTGCAACCATTTGAGA	415	Kurita and Nakajima, 2012
		MCP-specI879-R3	ACGGGGTGACTGAACCTG		
3	ISKNV ATPase gene	ATP2DGf	CACCACCTGTGTGTATTTGTC	1542	Go *et al*., 2006
		ATP1076DGIVlater	ATGAACCCGCTGCACTATGC		
4	ISKNV/RSIV OIE	1F	CTCAAACACTCTGGCTCATC	570	Kurita *et al*., 1998; OIE 2019
		1R	GCACCAACACATCTCCTA		
5	RSIV OIE	4F	CGGGGGCAATGACGACTACA	568	Kurita *et al*., 1998; OIE2019
		4R	CCGCCTGTGCCTTTTCTGGA		
6	RSIV*PstI* fragment	2F	TACAACATGCTCCGCAAGA	564	Kurita *et al*., 1998
		2R	GCGTTAAAGTAGTGAGGGCA		
7	RSIV MCP	MCP-specR674-F4	CCCGCACTGACCAACGTGTCC	191	Rimmer *et al*., 2012
		MCP-specR888-R6	CACAGGGTGACTGAACCTCAGGTCG		
8	TRBIV MCP	MCP-specT37-F1	TTCATCGACATCTCCGCTTTC	419	Rimmer *et al*., 2012
		MCP-specT440-R1	TSTGACCGTTGGTGATACCGGAG		
9	EHNV MCP gene	M151	AACCCGGCTTTCGGGCAGCA	321	OIE EHNV, 2019
		M152	CGGGGCGGGGTTGATGAGAT		
10	SGIV DNA Pol gene	SGIVDNPOLFW	GTGTAYCAGTGGTTTTGCGAC	560	Holopainen *et al*., 2009
		SGIVDNPOLRE	TCGTCTCCGGGYCTGTCTTT		
11	LCDV MCP gene	LCDVs F	YTGGTTCAGTAAATTACCRG	609	Kitamura *et al*., 2006
		LCDVs R	GTAATCCATACTTGHACRTC		

further, an equal amount of DNA samples from each organ of an individual fish were pooled to form one sample.

PCR amplification: PCR was performed using five sets of published oligonucleotide primers for confirmation of ISKNV (two nested PCRs based on MCP gene and one PCR based on ATPase gene). Samples were also PCR screened for RSIV, TRBIV, EHNV, SGIV and LCDV using published primers and conditions (Table 1).

The samples were initially screened for MCP gene of MCV using two sets of published primers (Rimmer *et al*., 2012). In the first set, C1105 and C1106 (Table 1) primers were used in a final volume of 25 µL containing 1 µL of total DNA, 1.5 µL (10 pmol) of each primer, 0.25 µL of *Taq* DNA polymerase (5 U/µL), 2.5 µL of 10X *Taq* buffer A, 0.5 µL of dNTPs (2 mM) and ddH_2O to make final volume to 25 µL. The reaction mix was subjected to 30 temperature cycles (30s at 94°C, 30s at 55°C and 1 min at 72°C) after an initial denaturing step (15 min at 95°C) followed by a final extension step of 7 min at 72°C in a Veriti thermal cycler (Applied Biosystem). A nested PCR was performed using a second set primers C1073 and C1074 using similar conditions. These primers only confirm the presence of MCV at genus level.

After confirming the presence of MCV, samples were processed for molecular screening of ISKNV using a combination of published primers (Kurita and Nakajima, 2012; Rimmer *et al*., 2012) with partial modification. In the first step, primer C1105F and MCP-uni1108-R8 (unpublished pairing) were used in a final volume of 25 µL containing 1 µL of total DNA, 1.5 µL (10 pmol) of each primer, 0.25 µL of *Taq* DNA polymerase (5 U/µL1), 2.5 µL of 10X *Taq* buffer A, 0.5 µL of dNTPs (2 mM) and ddH_2O to make final volume to 25 µL. The reaction mix was subjected to 35 temperature cycles (60 sec at 95°C, 60 sec at 57°C and 60 sec at 72°C) after an initial denaturing step (300 sec at 95°C) followed by a final extension step of 300 sec at 72°C in a Veriti thermal

cycler to amplify genus MCV. Further, nested PCR was performed using ISKNV specific primers MCP-specI 465-F3 and MCP-specI 879-R3 using the above first step product to increase the sensitivity and specificity for detecting only ISKNV as primers don't amplify RSIV and TRBIV (Kurita and Nakajima, 2012). The reaction mix was subjected to 35 temperature cycles (1 min at 95°C, 1 min at 58°C and 1 min at 72°C) after an initial denaturing step (5 min at 95°C) followed by a final extension step of 5 min at 72°C.

The samples were also screened for ISKNV ATPase gene (Mohr *et al*., 2015). PCR was carried out in a final volume of 25 µL reaction mix, as mentioned above for the MCP gene. Amplification was programmed for a preliminary 15 min denaturation step at 95°C, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 90 s, and finally extension at 72°C for 7 min (Mohr *et al*., 2015). Several target amplicons were generated to strengthen confirmation of ISKNV as it would be the first report from India.

As described by OIE (2019), the samples were screened for ISKNV *Pst-I* restriction fragment using primer 1F and 1R, followed by RSIV DNA polymerase gene using primers 4F and 4R (Kurita *et al*., 1998). PCR was carried out in a final volume of 25 µl reaction mix, as mentioned above for the MCP gene and PCR conditions were set as described by Kurita *et al*. (1998) and OIE (2019).

Sequencing and phylogenetic study: The PCR amplicons (using primers C1105/C1106, C1073/C1074, MCP-specI465-F3/MCP-specI879-R3,; ATP2DGf/ATP1076DGIVlater and ISKNV/RSIV 1F/1R) from four positive samples were purified using QIAquick Gel Extraction kit, and purified products were commercially sequenced (AgriGenome Labs Pvt. Ltd, Kochi, India). The nucleotide sequences were analysed using the Basic Local Alignment Search Tool (BLAST) of NCBI (http://www.ncbi.nlm.nih.gov/blast) to find out the homology. The amino acid sequences of ISKNV MCP gene of different isolates were retrieved from the NCBI database and aligned with the amino acid sequences of Indian samples. Multiple alignments were performed with MEGA 6 by using the ClustalW algorithm (Tamura, 2013). Phylogenetic analysis of ISKNV MCP sequences was performed through Maximum Likelihood method available in MEGA 6, and the phylogenetic tree was constructed using the Maximum Likelihood method. The amino acid sequences of different *Megalocytiviruses* were aligned and differentiated with ISKNV. Meanwhile, the nucleic acid sequences of three subgroups of *Megalocytivirus* i.e., RSIV, ISKNV and TRBIV were aligned and compared.

Protein stability prediction: Along with the sequence study, I-MUTANT 2.0 server was used to predict the stability of the MCV major capsid protein sequences at pH 7.0 and temperature 25^{0}C (Capriotti *et al*., 2005). This software can evaluate the stability change upon single-site mutation, starting from the protein structure or the protein sequence. I-Mutant 2.0 correctly predicts whether the protein mutation stabilises or destabilises the protein when the protein sequence is available.

RESULTS

Identification of the virus in samples: Among 35 samples screened, seven numbers (20%) of samples were found to be positive for ISKNV. For detection of *Megalocytivirus*, MCP gene was screened using primers C1105/C1106 and C1073/C1074, and amplicons of 431bp and 168bp were obtained in first and second step PCRs, respectively (Fig. 1a and 1b). Aimed at detection

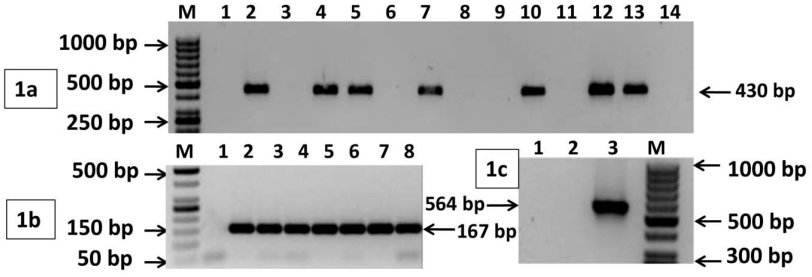

Fig. 1a. Samples amplified with C1105 and C1106 primers, with expected product sizes of 431bp. Lane M represents 50bp Ladder (Thermo Scientific); Lane 2 represent negative control; Lanes 3-15 represents targeted fish samples screened for the virus, 1b. Samples amplify with C1173 and C1174 primers; with expected product sizes of 167bp. Lanes M represents 50bp Ladder (Thermo Scientific) Lanes 2 represent negative control; Lanes 3-9 represents targeted fish samples screened for the virus, 1c. Samples amplification with RSIV 2F and 2R primer at expected size 564bp; Lane 1 represent negative control; Lane 2 targeted fish samples screened for the virus; Lane 3 represent RSIV Positive control (RSIV Kag YT-96 DNA received from Prof Y. Kawato, NRIA, JFREA, Mie, Japan); Lane M represent 50bp Ladder (Thermo Scientific)

of ISKNV, the tissues were screened with primers C1105/MCP-uni1108-R8 and MCP-specI465-F3/ MCP-specI879-R3 primers. The samples were screened for RSIV *Pst*I fragment gene were found to be negative (Fig 1c). In seven samples, amplified product of 415bp of ISKNV MCP gene were obtained in the nested PCR (Fig. 2a). Further, in same samples ISKNV ATPase gene were also amplified with amplicon size of 1542bp using ATP2DGf and ATP1076DGIVlater primers (Fig. 2b). Meanwhile, the same samples amplified a specific band of 570bp for *Pst*I gene by 1F and 1R (Fig. 2c)

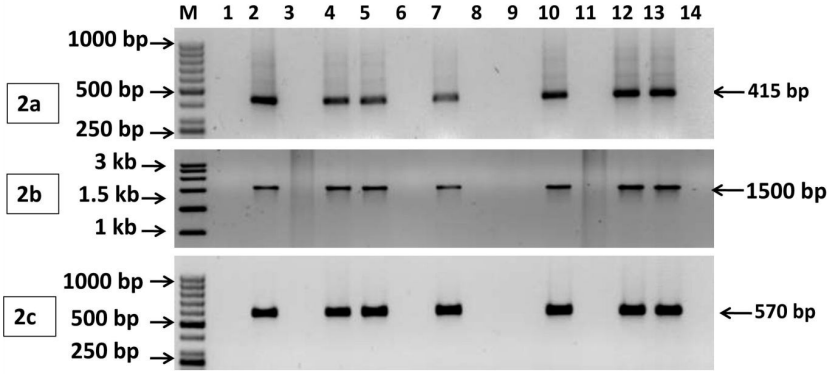

Fig. 2a. Samples amplified with MCP-specI465-F3 and MCP-specI879-R3 primers, with expected product sizes of 415bp, Lane M represent 50bp Ladder (Thermo Scientific), Lane 2 represents negative control and Lanes 3-15 represents targeted fish samples screened for the virus; 2b. Samples amplified with ATP2DGf and ATP1076DGIVlater primers; with expected product sizes of 1542bp. Lane M represent 50bp Ladder (Thermo Scientific), Lane 2 represents negative control and Lanes 3-15 represents fish samples; 2c. Samples amplified with ISKNV/RSIV OIE 1F and 1R primer at expected size 570bp, Lane M represent 50bp Ladder (Thermo Scientific), Lane 2 represent negative control and Lanes 3-15 represents fish samples

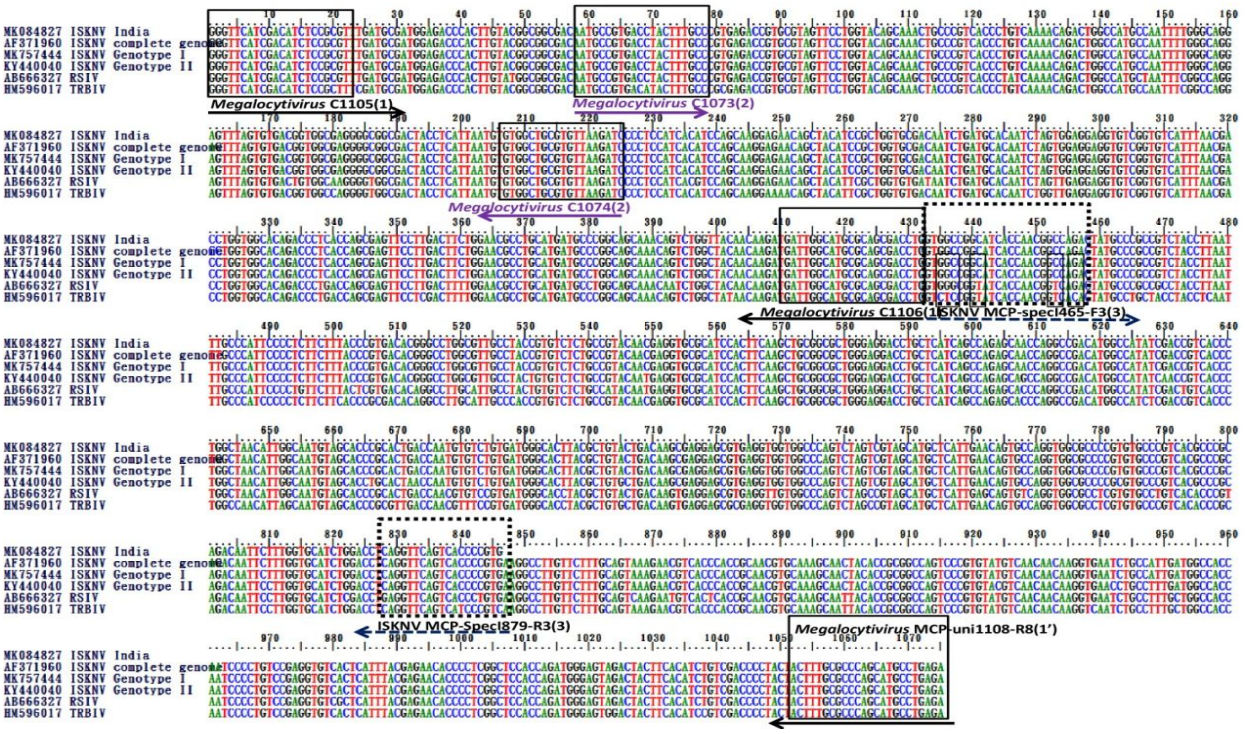

Fig. 3. Nucleotide sequence alignments of primer sets C1105/C1106, C1073/C1074, C1105/ MCP-uni1108-R8 and MCP-specI465-F3/MCP-specI879-R3 with major capsid protein (MCP) gene of infectious spleen and kidney necrosis virus (ISKNV) sequence of Indian isolate (accession: MK084827/MN518863), other published ISKNV isolates (accession: AF371960, MK757444, KY440040), red seabream iridovirus (RSIV) sequence (accession: AB666327) and turbot reddish body iridovirus (TRBIV) sequence (accession: HM596017). The nucleic acid variations are outlined in the ISKNV specific primer MCP-specI465-F3 alignment. The alignment result was obtained by graphic view of Bio Edit Sequence Alignment Editor

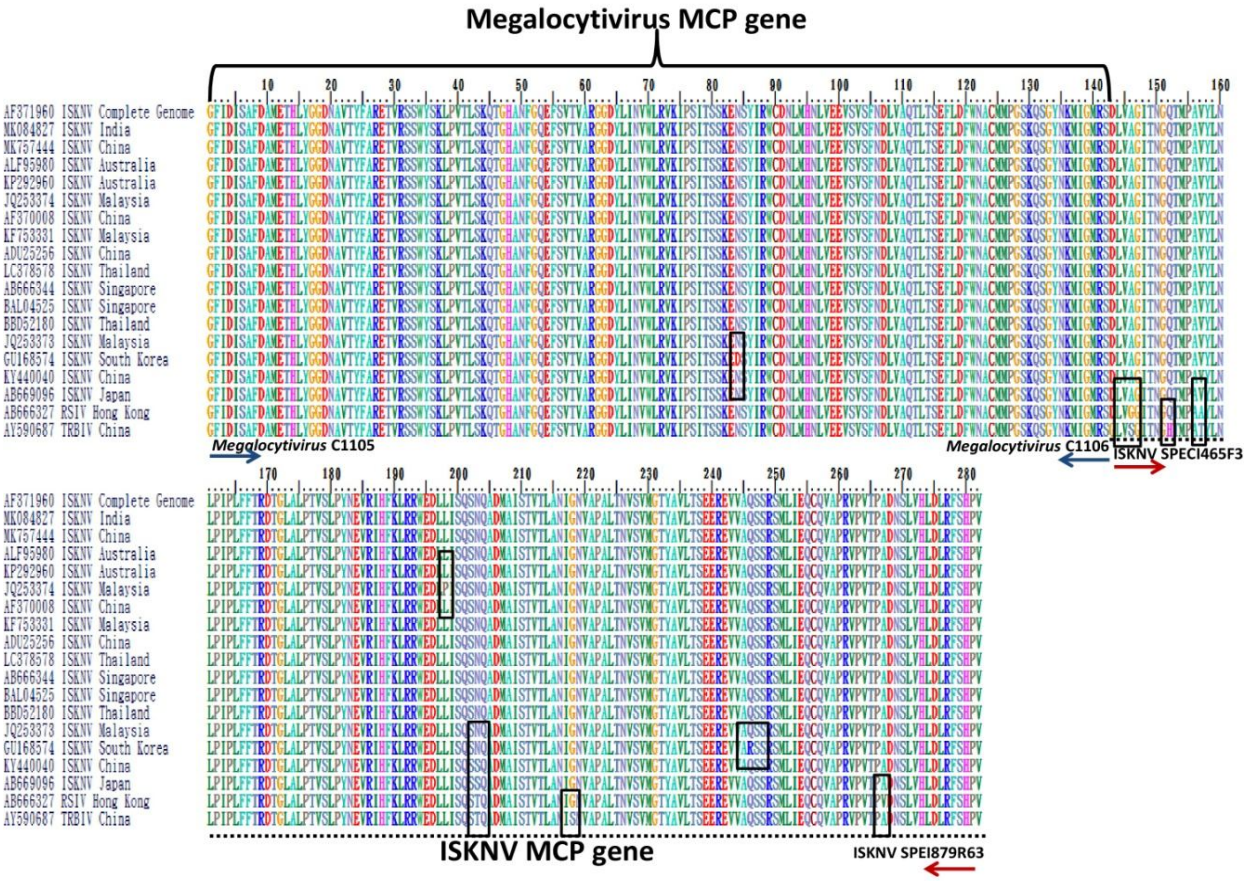

Fig. 4. Amino acid alignment of MCP sequences (1-282) of different ISKNV isolates including India, RSIV and TRBIV isolates retrieve from NCBI. The amino acid variations are outlined. The alignment result is obtained by graphic view of Bio Edit Sequence Alignment Editor

and no amplification of RSIV specific DNA polymerase gene by 4F and 4R, which indicated the presence of ISKNV infection in the samples. All the samples collected for this study were found to be negative for RSIV, TRBIV, EHNV, SGIV and LCDV using respective primer sets.

Sequencing, comparison of MCP gene and phylogenetic study: ISKNV MCP gene amplicons obtained (using primers pairsC1105/ C1106 and MCP-specI465-F3/MCP-specI879-R3) were sequenced, and a BLAST search of the sequence revealed 99% and 100% similarities with known previously published MCP genes of *Megalocytivirus* and ISKNV, respectively. Megalocytivirus MCP gene (primer C1105/C1106) sequence amplicon of 4 431bp encoding 143 amino acids has been submitted to GenBank (GenBank accession number: MK084827). The 431bp MCP gene fragment was found to be 99.77% similar with the complete genome or reference strain of ISKNV (AF371960) along with other ISKNV isolates (MK757444, KX354220, KT781098, AB666344, AB666337, AF370008), as well as ISKNV like viruses i.e., giant *seaperch iridovirus* isolate GSIV (JF264350) and African *lampeye iridovirus* (AY285745, AB109368) (Kurita and Nakajima, 2012). Also, the obtained sequence is 95.59% similar to RSIV (AB666327) and TRBIV (HM596017). Sequence alignment revealed a silent mutation observed at 399bp position (GGCGly to GGTGly) in the MCP gene of Indian isolate (Fig. 3). Despite changes in the nucleotide sequence, the multiple 143 amino acid alignments of the MCP gene have shown 100% similarity with all the sequences mentioned (Fig. 4).

The amplicon of 4 415bp, encoding 138 amino acids of MCP gene sequence was found to be specific for ISKNV. It has been subsequently submitted to GenBank (accession number: MN518863). Both of these gene fragments (first 431bp specific for *Megalocytivirus*, and next 415bp specific for ISKNV) were combined to form a total of 846 nucleotides and 282 amino acids (Fig. 3, 4). The primer binding sites of *Megalocytivirus* specific primer C1105/C1106 and C1073/C1074 are common

in the MCP gene of ISKNV, RSIV and TRBIV. To increase the sensitivity and confirmative diagnosis of ISKNV, the nested PCR using ISKNV specific MCP-specI465-F3 and MCP-specI879-R3 primer pair was successfully performed, that could differentiate ISKNV from the other *Megalocytivirus* species by amplifying only ISKNV (415bp). Further the diagnosis was more validated based on *in silico* analysis for all three *Megalocytivirus* sequence primer binding regions in this study and also based on earlier report of Kurita and Nakajima (2012) and Mohr *et al*. (2015) (Fig. 3).

A phylogeny tree was constructed based on the MCP gene amino acid sequence (MK084827). The Indian isolate in the present study was found to be ISKNV genotype I. It showed 100% MCP gene sequence similarity with that of China (MK757444, AF370008, ADU25256), Australia (ALF95980, KP292960), Malaysia (JQ253373, KF753331), Singapore (AB666344, BAL04525), and Thailand (BBD52180, LC378578.1), and was clustered within the same clade in the phylogeny tree (Fig. 5). There were variations at the positions corresponding to amino acids 84 (Asn-Asp) and 198 (Leu- Pro) in the MCP sequences of South Korea (GU168574) and Malaysia (JQ253374), respectively (Fig. 4). Even so, the later two strains clustered with the Indian strain within the same clade of ISKNV genotype I (Fig. 5). Meanwhile, two other isolates of ISKNV, i.e., Japan (AB669096) and China (KY440040) differed from other ISKNV at amino acid position 203 (Asn- Ser) and were found to have clustered together as ISKNV genotype II (Figs. 4,5). The two subspecies of Megalocytivirus, i.e., RSIV (AB666327) and TRBIV (AY590687) were segregated from ISKNV by amino acid position 146 (Ala-Gly), 157 (Val-Ala), 167 (Ala-Val) and 143(Asp-Gly), 146(Ala-Ser), 152 (Gln-His), 157 (Val-Thr), and 218 (Gla-Ser), respectively, and clustered at different clades (Figs. 4,5).

For further confirmation, the ATPase and *Pst*I genes amplicons obtained using primers pairs (ATP2DGf/ATP1076DGIVlater and RSIV/ISKNV OIE 1F/1R) were sequenced, and a BLAST search of the sequence revealed 99% similarities with known previously published ATPase and laminin-like protein genes of ISKNV.

Prediction of protein stability of MCP: The stability of the MCP of ISKNV of Indian strain was compared *in silico* with ISKNV, RSIV and TRBIV isolates using I-Mutant software. The stability of MCP was found to reduce due to amino acid changes in ISKNV isolates of South Korea (84; N-D, 246; Q-R), Malaysia (198; L-P), Japan and China (Genotype II) (203; N-S) (Fig. 6). MCP stability study indicated ISKNV MCP of having relatively better structural stability as compared to that of RSIV, whereas TRBIV MCP showed further decreased stability at 25°C and pH 7.0 (Fig. 7).

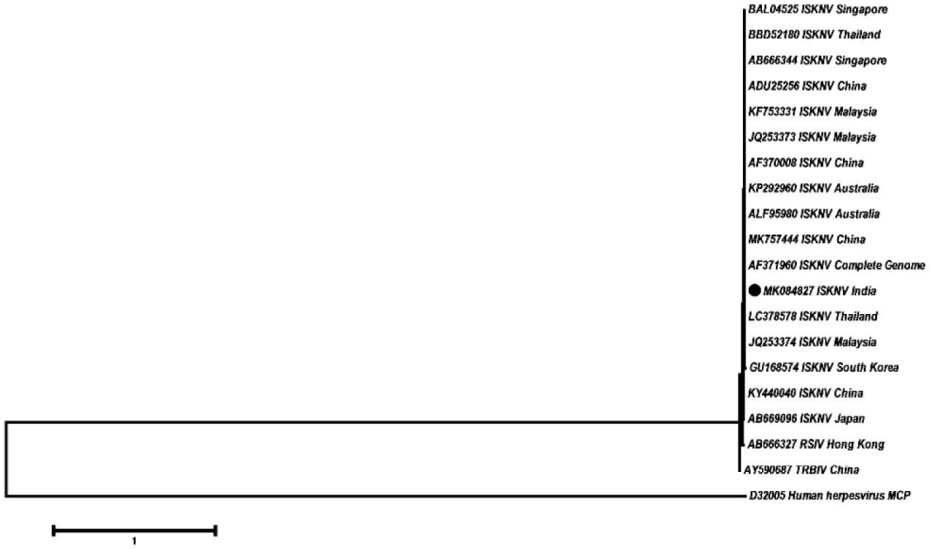

Fig. 5. Phylogenetic tree based on the MCP gene amino acid sequence of ISKNV, RSIV and TRBIV. The ISKNV sequence of the present study clustered closely with other ISKNV sequences reported earlier. The tree was generated by MEGA 6 using Maximum Likelihood method of Clustal W 2. 1. MCP gene of human herpes virus was used as an outgroup

DISCUSSION

The study reports presence of ISKNV in non-clinical samples of ornamental fishes. Further highlights a new combinational primer setto undertake a nested PCR (using C1105F/ MCP-uni1108-R8 and nested MCP-specI465-F3 and MCP-specI879-R3) to identify ISKNV without the need of sequencing. Further, an increase in the sensitivity and specificity of ISKNV-specific primer set published earlier (Kurita and Nakajima, 2012) was obtained to detect the virus in sub-clinical cases. Moreover,

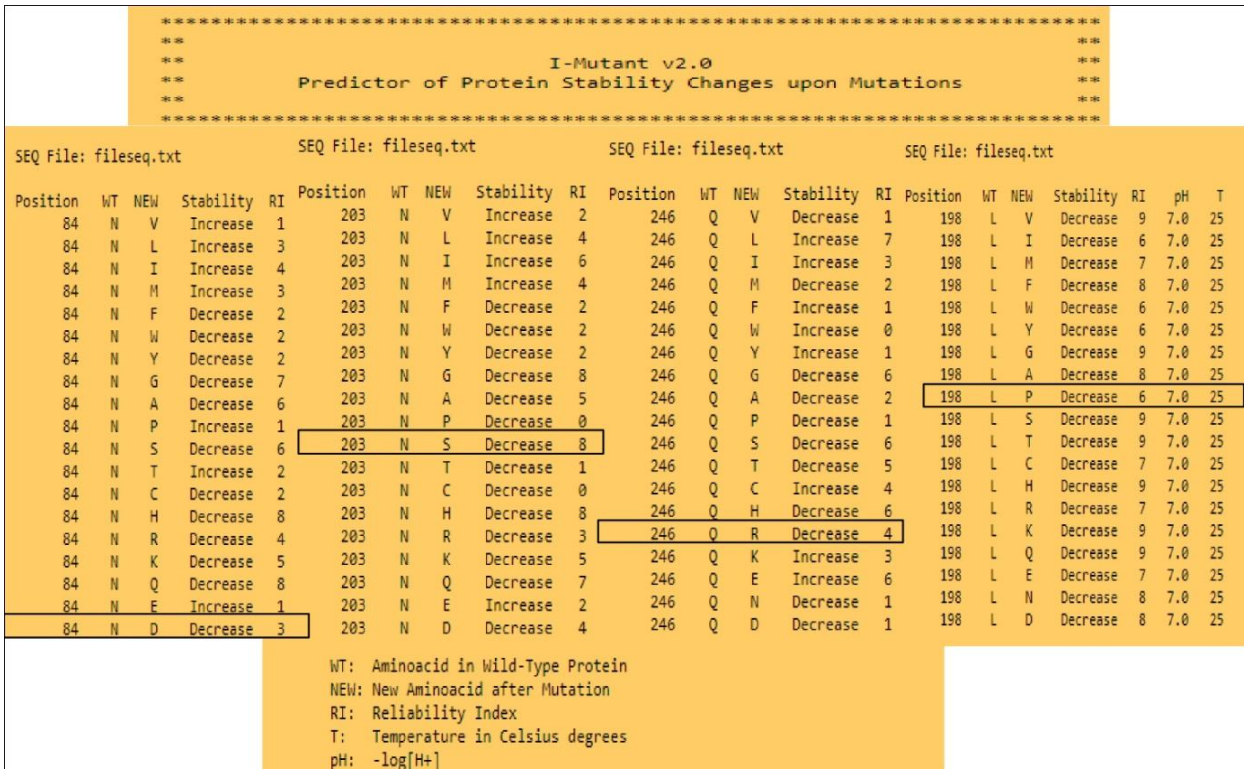

Fig. 6. Protein stability prediction of different ISKNV strain including Indian isolate. I-MUTANT 2.0 server was used to predict the stability of the protein sequences at pH 7.0 and temperature 25°C

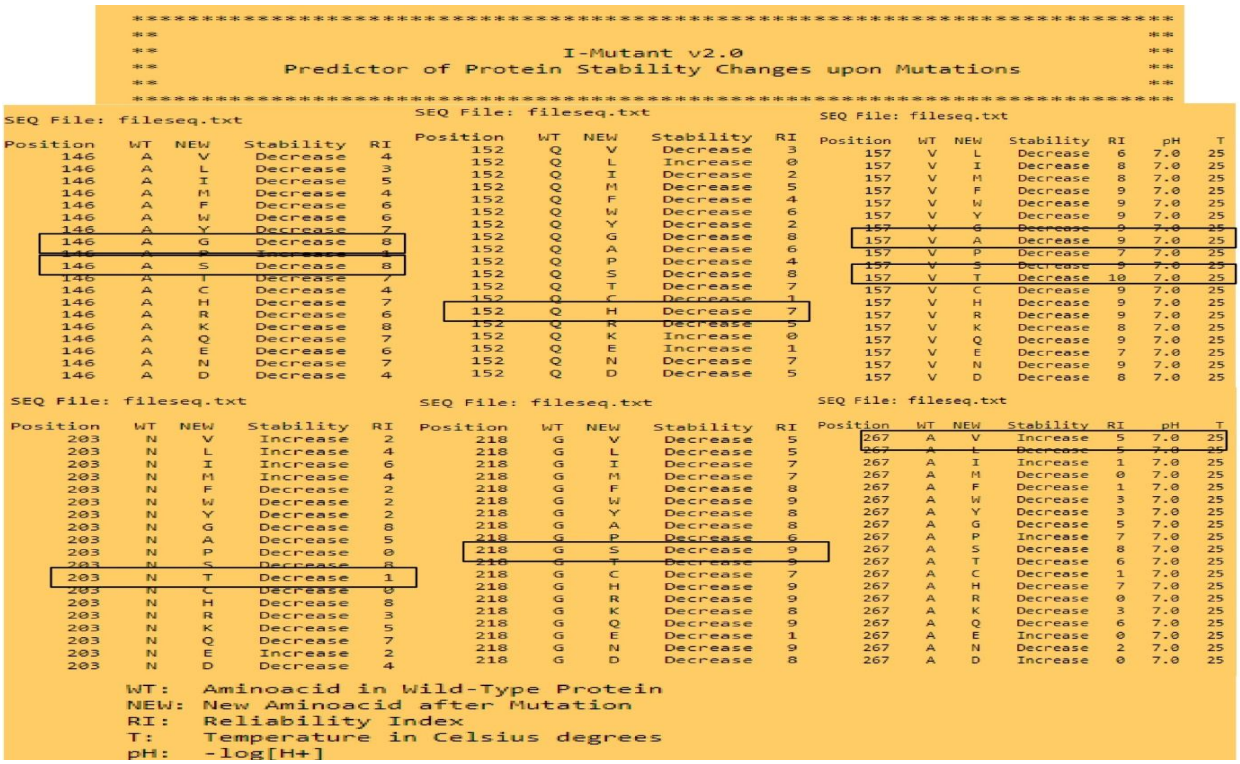

Fig. 7. Protein stability prediction and comparison of stability upon mutation at pH 7.0 and temperature 25°C, among Indian ISKNV isolate with RSIV and TRBIV using I-MUTANT 2.0 server

the samples tested for RSIV and TRBIV, were found to be negative thus indicating ISKNV as the associated virus in the processed samples. The samples were reconfirmed by OIE based ISKNV detection protocol (Kurita *et al*., 1998; OIE, 2019).

The hosts affected by ISKNV are relatively wide-ranging; nevertheless, freshwater fish species are the predominantly affected species (Kurita and Nakajima, 2012). Several outbreaks of ISKNV diseases in freshwater fishes have been reported in the ornamental fish of Germany (Jung-Schroers *et al*., 2016), Australia (Mohr *et al*., 2015; Rimmer *et al*., 2015), and Malaysia (Subramaniam *et al*. 2014; Zainathan *et al*., 2017) and more recently in Africa (Ramires *et al*., 2019) and India (Girisha *et al*., 2021). Detection of ISKNV in two ornamental fish species, molly (*P. sphenops*) and angelfish (*P. scalare*) obtained in this study were also reported earlier from other countries (Rodger *et al*., 1997; Yanong and Waltzek, 2010; Go *et al*., 2016; Zainathan *et al*., 2017). Subramaniam *et al*. (2014) reported many positive cases of ISKNV in ornamental fishes which did not show any clinical signs. This further confirmed the fact that *Megalocytivirus* infects visibly healthy fish species; this condition could be termed persistent or asymptomatic infection as reported by Jeong *et al*. (2006, 2008). In India also Girisha *et al*. (2021) reported ISKNV from infected ornamental fishes. In comparison to clinically infected fish tissue, DNA concentration of *Megalocytivirus* was approximately 10^5 times higher in asymptomatic fish, as proven by (Jeong *et al*., 2006). An earlier report finds the presence of ISKNV from apparently healthy fish at temperatures below 20°C (He *et al*., 2002). When water temperature was 28°C, juvenile Chinese perch challenged with ISKNV, displayed clinical signs with 100% mortality (He *et al*., 2000). This indicates *Megalocytivirus* requires high temperature to multiply (Liu *et al*., 2019) that needs further study after isolation of virus. Further, recent report of emergence in causing mass mortality in tilapia in Africa raise a big concern for tilapia farming (Ramires *et al*., 2019), if not taken proper care, likely entry of the virusfrom ornamental fish to tilapia species would be disastrous.

Currently, the OIE Manual for Diagnostic Tests for Aquatic Animals is not specific for identification of only ISKNV excluding other viruses of the same genus (Kurita *et al*., 1998; OIE, 2018, 2019). Out of the recommended criteria for confirmatory diagnosis, conventional PCR detects the two listed genotypes, whereas others require specialized reagents (monoclonal antibodies) or are not applicable to all ISKNV like isolates (eg. virus isolation)(Johnson *et al*., 2019). Better methods remain to be identified for the specific diagnosis of ISKNV infections with particular reference to the differentiation of gene sequence, subclinical infections and clinical disease. Although there are several reports of screening the ISKNV virus by PCR and sequencing using a *Megalocytivirus* genus primer (C1105/C1106), none of these reports used ISKNV species specific primers described by Kurita and Nakajima (2012) and Mohr *et al*. (2015) (Go *et al*., 2006; Xu *et al*., 2008; Rimmer *et al*., 2012; Subramaniam *et al*., 2014; Razak *et al*., 2014; Mohr *et al*., 2015; Rimmer *et al*., 2017: Dong *et al*., 2017; Bobby *et al*., 2018; Johnson *et al*., 2019; Zainathan *et al*., 2019). Hence in this investigation, a nested PCR assay was standardised to detect and differentiate ISKNV infections within the *Megalocytivirus* genus of the family *Iridoviridae*.

The major capsid protein (MCP) gene is relatively conserved among the viruses of the family *Iridoviridae*, for which it can be considered as one of the most important genes for the analysis of the genetic relationships among this family (Williams *et al*., 1996; Tidona *et al*., 1998; Liu *et al*., 2019). The amino acid sequence analysis of *Megalocytivirus* specific MCP gene regions revealed 100%similarity with all the three *Megalocytivirus* subgroup. However, subgrouping issue was resolved by use of new combinational nested ISKNV PCR with internal primer set of ISKNV (Kurita and Nakajima, 2012), as it was100% similar with only ISKNV genome with the produced amplicons, that emphasizes upon ISKNV subtyping with the current PCR system in one go.

ISKNV viruses can be further divided into two sub-clusters: genotypes I and II (Rimmer *et al*., 2012; Dong *et al*., 2017). Phylogenetic analysis revealed that the Indian isolate belonged to genotype I, which was more closely related to ISKNV isolates of China (MK757444, AF370008, ADU25256), Australia (ALF95980, KP292960), Malaysia (JQ253373, KF753331), Singapore (AB666344, BAL04525), and Thailand (BBD52180, LC378578.1), thus forming a single cluster. This isolate formed a different cluster with Japan (AB669096) and China (KY440040) isolates which belonged to ISKNV genotype II (Dong *et al*., 2017).

A proteomic study of ISKNV demonstrated that ISKNV virions possess 38 viral structural proteins, where MCP is the main structural component of the virus particle, comprising 40 to 45% of the total particle polypeptide (Dong *et al*., 2011; Jia *et al*., 2013). Jia *et al*. (2013) reported first time, ISKNV MCP interacted with Mandarin fish caveolin 1 (mCav-1), which gives an evidence of a viral structural protein interacted with a host protein in the family *Iridoviridae* for its primary infection. This information indicates that, major capsid proteins play an essential role in the ISKNV infection

by virus-host protein interactions. Further study can help us to understand the mechanisms of viral pathogenicity, which may be related to the structural proteins and virus-induced immunological responses. Keeping in view of the above study, we investigated structural stability of the MCP of ISKNV, RSIV and TRBIV using I-Mutant software, and it was found that the ISKNV is more stable at 25°C and pH 7.0. Among different ISKNV isolates, Indian isolate along with similar MCP sequence isolates were found more structurally stable. Furthermore, ISKNV genotype I (Indian isolate) of MCP was found to be having more structural stability than ISKNV genotype II. This study suggests that ISKNV genotype I might be more virulent, but further investigation is needed to establish its probable role in influencing viral pathogenicity at different water temperatures or stressors.

The current investigation reports presence of ISKNV in non-clinical ornamental fishes and an improved molecular diagnostic tool was developed for detection of ISKNV without need for sequencing, even from sub-clinical cases. Given the promiscuous nature of *Megalocytiviruses*, a strict biosecurity seems to be essential for its further spread to cultured fish species and also equally important to go for pan-India surveillance for this virus.

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