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1 **Detection and genetic analysis of infectious spleen and kidney necrosis virus (ISKNV) in**
2 **ornamental fish from non-clinical cases: First report from India**
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24 **Abstract**

25 Infectious spleen and kidney necrosis virus (ISKNV), a type species of the genus
26 *Megalocytivirus*, poses a threat to ornamental fish trade as most cases show nonspecific
27 symptoms, thus making timely diagnosis challenging. Apparently health molly (*Poecilia*
28 *sphenops*) and angelfish (*Pterophyllum scalare*) collected from two distinct geographic
29 localities of India were screened for four genera under *Iridoviridae*, *Megalocytivirus* i.e,
30 ISKNV, turbot reddish body iridovirus (TRBIV) and red seabream iridovirus (RSIV);
31 ranaviruses and Singapore grouper iridovirus; and Lymphocystivirus through molecular
32 approach. In total five numbers out of 17 samples (29.4%), ISKNV genome fragments were
33 detected. A PCR assay using major capsid protein (MCP) gene was standardised to detect and
34 differentiate infections within the *Megalocytivirus* genus, even without aid of sequencing.
35 This forms the first report of ISKNV infection in ornamental fish from India. Sequence
36 analysis of MCP gene showed that Indian isolate being 100% similar to the complete genome
37 or reference strain of ISKNV. Phylogenetic reconstruction demonstrated the present strain
38 belonging to ISKNV genotype I. Furthermore, structural stability of the MCP revealed this
39 strain was more stable than ISKNV genotype II, RSIV and TRBIV at 25°C and pH 7.0. Thus
40 a strong pan-India surveillance is recommended to reduce trade risk.

41

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

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47 ## 1. Introduction

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

58 On the flip side, the global ornamental fish trade poses a potential source for the
59 spread of many exotic pathogens, particularly viruses causing both morbidity and mortality in
60 fishes (Oyamatsu et al., 1997; George et al., 2015; Pragyan et al., 2019; Sahoo et al., 2016,
61 2020a, 2020b). Similarly, Sahoo et al. (2020b) investigated the infectious diseases in
62 freshwater aquaculture farms of eastern India and found that the incidences of viral infections
63 are more in ornamental fish. To-date, viral infections viz., viral nervous necrosis (VNN) in
64 freshwater aquarium fishes 'goldfish and rainbow shark' (Jithendran et al., 2011), Cyprinid
65 herpes virus-2 (CyHV-2) in goldfish (Sahoo et al., 2016), carp edema virus (CEV) in koi carp
66 (Swaminathan et al., 2016; Sahoo et al., 2020a), ranavirus in koicarp (George et al., 2015)
67 and marine ornamental similar damselfish (Sivasankar et al., 2017) have been reported from
68 Indian ornamental fishes.

69 The family *Iridoviridae* consists of a large group of enveloped viruses possessing
70 double-stranded DNA (Jancovich, 2012), which are known to cause large scale mortalities in
71 fish. Taxonomists have divided this family into five genera viz. *Megalocytivirus*, *Iridovirus,*

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72 *Ranavirus*, *Lymphocystivirus* and *Chloriridovirus* (Kurita and Nakajima, 2012). Viruses
73 belonging to the *Iridoviridae* family are typically vulnerable to both wild as well as
74 freshwater fish, including ornamental fish. Iridoviruses capable of infecting fish have been
75 accommodated in three major groups or genera
76 viz., *Ranavirus*, *Lymphocystivirus* and *Megalocytivirus* (Chinchar et al., 2009). Since their
77 discovery in the early 1990s, the group of Megalocytiviruses, in particular, have caused
78 significant morbidity as well as financial losses in marine and freshwater fish culture,
79 predominantly in Asia. Megalocytiviruses have been broadly distributed into three clusters,
80 i.e., RSIV, infectious spleen and kidney necrosis virus (ISKNV) and turbot reddish body
81 iridovirus (TRBIV), based on their major capsid protein (MCP) and ATPase gene
82 phylogenetic analysis (Kurita and Nakajima, 2012), and recently another strain, threespine
83 stickleback iridovirus (TSIV) have also been described (Murwantoko et al., 2018). He et al.
84 (1998) first isolated ISKNV, a species of the genus *Megalocytivirus*, from mandarin fish in
85 China. Subsequently ISKNV has also been reported from Japan (Tanaka et al., 2014),
86 Malaysia (Subramaniam et al., 2014), Australia (Mohr et al., 2015), USA (Subramaniam et
87 al., 2016), Germany (Jung-Schroers et al., 2016), Vietnam (Dong et al., 2017), Africa
88 (Ramires et al., 2019) and Thailand (Thanasaksiri et al., 2019) in both freshwater and marine
89 ornamental fishes. Phylogenetic analysis of the *Megalocytivirus* MCP gene sequence
90 revealed the existence of two genotypes I and II of ISKNV (Kurita and Nakajima, 2012;
91 Dong et al., 2017; Murwantoko et al., 2018).

92 Ornamental fish viruses have inherent inability to exhibit growth in commonly used
93 fish cell lines, thus making diagnosis difficult. ISKNV like isolates, mostly those from
94 freshwater ornamental fish, are challenging to culture *in vitro* (Kurita and Nakajima 2012,
95 Rimmer et al., 2016) and CEV also being reported not to grow in any studied cell lines
96 (Swaminathan et al., 2016). On multiple occasions, viral infection may remain latent until

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97 adverse environmental conditions like poor quality of water, rough handling of fish, or
98 overcrowding, activate the disease (Sahoo et al., 2016, 2020a). ISKNV poses a threat to
99 ornamental fish trade because *Megalocytiviral* infections have non-specific symptoms, thus
100 making timely diagnosis challenging (Jeong et al., 2006, 2008; Subramaniam et al., 2014;
101 Zainathan et al., 2017) and persistence of sub-clinical detected level of virus at the retailer
102 site (Rimmer et al., 2015). Viral diagnosis based on candidate gene PCR-sequencing seems to
103 be more accurate, reliable and faster than traditional phenotypic methods. Despite different
104 molecular techniques like PCR, nested PCR, real-time PCR (Xu et al., 2008), and LAMP
105 assay (Suebsing et al., 2016) being used for detection of ISKNV, the specificity for ISKNV
106 has usually been low (Razak et al., 2014; Bobby et a., 2018) thereby failing to reliably
107 differentiate ISKNV from other subgroups of *Megalocytiviruses*, i.e. RSIV and TRBIV.
108 Hence, the current investigation was aimed at detecting the presence of *Megalocytiviruses* in
109 two species of ornamental fishes, i.e., molly (*Poecilia sphenops*) and angelfish (*Pterophyllum*
110 *scalare*) collected from two geographically distinct Indian ornamental fish markets from
111 apparently healthy fish. Further, we standardised a precise and reliable nested PCR-based
112 approach to detect ISKNV and segregate it from other species of *Megalocytiviruses*.
113

114 ## 2. Materials and methods

115 ### *2.1. Case history and sample collection*

116 Seventeen numbers of apparently healthy fish (Molly, *Poecilia sphenops* and
117 angelfish, *Pterophyllum scalare*) were collected from five different ornamental retailer shops
118 of Kurla, Maharashtra and Bhubaneswar, Odisha states of India during the year 2018-2019
119 for routine screening of viruses in the National Referral Laboratory of Freshwater Fish
120 Diseases at ICAR-Central Institute of Freshwater Aquaculture, Bhubaneswar, India. The fish
121 were euthanized with overdose of MS222 (Sigma, USA), and liver, kidney, gills, brain and

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122 eye samples of individual fish were collected in 100% ethanol. The experiments were
123 performed following the approval of the Institute Animal Ethics Committee.

124 *2.2. Molecular screening for Megalocytiviruses*

125 *2.2.1. DNA isolation*

126 Tissue samples (~ 10 mg) were treated with proteinase K in lysis buffer (50 mM
127 Tris/HCl, 100 mM NaCl, 100 mM EDTA, 1% [w/v] SDS, pH 8.0) and subjected to extraction
128 with phenol/chloroform/isoamyl alcohol, followed by ethanol precipitation. The isolated
129 DNA was diluted in TE (50 mM Tris/HCl, one mM EDTA, pH 7.5) buffer. Concentration
130 and purity of the extracted DNA was determined by measuring OD at 260 and 280 nm using
131 a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies Inc., USA). The samples
132 were stored at –20ºC for further analysis. Before processing further, an equal amount of DNA
133 samples from each organ of an individual fish were pooled to form one sample.

134 *2.2.2. PCR amplification*

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

139 The samples were initially screened for MCP gene of MCV using two sets of
140 published primers (Rimmer et al., 2012). In the first set, C1105 and C1106 (Table 1) primers
141 were used in a final volume of 25 µL containing 1 µL of total DNA, 1.5 µL (10 pmol) of each
142 primer, 0.25 µL of *Taq* DNA polymerase (5 U/µL), 2.5 µL of 10X *Taq* buffer A, 0.5 µL of
143 dNTPs (2 mM) and ddH_2O to make final volume to 25 µL. The reaction mix was subjected to
144 30 temperature cycles (30s at 94 °C, 30s at 55 °C and 1 min at 72 °C) after an initial

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145 denaturing step (15 min at 95 °C) followed by a final extension step of 7 min at 72 °C in a
146 Veriti thermal cycler (Applied Biosystem). A nested PCR was performed using a second set
147 primers C1073 and C1074 using similar conditions. These primers only confirm the presence
148 of MCV at genus level.

149 After confirming the presence of MCV, samples were processed for molecular
150 screening of ISKNV using a combination of published primers (Kurita and Nakajima, 2012;
151 Rimmer et al., 2012) with partial modification. In the first step, primer C1105F and MCP-
152 uni1108-R8 (unpublished pairing) were used in a final volume of 25 μL containing 1 μL of
153 total DNA, 1.5 μL (10 pmol) of each primer, 0.25 μL of *Taq* DNA polymerase (5 U/μL1),
154 2.5 μL of 10X *Taq* buffer A, 0.5 μL of dNTPs (2 mM) and ddH_2O to make final volume to
155 25 μL. The reaction mix was subjected to 35 temperature cycles (60 sec at 95 °C, 60 sec at 57
156 °C and 60 sec at 72 °C) after an initial denaturing step (300 sec at 95 °C) followed by a final
157 extension step of 300 sec at 72 °C in a Veriti thermal cycler to amplify genus MCV. Further,
158 nested PCR was performed using ISKNV specific primers MCP-specI 465-F3 and MCP-
159 specI 879-R3 using the above first step product to increase the sensitivity and specificity for
160 detecting only ISKNV as primers don't amplify RSIV and TRBIV (Kurita and Nakajima,
161 2012). The reaction mix was subjected to 35 temperature cycles (1 min at 95 °C, 1 min at 58
162 °C and 1 min at 72 °C) after an initial denaturing step (5 min at 95 °C) followed by a final
163 extension step of 5 min at 72 °C.

164 The samples were also screened for ISKNV ATPase gene (Mohr et al., 2015). PCR was
165 carried out in a final volume of 25 μl reaction mix, as mentioned above for the MCP gene.
166 Amplification was programmed for a preliminary 15 min denaturation step at 95 °C, followed
167 by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C
168 for 90 s, and finally extension at 72 °C for 7 min (Mohr et al., 2015). Several target

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169 amplicons were generated to strengthen confirmation of ISKNV as it would be the first report
170 from India.

171 *2.3.Sequencing and phylogenetic study*

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

186 *2.4.Protein stability prediction*

187 Along with the sequence study, I-MUTANT 2.0 server was used to predict the
188 stability of the MCV major capsid protein sequences at pH 7.0 and temperature 25 ˚C
189 (Capriotti et al., 2005). This software can evaluate the stability change upon single-site
190 mutation, starting from the protein structure or the protein sequence. I-Mutant 2.0 correctly
191 predicts whether the protein mutation stabilises or destabilises the protein when the protein
192 sequence is available.

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193 ## 3. Results

194 ### *3.1. Identification of the virus in samples*

195 Among seventeen samples screened, five numbers (29.4%) of samples were found to
196 be positive for ISKNV. For detection of *Megalocytivirus*, MCP gene was screened using
197 primers C1105/C1106 and C1073/C1074, and amplicons of 431 bp and 168 bp were obtained
198 in first and second step PCRs, respectively (Figs. 1a and 1b). Aimed at detection of ISKNV,
199 the tissues were screened with primers C1105/MCP-uni1108-R8 and MCP-specI465-
200 F3/MCP-specI879-R3 primers. In five samples, amplified product of 415 bp of ISKNV MCP
201 gene were obtained in the nested PCR (Fig. 2a). Further, in same samples ISKNV ATPase
202 gene were also amplified with amplicon size of 1542 bp using ATP2DGf and
203 ATP1076DGIVlater primers (Fig. 2b). All the samples collected for this study were found to
204 be negative for RSIV, EHNV, SGIV and LCDV using respective primer sets.

205 ### *3.2.Sequencing, comparison of MCP gene and phylogenetic study*

206 ISKNV MCP gene amplicons obtained (using primers pairs C1105/C1106 and MCP-
207 specI465-F3/MCP-specI879-R3) were sequenced, and a BLAST search of the sequence
208 revealed 99% and 100% similarities with known previously published MCP genes of
209 *Megalocytivirus* and ISKNV, respectively. Megalocytivirus MCP gene (primer
210 C1105/C1106) sequence amplicon of□ 431 bp encoding 143 amino acids has been submitted
211 to GenBank (GenBank accession number: MK084827). The 431 bp MCP gene fragment was
212 found to be 99.77% similar with the complete genome or reference strain of ISKNV
213 (AF371960) along with other ISKNV isolates
214 (MK757444, KX354220, KT781098, AB666344, AB666337, AF370008), as well as ISKNV
215 like viruses i.e., giant seaperch iridovirus isolate GSIV (JF264350) and African lampeye
216 iridovirus (AY285745, AB109368) (Kurita and Nakajima, 2012). Also, the obtained

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217 sequence is 95.59% similar to RSIV (AB666327) and TRBIV (HM596017). Sequence
218 alignment revealed a silent mutation observed at 399 bp position (GGCGly to GGTGly) in
219 the MCP gene of Indian isolate (Fig. 3). Despite changes in the nucleotide sequence, the
220 multiple 143 amino acid alignments of the MCP gene have shown 100% similarity with all
221 the sequences mentioned (Fig. 4).

222 The amplicon of 415 bp, encoding 138 amino acids of MCP gene sequence was
223 found to be specific for ISKNV. It has been subsequently submitted to GenBank (GenBank
224 accession number: MN518863). Both of these gene fragments (first 431 bp specific
225 for Megalocytivirus, and next 415 bp specific for ISKNV) were combined to form a total of
226 846 nucleotides and 282 amino acids (Figs. 3, 4). The primer binding sites
227 of *Megalocytivirus* specific primer C1105/C1106 and C1073/C1074 are common in the MCP
228 gene of ISKNV, RSIV and TRBIV. To increase the sensitivity and confirmative diagnosis of
229 ISKNV, the nested PCR using ISKNV specific MCP-specI465-F3 and MCP-specI879-R3
230 primer pair was successfully performed, that could differentiate ISKNV from the other
231 *Megalocytivirus* species by amplifying only ISKNV (415 bp). Further the diagnosis was more
232 validated based on *in silico* analysis for all three *Megalocytivirus* sequence primer binding
233 regions in this study and also based on earlier report of Kurita and Nakajima (2012) and
234 Mohr et al. (2015) (Fig. 3).

235 A phylogeny tree was constructed based on the MCP gene amino acid sequence
236 (MK084827). The Indian isolate in the present study was found to be ISKNV genotype I. It
237 showed 100% MCP gene sequence similarity with that of China
238 (MK757444, AF370008, ADU25256), Australia (ALF95980, KP292960), Malaysia
239 (JQ253373, KF753331), Singapore (AB666344, BAL04525), and Thailand (BBD52180,
240 LC378578.1), and was clustered within the same clade in the phylogeny tree (Fig. 5). There
241 were variations at the positions corresponding to amino acids 84 (Asn-Asp) and 198 (Leu-

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242 Pro) in the MCP sequences of South Korea (GU168574) and Malaysia (JQ253374),
243 respectively (Fig. 4). Even so, the later two strains clustered with the Indian strain within the
244 same clade of ISKNV genotype I (Fig. 5). Meanwhile, two other isolates of ISKNV, i.e.,
245 Japan (AB669096) and China (KY440040) differed from other ISKNV at amino acid position
246 203 (Asn- Ser) and were found to have clustered together as ISKNV genotype II (Figs. 4, 5).
247 The two subspecies of Megalocytivirus, i.e., RSIV (AB666327) and TRBIV (AY590687)
248 were segregated from ISKNV by amino acid position 146 (Ala-Gly), 157 (Val-Ala),
249 167 (Ala-Val) and 143 (Asp-Gly), 146 (Ala-Ser), 152 (Gln-His), 157 (Val-Thr), and
250 218 (Gla-Ser), respectively, and clustered at different clades (Figs. 4, 5).

251 *3.3.Prediction of protein stability of MCP*

252 The stability of the MCP of ISKNV of Indian strain was compared *in silico* with
253 ISKNV, RSIV and TRBIV isolates using I-Mutant software. The stability of MCP was found
254 to reduce due to amino acid changes in ISKNV isolates of South Korea (84; N-D, 246; Q-R),
255 Malaysia (198; L-P), Japan and China (Genotype II) (203; N-S) (Supplementary Fig. 1).
256 MCP stability study indicated ISKNV MCP of having relatively better structural stability as
257 compared to that of RSIV, whereas TRBIV MCP showed further decreased stability at 25°C
258 and pH 7.0 (Supplementary Fig. 2).

259 **4. Discussion**

260 To our knowledge, it seems to be the first report of ISKNV infection in traded
261 ornamental fish in India. The study further highlights a new combinational primer set to
262 undertake a nested PCR (using C1105F/ MCP-uni1108-R8 and its nested MCP-specI465-F3
263 and MCP-specI879-R3) to directly identify ISKNV in PCR without ambiguity in one go,
264 thereby delineating needs for further immediate need of sequence confirmation. Further, an
265 increase in the sensitivity and specificity of only ISKNV-specific primer set published earlier

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266 (Kurita and Nakajima, 2012) was obtained to detect the virus in sub-clinical cases. This study
267 simultaneously shows that use of primers (C1105/C1106) to screen ISKNV may not
268 distinguish ISKNV from genus *Megalocytivirus* without the aid of sequencing.

269 The hosts affected by ISKNV are relatively wide-ranging; nevertheless, freshwater
270 fish species are the predominantly affected species (Kurita and Nakajima, 2012; Sihu et al.,
271 2017). Several outbreaks of ISKNV diseases in freshwater fishes have been reported in the
272 ornamental fish of Germany (Jung-Schroers et al. 2016), Australia (Mohr et al. 2015; Rimmer
273 et al. 2017), and Malaysia (Subramaniam et al. 2014; Zainathan et al. 2017) and more
274 recently in Africa (Ramirez-Paredes et al., 2019). Detection of ISKNV in two ornamental fish
275 species, molly (*P. sphenops*) and angelfish (*P. scalare*) obtained in this study were also
276 reported earlier from other countries (Rodger et al., 1997; Rodger et al., 2003; Yanong and
277 Waltzek, 2010; Go et al., 2016; Zainathan et al., 2017). Subramaniam et al. (2014) reported
278 many positive cases of ISKNV in ornamental fishes which did not show any clinical signs.
279 This further confirmed the fact that Megalocytivirus infects visibly healthy fish species; this
280 condition could be termed persistent or asymptomatic infection as reported by Jeong et al.
281 (2006, 2008). In comparison to clinically infected fish tissue, DNA concentration
282 of *Megalocytivirus* was approximately 10^5 times higher in asymptomatic fish, as proven by
283 (Jeong et al., 2006). An earlier report finds the presence of ISKNV from apparently healthy
284 fish at temperatures below 20 °C (He et al., 2002). When water temperature was 28 °C,
285 juvenile Chinese perch challenged with ISKNV, displayed clinical signs with 100% mortality
286 (He et al., 2000). It seems *Megalocytivirus* requires high temperature to multiply (Liu et al.,
287 2019) that needs further study after isolation of virus. Further, recent report of emergence in
288 causing mass mortality in tilapia in Africa raise a big concern for tilapia farming (Ramirez-
289 Paredes et al., 2019), if not taken proper care, likely entry of it from ornamental fish to tilapia
290 species would be disastrous.

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291 Currently, the OIE Manual for Diagnostic Tests for Aquatic Animals does not identify
292 a single test that would be suitable for accurate and specific detection of ISKNV (Kurita and
293 Nakajima, 2012; OIE, 2018) . Out of the recommended criteria for confirmatory diagnosis,
294 conventional PCR detects the two listed genotypes, whereas others require specialized
295 reagents (monoclonal antibodies) or are not applicable to all ISKNV like isolates (eg. virus
296 isolation) (Johnson et al., 2019). Better methods remain to be identified for the specific
297 diagnosis of ISKNV infections with particular reference to the differentiation of gene
298 sequence, subclinical infections and clinical disease. Although there are several reports of
299 screening the ISKNV virus by PCR and sequencing using a *Megalocytivirus* genus primer
300 (C1105/C1106), no reports of detection for ISKNV by using ISKNV species specific primer
301 described by Kurita and Nakajima (2012) and Mohr et al. (2015) (Go et al., 2006; Xu et al.,
302 2008; Rimmer et al., 2012; Subramaniam et al., 2014; Razak et al., 2014; Mohr et al., 2015;
303 Rimmer et al., 2017: Dong et al., 2017; Bobby et a., 2018; Johnson et al., 2019; Zainathan et
304 al., 2019). Hence in this investigation, a nested PCR assay was standardised to detect and
305 differentiate ISKNV infections within the *Megalocytivirus* genus of the family *Iridoviridae*.

306 The major capsid protein (MCP) gene is relatively conserved among the viruses of the
307 family *Iridoviridae*, for which it can be considered as one of the most important genes for the
308 analysis of the genetic relationships among this family (Williams et al., 1996; Tidona et al.,
309 1998; Liu et al., 2019). The amino acid sequence analysis of *Megalocytivirus* specific MCP
310 gene regions revealed 100% similarity with all the three *Megalocytivirus* subgroup.
311 However, subgrouping issue was resolved by use of new combinational nested ISKNV PCR
312 with internal primer set of ISKNV (Kurita and Nakajima, 2012), as it is100% similar with
313 only ISKNV genome was obtained with the produced amplicons, that emphasizes upon
314 ISKNV subtyping with the current PCR system in one go.

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

323 A proteomic study of ISKNV demonstrated that ISKNV virions possess 38 viral
324 structural proteins, where MCP is the main structural component of the virus particle,
325 comprising 40 to 45% of the total particle polypeptide (Dong et al., 2011; Jia et al., 2013).
326 Jia et al. (2013) reported first time, ISKNV MCP interacted with Mandarin fish caveolin
327 1 (mCav-1), which gives an evidence of a viral structural protein interacted with a host
328 protein in the family *Iridoviridae* for its primary infection. This information indicates that,
329 major capsid proteins play an essential role in the ISKNV infection by virus-host protein
330 interactions. Further study can help us to understand the mechanisms of viral pathogenicity,
331 which may be related to the structural proteins and virus-induced immunological responses.
332 Keeping in view of the above study, we investigated structural stability of the MCP of
333 ISKNV, RSIV and TRBIV using I-Mutant software, and it was found that the ISKNV is more
334 stable at 25°C and pH 7.0. Among different ISKNV isolates, Indian isolate along with similar
335 MCP sequence isolates were found more structurally stable. Furthermore, ISKNV genotype I
336 (Indian isolate) of MCP was found to be having more structural stability than ISKNV
337 genotype II. This study suggests that ISKNV genotype I might be more virulent, but further
338 investigation is needed to establish its probable role in influencing viral pathogenicity at
339 different water temperatures or stressors.

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340 To summarize, it seems to be the first report of ISKNV from ornamental fish meant
341 for either internal or external trade. The current investigation also established an improved
342 molecular diagnostic tool for detection of ISKNV pending sequencing confirmation, even
343 from sub-clinical cases. Given the promiscuous nature of Megalocytiviruses, a strict
344 biosecurity seems to be essential for its further spread to cultured fish species and also
345 equally important to go for pan-India surveillance for this virus.

346 **Conflict of interests**

347 There is no conflict of interests involved in this manuscript.

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352 **Data availability statement**

353

354 The data that supports the findings of this study are available in the supplementary material
355 of this article (supplementary Figs. 1 and 2).

356

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533

534 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	Megalocytivirus nPCR MCP gene	C1105	GGGTTCATCGACATCTCCGCG	430	Rimmer et al., 2012
		C1106	AGGTCGCTGCGCATGCCAATC		
		C1173	AATGCCGTGACCTACTTTGC	167	Rimmer et al., 2012
		C1174	GATCTTAACACGCAGCCACA		
2	ISKNV nPCR MCP gene	C1105	GGGTTCATCGACATCTCCGCG	1075	Current Study
		MCP-uni1108-R8	TCTCAGGCATGCTGGGCGCAAAG		
		MCP-specI465-F3	GCTGTTGCAACCATTTGAGA	415	Kurita & Nakajima, 2012
		MCP-specI879-R3	ACGGGGTGACTGAACCTG		
3	ISKNV ATPase gene	ATP2DGf	CACCACCTGTGTGTATTTGTC	1542	Go et al., 2006
		ATP1076DGIVlater	ATGAACCCGCTGCACTATGC		
4	RSIV *pstI* fragment	2F	TACAACATGCTCCGCAAGA	564	Kurita et al., 1998
		2R	GCGTTAAAGTAGTGAGGGCA		
5	EHNV MCP gene	M151	AACCCGGCTTTCGGGCAGCA	321	OIE EHNV, 2019
		M152	CGGGGCGGGGTTGATGAGAT		
6	SGIV DNA Pol gene	SGIVDNPOLFW	GTGTAYCAGTGGTTTTGCGAC	560	Holopainen et al.,2009
		SGIVDNPOLRE	TCGTCTCCGGGYCTGTCTTT		
7	LCDV MCP gene	LCDVs F	YTGGTTCAGTAAATTACCRG	609	Kitamura et al. 2006
		LCDVs R	GTAATCCATACTTGHACRTC		

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536 **Legend to the figures:**

537 **Fig: 1a.** Samples amplified with C1105 and C1106 primers, with expected product sizes of
538 431 bp. Lanes 1 and 2 represent fish samples, Lane 3 represents negative control; **1b.**
539 Samples amplified with C1173 and C1174 primers; with expected product sizes of 167 bp.
540 Lanes 1-5 represents fish samples, Lanes 7 represent negative control. M, 50 bp Ladder
541 (Thermo Scientific).

542 **Fig: 2a.** Samples amplified with MCP-specI465-F3 and MCP-specI879-R3 primers, with
543 expected product sizes of 415 bp. Lane 1 represents negative control and Lanes 2-15
544 represents fish samples; **2b.** Samples amplified with ATP2DGf and ATP1076DGIVlater
545 primers; with expected product sizes of 1542 bp. Lane 1-5 represents fish samples, Lanes 7
546 represent negative control. M, 3 kb Ladder (Thermo Scientific).

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

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

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560 **Fig: 5.** Phylogenetic tree based on the MCP gene amino acid sequence of ISKNV, RSIV and
561 TRBIV. The tree was generated by MEGA 6 using Maximum Likelihood method of Clustal
562 W 2. 1. MCP gene of human herpes virus was used as an outgroup.

563 **Supplementary Fig. 1.** Protein stability prediction of different ISKNV strain including
564 Indian isolate. I-MUTANT 2.0 server was used to predict the stability of the protein
565 sequences at pH 7.0 and temperature 25˚C.

566 **Supplementary Fig. 2.** Protein stability prediction and comparison of stability upon mutation
567 at pH 7.0 and temperature 25˚C, among Indian ISKNV isolate with RSIV and TRBIV using
568 I-MUTANT 2.0 server.

Fig: 1a

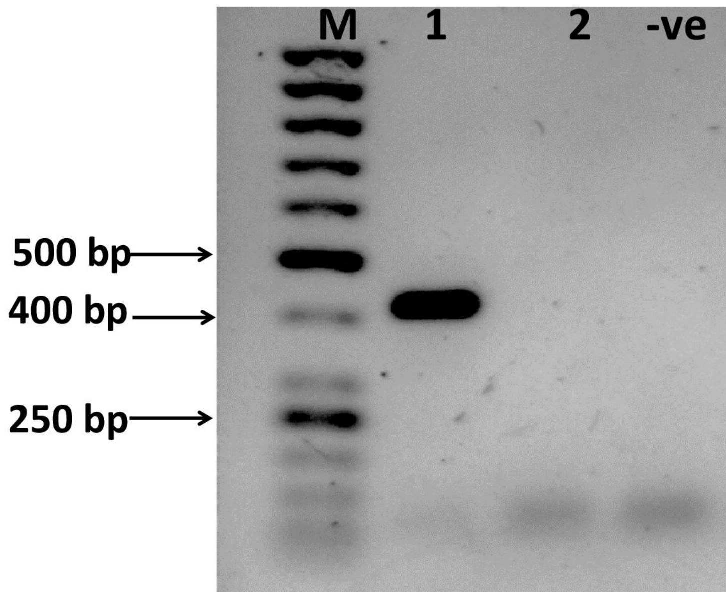

Fig: 1b

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2
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4
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M
-ve
1000 bp
250 bp
200 bp
150 bp
167 bp

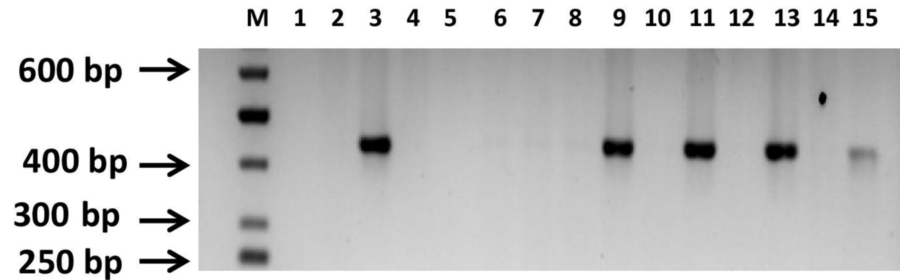


Fig: 2a
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
600 bp
400 bp
300 bp
250 bp

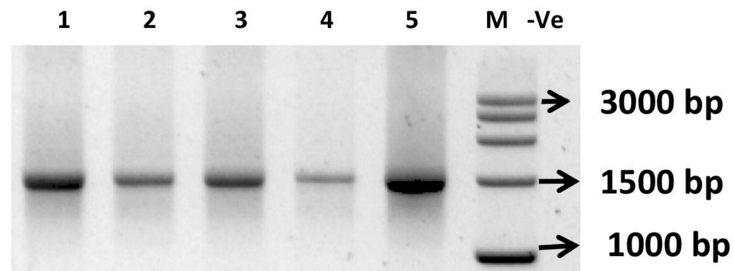
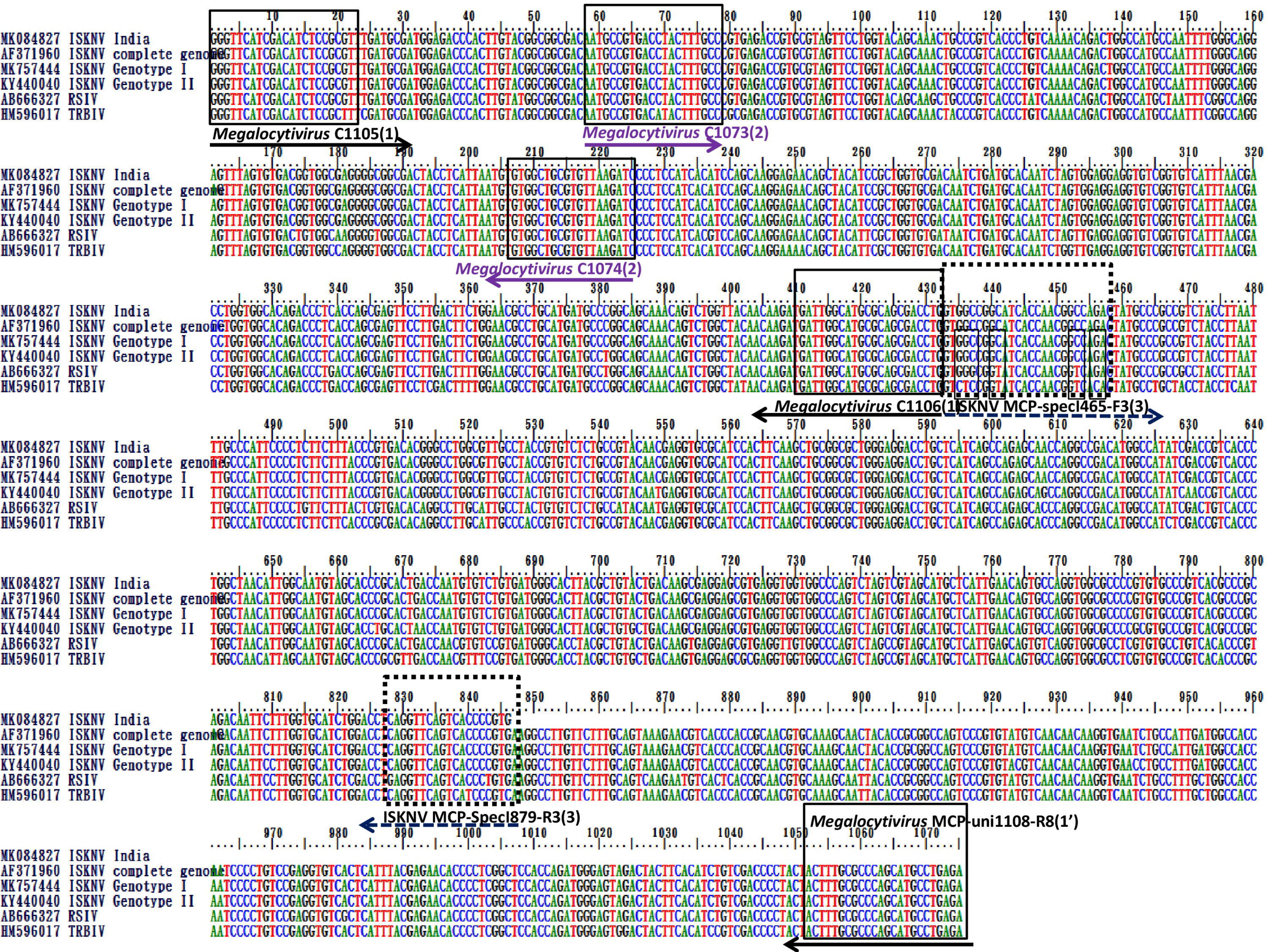


Fig: 2b
1 2 3 4 5 M -Ve
3000 bp
1500 bp
1000 bp



MK084827 ISKNV India
AF371960 ISKNV complete genome
MK757444 ISKNV Genotype I
KY440040 ISKNV Genotype II
AB666327 RSIV
HM596017 TRBIV
Megalocytivirus C1105(1)
Megalocytivirus C1073(2)
Megalocytivirus C1074(2)
Megalocytivirus C1106(1)
ISKNV MCP-specI465-F3(3)
ISKNV MCP-SpecI879-R3(3)
Megalocytivirus MCP-uni1108-R8(1')

Megalocytivirus MCP gene

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MK084827 ISKNV India           GFIDISAFDAMETHLYGGDNAVTYFARETVRSSWYSKLPVTLSKQTGHANFGQEFSVTVARGGDYLINVWLRVKIPSITSSKENSYIRWCDNLMHNLVEEVSVSFNDLVAQTLTSEFLDFWNACMMPGSKQSGYNKMIGMRSDLVAGITNGQTMPAVYLN
MK757444 ISKNV China           GFIDISAFDAMETHLYGGDNAVTYFARETVRSSWYSKLPVTLSKQTGHANFGQEFSVTVARGGDYLINVWLRVKIPSITSSKENSYIRWCDNLMHNLVEEVSVSFNDLVAQTLTSEFLDFWNACMMPGSKQSGYNKMIGMRSDLVAGITNGQTMPAVYLN
ALF95980 ISKNV Australia       GFIDISAFDAMETHLYGGDNAVTYFARETVRSSWYSKLPVTLSKQTGHANFGQEFSVTVARGGDYLINVWLRVKIPSITSSKENSYIRWCDNLMHNLVEEVSVSFNDLVAQTLTSEFLDFWNACMMPGSKQSGYNKMIGMRSDLVAGITNGQTMPAVYLN
KP292960 ISKNV Australia       GFIDISAFDAMETHLYGGDNAVTYFARETVRSSWYSKLPVTLSKQTGHANFGQEFSVTVARGGDYLINVWLRVKIPSITSSKENSYIRWCDNLMHNLVEEVSVSFNDLVAQTLTSEFLDFWNACMMPGSKQSGYNKMIGMRSDLVAGITNGQTMPAVYLN
JQ253374 ISKNV Malaysia        GFIDISAFDAMETHLYGGDNAVTYFARETVRSSWYSKLPVTLSKQTGHANFGQEFSVTVARGGDYLINVWLRVKIPSITSSKENSYIRWCDNLMHNLVEEVSVSFNDLVAQTLTSEFLDFWNACMMPGSKQSGYNKMIGMRSDLVAGITNGQTMPAVYLN
AF370008 ISKNV China           GFIDISAFDAMETHLYGGDNAVTYFARETVRSSWYSKLPVTLSKQTGHANFGQEFSVTVARGGDYLINVWLRVKIPSITSSKENSYIRWCDNLMHNLVEEVSVSFNDLVAQTLTSEFLDFWNACMMPGSKQSGYNKMIGMRSDLVAGITNGQTMPAVYLN
KF753331 ISKNV Malaysia        GFIDISAFDAMETHLYGGDNAVTYFARETVRSSWYSKLPVTLSKQTGHANFGQEFSVTVARGGDYLINVWLRVKIPSITSSKENSYIRWCDNLMHNLVEEVSVSFNDLVAQTLTSEFLDFWNACMMPGSKQSGYNKMIGMRSDLVAGITNGQTMPAVYLN
ADU25256 ISKNV China           GFIDISAFDAMETHLYGGDNAVTYFARETVRSSWYSKLPVTLSKQTGHANFGQEFSVTVARGGDYLINVWLRVKIPSITSSKENSYIRWCDNLMHNLVEEVSVSFNDLVAQTLTSEFLDFWNACMMPGSKQSGYNKMIGMRSDLVAGITNGQTMPAVYLN
LC378578 ISKNV Thailand        GFIDISAFDAMETHLYGGDNAVTYFARETVRSSWYSKLPVTLSKQTGHANFGQEFSVTVARGGDYLINVWLRVKIPSITSSKENSYIRWCDNLMHNLVEEVSVSFNDLVAQTLTSEFLDFWNACMMPGSKQSGYNKMIGMRSDLVAGITNGQTMPAVYLN
AB666344 ISKNV Singapore       GFIDISAFDAMETHLYGGDNAVTYFARETVRSSWYSKLPVTLSKQTGHANFGQEFSVTVARGGDYLINVWLRVKIPSITSSKENSYIRWCDNLMHNLVEEVSVSFNDLVAQTLTSEFLDFWNACMMPGSKQSGYNKMIGMRSDLVAGITNGQTMPAVYLN
BAL04525 ISKNV Singapore       GFIDISAFDAMETHLYGGDNAVTYFARETVRSSWYSKLPVTLSKQTGHANFGQEFSVTVARGGDYLINVWLRVKIPSITSSKENSYIRWCDNLMHNLVEEVSVSFNDLVAQTLTSEFLDFWNACMMPGSKQSGYNKMIGMRSDLVAGITNGQTMPAVYLN
BBD52180 ISKNV Thailand        GFIDISAFDAMETHLYGGDNAVTYFARETVRSSWYSKLPVTLSKQTGHANFGQEFSVTVARGGDYLINVWLRVKIPSITSSKENSYIRWCDNLMHNLVEEVSVSFNDLVAQTLTSEFLDFWNACMMPGSKQSGYNKMIGMRSDLVAGITNGQTMPAVYLN
JQ253373 ISKNV Malaysia        GFIDISAFDAMETHLYGGDNAVTYFARETVRSSWYSKLPVTLSKQTGHANFGQEFSVTVARGGDYLINVWLRVKIPSITSSKENSYIRWCDNLMHNLVEEVSVSFNDLVAQTLTSEFLDFWNACMMPGSKQSGYNKMIGMRSDLVAGITNGQTMPAVYLN
GU168574 ISKNV South Korea     GFIDISAFDAMETHLYGGDNAVTYFARETVRSSWYSKLPVTLSKQTGHANFGQEFSVTVARGGDYLINVWLRVKIPSITSSKEDSYIRWCDNLMHNLVEEVSVSFNDLVAQTLTSEFLDFWNACMMPGSKQSGYNKMIGMRSDLVAGITNGQTMPAVYLN
KY440040 ISKNV China           GFIDISAFDAMETHLYGGDNAVTYFARETVRSSWYSKLPVTLSKQTGHANFGQEFSVTVARGGDYLINVWLRVKIPSITSSKENSYIRWCDNLMHNLVEEVSVSFNDLVAQTLTSEFLDFWNACMMPGSKQSGYNKMIGMRSDLVAGITNGQTMPAVYLN
AB669096 ISKNV Japan           GFIDISAFDAMETHLYGGDNAVTYFARETVRSSWYSKLPVTLSKQTGHANFGQEFSVTVARGGDYLINVWLRVKIPSITSSKENSYIRWCDNLMHNLVEEVSVSFNDLVAQTLTSEFLDFWNACMMPGSKQSGYNKMIGMRSDLVAGITNGQTMPAVYLN
AB666327 RSIV Hong Kong        GFIDISAFDAMETHLYGGDNAVTYFARETVRSSWYSKLPVTLSKQTGHANFGQEFSVTVARGGDYLINVWLRVKIPSITSSKENSYIRWCDNLMHNLVEEVSVSFNDLVAQTLTSEFLDFWNACMMPGSKQSGYNKMIGMRSDLVGGITNGQTMPAAYLN
AY590687 TRBIV China           GFIDISAFDAMETHLYGGDNAVTYFARETVRSSWYSKLPVTLSKQTGHANFGQEFSVTVARGGDYLINVWLRVKIPSITSSKENSYIRWCDNLMHNLVEEVSVSFNDLVAQTLTSEFLDFWNACMMPGSKQSGYNKMIGMRSGLVSGITNGHTMPATYLN
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Megalocytivirus C1105 →

← *Megalocytivirus* C1106

ISKNV SPECI465F3 →

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                             170       180       190       200       210       220       230       240       250       260       270       280
AF371960 ISKNV Complete Genome LPIPLFFTRDTGLALPTVSLPYNEVRIHFKLRRWEDLLISQSNQADMAISTVTLANIGNVAPALTNVSVMGTYAVLTSEEREVVAQSSRSMLIEQCQVAPRVPVTPADNSLVHLDLRFSHPV
MK084827 ISKNV India           LPIPLFFTRDTGLALPTVSLPYNEVRIHFKLRRWEDLLISQSNQADMAISTVTLANIGNVAPALTNVSVMGTYAVLTSEEREVVAQSSRSMLIEQCQVAPRVPVTPADNSLVHLDLRFSHPV
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ALF95980 ISKNV Australia       LPIPLFFTRDTGLALPTVSLPYNEVRIHFKLRRWEDLLISQSNQADMAISTVTLANIGNVAPALTNVSVMGTYAVLTSEEREVVAQSSRSMLIEQCQVAPRVPVTPADNSLVHLDLRFSHPV
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LC378578 ISKNV Thailand        LPIPLFFTRDTGLALPTVSLPYNEVRIHFKLRRWEDLLISQSNQADMAISTVTLANIGNVAPALTNVSVMGTYAVLTSEEREVVAQSSRSMLIEQCQVAPRVPVTPADNSLVHLDLRFSHPV
AB666344 ISKNV Singapore       LPIPLFFTRDTGLALPTVSLPYNEVRIHFKLRRWEDLLISQSNQADMAISTVTLANIGNVAPALTNVSVMGTYAVLTSEEREVVAQSSRSMLIEQCQVAPRVPVTPADNSLVHLDLRFSHPV
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GU168574 ISKNV South Korea     LPIPLFFTRDTGLALPTVSLPYNEVRIHFKLRRWEDLLISQSNQADMAISTVTLANIGNVAPALTNVSVMGTYAVLTSEEREVVARSSRSMLIEQCQVAPRVPVTPADNSLVHLDLRFSHPV
KY440040 ISKNV China           LPIPLFFTRDTGLALPTVSLPYNEVRIHFKLRRWEDLLISQSSQADMAISTVTLANIGNVAPALTNVSVMGTYAVLTSEEREVVAQSSRSMLIEQCQVAPRVPVTPADNSLVHLDLRFSHPV
AB669096 ISKNV Japan           LPIPLFFTRDTGLALPTVSLPYNEVRIHFKLRRWEDLLISQSSQADMAISTVTLANIGNVAPALTNVSVMGTYAVLTSEEREVVAQSSRSMLIEQCQVAPRVPVTPADNSLVHLDLRFSHPV
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ISKNV MCP gene

ISKNV SPEI879R63 ←

BAL04525 ISKNV Singapore

BBD52180 ISKNV Thailand

AB666344 ISKNV Singapore

ADU25256 ISKNV China

KF753331 ISKNV Malaysia

JQ253373 ISKNV Malaysia

AF370008 ISKNV China

KP292960 ISKNV Australia

ALF95980 ISKNV Australia

MK757444 ISKNV China

AF371960 ISKNV Complete Genome

● MK084827 ISKNV India

LC378578 ISKNV Thailand

JQ253374 ISKNV Malaysia

GU168574 ISKNV South Korea

KY440040 ISKNV China

AB669096 ISKNV Japan

AB666327 RSIV Hong Kong

AY590687 TRBIV China

D32005 Human herpesvirus MCP

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