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Citation	Ticks and Tick-Borne Diseases, 8(1), 103-111 https://doi.org/10.1016/j.ttbdis.2016.10.005
Issue Date	2018-01-22
Doc URL	http://hdl.handle.net/2115/68171
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Rights(URL)	http://creativecommons.org/licenses/by-nc-nd/4.0/
Type	article (author version)
File Information	Ticks Tick Borne Dis._v.8_p.103-111.pdf



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1 **Putative RNA viral sequences detected in an *Ixodes scapularis*-derived cell line**

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27

Abstract

28 Ticks harbour various microorganisms, some of which act as pathogens of
29 humans and animals. The recent advancement of genome sequencing technologies
30 revealed that a wide range of previously unrecognised microorganisms exist in ticks.
31 Continuous cell lines established from ticks could play a key role in the isolation of
32 such microorganisms; however, tick cells themselves have been known to harbour
33 symbiotic microorganisms. The present study aimed to characterise putative RNA viral
34 sequences detected in the culture supernatant of one of the most frequently used tick
35 cell lines, ISE6, which was derived from embryos of the blacklegged tick *Ixodes*
36 *scapularis*. Viral particles purified from the culture supernatant were used for RNA
37 extraction, followed by Illumina sequencing. The reads were *de novo* assembled and the
38 resulting contigs were annotated by tBLASTx search. The results suggested that there
39 were at least five putative viral sequences of four phylogenetically distinct lineages in
40 ISE6 cells. The predominant viral sequence found in ISE6 cells, designated *I. scapularis*
41 iflavirus, was a member of the family Iflaviridae, which is an arthropod-infecting virus
42 group. We also identified L and M segments of the family Bunyaviridae, which could

43 not be classified into any of the five known genera, and a potential capsid protein
44 related to Drosophila A virus. In addition to these previously unrecognised viruses, ISE6
45 was revealed to harbour a putative genome sequence of *I. scapularis*-associated virus-1,
46 which was reported in a recent metagenomic study of *I. scapularis* itself. All the five
47 putative viral sequences were detected by RT-PCR in both ISE6 cells and the culture
48 supernatant. Electron microscopic analysis showed the existence of spherical virions
49 with a varying diameter of 50 to 70 nm in the culture supernatant of ISE6 cells. Further
50 studies are required to investigate the potential roles of ISE6-associated viruses in ticks.

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Keywords

54 bunyavirus; iflavirus; ISE6; *Ixodes scapularis*; *Ixodes scapularis*-associated virus-1

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Introduction

59 Ticks harbour various microorganisms including viruses, bacteria and
60 protozoa, some of which act as pathogens of humans and animals. The recent
61 advancement of genome sequencing technologies enabled microbial community
62 analysis in a culture-independent manner (so-called metagenomic analysis) and revealed
63 that a wide range of previously unrecognised microorganisms exist in ticks (Carpi et al.,
64 2011; Nakao et al., 2013; Qiu et al., 2014; Tokarz et al., 2014). One of the requirements
65 for post-metagenomic studies is the characterisation of individual microorganisms in
66 terms of their potential pathogenicity to humans and animals, their influence on tick
67 biology and their interaction with other microbial lineages.

68 Tick cell lines are key to achieving the above-mentioned objective. First, tick
69 cells are useful for isolation and molecular characterisation of microorganisms
70 harboured by ticks (Bell-Sakyi et al., 2012; 2007). Of note, prolonged culture of some
71 tick-borne pathogens in tick cells facilitates adaptation to mammalian systems (Bekker
72 et al., 2002; Munderloh et al., 2004), indicating that tick cell lines can be better tools for
73 the isolation of microorganisms than mammalian cells. Second, tick cell lines are useful
74 to investigate the interaction between tick-borne microorganisms and ticks *in vitro*, and

75 can be valuable tools to evaluate the vector competence of ticks for pathogens
76 (Bell-Sakyi et al., 2012). *In vitro* observation of the replication cycles and intracellular
77 localisation of microorganisms may help with the study of their survival mechanisms in
78 ticks and transmission dynamics in nature.

79 Since the establishment of the first tick cell line from *Rhipicephalus*
80 *appendiculatus* (Varma et al., 1975), nearly 60 different cell lines originating from 16
81 different tick species are now available (Bell-Sakyi et al., 2012). Alberdi et al. (2012)
82 screened for bacterial endosymbionts and endogenous viruses in over 50 tick cell lines.
83 Despite the detection of virus-like particles by transmission electron microscopy (TEM),
84 PCR assays amplifying some known tick-borne viruses failed to detect viral genome
85 sequence in many of the cell lines (Alberdi et al., 2012), suggesting that uncharacterised
86 viruses exist in tick cell lines. In fact, the authors observed reovirus-like particles in
87 ISE6 cells by TEM, while PCR was negative for St Croix River virus (SCRV), the only
88 previously characterised tick virus (Alberdi et al., 2012; Attoui et al., 2001).

89 This paper reports viral genome sequences obtained from one of the most
90 frequently used tick cell lines, ISE6, which was derived from embryos of the

91 blacklegged tick *Ixodes scapularis* (Kurtti et al., 1996). Initially, we recognised the
92 existence of some virus sequences in the culture supernatant of ISE6 cells when this cell
93 line was employed for isolation and characterisation of novel tick-borne viruses in
94 field-collected ticks. High-throughput RNA sequencing analysis revealed that there are
95 at least five putative virus sequences from four phylogenetically different virus lineages
96 in this cell line.

97

98 **Materials and Methods**

99 **Cell line.**

100 The ISE6 cell line was kindly provided by the CEH Institute of Virology and
101 Environmental Microbiology (Oxford, UK). The use of ISE6 cells was authorized by
102 the University of Minnesota, the original provider of the cell line, under a material
103 transfer agreement between the University of Minnesota and Hokkaido University. The
104 cells were cultured in L-15B medium supplemented with 10% fetal calf serum, 5%
105 tryptose phosphate broth (Sigma–Aldrich, St. Louis, MO, USA) and 0.1% bovine
106 lipoprotein concentrate (MP Biomedicals, Irvine, CA, USA) at 32°C as described by

107 Munderloh and Kurtti (1989).

108 **Virus purification.**

109 A total of 200 mL of the culture supernatant of ISE6 cells was obtained at 7
110 days after the last passage. The supernatant was centrifuged at 190 g for 10 min at 4°C
111 to remove cell debris. The supernatant was collected and the virus particles were
112 pelleted through a 20% sucrose cushion at 28,000 rpm for 1.5 h with a Beckman SW41
113 rotor at 4°C. The pellet was re-suspended in phosphate-buffered saline and the purified
114 virus was stored at -80°C until use.

115 **TEM.**

116 ISE6 culture supernatant and culture medium (as a negative control) were
117 subjected to low-speed centrifugation followed by ultracentrifugation as described
118 above. The pelleted was fixed with 0.25% glutaraldehyde in PBS for 1 week at 4°C.
119 Fixed samples supplemented with 0.1% bovine serum albumin were adsorbed to
120 collodion-carbon-coated copper grids and negatively stained for 30 s with 2%
121 phosphotungstic acid solution (pH 5.8). Virus particles were observed under an H-7650
122 TEM (Hitachi, Tokyo, Japan) at 100 kV. For ultrathin sections, ISE6 cells were pelleted

123 by centrifugation at 190 g for 10 min at 4°C, and fixed with 2.5% glutaraldehyde in 0.1
124 M cacodylate buffer (pH 7.4) for 30 minutes. The fixed pellet was washed with
125 cacodylate buffer, postfixed with 2% osmium tetroxide in the cacodylate buffer for 1
126 hour at 4°C, dehydrated with a series of ethanol gradients followed by propylene oxide,
127 embedded in Epon 812 Resin mixture (TAAB Laboratories Equipment Ltd.,
128 Aldermaston, UK), and polymerized at 60°C for 2 days. Ultrathin sections (70 nm) were
129 stained with EM stainer (Nissin EM, Tokyo, Japan) and lead citrate and examined with
130 an H-7650 TEM at 80 kV.

131 **High-throughput RNA sequencing.**

132 RNA extracted from the pellet using the QIAamp Viral RNA Mini Kit
133 (Qiagen, Hilden, Germany) was subjected to double-stranded complementary DNA
134 (cDNA) synthesis using the cDNA Synthesis Kit (M-MLV Version; TaKaRa Bio Inc.,
135 Shiga, Japan). The double-stranded cDNA was sequenced using the Illumina MiSeq v3
136 system (Illumina, San Diego, CA, USA) with paired-end reads (2 × 300 nucleotides
137 (nt)) following the manufacturer's protocol. The Illumina sequencing data were
138 deposited in EMBL/GenBank/DDBJ under the accession number DRA003818.

139 **PCR and Sanger sequencing.**

140 To examine the existence of putative viral sequences in the ISE6 cells and
141 the culture supernatant, conventional PCR was performed on the cDNA generated from
142 the total RNA extracted from both ISE6 cells and the culture supernatant. As a negative
143 control, total RNA was extracted from the culture medium prior to use in maintenance
144 of ISE6 cells. PCR was also performed using genomic DNA (gDNA) extracted from
145 ISE6 cells to investigate whether the viral sequences are incorporated into the tick
146 genome. Total RNA was extracted from the ISE6 cells and the culture
147 supernatant/medium using, respectively, the NucleoSpin RNA II (TaKaRa Bio Inc.) and
148 the QIAamp Viral RNA Mini Kit. The RNA was treated with DNase I (New England
149 Biolabs, Ipswich, MA, USA) and subjected to cDNA synthesis using the PrimeScript II
150 1st strand cDNA Synthesis Kit (TaKaRa Bio Inc.). gDNA was extracted from ISE6 cells
151 using the NucleoSpin Tissue kit (TaKaRa Bio Inc.). The PCR primers used in this study
152 are listed in Table 1. All PCR reactions were performed in a total volume of 25 μ L
153 containing 12.5 μ L of 2 \times Gflex PCR Buffer (TaKaRa Bio Inc.), 0.5 μ L of each primer
154 (10 μ M), 0.5 μ L of Tks Gflex DNA Polymerase (TaKaRa Bio Inc.) and 1.0 μ L of

155 template cDNA or gDNA. Sanger sequencing of the amplified products was performed
156 using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems,
157 Foster City, CA, USA) and an ABI Prism 3130x genetic analyser (Applied Biosystems).

158 **Data analysis.**

159 After trimming low-quality reads and PhiX control reads, the resulting reads
160 were *de novo* assembled using CLC Genomics Workbench (version 4.9). The contigs
161 with high coverage ($> \times 100$) were initially screened for homology to known viruses by
162 local BLASTn and tBLASTx searches against the NCBI Reference Sequence (RefSeq)
163 database (<http://www.ncbi.nlm.nih.gov/>) (e-value $< 10^{-4}$). The potential viral sequences
164 were then compared with the draft genome sequence of *I. scapularis* (GenBank no.
165 NZ_ABJB010000000) to filter out sequences possibly derived from the tick genome. In
166 the search for sequences of M and S segments of a novel bunyavirus, a custom database
167 comprising the sequences of all known Bunyaviridae deposited in GenBank was
168 employed.

169 **Multiple sequence alignment and phylogenetic analysis.**

170 The nucleotide sequences were aligned together with representative sequences

171 of other known relevant viruses available in GenBank using MUSCLE as implemented
172 in MEGA (version 6) (Tamura et al., 2013). After manual modification of the
173 alignments, phylogenetic trees were constructed using the neighbour-joining method
174 with 1,000 bootstrap replicates, using MEGA.

175

176 **Results**

177 **Detection of putative viral sequences.**

178 Out of 5,316,590 raw sequence reads (average length = 289.6 nt) generated
179 from Miseq, 4,873,495 (average length = 238.2 nt) were used for *de novo* assembly
180 after trimming low-quality reads/bases and PhiX control reads. The number of contigs
181 generated with high coverage ($> \times 100$) was 84. A total of 19 contigs showed similarity
182 with the reference sequences of the virus database by tBLASTx search. After
183 comparison with the *I. scapularis* genome, four contigs were initially determined as
184 virus sequences (Table 2). These four contigs were not annotated by BLASTn search,
185 indicating that nucleotide homologies with viruses deposited in the RefSeq database are
186 low.

187 **TEM.**

188 TEM analysis showed the existence of spherical virions with diameters
189 ranging from 50 to 70 nm in the culture supernatant of ISE6 cells. These virions had
190 spikes on their surface and apparently lacked an envelope (Figures 1A and 1B). It was
191 difficult to discriminate between virions by their size and surface structure observed by
192 TEM. No similar structures were observed in the culture medium (Figures 1C and 1D).
193 In ultrathin sections of ISE6 cells, numerous spherical virion-like structures were
194 observed in the cytoplasm (Figures 1E and 1F).

195 **Iflavirus.**

196 Approximately 20% of the reads were mapped to contig 1, which was
197 predicted to be a member of the Iflavirus genus by tBLASTx search (Table 2). This
198 contig is composed of 9,252 nt, excluding the 3'-terminal poly(A), and contains a single
199 open reading frame (ORF) encoding a polyprotein of 2,991 amino acids (aa) following
200 an incomplete internal ribosome entry site. Six conserved domains associated with viral
201 RNA replication were identified by InterProScan (Jones et al., 2014): three picornavirus
202 capsid-like domains at aa positions 405–620, 698–903 and 1,071–1,288, a RNA

203 helicase domain at aa positions 1,650–1,820, a peptidase domain at aa positions 2,223–
204 2,423 and an RNA-dependent RNA polymerase (RdRp) domain at aa positions 2,471–
205 2,956 (Figure 2A), which are well conserved throughout the genus Iflavirus. The
206 phylogenetic analysis also grouped this novel virus, tentatively designated *Ixodes*
207 *scapularis* iflavirus (ISIV), into the genus Iflavirus (Figure 3). Because the aa sequence
208 identity between the capsid proteins of ISIV and the other iflaviruses is less than 90%
209 (data not shown), ISIV would constitute a novel species in the genus Iflavirus following
210 the criteria described elsewhere (Chen et al., 2012).

211 **Bunyavirus.**

212 Contig 2 (9,147 nt) was assigned as a putative viral sequence related to the L
213 segment of a bunyavirus (Table 2). Contig 2 encodes a single ORF (2,978 aa), which
214 comprises a bunyavirus RdRp domain at aa positions 880–1,636 (Figure 2B). This
215 putative viral sequence was clustered together with an unclassified virus, Wenzhou
216 Shrimp Virus 2 (LC094424) which was recognised in RNA deep sequencing of shrimps
217 (*Penaeus monodon* and *Exopalaemon carinicauda*) (Li et al., 2015) in a phylogenetic
218 tree (Figure 4A). To detect the M and S segments of the virus tentatively designated

219 *Ixodes scapularis* bunyavirus (ISBV), all the contigs (n = 3,075), including those with
220 low coverage, were compared with all the sequences of known Bunyaviridae deposited
221 in GenBank. This successfully recovered the M segment (contig 5 (coverage = ×93)),
222 while the S segment was not detected. Contig 5 is 4,321 nt in length, encoding a single
223 coding sequence of the putative glycoprotein precursor (1,350 aa) (Figure 2B), which is
224 also grouped together with Wenzhou Shrimp Virus 2 in phylogenetic analysis (Figure
225 4B).

226 ***Ixodes scapularis*-associated virus-1 (ISAV-1).**

227 Contig 3 was initially associated with turnip rosette virus (NC_004553.3) in
228 the genus Sobemovirus by tBLASTx search against the RefSeq database. A further
229 similarity search against all sequences deposited in GenBank revealed that this sequence
230 has the highest similarity (98% nucleotide identity) with the partial genome sequence of
231 ISAV-1 isolate K13 (KM048318) in the same genus, which was recovered from
232 field-captured *I. scapularis* through a metagenomic study (Tokarz et al., 2014). The
233 contig sequence encodes a putative protease (540 aa) and RdRp (313 aa), both of which
234 showed the highest similarities (97%) with those encoded by ISAV-1.

235 **Unclassified virus.**

236 We identified that contig 4 of 1,485 nt encodes a putative viral capsid protein
237 (411 aa) with similarity to an unclassified positive-strand RNA virus, Drosophila A
238 virus (DAV). We could not find other fragments of this virus among all the contigs,
239 including those with low coverage ($< \times 100$).

240 **PCR.**

241 PCR was performed on the cDNA generated from the total RNA extracted
242 from ISE6 cells, the culture supernatant, and the culture medium and gDNA extracted
243 from ISE6 cells. All the putative viral sequences were detected in both ISE6 cells and
244 the culture supernatant but not in the culture medium (Figure 5). When gDNA was
245 included as a template, no amplification was observed except for the reaction with
246 primers amplifying tick mitochondrial 16S rRNA gene (rDNA). Sanger sequencing of
247 the PCR products confirmed the specific amplification of each target (data not shown).

248

249

Discussion

250 The present study demonstrated that at least five distinct putative viral

251 sequences existed in a tick cell line, ISE6. In comparison with bacterial detection, where
252 universal primers are available to amplify variable regions of bacterial 16S rDNA, a
253 search for previously uncharacterised viruses has been hampered by the lack of proper
254 detection tools, and little is known about viruses infecting ticks and tick cell lines
255 (Alberdi et al., 2012; Attoui et al., 2001). The use of high-throughput sequencing
256 technology sheds light on these poorly characterised viruses.

257 To our surprise, we recovered the sequences of L and M segments of one or
258 more putative viruses of the family Bunyaviridae from the culture supernatant of ISE6
259 cells (Table 2). Several members of this virus family have been recognised as tick-borne
260 viruses of medical and veterinary importance. The family is currently divided into five
261 genera (Orthobunyavirus, Hantavirus, Nairovirus, Phlebovirus and Tospovirus) based
262 on antigenic, structural and genetic characteristics (Plyusnin et al., 2012). As evidenced
263 by the finding of Gouléako virus in mosquitoes in Côte d'Ivoire (Marklewitz et al.,
264 2011), bunyaviruses that cannot be classified into any of the five known genera have
265 also come to attention. Recent metagenomic studies of arthropod-borne viruses led to
266 the discovery of many such unclassified bunyaviruses (Ballinger et al., 2014; Webster et

267 al., 2015). Based on the sequences found, the closest relative of ISBV is Wenzhou
268 Shrimp Virus 2, which is one of the unclassified bunyaviruses found in a metagenomic
269 study of Chinese shrimps (Li et al., 2015), and thus its phylogenetic position is not
270 clearly understood. Despite an intensive search using a custom database comprising the
271 sequences of all known Bunyaviridae deposited in GenBank, we could not find the
272 sequence of the potential S segment of ISBV. Similarly, Li et al. (2015) failed to detect
273 the sequence of putative S segment of Wenzhou Shrimp Virus 2 in the deep sequencing
274 data.

275 The predominant putative virus found in ISE6 cells was a member of the
276 family Iflaviridae, which is an arthropod-infecting virus group with a single-stranded
277 RNA genome. Along with the advancement of next-generation sequencing techniques,
278 an increasing number of iflaviruses are being reported in various arthropod species even
279 in laboratory colonies (Liu et al., 2015); however, there is no report to date on the
280 detection of this virus group from ticks. Some iflaviruses have occasionally been
281 associated with adverse effects on the survival of arthropod hosts (Martin et al., 2012;
282 Silva et al., 2015; Valles and Hashimoto 2009). For example, deformed wing virus,

283 which is an iflavirus commonly infecting the European honeybee (*Apis mellifera*)
284 asymptotomatically with low viral loads (de Miranda and Genersch, 2010), causes
285 morphological deformity and reduces the life span with elevated virus loads when
286 transmitted by the ectoparasitic mite *Varroa destructor* (de Miranda and Fries, 2008;
287 Dainat et al., 2012). Further studies are required to investigate the role of ISIV *in vivo*
288 and to assess the prevalence of ISIV and its related viruses in natural populations of
289 ticks.

290 In addition to the previously unrecognised viruses, ISE6 was revealed to
291 harbour partial sequence of the putative virus ISAV-1, which was recently discovered in
292 *I. scapularis* collected at Heckscher State Park in the United States (Suffolk County,
293 NY) (Tokarz et al., 2014), where the authors identified a partial genome sequence (2.8
294 kb) of ISAV-1 encompassing the majority of a putative protease and an RdRp without
295 information on its capsid protein. A recent metagenomic study reported the detection of
296 a virus closely related to ISAV-1, designated *Humaita-Tubiacanga* virus (HTV), in a
297 laboratory colony of the mosquito *Aedes aegypti* (Aguiar et al., 2015). The authors
298 detected a sequence corresponding to the RdRp of HTV together with one viral segment

299 (1,609 nt) predicted to encode a protein with a coat domain, with the highest similarity
300 to a capsid protein of DAV. As supported by further evidence of small RNA profiles and
301 simultaneous detection by RT-PCR in individual mosquitoes, the authors proposed that
302 the capsid protein related to DAV is associated with the RdRp of HTV. Likewise, we
303 detected one virus sequence encoding a putative viral capsid protein with the highest
304 similarity to DAV in ISE6 cells, while a sequence corresponding to the RdRp of DAV
305 was absent. These facts collectively support the possible association of ISAV-1 with a
306 capsid protein related to DAV.

307 An initial scan for potential virus sequences detected 19 contigs. Because the
308 rest of the virus-like sequences showed high similarity with draft genome sequences of *I.*
309 *scapularis*, we suspected that they are integrated in the genome of *I. scapularis* and thus
310 did not include them in the further analysis. It is, however, possible that some of them
311 were derived from endogenous viruses infecting the *I. scapularis* Wikel strain used in
312 the whole genome sequencing project (Gulia-Nuss et al., 2016). As reported elsewhere,
313 some arthropods have acquired a number of virus-like sequences in their genomes
314 during evolution (Ballinger et al., 2014; Katzourakis and Gifford, 2010; Liu et al., 2010).

315 It is therefore important to perform paleovirological analysis of arthropods with a
316 process of *in silico* identification of endogenous virus elements incorporated into the
317 host genome.

318 Considering the high abundance of viral sequences associated with ISIV in
319 the sequencing data, the majority of the virions observed in the culture supernatant by
320 TEM might be expected to be ISIV (Figure 1). However, the morphological features of
321 the particles were different from the virion properties of the genus I flavivirus, which are
322 approximately 30 nm in diameter with no distinctive surface structures (Chen et al.,
323 2012). Since ISAV-1 was only reported from a metagenomic study (Tokarz et al., 2014),
324 the morphological features of ISAV-1 are completely unknown. The size of the only
325 characterised tick virus, SCRV, is approximately 55 nm in diameter (Attoui et al., 2001),
326 which is comparable to that of the virions observed in this study (between 50 and 70 nm
327 in diameter). It is unfortunate that we could not differentiate the virions with a varying
328 diameter due to the similarity of their surface structures and the resolution limits of
329 TEM. Further studies using immune-electron microscopy are required to determine the
330 morphological structure of each virion in ISE6 cells.

331 The role of viruses in tick cells is totally unknown. There is some evidence in
332 mosquito cell lines that some persistently infecting viruses, for example C6/36
333 densovirus (Parvoviridae), cell-fusing agent virus (Flaviviridae) and *Aedes*
334 *pseudoscutellaris* reovirus (Reoviridae), modulate exogenous viruses' replication and
335 vice versa, possibly in part through regulation of host antiviral immune responses
336 (Morazzani et al., 2012). Hence, the experimental outcomes using ISE6 cells, which
337 might contain several putative endogenous viruses, should be carefully considered.
338 Because we characterised the putative viral sequences in ISE6 cells, it becomes possible
339 to determine the genome sequences of the viruses isolated in ISE6 cells by removing the
340 sequencing reads from putative viruses by *in silico* analysis. In fact, we have determined
341 whole genomes of previously uncharacterized bunyaviruses, which can be propagate in
342 ISE6 cells but not in mammalian cell lines (manuscript in preparation). It might be
343 worth trying to generate virus-free cell lines by interfering with replication of putative
344 viruses in ISE6 cells based on siRNA silencing technology (Bell-Sakyi and Attoui,
345 2013).

346 In conclusion, the present study reports five putative viral sequences from

347 four phylogenetically different virus lineages in ISE6 cells. Unfortunately, since we
348 could not test the presence of these putative viral sequences in ISE6 cells maintained in
349 other laboratories, we cannot rule out the possibility that the ISE6 cells used in this
350 study had been contaminated with the putative viruses during passage in our laboratory
351 or the provider's laboratory. Considering the increasing importance of tick cell lines in a
352 wide range of research on ticks and tick-borne pathogens, a comprehensive search for
353 tick-associated viruses not only in ISE6 cells but also in other tick cell lines is required.
354 Expanding knowledge of the diversity of tick-borne pathogens as well as tick-associated
355 viruses may lead to better understanding of the tripartite interaction between pathogenic
356 microorganisms, non-pathogenic microorganisms and ticks.

357

358

Acknowledgements

359 We acknowledge Dr. Ulrike Mundrloh of the University of Minnesota for kindly giving
360 permission to use ISE6 cells. This work was supported by JSPS KAKENHI
361 Grant-in-Aid for Young Scientists (B) (25850195) and (A) (15H05633), and for
362 Scientific Research on Innovative Areas (16H06431) and Japan Science and

363 Technology Agency (JST)/Japan International Cooperation Agency (JICA) within the
364 framework of the Science and Technology Research Partnership for Sustainable
365 Development (SATREPS).

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493

494 **Legends to Figures and Tables**

495 **Figure 1. Electron micrographs of virions observed in the supernatant and**
496 **ultrathin section of ISE6 cells.**

497 (A) Virions purified from the culture supernatant of ISE6 cells, (B) enlarged image of a
498 virion, (C) cell culture medium only, and (D) enlarged image of culture medium only
499 were observed by an H-7650 transmission electron microscope (Hitachi, Tokyo, Japan)
500 at 100 kV. (E) Ultrathin section of an ISE6 cell and (F) enlarged image of virion-like
501 structures in the ISE6 cell. The arrowheads indicate the virions.

502

503 **Figure 2. Predicted genome organisations of *Ixodes scapularis* iflavirus (A) and**
504 ***Ixodes scapularis* bunyavirus (B).**

505 *De novo* assembled contigs were subjected to an ORF search, and the putative amino
506 acid sequences were used for a conserved domain search with InterProScan. Each
507 domain is indicated by a grey bar, and the name is shown beneath the bar.

508

509 **Figure 3. Phylogenetic tree of the genus Iflavirus.**

510 The full-length sequence of the *Ixodes scapularis* iflavirus (contig 1) (shown
511 underlined) was used for phylogenetic analysis with the other iflaviruses. *Drosophila* C
512 virus in the family Dicistroviridae was deployed as an outgroup. Bootstrap probabilities
513 higher than 70% are indicated near the branches. The scale bar indicates 0.5 changes per
514 nucleotide position.

515

516 **Figure 4. Phylogenetic trees of the family Bunyaviridae.**

517 The full-length sequences of the *Ixodes scapularis* bunyavirus (contigs 2 and 5) (shown

518 underlined) were used for phylogenetic analyses with the other bunyavirus L (A) and M
519 segments (B), respectively. Bootstrap probabilities higher than 70% are indicated near
520 the branches. The scale bars indicate 0.5 changes per nucleotide position.

521

522 **Figure 5. PCR amplification of each putative viral sequence and tick**
523 **mitochondrial 16S rRNA gene.**

524 PCR was performed on the cDNA generated from the total RNA extracted from ISE6
525 cells (1), the culture supernatant (2), and the culture medium (3) and gDNA extracted
526 from ISE6 cells (4). ISIV, ISB_L, ISB_M, ISAV-1, ISUV, and 16S rDNA represent PCR
527 reactions with primers amplifying *Ixodes scapularis* iflavivirus, L segment of *Ixodes*
528 *scapularis* bunyavirus, M segment of *Ixodes scapularis* bunyavirus, *Ixodes scapularis*
529 associated virus-1, unclassified virus related to Drosophila A virus, and tick
530 mitochondrial 16S rRNA gene (rDNA), respectively. All the five putative viral
531 sequences were detected in both ISE6 cells and the culture supernatant while only tick
532 mitochondrial 16S rDNA was amplified from gDNA. Lane M shows a 100-bp DNA
533 marker.

534

535 **Table 1. Oligonucleotide primers used for PCR amplification of each putative viral**
536 **sequence and tick mitochondrial 16S rRNA gene sequence.**

537

538 **Table 2. Summary of putative partial viral genome sequences identified in ISE6**
539 **cells.**

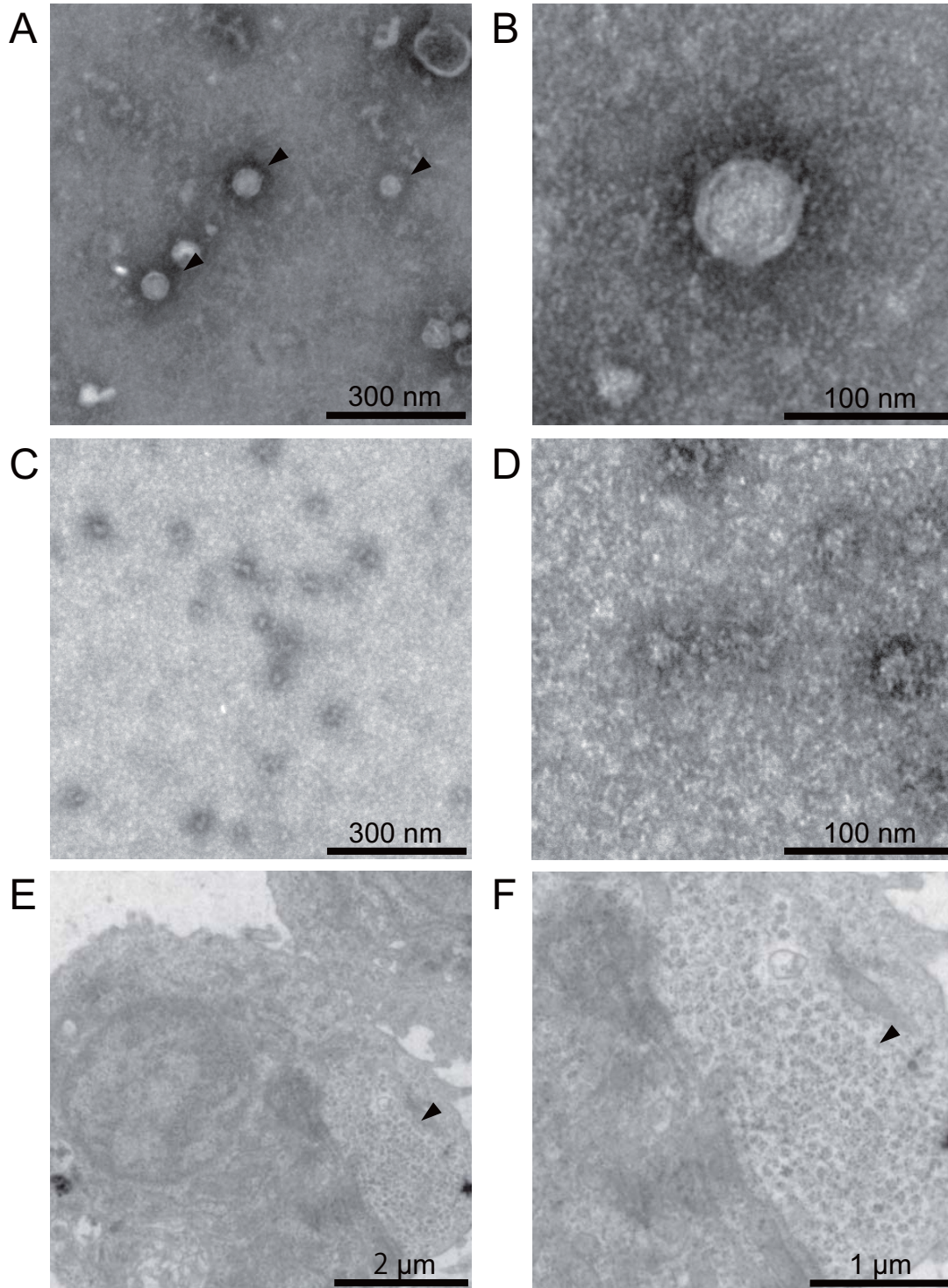
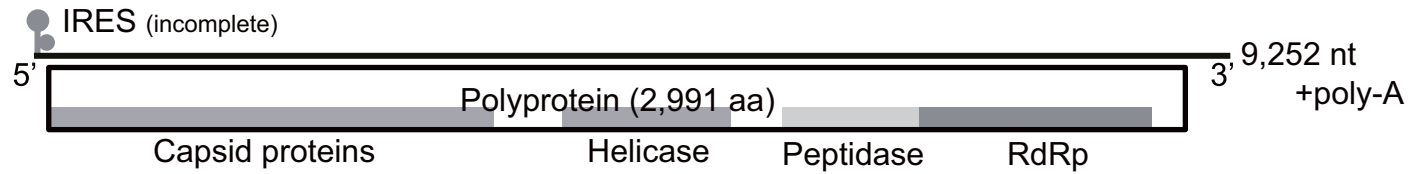


Fig. 1

A

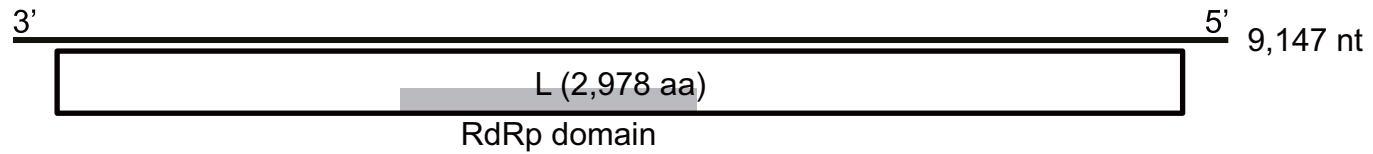
Ixodes scapularis iflavivirus



B

Ixodes scapularis bunyavirus

L segment



M segment

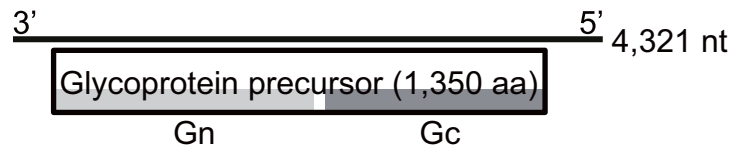


Fig. 2

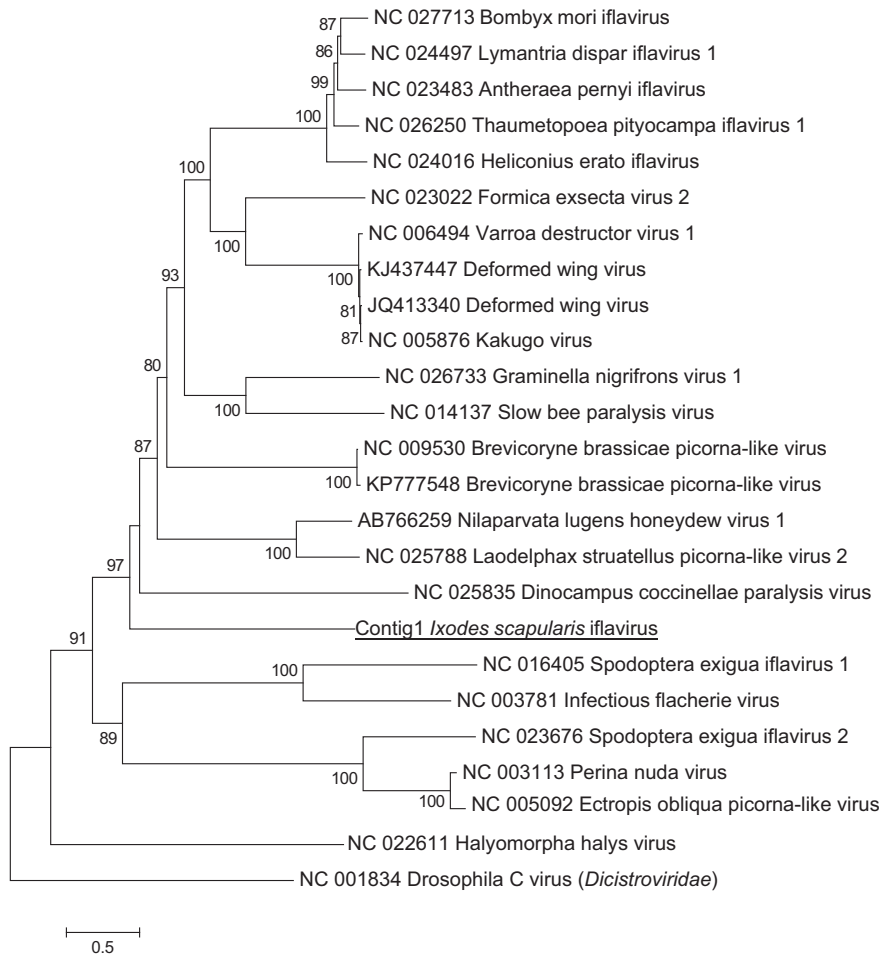


Fig. 3

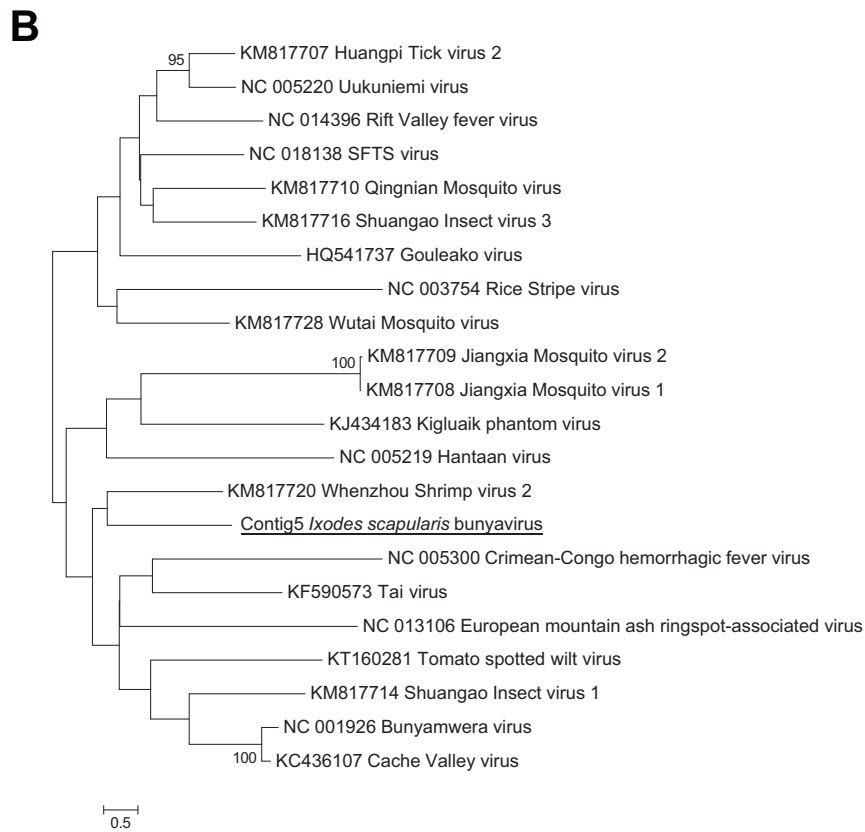
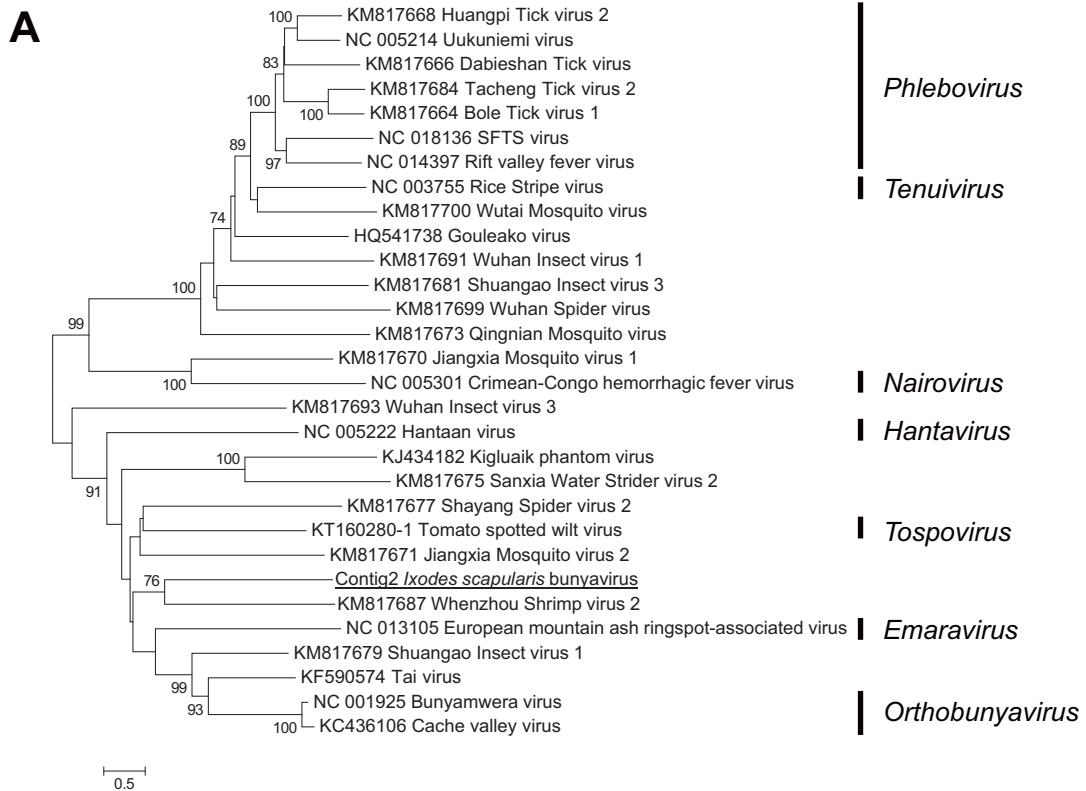


Fig. 4

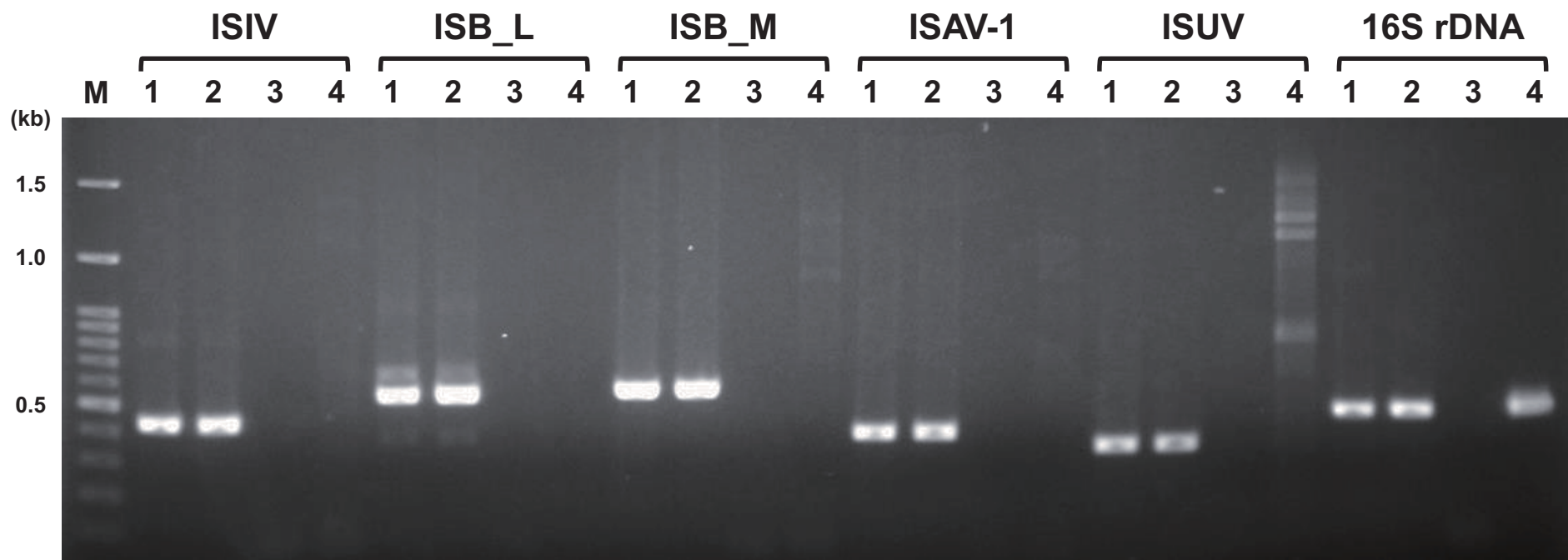


Fig. 5

Table 1. Oligonucleotide primers used for PCR amplification of each putative viral sequence and tick mitochondrial 16S rRNA gene sequence.

Primer name	Sequence (5' to 3')	Target	Amplicon size (bp)	Reference
ISIV_F	GGGGCGTGGTAAATGTGGTA	<i>Ixodes scapularis</i> iflavirus	437	This study
ISIV_R	ACACTCCGCGATGAAAGGTT			
ISB_L_F	GCATTGTGTCCAGCTCTTCA	<i>Ixodes scapularis</i> bunyavirus, L segment	547	This study
ISB_L_R	GTCTCCAGAAACAGGCTTGC			
ISB_M_F	CTGGCACAATCCTTTTGGTT	<i>Ixodes scapularis</i> bunyavirus, M segment	571	This study
ISB_M_R	TTCTCTCTCAGCCCTGGTGT			
ISAV-1_F	AACGTGTACGGAATGCACCT	<i>Ixodes scapularis</i> associated virus-1, RdRP	383	This study
ISAV-1_R	GTTTGGCCAGAACTTGGCAG			
ISUV_F	GACCACGGACACCAGTTTCT	Unclassified virus, capsid precursor	338	This study
ISUV_R	GGTCAGACAGATAGGTCGCC			
16S+1	CTGCTCAATGATTTTTTAAATTGCTGTGG	Tick mitochondrial 16S rRNA gene	460	Black and Piesman, 1994
16S-1	CCGGTCTGAACTCAGATCAAGTA			

Table 2. Summary of putative partial viral genome sequences identified in ISE6 cells.

Contig ID	Length (bp)	Average coverage	Virus family	Provisional virus name	Closest relative (aa identity)	Accession no.
1	9,252 ^a	24,785	<i>Iflaviridae</i>	<i>Ixodes scapularis</i> iflavirus	<i>Formica exsecta</i> virus 2 (25%)	LC094426
2	9,147	172	<i>Bunyaviridae</i>	<i>Ixodes scapularis</i> bunyavirus, L segment	Wenzhou Shrimp Virus 2 (25%)	LC094424
3	2,686	934	Unassigned	<i>Ixodes scapularis</i> -associated virus-1, peptidase and RdRp	<i>Ixodes scapularis</i> -associated virus-1 (98%)	LC094964
4	1,485	378	Unassigned	Unclassified virus, capsid precursor	Drosophila A virus (36%)	LC094965
5	4,321	93	<i>Bunyaviridae</i>	<i>Ixodes scapularis</i> bunyavirus, M segment	Wenzhou Shrimp Virus 2 (25%)	LC094425

^aContig size without polyA.