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

Ryo Nakao^{1,2}, Keita Matsuno^{3,4}, Yongjin Qiu⁵, Junki Maruyama⁶, Nao Eguchi⁶, Naganori
Nao⁶, Masahiro Kajihara⁶, Kentaro Yoshii⁷, Hirofumi Sawa^{4,8,9,10}, Ayato Takada^{4,6,9},
Chihiro Sugimoto^{4,5,9}

¹Unit of Risk Analysis and Management, Hokkaido University Research Center for
Zoonosis Control, Sapporo, Japan

²Laboratory of Parasitology, Department of Disease Control, Graduate School of
Veterinary Medicine, Hokkaido University, Sapporo, Japan

³Laboratory of Microbiology, Department of Disease Control, Graduate School of
Veterinary Medicine, Hokkaido University, Sapporo, Japan

⁴Global Institution for Collaborative Research and Education, Hokkaido University,
Sapporo, Japan

⁵Division of Collaboration and Education, Hokkaido University Research Center for
Zoonosis Control, Sapporo, Japan

- 17 ⁶Division of Global Epidemiology, Hokkaido University Research Center for Zoonosis
18 Control, Sapporo, Japan
- 19 ⁷Laboratory of Public Health, Department of Disease Control, Graduate School of
20 Veterinary Medicine, Hokkaido University, Sapporo, Japan
- 21 ⁸Division of Molecular Pathobiology, Hokkaido University Research Center for Zoonosis
22 Control, Sapporo, Japan
- 23 ⁹Department of Disease Control, School of Veterinary Medicine, University of Zambia,
24 Lusaka, Zambia
- 25 ¹⁰Global Virus Network, Baltimore, USA
- 26

Abstract

Ticks harbour various microorganisms, some of which act as pathogens of humans and animals. The recent advancement of genome sequencing technologies revealed that a wide range of previously unrecognised microorganisms exist in ticks. Continuous cell lines established from ticks could play a key role in the isolation of such microorganisms; however, tick cells themselves have been known to harbour symbiotic microorganisms. The present study aimed to characterise putative RNA viral sequences detected in the culture supernatant of one of the most frequently used tick cell lines, ISE6, which was derived from embryos of the blacklegged tick *Ixodes scapularis*. Viral particles purified from the culture supernatant were used for RNA extraction, followed by Illumina sequencing. The reads were *de novo* assembled and the resulting contigs were annotated by tBLASTx search. The results suggested that there were at least five putative viral sequences of four phylogenetically distinct lineages in ISE6 cells. The predominant viral sequence found in ISE6 cells, designated *I. scapularis* iflavivirus, was a member of the family Iflaviridae, which is an arthropod-infecting virus group. We also identified L and M segments of the family Bunyaviridae, which could not be classified into any of the

five known genera, and a potential capsid protein related to Drosophila A virus. In addition to these previously unrecognised viruses, ISE6 was revealed to harbour a putative genome sequence of *I. scapularis*-associated virus-1, which was reported in a recent metagenomic study of *I. scapularis* itself. All the five putative viral sequences were detected by RT-PCR in both ISE6 cells and the culture supernatant. Electron microscopic analysis showed the existence of spherical virions with a varying diameter of 50 to 70 nm in the culture supernatant of ISE6 cells. Further studies are required to investigate the potential roles of ISE6-associated viruses in ticks.

Keywords

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

Introduction

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

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

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

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

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

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

Materials and Methods

Cell line.

The ISE6 cell line was kindly provided by the CEH Institute of Virology and Environmental Microbiology (Oxford, UK). The use of ISE6 cells was authorized by the University of Minnesota, the original provider of the cell line, under a material transfer agreement between the University of Minnesota and Hokkaido University. The cells were cultured in L-15B medium supplemented with 10% fetal calf serum, 5% tryptose phosphate broth (Sigma–Aldrich, St. Louis, MO, USA) and 0.1% bovine lipoprotein concentrate (MP Biomedicals, Irvine, CA, USA) at 32°C as described by Munderloh and Kurtti (1989).

107 **Virus purification.**

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

114 **TEM.**

115 ISE6 culture supernatant and culture medium (as a negative control) were
116 subjected to low-speed centrifugation followed by ultracentrifugation as described above.

117 The pelleted was fixed with 0.25% glutaraldehyde in PBS for 1 week at 4°C. Fixed
118 samples supplemented with 0.1% bovine serum albumin were adsorbed to
119 collodion-carbon-coated copper grids and negatively stained for 30 s with 2%
120 phosphotungstic acid solution (pH 5.8). Virus particles were observed under an H-7650
121 TEM (Hitachi, Tokyo, Japan) at 100 kV. For ultrathin sections, ISE6 cells were pelleted
122 by centrifugation at 190 g for 10 min at 4°C, and fixed with 2.5% glutaraldehyde in 0.1 M

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

129 **High-throughput RNA sequencing.**

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

137 **PCR and Sanger sequencing.**

138 To examine the existence of putative viral sequences in the ISE6 cells and the

139 culture supernatant, conventional PCR was performed on the cDNA generated from the
140 total RNA extracted from both ISE6 cells and the culture supernatant. As a negative
141 control, total RNA was extracted from the culture medium prior to use in maintenance of
142 ISE6 cells. PCR was also performed using genomic DNA (gDNA) extracted from ISE6
143 cells to investigate whether the viral sequences are incorporated into the tick genome.
144 Total RNA was extracted from the ISE6 cells and the culture supernatant/medium using,
145 respectively, the NucleoSpin RNA II (TaKaRa Bio Inc.) and the QIAamp Viral RNA Mini
146 Kit. The RNA was treated with DNase I (New England Biolabs, Ipswich, MA, USA) and
147 subjected to cDNA synthesis using the PrimeScript II 1st strand cDNA Synthesis Kit
148 (TaKaRa Bio Inc.). gDNA was extracted from ISE6 cells using the NucleoSpin Tissue kit
149 (TaKaRa Bio Inc.). The PCR primers used in this study are listed in Table 1. All PCR
150 reactions were performed in a total volume of 25 μ L containing 12.5 μ L of 2 $\times$ Gflex PCR
151 Buffer (TaKaRa Bio Inc.), 0.5 μ L of each primer (10 μ M), 0.5 μ L of Tks Gflex DNA
152 Polymerase (TaKaRa Bio Inc.) and 1.0 μ L of template cDNA or gDNA. Sanger
153 sequencing of the amplified products was performed using the BigDye Terminator
154 version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an

155 ABI Prism 3130x genetic analyser (Applied Biosystems).

156 **Data analysis.**

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

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

168 The nucleotide sequences were aligned together with representative sequences
169 of other known relevant viruses available in GenBank using MUSCLE as implemented in
170 MEGA (version 6) (Tamura et al., 2013). After manual modification of the alignments,

phylogenetic trees were constructed using the neighbour-joining method with 1,000 bootstrap replicates, using MEGA.

Results

Detection of putative viral sequences.

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

TEM.

TEM analysis showed the existence of spherical virions with diameters ranging from 50 to 70 nm in the culture supernatant of ISE6 cells. These virions had spikes on

187 their surface and apparently lacked an envelope (Figures 1A and 1B). It was difficult to
188 discriminate between virions by their size and surface structure observed by TEM. No
189 similar structures were observed in the culture medium (Figures 1C and 1D). In ultrathin
190 sections of ISE6 cells, numerous spherical virion-like structures were observed in the
191 cytoplasm (Figures 1E and 1F).

192 **Iflavirus.**

193 Approximately 20% of the reads were mapped to contig 1, which was predicted
194 to be a member of the Iflavirus genus by tBLASTx search (Table 2). This contig is
195 composed of 9,252 nt, excluding the 3'-terminal poly(A), and contains a single open
196 reading frame (ORF) encoding a polyprotein of 2,991 amino acids (aa) following an
197 incomplete internal ribosome entry site. Six conserved domains associated with viral
198 RNA replication were identified by InterProScan (Jones et al., 2014): three picornavirus
199 capsid-like domains at aa positions 405–620, 698–903 and 1,071–1,288, a RNA helicase
200 domain at aa positions 1,650–1,820, a peptidase domain at aa positions 2,223–2,423 and
201 an RNA-dependent RNA polymerase (RdRp) domain at aa positions 2,471–2,956 (Figure
202 2A), which are well conserved throughout the genus Iflavirus. The phylogenetic analysis

203 also grouped this novel virus, tentatively designated *Ixodes scapularis* iflavirus (ISIV),
204 into the genus Iflavirus (Figure 3). Because the aa sequence identity between the capsid
205 proteins of ISIV and the other iflaviruses is less than 90% (data not shown), ISIV would
206 constitute a novel species in the genus Iflavirus following the criteria described elsewhere
207 (Chen et al., 2012).

208 **Bunyavirus.**

209 Contig 2 (9,147 nt) was assigned as a putative viral sequence related to the L
210 segment of a bunyavirus (Table 2). Contig 2 encodes a single ORF (2,978 aa), which
211 comprises a bunyavirus RdRp domain at aa positions 880–1,636 (Figure 2B). This
212 putative viral sequence was clustered together with an unclassified virus, Wenzhou
213 Shrimp Virus 2 (LC094424) which was recognised in RNA deep sequencing of shrimps
214 (*Penaeus monodon* and *Exopalaemon carinicauda*) (Li et al., 2015) in a phylogenetic tree
215 (Figure 4A). To detect the M and S segments of the virus tentatively designated *Ixodes*
216 *scapularis* bunyavirus (ISBV), all the contigs (n = 3,075), including those with low
217 coverage, were compared with all the sequences of known Bunyaviridae deposited in
218 GenBank. This successfully recovered the M segment (contig 5 (coverage = ×93)), while

the S segment was not detected. Contig 5 is 4,321 nt in length, encoding a single coding sequence of the putative glycoprotein precursor (1,350 aa) (Figure 2B), which is also grouped together with Wenzhou Shrimp Virus 2 in phylogenetic analysis (Figure 4B).

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

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

Unclassified virus.

We identified that contig 4 of 1,485 nt encodes a putative viral capsid protein (411 aa) with similarity to an unclassified positive-strand RNA virus, *Drosophila A virus* (DAV). We could not find other fragments of this virus among all the contigs, including

235 those with low coverage ($< \times 100$).

236 **PCR.**

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

244

245 **Discussion**

246 The present study demonstrated that at least five distinct putative viral
247 sequences existed in a tick cell line, ISE6. In comparison with bacterial detection, where
248 universal primers are available to amplify variable regions of bacterial 16S rDNA, a
249 search for previously uncharacterised viruses has been hampered by the lack of proper
250 detection tools, and little is known about viruses infecting ticks and tick cell lines

(Alberdi et al., 2012; Attoui et al., 2001). The use of high-throughput sequencing technology sheds light on these poorly characterised viruses.

To our surprise, we recovered the sequences of L and M segments of one or more putative viruses of the family Bunyaviridae from the culture supernatant of ISE6 cells (Table 2). Several members of this virus family have been recognised as tick-borne viruses of medical and veterinary importance. The family is currently divided into five genera (Orthobunyavirus, Hantavirus, Nairovirus, Phlebovirus and Tospovirus) based on antigenic, structural and genetic characteristics (Plyusnin et al., 2012). As evidenced by the finding of Gouléako virus in mosquitoes in Côte d'Ivoire (Marklewitz et al., 2011), bunyaviruses that cannot be classified into any of the five known genera have also come to attention. Recent metagenomic studies of arthropod-borne viruses led to the discovery of many such unclassified bunyaviruses (Ballinger et al., 2014; Webster et al., 2015). Based on the sequences found, the closest relative of ISBV is Wenzhou Shrimp Virus 2, which is one of the unclassified bunyaviruses found in a metagenomic study of Chinese shrimps (Li et al., 2015), and thus its phylogenetic position is not clearly understood. Despite an intensive search using a custom database comprising the sequences of all

known Bunyaviridae deposited in GenBank, we could not find the sequence of the potential S segment of ISBV. Similarly, Li et al. (2015) failed to detect the sequence of putative S segment of Wenzhou Shrimp Virus 2 in the deep sequencing data.

The predominant putative virus found in ISE6 cells was a member of the family Iflaviridae, which is an arthropod-infecting virus group with a single-stranded RNA genome. Along with the advancement of next-generation sequencing techniques, an increasing number of iflaviruses are being reported in various arthropod species even in laboratory colonies (Liu et al., 2015); however, there is no report to date on the detection of this virus group from ticks. Some iflaviruses have occasionally been associated with adverse effects on the survival of arthropod hosts (Martin et al., 2012; Silva et al., 2015; Valles and Hashimoto 2009). For example, deformed wing virus, which is an iflavirus commonly infecting the European honeybee (*Apis mellifera*) asymptotically with low viral loads (de Miranda and Genersch, 2010), causes morphological deformity and reduces the life span with elevated virus loads when transmitted by the ectoparasitic mite *Varroa destructor* (de Miranda and Fries, 2008; Dainat et al., 2012). Further studies are required to investigate the role of ISIV *in vivo* and to assess the prevalence of ISIV and its

283 related viruses in natural populations of ticks.

284 In addition to the previously unrecognised viruses, ISE6 was revealed to
285 harbour **partial sequence of the** putative **virus** ISAV-1, which was recently discovered **in** *I.*
286 *scapularis* collected at Heckscher State Park in the United States (Suffolk County, NY)
287 (Tokarz et al., 2014), where the authors identified a partial genome sequence (2.8 kb) of
288 ISAV-1 encompassing the majority of a putative protease and an RdRp without
289 information on its capsid protein. A recent metagenomic study reported the detection of a
290 virus closely related to ISAV-1, designated *Humaita-Tubiacanga* virus (HTV), in a
291 laboratory colony of the mosquito *Aedes aegypti* (Aguiar et al., 2015). The authors
292 detected a sequence corresponding to the RdRp of HTV together with one viral segment
293 (1,609 nt) predicted to encode a protein with a coat domain, with the highest similarity to
294 a capsid protein of DAV. As supported by further evidence of small RNA profiles and
295 simultaneous detection by RT-PCR in individual mosquitoes, the authors proposed that
296 the capsid protein related to DAV is associated with the RdRp of HTV. Likewise, we
297 detected one virus sequence encoding a putative viral capsid protein with the highest
298 similarity to DAV in ISE6 cells, while a sequence corresponding to the RdRp of DAV was

absent. These facts collectively support the possible association of ISAV-1 with a capsid protein related to DAV.

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

Considering the high abundance of viral sequences associated with ISIV in the sequencing data, the majority of the virions observed in the culture supernatant by TEM might be expected to be ISIV (Figure 1). However, the morphological features of the particles were different from the virion properties of the genus Iflavirus, which are

315 approximately 30 nm in diameter with no distinctive surface structures (Chen et al., 2012).
316 Since ISAV-1 was only reported from a metagenomic study (Tokarz et al., 2014), the
317 morphological features of ISAV-1 are completely unknown. The size of the only
318 characterised tick virus, SCRNV, is approximately 55 nm in diameter (Attoui et al., 2001),
319 which is comparable to that of the virions observed in this study (between 50 and 70 nm
320 in diameter). It is unfortunate that we could not differentiate the virions with a varying
321 diameter due to the similarity of their surface structures and the resolution limits of TEM.
322 Further studies using immune-electron microscopy are required to determine the
323 morphological structure of each virion in ISE6 cells.

324 The role of viruses in tick cells is totally unknown. There is some evidence in
325 mosquito cell lines that some persistently infecting viruses, for example C6/36
326 densovirus (Parvoviridae), cell-fusing agent virus (Flaviviridae) and *Aedes*
327 *pseudoscutellaris* reovirus (Reoviridae), modulate exogenous viruses' replication and
328 vice versa, possibly in part through regulation of host antiviral immune responses
329 (Morazzani et al., 2012). Hence, the experimental outcomes using ISE6 cells, which
330 might contain several putative endogenous viruses, should be carefully considered.

331 Because we characterised the putative viral sequences in ISE6 cells, it becomes possible
332 to determine the genome sequences of the viruses isolated in ISE6 cells by removing the
333 sequencing reads from putative viruses by *in silico* analysis. In fact, we have determined
334 whole genomes of previously uncharacterized bunyaviruses, which can be propagate in
335 ISE6 cells but not in mammalian cell lines (manuscript in preparation). It might be worth
336 trying to generate virus-free cell lines by interfering with replication of putative viruses in
337 ISE6 cells based on siRNA silencing technology (Bell-Sakyi and Attoui, 2013).

338 In conclusion, the present study reports five putative viral sequences from four
339 phylogenetically different virus lineages in ISE6 cells. Unfortunately, since we could not
340 test the presence of these putative viral sequences in ISE6 cells maintained in other
341 laboratories, we cannot rule out the possibility that the ISE6 cells used in this study had
342 been contaminated with the putative viruses during passage in our laboratory or the
343 provider's laboratory. Considering the increasing importance of tick cell lines in a wide
344 range of research on ticks and tick-borne pathogens, a comprehensive search for
345 tick-associated viruses not only in ISE6 cells but also in other tick cell lines is required.
346 Expanding knowledge of the diversity of tick-borne pathogens as well as tick-associated

viruses may lead to better understanding of the tripartite interaction between pathogenic microorganisms, non-pathogenic microorganisms and ticks.

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481

482 **Legends to Figures and Tables**

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

485 (A) Virions purified from the culture supernatant of ISE6 cells, (B) enlarged image of a
486 virion, (C) cell culture medium only, and (D) enlarged image of culture medium only

were observed by an H-7650 transmission electron microscope (Hitachi, Tokyo, Japan) at 100 kV. (E) Ultrathin section of an ISE6 cell and (F) enlarged image of virion-like structures in the ISE6 cell. The arrowheads indicate the virions.

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

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

Figure 3. Phylogenetic tree of the genus Iflavirus.

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

503

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

505 The full-length sequences of the *Ixodes scapularis* bunyavirus (contigs 2 and 5) (shown
506 underlined) were used for phylogenetic analyses with the other bunyavirus L (A) and M
507 segments (B), respectively. Bootstrap probabilities higher than 70% are indicated near the
508 branches. The scale bars indicate 0.5 changes per nucleotide position.

509

510 **Figure 5. PCR amplification of each putative viral sequence and tick mitochondrial**
511 **16S rRNA gene.**

512 PCR was performed on the cDNA generated from the total RNA extracted from ISE6
513 cells (1), the culture supernatant (2), and the culture medium (3) and gDNA extracted
514 from ISE6 cells (4). ISIV, ISB_L, ISB_M, ISAV-1, ISUV, and 16S rDNA represent PCR
515 reactions with primers amplifying *Ixodes scapularis* iflavirus, L segment of *Ixodes*
516 *scapularis* bunyavirus, M segment of *Ixodes scapularis* bunyavirus, *Ixodes scapularis*
517 associated virus-1, unclassified virus related to *Drosophila* A virus, and tick
518 mitochondrial 16S rRNA gene (rDNA), respectively. All the five putative viral sequences

519 were detected in both ISE6 cells and the culture supernatant while only tick
520 mitochondrial 16S rDNA was amplified from gDNA. Lane M shows a 100-bp DNA
521 marker.

522

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

525

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

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.

Figure %

Figure 2

A

Ixodes scapularis iflavivirus

B

Ixodes scapularis bunyavirus

Figure 3

Figure 4

A

B

Figure 5