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Original article

Infection of newly identified phleboviruses in ticks and wild animals in Hokkaido, Japan indicating tick-borne life cycles

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ABSTRACT

Recent discoveries of tick-borne pathogens have raised public health concerns on tick-borne infectious diseases and emphasize the need to assess potential risks of unrecognized tick-borne pathogens. First, to determine the existence of tick-borne phleboviruses (TBPVs), genetic surveillance of phleboviruses in ticks was conducted mainly in Hokkaido, the northernmost island in Japan from 2013 to 2015. Genes of two TBPVs, previously reported as Mukawa virus (MKWV) and a newly identified relative of MKWV, Kuriyama virus (KURV), were detected and the viruses were isolated from *Ixodes persulcatus* collected in Hokkaido, but not in *I. persulcatus* collected from other areas of Japan. These viruses were phylogenetically and antigenically similar to each other. Next, to investigate the infection of MKWV in mammals, serum samples from wildlife captured in Hokkaido from 2007 to 2011 were used for serological screening. Neutralizing antibodies against MKWV were detected in both Yezo-deer (*Cervus nippon yesoensis*) (2/50) and raccoons (*Procyon lotor*) (16/64). However, no infectious MKWV was recovered from laboratory mice in experimental infections, though viral RNAs were detected in their tissues. Thus, MKWV and KURV may maintain tick-mammalian life cycles in Hokkaido, suggesting their potential as causative agents of tick-borne diseases in mammals.

1. Introduction

The recent discoveries of human-pathogenic tick-borne viruses have impacted public health by revealing the silent risks of emerging pathogens transmitted by ticks (Kosoy et al., 2015; McMullan et al., 2012; Yu et al., 2011). Ticks hardly spread disease outside their local habitats (Eisen and Eisen, 2018; Eisen et al., 2017; Jongejan and Uilenberg, 2004), and thus, early detection and characterization of novel viruses in local ticks is important to highlight the endemic threats of unrecognized pathogens. In the genus *Phlebovirus*, family *Phenuiviridae* [previously known as *Bunyaviridae*] (King et al., 2018), two human-pathogenic tick-borne phleboviruses (TBPVs) have been identified in the 2010s; severe fever with thrombocytopenia syndrome (SFTS) virus emerged in east Asian countries (Kim et al., 2013; Takahashi et al., 2014; Yu et al., 2011) and Heartland virus in the United States (McMullan et al., 2012), followed by several discoveries of RNAs from novel TBPVs in ticks using pan-phlebovirus RT-PCR (Matsuno et al., 2015; Papa et al., 2016, 2017; Pereira et al., 2017; Prinz et al., 2017) and/or next generation sequencing (Li et al., 2015; Tokarz et al., 2014). Since epidemiological information of these novel viruses that have been identified only in

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ticks are limited, their potential risks of spillover into the human population have not been fully understood.

Screening of novel TBPVs in ticks is required especially in endemic countries of tick-borne diseases for differentiating novel TBPV infections from known diseases, as replication of a novel TBPV may cause nonspecific febrile illness. In Japan, SFTS and tick-borne encephalitis (Lindquist and Vapalahti, 2008; Takashima, 1998; Yoshii et al., 2017) are reported as viral tick-borne diseases, and tick-borne diseases caused by bacteria or parasites such as Lyme disease (Saito et al., 2007; Sato et al., 2014; Takano et al., 2014), rickettsiosis (Ando et al., 2010; Imaoka et al., 2011; National Institute of Infectious Diseases, 2017), and human granulocytic anaplasmosis (Ohashi et al., 2013), are also recognized. These diseases present non-specific febrile illness (Bakken and Dumler, 2006; Parola and Raoult, 2001; Suttinont et al., 2006). To identify the potential causes of febrile illness by novel TBPVs, clarification of TBPV's distribution and pathogenicity will be beneficial in case of their future emergence.

In the present study, we report the ecological characteristic of a previously reported TBPV, Mukawa virus (MKWV) (Matsuno et al., 2018). Our previous study discussed the unique genetic and biological characteristics of MKWV, a tick-derived virus genetically similar to mosquito/sandfly-borne phleboviruses rather than other TBPVs. Even though the potential of MKWV to adapt to a human cell line has been shown, the life cycle of MKWV and its infectivity and pathogenicity in mammals is unknown. Here, serological screening and experimental infection was performed to understand the ecological life cycle as well as the pathogenicity of MKWV in nature.

The current epidemiological landscape of tick-borne diseases is complicated, as studies of known and novel pathogens in ticks as well as their correlations with each other in nature are limited. Thus, potential pathogens in ticks should be discovered using a comprehensive method in addition to studying each pathogen specifically. The present study also aimed to reveal a complete picture of TBPV distribution in a limited area in Japan (i.e. Hokkaido) by genetic screening of ticks using pan-phlebovirus RT-PCR (Matsuno et al., 2015).

2. Materials and methods

2.1. Study site and sample collection

Only adult ticks (n = 1,481) were collected from various locations in Hokkaido, the northernmost island in Japan from 2013 to 2015 and used for genetic surveillance of TBPVs. Ticks (n = 67) collected in Mukawa area in the previous study were also included. Questing ticks (n = 1,217) were captured by the flagging method, and blood-sucking ticks were removed from wild Yezo-deer (*Cervus nippon yesoensis*) captured by hunters in 2013 (n = 54) and from wild raccoons (*Procyon lotor*) captured by local veterinary offices in 2015 (n = 210). Tick species were identified under a stereomicroscope based on morphologic features (Sasa and Aoki, 1977).

Serum samples of Yezo-deer and raccoons were used for serological testing for MKWV. Serum samples of Yezo-deer (n = 50) hunted in Hidaka area from 2010 to 2011 (Fig. 1) were a part of the samples previously used for a serological study targeting hepatitis E virus (Tomiyama et al., 2009). Serum samples were also obtained from raccoons (n = 64) captured in Mukawa, Kuriyama, Atsuma, and Ebetsu areas from 2007 to 2010 as a part of raccoon population control programs implemented by the Hokkaido Government (Fig. 1). These samples were stored at −80 °C until use.

2.2. RNA extraction from ticks

Each tick was washed once in 70% ethanol and soaked twice in distilled sterile water for 10 min. The samples were then homogenized with 100 µl of Dulbecco's modified Eagle's medium (DMEM) (Nissui, Tokyo, Japan) using a homogenizer (Tomy Seiko, Tokyo, Japan) twice

Fig. 1. Map of the present study sites. Hokkaido is the second largest island in Japan and is geographically isolated from other prefectures. The areas where Mukawa virus or Kuriyama virus-positive ticks were captured from 2013 to 2015 (Mukawa, Kuriyama, Obihiro, and Shimizu) are indicated by black stars, areas where Yezo-deer (*Cervus nippon yesoensis*) were captured from 2010 to 2011 (Hidaka) are colored in dark gray, and areas where raccoons (*Procyon lotor*) were captured from 2007 to 2010 (Mukawa, Kuriyama, Atsuma, and Ebetsu) are colored in light gray.

at 3000 rpm. Total RNA was extracted from 50 µl of the homogenate using blackPREP Tick DNA/RNA Kit (Analytic Jena, Jena, Germany) according to the manufacturer's protocol and the remaining homogenized samples were stored at −80 °C until use for virus isolation.

2.3. Detection of TBPV RNAs

A one-step RT-PCR system reported previously (Matsuno et al., 2015) was employed to detect a wide range of TBPVs in the extracted tick RNAs. Briefly, around a 500-bp fragment of the L segment RNA was amplified using a PrimeScript One step RT-PCR Kit Ver. 2 (Dye Plus) (TAKARA, Shiga, Japan) from the tick RNA using the primer set; HRT-GOUL2759 F (5′-CAGCATGGIGGIYTIAGRGAAATYTATGT-3′) and HRT-GOUL3276R (5′-GAWGTRWARTGCAGGATICCYTGCATCAT-3′). The amplified products were sequenced using the ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit on a 3500 Genetic Analyzer (ABI3500) (Thermo Fisher Scientific, Waltham, MA) using the HRT-GOUL2759 F or HRT-GOUL3276R primer. The nucleotide sequences were determined using GENETYX version 13 (GENETYX, Tokyo, Japan).

2.4. Virus isolation

The remaining homogenates of RT-PCR-positive ticks were subjected to virus isolation. The *Ixodes scapularis* embryo-derived ISE6 cells were cultured in modified L-15B medium supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific), 5% tryptose phosphate broth (TPB) (Sigma, St. Louis, MO), 0.1% bovine lipoprotein concentrate (MP Biomedicals, Irvine, CA), and 2% penicillin-streptomycin (Thermo Fisher Scientific) at 34 °C as reported previously (Munderloh and Kurtti, 1989). After ISE6 cells were cultured in 12-well plates (Becton Dickinson and Co, Franklin Lakes, NJ) for one day, the media were removed and 20 µl of tick homogenates were inoculated into cultured cells in 1 ml of the modified L-15B medium. One hour after inoculation, the media were changed, and the cells were cultured for seven days at 34 °C under 5% CO_2. Following three blind passages (i.e. four weeks after the inoculation), viral production in the supernatant was confirmed using the RT-PCR described above.

2.5. Electron microscopy

Supernatants of virus-inoculated ISE6 cells were subjected to ultracentrifugation (27,000 rpm, 90 min) through 25% sucrose in PBS, following low-speed centrifugation to remove debris. The ultracentrifuged sample was adsorbed to collodion-carbon-coated copper grids and negatively stained with 2% phosphotungstic acid solution (pH 5.8). Virus particles were observed under an H-7650 transmission electron microscope (Hitachi, Tokyo, Japan) at 80 kV.

2.6. Determination of nucleotide sequences of full-length viral genome

Viral RNA was extracted from the supernatants of ISE6 cells using QIAamp Viral RNA Mini Kit (QIAGEN, Venlo, Netherland) and the viral genome was amplified using PrimeScript One Step RT-PCR kit with specific primer pairs, which were designed based on the nucleotide sequence of MKWV (primer sequences available upon request). The 5′ and 3′ termini of each segment RNA were respectively amplified using the RACE method (Li et al., 2005) with specific primers for MKWV. Amplified products were directly sequenced in both directions on the ABI3500. The full-genome sequence of each segment RNA was determined, and the amino acid sequences were predicted using GENETYX version 13.

2.7. Phylogenetic analysis

The determined full-length nucleotide sequences of the isolated viruses and those of other known phleboviruses available at GenBank, were aligned using MUSCLE implemented in MEGA version 6.0 (Tamura et al., 2013). Multiple sequence alignments were modified manually. Phylogenetic trees based on three segment RNAs were constructed by the maximum-likelihood method using MEGA version 6.0. The robustness of the nodes was tested using 1000 bootstrap replications.

2.8. Detection of viral antigens by immunofluorescence assay

Monolayers of ISE6 cells infected with TBPVs were fixed in ice cold acetone at three days post infection. After fixation, the cells were incubated with 1:1,000 diluted MKWV-infected mouse serum in the antibody dilution buffer: phosphate buffered saline (PBS) containing 1.0% bovine serum albumin fraction V (Roche, Basel, Schweiz), and 0.05% Tween 20 (Nacalai Tesque, Kyoto, Japan), for one hour at room temperature (approximately 24 °C), followed by washing three times in PBS. The viral antigens were visualized by incubation with 1:1,000 diluted Fluorescein isothiocyanate (FITC)-conjugated Goat IgG Fraction to Mouse IgG (MP Biomedicals) in the antibody dilution buffer for one hour at room temperature, followed by staining with DAPI (Thermo Fisher Scientific) (1:2,000 dilution) for 10 min. The cells were examined for staining under a fluorescence microscope (Olympus, Tokyo, Japan) with an appropriate barrier and excitation filters for visualizing FITC and DAPI (Chroma, Bellows Falls, VT).

2.9. Detection of antibodies to MKWV by indirect immunofluorescence (IIF) assay

MKWV-infected and mock-infected Huh-7 cells (human liver carcinoma cells) were prepared for the IIF assay. Huh-7 cells were cultured in DMEM with 2% FBS, 2 mM L-glutamine, and 2% penicillin-streptomycin at 37 °C with 5% CO_2. Three days after MKWV infection, cells were fixed with 4% paraformaldehyde at room temperature for 10 min, followed by permeabilization with PBS containing 0.1% Triton X-100 (Nacalai Tesque) for 10 min. Serum samples were serially diluted 2-fold from 1:20 to 1:2,560 and added to both the fixed MKWV-infected and mock-infected cells. The cells were incubated at 4 °C overnight. After washing with PBS, the antigens on the slides were treated with Protein A/G-FITC (BioVision, Milpitas, CA) at a dilution of 1:5,000 for one hour at room temperature. After another wash with PBS, the cells were examined under a fluorescence microscope with appropriate barrier and excitation filters for FITC. The titers of tested samples were recorded as the reciprocals of the highest dilutions showing positive staining.

2.10. Neutralization assay

IIF-positive serum samples were then used to detect neutralizing antibodies against MKWV. Serum samples were serially diluted 2-fold from 1:40 to 1:5,120 and mixed with an equal volume of MKWV at 500 50% tissue culture infectious dose ($TCID_{50}$). The mixtures were incubated at 37 °C for one hour, and then added to cultured Huh-7 cells in 96-well plates (Corning, Corning, NY). Cells were incubated at 37 °C with 5% CO_2 for four days, as inhibition of viral infection by MKWV-infected mouse serum used for positive serum control was clearly observed by the immunofluorescence assay. The titers were recorded as the reciprocals of the highest dilutions of serum samples that prevent infection. Titers of 1:40 or greater were considered positive results.

2.11. Experimental inoculation of MKWV into eight-week-old C57BL/6J mice

For the animal experiment, MKWV passaged four times in ISE6 cells (ISE6 cell-passaged) and MKWV passaged eight times in Huh-7 cells after the four passages in ISE6 cells (Huh-7 cell-passaged) were used. Huh-7 cell-passaged MKWV was inoculated intramuscularly (Group 1) or subcutaneously (Group 2), and ISE6 cell-passaged MKWV was inoculated subcutaneously (Group 3) at 10^5 $TCID_{50}$/mouse in a group of fifteen eight-week-old female C57BL/6 J mice (Japan SLC, Shizuoka, Japan). In total, 18 mice were inoculated with supernatants of ISE6 cells or Huh-7 cells as negative controls. To analyze viral distribution in the blood and tissues of MKWV-inoculated mice, five mice from each group (three mice of each negative-control group) were sacrificed at 3, 7, and 14 days post inoculation (d.p.i.). The remaining mice were observed for their clinical signs, and their body weights were recorded for 14 days.

2.12. Detection of viral RNA and infectious titers in blood and tissues

Tissues (liver, spleen, and kidney) were collected individually from the mice and homogenized in DMEM supplemented with 2% FBS. Homogenates and whole blood of the three representative individuals in each group were serially diluted 10-fold with DMEM containing 2% FBS and inoculated into Huh-7 cells for titration. The remaining homogenates and whole blood were also subjected to RNA extraction with TRIzol LS (Thermo Fisher Scientific). After generation of cDNAs from the extracted RNAs with Superscript IV Reverse Transcriptase (Thermo Fisher Scientific), a quantitative PCR (qPCR) assay targeting MKWV was performed with the generated cDNA and a MKWV-specific primer set; Mukawa-L-6314 F (5′-AGATCTTGTTGGGAAACACC-3′) and Mukawa-L6414R (5′-ACACAAAGTCCGCCCATTACCAATGAGATG-3′) using THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan). The reaction was performed on a Bio-Rad CFX96 system (Bio-Rad, Hercules, CA) and the MKWV titers were calculated as the $TCID_{50}$/g-equivalents of tissues or $TCID_{50}$/ml-equivalents of whole blood.

3. Results

3.1. Genetic surveillance of TBPVs in ticks collected in Hokkaido

To reveal the distribution of TBPVs in Hokkaido, ticks were collected from various locations and examined for TBPV genes by one-step RT-PCR. MKWV was identified in a part of this screening (Matsuno et al., 2018). Based on morphological identification, ticks (n = 625) collected in 2013 comprised nine species in two genera, ticks (n = 109)

Table 1
Screening of ticks for tick-borne phleboviruses in Hokkaido, Japan.

Place	Year	Tick species	Source	Total	TBPV-positive	MKWV-positive[*1]
Hokkaido	2013	*Haemaphysalis concinna*	Flagging[*2]	7	0	0
		H. flava	Yezo-deer[*3]	8	0	0
		H. japonica	Flagging	27	0	0
			Yezo-deer	32	0	0
		H. longicornis	Flagging	4	0	0
		H. megaspinosa	Flagging	24	0	0
			Yezo-deer	3	0	0
		Ixodes ovatus	Flagging	323	0	0
			Yezo-deer	11	0	0
		I. pavlovskyi	Flagging	2	0	0
		I. persulcatus	Flagging	183	17	5
		I. tanuki	Flagging	1	0	0
	2014	*H. japonica*	Flagging	1	0	0
		H. megaspinosa	Flagging	1	0	0
		I. ovatus	Flagging	53	0	0
		I. pavlovskyi	Flagging	13	0	0
		I. persulcatus	Flagging	41	3	0
	2015	*H. flava*	Flagging	10	0	0
			Raccoons[*4]	4	1	0
		H. japonica	Flagging	33	0	0
		H. megaspinosa	Flagging	111	1	0
			Raccoons	10	0	0
		I. ovatus	Flagging	144	0	0
			Raccoons	136	0	0
		I. pavlovskyi	Flagging	34	0	0
			Raccoons	6	0	0
		I. persulcatus	Flagging	204	10	2
			Raccoons	15	0	0
		I. tanuki	Flagging	1	0	0
			Raccoons	39	0	0
Honshu island	2013	*I. persulcatus*	Flagging	12	1	0
	2014	*I. persulcatus*	Flagging	61	3	0

*1 TBPV-positive samples with > 80% nucleotide sequence identity to MKWV strain MKW73.
*2 Questing ticks captured by the flagging method.
*3 Blood-sucking ticks removed from wild Yezo-deer (*Cervus nippon yesoensis*).
*4 Blood-sucking ticks removed from wild raccoons (*Procyon lotor*).

collected in 2014 comprised five species in two genera, and ticks (n = 747) collected in 2015 comprised seven species in two genera (Table 1). TBPV genes were detected in *I. persulcatus* in 2013 (17/183); *I. persulcatus* in 2014 (3/41); and *Haemaphysalis flava* (1/14), *H. megaspinosa* (1/121), and *I. persulcatus* (10/219) in 2015. Only one TBPV was detected in an engorged *H. flava* tick collected from a raccoon in 2015 among engorged ticks. Although *I. ovatus* was dominant in Hokkaido throughout all years, no TBPV genes were detected. The homogenates of TBPV-positive ticks were further investigated based on the nucleotide sequences amplified by one-step RT-PCR. The homology values between nucleotide sequences were calculated with the corresponding partial sequences of MKWV L segment RNA. No SFTS virus-positive ticks were identified, and the nucleotide sequences derived from five *I. persulcatus* samples collected in Mukawa and Shimizu areas in 2013 [including MKW73 from which MKWV has been isolated (Matsuno et al., 2018)] were 100% matched to the corresponding partial sequences of MKWV MKW73 (Table S1). Interestingly, the nucleotide sequences in ticks collected from Kuriyama and Obihiro areas in 2015 demonstrated an 84% identity to the MKWV sequence and 100% identity with each other (Table S1). The putative MKWV-like virus has been named Kuriyama virus (KURV). MKWV and KURV RNAs were not detected in *I. persulcatus* collected from Honshu island, the largest island of Japan (Table 1).

3.2. Isolation and characterization of KURV

Homogenates of TBPV-positive samples were inoculated into ISE6 cells for virus isolation. Successful isolation of KURV was confirmed after three passages in ISE6 cells, without any cytopathic effects (CPE). No other putative TBPVs apart from KURV and MKWV were isolated. The KURV isolate KUR80Q originated from the tick CZC80Q collected in Kuriyama and was used in the following experiments. The virions of KURV KUR80Q were enveloped spherical particles (100-nm average in diameter) with surface spike proteins (Fig. 2A), indicating morphological similarity to MKWV and other bunyaviruses (Plyusnin et al., 2012).

Full-genome sequences of KURV RNA segments were determined by MKWV-specific primer sets followed by primer walking and the RACE method. KURV genome was consisting of three RNA segments: L segment, 6,444 base (b); M segment, 3,328 b; and S segment, 1,908 b. Each L or M segment RNA encoded one open reading flame (ORF) in the negative sense and the S segment RNA encoded two ORFs in the ambisense orientation. BLAST search indicated that the deduced amino acid sequences of KURV ORFs were related to those of proteins encoded in each segment from phleboviruses: RNA-dependent RNA polymerase (L) on L segment, glycoproteins (Gn and Gc) on M segment, and nonstructural and nucleocapsid proteins (NSs and N, respectively) on S segment RNAs, respectively. The pairwise amino acid identities of KURV were 95% (L), 93% (glycoprotein precursor), 93% (N), and 74% (NSs) with MKWV. In the phylogenetic trees constructed based on all three segment RNAs, KURV was classified into the same phylogroup as MKWV (Fig. 2B). In all phylogenetic trees, the phylogroup of MKWV and KURV was branched together with the mosquito/sandfly-borne phlebovirus group and was closely related to mosquito/sandfly-borne phleboviruses rather than TBPVs.

To compare the serological reactivity of KURV and MKWV, an immunofluorescence assay was conducted using MKWV-infected mouse serum. Immunofluorescence signals were mainly detected in the cytoplasm of both KURV- and MKWV-infected cells and the intensity of immunofluorescence signals was nearly identical to each other

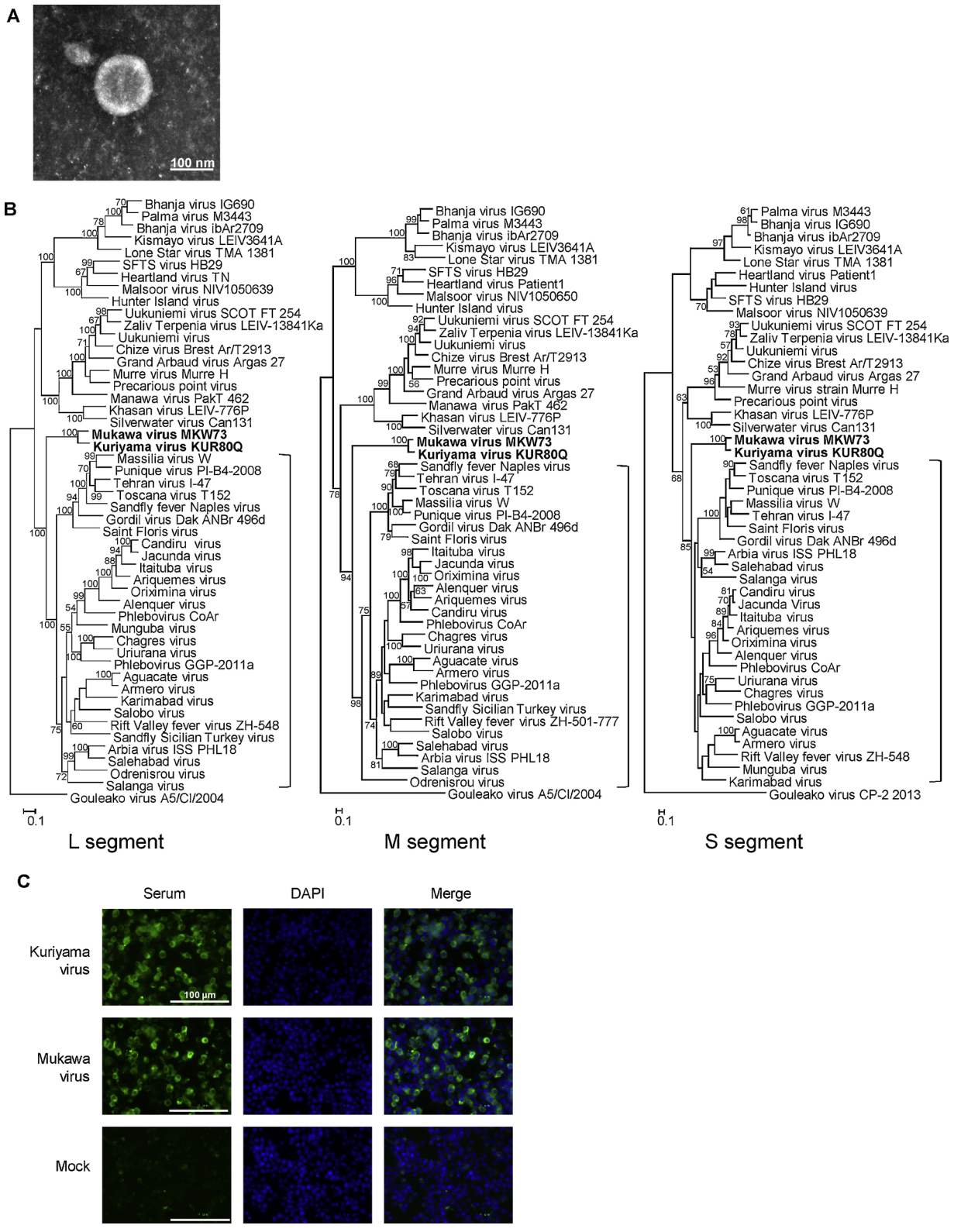

Fig. 2. Isolation and characterization of Kuriyama virus. (A) Electron micrograph of a Kuriyama virus particle. (B) Phylogenetic trees constructed based on the full-length nucleotide sequences of three RNA segments of phleboviruses using the maximum-likelihood method in MEGA version 6.0. Bootstrap values greater than 50 are shown near the branch nodes and the scale bar indicates the number of substitutions per site. A square bracket on the right side of each tree indicates mosquito/sandfly-borne phleboviruses. (C) The antigens of Kuriyama and Mukawa viruses were detected in tick cell monolayer at 7 days post-inoculation using Mukawa virus-infected mouse serum with indirect fluorescence-labelled antibodies. The antigens reacting with the serum were visualized in green; the blue color indicates nuclei stained with DAPI. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 2
Seropositivity against MKWV in wildlife captured in Hokkaido.

Animal species	Area	Year	IIF[*1]	NT[*2]
Yezo-deer	Hidaka	2010	5/27	1/5
(*Cervus nippon yesoensis*)		2011	1/23	1/1
Raccoons	Mukawa	2007	1/7	1/1
(*Procyon lotor*)		2010	0/6	-/-
	Kuriyama	2007	3/8	3/3
		2008	1/10	1/1
	Atsuma	2008	1/4	1/1
	Ebetsu	2007	5/10	5/5
		2008	3/10	2/3
		2010	3/9	3/3

*1 IIF: Indirect immunofluorescence assay (positive/tested) tpters of 1:20 or greater were considered as positive results.

*2 NT: Neutralization assay (positive/tested) titers of 1:40 or greater were considered as positive results.

(Fig. 2C). The results indicated that the anti-MKWV serum cross-reacted with KURV antigens.

3.3. Serological survey

IIF assay was performed using serum samples from Yezo-deer and raccoons to investigate MKWV infections in wild animals in Hokkaido. Antibodies recognizing MKWV antigens were detected in 12% (6/50) of Yezo-deer and 27% (17/64) of raccoons, respectively (Table 2). Titers of positive sera ranged from 20 to 2560 (Table S2). IIF-positive serum samples were then used for the neutralization assay to confirm MKWV infection. Serum samples of Yezo-deer (2/6) and raccoons (16/17) could neutralize MKWV; the seropositivity of anti-MKWV neutralizing antibodies was 4% (2/50) in Yezo-deer and 25% (16/64) in raccoons (Table 2). Neutralizing titers ranged from 40 to 640 (Table S2). The neutralizing antibody-positive animals were found at least in four different areas in Hokkaido during the four-year period.

3.4. Evaluation of MKWV pathogenicity in eight-week-old C57BL/6J mice

To explore MKWV pathogenesis in adult mice, the MKWV isolate MKW73 was inoculated into groups of eight-week-old C57BL/6 J mice, and their clinical signs were monitored until 14 d.p.i. None of the mice inoculated with MKWV demonstrated any clinical signs such as severe weight loss for 14 days. No infectious MKWVs were recovered from whole blood or tissues collected on 3, 7, and 14 d.p.i., and MKWV RNA genomes were detected in some tissues but not in whole blood (Fig. 3). On 7 and 14 d.p.i., MKWV RNA was detected in a limited number of organs that were inconsistent between animals.

4. Discussion

Recently, an increasing number of viruses has been identified in ticks (Mansfield et al., 2017; Paules et al., 2018), and their potential to cause diseases in humans as well as animals is seriously discussed to disclose the impact on our public health. However, the ecological feature of these tick viruses has not fully addressed due to lack of comprehensive tick screening. Our comprehensive screening of TBPVs in ticks in the present study was conducted on ticks collected in a limited area, i.e., Hokkaido, Japan, and discovery of novel viral RNAs suggested that a variety of TBPVs are still undiscovered in ticks worldwide. We detected the genes of two putative TBPVs, MKWV and KURV, from

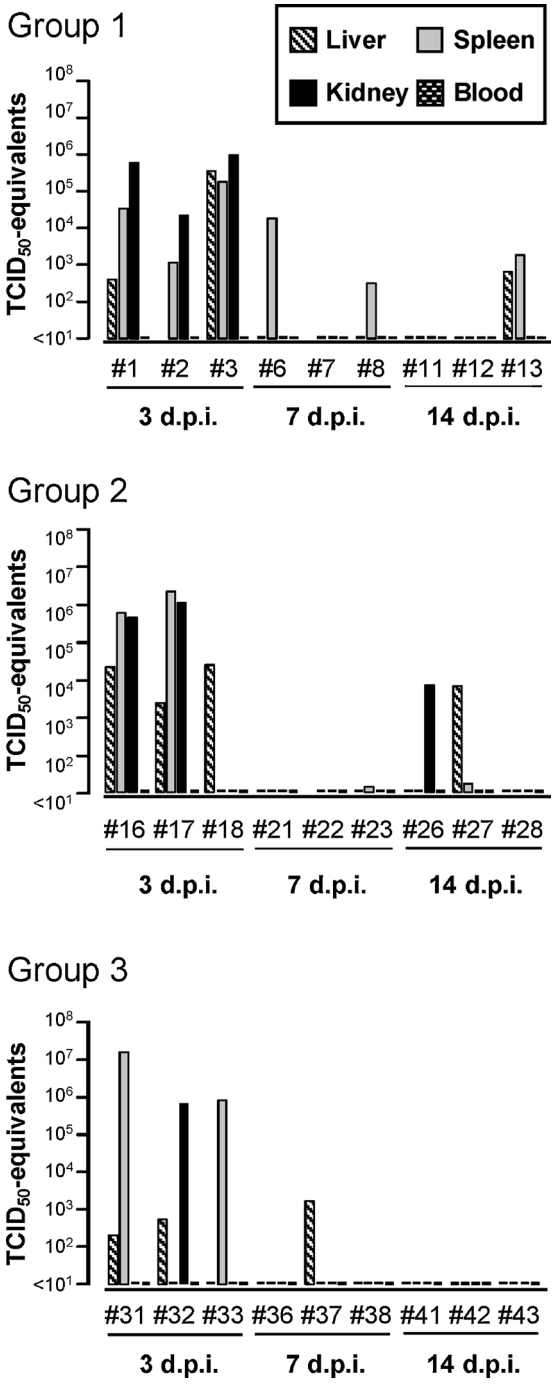

Fig. 3. Viral RNA loads in the Mukawa virus-infected C57BL/6 J mice. Nine mice in each group were inoculated with 10^5 $TCID_{50}$ of Mukawa virus as follows: intramuscular inoculation with Huh-7 cell-passaged Mukawa virus (group 1), subcutaneous inoculation (group 2), or inoculation with ISE6 cell-passaged Mukawa virus (group 3). At 3, 7, and 14 days post inoculation (d.p.i.), tissues (liver, spleen, and kidney) and whole blood collected from three individuals were subjected to reverse transcription followed by qPCR to quantify the viral RNA load. The load of Mukawa virus RNA was indicated as the $TCID_{50}$/g-equivalents in tissues or $TCID_{50}$/ml-equivalents in whole blood. The IDs of mice are indicated as #number (1–63) below each graph.

I. persulcatus ticks and isolated them using the tick-derived ISE6 cell line. As MKWV and KURV replicated in ISE6 cells but not in mosquito-derived C6/36 cells like the tick-borne Uukuniemi virus (Matsuno et al., 2018) (data not shown for KURV in C6/36 cells), the major vectors of these viruses may be ticks. Infection of MKWV in mammals was also suggested by serological tests in wildlife and detection of viral RNA in animal experiments, while cross reactivity of antisera against MKWV to other endemic TBPVs may need to investigate carefully. Taken together, a tick-borne life cycle can be proposed for MKWV. Interestingly, the distribution of MKWV and KURV in ticks seemed sporadic and was not identical to the areas where antibody-positive animals were captured. Further investigation of *I. persulcatus* ticks using more sensitive detection methods for MKWV and KURV may be necessary to confirm their distributions and transmission between ticks and mammals.

So far, both MKWV and its close relative KURV were detected only from *I. persulcatus* collected in Hokkaido, but not in Honshu island. While the number of *I. persulcatus* ticks collected in Honshu island was insufficient to prove the absence of MKWV and KURV using the panphlebovirus RT-PCR, the sporadic discoveries of MKWV and KURV in only a single tick species in limited areas suggested that their distribution could be restricted by host ticks as well as host animals. Testing on individual ticks allowed us to determine the detailed spatiotemporal distribution of MKWV and KURV rather than testing on pooled samples because a number of ticks collected in a single site is limited. So far, no study has addressed the mechanism(s) underlying the emergence of similar viruses such as SFTS virus and Heartland virus, which are genetically related but discovered in different continents. Furthermore, selection pressure(s) to produce divergence in each TBPV species has also not been clarified. Since MKWV and KURV are closely related and were identified in a limited island, a spatiotemporal approach to reveal the evolutional pressures defining and maintaining the divergence of MKWV and KURV may be crucial to understand process of TBPV evolution.

To assess the potential risk of MKWV, understanding its virulence in humans and livestock animals is essential. Although serological evidences indicate that MKWV infection occurred in wild animals, no infectious virus was recovered upon experimental infection in adult C57BL/6 mice, which show no visible clinical signs. However, as the virulence of a TBPV may vary depending on the host animal species, it is necessary to confirm the virulence of MKWV using other models. An immunocompromised mouse model such as interferon-α receptor knockout mice (Liu et al., 2014; Müller et al., 1994), may be a useful fatal model for assessing the potential of these viruses to replicate in an animal model. Infection of MKWV in mammals as well as arthropods suggested the potential risk of spillover to humans in the future. Thus, the seroprevalence of MKWV in humans and/or domestic animals is also interesting to assess the public health concerns.

In the present study, we revealed that divergent TBPVs have been maintained in ticks in Hokkaido, and demonstrated possible infection of one of the TBPVs in wildlife animals. Virus isolation is essential to conduct downstream experiments such as serological assays, experimental infections to laboratory animals, and biological characterization. Since most of recently reported novel TBPVs have not been isolated, establishment of isolation methods for these TBPVs is essential for comprehensively understanding their potential risks to public health.

Conflicts of interest

The authors declare no conflicts of interest.

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Ethics statement

Animal experiments were approved by the Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University (approval numbers: 14-0053).

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2018.11.012.

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