



NOTE

Virology

Detection and phylogenetic analysis of phlebovirus, including severe fever with thrombocytopenia syndrome virus, in ticks collected from Tokyo, Japan

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ABSTRACT. Severe fever with thrombocytopenia syndrome (SFTS) was detected for the first time in China in 2011. Since then, human cases have been reported in endemic regions, including Japan. To investigate the presence of tick-borne pathogens in Tokyo, 551 ticks (266 samples) were collected from October 2015 to October 2016. Although the SFTS virus was not detected by RT-PCR, a novel phlebovirus was detected in one sample. In a phylogenetic analysis based on the partial nucleotide sequences of the L and S segments of the virus, the virus clustered with Lesvos virus (Greece), Yongjia tick virus, and Dabieshan tick virus (China). Further studies involving virus isolation are required to characterize this novel phlebovirus and to expand the epidemiological knowledge of related pathogens.

KEY WORDS: phlebovirus, tick, tick-borne pathogen, Tokyo

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The genus *Phlebovirus*, which includes the causative pathogen for severe fever with thrombocytopenia syndrome (SFTS), was classified in the order *Bunyavirales*, family *Phenuviridae* in the 10th Report of the International Committee for Taxonomy of Virus (ICTV) (<https://talk.ictvonline.org/taxonomy/>). SFTS is one of the most serious tick-borne diseases. The SFTS virus was first isolated in China in 2011 [16]. Fever, thrombocytopenia, gastrointestinal symptoms, multiple organ failure, muscular symptoms, neurological abnormalities, and coagulopathy are observed in SFTS patients. The fatality rate is 10 to 30% [16]. In 2012, the Heartland virus, which is closely related to the SFTS virus, was isolated in the United States [7]. The previously reported but uncharacterized Bhanja and Uukuniemi viruses were classified to the genus *Phlebovirus* in 1990 and 2013, respectively [6, 10, 12–14].

By 2016, 229 cases of human SFTS, including 53 fatal cases, had been reported in Japan [9]. There has not been a case report of human SFTS in Tokyo. However, the presence of SFTS virus in ticks have been reported in neighboring prefectures [8]. To investigate the presence of SFTS virus in Tokyo, we conducted a molecular epidemiological survey of SFTS virus and other tick-borne phleboviruses in Tokyo.

From October 2015 to October 2016, ticks were collected using the drag-flag method from two areas (35°48' 20.2" N, 139°10' 36.2" E and 35°58' 85.34" N, 139°66' 45.48" E) along the Tamagawa River, which flows along the border of Tokyo and Kanagawa prefecture. Tick species were identified by morphology using a stereo-microscope (Nikon, Tokyo, Japan) and taxonomic criteria [2]. One adult or nymph of each tick species was individually put in a separate tube, and one to six larvae were combined in one tube as a pooled sample. The ticks were stored at –80°C until further testing.

Ticks were thawed and homogenized using a BEAD CRUSHER (TAITEC, Nagoya, Japan) with a Ceramic Sphere (MP Biomedicals, Santa Ana, CA, U.S.A.; diameter, 6.35 mm) and one dispensing spoonful of Garnet Matrix A Bulk (MP Biomedicals). Total RNA was extracted from ticks using ISOGEN (Nippongene, Tokyo, Japan) according to the manual of National Institute of Infectious Diseases in Japan (<http://www0.nih.go.jp/~auda/tick-SFTS-manual.pdf>). A total of 20 µl of the extracted RNA was converted to cDNA using 5 µl of PrimeScript RT Master Mix Perfect Real Time including random primers (TaKaRa Bio, Kusatsu, Japan) at 37°C for 15 min.

Detection of the SFTS virus was conducted by nested PCR using the SFTSV NP-2F and SFTSV NP-1Rd primers for the first reaction and the SFTSV NP-1F and SFTSV NP-2R primers for the second reaction (35 cycles of 94°C for 30 sec, 52°C for 30 sec,

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Table 1. Primer sequences used in this study

Targets	Primer names	Sequences (5'-3')	Product sizes (bp)	References
SFTS virus	SFTSV NP-2F	CATCATGTGCTTTGCCCTGA	420	[15]
	SFTSV NP-1Rd	TTCAGCCACTTCACCCGRA		
	SFTSV NP-1F	ATCGTCAAGGCATCAGGGAA	490	
	SFTSV NP-2R	AGAAGACAGAGTTCACAGCA		
Phlebovirus L segment	TBPVL2759F	CAGCATGGIGGICTIAGAGAGAT	514	[5]
	TBPVL3267R	TGIAGIATSCCYTGCATCAT		
Phlebovirus S segment	PLBV Sseg1F	CCTRCACATCTGCCGSGGGAC	710	This study
	PLBV SsegR	GTGTMCCCAGTCTGTCCTAA		
	PLBV Sseg2F	GCGACGACATCACCCCTG	590	
	PLBV SsegR	GTGTMCCCAGTCTGTCCTAA		

Table 2. Collected tick species and positive rates for SFTS virus and phlebovirus in Tokyo, Japan

	<i>H. megaspinosa</i>		<i>H. flava</i>		<i>H. longicornis</i>		<i>H. kitaokai</i>		<i>I. ovatus</i>		Larvae	Total
	Adults	Nymphs	Adults	Nymphs	Adults	Nymphs	Adults	Nymphs	Adults	Nymphs		
SFTS virus	0/1	0/20	0/12	0/56	0/7	0/95	0/1	0/0	0/2	0/0	0/72	0/266
Phlebovirus	0/1	0/20	0/12	1/56	0/7	0/95	0/1	0/0	0/2	0/0	0/72	1/266

Results are expressed as number of positive tick samples / total.

and 72°C for 30 sec for both reactions) [15]. For phlebovirus detection, the L segment of virus was amplified by PCR using the TBPVL2759F and TBPVL3267R primers (40 cycles of 94°C for 30 sec, 46°C for 30 sec, and 72°C for 30 sec) [5]. Samples positive for the L segment were also amplified for the S segment of the phlebovirus by semi-nested PCR. The PLBV Sseg1F and PLBV SsegR primers were used for the first reaction (40 cycles of 94°C for 30 sec, 47°C for 40 sec, and 72°C for 60 sec) and the PLBV Sseg2F and PLBV SsegR primers were used for the second reaction (40 cycles of 94°C for 30 sec, 45°C for 40 sec, and 72°C for 60 sec). The S segment primers, PLBV Sseg1F, PLBV Sseg2F, and PLBV SsegR, were designed by selection from the highly homologous gene sequences between Yongjia (KM817764) and Dabieshan (KM817733) tick viruses using the primer 3 program in GENETYX-MAC software (Genetyx Co.). All PCR reactions were performed using 25 µl of 1x Go Taq Master Mix (TaKaRa Bio) containing 1 pmol of each primer and 2.5 µl of cDNA. The primers used for PCR and subsequent nucleotide sequencing are listed in Table 1 [5, 15].

The PCR products were purified using the QIAquick Gel Extraction kit (Qiagen, Venlo, Netherlands) and sequenced using an ABI Prism 3130 × 1 Genetic Analyzer with the ABI Prism BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Waltham, MA, U.S.A.). Multiple sequence alignments were performed using ATGC software (Genetyx Co.) and GENETYX-MAC software. Complete or partial nucleotide sequences of the L and S segments of phlebovirus were obtained from GenBank. A phylogenetic tree was constructed using the maximum likelihood method in MEGA7 software [3].

In total, 551 ticks were collected in this study. Of these, 194 samples belonged to the genus *Haemaphysalis*: 21 *H. megaspinosa* (20 nymphs and 1 male adult), 68 *H. flava* (56 nymphs, 10 male adults, and 2 female adults), 102 *H. longicornis* (95 nymphs, 1 male adult, and 6 female adults), 1 *H. kitaokai* (1 male adult), and 2 *Ixodes ovatus* (1 male adult and 1 female adult) (Table 2). The remaining 357 ticks were identified as larvae of *Haemaphysalis* spp. and divided into 72 pooled samples. All 266 samples were tested for tick-borne pathogens.

None of the ticks collected tested positive for the SFTS virus gene by nested PCR (Table 2). A 465-bp fragment of the L segment gene of phlebovirus was detected in a nymph of *H. flava*. The nucleotide sequence of this 465-bp fragment showed 75.9% identity with that of Yongjia tick virus (KM817704), 74.6% with that of Lesvos virus (KX452150), and 74% with that of Dabieshan tick virus (KM817666) (Table 3). The virus detected using phlebovirus genetic analysis in this study is hereafter referred to as Okutama tick virus. When compared with reference viruses (Uukuniemi, SFTS, and Bhanja viruses), the nucleotide sequence identity varied from 47.7 to 56.8%. A 554-bp fragment of the S segment gene was also successfully obtained. The nucleotide sequence of this fragment showed 76.1 and 68% identity with the Yongjia and Dabieshan tick virus, respectively. The identity to the aforementioned reference viruses was less than 50% (Table 4). The putative percentage of amino acid identity between Okutama tick virus and the reference virus strains are summarized in Tables 3 and 4. The nucleotide sequences of Okutama tick virus are deposited in GenBank under the accession numbers LC259521 and LC259522.

In the phylogenetic analysis using a part of the L segment sequences of Okutama tick virus and the reference viruses, the Okutama tick virus clustered with the Yongjia tick virus, Lesvos virus, and Dabieshan tick virus sequences isolated from *H. longicornis* in China, *H. parva* in Greece, and *H. hystricis* in China, respectively [4, 11] (Fig. 1a). A phylogenetic tree constructed using sequences of the S segment also revealed that the Okutama tick virus was in the same cluster as the Yongjia and Dabieshan tick viruses (Fig. 1b). To date, any virus in this cluster has not been reported in Japan. At the nucleotide level, the L segment of the Okutama tick virus differed from that of Yongjia, Lesvos, and Dabieshan tick viruses by 24.1, 25.4 and 26%, respectively.

Table 3. Pairwise comparison (%) of nucleotide identity (upper diagonal) and amino acid identity (lower diagonal) for the L segment of phleboviruses in the study

Strains	1	2	3	4	5	6	7
1. Okutama tick virus (LC259521)		74.0	75.9	74.6	56.8	47.7	49.6
2. DBV (KM817666)	98.7		73.5	75.6	57.4	46.8	52.6
3. YJV (KM817704)	98.7	96.7		72.6	55.9	45.5	46.2
4. LVV (KX452150)	97.4	98.7	96.7		57.4	49.8	47.7
5. UUKV (NC_005214)	83.2	83.2	82.5	81.9		51.8	53.7
6. SFTSV (NC_018136)	75.4	74.8	75.4	75.4	76.7		53.3
7. BHAV (JX961619)	74.8	77.4	75.4	73.5	75.4	80.0	

BHAV (Bhanja virus); DBV (Dabieshan tick virus); LVV (Lesvos virus); SFTSV (Severe fever with thrombocytopenia syndrome virus); UUKV (Uukuniemi virus); YJV (Yongjia tick virus).

Table 4. Pairwise comparison (%) of nucleotide identity (upper diagonal) and amino acid identity (lower diagonal) for the S segment of phleboviruses in the study

Strains	1	2	3	4	5	6
1. Okutama tick virus (LC259522)		68.0	76.1	49.5	44.4	43.3
2. DBV (KM817733)	91.8		68.4	49.3	47.7	47.2
3. YJV (KM817764)	96.5	92.6		50.9	43.5	48.9
4. UUKV (NC_005221)	79.8	77.7	81.6		49.4	44.9
5. SFTSV (NC_018137)	71.8	70.1	75.0	72.2		44.5
6. BHAV (KC521442)	76.0	72.1	76.1	71.9	70.1	

BHAV (Bhanja virus); DBV (Dabieshan tick virus); SFTSV (Severe fever with thrombocytopenia syndrome virus); UUKV (Uukuniemi virus); YJV (Yongjia tick virus).

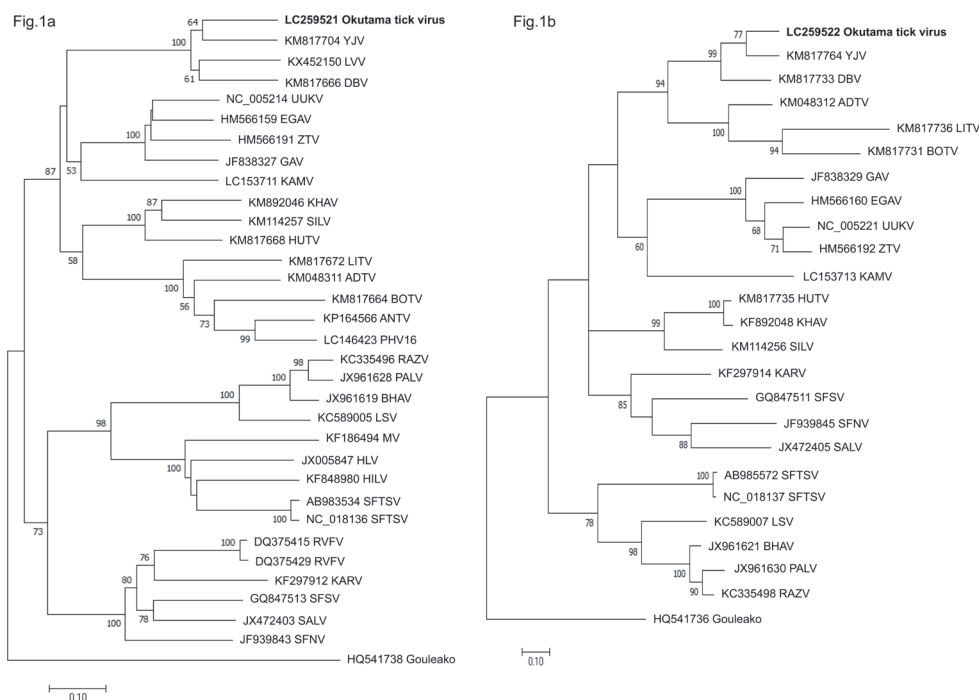


Fig. 1. Maximum Likelihood phylogenetic trees based on a 465-bp nucleotide sequence of the L segment (a) and on a 554-bp nucleotide sequence of the S segment (b). The tests of nucleotide sequences based on the Tamura-Nei model. The numbers at the nodes represent bootstrap values of 1,000 replicates. Bootstrap probabilities above 50% are indicated near the branches. Sequences in the trees are indicated as GenBank accession number and strain name. Sequences of the present study are shown in bold. ADTV (American dog tick virus); ANTV (Antigone tick virus); BHAV (Bhanja virus); BOTV (Bole Tick virus); DBV (Dabieshan tick virus); EGAV (EgAN 1825-61 virus); GAV (Grand Arbaud virus); HILV (Hunter Island Group virus); HLV (Heartland virus); HUTV (Huangpi Tick virus); KARV (Karimabad virus); KAMV (Kabuto Mountain virus); KHAV (Khasan virus); LVV (Lesvos virus); LITV (Lihan Tick virus); LSV (Lone Star virus); MV (Malsoor virus); PALV (Palma virus); PHV16 (Phlebovirus sp. 16); RAZV (Razdan virus); RVFV (Valley fever virus); SALV (Salehabad virus); SFNV (Sandfly fever Naples virus); SFSV (Sandfly fever Sicilian virus); SFTSV (Severe fever with thrombocytopenia syndrome virus); SILV (Silverwater virus); UUKV (Uukuniemi virus); YJV (Yongjia virus); ZTV (Zaliv Terpenia virus). Gouleako virus was used as outgroup.

Differences in the S segments were 23.9 and 32% compared with the Yongjia and Dabieshan tick viruses, respectively. The Okutama tick virus was most distantly related to other viruses in the same cluster. The human health burdens posed by the Yongjia, Lesvos, and Dabieshan tick viruses as well as Okutama tick virus remain to be elucidated. Furthermore, a novel tick-borne phlebovirus, designated as Kabuto Mountain virus (KAMV), was isolated from *H. flava* in Hyogo, Japan. KAMV clustered with the Uukuniemi virus, but not with the Okutama tick virus (Fig. 1a and 1b) [1].

A recent report concerning KAMV and this study provide evidence of undiscovered tick-borne phleboviruses in Japan. Further studies of Okutama tick virus, including virus isolation and characterization, are necessary. The finding of Okutama tick virus in Japan may help classify phleboviruses in further detail and elucidate the evolutionary relationships among phleboviruses.

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