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Isolation of the Thogoto virus from a *Haemaphysalis longicornis* in Kyoto city, Japan.

Running title: Thogoto virus was first isolated from ticks in Japan.

The Content Category: Animal Viruses-Negative-strand RNA

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The GenBank[/EMBL/DDBJ] accession number for the PB2, PB1, PA, GP, NP and Matrix sequence of Thogoto_virus strain Hl-Kamigamo-25 is LC010981, LC010982, LC010983, LC010984, LC010985 and LC010986, respectively.

1

Summary

Ticks transmit viruses responsible for severe emerging and re-emerging infectious diseases, some of which have a significant impact on public health. In Japan, little is known about the distribution of tick-borne viruses. In this study, we collected and tested ticks to investigate the distribution of tick-borne arboviruses in Kyoto, Japan, and isolated the first Thogoto virus (THOV) to our knowledge from a *Haemaphysalis longicornis* in the far-eastern Asia. The Japanese isolate was genetically distinct from the cluster of the other isolates from Africa, Europe and the Middle East. Various cell lines derived from mammals and ticks were susceptible to the isolate, but it was not pathogenic in mice. These results advance understanding of the distribution and ecology of THOV.

Text

Ticks transmit viruses responsible for severe emerging and re-emerging infectious diseases, some of which have a significant impact on public health. Several tick-borne viruses such as tick-borne encephalitis virus (TBEV), Crimean-Congo hemorrhagic fever virus, and African swine fever virus, are known to cause severe clinical symptoms in humans and domestic animals, but most cause asymptomatic infection or a non-specific flu-like syndrome. The diversity of tick-borne viruses has been less thoroughly studied than that of mosquito-borne viruses.

The only tick-borne viruses known to be endemic in Japan are TBEV and severe fever with thrombocytopenia syndrome virus (SFTSV) (Kentaro *et al.*, 2013; Takahashi *et al.*, 2014; Takashima *et al.*, 1997; Yoshii *et al.*, 2011); the distribution of others has not been investigated. In this study, we collected and tested ticks to investigate the distribution of tick-borne arboviruses in Kyoto, Japan, and isolated Thogoto virus (THOV), which has not previously been reported in far-eastern Asia.

To investigate the distribution of tick-borne pathogens, 363 host-questing ticks were captured by sweeping flannel sheets over the vegetation on mountainsides of the northern parts of Kyoto city (N: 35°04’03.29”, E: 135°45’23.13”, Altitude: 140 m) (Table S1). Ticks were collected every week from September, 2013 to August, 2014. Each tick species was determined by its morphological index (Fujita & Takada, 2007; Yamaguti *et al.*, 1971). Individual ticks were disinfected with 70 to 80% ethanol, and washed with PBS three times. Then they were homogenized in Dulbecco’s modified minimum essential medium (DMEM) without antibiotics using pestles. Part of the homogenized suspensions from 50 adult *Haemaphysalis* (*H.*) *longicornis* was individually used for infection of VeroE6 cells to isolate pathogens. Cells inoculated with homogenate from one *H. longicornis* displayed cytopathic effects (CPE) (data not shown). Inoculation of the supernatant of the CPE showing cells into fresh VeroE6 cells or Baby hamster kidney (BHK)-21 cells also induced CPE, and

clear plaque formation was observed in the BHK cells, indicating involvement of an infectious microbe. Induction of CPE was insensitive to the antibiotic penicillin, suggesting that the infectious agent was Rickettsia or a virus.

To identify the microbe, DNA and RNA extracted from infected cells, was subjected to PCR or RT-PCR for rickettsia, TBEV, or SFTSV, which are known tick-borne pathogens endemic in Japan, but no genomic sequence for any of these pathogens was detected in the Vero cells inoculated with the *H. longicornis* homogenate. Thus, we sought to identify the microbe by next generation sequencing (NGS). Supernatant from the inoculated cells was harvested and centrifuged at 160,000 x *g* for 2 h, and the pellet was resuspended in PBS followed by RNA extraction using Isogen-LS (Nippon Gene, Tokyo, Japan). Double-stranded complementary DNA (cDNA) was synthesized from total RNA using random primers and the cDNA Synthesis Kit (M-MLV Version; TaKaRa Bio Inc., Shiga, Japan). NGS analysis was performed on a 454 GS Junior platform (Roche) following the manufacturer's protocol. The resulting reads were *de novo* assembled using the Newbler assembler (Roche) with default settings. Contiguous sequences derived from microorganisms were identified by BLAST search. The average coverage of viral sequences was calculated by mapping raw sequencing reads against target sequences using CLC Genomics Work Bench (CLC Bio, Tokyo, Japan). The pyrosequencing data obtained in this study were deposited in DDBJ with accession no. DRA002619. BLAST analysis identified six contiguous sequences related to the genome of Thogoto virus (THOV) in the family *Orthomyxoviridae*, genus *thogotovirus*. The average coverages for segments 1, 2, 3, 4, 5, and 6 were x130, x188, x107, x209, x138, and x88, respectively (data not shown).

To investigate which tick species carry THOV at the research area, all tick homogenates were used for RNA extraction using ISOGEN (Nippon Gene, Tokyo, Japan) and were subjected to RT-PCR for segment 6 (Matrix protein) using the forward primer 5'-AGCAGCGCCACCTTATTGCT-3' and reverse primer 5'-TCCCTCTGCAGTCATGTACA-3'.

64 The THOV genome was detected in both *H. flava* and *H. longicornis*, and their infection rates were
65 1.92% (2 adult female ticks /104 ticks) and 0.39% (1 adult female tick /259 ticks), respectively.
66
67 The sequence of each segment of the newly isolated THOV (designated as Hl-Kamigamo-25)
68 genome was compared with that of other strains available in GeneBank. Nucleotides and amino acids
69 in the complete open reading frame of each segment of Hl-Kamigamo-25 showed 72-80% and
70 79-91% homology, respectively, with those of the Italian strain SiAr126 (Table 1).
71 A phylogenetic analysis was performed using partial coding sequences of the PB1 (segment 2),
72 PA (segment 3), and glycoprotein (GP: segment 4) genes of THOV strains. Accession numbers of
73 strains used in this study are shown in Table S2. Multiple alignments were generated using ClustalX
74 version 2.1 (Thompson *et al.*, 1997). Phylogenetic trees were generated using MEGA 4
75 (http://www.megasoftware.net/mega.html) by the maximum-likelihood method with 1000 bootstrap
76 replicates. The Japanese isolate Hl-Kamigamo-25 was genetically distinct from the African and
77 European/Middle Eastern isolate clusters (Fig. 1).
78
79 To examine the range of Thogotovirus infectivity, several cell lines were infected with the
80 Hl-Kamigamo-25 strain (Table 2). The virus in the original *H. longicornis* homogenate was
81 amplified once in VeroE6 cells and then once in BHK cells. Subconfluent cells were grown in
82 24-well plates and inoculated with THOV at a multiplicity of infection (MOI) of 0.1 plaque-forming
83 units (pfu). Cells were incubated for over 48 h, and susceptibility to THOV was evaluated via
84 induction of CPE or progeny virus production as measured by titration. All infected cell lines
85 produced progeny viruses regardless of species and tissue type. While HI-Kamigamo-25 showed
86 CPE in most mammalian cells used in this study, it did not cause any pathological change in
87 tick-derived ISE6 cells at time points up to two weeks.
88 European THOV (strain SiAr 126) has been reported to cause lethal infection in adult mice when

given intraperitoneally (LD_{50}: 3 pfu mL^{-1}) (Haller *et al.*, 1995; Pichlmair *et al.*, 2004). To assess the pathogenicity of the Japanese isolate of THOV, 100, 1,000 or 10,000 pfu of the Hl-Kamigamo-25 strain were inoculated intraperitoneally into 8-week-old female BALB/c mice. Animal experiments were performed in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology. Experimental protocols were approved by the Animal Care and Use Committee of Hokkaido University. All mice survived without any symptoms to signs, and high titers of neutralizing antibodies (>1,600) were detected in all surviving mice. These data indicate that the mice were infected, but that this strain's pathogenicity is much lower than that of the European strain in a mouse model.

This survey is the first to identify THOV in far-eastern Asia to our knowledge. THOV belongs to the family *Orthomyxoviridae*, genus *Thogotovirus*, and is a negative-sense, single-stranded RNA virus with six segments (Staunton *et al.*, 1989). THOV has been isolated in Kenya, West Africa, Southern Europe, and Iran (Calisher *et al.*, 1987), and has not been reported in other regions. The Japanese isolate is genetically distinct from other isolates but has high amino acid homology with the Italian isolate. These results indicate the possibility that THOV circulates widely in the Eurasian continent.

Little is known about the ecology of THOV. THOV was first isolated from *Rhipicephalus* species and *Boophilus decoloratus* (Karabatsos, 1985), and has been isolated repeatedly from various tick species (Calisher *et al.*, 1987; Sang *et al.*, 2006). In this study, the Japanese isolates obtained from *Haemaphysalis* ticks, the major tick species in the area, indicating that they might be the principal vectors in the area. THOV is also known to infect domestic animals (cattle and sheep) and a mongoose (Davies *et al.*, 1984; Ogen-Odoi *et al.*, 1999; Sang *et al.*, 2006). However, the primary vertebrate hosts in nature are still unclear. The area in which the Japanese strain of THOV was

isolated contains no domestic animals, but wild animal inhabitants include small rodents, deer, boars, bears, and monkeys. Further ecological research is required to elucidate the mode of natural transmission of the Japanese strain of THOV.

Natural infection with THOV has been reported in two human cases, with one fatality (Moore *et al.*, 1975), and infection in sheep is associated with high rates of abortion (Davies *et al.*, 1984). The pathogenicity of Japanese THOV was not clear, but it appears to have no or very low virulence in mice, in contrast to the Italian isolate, which causes lethal infection in mice (Filipe *et al.*, 1986; Haller *et al.*, 1995; Pichlmair *et al.*, 2004). Although pathogenicity in mice is not directly related to that in humans and other animals, identification of the factors (genes) responsible for varying pathogenicity between strains could help elucidate the pathogenic mechanism of THOV and lead to development of prevention and therapeutic strategies for THOV infection.

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Table 1. Identities of nucleotide (ORF region) and amino acid sequences between THOV Hl-Kamigamo-25 strain and other related viruses

Virus-strain	Viral proteins											
	PB2 (Segment 1)		PB1 (Segment 2)		PA (Segment 3)		GP (Segment 4)		NP (Segment 5)		Matrix (Segment 6)	
	nt (%)	aa (%)	nt (%)	aa (%)	nt (%)	aa (%)	nt (%)	aa (%)	nt (%)	aa (%)	nt (%)	aa (%)
THOV-SiAr126	75.13	86.12	77.87	88.61	72.55	79.13	79.22	90.06	78.68	89.45	75.18	90.82
Jos virus-IBAn-17854	61.63	60.75	68.11	71.77	58.81	53.25	53.78	40.65	62.49	62.2	59.56	49.52
Dhori virus-Dhori/1313/61	53.47	35.34	63.01	61.72	54.61	38.63	51.99	34.55	55.22	33.06	48.86	20.06

197

Table 2. Susceptibility of cultured cells to the THOV Hl-Kamigamo-25 strain

Cell line		CPE	Progeny virus*
BHK	(hamster: kidney)	+	+
Vero E6	(African green monkey: kidney)	+	+
HEK293T	(human: kidney)	n.d.[#]	+
A549	(human: lung)	+	+
NA	(mouse: neuroblastoma)	n.d.	+
ISE6	(*Ixodes scapularis*: ovary)	-	+

* Production of progeny viruses were confirmed by the virus titer in the medium above that of input viruses

[#] Induction of CPE could not be determined due to the short incubation time of the cells.

198

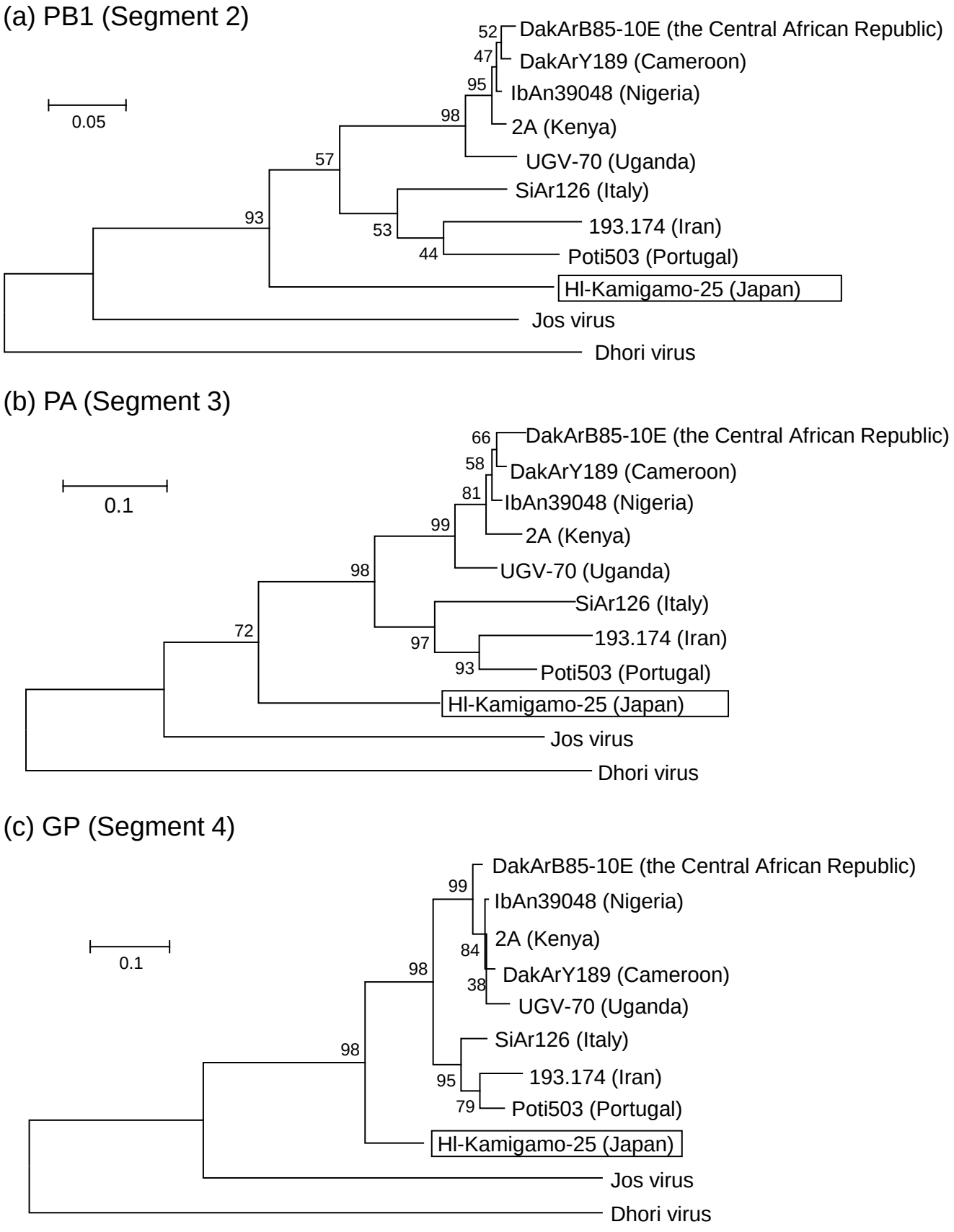
199 Figure legend

200

201 Figure 1. Phylogenetic analysis of nucleotide sequences for partial coding regions of GP (a), PA (b),
202 and PB1 (c). The percentage of trees in which the associated taxa cluster together is shown next to
203 the branches. Bars, 0.02 nucleotide substitutions per site.

204

Figure 1


(a) PB1 (Segment 2)
0.05
52
47
95
98
57
93
53
44
DakArB85-10E (the Central African Republic)
DakArY189 (Cameroon)
IbAn39048 (Nigeria)
2A (Kenya)
UGV-70 (Uganda)
SiAr126 (Italy)
193.174 (Iran)
Poti503 (Portugal)
Hl-Kamigamo-25 (Japan)
Jos virus
Dhori virus
(b) PA (Segment 3)
0.1
66
58
81
99
98
72
97
93
DakArB85-10E (the Central African Republic)
DakArY189 (Cameroon)
IbAn39048 (Nigeria)
2A (Kenya)
UGV-70 (Uganda)
SiAr126 (Italy)
193.174 (Iran)
Poti503 (Portugal)
Hl-Kamigamo-25 (Japan)
Jos virus
Dhori virus
(c) GP (Segment 4)
0.1
99
98
84
38
98
95
79
DakArB85-10E (the Central African Republic)
IbAn39048 (Nigeria)
2A (Kenya)
DakArY189 (Cameroon)
UGV-70 (Uganda)
SiAr126 (Italy)
193.174 (Iran)
Poti503 (Portugal)
Hl-Kamigamo-25 (Japan)
Jos virus
Dhori virus