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Detection of a novel tick-borne flavivirus and its serological surveillance

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ABSTRACT

Tick-borne encephalitis virus (TBEV), a flavivirus that causes severe neurological symptoms in humans, has been found in Hokkaido, Japan. In the present study, we detected sequences from a novel tick-borne flavivirus, designated Yamaguchi virus (YGV), in liver and serum samples obtained from a wild boar in the Yamaguchi prefecture, Japan. Phylogenetic analysis revealed that YGV belongs to the TBEV complex and is closely related to Langat virus (LGTV). YGV was also detected by specific RT-PCR from 20 in 378 pools of ticks (2923 ticks) collected in Yamaguchi and Wakayama prefectures and from seven in 46 wild boar captured in Wakayama. The major ticks infected with YGV belong to the genus *Haemaphysalis*. Unfortunately, YGV could not be isolated from any samples from the RT-PCR positive wild boar or ticks. Therefore, ELISA for detection of antibodies against YGV was established using LGTV, and surveillance was performed among wild boar in 10 different prefectures on Honshu Island, the main island of Japan. The results showed that the seroprevalence of tick-borne flavivirus infection in the Wakayama and Hyogo prefectures of western Japan was significantly higher than that in the other prefectures, while antibodies against tick-borne flavivirus were not detected in any wild boar in the Tochigi prefecture in the eastern part of Japan. In addition, wild raccoons or masked palm civets in the Hyogo prefecture did not possess detectable antibodies against tick-borne flaviviruses. In conclusion, YGV appears to be maintained primarily among wild boar and ticks in the western part of Japan. YGV is the second flavivirus (after Japanese encephalitis virus) shown to be circulating on Honshu Island in Japan.

1. Introduction

Tick-borne encephalitis virus (TBEV) is a member of the genus *Flavivirus* in the family *Flaviviridae*; this virus causes serious disease in humans, potentially resulting in encephalitis or meningoencephalitis with severe sequelae (Gritsun et al., 2003). In 1993, the first tick-borne encephalitis (TBE) case was confirmed in Hokkaido prefecture, Japan, followed by 2 fatal cases in Hokkaido in 2016 and 2017 (IASR, 2017; Takashima et al., 1997). TBEV also has been isolated from dogs, ticks, and rodents in the area where the first TBE patient was found (Takashima et al., 1997; Takeda et al., 1998, 1999). On the other hand, although no human case has been reported on Honshu Island, Japan, some rodents that were seropositive for TBEV have been detected by ELISA in the Shimane prefecture on Honshu Island, the main island of Japan (Yoshii et al., 2011). Additionally, the Negishi virus was isolated from a fatal case of encephalitis in Tokyo (Ando et al., 1952); antigenic analysis subsequently identified the Negishi virus as a member of the TBE complex. The nucleotide sequence of the Negishi virus, which was determined several decades after its discovery, revealed that the Negishi virus shows high homology with the louping ill virus (LIV) and could be classified as a strain of LIV (Venugopal et al., 1992). However, little information is available regarding the distribution of tick-borne flaviviruses on Honshu Island, Japan.

Japanese encephalitis (JE) virus, which is a mosquito-borne flavivirus, causes severe encephalitis in humans and is broadly endemic on Honshu Island. Although the annual number of human JE cases have

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fewer than 10 since 1992, as the result of an ongoing vaccination program, our surveillance of domestic and wild animals indicated that JEV remains endemic in Japan, especially in the western part of Japan (Hamano et al., 2007; Ohno et al., 2009; Shimoda et al., 2010, 2014). In 1971, a novel flavivirus, Yokose virus, was isolated in Oita, Japan, from a *Miniopterus fuliginosus* bat. Yokose virus is closely related to Entebbe bat virus, Sokuluk virus, Sepik virus, and yellow fever virus (Kuno et al., 1998). JEV, which is endemic to Honshu Island, is the main flavivirus found on this island; however, the etiology of the other flaviviruses in Japan remains unclear.

Screening of blood from dogs in TBEV-affected areas of Japan showed that the sera had strong cross-reactivity against Langat virus (LGTV) and weaker cross-reactivity against JEV (Takashima et al., 1997). Mice immunized with LGTV or JEV produced IgM and IgG antibodies that were cross-reactive to the other virus (Garciá García et al., 1994). Antibodies raised against JEV or Negishi virus were cross-reactive against the other virus by ELISA, but not by a virus-neutralization (VN) test or by a complement fixation test (Miyata et al., 1982; Okuno et al., 1961). Human sera immunized with yellow fever virus cross-reacted with Yokose virus, and exhibited some VN activity against Yokose virus (Tajima et al., 2005). Considered together, these data indicate the importance of assessing immune cross-reactivity among flaviviruses when performing serological surveillance of flavivirus infections in Japan.

To elucidate flavivirus infections in Japan, we surveyed for flaviviruses among wild boar in the Yamaguchi Prefecture (the western part of Honshu Island) by RT-PCR using flavivirus consensus primers. As we report here, we detected a novel flavivirus from the serum and liver samples obtained from one wild boar.

2. Materials and methods

2.1. Collection of samples from wild mammals

Serum samples were collected from wild boar (*Sus scrofa*), raccoons (*Procyon lotor*), and masked palm civets (*Paguma larvata*). A total of 743 sera were collected from wild boar captured in the Tochigi (n = 123), Chiba (n = 30), Toyama (n = 78), Gifu (n = 76), Hyogo (n = 67), Wakayama (n = 90), Kagawa (n = 16), Ehime (n = 25), Yamaguchi (n = 208), and Oita (n = 30) prefectures, and 206 and 71 sera were collected from raccoons and masked palm civets, respectively, in the Hyogo prefecture (Fig. 1). The blood samples of wild boar, raccoons and masked palm civets were used in this study. These wild boar were hunter-harvested or culled for nuisance control under the program of prevention from the bird and animal damages by the local governments from 2007 to 2016. Wild raccoons and masked palm civets were captured and culled for invasive pest control in Wakayama and Hyogo prefectures under the program of prevention from the bird and animal damages by each local government from 2009 to 2014. Blood was collected directly from their heart after the death by shooting or by carbon dioxide. No animals were killed only for this study. Hunters received permission from each local government. All collected sera were stored at −20 °C until use. Livers were collected from 48 wild boar captured in the Yamaguchi prefecture in 2012 and stored at −80 °C until use.

2.2. Collection of ticks

Ticks were collected from vegetation by flagging in the Wakayama and Yamaguchi prefectures from February 2015 to March 2016 (Fig. 1). In these areas, no specific permission was required for collection of ticks, and this study did not involve endangered or protected species. The collected ticks were identified to the species level and stage based on their morphological features (Yamaguti et al., 1971). The collected ticks in this study were listed in the Tables 1 and 2.

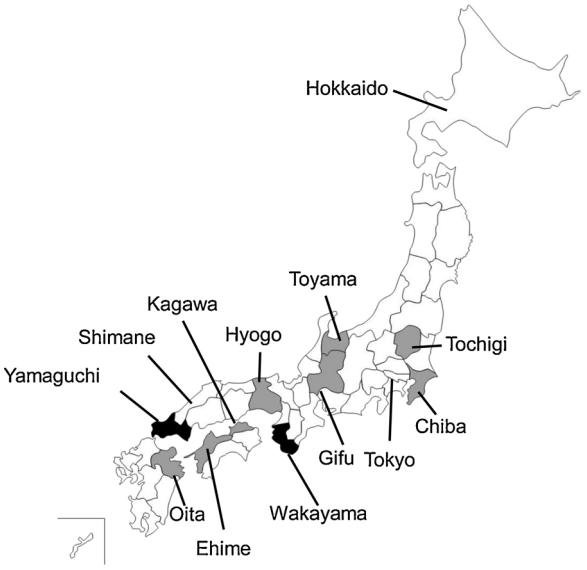

Fig. 1. Collection sites of ticks and wild boar in Japan. The black and gray regions in the map indicate collection sites of both ticks and wild boar, and of wild boar, respectively.

Table 1
Detection of Yamaguchi virus from ticks in Yamaguchi prefecture.

Species	Stages	Number of Samples	Number of pools	Number of positive
Ixodes ovatus	Female	1	1	0
Ixodes turdus	Female	12	5	0
	Nymph	77	6	0
	Larva	102	3	0
Haemaphysalis flava	Male	19	10	0
	Female	23	10	0
	Nymph	459	31	0
Haemaphysalis formosensis	Male	4	2	0
	Female	4	3	0
Haempahysalis hystricis	Male	10	5	0
	Female	2	2	0
	Nymph	2	2	0
Haemaphysalis longicornis	Male	2	2	1
	Female	67	24	0
	Nymph	725	44	3
	Larva	19	2	0
Haemaphysalis spp.	Larva	150	3	0
Total		1678	155	4

2.3. Cells

BHK-21 cells (JCRB number: JCRB9020), which are derived from baby hamster kidney cells, were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, NY, U.S.A) supplemented with 10% heat-inactivated fetal calf serum (FCS; Hyclone Laboratories, UT, USA), 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37 °C in 5% CO_2. *Aedes albopictus* mosquito-derived C6/36 cells (JCRB number: IFO 50,010) were cultured at 28 °C and 5% CO_2 in DMEM supplemented with 10% heat-inactivated FCS, 100 U/ml of penicillin and 100 μg/ml of streptomycin.

2.4. Viruses

JEV/sw/Chiba/88/2002, which was isolated from the peripheral blood mononuclear cells of a healthy pig in 2002 (Nerome et al., 2007), was kindly provided by Dr. Takasaki of the National Institute of Infectious Diseases. The virus was propagated in C6/36 cells in DMEM supplemented with 2% FCS at 28 °C. The LGTV TP-21 strain was

Table 2
Detection of Yamaguchi virus from ticks in Wakayama prefecture.

Species	Stage	Number of samples	Number of pools	Number of positive
Amblyomma testudinarium	Male	1	1	0
	Nymph	64	22	0
Dermacentor taiwanesis	Male	3	3	0
	Female	2	2	0
Ixodes ovatus	Male	6	5	0
	Female	6	5	0
Ixodes turdus	Nymph	2	1	0
Haemaphysalis cornigera	Male	6	3	0
	Female	4	3	0
	Nymph	32	10	0
Haemaphysalis flava	Male	70	20	3
	Female	67	17	3
	Nymph	249	20	1
Haemaphysalis formosensis	Male	27	10	0
	Female	24	7	0
	Nymph	117	14	3
Haemaphysalis hystricis	Male	2	2	0
	Female	7	5	0
Haemaphysalis kitaokai	Male	26	12	0
	Female	26	10	0
Haemaphysalis longicornis	Male	19	7	1
	Female	6	2	0
	Nymph	281	17	2
Haemaphysalis megaspinosa	Male	14	6	1
	Female	18	6	0
	Nymph	166	13	2
Total		1245	223	16

isolated from *Ixodes granulatus* ticks in Malaysia (Smith, 1956) and propagated in BHK-21 cells in DMEM supplemented with 2% FCS at 37 °C. All viruses were stored at −80 °C until use.

2.5. VN test

Heat-inactivated wild boar sera were subjected to serial 2-fold dilutions (ranging from 1:5 to 1:320) in DMEM containing 10% FCS and distributed at 50 µl/well in duplicate wells in 96-well culture plates (Sumitomo Bakelite, Inc., Tokyo, Japan). An equal volume of 100 $TCID_{50}$ of LGTV or JEV diluted in DMEM containing 10% FCS was added to each well, and the plates were incubated for 1.5 h at 37 °C in a 5% CO_2 incubator. After incubation, 100 µl of BHK-21 cell suspension (2×10^5 cells/ml) was added to each well, and the plates were incubated at 37 °C for 5 days. The cells then were observed every day for cytopathic effect (CPE). The VN titer was defined as the reciprocal of the highest serum dilution that inhibited the CPE in both wells.

2.6. ELISA

JEV-, LGTV-, or mock-infected BHK-21 cells were lysed with RIPA [25 mM Tris-HCl (pH 7.6), 150 mM sodium chloride, 1% sodium dodecyl sulfate, 1% sodium deoxycholate, and 1% Triton X-100] and used as antigens for ELISA (Suzuki et al., 2015). The concentration of antigens was determined using Protein Assay (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions, and was adjusted to 5 µg/ml with adsorption buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6). Diluted antigen was added to 96-well microplates at 100 µl/well (Maxisorp; Nunc, Roskilde, Denmark). After incubation at 37 °C for 2 h, plates were placed at 4 °C until use. Non-adsorbed antigens were discarded; 1% Block Ace (Dainippon Pharmaceutical, Osaka, Japan) in PBS then was added at 200 µl/well and the plates were incubated at 37 °C for 30 min. After washing the wells three times with PBS containing 0.05% Tween 20 (PBS-T), diluted serum (1:100 in PBS-T containing 0.4% Block Ace) was added at 100 µl/well to duplicate wells and the plates were incubated at 37 °C for 30 min. Subsequently, wells were washed three times with PBS-T before addition of 100 µl/well of Peroxidase Conjugated Purified Recomb® Protein A/G (Thermo Fisher Scientific, Waltham, MA, U.S.A.) diluted with PBS-T containing 0.4% Block Ace, and plates were incubated at 37 °C for 30 min. Following three washes with PBS-T, 100 µl of Horseradish Peroxidase Substrate (Bio-Rad) was added to each well. After incubation at room temperature for 30 min, the enzymatic reaction was stopped by adding 100 µl of 2% oxalic acid to each well. The absorbance was measured using a spectrophotometer (Bio-Rad) with a 415-nm filter. The resulting values were corrected by subtracting the value of control wells with extract from mock-infected cells.

2.7. Semi-nested RT-PCR for detection of flavivirus genes

RNA was extracted from homogenates of liver samples using an RNeasy Mini Kit (QIAGEN, Hilden, Germany). RT-PCR was performed using a QIAGEN OneStep RT-PCR Kit (QIAGEN) and flavivirus consensus primers targeting NS5 protein-encoding sequences (Scaramozzino et al., 2001), cFD2 (5′-GTG TCC CAG CCG GCG GTG TCA TCA GC-3′) and MAMD (5′-AAC ATG ATG GGR AAR AGR GAR AA-3′), for the first PCR. The RT-PCR conditions were as follows: an initial step of 30 min at 50 °C for reverse transcription; 15 min at 95 °C for denaturation; 40 cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 1 min, and extension at 72 °C for 1 min; and a final extension step at 72 °C for 10 min. The second PCR was performed using TaKaRa Ex Taq (Takara Bio Inc., Shiga, Japan) and primers cFD2 and FS778 (5′-AAR GGH AGY MCD GCH ATH TGG T-3′) under the following conditions: 1 min at 94 °C for denaturation; 40 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min; and final extension step at 72 °C for 10 min. PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and visualized under UV illumination. The length of products amplified by RT-PCR using flavivirus consensus primer set targeting NS5 was 215 bp.

2.8. Nested RT-PCR for detection of Yamaguchi virus

RNA was extracted from serum samples of wild boar and tick samples homogenized using a Multi-beads Shocker (Yasui Kikai Co., Osaka, Japan) using a QIAamp® Viral RNA Mini Kit (QIAGEN, Hilden, Germany). For detection of Yamaguchi virus (YGV), YGV-specific primer pairs were designed based on the identified sequences, yielding an outer primer pair consisting of WB1267-1 F (5′- ACA TGT GGC TAG GGA GCC GT-3′) and WB1267-1R (5′-TAG AAC AGC CCT CCC GTG GT-3′), and an inner primer pair consisting of WB1267-2 F (5′-AGG GAG CCG TTT CTT GGA AT-3′) and WB1267-2R (5′-TGG TGG CTG CCA AGT TCT TC-3′). RT-PCR and nested PCR were performed as above but with annealing temperature of 64 °C and 60 °C, respectively. The length of products amplified by nested RT-PCR was 140 bp.

2.9. Sequence analysis

Amplified fragments were purified using a MinElute® PCR Purification Kit (QIAGEN, Germantown, MD, U.S.A.) and directly sequenced using a BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Austin, TX, U.S.A.) according to the manufacturer's instructions. Nucleotide sequences of YGV and strains detected from wild boar and ticks were deposited in the DDBJ/EMBL/GenBank databases under accession no. LC390172-LC390199.

2.10. Phylogenetic analysis of detected flaviviruses

Phylogenetic analysis was performed using the MEGA 7 program (Kumar et al., 2016). Sequence data were aligned using the ClustalW method (Thompson et al., 1994). Genetic distances were calculated using the p-distance model (Nei and Kumar, 2000), and the phylogenetic tree was constructed using neighbor-joining methods (Saitou and Nei, 1987); the reliability of the branching was evaluated by

bootstrapping with 1000 replicates (Felsenstein, 1985).

2.11. Statistical analysis

Fisher's exact test was performed to assess statistically the seroprevalence of JEV and LGTV infections using ELISA, and Pearson's correlation coefficient was calculated to determine the correlation between the VN test and ELISA. p values of < 0.05 were considered statistically significant.

3. Results

3.1. Detection of a novel flavivirus from wild boar

Liver samples from 48 wild boar were used for the detection of flavivirus genes. RNA was extracted from liver samples, and flavivirus genes were detected by semi-nested RT-PCR using flavivirus consensus primer pairs. As a result, a novel flavivirus gene was detected from one male piglet (body weight 12.5 kg) captured in December 2012 in Shimonoseki, Yamaguchi prefecture. Phylogenetic analysis of the detected gene revealed that this sequence belonged to the TBEV complex and was closely related to LGTV (Fig. 2). Pairwise sequence comparison among tick-borne flavivirus also revealed that detected virus was most closely related to LGTV by amino acid, and distinct from the other tick-borne flaviviruses (68.9%–77.8% nucleotide sequence homology) (Table 3). The detected virus was designated as Yamaguchi virus (YGV). Attempts to isolate YGV from wild boar samples and ticks were unsuccessful.

3.2. Detection of YGV from ticks and wild boar

Nested RT-PCR using YGV-specific primer pairs was carried out using ticks collected in the Yamaguchi and Wakayama prefectures. As a result, YGV was detected from ticks captured in both the Yamaguchi (4 pools out of 155 pools; total of 1678 ticks) and Wakayama (16 pools out of 223 pools; total of 1245 ticks) prefectures (Tables 1 and 2). Only one species, *Haemaphysalis* longicornis, tested positive for YGV in the Yamaguchi prefecture; in contrast, four species (H. flava, H. longicornis, H. formosensis and H. megaspinosa) tested positive in the Wakayama prefecture. Minimal infection rates of ticks with YGV were 0.2% and 1.3% in Yamaguchi and Wakayama, respectively. Semi-nested RT-PCR for YGV also was performed using 46 sera from wild boar captured in the Yamaguchi prefecture in 2014, revealing that seven (15%) were positive for YGV (data not shown).

3.3. Determination of cut-off value of ELISA

Given our inability to isolate YGV in field samples, we instead used serological screening based on the serological similarity of YGV to LGTV. In addition, since JEV has been spreading among wild animals in Japan (Hamano et al., 2007; Ohno et al., 2009; Sugiyama et al., 2009), cross-reaction of antibody against JEV had to be considered in any sero-survey of LGTV infection. Therefore, serological surveys of both LGTV and JEV infections were performed as part of the study described here. Although the VN test is the "gold standard" for the serological surveillance for flavivirus infection (Maeda and Maeda, 2013), the VN test requires a large volume of clear serum samples. The volume of some sera from wild animals were not sufficient for the VN test and not suitable for VN test because of hemolysis. Therefore, we established an ELISA for surveillance of JEV and LGTV. To determine the cut-off value, 160 sera of wild boar from the Hyogo and Tochigi prefectures were examined by ELISA and by VN test for both JEV and LGTV. Using the results from the VN test, we analyzed the sensitivity and specificity of the ELISA to optimize predictive accuracy (Kanda, 2013). The ELISA cut-off values were calculated as 0.623 and 0.622 for JEV and LGTV, respectively (Fig. 3).

3.4. Comparison of ELISA and VN test

To investigate the specificity and sensitivity of ELISA for JEV and LGTV, 54 serum samples collected from wild boar in the Hyogo prefecture were examined by both ELISA and VN test. Of 38 serum samples positive for JEV by VN test, 33 and 5 tested positive and negative for JEV ELISA, respectively. Of 16 serum samples judged negative for JEV

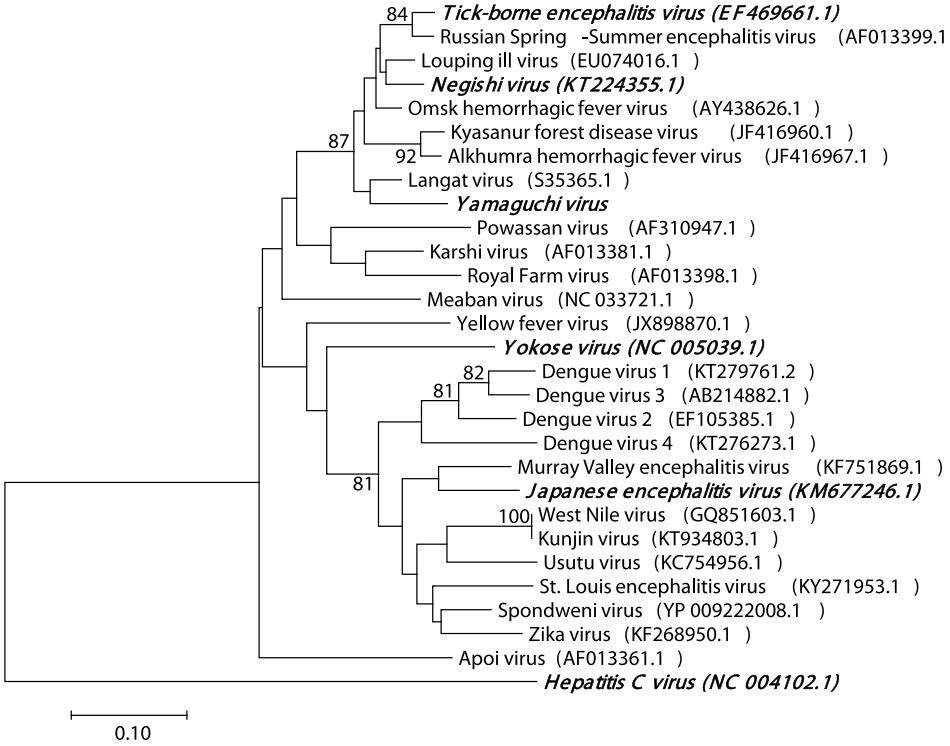

Fig. 2. Phylogenetic tree based on the partial amino acid sequences of nonstructural protein 5. This phylogenetic tree was constructed based on 55 amino acids of NS5. Sequences for the YGV NS5 are shown in bold. Sequences for other flaviviruses detected in Japan are shown in bold and italics. GenBank accession numbers of the listed viruses are shown in parentheses.

Table 3
Pairwise sequence comparison of the partial genome of NS5 protein.

	YGV[a]	LGTV	LIV	OHFV	NEGV	TBEV	AHFV	KFDV
LGTV	91% (76.6%)[b]	–	–	–	–	–	–	–
LIV	87% (76.6%)	93% (79.0%)	–	–	–	–	–	–
OHFV	87% (75.4%)	93% (79.6%)	95% (82.6%)	–	–	–	–	–
NEGV	85% (77.2%)	91% (78.4%)	95% (92.8%)	95% (81.4%)	–	–	–	–
TBEV	85% (77.8%)	89% (76.0%)	93% (82.0%)	93% (78.4%)	91% (82.0%)	–	–	–
AHFV	85% (75.4%)	87% (79.6%)	91% (77.8%)	91% (79.6%)	87% (77.2%)	89% (74.9%)	–	–
KFDV	84% (77.2%)	87% (78.1%)	87% (77.8%)	91% (83.2%)	87% (77.2%)	89% (74.9%)	96% (91.6%)	–
POWV	76% (68.9%)	75% (71.3%)	75% (73.1%)	75% (73.1%)	73% (73.7%)	75% (73.7%)	75% (71.3%)	73% (67.1%)

[a] Each abbreviation of virus is listed: YGV = Yamaguchi virus, LGTV = langat virus, LIV = Louping ill virus, OHFV = Omsk hemorrhagic fever virus, NEGV = Negishi virus, TBEV = Tick-borne encephalitis virus, AHFV = Alkhurma hemorrhagic fever virus, KFDV = Kyasanur forest disease virus and POWV = Powassan virus.
[b] The number without and with parentheses indicate percentage of homology of amino acid (55aa) and nucleotide (167bp), respectively.

by VN test, none tested positive by the JEV ELISA (Fig. 3a). Of 40 serum samples judged positive for LGTV by VN test, 37 and 3 tested positive and negative for LGTV by ELISA, respectively. Of 14 serum samples judged negative for LGTV by VN test, only one was positive in the LGTV ELISA (Fig. 3b). In comparison with the VN test, the sensitivity and specificity of ELISA were 87% and 100% (respectively) for JEV, and 93% and 93% for LGTV. The correlation coefficient between ELISA and VN test was 0.801 for JEV ($n = 54$, $p < 0.01$) and 0.662 for LGTV ($n = 54$, $p < 0.01$). These results indicated that this ELISA was applicable for screening of JEV and LGTV, and could distinguish infection by these two viruses.

3.5. Serological surveillance of Yamaguchi virus in Japan

Sera from 743 wild boar captured in 10 different prefectures were tested for antibodies against JEV and LGTV by ELISA. The results showed that 19% of wild boar in Yamaguchi, 36% in Wakayama, 57% in Hyogo, 7% in Oita, 16% in Ehime, 12% in Gifu, 3% in Toyama, and 3% of Chiba were seropositive for LGTV, while no positive wild boar were found in the Kagawa and Tochigi prefectures (Table 4). Additionally, wild boar that are positive only for LGTV, but not for JEV, were found in many prefectures, indicating that these wild boar have antibodies against LGTV, but not cross-reactive antibodies against JEV. Seroprevalence of anti-LGTV antibodies among wild boar in the Hyogo and Wakayama prefectures were significantly higher ($p < 0.05$) than in the other prefectures. Interestingly, sera from 206 raccoons and 71 masked palm civets captured in the Hyogo prefecture, where high seroprevalence of LGTV infection was observed among wild boar, also were tested for LGTV. This screen detected no raccoons or masked palm civets that were positive for antibody against LGTV, even among those animals harboring anti-JEV antibody (data not shown).

Table 4
Seroprevalence of JEV and LGTV among wild boar in Japan.

Prefectures	Year of collection	No. of animals	No. of positive animals (%)		
			Only JEV[a]	Only LGTV[b]	Both[c]
Yamaguchi	2012–2016	208	81(39%)	11(5%)	29(14%)
Tochigi	2011–2012	123	0(0%)	0(0%)	0(0%)
Wakayama	2007–2014	90	19(21%)	19(21%)	13(14%)
Hyogo	2011–2015	67	6(9%)	11(16%)	27(40%)
Oita	2011–2012	30	8(27%)	2(7%)	0(0%)
Ehime	2016	25	14(56%)	0(0%)	4(16%)
Kagawa	2016	16	1(6%)	0(0%)	0(0%)
Gifu	2014–2016	76	3(4%)	6(8%)	3(4%)
Toyama	2014–2015	78	12(15%)	1(1%)	1(1%)
Chiba	2015	30	14(47%)	1(3%)	0(0%)

[a] Wild boar that are seropositive against only JEV (OD > 0.623).
[b] Wild boar that are seropositive against only LGTV (OD > 0.622).
[c] Wild boar that are seropositive against both JEV and LGTV.

4. Discussion

In this study, we successfully detected a novel tick-borne flavivirus (designated Yamaguchi virus) that is closely related to LGTV; YGV was detected in the Yamaguchi prefecture on Honshu Island, Japan. To our knowledge, this result represents the first report of an epizootic tick-borne flavivirus infection in Japan outside of the Hokkaido prefecture.

In this study, we detected only partial NS5 gene in spite of the attempt on detecting the other genome using many flavivirus-consensus or tick-borne flaviviruses-specific primer pairs. Pairwise sequence

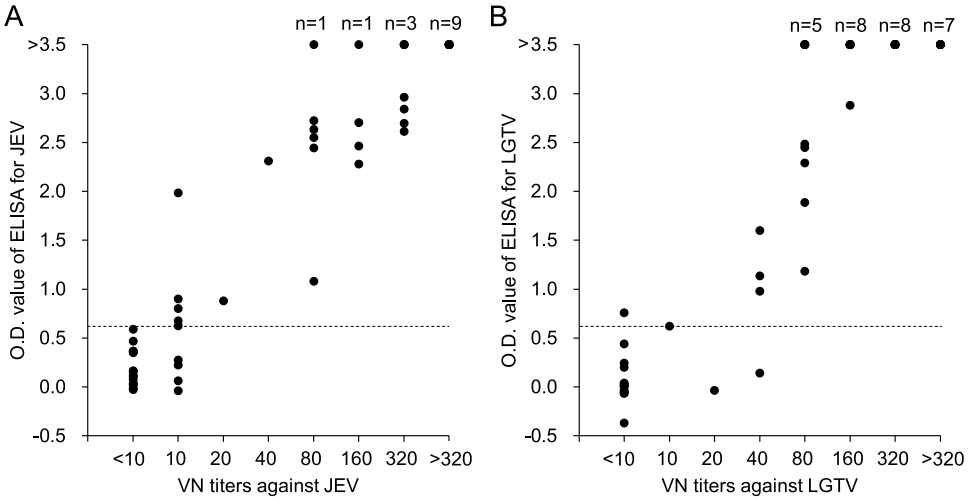

Fig. 3. Comparison of VN test and ELISA against JEV and LGTV. Dot plot analysis of VN titers and optical density (O.D.) values for JEV (A) and LGTV (B) among wild boar in the Hyogo prefecture (n = 54). X- and Y-axes indicate VN titers and O.D. values, respectively. Dotted lines indicate the tentative cut-off values (JEV: O.D.value = 0.623, LGTV: O.D. value = 0.622). The numbers above dots indicate the number of samples that showed absorbance of over 3.5 and same VN titer.

comparison among tick-borne flaviviruses revealed that YGV is distinct from the other tick-borne flaviviruses, suggesting that YGV might be the novel tick-borne flavivirus. However, the gold standard of phylogenetic analysis of flaviviruses is performed using E protein. Also, the previous study reported that Jingmen tick virus, segmented tick-borne virus containing partial flavivirus in its one segment (Qin et al., 2014). Therefore, we have to perform further research including virus isolation of YGV to confirm the phylogenetic relationship with the other tick-borne flaviviruses.

LGTV has been isolated from *I. granulatus* ticks infesting rodents (in Malaysia) and from *H. papuana* ticks collected by flagging (in Thailand) (Bancroft et al., 1976; Smith, 1956). Although LGTV is not known to cause any diseases in rodents in their natural environment, young laboratory mice inoculated intracerebrally with this virus were shown to develop encephalitis (Chiba et al., 1996). Anti-TBEV antibodies were detected in human sera in Malaysia, suggesting LGTV infection in humans in that country, given that LGTV, but not TBEV, is endemic in Malaysia (Muhd Radzi et al., 2015). Although LGTV is completely nonvirulent in primates following intracerebral inoculation, a small number of cases of encephalitis have been reported in humans following vaccination with live–attenuated LGTV-based vaccines; this encephalitis was similar to that observed in patients infected with the typical European strain of TBEV (Gritsun et al., 2003; Price et al., 1970). We were unable to isolate (from the serum or liver of YGV-positive wild boar, or from YGV-positive ticks) YGV using Vero and BHK-21 cell lines or suckling mice. Further attempts for isolation of YGV using the other cell lines, such as tick-derived ISE6 cell line, as well as analysis of YGV sequences and of YGV-infected animals, will be needed to clarify this virus' virulence and pathogenicity in humans.

Our serological surveillance of LGTV detected no LGTV-positive wild boar in the Tochigi prefecture, a region located on the eastern part of Honshu Island, despite this virus' high prevalence in the western part of Honshu Island. In addition, a high prevalence of LGTV infection (56%) was observed among wild boar, but not raccoons and masked palm civets, in the Hyogo prefecture. Therefore, these findings suggested that YGV, like LGTV, appears to be epizootic primarily in the western part of Japan, and that this virus appears to be maintained among ticks and wild boar. Consequently, YGV appears not to be able to distribute in Hokkaido, given the lack of wild boar in Hokkaido. A positive serology of a given tick host, here wild boar, is not indicative of its reservoir competence for the microorganism involved. This can only be shown by carrying out a conclusive transmission experiment.

Cross-reactions among flaviviruses have been reported in many previous studies. On Honshu Island, JEV has been epidemic during every summer season. In 1994, serological examinations of dogs from TBEV-affected areas of Japan showed strong cross-reaction against LGTV, but weaker cross-reaction against JEV (Takashima et al., 1997). Since many pigs and wild boar were infected with JEV and possessed antibody against JEV, the anti-JEV antibody in these animals may have exhibited cross-reactivity with LGTV, TBEV, and YGV. However, our results indicated that our established ELISA could distinguish between LGTV and JEV infection as well as the VN test does. Notably, for this ELISA, the antigens were prepared in RIPA to ensure maintenance of protein structure, and sera were highly diluted (1:100). This processing may have kept the cross-reactivity low enough to remain below the limit of detection. We expect that our novel ELISA will be of great value for screening JEV and tick-borne virus infections in Japan, in place of the VN test.

Many wild boar in the western part of Japan were seropositive for LGTV, indicating that YGV, but not LGTV, appears to be epizootic mainly in the western part of Japan. YGV was detected in *Haemaphysalis* ticks, including *H. flava*, *H. longicornis*, *H. formosensis*, and *H. megaspinosa*. Since these *Haemaphysalis* ticks are found throughout Japan (Yamaguti et al., 1971), YGV infection may be able to spread in the eastern part of Japan.

TBEV is transmitted primarily by *Ixodes* ticks, and rodents are thought to be a natural host. LGTV has been isolated from both *Ixodes* and *Haemaphysalis* ticks collected from rodents (Gritsun et al., 2003). In the present study, we successfully detected a novel flavivirus (designated YGV) in questing *Haemaphysalis* ticks and wild boar. These data suggested that YGV appears to be maintained by a reservoir of mammals and ticks distinct from that used by TBEV and LGTV. Further analysis will be needed to clarify the transmission cycle of YGV in the field.

5. Conclusions

We showed strong evidence for the presence of a novel tick-borne flavivirus in Japan outside of the Hokkaido area.

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Conflict of interest

The authors declare no conflict of interest.

Ethical statements

All animal samples used in this study were collected under hunting permits issued by the respective local government. This article does not contain any studies with live animals.

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