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Survey of tick-borne zoonotic viruses in wild deer in Hokkaido, Japan

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ABSTRACT. Tick-borne encephalitis (TBE) and severe fever with thrombocytopenia syndrome (SFTS) are both tick-borne zoonotic diseases caused by TBE virus (TBEV) and SFTS phlebovirus (SFTSV). In 2016, a second domestic TBE case was reported in Hokkaido, Japan, after an absence of 23 years. We conducted IgG ELISA for TBEV and SFTSV on 314 deer (*Cervus nippon yesoensis*) serum samples collected from 3 places in Hokkaido. There were 7 seropositive samples for TBEV but none for SFTSV by ELISA. The specificity of the 7 positive samples was confirmed by neutralization tests against TBEV, and 5 sera showed 320 to 640 of 50% focus reduction endpoint titers. Our results provide information about the infectious status of TBEV in wild deer in Hokkaido, Japan.

KEY WORDS: serosurvey, severe fever with thrombocytopenia syndrome virus, tick-borne encephalitis virus, wild deer, zoonotic disease

Tick-borne encephalitis (TBE) is one of the zoonotic diseases caused by tick-borne encephalitis virus (TBEV), which belongs to family *Flaviviridae*. The virus is clustered into three subtypes: Western or European subtype, Siberian subtype, and Far Eastern subtype [4]. All subtypes can cause symptoms ranging from a mild fever to severe neurological disorders, depending on the subtypes. In 1993, the first domestic human case of TBE in Japan was found in the southern area of Hokkaido and was caused by the Far Eastern subtype [19]. Virus has been isolated from ticks, sentinel dogs and small rodents in the same region [19–21]. Over the next 23 years, there were no confirmed human cases of TBE in Japan; however, more recently, 3 cases have been reported in 2016 and 2017 in Hokkaido [8, 9]. TBE is becoming a public health concern in Hokkaido, and the assessment of the distribution of TBEV is urgently required.

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging tick-borne infectious disease, which was first reported from China in 2011 [27]. The causative agent, SFTS phlebovirus (SFTSV) belongs to the order *Bunyavirales*, family *Phenuiviridae*. Thrombocytopenia is observed as a common symptom of SFTS in humans and mortality rates range from 6.3 to 30.0% [14]. In people, domestic SFTS human cases have been reported in western Japan, mainly where there is an overlap in the distribution of several seropositive wild animals, such as deer, boars and raccoons that are known to be natural hosts of the virus [3, 7]. These wild animals and ticks are considered to contribute to the circulation of SFTSV in the environment.

Wild deer (*Cervus* spp.) are known to be susceptible to the both viruses and serosurveys have been conducted using deer sera to evaluate the virus distribution [2, 7, 17]. TBEV infection of wild deer has been reported in Austria [2], Denmark [17], Germany [1], Norway [25], and Croatia [10]. However the infectious status of wild deer for TBEV has not been elucidated in Hokkaido, Japan. In this study, we aimed to investigate the prevalence of antibodies against TBEV and SFTSV, using wild deer sera. Our data provide the information about seroprevalence of these viruses in the wild deer population in the study sites.

Sera from wild deer (*Cervus nippon yesoensis*) were collected between 2011 and 2016, from 3 places in Hokkaido, Japan (Fig. 1). In total 314 samples, 100 from Toyotomi, 100 sera from Hidaka and 114 sera from 5 towns in the eastern area of Hokkaido, were collected and kept at −30°C until further use. BHK-21 and mosquito-derived C6/36 cells were maintained in a minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and 0.2 mM nonessential amino acids. The BHK-21 cells were grown at 37°C with 5% CO_2, and the C6/36 cells were grown at 28°C without CO_2. TBEV strain Oshima [19]
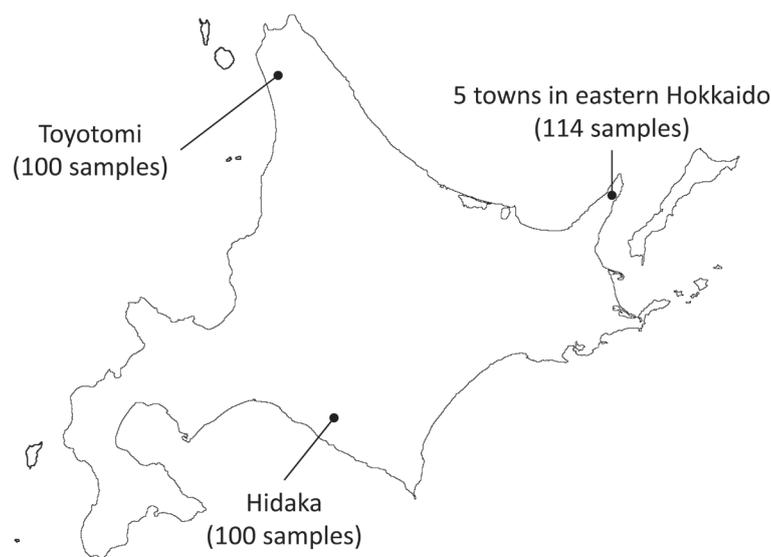
Fig. 1. Map of Hokkaido Prefecture showing the sample collection sites and number of samples collected at each site.
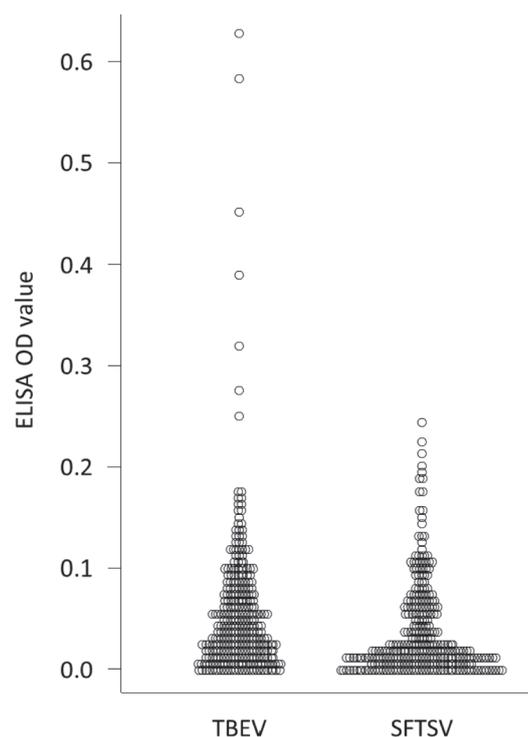

Fig. 2. Distribution of OD values obtained by IgG indirect ELISA. The dots indicate the OD values at 415 nm of IgG indirect ELISA for Tick-borne encephalitis virus (TBEV) and Severe fever with thrombocytopenia syndrome virus (SFTSV). OD: optical density. Means of OD value were obtained from duplicate experiments.

and JEV strain JaOArS982 [18] were propagated in BHK-21 and C6/36 cells, respectively. Virus titers were determined by focus assay using BHK-21 cells, as described in a previous study [23].

IgG indirect ELISA was performed according to previous studies [22, 26]. In brief, 250 *n*g/well of formalin-inactivated whole TBEV [22], 100 *n*g/well of recombinant SFTSV nucleocapsid [26], or 50-times diluted commercial Japanese encephalitis virus (JEV) vaccine (Mitsubishi Tanabe Pharma Corp., Osaka, Japan) were diluted in the ELISA coating buffer (15 mM Na_2CO_3, 34.8 mM $NaHCO_3$ and 0.02% NaN_3) and incubated on the plate (Cat No. 3355, Thermo Fisher Scientific, Waltham, MA, U.S.A.), at 4°C for overnight. Five hundred times diluted sera in 0.4% Block Ace (DS Pharma Biomedical Co., Osaka, Japan) were used for the test. The deer IgG was detected by 250 times diluted HRP-conjugated anti-deer antibody (SeraCare, Milford, MA, U.S.A.), and 2,2'-azino-di-[3-ethylbenzthiazoline sulfonic acid] (ABTS) was used for the substrate. Twenty min after adding the substrate, the optical density (OD) value at 415 nm was measured by iMark™ Microplate Absorbance Reader (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The JEV ELISA system was assessed by using some pig sera, which had been infected with JEV. Smirnov-Grubbs rejection test was performed using statistical analysis software EZR (Easy R) [12], to distinguish the significantly higher optical density (OD) values of the IgG indirect ELISA (i.e., outliers) [16].

Focus reduction neutralization tests (FRNT) for TBEV and JEV were performed according to a previous study [15]. Sera were heat-inactivated at 56°C for 30 min, followed by filtration with a 0.22 μm filter membrane before use. Two-fold serially-diluted sera were mixed with equal volumes of TBEV or JEV stock solution containing 50 focus formation units (FFU) of the viruses. The mixture was incubated at 37°C for 60 min and inoculated to the BHK-21 monolayer cells in a 96-well plate. After 90 min of virus absorption, the overlay medium was applied to the cells. At 2 days post-infection, the viral foci were stained and counted as described above.

In total, 7 deer samples had a significantly higher ELISA OD values against TBEV antigen ($P<0.001$) (Fig. 2). The OD values of positive sera ranged from 0.252 to 0.629. There were 2 positive samples from Hidaka and 5 positive samples from Toyotomi. All sera from 5 towns in the eastern area of Hokkaido had lower OD values and were considered as negative for TBEV. There were no positive sera against SFTSV antigen identified in this study.

To confirm the specificity of the TBEV-positive sera, neutralization activity for the 7 positive sera (samples H24, H250, T26, T33, T 40, T64 and T110) and 5 negative sera (samples T56, T74, T76, T100 and T103) were assessed using FRNT. Fifty percent focus reduction neutralization endpoint titers ($FRNT_{50}$) were calculated based on the number of foci (Table 1). In total, 5 samples demonstrated neutralization activity for TBEV, ranging from 320 to 640 of $FRNT_{50}$. Conversely, $FRNT_{50}$ titers for the 2 ELISA-

Table 1. OD values of IgG indirect ELISA and 50% focus reduction neutralization endpoint titers against TBEV and JEV

Serum No.	Collection site	Capture year	ELISA OD value		FRNT$_{50}$	
			TBEV	JEV	TBEV	JEV
H24	Hidaka	2011	0.279*	0.013	<10	<10
H250	Hidaka	2012	0.252*	0.034	<10	20
T26	Toyotomi	2013	0.629*	0.014	320	<10
T33	Toyotomi	2013	0.388*	0.070	320	<10
T40	Toyotomi	2013	0.585*	0.058	640	<10
T56	Toyotomi	2013	0.005	0.052	<10	<10
T64	Toyotomi	2013	0.324*	0.006	640	<10
T74	Toyotomi	2013	0.001	<0.001	<10	<10
T76	Toyotomi	2013	0.001	<0.001	<10	<10
T100	Toyotomi	2013	<0.001	0.017	<10	<10
T103	Toyotomi	2013	<0.001	0.002	<10	20
T110	Toyotomi	2014	0.453*	0.051	640	<10

ELISA OD values marked with an asterisk (*) are significantly higher OD values, determined by a Smirnov-Grubbs rejection test ($P<0.001$). OD: optical density. FRNT$_{50}$: 50% focus reduction neutralization endpoint titer. Means of OD values and FRNT$_{50}$ were obtained from duplicate experiments.

positive samples were less than 10. The 5 samples that demonstrated neutralization activity were obtained from Toyotomi, whereas the 2 negative samples were from Hidaka. The antibody specificity was confirmed by comparing with JEV, which belongs to same genus of *Flavivirus* as TBEV. None of the sera were recognized by the JEV antigen using IgG indirect ELISA and did not neutralize JEV by FRNT (Table 1), indicating that these 5 samples contained a specific antibody for TBEV.

To date, TBEV has been isolated from dogs, small rodents, and *Ixodes* ticks [19–21], and positive sera against TBEV has been reported in dogs, small rodents, and horses [19–21, 24] in Japan. However, the infectious status of large wild animals, such as wild deer was unclear. Our study clarified that a small proportion of wild deer (*C. nippon yesoensis*) were infected with TBEV in Hokkaido, Japan. The TBEV infection rate of wild deer in European countries ranges from 1.1 to 22.9%, while our study identified a prevalence of 5% in Toyotomi. Another study has reported that the seroprevalence of small rodents in the southern area of Hokkaido was 7.6% [24], indicating a similar prevalence to our study.

Previously, TBEV and seropositive animals were reported in the southern and central area of Hokkaido, but not in the northern area of Hokkaido yet. Our results indicate that the northern area of Hokkaido is also an area at risk of TBEV infection. Some *C. nippon yesoensis* can migrate up to 100 km [5], therefore it is possible the seropositive deer came from outside Toyotomi. Additionally, wild deer could be acting as a potential transporter of TBEV, by carrying TBEV-positive ticks, even though the viremia in deer appears to be low [6]. Spatial analysis revealed the correlation between higher red deer (*Cervus elaphus*) densities and higher odds of TBE incidence in Slovenia [13]. In addition to a serosurvey of wild life, surveillance of field-collected ticks is important for a more accurate evaluation of TBEV distribution.

According to data from the National Institute of Infectious Diseases, Japan, there have been no SFTS cases reported in Hokkaido to date [7]. The disease is common in western Japan, where the mean seroprevalence in deer is 37.0%, whereas seropositive deer have not been reported in Hokkaido [7], agreeing with the results from our study. SFTSV-positive ticks have been collected in Hokkaido [7], suggesting there is a possibility that other wildlife such as raccoons, contribute to the maintenance of SFTSV in the environment.

In this study, we found TBEV-positive deer sera in the northern area of Hokkaido, where the existence of the virus had not previously been identified. However, the role of wild deer in the transmission of TBEV-infected ticks remains unclear. Recently, the number and distribution of wild deer has increased in Hokkaido, and they have become a social and ecological concerns [11]. Further assessment of the relationship between deer distribution and density and disease occurrence in humans is required.

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