Epidemiological survey of tick-borne encephalitis virus in wild animals and livestock, 1998–2018, Japan

2018

Jamsransuren DULAMJAV

Doctoral Program in Animal and Food Hygiene

Graduate School of Animal Husbandry

Obihiro University of Agriculture and Veterinary Medicine

日本において 1998年～2018年に行われた野生動物と家畜におけるダニ媒介脳炎ウイルスの疫学的調査

平成30年
（2018）

帯広畜産大学大学院畜産学研究科
博士後期課程　畜産衛生学専攻

ジャムスランスレン ドラムジャブ

Contents

Contents ... i

Abbreviation ... iii

General introduction ... 1

Chapter I ... 13

Optimization of Strep-SP ELISA for TBEV antibody detection in sera from large wild animals ... 13

1.1. Introduction ... 13

1.2. Materials and methods ... 14

1.3. Results ... 18

1.4. Discussion ... 19

1.5. Conclusion ... 21

Chapter II ... 29

Epidemiological survey for TBEV infection in large wild animals on Hokkaido and Honshu island, 2003–2018, Japan ... 29

2.1. Introduction ... 29

2.2. Materials and methods ... 31

2.3. Results ... 34

2.4. Discussion ... 35

2.5. Conclusion ... 39

Chapter III ... 50

Serological survey for TBEV in domestic ruminants, Japan, 1998–1999 ... 50

3.1. Introduction 50

3.2. Material and methods 52

3.3. Results 53

3.4. Discussion 53

3.5. Conclusion 54

General discussion 56

General summary 59

Acknowledgments 63

References 72

Abbreviation

A	AP	Alkaline phosphatase
B	BHK-21	Baby hamster kidney cell 21
	BSA	Bovine serum albumin
	BSL	Biosafety level
C	C protein	Capsid protein
	CBB	Carbonate-bicarbonate buffer
	CPE	Cytopathogenic effect
D	DMEM	Dulbecco’s modified Eagle’s medium
E	E protein	Envelope protein
	ELISA	Enzyme-linked immunosorbent assay
	EMEM	Eagle’s minimum essential medium
	ER	Endoplasmic reticulum
F	FBS	Fetal bovine serum
H	HEK293T	Human embryonic kidney cell 293
	HRP	Horseradish peroxidase
I	IF	Immunofluorescence
	Ig	Immunoglobulin
J	JEV	Japanese encephalitis virus
M	M protein	Membrane protein
O	OD	Optical density
P	PBS	Phosphate buffered saline
	PBST	PBS containing 0.05% Tween 20
	PCR	Polymerase chain reaction
	PEG	Polyethylene glycol

	PFU	Plaque forming units
	P/N ratio	Positive/negative ratio
	prM protein	Precursor membrane protein
	PRNT	Plaque reduction neutralization test
	PRNT50	A 50% plaque reduction neutralization test
	$PRNT_{50}$	A 50% plaque reduction neutralization titer
R	Real-time RT-PCR	Real-time reverse transcription PCR
	RNA	Ribonucleic acid
	RT-PCR	Reverse transcription PCR
S	S/N ratio	Signal/noise ratio
	SP	Subviral particle
	Strep-SP	SP with Strep-tag
	Strep-SP ELISA	ELISA using Strep-SP
T	TBE	Tick-borne encephalitis
	TBEV	TBE virus
	TBEV-FE	Far-Eastern subtype of TBEV
	TBEV-SP	SP of TBEV
	TMB	3,3′,5,5′-Tetramethylbenzidine
V	VTM	virus transfer medium
W	WBC	White blood cell
	WHO	World Health Organization
	WNV	West Nile virus

General introduction

Tick-borne encephalitis (TBE)

TBE is a tick-transmitted viral neuroinfectious disease of humans and infectious to animals. The etiology of TBE has been notified in 27 European and five north Asian countries, with different epidemiological features (Gritsun et al., 2003; Petri et al., 2010; Süss, 2011).

The etiologic agent is TBE virus (TBEV), which was first isolated in Russian Far Eastern in 1937 (Smorodintsev, 1958; Zlobin et al., 2017). TBEV is currently classified into three subtypes: the Western/European subtype, the Siberian subtype, and the Far-Eastern (TBEV-FE) subtype (Süss, 2003; Gubler et al., 2007; Gritsun and Gould, 2008); however, several viral isolates are suggested to be the fourth or fifth subtype (Morozova et al., 2014). TBEV subtypes slightly differ by their antigenic and phylogenetic properties associated with their natural transmission cycle and geographical distribution which correspond to the clinical pictures of TBE (Heinz, 2007; Demina et al., 2010; Süss, 2011). Besides, antibodies against one TBEV subtype can induce cross-reactivity to other two subtypes (Fritz et al., 2012). TBE caused by the TBEV-FE subtype (which is more pathogenic for humans than other two subtypes) leads to chronic TBE and long-lasting sequelae, with 20–40% case fatality. The TBEV-FE subtype is prevalent in Far Eastern Russia, China, Japan, and Mongolia (Gubler et al., 2007; Gritsun and Gould, 2008; Lindquist and Vapalahti, 2008; Petri et al., 2010; Bogovic and Strle, 2015).

TBEV transmission cycle in nature is maintained by the interactions between virus, vector ticks and their vertebrate hosts (Nuttall, 1999; Süss, 2003; Havlikova et al., 2013; Pfeffer and Leschnik, 2017), mainly forest animals distributed in the vegetation of Ixodid ticks.

Ixodid ticks (*Ixodes* spp.) are principal vectors of TBEV, although *Dermacentor* spp. and *Haemaphysalis* spp. can be infected with the virus in endemic areas (Lindquist and Vapalahti, 2008; Donoso-Mantke et al., 2011). Ticks, once infected, carry the virus forever and the virus prevalence increases during their life stage from larvae to adult (Süss, 2008). The virus

prevalence in vector ticks varies in endemic areas, perhaps depending on the seasonal activity of locally distributed tick populations. For instance, the virus prevalence in host-seeking nymphal ticks less than or equal to 0.5% is in the majority, but up to 20% in Siberian TBEV foci (Süss, 2003).

Eco-epidemiologically, vertebrates acquire TBEV infection by the following natural transmission routes: 1) biological transmission that is caused via infected tick-bites, 2) non-viremic or co-feeding transmission between tick-to-tick using vertebrate hosts, 3) direct transmission to humans via consuming TBEV contaminated milk and milk products from infected livestock (Labuda et al., 1993; Süss, 2003; Gubler et al., 2007; Pfeffer and Leschnik, 2017). Laboratory cases associated with intranasal infection and needle-stick injuries with the virus were previously reported when laboratory facilities and personal protection equipment was poor (Gritsun et al., 2003; Dobler et al., 2012). TBE cases caused via an infected tick-bite are in the majority. Humans and domestic animals are usually dead-end hosts for TBEV. There has been no human-to-human transmission reported (Martina et al., 2017).

Approximately 10,000–12,000 clinical cases of TBE are reported annually (Gritsun et al., 2003; Mandl, 2005; Kunz, 2007; Petri et al., 2010; World Health Organization (WHO), 2011; Valarcher et al., 2015), of which children and the elderly generally present a severe illness (Bogovic and Strle, 2015; Kunze, 2016). The illness is typically biphasic, with an initial period of flu-like symptoms, followed by the severe encephalitic syndromes. Approximately one-third of meningitis cases develop meningoencephalitis or meningomyelitis (Kaiser, 2007; Petri et al., 2010; Holbrook, 2017). Most encephalitic patients leave with long-term sequelae (in 35–60% of survivors) because the primary targets of TBEV in the central nervous system are neurons. No therapy for TBE is available to date (Kaiser, 2008; Růžek et al., 2010; Bogovic and Strle, 2015).

About 70–90% of TBE cases remain underdiagnosed due to the diagnosis challenging with asymptomatic TBEV infection and insufficiently facilitated diagnostic laboratories (Gritsun et al., 2003; Mandl, 2005; Gubler et al., 2007). The most common laboratory diagnostic method

of detecting TBEV infection is serodiagnostic tests; mainly an enzyme-linked immunosorbent assay (ELISA), immunofluorescence assay (IF), and neutralization test (NT), but the ELISA is a preferable assay. When neurological symptoms were expressed in the second week of illness after the viral infection, the virus and viral-specific nucleic acid are rarely detectable in blood or cerebral spinal fluid of TBE patients using polymerase chain reaction (PCR) assays and virus isolation methods (Dobler 1996 and 2017; Holzmann, 2003; Roelandt et al., 2010; Růžek et al., 2010; Levanov et al., 2014; Borde and Zajkowska, 2017). Therefore, detection of TBEV IgM and IgG antibodies in convalescence sera from patients by ELISA or IF followed by confirmation via NT are required for a TBE case diagnosis. Other serological tests such as a hemagglutination inhibition test and immunoblot analysis are also available (Günther and Lindquist, 2005; Donoso-Mantke, 2011; Süss, 2011; WHO, 2011). Moreover, co-infection with other tick-borne bacterial pathogens such as *B. burgdorferi, A. phagocytophilum*, and rickettsiae, is another challenge in the disease diagnosis (Gritsun et al., 2003; Kaiser, 2007; Kunz, 2007; Bogovic and Strle, 2015).

TBE is a vaccine-preventable disease. Public awareness towards personal protection and vaccination as prevention measures are highly recommended. WHO recommends vaccination of all age groups in the endemic countries and also for travelers. There are five TBE vaccine manufacturers in Austria, Germany, Russia, and China. All are cell-cultured and formalin-inactivated whole-virus vaccines. The vaccines are approved to use for children older than 1 year and adults with 2 or 3 doses schedule, with a few cases of vaccination failure (Gritsun et al., 2003; WHO, 2011; Kollaritsch et al., 2012; Amicizia et al., 2013; Morozova et al., 2014; Bogovic and Strle, 2015; Pöllabauer and Kollaritsch, 2017). Intravenous Ig therapy or passive immunization against TBEV have been used as postexposure prophylaxis in Russia and the former Soviet republic countries, but no longer recommended in the European countries because of the weak effectiveness in children (Lindquist and Vapalahti, 2008; WHO, 2011; Bogovic and Strle, 2015; Borde and Zajkowska, 2017; Pöllabauer and Kollaritsch, 2017).

TBEV

Taxonomically, TBEV belongs to the mammalian tick-borne virus group in the genus *Flavivirus* within the family *Flaviviridae*. TBEV in the group is comprised of the closely related agents causing encephalitis (Louping ill virus, Powasson virus, etc.) and hemorrhagic fever (Omsk hemorrhagic fever virus, Kyasnur Forest disease virus, etc.) in humans and animals (Gritsun et al., 2003; Mansfield et al., 2009; Donoso-Mantke et al., 2011; Heinz and Stiasny, 2017).

TBEV (mature virion) is a lipid-enveloped and positive-sense single-stranded RNA virus with a diameter of approximately 50 nm. The mature TBEV particle is consisted of a core (30 nm) encircled by a lipid bilayer containing two membrane-associated proteins, the envelope glycoprotein E (molecular mass, 52 kDa) and membrane protein M. The core consists of a genomic RNA of approximately 11 kilobases in length and a capsid protein (C). The genomic RNA encodes one large polyprotein which is cleaved by viral and cellular proteases into three structural and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins required for virus replication in the cell (Gritsun et al., 2003; Lorenz et al., 2003; Lindquist and Vapalahti, 2008; Heinz and Stiasny, 2017).

TBEV particles are intracellularly assembled by budding into the endoplasmic reticulum (ER) of the infected cell (Figure 1). The intracellular (immature) virion contains a precursor membrane (prM; 7–8 kDa), which is the immature form of M protein. Transformation of prM to M occurs during the budding of virions from the affected cells (Gritsun et al., 2003; Heinz and Stiasny, 2017).

The structural proteins are essential for the survival, transmission, and replication of TBEV in both vertebrate and invertebrate hosts (Mansfield et al., 2009; Donoso-Mantke et al., 2011). For instance, the E protein is responsible for viral entry functions, including receptor binding and membrane fusion steps that are resulting in the virus infectivity.

The E protein is the preeminent antigen that is responsible for hemagglutination and neutralization, resulting in the protective immune response in the hosts (Heinz et al., 1995;

Ferlenghi et al., 2001; Gritsun et al., 2003; Stiasny and Heinz, 2006; Füzik et al., 2018). However, both prM/M protein aids the E protein to be matured (Allison et al., 1995; Heinz, 2007).

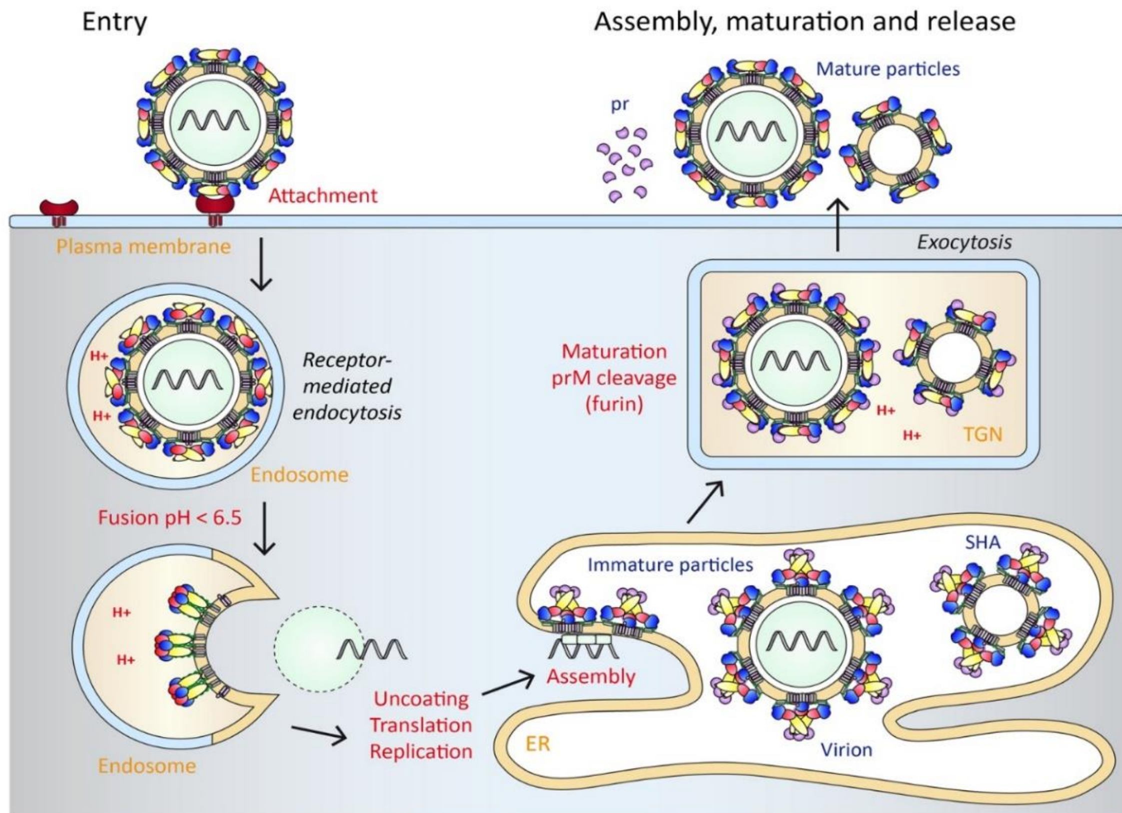

Figure 1. Life cycle of flaviviruses: Virus enters by receptor-mediated endocytosis, and viral membrane fusions are discomposed, resulting in the viral genome to get free in the ER that leads to protein translation and RNA replication. A budding process forms immature virions into the ER that leads to subviral particles (or slowly sedimenting hemagglutinin (SHA)) lacking C protein. The viral particles are formed in mature through a combination of prM and E proteins in the trans-Golgi network (TGN) by the cellular protease furin, which results in the generation of protein M while transported towards the exocytosis pathway. The figure is adapted from Heinz and Stiasny (2017).

During the virus replication, the particles with only the membrane proteins E and M or lacking the core particle, which is known to be virus subviral particles (SPs; approximately 30 nm in a diameter) having similar properties and fusion activities to intact virions, are produced while immature virions are fully maturing (Lorenz et al., 2003; Zeltins, 2013; Pulkkinen et al., 2018).

TBEV-SPs

TBEV-SPs are capsidless virus-like icosahedral particles of 30 nm with co-expressed two membrane proteins prM and E that have crucial roles in the assembly and secretion of the virions. Although the SPs are non-infectious and lacking in C protein and genomic RNA, they are similar to the intact virus reflecting its viral structures and antigenic properties (Ferlenghi et al., 2001; Heinz, 2003; Mandl, 2005). The SPs, therefore, can be used as a diagnostic antigen of immunoassays and an immunogen for producing monoclonal and polyclonal antibodies (Heinz et al., 1995; Jääskeläinen et al., 2003; Yoshii et al., 2009). The E protein in TBEV-SPs is identical with its intact particle's feature (Allison et al., 2003).

In the thesis study, recombinant TBEV-SPs are used as a diagnostic antigen that were applied to an indirect ELISA for detecting immunoglobulin (Ig) IgG antibodies to TBEV in wild animals and domestic ruminants. The details about the TBEV-SPs used in this study are described in Chapter 1.

Natural foci of TBEV

The virus persists in nature, which is called natural foci, circulating among its vertebrate hosts and vector ticks. Approximately 30,000 foci have been recorded at least in 32 Eurasian countries, with individual spatial distribution from a few square meters (500 m^2) to several square kilometers (Zeman, 1997; Korenberg and Kovalevskii, 1999; Gritsun et al., 2003; Süss, 2003; Mandl, 2005; Lindquist and Vapalahti, 2008; Dobler et al., 2011 and 2012). This etiologic area spans across almost the entire southern part of non-tropical Eurasian forest zone where

relative humidity is high (about 85–92%), with dense and moist undergrowth, and rich in forest ticks and small rodents (Heinz, 2007; Lindquist and Vapalahti, 2008; Petri et al., 2010; Donoso-Mantke et al., 2011; Valarcher et al., 2015).

Eco-epidemiologically, the existence of TBEV foci can be inactivated for an extended period (over a decade) and re-emerged (Stefanoff et al., 2013; Frimmel et al., 2014). TBEV foci remain active in the area depending on the interactions of the virus survival in vector ticks and vertebrate hosts on which the ticks can be infected or maintained themselves (Süss, 2003; Pfeffer and Leschnik, 2017).

Vector ticks

TBE is a seasonal disease linking to a questing activity of vector ticks with two peaks in spring and autumn which is associated with the distribution of annual cases in the TBEV-endemic countries (Lindquist and Vapalahti, 2008; Bogovic and Strle, 2015). *Ixodes* spp. are primary vectors of TBEV, of which *Ixodes ricinus* (*I. ricinus*) and *I. persulcatus* are the most prominent species, which are also taxonomically closely related (Süss, 2003). The infection rate in ticks rarely exceeds 1% in TBEV affected areas, regardless of whether testing with a large number (Imhoff et al., 2015). Moreover, *I. ovatus* tick species is recently known to be a vector of TBEV found in Japan and China (Süss, 2011; Yoshii et al., 2017a). In recent years, new vector ticks have been revealed, for instance, *Dermacentor reticulatus* with the virus prevalence of about 10% (Valarcher et al., 2015; Földvári et al., 2016; Kunze, 2018).

The virus is transmitted from saliva and salivary glands of an infected tick during the first minutes of the tick-bite. Once infected, ticks maintain TBEV in nature for their lifespan of about three years, so that the virus usually remains detectable (Süss, 2003; Heinz, 2007; Valarcher et al., 2015). Ixoded ticks have three life stages: larvae, nymph, and adult, and each feeds on different hosts from small-sized to large. Questing ticks transmit the virus to their competent vertebrate hosts in their environments while feeding on them, and allow the virus to circulate in nature involving infected feeding hosts. The co-feeding transmission of TBEV from infected

ticks to naive ticks on the hosts is crucial for the virus survival in nature. During the active season, ticks are maintained in a large portion on large wild animals such as abundant cervid species or others, and the following winter they move to small rodents as a blood-source for the virus overwintering (Tonteri et al., 2011; Jaenson et al., 2012).

Consequently, ticks not only serve as vectors but also play a role as reservoirs (Süss, 2003; Lindquist and Vapalahti, 2008; Donoso-Mantke et al., 2011; Bogovic and Strle, 2015). The virus prevalence detected in the field-collected questing ticks confirms the newly established TBEV foci (risk area) in previously unknown areas (Imhoff et al., 2015).

TBEV infected ticks are often found to be co-infected by other tick-transmitted pathogens, such as *Borrelia burgdorferi* (*B. burgdorferi*) sensu lato, *Anaplasma phagocytophilum* (*A. phagocytophilum*), and *Babesia* spp. (Süss, 2003 and 2011).

Vertebrate hosts

The principal vertebrate hosts are small rodents, wild and domestic ruminants, and forest animals such as wild boars and foxes. Vertebrate hosts are mainly infected with TBEV subclinically and remain lifetime immunity against reinfection (Heinz, 2007). Natural infection of TBEV in dogs, horses, sheep, and monkeys has been also reported (Süss et al., 2008; Pfeffer and Dobler, 2011; Klaus et al., 2013; Böhm et al., 2017).

Small rodents develop a viremic transmission to vector ticks (usually larvae and nymphs) as reservoirs and amplifying hosts. They also establish persistent infections, leading to the way of tick survival during the cold season in TBEV foci for years (Süss, 2003; Dobler et al., 2012).

Of large wild animals, cervid species and wild boars are well-established and sensitive indicators of TBEV circulation in their habitats with the probable infection rate higher than small rodents. Through being fed via infected ticks to maintain tick populations at sufficient level, these animal species in forested area stimulate the transmission cycle of TBEV despite a brief viremia with low virus growth level (Heinz, 2007; Dobler et al., 2012). The following

epidemiological aspects make large animals become indicator hosts or sentinels in the ecology of TBEV: 1) due to their large-sized body, they are often infested with ticks in a high number, 2) they may support non-viremic transmission among co-feeding ticks during their blood mealing, and 3) their migration and habitation cause the virus to distribute from endemic to non-endemic areas (Donoso-Mantke et al., 2011; Jaenson et al., 2012; Mlera and Bloom, 2018).

Domestic ruminants, once infected, excrete the virus in their milk that direct humans getting infected with TBEV through alimentary infection causing a biphasic milk fever (Vereta et al., 1991; Gritsun et al., 2003; Holzmann et al., 2009; Donoso-Mantke et al., 2011). The virus particles are stable in milk for several days (Imhoff et al., 2015). Outbreaks caused via the consumption of the virus contaminated unpasteurized dairy products (raw milk, cheese, yogurt, and butter) are often recorded in eastern Europe and Baltic states, with infection rate up to 35% (Gritsun et al., 2003; Süss, 2003; Balogh, 2010; Růžek et al., 2010; Bogovic and Strle, 2015; Imhoff et al., 2015; Valarcher et al., 2015). Not only do these grazers, but also unvaccinated mothers shed the virus in their breast milk so that their infants can be infected with TBEV through breastfeeding (Dobler et al., 2012; Havlikova et al., 2013).

Veterinary surveillance

Although tick testing indicates a local TBEV circulation in the area as a standard method to monitor TBE risk to humans (Süss, 2003; Donoso-Mantke et al., 2008), studies in the endemic countries often failed to detect the virus in vector ticks sampled even in large numbers (i.e., more than 65,000 ticks) using RT-PCR assays (Klaus et al., 2010a; Stefanoff et al., 2013). The infection rate in nymph, which is an epidemiologically important stage due to its large population, frequently ranges from 0% to 5% but rarely exceeding 1% in the endemic areas (Lindquist and Vapalahti, 2008; Imhoff et al., 2015; Valarcher et al., 2015).

TBEV serological testing in wild animals is therefore a preferable way to monitor the distribution of TBEV in the risk areas where the prevalence of infected ticks is low (Stefanoff

et al., 2013; Valancher et al., 2015). In the veterinary surveillance, the most determined species as sentinel hosts are cervid species (with infection rate between 1.1–39.0%) and wild boars (2.9–35.7%) (Borcić et al., 1990; Cisak et al., 2012; Balling et al., 2014; Jemeršić et al., 2014; Roelandt et al., 2016) because they tend to be largely infested with vector ticks of TBEV in their habitats. As a result, seroepidemiological investigations of regions with TBEV risk are widely performed using these animal species as sentinels indicating the presence and transmission of the virus in their habitats.

Infection in dogs and livestock is the leading cause of TBE in humans in the endemic countries. There are canine TBEV infection associated with subclinical infection or neurological disease that is a frequent event, whereas the clinical TBE in horses is rare (Leschnik and Thalhammer, 2002; Pfeffer and Dobler, 2011; Baneth, 2014; Pfeffer and Leschnik, 2017). Domestic ruminants can be also sentinels to reveal the risk of TBE to humans, with seroprevalence around 1% to 43% (Dantas-Torres et al., 2012; Imhoff et al., 2015).

Consequently, in veterinary surveillance, most studies use an ELISA as a screening assay to detect TBEV antibodies in testing a large number of samples in a short time, and the results are usually confirmed by detecting neutralizing antibodies against TBEV as a test validation. Coupling of ELISA and NT is unique to flaviviruses on surveillance, differentiating one flavivirus from another which is circulating among a huge animal population. As for TBEV, the assay combination is as a likely method to differentiate exposed vertebrate hosts from non-exposed in order to seek the presence of the virus circulation in their environment (Yoshii et al., 2003; Donoso-Mantke et al., 2008; Klaus et al., 2014; Litzba et al., 2014; Imhoff et al., 2015). Also, determining the end-point titer of TBEV-neutralizing antibodies in defined antibody-positive sera can interpret the level of viremic capacity against the virus in hosts, which is linked to the disease development in the animal species (Heinz, 2003).

The presence of neutralizing antibody to TBEV E protein provides the evidence that protective immunity was developed in infected hosts; therefore, the NT is the most specific

assay available. Although a plaque-reduction neutralization test (PRNT) and virus infectivity assay are both available assays to determine the neutralizing antibody titers in testing host serum against TBEV (Stiasny and Heinz, 2006; Maeda and Maeda, 2013). The PRNT is not a standardized test, however (Litza et al., 2014). Laboratories which can afford the assay use their in-house procedures with different level of plaque reduction for assessing the neutralizing activity against TBEV in test serum. For instance, a 50% PRNT (PRNT50) can detect TBEV-neutralizing antibodies with low titers than ELISA (Holzmann et al., 1996), and TBEV-neutralizing antibody titers ($PRNT_{50}$ titer) higher than or equals to 1:10 confirms the diagnosis according to the European Centre for Disease Prevention and Control (Pfeffer and Leschnik, 2017).

Several indirect ELISA detecting TBEV antibodies (IgM and IgG) in some animal species (horses, cattle, sheep, goats, pigs, dogs, rabbits, mice, and monkeys) are commercially available today in veterinary medicine (Klaus et al., 2011 and 2014). Different types of ELISA with an indirect or competitive format have been established to increase the assay performance.

Current knowledge of TBE in Japan

TBE is a nationally notifiable disease. The first clinical case was reported in 1993 in the southern part of Hokkaido, the northernmost prefecture of Japan. TBEV is a biosafety level (BSL) 3 pathogen in Japan, which requires BSL3 facility for working with TBEV strains (Takashima et al., 1997; Yoshii et al., 2017a).

The geographic distribution of TBEV seems to be expanding throughout Hokkaido. In 2016–2018, four human cases of TBE including two deaths were reported not only in the southern part of Hokkaido but also in the central and northern parts (Yoshii et al., 2017a; http//www.city.asahikawa.hokkaido.jp/kurashi/135/136/150/d064126.html; in Japanese).

Until recent reporting the second case of TBE in 2016, few studies to investigate TBEV infection in certain animal species in Hokkaido were performed. The evidence in the southern

part was as follows: the TBEV-FE strains from *I. ovatus* ticks (0.3%), *Apodemus speciosus* (*A. speciosus*) and *Clethrionomys rufocanus* (*C. rufacanus*) rodents (1.3–9.1%), and dogs (30.0%), as well as serological evidence of TBEV infection among rodents (3.7–15.9%; also mainly *A. speciosus* and *C. rufacanus*), dogs (76.2%), and horses (4.0%) (Takeda et al., 1999; Yoshii et al., 2011) were demonstrated.

In other parts of Hokkaido, seropositive dogs (12.5%) and horses (2.0%) were identified in the central part (Yoshii et al., 2011). Recently in 2018, five Hokkaido sika deer were tested positive for TBEV antibodies in the northern part of Hokkaido (Uchida et al., 2018). Furthermore, retrospective seroepidemiological studies in 2017 and 2018 revealed subclinical cases of TBE among the members of the Japan Self-Defense Force in Hokkaido (0.6%) and the patients with Lyme disease (1.2%), with a history of tick-bites in Hokkaido within the previous 10 years (Yoshii et al., 2017b; 2018).

Outside Hokkaido, Shimane Prefecture in Honshu island (the main island of Japan) was reported to be the only area with TBEV foci with prevalence rate of 3.4% among *A. speciosus* rodents (Yoshii et al., 2011).

In summary, a dearth of information regarding the eco-epidemiological aspects of TBEV in this country necessitates updating the current knowledge of TBEV infection through veterinary control covering different faunas.

Chapter I

Optimization of Strep-SP ELISA for TBEV antibody detection in sera from large wild animals

1.1. Introduction

The ecology of TBEV in Japan is generally unknown. Information on infection in large wild animals is very limited. Few investigations on exposure to TBEV in large wild animals have not been conducted, excluding a small survey detecting TBEV-seropositive Hokkaido sika deer in (Uchida et al., 2018).

Although large-sized animals are not responsible for a direct transmission of TBEV to humans, their roles are essential for the maintenance and dispersal of TBEV foci, as a feeder of adult ticks growing in a high number on their bodies, leading to transovarial transmission of TBEV, supporters of the virus circulating pathway between ticks co-feeding, and distributors of TBEV infected ticks to new areas (Labuda et al., 1993; Mansfield et al., 2009; Donoso-Mantke et al., 2011; Dobler et al, 2012; Jaenson et al., 2012; Mlera and Bloom, 2018). Thus, infected large wild animals can be sentinels indicating the virus circulation in their habitats and the risk of TBEV to humans (Heinz, 2007; Dobler et al., 2012).

The majority of tick-borne zoonoses are usually identified by serological screening. In case of TBE, ELISA is a preferable test, not only for epidemiological purposes but also for the diagnosis; therefore, it has been broadly applied to vertebrate species with a variety of formats (Roelandt et al., 2010; Donoso-Mantke, 2011; Klaus et al., 2011 and 2014).

The use of ELISA in veterinary surveillance is cost-effective, because most vertebrates around forested areas in Eurasian continents are likely to become TBEV-seropositive (Heinz, 2007) for an extended period so that adequate sample numbers being tested is preferred. However, most ELISAs to detect anti-TBEV IgG antibodies in animal species are based on whole-virus antigens (Dobler et al., 1996; Yohsii et al., 2003), causing cross-reactivity in

related-flavivirus serology, such as dengue viruses, Japanese encephalitis virus (JEV) and West Nile virus (WNV) (Klaus et al., 2014; Litzba et al., 2014).

Recently, the indirect ELISA using recombinant TBEV-SPs with Strep-tag as a diagnostic antigen (Strep-SPs) that called Strep-SP ELISA was developed (Inagaki et al., 2016). The assay is intended to screen multiple animal species for anti-TBEV IgG antibodies using Strep-SPs through its affinity toward Strep-tactin as a coating parameter, with the binding ability of horseradish peroxidase (HRP) conjugated Protein A/G. Strep-SP ELISA was evaluated using sera from TBE patients and TBEV infected rodents with the assay validation via PRNT50, and showed high sensitivity and no cross-reactivity with antibodies to JEV. Another advantage of Strep-SP ELISA in use is its accessibility of performance under the BSL1 facilitated laboratory, eliminating the extreme caution as working with BSL3 pathogen. However, this ELISA system has not yet been applied to animal species, except rodents.

Japan is rich in wild animals. In recent years, the population and distribution of wild species such as wild boars, sika deer, and raccoons have rapidly raised nation-wide. Raccoons are recently more inhabited throughout the country. To my knowledge, interestingly, there is no documented serological evidence of TBEV infection in raccoons.

Therefore, the thesis study primarily aimed to define the presence of TBEV infection in raccoons, sika deer, and wild boars in certain parts of Hokkaido and Honshu islands. To conduct seroepidemiological surveillance for TBEV in these animal species, the study described in this chapter is associated with setting up the optimum conditions of Strep-SP ELISA to detect the exposed raccoons and sika deer.

1.2. Materials and methods

1.2.1. Virus

A TBEV Oshima 5-10 strain (Takashima et al., 1997) was used for PRNT50 as previously described (Inagaki et al., 2016).

1.2.2. Cells

293T-OSF cells which were kindly provided by Dr. K. Yoshii, Hokkaido University are human embryonic kidney 293T (HEK293T) cells transfected with the pCAG-TBE-M-StrepE plasmid (Inagaki et al., 2016), which is the pCAGGS plasmid encoding the TBEV-Oshima prM and E gene signal sequences with Strep-tag peptide sequence (WSHPQFEK) as shown in Figures 1-1 and 1-2. 293T-OSF cells were grown at 37°C in Dulbecco's Modified Eagle's Medium (DMEM; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 4.5 g/L D-glucose, 10% fetal bovine serum (FBS), L-glutamine (2 mM), amphotericin B (2.5 µg/ml), and kanamycin (100 µg/ml), and used for the preparation of a positive antigen in Strep-SP ELISA as described below.

Normal HEK293T cells were cultured using the same growth medium as described above and used for preparation of a negative antigen in Strep-SP ELISA as described below.

Baby hamster kidney (BHK-21) cells were grown at 37°C in Eagle's Minimum Essential Medium (EMEM; FUJIFILM Wako Pure Chemical Corp., Osaka, Japan.) containing 8% FBS and used for PRNT50 as described below.

1.2.3. Serum samples

A rabbit anti-TBEV E antiserum as a positive control serum which was kindly provided by Dr. K. Yoshii, Hokkaido University and a normal rabbit serum as a negative control were used for optimizing Strep-SP ELISA.

A total of 411 sera, including 368 raccoons and 43 Hokkaido sika deer were tested for TBEV antibodies using the optimized Strep-SP ELISA. The results were confirmed using PRNT50. All of the sera were inactivated at 56°C for 30 min before testing.

1.2.4. Preparation of ELISA antigens

293T-OSF cells were cultured for 2 days at 37°C using DMEM as described above. The supernatants of 293T-OSF cells were collected in 500 ml centrifuge tubes. The cell debris in

the supernatants was removed by centrifuging at 8,000 x g for 10 min at 4°C, and then the supernatants were treated with 10% polyethylene glycol (PEG) 8,000 (MP Biomedicals, Santa Ana, USA) and 1.9% NaCl for 2 h at 4°C with constant agitation. The PEG-treated supernatant was centrifuged at 10,000 x g for 30 min at 4°C using an R10A3 rotor in Himac CR20G centrifuge (Koki Holdings Co., Ltd., Tokyo, Japan). The pellets were resuspended in phosphate-buffered saline (PBS; pH 7.4) to make a 100X concentrated volume of the PEG-treated supernatant and stored at –80°C as Strep-SPs stock solution. The stock Strep-SPs were further diluted in 1:5 which equals 20X concentrated Strep-SPs from the PEG-treated supernatant in use for Strep-SP ELISA.

The supernatant of normal HEK293T cells were treated with PEG 8,000 and 1.9% NaCl, and negative antigens were prepared using the PEG-treated supernatant as described above.

1.2.5. Strep-SP ELISA

The optimum conditions for Strep-SP ELISA were examined as follows:

Selection of a blocking buffer: Four percent of bovine serum albumin (BSA; FUJIFILM Wako Pure Chemical Corp.) and 5% skimmed milk (FUJIFILM Wako Pure Chemical Corp.) in PBS containing 0.05% Tween 20 (PBST), and 1% alkaline-soluble casein (Novagen, Darmstadt, Germany) or 1% BlockAce (DS Pharma Biomedical, Osaka, Japan) in ultrapure water were evaluated using the rabbit antiserum and normal serum. For examination, the 96-well plates were coated with Strep-tactin labeled with alkaline phosphate (Strep-tactin AP; 1:1,000) in carbonate-bicarbonate buffer (CBB) to capture 20X concentrated Strep-SPs on the well surface of the ELISA plate and incubated for overnight. On next day, the wells were incubated with blocking buffers (200 µl/well) for 2 h at 37°C. After incubation, Strep-SPs and negative antigens were added in duplicate wells and incubated for 1 h at 37°C. A pair of wells with 20X concentrated Strep-SPs and negative antigens were incubated with the rabbit antiserum for another 1 h at 37°C, followed by HRP-conjugated Protein A/G (Thermo Fisher Scientific, Waltham, USA) for 30 min at 37°C. After 15 min of incubation with TMB solution,

the reaction was stopped and measured at 450 nm. Blocking buffers showing a big difference in optical densities (OD) between the wells with Strep-SPs as a positive reaction and negative antigens as a negative reaction was selected as a blocking buffer in the optimized Strep-SP ELISA.

Selection of diluents: One and 3% BSA/PBST for a test serum diluent, and 1% BSA/PBST and BlockAce for a conjugate diluent were tested using the rabbit antiserum as described above. To detect antibodies to TBEV in sera from raccoons, HRP-conjugated Protein A/G at dilutions from 1:1,000 to 1:4,000 were evaluated.

Evaluation of Strep-SP ELISA: To detect antibodies to TBEV in test sera, a 1:100 diluted raccoons and sika deer sera were incubated with the 20X concentrated Strep-SPs and negative antigens in a pair of wells each, and then with HRP-conjugated Protein A/G with a 1:2,000 dilution and rabbit anti-deer IgG (KPL, Gaithersburg, USA) at a dilution of 1:500 (according to the company recommendation). The ELISA results were recorded as the positive to negative (P/N) ratio (i.e., the ratio of the OD values with Strep-SPs to that the negative antigens).

1.2.6. PRNT50

A PRNT50 (Inagaki et al., 2016) was used to detect neutralizing antibodies against TBEV-Oshima in raccoon and sika deer sera tested by the optimized Strep-SP ELISA. Briefly, a serum diluted 1:25 and 1:50 in 2% EMEM was mixed with an equal volume of TBEV (approximately 50 plaque-forming units (PFU) per 0.1 ml) and incubated at 37°C for 1 h. The serum-virus mixture (100 µl/well) was inoculated onto BHK-21 cells seeded in 12-well plates followed by an incubation of 1 h. The maintenance medium instead of the virus was used in virus controls. Then the overlay medium containing 2% FBS and 1.5% carboxymethyl cellulose solution was added and incubated for 4 days. Then, the plates were incubated with 10% formalin with 0.1% crystal violet for 30 min at room temperature. After removing the fixation and staining solution, the plates were washed with tap water and dried up. Plaques were

counted and the test serum showing a 50% plaque reduction of the virus control plaque count was judged as neutralizing antibody positive. Furthermore, PRNT50-positive sera were retested for determining the end-point titers of the test sera.

1.3. Results

1.3.1. Strep-SP ELISA optimization

One percent of BlockAce in ultrapure water as a blocking buffer had the best signal/noise (S/N) ratio than 5% skimmed milk, 4% BSA, and 1% Casein (Figure 1-3a).

As a serum diluent, 1% BSA/PBST was chosen, because the difference between the positive and negative reactions (Strep-SPs and negative antigens against antiserum) was larger than 3% BSA/PBST. In 3% BSA/PBST, the antiserum against Strep-SPs had a lower reaction, but a higher against negative antigens than in 1% BSA/PBST (Figure 1-3b).

As an HRP-conjugate diluent, the blocking buffer (1% BlockAce) lowered the non-specific binding of antiserum to negative antigens compared with 1% BSA/PBST (Figure 1-3c).

Raccoon sera diluted 1:100 in 1% BSA/PBST with a 1:2,000 dilution of HRP-conjugated Protein A/G gave the best response (Figure 1-3d). The incubation with a 45 min for both antigens and test sera was chosen, because an extended time about 1 h often showed edge effects (data not shown).

To evaluate the optimized Strep-SP ELISA for raccoon sera, 19 out of 368 that showed the P/N ratios of equals to or higher than 0.5 were analyzed for TBEV-neutralizing antibodies using the PRNT50. For sika deer testing, seven out of 43 sera that showed the P/N ratios of approximately from 0.4 to 1.3 were selected for confirming by the PRNT50.

1.3.2. PRNT50

The PRNT50 tested 26 sera including 19 raccoons and seven Hokkaido sika deer. All nineteen raccoon sera showed TBEV $PRNT_{50}$ titers ranging from 1:25 to 1:800 (Table 1.1.), but all sika deer sera were PRNT50-negative.

The end-point titers of PRNT50-positive raccoon sera were as follows; $PRNT_{50}$ titer of 1:25 (eight sera), 1:50 (seven), 1:200 (three), and 1:800 (one) (Table 1.1.). The eight sera with the lowest $PRNT_{50}$ titer (1:25) showed a lower P/N ratio (≤1.5) than other TBEV PRNT50-positive sera. Also, $PRNT_{50}$ titer of 1:25 often presents non-specific background or cross-reactivity with other antigenically-related flaviviruses (Dr. K. Yoshii's personal communication). As a result, the $PRNT_{50}$ titer of ≥1:50 was considered as TBEV-neutralizing antibody positive. By the PRNT50 result, 11 raccoons were determined to be TBEV-seropositive. According to this interpretation, the cut-off value of Strep-SP ELISA for raccoon testing was determined to be the P/N ratio of ≥1.5 (Table 1.1.).

1.3.3. Optimized Strep-SP ELISA protocol

The ELISA plates were coated with a 1:1,000 dilution of Strep-tactin AP (50 μl/well) in 0.1 M CBB (pH 9.6), and incubated overnight at 4°C. The washing steps were performed with 0.05% PBST (350 μl/well) five times each. The plates were blocked with 1% BlockAce (200 μl/well) in ultrapure water for 2 h at 37°C. Then, 20X concentrated Strep-SPs and negative antigens (50 μl/well) were added in duplicate and incubated for 45 min at 37°C. Test sera (50 μl/well) in 1% BSA/PBST were added and incubated for a further 45 min at 37°C. HRP-conjugated Protein A/G with a 1:2,000 dilution and anti-deer IgG (200 μl/well) at a 1:500 dilution in 1% BlockAce were added, and incubated for 30 min at 37°C. Then TMB substrate (100 μl/well) was added and incubated for 15 min at 37°C. The reaction was stopped using 2N H_2SO_4 (50 μl/well). The OD values were measured at 450 nm. The P/N ratio of each test serum was calculated, and the P/N ratio of about ≥1.5 was determined as Strep-SP ELISA positive.

1.4. Discussion

This study aimed to obtain the optimum condition of Strep-SP ELISA in order to detect TBEV antibodies in wild animals. The optimized ELISA was evaluated by testing sera from

raccoons and sika deer, and the result was confirmed with PRNT50, which can detect the antibodies with low titers (Holzmann et al., 1996).

The optimized Strep-SP ELISA without TBEV antisera raised in raccoons and deer were challenging. Once the Strep-SP ELISA optimized, raccoons and sika deer sera were tested against 20X concentrated Strep-SPs using Protein A/G or anti-deer IgG conjugates. Of the all tested, the sera showing the P/N ratios of equals to or higher than 0.4 were confirmed with PRNT50 for TBEV-neutralizing antibodies.

Although the $PRNT_{50}$ titer of 1:25 is used as the cut-off titer to demonstrate TBEV-neutralizing antibodies in sera from sentinel animals in TBE-endemic European countries (Litzba et al., 2014; Frimmel et al., 2016), the $PRNT_{50}$ titer ≥1:50 was adopted in this study, because cross-reactivity with other flaviviruses could not be necessarily denied when the PRNT50 showed the $PRNT_{50}$ titer of 1:25 (Dr. K. Yoshii's personal communication).

However, I did not examine the presence of any other flaviviruses in the sera from both raccoons and sika deer by the PRNT50. Inagaki et al. (2016) reported that Strep-SP ELISA could detect TBEV IgG antibodies in humans and rodent species without cross-reactivity with antigenically related-flavivirus JEV. Hokkaido has been known to be JEV-free, which was proved by the previous studies. For instance, Uchida et al. (2018) showed the negative results in TBEV-seropositive Hokkaido sika deer sera tested by JEV ELISA and PRNT50. Ohno et al. (2009) also did not detect JEV-neutralizing antibodies in sera collected from raccoons in Hokkaido. Therefore, it is unknown whether flaviviruses other than JEV with cross-reactivity to TBEV are contained in the tested sera.

By the PRNT50, the cut-off value of Strep-SP ELISA for raccoon sera was determined as the P/N ratio of ≥1.5 (Table 1.1.). In contrast, a sika deer serum that had the highest P/N ratio of 1.3 was TBEV PRNT50-negative.

In Japan, TBEV infection in large wild animals is unknown. In TBEV-endemic European countries, cervid species and wild boars are used as well-established sentinels to monitor the distribution of TBEV in their environment, using mostly commercially available species-specific

ELISA tools. However, the raccoon is a new host species in this field, with a dearth of TBEV seroprevalence study and diagnostic tools. Introducing Strep-SP ELISA in TBEV veterinary surveillance can be a useful tool to monitor as many species populations as possible with low cost and safety procedures.

1.5. Conclusion

The optimized Strep-SP ELISA with the P/N ratio of ≥1.5 as a screening assay was established to detect antibodies against TBEV in sera from raccoons.

In this study the cut-off value of Strep-SP ELISA was not determined for sera from sika deer.

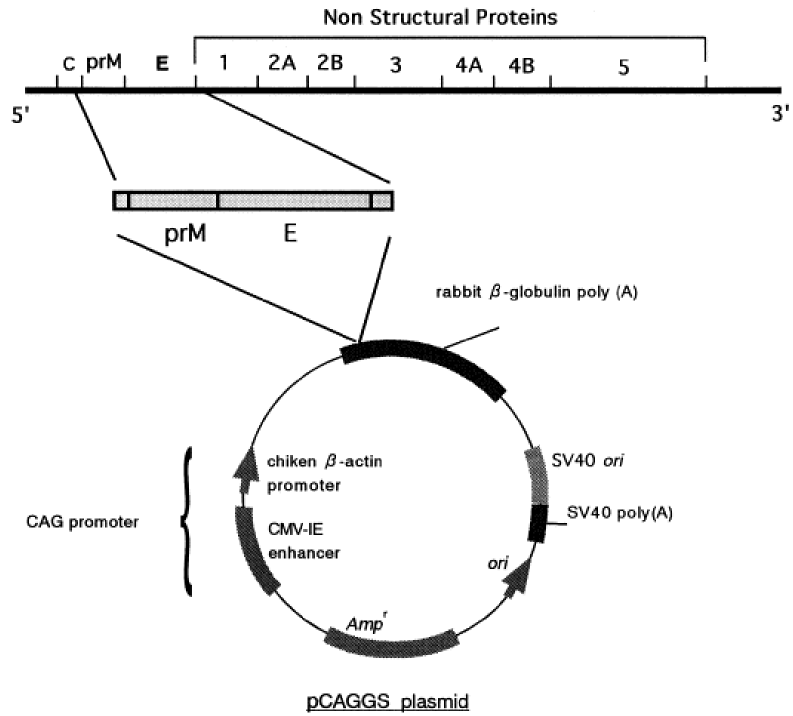

Figure 1-1.

Construction of the pCAGGs plasmid encoding the TBEV-Oshima signal sequences of prM and E genes. The figure is adapted from Yoshii et al. (2003).

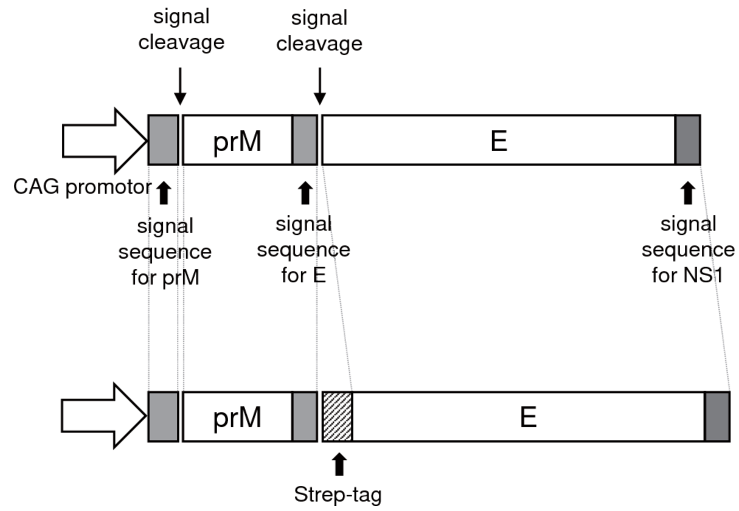

Figure 1-2.

Construction of the pCAG-TBE-M-StrepE plasmid encoding the TBEV-Oshima signal sequences of prM and E genes together with Strep-tagged signal sequence. The figure is adapted from Inagaki et al. (2016).

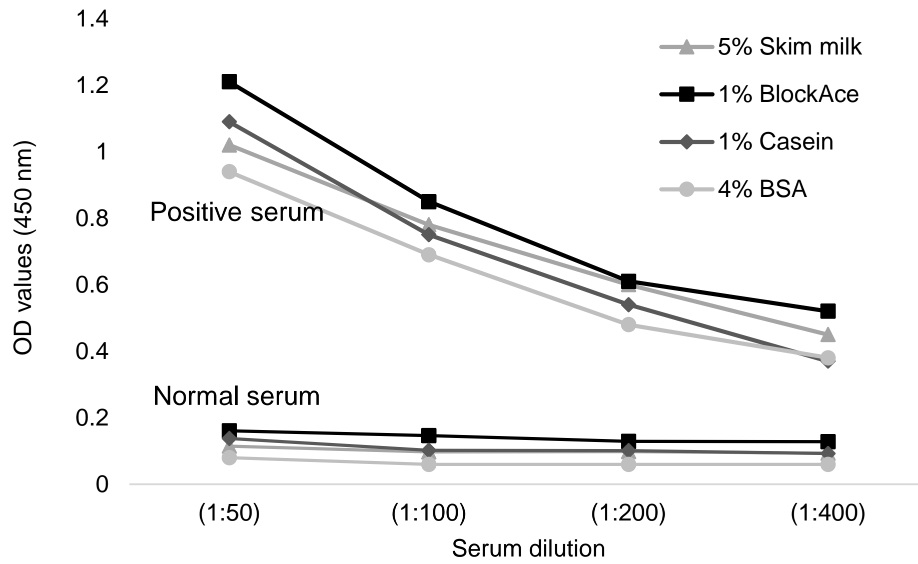

Figure 1-3.

Strep-SP ELISA optimization

a). Selection of a blocking buffer: 4% BSA and 5% skimmed milk in 0.05% PBST, and 1% casein and 1% BlockAce in ultrapure water were tested using a serial dilution of rabbit antiserum and normal serum. 1% BlockAce in ultrapure water showed the best signal/noise (S/N) ratio than other factors tested.

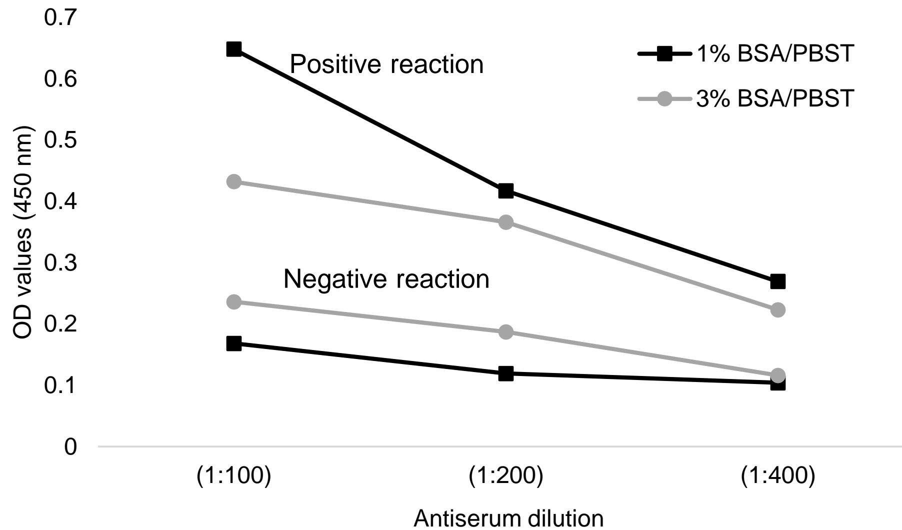

Figure 1-3.

Strep-SP ELISA optimization

b). Selection of a test serum diluent: 1% and 3% BSA/PBST were considered to be tested. At a BSA concentration of 1%, the OD value distances between Strep-SPs (positive reaction) and negative antigens (negative reaction) against antiserum diluted at 1:100, 1:200, and 1:400 were larger than 3% BSA/PBST. At 3% BSA, associated with the increase in the concentration of BSA, the antiserum against Strep-SPs had a lower reaction, but higher against the negative antigens than 1% BSA/PBST.

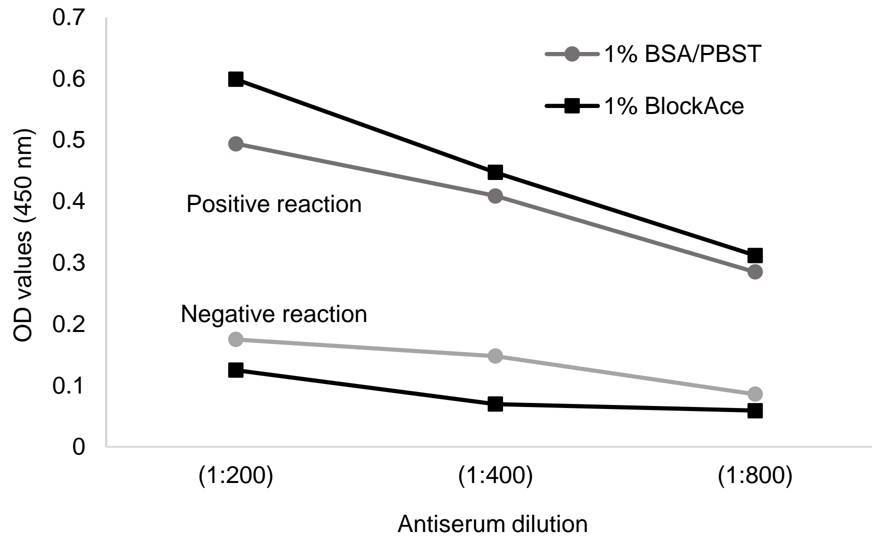

Figure 1-3.

Strep-SP ELISA optimization

c). Selection of a HRP-conjugate diluent: HRP-conjugated Protein A/G was diluted in 1% BSA/PBST and BlockAce in ultrapure water. The blocking buffer (1% BlockAce) lowered the non-specific binding of the negative antigen against serially diluted antiserum than 1% BSA/PBST.

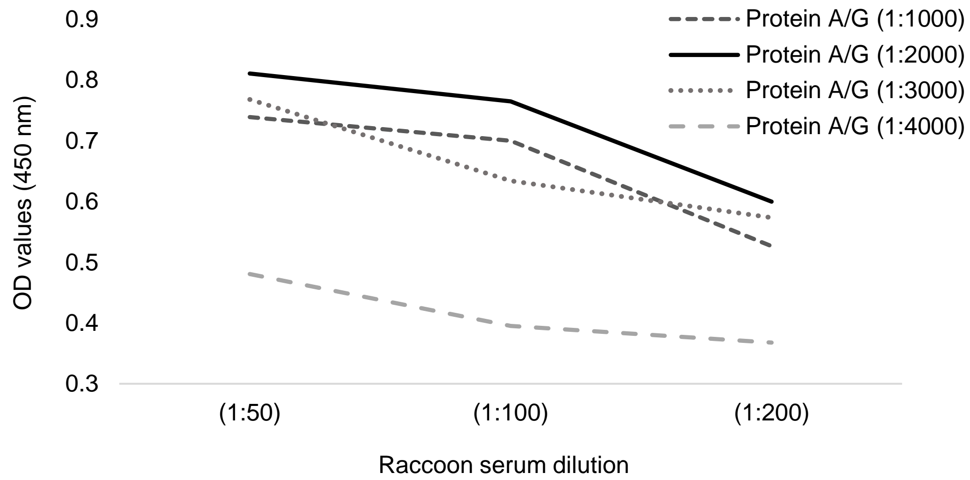

Figure 1-3.

Strep-SP ELISA optimization

d). The optimal concentration of HRP-conjugated Protein A/G: Serially diluted conjugate in 1% BlockAce were tested. Combination of raccoon sera at a 1:100 dilution and HRP-conjugated Protein A/G diluted in 1:2,000 were selected.

Table 1.1.

PRNT50 confirmation of the optimized Strep-SP ELISA tested raccoon sera

Serum ID	P/N ratio of ≥0.5[a]	$PRNT_{50}$[b]	PRNT50 interpretation
1	0.5	1:25	Negative
2	0.6	1:25	Negative
3	0.9	1:25	Negative
4	1.0	1:25	Negative
5	1.0	1:25	Negative
6	1.1	1:25	Negative
7	1.2	1:25	Negative
8	1.5	1:25	Negative
9	1.7	1:50	Positive
10	2.3	1:50	Positive
11	2.7	1:50	Positive
12	3.2	1:50	Positive
13	4.7	1:50	Positive
14	5.0	1:50	Positive
15	6.9	1:50	Positive
16	2.7	1:200	Positive
17	2.9	1:200	Positive
18	4.2	1:200	Positive
19	2.5	1:800	Positive

[a] P/N ratio: Positive to negative ratio of test serum using OD values against the Strep-SPs and negative antigens. Nineteen sera that showed P/N ratios about ≥0.5 were tested by PRNT50 for TBEV.

[b] $PRNT_{50}$: TBEV-neutralizing antibody titers of test serum with a 50% plaque reduction. The $PRNT_{50}$ titer of ≥1:50 was described as TBEV PRNT50 positive with a P/N ratio of ≥1.5, whereas a $PRNT_{50}$ titer of 1:25 was decided to be negative with a P/N ratio of ≤1.5.

Chapter II

Epidemiological survey for TBEV infection in large wild animals on Hokkaido and Honshu island, 2003–2018, Japan

2.1. Introduction

The unknown distribution of TBEV might have widely spread throughout Hokkaido; therefore, vigilant monitoring including identifying the burden of TBEV in the country is essential (Yoshii et al., 2017a). The role of large wild animals in the circulation of TBEV in Japan has not been investigated yet. However, it is well-known that infectious diseases of wild animal present risk to domestic animals and humans. On TBEV transmission, wild animals with their abundant populations can directly affect the spread of the virus in their habitats (Baneth, 2014; Imhoff et al., 2015; Pfeffer and Leschnik, 2017). Japan is rich in wild animal species, and overabundance of sika deer (*Cervus nippon*), wild boars (*Sus scrofa*), and raccoons (*Procyon lotor*) are controlled under the national eradication programs to minimize their impacts on forestry and agriculture damages associating with the country's economic losses and ecological deviations (Ministry of the Environment, Japan, 2015).

In the TBEV-endemic countries, cervid species are the most identified sentinel animals of TBEV infection, followed by wild boars, with infection rates up to 40% (Borcić et al., 1990; Gerth et al., 1995; Roelandt et al., 2010; Ytrehus et al., 2013; Balling et al., 2014; Imhoff et al., 2015; Frimmel et al., 2016; Roelandt et al., 2017). The correlation between seroprevalence in the sentinels and TBE incidence is often observed; additionally, a significant relationship between deer density and vector tick abundance in endemic areas is observed (Carpi et al., 2008; Rizzoli et al., 2009; Jaenson et al., 2012; Imhoff et al., 2015). For example, large forest animals such as deer species can carry a vast number of ticks on their big framed body that is likely to be equal to the activation of the virus transmission between ticks, which also equals the probability of TBEV risk in their habituated areas.

In contrast, TBEV infection in raccoons remains unknown even in TBE-endemic European countries where the population is an overabundance. It has been known that raccoons can carry the TBEV related flaviviruses, with relatively high prevalence, however (Artsob et al., 1986; Blitvich et al., 2009; Ohno et al., 2009). In Japan, the raccoon is one of the alien species with a high population density, being widespread throughout the country.

Although serological evidence of TBEV infections in rodents and domestic animals such as dogs and horses were demonstrated throughout Japan (Takashima, 1998; Takeda et al., 1999; Yoshii et al., 2011), no large wild populations have been tested for TBEV antibodies, excluding a Hokkaido sika deer (*C. n. yesoensis*) survey with prevalence rate of 5 % (5/100 head) in the northern part of Hokkaido in 2018 (Uchida et al., 2018). Thus, large wild population testing would be helpful and necessary to map or estimate the risk of TBEV infection in this country by determining the host ranges and their exposures, particularly in Hokkaido where TBE cases have been reported.

Several seroepidemiological surveillances for TBEV among rodents and domestic animals were conducted during 1997–2011 in Japan, and a 2018 survey testing Hokkaido sika deer is the only survey testing large wild animals. The southern part of Hokkaido was the most tested areas, TBEV antibodies were detected in 76.2% of the dogs and 7.6% of the rodents tested. In a survey in all 14 districts of Hokkaido in 2011, TBEV seropositive horses (3.0%) and dogs (12.5%) were detected in the central part of Hokkaido only. Outside Hokkaido, a 2011 large-scale survey in rodents sampled from other three islands of Japan (Honshu, Shikoku, and Tsushima), TBEV-seropositive rodents with infection rate of 3.4% were detected in Shimane Prefecture on Honshu only. Altogether, it is indicated that a patch of TBEV foci might have occurred throughout the country.

In this study, raccoons, Hokkaido sika deer, and ticks were serologically and virologically examined to define TBEV-endemic regions in Hokkaido Prefecture. Serological survey testing raccoons, Honshu sika deer, and wild boars was conducted in Fukushima and Tochigi Prefectures in Honshu to detect other the virus endemic areas.

2.2. Materials and methods

2.2.1. Sampling location

Serum samples from raccoons and Hokkaido sika deer were collected in both the central (Iburi, Ishikari, Sorachi, and Hidaka Districts) and the eastern (Kushiro and Tokachi Districts) parts of Hokkaido in 2003–2018. On Honshu island, three animal species (raccoons, Honshu sika deer, and wild boars) were sampled in Fukushima Prefecture (Aizu, Hamadori, and Nakadori Districts) in 2016 and 2017, and Honshu sika deer and wild boars were sampled in Tochigi Prefecture (Kenhoku and Kennan Districts) during 2016–2018 (Table 2.1., Figures 2-1. and 2-2.).

Ticks were dragged only in the eastern part of Hokkaido (Tokachi District) in 2018 as described below.

2.2.2. Serum samples

A total of 1,649 sera from 1,072 raccoons, 519 sika deer, and 58 wild boars collected during 2003–2018 were tested for antibodies to TBEV (Table 2.1.). All sera were inactivated at 56°C for 30 min, and stored at -20°C.

2.2.3. Tick dragging and processing

A total of 507 questing adult ticks (420 *I. ovatus* and 87 *I. persulcatus*) from the forested area in Tokachi District in the eastern part of Hokkaido were picked up by dragging (a drag cloth) in early May in 2018 (Table 2.1.). Ticks were grouped by species and sex into 52 pools with 7–10 ticks each. Tick homogenizing was done by crashing the pooled ticks using a sterilized mortar and pestle with sea sands in 1 ml of virus transfer medium (VTM), DMEM supplemented with 10% BSA, penicillin (1,000 units/ml), streptomycin (1 mg/ml), gentamycin (100 μg/ml), and amphotericin B (20 μg/ml). The homogenates were centrifuged, and the supernatants were collected and used for virus and gene detection, as described below. The samples were stored at –80°C until testing.

2.2.4. Blood samples and processing

Twenty-two plasma heparinized blood samples (approximately 0.6–9.0 ml/each) from raccoons captured in Tokachi District in 2018 were tested for TBEV isolation and viral RNA detection. White blood cells (WBCs) were separated by lysing red blood cells using a cell lysis buffer containing ammonium chloride (154.4 mM), potassium bicarbonate (10 mM), and tetrasodium ethylenediaminetetraacetic acid (97.3 µM) in ultrapure water. After washing three times with PBS, WBCs were resuspended in VTM containing 1% BSA and stored at 4°C until testing as described below.

2.2.5. Strep-SP ELISA

The 96-well ELISA plates were coated with Strep-tactin AP in CBB (1:1,000) and incubated overnight. Before blocking the plate with 1% BlockAce (200 µl/well) for 2 h at 37°C, washing was done with 350 µl/well of 0.05% PBST 5 times. Both positive and negative antigens (50 µl/well) were applied in duplicate and incubated for 45 min at 37°C, followed by an incubation of serum samples with a 1:100 dilution in 1% BSA/PBST (50 µl/well) for 45 min at 37°C. After washing, 50 µl/well of HRP-conjugates that Protein A/G (1:2,000 in 1% BlockAce) for sera from raccoons and wild boars, and rabbit anti-deer IgG (1:500) for sika deer sera were added and incubated for 30 min at 37°C. By adding 100 µl/well of TMB substrate, the conjugated reactions were developed after 15 min at room temperature, and the reactions stopping were with 50 µl/well of 2N H_2SO_4 solution. The OD was measured at 450 nm, and the P/N ratios were calculated. Sera with the P/N ratios of ≥1.5 were chosen for the assay confirmation by PRNT50.

2.2.6. PRNT50

The PRNT50 was performed as previously described in Chapter 1. Briefly, BHK-21 cells in 12-well plates were incubated with the mixtures of serially 2-fold diluted sera with an equal volume of 50 PFU/ 0.1 ml of the TBEV-Oshima strain at 37°C for 1 h. An overlay medium

containing 1.5% carboxymethyl cellulose was then added to the cells, and the plates were incubated for 4 days at 37°C. At the end of incubation, the cells were fixed with 10% formalin containing 0.1% crystal violet. The $PRNT_{50}$ titer of ≥1:50 was determined to be seropositive against TBEV.

2.2.7. Virus isolation

BHK-21 cells grown in 24-well plates were inoculated with 0.1 ml of the tick homogenates or WBC suspensions/ well, and incubated for 3–4 days at 37°C. During the incubation period, the inoculated cells were checked for cytopathogenic effect (CPE). The culture supernatant was passaged two times when CPE was not observed and tested for TBEV RNA detection using a real-time RT-PCR assay as described below.

2.2.8. Real-time RT-PCR

Real-time RT-PCR was settled as a previously described TaqMan-based real-time RT-PCR by Schwaiger and Cassinotti (2003). Total RNA from the tick homogenates and their culture supernatants of the BHK-21 cells, and WBCs suspension was extracted using ISOGEN LS (Nippon Gene, Tokyo, Japan). A cDNA synthesis from the RNA samples was performed using Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen, Carlsbad, USA). The assay was carried out with 25 µl RT-PCR mixture, including 12.5 µl of EagleTaq Universal Master Mix (Roche Diagnostics, Mannheim, Germany), 0.6 µl of TBEV specific forward primer F-TBE 1 (5'-GGG CGG TTC TTG TTC TCC-3') and reverse primer R-TBE 1 (5'-ACA CAT CAC CTC CTT GTC AGA CT-3'), 0.3 µl of TaqMan probe TBE-Probe-WT (5'-TGA GCC ACC ATC ACC CAG ACA CA-3'), 6 µl of RNase-free water, and 5 µl of the cDNA sample. Reverse transcription at 50°C for 2 min and 95°C for 10 min, followed by amplification at 95°C for 15 s and 60°C for 1 min during 45 cycles were performed using a LightCycler® Nano (Roche Diagnostics). Viral RNA extracted from TBEV infected BHK-21 cells using ISOGEN-II RNA extraction reagent (Nippon Gene) was used as a positive control and RNase-free water as a

negative control. The cycle threshold value lower than 35 cycles was estimated to be a positive for TBEV RNA.

2.2.9. Statistical analysis

The significant differences in the prevalence rates of districts were calculated using a Fisher's exact test with a *p*-value of <0.05 and a confidence interval (CI) with 95% at www.openepi.com.

2.3. Results

2.3.1. Serological study

Hundred-seven sera, including 81 (7.6%) out of 1,072 raccoons, 18 (3.8%) out of 474 Hokkaido sika deer, and 8 (17.8) out of 45 Honshu sika deer tested were Strep-SP ELISA positive with P/N ratios of ≥1.5 (Tables 2.2. and 2.3.), but none of the wild boars tested positive (data not shown).

Out of 107 Strep-SP ELISA positive sera, 45 were found to be positive for TBEV PRNT50 with $PRNT_{50}$ titers ranging from 1:50 to ≥1:800, including 42 (3.9%) raccoons (Table 2.2.), 2 (0.4%) Hokkaido sika deer, and 1 (2.2%) Honshu sika deer (Table 2.3.).

Raccoons were the most seropositive species with 3.9% (42/1,072) positivity; the overall prevalence rate was 5.9% (39/662) in the central part of Hokkaido, but it was 0.8% (3/368) in the eastern part (Table 2.3.). The seroprevalence in raccoons in Sorachi District (9.5%) in the central part of Hokkaido was significantly higher than that in other districts; Iburi (2.1%; $p < 0.05$), Ishikari (2.1%; $p < 0.01$), and Tokachi District in (0.8%; $p < 0.01$; Table 2.2.). None of the raccoons in Honshu island was positive for TBEV (Table 2.2.). Raccoons in the central part of Hokkaido had relatively high TBEV-neutralizing antibody titers with $PRNT_{50}$ titers of 1:200 to ≥1:800 (64.1%, 25/39). Of these, eight (32%) raccoons showed the highest $PRNT_{50}$ titer of ≥1:800 antibodies, and six out of these eight were sampled in Sorachi District. In

contrast, all three raccoons in Tokachi District had TBEV-neutralizing antibody titer of 1:50 (Table 2.4.).

Sika deer species were found to have lower TBEV-seropositivity than raccoons. Honshu sika deer had rather high TBEV-seropositivity with a seroprevalence rate of 2.4% (1/42) in Kenhoku District in Tochigi Prefecture, whereas Hokkaido sika deer was 0.5% (2/401) in Tokachi District in the eastern part of Hokkaido (Table 2.3.). The TBEV-seropositive serum from Honshu sika deer in Tochigi Prefecture were confirmed to be negative for JEV by PRNT50 (Dr. K. Yoshii's personal communication). In sika deer species, the Honshu sika deer in Kenhoku District in Tochigi Prefecture had TBEV-neutralizing antibodies with a $PRNT_{50}$ titer of 1:50 which was the same as the one of two Hokkaido sika deer in Tokachi District in the eastern part of Hokkaido, but another sika deer in Tokachi District had a $PRNT_{50}$ titer of 1:200 (Table 2.5.).

By the study results, TBEV foci were newly discovered in Tokachi District in the eastern part of Hokkaido with TBEV seroprevalence in raccoons (0.8%; 3/368) and Hokkaido sika deer (0.5%; 2/401), and in Tochigi Prefecture with TBEV seroprevalence in Honshu sika deer (2.4%; 1/42) (Tables 2.6. and 2.7.).

Although none of the sera from wild boar showed a P/N ratio of ≥1.5, one with the highest P/N ratio of 1.0 was negative by PRNT50 (data not shown).

2.3.2. Virus isolation and TBEV RNA detection

Neither TBEV nor its viral RNA was detected in all 52 tick pools and 22 raccoon blood samples collected in Tokachi District in the eastern part of Hokkaido where TBEV seropositive sika deer and raccoons were sampled.

2.4. Discussion

TBE seems to be reemerging on Hokkaido. Although only four TBE cases have been reported in Hokkaido during 2016–2018 since the first case in 1993, the retrospective studies

concluded that an actual number of TBE cases would have been underestimated, because of misdiagnosis with other tick-borne diseases, such as Lyme disease (Yoshii et al., 2017a; 2017b; and 2018). According to the reported TBE cases, it has been strongly suggested that TBEV foci is probably widely distributed throughout Hokkaido, not only in southern part of the island.

Wild animals are indicators for the risk of TBEV in their habitats (Donoso-Mantke et al., 2011; Imhoff et al., 2015; Pfeffer and Leschnik, 2017). Deer species and wild boars are well-known sentinel animals in TBEV-endemic countries. However, there has been no reported evidence of TBEV infection in raccoons. In this study, seropositive raccoons were detected in the samples obtained in 2003–2005 in the central part of Hokkaido, and also in the eastern part that sampled in 2017. These findings could indicate that raccoons can be a sensitive sentinel to disclose the possible risk of TBE to humans, contributing to better understanding the distribution of TBEV in nature.

In the central part of Hokkaido, the risk of TBEV infection has been defined. Actually, two TBE cases, and subclinical cases among residents were reported in 2016 and 2017 (Yoshii et al., 2017b and 2018). A previous study found seropositive horses sampled in 1992 in Shiribeshi District and dogs in 1994–1997 in Iburi District (Takeda et al., 1999). In the present study, I found the serological evidence of TBEV infection in raccoons in Iburi (2.1%), Ishikari (2.1%), and Sorachi (9.5%) Districts, with relatively high $PRNT_{50}$ titers (Tables 2.2. and 2.4.). In these surveys, Iburi District was re-identified to be the TBEV risk area after about ten years of the previous sampling, albeit Ishikari and Sorachi Districts were determined as non-risk TBE area in a 1999 survey with seronegative horses. Therefore, I may speculate that the transmission cycle of TBEV may have been distributed throughout the central part of Hokkaido. In addition, the present study findings in Sorachi District may provide the epidemiological link to a 2018 TBE case reported in Asahikawa District adjoining Sorachi District, where there is no evidence regarding TBEV infection.

In contrast, the prevalence of TBEV in the eastern part of Hokkaido has been unidentified, although a small number of dogs and horses were tested and found to be seronegative in a 1999 survey (Takeda et al., 1999). However, this study suggested that the eastern part of Hokkaido may be the TBEV risk area, because natural exposure to TBEV of two different animal species (raccoons and sika deer) were found in Tokachi District. Although tested sera from both raccoons and sika deer were collected in 2010–2018, seropositive sera were found only in 2017 and 2016, respectively (Tables 2.4. and 2.5.), with a low risk of TBEV infection (Table 2.6.). This data may also indicate the current distribution of TBEV in the area around Tokachi District.

In response to the serological findings in the eastern part of Hokkaido, the survey was expanded in 2018 by testing ticks and raccoon blood samples in order to find the TBEV infected area in Tokachi District. The negative results of TBEV detection obtained might be due to the limited testing number of ticks and raccoons or the area was not the place to seek ticks infected with TBEV. It has been reported that questing ticks were found to be negative for TBEV for over decade or with a low virus prevalence (0–1%) even in the affected areas with a high TBE incidence (Klaus et al., 2010a; Frimmel et al., 2014; Jemeršić et al., 2014; Imhoff et al., 2015; Valarcher et al., 2015). For instance, Stefanoff et al. (2013) tested a large number of field-questing ticks (n=7,436) by real-time RT-PCR, and all ticks were negative for TBEV RNA, suggesting that serological monitoring of wild animals can be an alternative method to tick hunting for TBEV in veterinary surveillance. On the other hand, tick testing using PCR assays, which often show a dearth of the sensitivity, cannot directly apply to disclosure of the risk of TBEV to local residents without awareness of the seroprevalence in animals in a defined area (Süss, 2003; Gäumann et al., 2010; Stefanoff et al., 2013). Hence, the seroprevalence in wild animals can reflect further dynamics of TBEV distribution in the area where the virus prevalence in ticks is low (Klaus et al., 2010b; Süss, 2011).

On Honshu island, there is TBEV serological evidence only among rodents (3.4%) in Shimane Prefecture (Yoshii et al., 2011), showing the possibility that the geographical

distribution of TBEV foci may have widely occurred on Honshu and/or other islands. The number of sera collected in Fukushima and Tochigi Prefectures on Honshu was smaller than that on Hokkaido, but Honshu sika deer sampled in 2018 in Kenhoku District in Tochigi Prefecture was determined as TBEV-seropositive (Table 2.5.). The evidence of TBEV infection in Honshu sika deer in Kenhoku District of Tochigi Prefecture was shown for the first time. Regarding the infection rate of 2.4% (1/42) in Kenhoku District, the ratio of TBEV infection in the area was higher than the infection rate (0.4%; 2/451) of Hokkaido sika deer in Tokachi District in the eastern part of Hokkaido (Tables 2.3. and 2.5.). Although wild boar sera sampled in 2016–2017 in both prefectures were all negative for TBEV serological assays, the results might be due to a few number of samples tested that were not enough to present the presence of the virus circulation in the areas (Table 2.1.).

The majority (3.9%; 42/1,072) of the infected species was raccoons, and the number of tested samples (65.0%; 1,072/1,649) was likely to influence the results (Tables 2.1. and 2.2.), although a high expectation of TBEV infection was in sika deer as a well-established sentinel for TBEV transmission in TBEV-endemic countries. It was surprising that the majority (59.5%; 25/42) of infected raccoons had the relatively high TBEV-neutralizing antibody titers, which may be associated with the development of high viremic level of TBEV so that naturally infected raccoons could be one of the reservoirs in the virus transmission cycle. Similarly, Blitvich et al. (2009) concluded that further research was required to clarify that raccoons could be a reservoir of WNV, which has a close-antigenic relationship with TBEV, as a high viremic level of the virus was shown.

Raccoons may have an important role in the ecology of TBEV similar to dogs from which TBEV was often isolated (Takashima et al., 1997; Leschnik et al., 2002; Pfeffer and Dobler, 2011; Böhm et al., 2017; Pfeffer and Leschnik, 2017). However, as dogs are dead-end hosts they are unlikely to distribute the virus or infected ticks to new areas (Gritsun et al., 2003; Růžek et al., 2010; Bogovic and Strle, 2015; Imhoff et al., 2015).

In Japan, the knowledge about raccoons as an invasive alien species have been well-established; for instance, their home range was estimated about 290 ha throughout the country but in reduced size in Hokkaido because of the climate (Ikeda et al., 2004) and the mean number of infested ticks on a single raccoon was about 140 and up to ten times (Doi et al., 2018). Valarcher et al. (2015) stated that the maintenance of TBEV circulation in the area demanded suitable hosts that have a large population and high and prolonged viremia. Thus, it is worth noting that raccoon can be a reservoir of TBEV in their environment with an overabundant population in Japan, particularly in Hokkaido.

Hokkaido sika deer in Tokachi District was the majority (77.3%, 401/519) in the tested deer, but with low seroprevalence (0.5%, 2/401), which was significantly lower ($p < 0.01$; Table 2.2) than in Toyotomi town (5%) in the far northern part of Hokkaido (Uchida et al., 2018). Results negative for TBEV in the sika deer testing in Hidaka District in the central part of Hokkaido matched with the data of Uchida et al. (2018) who found that 100 sika deer tested were all negative for TBEV antibodies, indicating that Hidaka District could be the TBEV low- or non-risk area. Seropositivity in deer species as sentinels was highly expected than in raccoons at first, but this study suggested that raccoons may have more close host-pathogen interactions in TBEV circulation.

Similarity to raccoon testing, Strep-SP ELISA in sika deer was sufficiently imitated, with a P/N ratio of ≥1.5 as the cut-off value. However, no correlation between the $PRNT_{50}$ and P/N ratios of Strep-SP ELISA was observed.

2.5. Conclusion

Anti-TBEV antibodies were detected in raccoons in the central and eastern parts of Hokkaido, Hokkaido sika deer in the eastern part of Hokkaido, and Honshu sika deer in Tochigi Prefecture on Honshu island. The present study provided new information on the presence of TBEV foci on both eastern part of Hokkaido and eastern Honshu. The serological evidence of TBEV infection in Honshu sika deer was reported for the first time.

This study also reported that raccoons infected with TBEV had high $PRNT_{50}$ titers of ≥1:800, predicting that the species can serve as both a reservoir and indicator hosts of the circulation of TBEV in nature. However, the further study should be needed to clarify this assumption.

Seroprevalence in the central part of Hokkaido (5.7%) was markedly higher than all other investigated areas, the eastern part of Hokkaido (0.6%) and Tochigi Prefecture (2.0%) on Honshu (Table 2.7.).

Further investigations regarding the risk of TBEV infection to humans, a comprehensive eco-epidemiological survey to find out the exact location of TBEV foci in these defined areas, and experimental or field study targeting raccoons' suitability as a reservoir of TBEV are required.

Table 2.1.

Sampling locations, years, and numbers of wild animal sera and ticks tested in this study

Species	Island	Prefecture	District	Years collected	No. of animals tested
Raccoon	Hokkaido	Hokkaido			
		Central	Iburi	2003–2005	94
(*Procyon lotor*)			Ishikari	2003–2005	242
			Sorachi	2003–2005	304
			Unknown[a]	2003–2005	22
		Eastern	Tokachi	2010–2018	368
	Honshu	Fukushima	Hamadori	2016–2017	35
			Nakadori	2016–2017	7
				Subtotal	1,072
Hokkaido sika deer	Hokkaido	Hokkaido			
		Central	Hidaka	2010–2017	23
(*Cervus nippon yesoensis*)		Eastern	Kushiro	2010–2016	13
			Tokachi	2010–2017	401
			Unknown[a]	2010–2017	37
				Subtotal	474
Honshu sika deer	Honshu	Fukushima	Aizu	2017	1
			Hamadori	2016	2
(*C. n. centralis*)		Tochigi	Kenhoku	2016–2018	42
				Subtotal	45
Wild boar	Honshu	Fukushima	Hamadori	2016	48
(*Sus scrofa leucomystax*)			Nakadori	2016	2
		Tochigi	Kennan	2016–2017	8
				Subtotal	58
				Total	1,649
Ticks	Hokkaido	Hokkaido			
I. ovatus		Eastern	Tokachi	2018	420
I. persulcatus				2018	87
				Total	507

[a] Information was unavailable.

Table 2.2.

Seroprevalence of TBEV-neutralizing antibodies in raccoons captured on Hokkaido and Honshu islands, Japan

Island	Prefecture	District	No. of sera tested	No. of sera with[a]	
				P/N ratio of ≥1.5 (%)	PRNT50 positivity (%)[b]
Hokkaido	Hokkaido	Iburi	94	4 (4.3)	2 (2.1)
	Central	Ishikari	242	10 (4.1)	5 (2.1)
		Sorachi	304	41 (13.5)	29 (9.5)
		Unknown[c]	22	3 (13.6)	3 (13.6)
		Subtotal	662	58 (8.7)	39 (5.9)
	Eastern	Tokachi	368	20 (5.4)	3 (0.8)
		Total	1,030	78 (7.6)	42 (4.1)
Honshu	Fukushima	Hamadori	35	3 (8.6)	0 (0.0)
		Nakadori	7	0 (0.0)	0 (0.0)
		Total	42	3 (7.1)	0 (0.0)
		Grand total	1,072	81 (7.6)	42 (3.9)

[a] A total of 81 sera with positive/negative (P/N) ratios of approximately ≥1.5 out of 1,072 were tested using PRNT50.

[b] Data were significant different between central Hokkaido (5.9%) and eastern Hokkiaod (0.8%) ($p < 0.01$); Sorachi District (9.5%) and Iburi District (2.1%) ($p < 0.05$); and Sorachi District and Ishikari (2.1%) and Tockachi (0.8%) Districts ($p < 0.01$).

[c] Information was unavailable.

Table 2.3.

Seroprevalence of TBEV-neutralizing antibodies in sika deer captured on Hokkaido and Honshu islands,Japan

Species	Island	Prefecture	District	No. of sera tested	No. of sera with[a]	
					P/N ratio of ≥1.5 (%)	PRNT50 positivity (%)
Hokkaido sika deer	Hokkaido	Hokkaido Central	Hidaka	23	0 (0.0)	0 (0.0)
		Eastern	Kushiro	13	0 (0.0)	0 (0.0)
			Tokachi	401	18 (4.5)	2 (0.5)
			Unknown[b]	37	0 (0.0)	0 (0.0)
			Subtotal	451	18 (3.8)	2 (0.4)
			Total	474	18 (3.8)	2 (0.4)
Honshu sika deer	Honshu	Fukushima	Aizu	1	0 (0.0)	0 (0.0)
			Hamadori	2	0 (0.0)	0 (0.0)
		Tochigi	Kenhoku	42	8 (19.0)	1 (2.4)
			Subtotal	45	8 (17.8)	1 (2.2)
			Total	519	26 (5.0)	3 (0.6)

[a] A total of 26 sera with positive/negative (P/N) ratios of approximately ≥1.5 out of 519 sera were tested using PRNT50.

[b] Information was unavailable.

Table 2.4.

TBEV-neutralizing antibody titers ($PRNT_{50}$) in the raccoon sera in Hokkaido

Prefecture	District	PRNT50 positivity (%)	Years collected	$PRNT_{50}$		
				1:50	1:200	≥1:800
Hokkaido	Iburi	2 (2.1)	2004–2005	1	1	0
Central	Ishikari	5 (2.1)	2003–2005	3	1	1
	Sorachi	29 (9.5)	2003–2005	9	14	6
	Unknown [a]	3 (13.6)	2005	1	1	1
	Subtotal	39 (5.9)	-	14	17	8
Eastern	Tokachi	3 (0.8)	2017	3	0	0
	Total	42 (4.1)	-	17	17	8

[a] Information was unavailable.

Table 2.5.

TBEV-neutralizing antibody titers ($PRNT_{50}$) in the sera from Hokkaido and Honshu sika deer

Species	Prefecture	District	PRNT positivity (%)	Years collected	$PRNT_{50}$	
					1:50	1:200
Hokkaido sika deer	Hokkaido Eastern	Tokachi	2 (0.5)	2016	1	1
Honshu sika deer	Tochigi	Kenhoku	1 (2.4)	2018	1	0
		Total	3 (0.7)	-	2	1

Table 2.6.

Maximum likelihood estimation of TBEV seroprevalence in the defined districts on Hokkaido and Honshu islands

Island	Prefecture	District	No. of sera tested	Seroprevalence (%)	95% CI (%)[a]	
					Lower	Upper
Hokkaido	Hokkaido	Iburi	94	2.12	0.59	7.43
	Central	Ishikari	242	2.06	0.89	4.74
		Sorachi	304	9.53	6.72	13.36
	Eastern	Tokachi	769	0.65	0.28	1.51
Honshu	Tochigi	Kenhoku	42	2.38	0.42	12.32

[a] Confidence interval = 95%.

Table 2.7.

Summary of TBEV seroprevalence on Hokkaido and Honshu islands

Island	Prefecture	Species tested	District	No. of sera tested	PRNT50 positivity (%)
Hokkaido	Central	Raccoons	Iburi	94	2 (2.1)
			Ishikari	242	5 (2.1)
			Sorachi	304	29 (9.5)
			Unknown[a]	22	3 (13.6)
		Sika deer	Hidaka	23	0 (0.0)
			Subtotal	685	39 (5.7)
	Eastern	Raccoons	Tokachi	368	3 (0.8)
		Sika deer	Kushiro	13	0 (0.0)
			Tokachi	401	2 (0.5)
			Unknown[a]	37	0 (0.0)
			Subtotal	819	5 (0.6)
			Total	1,504	44 (3.0)
Honshu	Fukushima	Raccoons	Hamadori	35	0 (0.0)
			Nakadori	7	0 (0.0)
		Wild boars	Hamadori	48	0 (0.0)
			Nakadori	2	0 (0.0)
		Sika deer	Aizu	1	0 (0.0)
			Hamadori	2	0 (0.0)
			Subtotal	95	0 (0.0)
	Tochigi	Sika deer	Kenhoku	42	1 (2.4)
		Wild boars	Kennan	8	0 (0.0)
			Subtotal	50	1 (2.0)
			Total	145	1 (0.7)
			Grand total	1,649	45 (2.7)

[a] Information was unavailable.

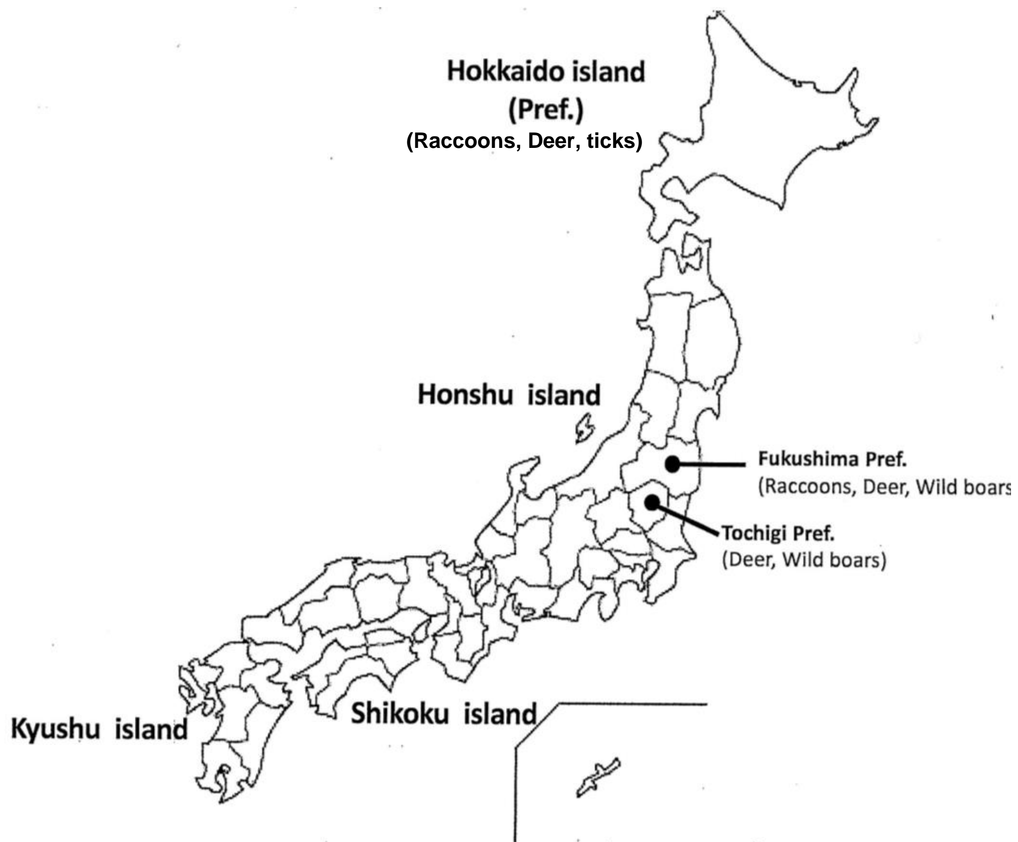

Figure 2-1.

Sampling locations on Hokkaido and Honshu islands, Japan. Tested species were indicated in parentheses.

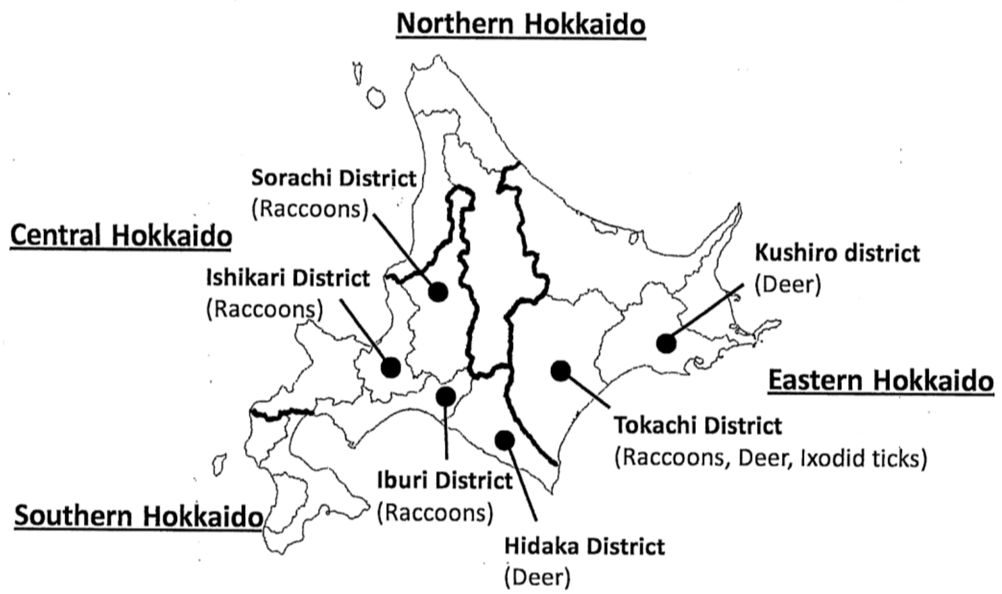

Figure 2-2.

Sampling locations on Hokkaido. The sites were underlined. Tested wild animal and tick species were indicated in parentheses.

Chapter III

Serological survey for TBEV in domestic ruminants, Japan, 1998–1999

3.1. Introduction

TBEV circulates between wild animals and its vector ticks and affects humans and domestic animals, both of which are a dead-end and accidental hosts since they are not probably a source of the infection for vector ticks (Süss, 2011). The geographical prevalence of TBEV has been increased, with newly notified endemic foci, vector ticks, and sentinels (Dobler et al., 2012; Pfeffer and Leschnik, 2017; Kunze, 2018). TBEV infection in livestock may cause chronic illness and lead to diminished producing milk which associates with the economic losses in the country.

Alimentary TBEV infection is one of two direct transmission routes of the virus affecting humans through unpasteurized milk consuming, causing the illness named biphasic milk fever of TBE (Lindquist and Vapalahti, 2008; Kriz et al., 2009; Dobler et al., 2012). The milk fever starts suddenly, with high biphasic fever up to 40°C during about ten days' interval in each phase, but with high level recovering and without neurological sequelae (Gritsun et al., 2012). In eastern Europe and Baltic states, the alimentary infection of TBEV in humans has been growing as a public health issue. Associated with people's lifestyle or food culture in these countries, they routinely consume unpasteurized milk and dairy products (cheese, yogurt, and butter) made from raw milk, which is often linked with the alimentary transmission of TBEV from viremic cattle, goats, and sheep (Vereta et al., 1991; Gritsun et al., 2003; Süss, 2003). Some countries are highly endemic with TBEV alimentary infection, for instance, more than 150 cases caused by 22 outbreaks have been reported in Slovakia in the past 5 years (Kerlic et al., 2018; Kunze, 2018), and TBE lethal cases caused via the virus-contaminated cow milk

and milk product were reported in Russian Siberia and Far Eastern parts (Vereta et al., 1991; Gritsun et al., 2003).

Domestic ruminants can serve as the reservoirs of TBEV but produce a low-level viremia only for a few days, and rarely show clinical signs. They are infected with TBEV via tick bites, and once infected, the virus is shielded from the blood of the infected cows or sheep into their mammary glands during the viremic phase (Estrada-Peña and de la Fuente, 2014; Böhm et al., 2017; Rieille et al., 2017). TBEV is relatively resistant in environmental conditions; at pH of less than 7, and heating for 10 min at 60°C. This stability may allow TBEV to survive in milk, and the virus can be detectable in cows' milk up to 25 days (Gritsun et al., 2003; Valancher et al., 2015), but the survival only between 2 and 8 days in goat and sheep' milk (Cisak et al., 2010; Süss, 2011). A study in Poland carried out by Cisak et al. (2010) found the viral RNA in cow's milk with infection rate at 11.1% by RT-PCR and TBEV antibodies with infection rate at 3.2% using a commercial anti-TBEV ELISA IgG. However, in humans, the infection caused via virus-contaminated milk is more acute than the infection via tick-bites (Dobler et al., 2012).

Through sharing the grassing land with wild animals and vector ticks, these domestic ruminants can bring infected ticks to the human environment from their fields. Therefore, they also can be sentinels for TBEV in the area (Imhoff et al., 2015). The infection rate of TBEV in cows is reported less, but about 0–26% in the endemic areas, and 0–58% in goats and sheep (Holzmann et al., 2009; Klaus et al., 2010b; Imhoff et al., 2015; Böhm et al., 2017; Rieille et al., 2017; Salat et al., 2017).

Before diagnosing the TBE case in 1993, Takashima et al. (1997) had suspected the presence of scattered TBEV foci in Hokkaido through the seroepidemiological surveys in cattle in 1978. In 1994, they reported TBEV-seronegative cattle sera and cows' milk collected from both the farm of the TBE patient and an adjacent farm. Since then, there has been no published study of cattle for TBEV infection. In Japan, there is a quite low possibility that TBE cases are caused via taking unpasteurized TBEV contaminated cow milk or dairy products. In TBEV-endemic European countries, grazing animals such as sheep, goats, and cattle have been

shown to be suitable sentinels for detecting the virus endemic areas (Gresikova et al., 1975; Leutloff et al., 2006; Klaus et al., 2012 and 2014). In Japan, many cattle are kept. Therefore, it would be worth examining cattle serologically whether they serve as specific sentinels for uncovering TBEV related risk areas. In contrast to endemic foreign countries, there is no study on TBEV infection in sheep in this country.

This study sought to reveal the presence or absence of antibodies to TBEV infection in sera from cattle and sheep using Strep-SP ELISA.

3.2. Material and methods

3.2.1. Serum samples

A total of 728 cattle sera collected in 1998–1999 from 13 prefectures in different islands (Table 3.1.) and 177 sheep sera collected from the central part of Hokkaido were tested. Serum samples used in this study were kindly provided by National Institute of Animal Health, Japan.

3.2.2. Serological methods

Strep-SP ELISA was performed as previously described. Briefly, the ELISA plates were coated with 50/well µl of Strep-tactin AP (1:1,000 in CBB) and incubated overnight at 4°C. After washing of the plates with 350/well µl of 0.05% PBST, the plates were blocked with 1% BlockAce (200 µl/well) for 2h at 37°C, followed by incubations with antigens (50 µl/well) and sera (50 µl/well) for 45 min at 37°C, respectively. Then, the plates were incubated with 50 µl/well of HRP-conjugated Protein A/G (1:2,000) for 30 min at 37°C, and then OD was read at 450 nm by adding 100 µl/well of TMB. Sera showing P/N ratios of ≥1.5 were selected for confirmation of PRNT50.

PRNT50 was conducted as described in Chapter 1. Briefly, BHK-21 cells seeded in 12-well plates were inoculated with a mixture of 0.1 ml of two-fold diluted serum in 2% EMEM with TBEV-Oshima (50 PFU/0.1 ml) suspension and incubated for 4 days at 37°C. Then, the plates

were fixed with 10% formalin containing 0.1% crystal violet, and after removing the formalin solution, $PRNT_{50}$ was determined by counting 50% or more reduction in plaque counts.

3.3. Results

Of the 728 cattle sera, 70 (9.6%) showed P/N ratios of ≥1.5 (up to ≤6.0) in Strep-SP ELISA (Table 3.1.). The ELISA-positive sera were detectable in all prefectures examined, ranging from 4.1% to 25.0%. The positive rate higher than 20% was found in Nagano, Okayama, Ehime, and Saga Prefectures. However, none of the cattle sera had TBEV-neutralizing antibody titers higher than 1:50 (Table 3.1.). Only one serum sampled in Ishikari District in the central part of Hokkaido showed a $PRNT_{50}$ titer of 1:20 (data not shown).

Three out of 177 sheep sera had P/N ratios of 3.2–4.9 by Strep-SP ELISA but did not yet confirm the results by $PRNT_{50}$.

3.4. Discussion

Of the 728 cattle sera, 70 (9.6%) were estimated the P/N ratios of ≥1.5, and most of the ELISA-positive sera showed the P/N ratios ranging from 2.5 to 3.0. Surprisingly, however, such a big proportion of cattle sera testing was negative for TBEV PRNT50. In this study, it was revealed that Strep-SP ELISA could not be applied to detecting TBEV antibodies in cattle sera, albeit the assay was successfully applied to raccoons and sika deer.

One cattle serum with the P/N ratio of 3.0 had TBEV-neutralizing antibody titer of 1:20. Klaus et al. (2014) pointed out that cross-reactivity with other flaviviruses must be considered, especially for TBEV serology in the areas with a seroprevalence of Louping ill virus. However, the prevalence of other flaviviruses such as JEV and Louping ill virus has not been known in Hokkaido. The $PRNT_{50}$ of ≤1:20 was often observed due to non-specific reaction or cross-reactivity with related flaviviruses (Dr. K. Yoshii's personal communication). Therefore, this serum was judged to be TBEV-seronegative.

It was reported that Strep-SP ELISA could be successfully used for serological examination of TBEV infection in humans and rodents since this ELISA system showed over 95% sensitivity and specificity compared to PRNT results (Inagaki et al., 2016). In this study, the ELISA system was first applied to the examination of cattle sera. The reason for the nonspecific reaction of Strep-SP ELISA in cattle sera is unknown and indeterminate. As possible explanations for this unexpected results might be because of the non-specific IgG antibodies in cattle sera might have associated with the reaction of Strep-tag of TBEV-SPs or Strep-tactin (Skerra and Schmidt, 2000), or Strep-SPs might have bound to the non-specific antibodies against antigenically-related viruses which may spread across the country.

In contrast, only three out of 177 sheep sera showed P/N ratios of ≥1.5. However, the results of Strep-SP ELISA could not be confirmed using PRNT50.

The risk of the alimentary transmission of TBEV to humans in Japan would be quite low owing to the highly standardized cattle farming system, and milk or its products and consumption throughout the country. However, as the possibility that TBEV may affect domestic animals through sharing their environment with overabundance wild animals including raccoons and sika deer rather than vector ticks, continuous investigation of TBEV infection in domestic animals should be considered.

3.5. Conclusion

Although a large number of cattle sera were tested across Japan, none was seropositive for TBEV. This study indicated that Strep-SP ELISA could not test cattle sera for screening and uncovering TBEV foci. However, further studies are needed to reveal cross-reactivity with other flaviviruses and the cause of non-specific reaction in cattle.

Table 3.1.

Strep-SP ELISA examination in cattle sera

Island	Prefecture	No. of sera tested	No. of sera with P/N ratios of ≥1.5[a]	PRNT50 result
Hokkaido	Hokkaido	133	9 (6.8%)	Negative
Honshu	Aomori	49	2 (4.1%)	Negative
	Yamagata	47	2 (4.3%)	Negative
	Nagano	44	11 (25.0%)	Negative
	Gifu	73	3 (4.1%)	Negative
	Wakayama	44	4 (11.0%)	Negative
	Okayama	10	2 (20.0%)	Negative
	Hiroshima	61	4 (6.6%)	Negative
	Subtotal	328	28 (8.5%)	
Shikoku	Ehime	77	17 (22.1%)	Negative
Kyushu	Saga	24	5 (21.0%)	Negative
	Oita	50	5 (10.0%)	Negative
	Miyazaki	26	1 (3.8%)	Negative
	Subtotal	100	11 (11.0%)	
Ryukyu	Okinawa	90	5 (5.6%)	Negative
	Total	728	70 (9.6%)	

[a] Of the 728 sera, 70 with positive/negative (P/N) ratios of ≥1.5 were selected for PRNT50.

General discussion

Tick-borne encephalitis (TBE) is a tick-transmitted viral infection that causes severe human central nervous system diseases, but asymptomatic TBEV infection is in the majority (Gritsun et al., 2003; Mandl, 2005; Kunz, 2007). TBEV strains differs based on the viral antigenic variants, which resulting into the classification of three subtypes (the Western, Siberian, and Far-Eastern) that further associates with the differences in the geographical distribution, the natural transmission cycle, the disease ecology, and pathogenic potentials in humans and animals (Süss, 2003; Gubler et al., 2007; Dobler et al., 2012). TBEV natural cycle is maintained through the interactions between vector ticks and vertebrate hosts in the specific geographic biotypes that creates TBEV foci in a defined area, which represents the disease ecology of TBEV in local humans and animals (Zeman, 1997; Korenberg and Kovalevskii, 1999; Süss, 2003; Dobler et al., 2011; Pfeffer and Leschnik, 2017). The most identified vector ticks are Ixodid ticks, and vertebrate hosts of TBEV can vary depending on ticks' host-finding activity and animal species in the area. Rodents are the primary hosts, but large animals such as deer, wild boars, and livestock were often found to have TBEV antibodies indicating the presence of TBEV foci in their habitats.

Geographical distribution of TBEV foci spans throughout the Eurasian forest belt involving at least 32 countries including Japan, particularly in Hokkaido, with TBEV-Far Eastern strain circulation (Mandl, 2005; Lindquist and Vapalahti, 2008). TBE became familiar in this country by the first TBE case reported in the southern part of Hokkaido in 1993 (Takashima et al., 1997). Since then, the ecology of TBEV in this country seemed to be present with a low epizootic activity. However, in recent years TBEV foci seem to have been activated, because four TBE cases with two fatal cases were reported in Hokkaido between 2016 and 2018 (Yoshii et al., 2017a; http//www.city.asahikawa.hokkaido.jp/kurashi/135/136/150/ d064126. html; in Japanese). To monitor the distribution and transmission of TBEV, veterinary surveillance is a crucial to discover natural hosts of TBEV in the area to identify the exact location of TBEV foci

in order to prevent the risk to humans (Gritsun et al., 2003; Dobler et al., 2012; Imhoff et al., 2015). However, TBEV infection in large wild animals and livestock in Japan is generally unknown. In this thesis study, the infection of TBEV in selected wild animals and livestock sampled across the country was ascertained, and the prevalence of TBEV was calculated based on the serological findings.

This epidemiological surveillance was initiated to seek the evidence of TBEV infection in wild overabundance species such as raccoons, Hokkaido, and Honshu sika deer, and wild boars and livestock (cattle and sheep) using serological assays. In response to the serological findings in the eastern part of Hokkaido, the study was then expanded by testing ticks and raccoon blood for TBEV using virological and genetical examinations to investigate the area, where TBEV circulation is likely to be present. However, none of Ixodid ticks (*I. persulcatus* and *I. ovatus*) and raccoon blood samples collected around Tokachi District was positive for TBEV. Although it is difficult to conclude that the area is at the low-level activity, the negative results might be due to a small number of samples collected in a short time.

To detect TBEV antibodies in wild animals, Strep-SP ELISA (Inagaki et al., 2016) was first set up with optimized conditions using TBEV-possitive rabbit sera, and the test sera from raccoons and sika deer. A $PRNT_{50}$ titer of ≥1:50 in PRNT50 was considered to be TBEV-seropositive in this study, which was used to confirm the presence of TBEV antibodies in animal sera with a positive/negative (P/N) ratio of ≥1.5 in Strep-SP ELISA. Although Strep-SP ELISA was successfully applied to screening TBEV antibodies in the sera from raccoons, sika deer, wild boars and sheep, but it could not work with cattle sera. This unexpected results of Strep-SP ELISA in cattle demonstrated that the ELISA system could not detect TBEV antibodies in all vertebrate hosts of TBEV which have a binding affinity of IgG to Protein A/G conjugates.

Accordingly, the detection capability of the ELISA is required to be enhanced in its specificity and sensitivity.

To my knowledge, there were no documented studies reporting the evidence of TBEV infection in raccoons and Honshu sika deer, but this study found that both animal species are involved in the ecology of TBE in Japan. The central hypothesis of TBEV infection in wild animals was mainly for deer species as a well-known sentinel of TBEV risk in the endemic countries, but raccoons were found to be more seropositive for TBEV with $PRNT_{50}$ titer of up to ≥1:800 in this study. It may indicate that raccoons have a more prominent role than cervid species in the TBEV transmission cycle in their habitats. Therefore, further studies are needed to identify the host-specificity of TBEV to raccoons how they participate in the ecology of TBEV as reservoirs or excellent sentinels.

In the present study, TBEV foci were newly discovered in the eastern part of Hokkaido with TBEV seroprevalence in raccoons (0.8% (3/368)) and Hokkaido sika deer (0.4% (2/451)), and in Tochigi Prefecture on Honshu island with 2.4% (1/42) seropositivity in Honshu sika deer as shown in Table 2.7. From the sampling data of these TBEV seropositive sera collected during 2016–2018 (Tables 2.4. and 2.5.), both areas may have been recently exposed to TBEV, indicating the distribution of TBEV in a wide range not only in Hokkaido but also other islands, including Honshu.

Taken together, the study findings of TBEV in wild animals in addition to the previous evidences of TBEV infections signify that TBEV is endemic in Japan. This study provides a risk of TBEV infection associated with overabundance raccoons and sika deer on Hokkaido and Honshu islands, and a need for additional studies to ensure the focal distribution of TBEV in the defined areas to localize the TBEV foci.

General summary

TBEV is mainly maintained between Ixodid ticks and their vertebrate hosts. Feeding hosts of ticks are mainly forested and domestic animals that play an important role in TBEV transmission. Among the vertebrate hosts, rodents with persistent and high-level viremia act as both maintenance and amplifying hosts. Other large animals cannot transmit the virus to ticks, because of a short and low-level viremia. However, in masses they provide the place that ticks can feed and multiply on their bodies, resulting in support for virus circulation. In the TBEV-endemic countries, both large wild and domestic animals such as deer, wild boar, sheep, goat, and cattle are known to be available as valuable indicator hosts (sentinels) to monitor the distribution of vector ticks, because they long maintain antibodies to TBEV.

However, very little is known regarding TBEV infection in these sentinel species in Japan.

The first human case of TBE was recorded in the southern part of Hokkaido in 1993, and it was revealed that this region was a TBEV endemic area based on the isolation of TBEV from ticks, rodents, and dogs, as well as serological evidence in rodents, dogs, and horses. Recently four confirmed cases were reported in the southern, central and northern parts of Hokkaido during 2016–2018, suggesting the re-emergence of TBE and the geographical distribution of TBEV in Hokkaido. Seropositive Hokkaido sika deer were detected in the far northern part of Hokkaido before the occurrence of TBE in 2018. However, TBEV foci are unknown in other parts of Hokkaido. Outside Hokkaido, Shimane Prefecture was the only area where seropositive rodents were found in 2001.

Thus, it is an urgent issue to examine and clarify the distribution of TBEV foci in Japan to avoid the further occurrence of TBE. Therefore, the thesis aimed to uncover and identify the previously unknown TBEV risk areas by testing wild animal raccoons, sika deer and wild boars, and livestock (cattle and sheep) using ELISA and a 50% plaque reduction neutralization test (PRNT50).

The first chapter describes the optimization of Strep-SP ELISA in order to detect TBEV antibodies in sera from large wild animals. Several parameters such as the concentration of

Strep-SPs and Protein A/G conjugates, and selection of blocking buffers and diluents were evaluated using a rabbit anti-TBEV antiserum and normal rabbit serum. As the ELISA positive antigens, TBEV subviral particles (SPs) with Strep-tag (WSHPQFEK) that were secreted into the supernatants of HEK293T cells transfected with the plasmid expressing Strep-SPs were used (Strep-SP ELISA). Negative antigens were similarly prepared from the supernatants of normal HEK293T cells. As a blocking buffer, 1% BlockAce highly reduced background reaction compared with 4% bovine serum albumin (BSA), 5% skim milk or 1% casein. As serum and conjugate diluents,1% BSA was superior to 3% BSA, because of lower background. In the optimized ELISA, the results were recorded as the positive/negative (P/N) ratio (i.e., the ratio of the optimal density values at 450 nm of Strep-SPs to that of the negative antigens). A total of 411 sera including 368 raccoons and 43 sika deer were first tested to examine whether Strep-SP ELISA can be used to detect antibodies to TBEV in wild animals, and then the ELISA results were confirmed by PRNT50. By the PRNT50 results, nineteen raccoon sera showed TBEV $PRNT_{50}$ titers ranging from 1:25 to 1:800, but seven sika deer sera were PRNT50-negative. In this study, the PRNT50 with neutralizing antibody titers ($PRNT_{50}$) of ≥1:50 indicated Strep-SP ELISA positive when raccoon serum samples showed a P/N ratio of ≥1.5. However, the cut-off value of Strep-SP ELISA was not determined for sera from sika deer.

Chapter II describes an expanded, serological survey of TBEV on Hokkaido and Honshu islands by testing a total of 1,649 animal sera sampled in 2003–2018, including 1,072 raccoons, 519 sika deer, and 58 wild boars using Strep-SP ELISA and PRNT50. In addition, 507 Ixodid ticks and 22 raccoon bloods were examined by real-time RT-PCR and virus isolation. As a result of the serological survey, the most identified TBEV seropositive species was raccoons (3.9%; 42/1,072), followed by sika deer (0.6%; 3/519). The central part of Hokkaido had the highest seroprevalence at 5.7% (39/685), followed by the Kenhoku District of Tochigi Prefecture in Honshu at 2.4% (1/42), and the eastern part of Hokkaido at 0.6% (5/819). Significant differences in TBEV seroprevalence among raccoons were observed between Sorachi District (9.5%) in the central part of Hokkaido and Iburi (2.1%; $p < 0.05$),

Ishikari (2.1%; $p < 0.01$), and Tokachi District in (0.8%; $p < 0.01$). Neither raccoons nor wild boars in Fukushima and Tochigi Prefectures had TBEV antibodies. All Ixodid ticks and raccoon blood tested negative for TBEV.

In the study, TBEV foci were newly discovered in Tokachi District in the eastern part of Hokkaido with TBEV seroprevalence in raccoons (0.8%; 3/368) and Hokkaido sika deer (0.5%; 2/401), and in Tochigi Prefecture with TBEV seroprevalence in Honshu sika deer (2.4%; 1/42). From the data of these seropositive sera collected in 2016–2018, the areas may have been recently exposed to TBEV, which may suggest a possibility that TBEV is distributed in a wide range not only in Hokkaido but also other islands.

Chapter III includes a serological survey on cattle and sheep sera collected across the country using Strep-SP ELISA. A total of 728 cattle sampled in 1998–1999 from 13 prefectures on all islands of Japan, and 177 sheep sera in the central part of Hokkaido were tested. Of these, 70 cattle and three sheep sera showed P/N ratios of ≥1.5. Unexpectedly, however, TBEV-neutralizing antibodies were not detected in cattle sera. Only one cattle serum showed a low-level titer (1:20) against TBEV in PRNT50. In this study, Strep-SP ELISA was first applied to the examination of cattle sera. Although the reason of high nonspecific reaction in cattle sera is unknown, as a possible explanation for this unexpected result, non-specific IgG in cattle sera might have associated with the reaction of Strep-tag of TBEV-SPs or Strep-tactin AP. However, it may not deny the possibility that TBEV Strep-SPs might have reacted to antibodies against unknown flaviviruses that are antigenically related to TBEV.

In conclusion, this thesis study revealed the presence of new TBEV foci in the eastern part of Hokkaido and Tochigi Prefectures (Honshu island) through serological surveys in large wild animals. The present study may suggest that TBEV is widely distributed in Japan more than expected, especially in Hokkaido. For continuous large-scale monitoring of TBEV foci using large wild animals such as raccoons, sika deer or wild boars as known sentinels, a combination of Strep-SP ELISA with PRNT50 seems to be useful. To avoid emerging TBE, it is required to

find out the exact locations of TBEV foci in the areas where the serological evidence of TBEV infection is given.

和文要約

ダニ脳炎 (Tick-borne encephalitis, TBE) は、人の中枢神経系を冒す重篤なウイルス感染症の1つである。人におけるTBEウイルス（TBEV）の感染は多くの場合不顕性感染であるが、発症した場合には髄膜炎や脳炎といった特徴的な神経系の異常を示す。TBEVがエンデミックである国々では、毎年10,000人以上の患者が発生し、そのうちの約70%は後遺症残すと推定されている。

人はウイルスを保有するダニの吸血により感染するが、稀ではあるがウイルスに感染した母親や家畜のミルクを飲むことによっても感染が起こる。TBEはワクチンによって予防することができるが、TBEVがエンデミックである国々では、依然として人の発症が認められている。

自然界ではTBEVはダニと脊椎動物の間で維持されている。最もベクターとして知られているダニはマダニであり、吸血により感染した動物はTBEVの伝播や維持において重要な役割を果たす。脊椎動物宿主の中で、小型げっ歯類がウイルスの維持及び増殖の場となっており、また感染後にウイルスが体内に長く残り、また高いレベルのウイルス血症を示すために感染巣としての役割も果たしている。一方それよりも大型の動物は、ウイルス血症のレベルも低く期間も短いために、ダニにウイルスを伝達することができない。しかし、これらの動物は通常集団を形成し、ダニが吸血することによって多数のダニの繁殖を許し、その結果自然界でウイルスの循環を助ける役割を果たしている。加えて、TBEVがエンデミックであ

る国においては、大型の野生動物（鹿、イノシシ等）及び家畜（牛、羊、山羊等）が感染後に長く持続する抗体を産生するために、地域におけるベクターダニの分布やその個体数をモニターするのに非常に重要なindicator hostつまり歩哨動物として注目されている。このようなindicator host（歩哨動物）における血清学的調査は、人でのTBEリスクを未然に防ぐ上で貴重な情報を提供する。しかしながら、日本においてはindicator hostにおけるTBEV感染についてはほとんど知られていない。ベクターダニとげっ歯類などの野生動物で構成されるTBEV fociは、日本を含む32のユーラシアの国々に存在していることが報告されているが、約30,000の TBEV fociのあることが推測されている。

日本では、TBEは届出義務のある感染症である。最初の患者の発生は1993年に北海道の道南地方で報告されているが、その後この地域においてTBEVがダニ、げっ歯類、犬から分離されると共に、抗体が犬や馬から検出されたことから、道南地方がエンデミックエリアであることが判明した。その後長い間TBEの発生はなかったが、2016年から2018年にかけて4件のTBE患者の発生が、道央、道南および道北地方において報告された。道北地方においては、TBE発生の前にエゾシカから抗体が検出されたことが報告されている。これらの事実から、北海道において広くTBEVが存在していることが示唆された。しかし、その他の北海道の地域におけるTBEV fociの存在は知られていない。北海道以外では、2001年に島根県のみでげっ歯類から抗体が検出されている。TBEが再興感染症として再び問題になっていること

を考慮すると、さらなるTBEの発生を避けるために、日本におけるTBEV fociの分布を明らかにする事は緊急の課題である。

本研究は、野生動物（鹿、アライグマ、イノシシ）及び家畜（牛、羊）を血清学的に調べることにより、これまで知られていないTBE のリスクエリアを明らかにすることを目的に実施された。また、ダニ及びアライグマの血液からのウイルス検出も試みた。

第I章では、野生大型動物からのTBEV抗体検出に使用するELISAの反応条件の設定を兎抗TBEV血清及び正常兎血清を用いて行った。ELISA抗原として、Strep-tag (WSHPQFEK) が付加されているウイルス様粒子（Subviral particles, SPs）を用いた。Strep-tag 付加SP (Strep-SPs）を発現しているplasmid がトランスフェクトされたHEK293T細胞の培養上清中に分泌されているStrep-SPsをポリエチレングリコール8000で濃縮後に抗原として使用した。陰性抗原は、正常HEK293T細胞の培養上清を同様に処理して得られた。Alkaline phosphatase 標識Strep-Tactinの ELISAプレートへの吸着は、懸濁液として炭酸-重炭酸緩衝液を用いた方が PBSよりも高い吸着率を示した。Strep-SPsを含む培養上清の100倍濃縮液が、ELISA抗原として最も高い反応性を示した。ブロッキングバッファーとしては、1%BlockAceが4%牛血清アルブミン(BSA)、5%スキムミルクあるいは1%カゼインよりも低い非特異的反応を示した。血清及びコンジュゲートの希釈液としては、1%BSAが3%BSAより非特異的反応のレベルが低かった。ELISAの結果は、陽性抗原のOD値（P）と陰性抗原のOD値（N）の比（P/N比）として表した。本試験では、被験血清のP/N比が1.5以上を示

した場合をStrep-SP ELISA陽性とし、50%プラック減少中和試験 (50% plaque reduction neutralization test、PRNT50)を用いて被験血清中のTBEV特異抗体の存在を確認した。368検体のアライグマ血清及び43検体のシカ血清の計411検体の血清について、Strep-SP ELISAを用いてTBEVに対する抗体が検出可能か調べた。その結果、大凡P/N比が1.5以上を示した検体は、PRNT50においても陽性を示した。

第II章は、北海道と本州において捕獲された合計1,649検体の野生動物の血清を用いた抗体調査の結果を示している。その内訳は、1,072頭のアライグマ、519頭の鹿及び58頭のイノシシである。検査方法としてStrep-SP ELISAとPRNT50を用いた。また、抗体が検出された地域で採取されたダニ及びアライグマの血液についてウイルス分離及びリアルタイムRT-PCRによりウイルス遺伝子の検出を試みた。

抗体検査の結果、最も陽性率が高かった動物種はアライグマであった (3.9%、42/1,072)。次にシカであった(0.6%、3/519)。地域的に最も高い抗体陽性率を示したのは、アライグマとエゾシカ685頭中5.7%（３9頭）が 抗体陽性を示した北海道の道央地方であった。次に、ホンシュウジカ42頭中2.4%（1頭）が陽性を示した栃木県の県北地方、そしてアライグマとエゾシカ819頭中0.6%（5頭）が陽性を示した北海道の道東地方であった。北海道におけるアライグマの抗体陽性率を地域的に比較すると、空知地域における抗体陽性率（9.5%）は、胆振地域 (2.1%; $p < 0.05$)、石狩地域 (2.1%; $p < 0.01$) 及び十勝地域 (0.8%; $p < 0.01$) に比べて有意に高かった。

福島県と栃木県で捕獲されたアライグマやイノシシ、また福島県で捕獲されたホンシュウジカから抗体は検出されなかった。アライグマとエゾシカから抗体が検出された十勝地域で採取されたダニとアライグマの血液から、ウイルスは検出されなかった。

2016年〜2018年に北海道の道東地方で捕獲されたアライグマ及びエゾ鹿における抗体調査から道東地方にTBEV fociが存在することが、本研究において初めて明らかになった。また、本州においても栃木県で捕獲されたホンシュウジカから抗体が検出されたことから、鳥取県以外にもTBEV fociが存在することが初めて明らかにされた。これらの結果から、この両地域は近年になってウイルスの侵入を許した可能性が考えられたが、日本において広い範囲にわたってTBEVが分布していることが示唆された。

第III章は、牛と羊における抗体調査の結果を示している。最初に1998年〜1999年に北海道を含む日本全国の13の県において採取された728頭の牛血清及び北海道の道央地方の177頭の羊血清（採取時期は不明）について、Strep-SP ELISAを用いて調べた。その結果、70頭の牛血清がStrep-SP ELISA陽性を示した。しかし、予想に反して、TBEV中和抗体陽性（≥1:50）と判断される血清は認められなかった。一頭の牛のみがTBEVに対して非常に低い抗体価 (1:20) を示した。

Strep-SP ELISAは、今回の研究で用いた野生動物に加え、人や野生のげっ歯類の血清を用いた検査では良好な結果を示した報告がなされている。本研究において、初めてStrep-SP ELISAが牛血清の調査に使用されたが、今回認められた牛血清における高い非特異的反応の

原因は不明であるが、牛血清中の非特異的IgGとStrep-SP のStrep-tagあるいはStrep-Tactin AP に非特異的に結合した可能性が考えられた。一方、TBEV Strep-SPが牛血清中に存在しているTBEVと抗原的に交差する他のフラビウイルスの抗体と交差反応を起こした可能性も否定できない。羊の血清については、中和試験を実施できなかったので、今後検討したいと考えている。

本研究について総括すると、大型野生動物の血清学的検査により、北海道の道東地方と本州の栃木県において新たにTBEV fociの存在が明らかになった。本研究によって、予想以上に日本においてTBEVが、特に北海道において広く分布していることが示唆された。従って、日本に広く分布しているアライグマ、シカ、イノシシ等の大型の野生動物（歩哨動物）を使ってTBEV fociを継続的にモニタリングすることは、人でのTBEの発生を未然に防ぐためにも意義のあることである。この目的のために、まずスクリーニング法としてStrep-SP ELISAを用いて調査し、中和試験で確認することが、現時点では最も有用な方法と考えられた。また、血清学的にTBEVの存在が示唆される地域において、正確なTBEV fociの位置を特定することも人におけるTBEの発生を避ける上でも非常に重要である。

Acknowledgments

My thesis study could not have been completed without many people who contributed directly and indirectly to this research endeavor.

I owe all a lot.

I wish to express my sincere gratitude to:

My supervisor, Professor Kunitoshi Imai. Thank you very much Sensei for giving me this great opportunity to obtain a doctoral degree under your supervision and intellectual guidance. Thank you Sensei, for managing my thesis study through gathering such beautiful people who agreed on me to use their samples and diagnostic reagents to complete the program. Also, I have to thank you for your support and assistance for correcting and completing my manuscript and thesis.

My co-supervisor, Professor Haruko Ogawa. Thank you very much Sensei for giving me the chance to pursue my doctoral program under your mentorship and promoting me to state with the thesis topic. Your support is incomparable. With your immense knowledge, expertise, and feedback as your student, my study was much easy that made me feel comfortable during the program years.

I want to thank my co-supervisor Professor Ikuo Igarashi, for giving me valuable suggestions and critical discussions on my thesis study. Your given lectures and classes contributed a lot to the development of my foundation during my study period.

I am grateful to my thesis committee support for completing my thesis study with critical comments and corrections. Many thanks to Professor Xuenan Xuan and Associate Professor Shinya Fukumoto for consulting with constructive comments.

I wish to especially thank Professor Hiroaki Kariwa and Associate Professor Kentaro Yoshii who have kindly supported my study with their diagnostic reagents providing with laboratory techniques, protocols, and practical assistance. With their privileged support, I have pursued my program. Also, thank you Senseis for supervising and contributing my internship study at

your laboratory, Laboratory of Public Health, Faculty of Veterinary Medicine, Hokkaido University. I am also grateful to all the students and members of your laboratory, who helped me a lot during my days there.

I want to express my deep appreciation to my co-authors who provided me by their labor-intensive sample collections and the “tick hunter” project. Without all your support, I doubt it should ever have been completed.

- Professor Mitsuhiko Asakawa, School of Veterinary Medicine, Rakuno Gakuen University,
- Professor Kei Okuda, Faculty of Agriculture, Tokyo University of Agriculture and Technology,
- Kei Fujii, Ph.D., Animal Research Center, Hokkaido Research Organization,
- Associate Professor Shinya Fukumoto, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine (OUAVM),
- Assistant Professor Rika Umemiya-Shirafuji, National Research Center for Protozoan Diseases, OUAVM,
- Professor Motoki Sasaki, Department of Veterinary Medicine, OUAVM,
- Associate Professor Kotaro Matsumoto, Department of Veterinary Medicine, OUAVM,
- Emi Yamaguchi, Ph.D., Department of Veterinary Medicine, OUAVM.

Special thanks to a laboratory technician Ms. Sachiko Matsuda and all members of our laboratory, Diagnostic Center for Animal Health and Food Safety who have had the excellent skills to follow and the most talented to work together. I sincerely appreciate our membership under such collaborative support.

I wish to thank Graduate School of Animal Husbandry, Obihiro University of Agriculture and Veterinary Medicine for giving me the opportunity to study here in Japan under the MEXT scholarship.

My thanks extend to JICA-OBIC. I am proud of having attended to the JICA course “Advanced research course on control zoonosis food safety” in 2009-2010 because it led me

to join to the laboratory of Imai and Ogawa Senseis, who have made me this accompanied approach. You are the biggest supporters of my life. I will always appreciate it.

JICA-OBIC and Senseis, no words are enough to express my gratitude.

Finally, I would like to thank all my family members for supporting me all the way since the beginning of my thesis study.

I dedicate this thesis study to my beloved daughter Khuslen who is the most important person in my life.

References

Allison S.L., Stadler K., Mandl C.W., Kunz C., Heinz F.X. Synthesis and secretion of recombinant tick-borne encephalitis virus protein E in soluble and particulate form. J Virol 69, 5816–20, 1995.

Allison S.L., Tao Y.J., O'Riordain G., Mandl C.W., Harrison S.C., Heinz F.X. Two distinct size classes of immature and mature subviral particles from tick-borne encephalitis virus. J Virol 77, 11357–66, 2003.

Amicizia D., Domnich A., Panatto D., Lai P.L., Cristina M.L., Avio U., Gasparini R. Epidemiology of tick-borne encephalitis (TBE) in Europe and its prevention by available vaccines. Hum Vaccin Immunother 9, 1163–71, 2013.

Artsob H., Spence L., Th'ng C., Lampotang V., Johnston D., MacInnes C., Matejka F., Voigt D., Watt I. Arbovirus infections in several Ontario mammals, 1975–1980. Can J Vet Res 50, 42–6, 1986.

Balogh Z., Ferenczi E., Szeles K., Stefanoff P., Gut W., Szomor K., Takacs M., Berencsi G. Tick-borne encephalitis outbreak in Hungary due to consumption of raw goat milk. J Virol Methods 163, 481–5, 2010.

Balling A., Plessow U., Beer M., Pfeffer M. Prevalence of antibodies against tick-borne encephalitis virus in wild game from Saxony, Germany. Ticks Tick Borne Dis 5, 805–9, 2014.

Baneth G. Tick-borne infections of animals and humans: Common ground. Int J Parasitol 44, 591–6, 2014.

Blitvich B.J., Juarez L.I., Tucker B.J., Rowley W.A., Platt K.B. Antibodies to West Nile virus in raccoons and other wild peridomestic mammals in Iowa. J Wildl Dis 45, 1163–8, 2009.

Bogovic P., Strle F. Tick-borne encephalitis: A review of epidemiology, clinical characteristics, and management. World J Clin Cases 3, 430–41, 2015.

Borcić B., Raos B., Kranzelić D., Abu Eldan J., Filipović V. The role of large wildlife in the maintenance of natural foci of tick-borne meningoencephalitis in northern Croatia. Acta Med Jugosl 44, 399–406, 1990.

Borde J.P., Zajkowska J. TBE in adults. In: TBE-The Book, Global Health Press, Singapore 2017.

Böhm B., Schade B., Bauer B., Hoffmann B., Hoffmann D., Ziegler U., Beer M., Klaus C., Weissenböck H., Böttcher J. Tick-borne encephalitis in a naturally infected sheep. BMC Vet Res 13, 267, 2017.

Carpi G., Cagnacci F., Neteler M., Rizzoli A. Tick infestation on roe deer in relation to geographic and remotely sensed climatic variables in a tick-borne encephalitis endemic area. Epidemiol Infect 136, 1416–24, 2008.

Cisak E., Wojcik-Fatla A., Zajac V., Sroka J., Buczek A., Dutkiewicz J. Prevalence of tick-borne encephalitis virus (TBEV) in samples of raw milk taken randomly from cows, goats and sheep in eastern Poland. Ann Agric Environ Med 17, 283–6, 2010.

Cisak E., Wojcik-Fatla A., Sroka J., Zajac V., Bilska-Zajac E., Chmurzynska E., Dutkiewicz J. Prevalence of tick-borne encephalitis virus antibodies in domestic and game animals from eastern Poland. Bull Vet Inst Pulawy 56, 275–8, 2012.

Dantas-Torres F., Chomel B.B., Otranto D. Ticks and tick-borne diseases: A one health perspective. Trepar 1138, 1–10, 2012.

Demina T.V., Dzhioev Y.P., Verkhozina M.M., Kozlova I.V., Tkachev S.E., Plyusnin A., Doroshchenko E.K., Lisak O.V., Zlobin V.I. Genotyping and characterization of the geographical distribution of tick-borne encephalitis virus variants with a set of molecular probes. J Med Virol 82, 965–76, 2010.

Dobler G., Treib J., Kiessig S. T., Blohn W. V., Frosner G., Haass A. Diagnosis of tick-borne encephalitis: Evaluation of sera with borderline titers with the TBE-ELISA. Infect 24, 405–6, 1996.

Dobler G., Hufert F., Pfeffer M., Essbauer S. Tick-borne encephalitis: from microfocus to human disease. In: Progress in parasitology. Springer, New York, pp 323–31, 2011.

Dobler G., Gniel D., Petermann R., Pfeffer M. Epidemiology and distribution of tick-borne encephalitis. Wien Med Wochenschr 162, 230–8, 2012.

Dobler G. Diagnosis. In: TBE-The Book, Global Health Press, Singapore 2017.

Doi K., Kato T., Hayama S.I. Infestation of introduced raccoons (*Procyon lotor*) with indigenous Ixodid ticks on the Miura Peninsula, Kanagawa prefecture, Japan. Int J Parasitol Parasites Wildl 7, 355–9, 2018.

Donoso-Mantke O., Schadler R., Niedrig M. A survey on cases of tick-borne encephalitis in European countries. Euro Surveill 13, 2008.

Donoso-Mantke O., Karan L., Růžek D. Tick-borne encephalitis virus: A general overview. Flavivirus Encephalitis IntechOpen, pp 133–56, 2011.

Estrada-Peña A., de la Fuente J. The ecology of ticks and epidemiology of tick-borne viral diseases. Antiviral Res 108, 104–28, 2014.

Ferlenghi I., Clarke M., Ruttan T., Allison S.L., Schalich J., Heinz F.X. Molecular organization of a recombinant subviral particle from tick-borne encephalitis virus. Mol Cell 7, 593–602, 2001.

Földvári G., Široký P., Szekeres S., Majoros G., Sprong H. *Dermacentor reticulatus*: a vector on the rise. Parasit Vectors 9, 314, 2016.

Frimmel S., Krienke A., Riebold D., Loebermann M., Littmann M., Fiedler K., Klaus C., Süss J., Reisinger E.C. Tick-borne encephalitis virus habitats in North East Germany: reemergence of TBEV in ticks after 15 years of inactivity. Biomed Res Int 308371, 2014.

Frimmel S., Leister M., Löbermann M., Feldhusen F., Seelmann M., Süss J., Reisinger E.C. Seroprevalence of tick-borne encephalitis virus in wild game in Mecklenburg-Western Pomerania (north-eastern Germany). Ticks Tick Borne Dis 7, 1151–4, 2016.

Fritz R., Orlinger K. K., Hofmeister Y., Janecki K., Traweger A., Perez-Burgos L., Barrett P.N., Kreil T.R. Quantitative comparison of the cross-protection induced by tick-borne encephalitis virus vaccines based on European and Far Eastern virus subtypes. Vaccine 30, 1165–9, 2012.

Füzik T., Formanová P., Růžek D., Yoshii K., Niedrig M., Plevka P. Structure of tick-borne encephalitis virus and its neutralization by a monoclonal antibody. Nat Commun 9, 436, 2018.

Gäumann R., Mühlemann K., Strasser M., Beuret C.M. High-throughput procedure for tick surveys of tick-borne encephalitis virus and its application in a national surveillance study in Switzerland. Appl Environ Microbiol 76, 4241–9, 2010.

Gerth H.J., Grimshandl D., Stage B., Döller G., Kunz C. Roe deer as sentinels for endemicity of tick-borne encephalitis virus. Epidemiol Infect 115, 355–65, 1995.

Gresíková M., Sekeyová M., Stúpalová S., Necas S. Sheep milk-borne epidemic of tick-borne encephalitis in Slovakia. Intervirology 5, 57–61, 1975.

Gritsun T.S., Lashkevich V.A., Gould E.A. Tick-borne encephalitis. Antiviral Res 61, 129–46, 2003.

Gritsun T.S., Gould E.A. Tick-borne encephalitis viruses (Flaviviridae). In: Encyclopedia of Virology. Elsevier, pp 45–54, 2008.

Gubler D.J., Kuno G., Markoff L. Flaviviruses. In: Fields Virology. Fifth. Philadelphia: Lippincott Williams and Wilkins, pp 1153–1252, 2007.

Günther G., Lindquist L. Surveillance of tick-borne encephalitis in Europe and case definition. Euro Surveill 10, 23–5, 2005.

Havlikova S., Lickova M., Klempa B. Non-viraemic transmission of tick-borne viruses. Acta Virol 57, 123–9, 2013.

Heinz F.X., Allison S.L., Stiasny K., Schalich J., Holzmann H., Mandl C.W., Kunz C. Recombinant and virion-derived soluble and particulate immunogens for vaccination against tick-borne encephalitis. Vaccine 13, 1636–42, 1995.

Heinz F.X. Molecular aspects of TBE virus research. Vaccine 21, 3–10, 2003.

Heinz F.X. Etiology, Monograph: Tick-borne encephalitis (TBE, FSME): Baxter AG, Austria, pp 6–14, 2007.

Heinz F.X., Stiasny K. The molecular and antigenic structure of TBEV. In: TBE-The Book, Global Health Press, Singapore 2017.

Holbrook M.R. Historical perspectives on flavivirus research. Viruses 9, 2017.

Holzmann H., Kundi M., Stiasny K., Clement J., McKenna P., Kunz C., Heinz F.X. Correlation between ELISA, hemagglutination inhibition, and neutralization tests after vaccination against tick-borne encephalitis. J Med Virol 48,102–7, 1996.

Holzmann H. Diagnosis of tick-borne encephalitis. Vaccine 21, 36–40, 2003.

Holzmann H., Aberle S.W., Stiasny K., Werner P., Mischak A., Zainer B., Netzer M., Koppi S., Bechter E., Heinz F.X. Tick-borne encephalitis from eating goat cheese in a mountain region of Austria. Emerg Infect Dis 15, 1671–3, 2009.

Ikeda T., Asano M., Matoba Y., Go A. Present status of invasive alien raccoon and its impact in Japan. Glob Environ Res 8, 125–31, 2004.

Imhoff M., Hagedorn P., Schulze Y., Hellenbrand W., Pfeffer M., Niedrig M. Review: Sentinels of tick-borne encephalitis risk. Ticks Tick Borne Dis 6, 592–600, 2015.

Inagaki E., Sakai M., Hirano M., Muto M., Kobayashi S., Kariwa H., Yoshii K. Development of a serodiagnostic multi-species ELISA against tick-borne encephalitis virus using subviral particles. Ticks Tick Borne Dis 7, 723–9, 2016.

Jääskeläinen A., Han X., Niedrig M., Vaheri A., Vapalahti O. Diagnosis of tick-borne encephalitis by a μ-capture IgM-EIA based on secreted recombinant antigen produced in insect cells. J Clin Microbiol 41, 4336–42, 2003.

Jaenson T.G., Hjertqvist M., Bergstrom T., Lundkvist A. Why is tick-borne encephalitis increasing? A review of the key factors causing the increasing incidence of human TBE in Sweden. Parasit Vectors 5, 184, 2012.

Jemeršić L., Dežđek D., Brnić D., Prpić J., Janicki Z., Keros T., Roić B., Slavica A., Terzić S., Konjević D., Beck R. Detection and genetic characterization of tick-borne encephalitis virus (TBEV) derived from ticks removed from red foxes (*Vulpes vulpes*) and isolated from spleen samples of red deer (*Cervus elaphus*) in Croatia. Ticks Tick-Borne Dis 5, 7–13, 2014.

Kaiser R. Clinical description, Monograph: Tick-borne encephalitis (TBE, FSME): Baxter AG, Austria, pp 21–8, 2007.

Kaiser R. Tick-borne encephalitis. Infect Dis Clin North Am 22, 561–75, 2008.

Kerlik J., Avdičová M., Štefkovičová M., Tarkovská V., Pántiková Valachová M., Molčányi T., Mezencev R. Slovakia reported the highest occurrence of alimentary tick-borne encephalitis in Europe: Analysis of tick-borne encephalitis outbreaks in Slovakia during 2007–2016. Travel Med Infect Dis S1477–8939, 2018.

Klaus C., Hoffmann B., Hering U., Mielke B., Sachse K., Beer M., Süss J. Tick-borne encephalitis (TBE) virus prevalence and virus genome characterization in field-collected ticks (*Ixodes ricinus*) in risk, non-risk, and former risk areas of TBE, and in ticks removed from humans in Germany. Clin Microbiol Infect 16, 238–44, 2010a.

Klaus C., Hoffmann B., Moog U., Schau U., Beer M., Süss J. Can goats be used as sentinels for tick-borne encephalitis (TBE) in non-endemic areas? Experimental studies and epizootological observations. Berl Munch Tierärztl Wochenschr 123, 10–14, 2010b.

Klaus C., Beer M., Saier R., Schubert H., Bischoff S., Süss J. Evaluation of serological tests for detecting tick-borne encephalitis virus (TBEV) antibodies in animals. Berl Munch Tierarztl Wochenschr 124, 443–9, 2011.

Klaus C., Beer M., Saier R., Schau U., Moog U., Hoffmann B., Diller R., Süss J. Goats and sheep as sentinels for tick-borne encephalitis (TBE) virus – epidemiological studies in areas endemic and non-endemic for TBE virus in Germany. Ticks Tick Borne Dis 3, 27–37, 2012.

Klaus C., Hörügel U., Hoffmann B., Beer M. Tick-borne encephalitis virus (TBEV) infection in horses: clinical and laboratory findings and epidemiological investigations. Vet Microbiol 163, 368–72, 2013.

Klaus C., Ziegler U., Kalthoff D., Hoffman B., Beer M. Tick-borne encephalitis virus (TBEV) – findings on cross-reactivity and longevity of TBEV antibodies in animals sera. BMC Vet Res 10, 78, 2014.

Kollaritsch H., Paulke-Korinek M., Holzmann H., Hombach J., Bjorvatn B., Barrett A. Vaccines and vaccination against tick-borne encephalitis. Expert Rev Vaccines 11, 1103–19, 2012.

Korenberg E. I., Kovalevskii Y. V. Main features of tick-borne encephalitis eco-epidemiology in Russia. Zent Bakteriol 289, 525–39, 1999.

Kríz B., Benes C., Daniel M. Alimentary transmission of tick-borne encephalitis in the Czech Republic (1997–2008). Epidemiol Microbial Immunol 58, 98–103, 2009.

Kunz C. Introduction, Monograph: Tick-borne encephalitis (TBE, FSME): Baxter AG, Austria, pp 4–5, 2007.

Kunze U. The international scientific working group on tick-borne encephalitis (ISW-TBE): Review of 17 years of activity and commitment. Ticks Tick Borne Dis 7, 399–404, 2016.

Kunze U. The international scientific working group on tick-borne encephalitis (ISW-TBE) (2017) Report of the 19th Annual meeting of the International Scientific Working Group on Tick-Borne Encephalitis - TBE in a changing world. Ticks Tick Borne Dis 9, 146–50, 2018.

Labuda M., Jones L.D., Williams T., Danielova V., Nuttall P.A. Efficient transmission of tick-borne encephalitis virus between co-feeding ticks. J Med Entomol 30, 295–309, 1993.

Leschnik M.W., Kirtz G.C., Thalhammer J.G. Tick-borne encephalitis (TBE) in dogs. Int J Med Microbiol 33, 66–9, 2002.

Leutloff R., Nübling M., Neumann-Haefelin D., Rieger M.A. Cows as indicators for TBE endemic regions: suitability of testing for antibodies in serum and milk. Int J Med Microbiol 296, 87–8, 2006.

Levanov L., Kuivanen S., Matveev A., Swaminathan S., Hakala A.J., Vapalahti O. Diagnostic potential and antigenic properties of recombinant tick-borne encephalitis virus subviral particles expressed in mammalian cells from Semliki Forest virus replicon. J Clin Microbiol 52, 814–22, 2014.

Lindquist L., Vapalahti O. Tick-borne encephalitis. Lancet 371, 1861–71, 2008.

Litzba N., Zelená H., Kreil T.R., Niklasson B., Kühlmann-Rabens I., Remoli M.E., Niedrig M. Evaluation of different serological diagnostic methods for tick-borne encephalitis virus: enzyme-linked immunosorbent, immunofluorescence, and neutralization assay. Vector Borne Zoonotic Dis 14, 149–59, 2014.

Lorenz I.C., Kartenbeck J., Mezzacasa A., Allison S.L., Heinz F.X., Helenius A. Intracellular assembly and secretion of recombinant subviral particles from tick-borne encephalitis virus. J Virol 77, 4370–82, 2003.

Maeda A., Maeda J. Review of diagnostic plaque reduction neutralization tests for flavivirus infection. Vet J 195, 33–40, 2013.

Mandl C.W. Steps of the tick-borne encephalitis virus replication cycle that affect neuropathogenesis. Virus Res 111, 161–74, 2005.

Mansfield K. L., Johnson N., Phipps L. P., Stephenson J. R., Fooks A. R., Solomon T. Tick-borne encephalitis virus – a review of an emerging zoonosis. J Gen Virol 90, 1781–94, 2009.

Martina B.E., Barzon L., Pijlman G.P., de la Fuente., Rizzoli A., Wammes L.J., Takken W., van Rij R.P., Papa A. Human to human transmission of arthropod-borne pathogens. Curr Opin Virol 22, 13–21, 2017.

Ministry of the Environment, Japan. The wildlife in Japan. 2015.

Mlera L., Bloom M.E. The role of mammalian reservoir hosts in tick-borne flavivirus biology. Front Cell Infect Microbiol 8, 298, 2018.

Morozova O.V., Bakhvalova V.N., Potapova O.F., Grishechkin A.E., Isaeva E.I., Aldarov K.V., Klinov D.V., Vorovich M.E. Evaluation of immune response and protective effect of four vaccines against the tick-borne encephalitis virus. Vaccine 32, 3101–6, 2014.

Nuttall P. A. Pathogen-tick-host interactions: *Borrelia burgdorferi* and TBE virus. Zentralbl Bakteriol 289, 492–505, 1999.

Ohno Y., Sato H., Suzuki K., Yokoyama M., Uni S., Shibasaki T., Sashika M., Inokuma H., Kai K., Maeda K. Detection of antibodies against Japanese encephalitis virus in raccoons, raccoon dogs and wild boars in Japan. J Vet Med Sci 71, 1035–9, 2009.

Petri E., Gniel D., Zent O. Tick-borne encephalitis (TBE) trends in epidemiology and current and future management. Travel Med Infect Dis 8, 233–45, 2010.

Pfeffer M., Dobler G. Tick-borne encephalitis virus in dogs–is this an issue? Parasit Vectors 4, 59, 2011.

Pfeffer M., Leschnik M. TBE in animals. In: TBE-The Book, Global Health Press, Singapore 2017.

Pöllabauer E. M., Kollaritsch H. Prevention – Vaccines + Immunoglobulins. In: TBE-The Book, Global Health Press, Singapore 2017.

Pulkkinen L.I.A., Butcher S.J., Anastasia M. Tick-borne encephalitis virus: a structural view. Viruses 10, 350, 2018.

Rielle N., Klaus C., Hoffmann D., Péter O., Voordouw M. J. Goats as sentinel hosts for the detection of tick-borne encephalitis risk areas in the Canton of Valais, Switzerland. BMC Vet Res 13, 217, 2017.

Rizzoli A., Hauffe H.C., Tagliapietra V., Neteler M., Rosa R. Forest structure and roe deer abundance predict tick-borne encephalitis risk in Italy. PloS One 4, 4336, 2009.

Roelandt S., Heyman P., Tavernier P., Roels S. Tick-borne encephalitis in Europe: review of an emerging zoonosis. Vlaams Diergeneeskd Tijdschr 1, 23–31, 2010.

Roelandt S., Suin V., Van der Stede Y., Lamoral S., Marche S., Tignon M., Carlos Saiz J., Escribano-Romero E., Casaer J., Brochier B., Van Gucht S., Roels S., Vervaeke M. First tick-borne encephalitis virus (TBEV) serological screening in Flemish wild boar. Infect Ecol Epidemiol 6, 31099, 2016.

Roelandt S., Suin V., Gucht S.V., Stede Y.V., Roels S. Comparative tick-borne encephalitis (virus) surveillance in Belgium 2009–2015: Experiences with diagnostic tests, sentinel species and surveillance designs. J Zoonotic Dis Public Health 1, 4, 2017.

Růžek D., Dobler G., Donoso-Mantke O. Tick-borne encephalitis: Pathogenesis and clinical implications. Travel Med Infect Dis 8, 223–32, 2010.

Salat J., Mihalca A. D., Mihaiu M., Modrý D., Růžek D. Tick-borne encephalitis in sheep, Romania. Emerg Infect Dis 23, 2065–7, 2017.

Schwaiger M., Cassinotti P. Development of a quantitative real-time RT-PCR assay with internal control for the laboratory detection of tick-borne encephalitis virus (TBEV) RNA. J Clin Virol 27, 136–45, 2003.

Skerra A., Schmidt T. G. Use of the Strep-tag and streptavidin for detection and purification of recombinant proteins. Method Enzymol 326, 271–304. 2000.

Smorodintsev A.A. Tick-borne spring-summer encephalitis. In: Progress in medical virology, Basel: Karger 1, 210–47, 1958.

Stefanoff P., Pfeffer M., Hellenbrand W., Rogalska J., Rühe F., Makówka A., Michalik J., Wodecka B., Rymaszewska A., Kiewra D., Baumann-Popczyk A., Dobler C. Virus detection in questing ticks is not a sensitive indicator for risk assessment of tick-borne encephalitis in humans. Zoonoses Public Health 60, 215–26, 2013.

Stiasny K., Heinz F. X. Flavivirus membrane fusion. J Gen Virol 87, 2755–66, 2006.

Süss J. Epidemiology and ecology of TBE relevant to the production of effective vaccines. Vaccine 21, 19–35, 2003.

Süss J., Dobler G., Zöller G., Essbauer S., Pfeffer M., Klaus C., Liebler-Tenorio E.M., Gelpi E., Stark B., Hotzel H. Genetic characterization of a tick-borne encephalitis virus

isolated from the brain of a naturally exposed monkey (*Macaca sylvanus)*. Int J Med Microbiol 298, 295–300, 2008.

Süss J. Tick-borne encephalitis 2010: epidemiology, risk areas, and virus strains in Europe and Asia – an overview. Ticks Tick Borne Dis 2, 2–15, 2011.

Takashima I., Morita K., Chiba M., Hayasaka D., Sato T., Takezawa C., Igarashi A., Kariwa H., Yoshimatsu K., Arikawa J., Hashimoto N. A case of tick-borne encephalitis in Japan and isolation of the virus. J Clinical Microbiol 35, 1943–7, 1997.

Takashima I. Epidemiology of tick-borne encephalitis in Japan. Comp Immunol Microbiol Infect Dis 21, 81–90, 1998.

Takeda T., Ito T., Osada M., Takahashi K., Takashima I. Isolation of tick-borne encephalitis virus from wild rodents and a seroepizootiologic survey in Hokkaido, Japan. Am J Trop Med Hyg 60, 287–91, 1999.

Tonteri E., Jaaskelainen A. E., Tikkakoski T., Voutilainen L., Niemimaa J., Henttonen H., Vaheri A., Vapalahti O. Tick-borne encephalitis virus in wild rodents in winter, Finland, 2008–2009. Emerg Infect Dis 17, 72–5, 2011.

Uchida L., Hayasaka D., Ngwe Tun M.M., Morita K., Muramatsu Y., Hagiwara K. Survey of tick-borne zoonotic viruses in wild deer in Hokkaido, Japan. J Vet Med Sci 80, 985–8, 2018.

Valarcher J.F., Hagglund S., Juremalm M., Blomqvist G., Renstrom L., Zohari S., Leijon M., Chirico J. Tick-borne encephalitis. Rev Sci Tech 34, 453–66, 2015.

Vereta L.A., Skorobrekha V.Z., Nikolaeva S.P., Aleksandrov V.I., Tolstonogova V.I., Zakharycheva T.A., Red'ko A.P., Lev M.I., Savel'eva N.A. The transmission of the tick-borne encephalitis virus via cow’s milk. Med Parazitol 3, 54–6, 1991.

World Health Organization. Vaccines against TBE: WHO position paper. Geneva, Switzerland: WHO, 2011.

Yoshii K., Hayasaka D., Goto A., Obara M., Araki K., Yoshimatsu K., Arikawa J., Ivanov L., Mizutani T., Kariwa H., Takashima I. Enzyme-linked immunosorbent assay using

recombinant antigens expressed in mammalian cells for serodiagnosis of tick-borne encephalitis. J Virol Methods 108, 171–9, 2003.

Yoshii K., Ikawa A., Chiba Y., Omori Y., Maeda J., Murata R., Kariwa H., Takashima I. Establishment of a neutralization test involving reporter gene-expressing virus-like particles of tick-borne encephalitis virus. J Virol Methods 161, 173–6, 2009.

Yoshii K., Mottate K., Omori-Urabe Y., Chiba Y., Seto T., Sanada T., Maeda J., Obara M., Ando S., Ito N., Sugiyama M., Sato H., Fukushima H., Kariwa H., Takashima I. Epizootiological study of tick-borne encephalitis virus infection in Japan. J Vet Med Sci 73, 409–12, 2011.

Yoshii K., Song J.Y., Park S.B., Yang J., Schmitt H.J. Tick-borne encephalitis in Japan, Republic of Korea and China. Emerg Microbes Infect 6, 82, 2017a.

Yoshii K., Kojima R., Nishiura H. Unrecognized subclinical infection with tickborne encephalitis virus, Japan. Emerg Infect Dis 23, 1753–4, 2017b.

Yoshii K., Sato K., Ishizuka M., Kobayashi S., Kariwa H., Kawabata H. Serologic evidence of tick-borne encephalitis virus infection in a patient with suspected Lyme disease in Japan. Am J Trop Med Hyg 99, 180–1, 2018.

Ytrehus B., Vainio K., Dudman S. G., Gilray J., Willoughby K. Tick-borne encephalitis virus and Louping-ill virus may co-circulate in Southern Norway. Vector Borne Zoonotic Dis 13, 762–8, 2013.

Zeltins A. Construction and characterization of virus-like particles: a review. Mol Biotechnol 53, 92–107, 2013.

Zeman P. Objective assessment of risk maps of tick-borne encephalitis and Lyme borreliosis based on spatial patterns of located cases. Int J Epidemiol 26, 1121–9, 1997.

Zlobin V.I., Pogodina V.V., Kahl O. A brief history of the discovery of tick-borne encephalitis virus in the late 1930s (based on reminiscences of members of the expeditions, their colleagues, and relatives). Ticks Tick Borne Dis 8, 813–20, 2017.