

Integrative studies on emerging orthonairoviruses:
viral dissemination via ticks on migratory birds,
and pathogenicity in mouse models

新興オルソナイロウイルスの統合研究：
渡り鳥由来マダニを介したウイルスの伝播と
マウスにおける病原性の評価

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1. General introduction

1. 1. Orthonairoviruses

1. 1. 1. Taxonomy and phylogeny

Orthonairoviruses are arthropod-borne viruses classified within the order *Bunyavirales*, family *Nairoviridae*, and genus *Orthonairovirus*. The genus currently comprises 52 recognized species (International Committee on Taxonomy of Viruses 2024; Kuhn et al. 2022). Historically, classification relied on antigenic cross-reactivity assessed by hemagglutination-inhibition, neutralization, complement-fixation, and indirect immunofluorescence assays, which defined nine serogroups (Crimean–Congo haemorrhagic fever, Nairobi sheep disease, Dera Ghazi Khan, Qalyub, Hughes, Sakhalin, Thiafora, Kasokero, and Keterah) (Casals and Tignor 1980; Gould et al. 1983; Lasecka and Baron 2014; Walker et al. 2015; Walker et al. 2016; Zeller et al. 1989). In contrast, molecular phylogenetic analyses have revealed genogroups that more closely reflect vector associations, host range, and geographical distribution (Walker et al. 2016).

1. 1. 2. Molecular biology

Orthonairoviruses are enveloped viruses with a tripartite, negative-sense RNA genome comprising the L, M, and S segments (Walker et al. 2016). The L segment encodes the RNA-dependent RNA polymerase (RdRp), the M segment encodes the glycoprotein precursor that is cleaved into Gn and Gc, and the S segment encodes the nucleoprotein (Walker et al. 2016). Some orthonairoviruses additionally encode non-structural proteins (NSs and/or NSm) that modulate host cellular pathways to facilitate viral replication (Hawman and Feldmann 2023; Walker et al. 2016).

1. 1. 3. Epidemiology and ecology

These viruses are primarily transmitted by hard-bodied and soft ticks (*Ixodidae* and *Argasidae*) (Lasecka and Baron 2014). Natural reservoirs include ground-foraging birds, small mammals (rodents, shrews, hedgehogs, and lagomorphs), bats, and ungulates (Lasecka and Baron 2014). Several viruses are pathogenic to humans or animals, including Crimean-Congo

hemorrhagic fever virus (CCHFV) (Hawman and Feldmann 2023), Nairobi sheep disease virus (NSDV) (Krasteva et al. 2020), Dugbe virus (DUGV) (Burt et al. 1996), Kasokero virus (KASV) (Kalunda et al. 1986), Issyk-Kul virus (ISKV) (L'vov et al. 1984), Erve virus (ERVEV) (Treib et al. 1998), Wetland virus (WELV) (X.A. Zhang et al. 2024), as well as viruses belonging to the Tamdy and Sulina gene groups, including Yezo virus (YEZV) (Chen et al. 2024; Kodama et al. 2021; Lv et al. 2023; Ogata et al. 2024; Suzuki et al. 2024; M.Z. Zhang et al. 2025), which are described below.

CCHFV is the most clinically important member of the genus *Orthonairovirus* and is endemic across Africa, Asia, the Middle East, and parts of Europe (Messina et al. 2015). Although most human infections are asymptomatic, severe hemorrhagic fever develops in a minority of cases, with reported case fatality rates ranging from 10% to 40% (WHO 2025). The global annual burden is estimated at 10,000–15,000 infections, with 1,000–2,000 deaths, although the true incidence is likely underestimated due to limited surveillance in endemic countries (WHO 2025). Human infection occurs mainly through tick bites or contact with blood or tissues of viremic livestock, and humans are the only hosts known to develop severe disease (Spengler, Bergeron, and Rollin 2016). Currently, no licensed vaccine is available for CCHFV (Hawman and Feldmann 2023).

The ecology of CCHFV is closely associated with genus *Hyalomma* ticks, which act both as the principal vectors and as major reservoir (Gargili et al. 2017). A wide range of domestic and wild animals, including cattle, sheep, goats, and hares, develop transient, asymptomatic viraemia sufficient to infect feeding ticks and act as amplifying hosts (Hawman and Feldmann 2023).

NSDV is an orthonairovirus of major veterinary importance (Krasteva et al. 2020). It causes hemorrhagic gastroenteritis, fever, abortion, and high mortality in small ruminants (reviewed by Krasteva et al. 2020), and induces febrile illness, nausea, vomiting, and headache in humans (Yadav et al. 2011). Mortality in susceptible animals can exceed 90%, resulting in substantial economic losses for livestock production systems (reviewed by Krasteva et al. 2020). This

disease is listed as a notifiable disease by the World Organisation for Animal Health (WOAH) (WOAH 2025), and it has the potential to impose trade barriers, thereby posing significant impacts on small ruminant producers worldwide (Ibrahim 1998).

DUGV is a tick-borne virus widely distributed in Africa, with *Amblyomma variegatum* ticks and cattle considered its potential vector and natural hosts (Causey 1970; Steele and Nuttall 1989; Williams et al. 1972). Although human infection is relatively rare, DUGV has been associated with febrile illness and thrombocytopenia, particularly among children (Burt et al. 1996; Moore et al. 1975).

KASV was first isolated from *Rousettus aegyptiacus* bats in Uganda and has caused laboratory-associated human infections (Kalunda et al. 1986). Recent evidence indicates that KASV is maintained in an enzootic cycle involving *Ornithodoros (Reticulinasus) faini* ticks and bats, suggesting potential spillover to humans (Schuh et al. 2020).

ISKV is a bat-associated virus first isolated in Kyrgyzstan and later detected in Tajikistan and Kazakhstan (Atkinson et al. 2015; L'vov et al. 1973) and has been associated with sporadic febrile illness in humans, with transmission presumed to occur via tick bites and exposure to bat feces or urine (L'vov et al. 1984).

ERVEV is an orthonairovirus first isolated from a white-toothed shrew (*Crocidura russula*) in France and has subsequently been detected in several European countries, indicating the presence of a natural focus (Chastel et al. 1989; Zeller et al. 1989). Although ERVEV has been associated with intracerebral or subarachnoid hemorrhage in humans, its transmission route remains unclear (Treib et al. 1998; Woessner et al. 2000).

WELV was identified in 17 patients from northeastern China (Inner Mongolia, Heilongjiang, Jilin, and Liaoning) presenting with acute febrile illness accompanied by leukopenia and thrombocytopenia. WELV RNA has also been detected in five tick species and in domestic and wild animals, indicating a broad enzootic transmission cycle and potential zoonotic risk (X.A. Zhang et al. 2024).

1. 1. 4. Emerging orthonairoviruses in East Asia

In recent years, several emerging orthonairoviruses belonging to the Tamdy and Sulina genogroups have been identified as human pathogens in northern China and Japan. These include TAMV (Moming et al. 2021), TcTV-1 (Liu et al. 2020; Y. Zhang et al. 2021), SGLV (Ma et al. 2021), BJNV (Meng et al. 2019; Wang et al. 2021), and YEZV (Chen et al. 2024; Kodama et al. 2021; Lv et al. 2023; Ogata et al. 2024; Suzuki et al. 2024; M.Z. Zhang et al. 2025). These viruses have been detected in patients presenting with acute febrile illness following tick bites.

Although no human cases have been reported, several additional tick-borne bunyaviruses, Tofla virus (TFLV) (Shimada et al. 2016), Soft tick bunyavirus (STBV) (Oba et al. 2016), and Iwanai Valley virus (IWVV) (Matsumura et al. 2024), have recently been isolated from ticks in Japan. Moreover, TFLV-specific antibodies have been identified in wild boars (*Sus scrofa*) in Nagasaki, Japan (Luvai et al. 2022).

1. 1. 4. 1. Yezo virus

YEZV is a novel orthonairovirus belonging to the Sulina genogroup (Kodama et al. 2021). YEZV infection was first recognized as an emerging tick-borne infectious disease, detected in Hokkaido, Japan, in 2019, among patients who had febrile illness after a tick bite (Kodama et al. 2021). To date, more than nine autochthonous human cases have been documented in Hokkaido (Kodama et al. 2021; Ogata et al. 2024; Suzuki et al. 2024). Human YEZV infections have been reported from northeastern China as well. One patient was reported in Inner Mongolia, northeastern China, following a tick bite (Lv et al. 2023). Interestingly, retrospective screening of tick-bite patients from 2012 to 2016 in Heilongjiang province, northeastern China, traced YEZV infections as early as 2012, identifying four cases (Chen et al. 2024), and an additional study identified 18 human cases in Heilongjiang Province of northeast China, all cases associated with tick bite (M.Z. Zhang et al. 2025).

Based on the reports described above, the clinical manifestations of YEZV infection include fever, headache, dizziness, malaise, gastrointestinal symptoms such as loss of appetite,

neurological symptoms including fatigue, and musculoskeletal symptoms such as arthralgia and myalgia. In addition, laboratory tests revealed leukopenia, thrombocytopenia, elevated liver enzyme levels, and increased concentrations of lactate dehydrogenase or α -hydroxybutyrate dehydrogenase. No fatal cases have been reported to date.

In experimental infection models, a lethal YEZV infectious disease model has been established in AG129 mice, double knockout immunocompromised mice lacking both type I and type II interferon receptors (S129 background), in which intraperitoneal inoculation results in marked weight loss, severe hepatic injury, and 100% mortality within six days post-infection (Ariizumi et al. 2024). However, the pathogenic mechanisms and virulence determinants of other Tamdy- and Sulina-related orthonairoviruses remain poorly understood, and robust small-animal models for this viral group are still lacking.

Computational immunoinformatic studies have proposed multi-epitope vaccine candidates for YEZV, based on epitope prediction and in silico structural modeling (Ayaz et al. 2025; Rahman et al. 2024). These approaches provide an initial framework for vaccine development, though experimental validation is still required.

YEZV has been detected in ixodid ticks (family *Ixodidae*) collected from a wide geographical range in Northeast Asia, including Hokkaido, Japan, northeastern and southern China, northern Inner Mongolia, western and eastern Siberia, and the Russian Far East (Apanasevich et al. 2025; Chen et al. 2024; Ito et al. 2024; Kartashov et al. 2025; Kodama et al. 2021; Lv et al. 2023; Ni et al. 2023; Su et al. 2024; M.Z. Zhang et al. 2025) (Table 1-1). Most YEZV-positive ticks have been identified as *Ixodes persulcatus* (Apanasevich et al. 2025; Chen et al. 2024; Ito et al. 2024; Kartashov et al. 2025; Kodama et al. 2021; Lv et al. 2023; Ni et al. 2023; Su et al. 2024; M.Z. Zhang et al. 2025) (Table 1-1 and Figure 1-1). In addition to *I. persulcatus*, YEZV has been detected in several other ixodid tick species across its known range. In Hokkaido, Japan, YEZV has been detected in *I. ovatus* and *Haemaphysalis megaspinosus* (Ito et al. 2024; Kodama et al. 2021) (Table 1-1 and Figure 1-1). In China, detections have been reported from *Dermacentor nuttalli* in Heilongjiang and from *Rhipicephalus microplus* in Fujian

and Yunnan; moreover, it has also been detected in *H. japonica* and *D. silvarum*, although sampling locations were unspecified (Chen et al. 2024; Ni et al. 2023) (Table 1-1 and Figure 1-1). In Russia, YEZV-positive *I. pavlovskyi* have been collected in Tomsk in western Siberia (Apanasevich et al. 2025) (Table 1-1 and Figure 1-1).

The geographical distribution of *I. persulcatus* extends from scattered populations in Finland and Sweden to a continuous population stretching from eastern Latvia and eastern Estonia, through the taiga zone of European and Asian Russia, and on to Mongolia, China, Taiwan, and the Korean Peninsula, reaching as far as Hokkaido and Honshu in Japan (Jaenson et al. 2016; Katargina et al. 2013; Bormane et al. 2004; Swanson et al. 2006; G. Zhang et al. 2019; St John et al. 2021). *I. persulcatus* is predominantly associated with mixed deciduous–coniferous forests (Jaenson et al. 2016).

Regarding wildlife hosts, YEZV-specific antibodies have been detected in raccoons (*Procyon lotor*) and native raccoon dogs (tanuki; *Nyctereutes procyonoides albus*) in Hokkaido (Ito et al. 2024; Kodama et al. 2021). As the host range and ecological transmission dynamics in mammals of YEZV remain insufficiently characterized, further detailed research is essential to clarify its infection cycle

1. 1. 4. 2. Iwanai Valley virus

IWVV is a novel orthonairovirus isolated from *I. ovatus* collected in Obihiro city, Hokkaido, Japan (Matsumura et al. 2024). The amino acid sequence of its nucleoprotein shares 34–45% identity with those of known orthonairoviruses, and phylogenetic analyses indicate that IWVV is most closely related to orthonairoviruses within the Tamdy and Sulina genogroups, including YEZV, while forming a distinct lineage separate from any previously recognized groups (Matsumura et al. 2024).

IWVV replicates efficiently in multiple cell lines, including BHK-21 (derived from baby hamster kidney), Vero (derived from African green monkey), SW-13 (derived from human adrenal adenocarcinoma), HuH-7 (derived from human hepatocellular carcinoma), and ISE6 cells

(derived from an *I. scapularis* tick embryo). Notably, the virus achieves high titers in the human-derived SW-13 and HuH-7 cell lines and induces marked cytopathic effects, demonstrating its strong tropism for human cells (Matsumura et al. 2024).

Infections in humans or in other animals have not yet been documented, and the host range and pathogenic potential of IWVV remain largely unknown. However, the virus was isolated from adult *I. ovatus*, and immature *I. ovatus* preferentially feed on small mammals, particularly rodents, it is likely that small mammals serve as natural reservoir hosts for IWVV (Matsumura et al. 2024).

1. 1. 5. Mouse models

Experimental models for orthonairovirus infection in small animals have relied largely on immunocompromised mice, particularly those deficient in type I interferon signaling. Mouse models have been established for several human-pathogenic orthonairoviruses, including CCHFV, DUGV, ISKV, and YEZV using type I interferon receptor gene-knockout (IFNAR1^{-/-}) mice on C57BL/6 or 129 backgrounds, STAT1^{-/-} mice, and cyclophosphamide-treated mice (Ariizumi et al. 2024; Bente et al. 2010; Berezky et al. 2010; Coates and Sweet 1990; Lindquist et al. 2018; Sugimoto et al. 2022).

Infection of IFNAR1^{-/-} mice with orthonairoviruses results in characteristic pathological changes in the liver, spleen, gastrointestinal tract, and central nervous system (Berezky et al. 2010; Boyd, Fazakerley, and Bridgen 2006; Dowall et al. 2012; Shimada et al. 2016). Among these, liver injury is a consistently observed hallmark across multiple orthonairovirus infection models (Ariizumi et al. 2024; Bente et al. 2010; Berezky et al. 2010; Dowall et al. 2012; Ishii et al. 2014; Lindquist et al. 2018; Sugimoto et al. 2022; Zivcec et al. 2013). Similar hepatic pathology has also been reported in CCHF human patients (Cevik et al. 2008; Hawman and Feldmann 2023; Karakecili et al. 2018; Rathore et al. 2021).

1. 2. Migratory birds as dispersal vectors of ticks and tick-borne pathogens

Several species of birds migrate annually between their breeding areas and wintering areas. The major migration routes followed annually by migratory birds are referred to as flyways (BirdLife International n.d.b.). Eight principal flyways are generally recognized worldwide (BirdLife International n.d.b.). A flyway represents a large-scale ecological pathway that encompasses all stages of the annual cycle of birds, including breeding areas, migratory stopover sites used for rest and refueling during migration, and non-breeding grounds (BirdLife International n.d.b.). Although many landbirds and waterbirds migrate predominantly along north to south routes, east to west movements are also seen in some species (BirdLife International n.d.b.).

The primary flyway used by landbirds (i.e., terrestrial birds excluding waterbirds) passing through Japan is the East Asian- Australasian Flyway (EAAF) (BirdLife International n.d.a.). This flyway extends from the Russian Far East and Alaska southward through East Asia and Southeast Asia to Australia and New Zealand, covering 41 countries (BirdLife International n.d.a.; EAAFP n.d.). The EAAF supports 523 migratory bird species, of which 338 are migratory landbirds (BirdLife International n.d.a.).

Several hard-bodied tick species that parasitize migratory birds, including those belonging to the genera *Ixodes* and *Hyalomma*, are known to be competent vectors for a wide variety of pathogens (Table 1-2). Regarding viruses, studies conducted along the African–Western Palearctic flyway have reported the detection of several high-impact, human pathogenic tick-borne viruses such as CCHFV (Leblebicioglu et al. 2014; Lindeborg et al. 2012; Mancuso et al. 2019; Palomar et al. 2012), Alkhurma hemorrhagic fever virus (AHFV) (Hoffman et al. 2018), and tick-borne encephalitis virus (TBEV) (Mikryukova et al. 2014; Waldenström et al. 2007), in ticks attached to migratory birds (Table 1-2).

In Asia, there is evidence suggesting that Severe fever with thrombocytopenia syndrome virus (SFTSV) may also be transported via ticks carried by migratory birds (Yun et al. 2015). Additionally, in Japan, *Borrelia* species causing Lyme disease have been detected in ticks

collected from migratory birds in Nemuro City, Hokkaido, and in Fukui Prefecture (Ishiguro et al. 2000; Miyamoto et al. 1997).

Despite these observations, studies examining the role of migratory birds in the dissemination of ticks and tick-borne viruses *in vitro* and in Asia, including Japan, remain limited.

1. 3. Aim of this thesis

In recent years, several emerging orthonairoviruses belonging to the Tamdy and Sulina genotypes, including YEZV, have been reported from human patients in Japan and northern China, increasing public health concerns in East Asia. Nevertheless, the natural ecology, mechanisms of geographic dissemination, and pathogenic potential of these viruses in mammalian hosts, including humans, remain poorly understood.

Given the previously reported detection of YEZV in humans and ticks across East Asia and Russia, these findings suggest the possibility of broad geographic movement of the virus. Migratory birds are known to transport ticks harboring a variety of pathogens, including CCHFV, across international boundaries; therefore, it is plausible that migratory birds may also contribute to the dispersal of YEZV-infected ticks.

IWVV is a Tamdy genotype orthonairovirus isolated from ticks collected from vegetation in Hokkaido. Although the virus has not yet been detected in mammalian hosts, including humans, its close phylogenetic relationship to the Sulina genotype, which includes YEZV, highlights the need to characterize its pathogenicity to assess potential public health risks.

Accordingly, this thesis focuses on the following two aims:

1. To elucidate the role of migratory birds in the long-distance dispersal of YEZV-infected ticks.

This aim involves analyzing the presence of YEZV in ticks collected from migratory birds arriving in Hokkaido from northern regions, to determine whether bird-mediated transport contributes to the geographic spread of YEZV.

2. To experimentally characterize the pathogenicity of IWVV in mammalian hosts.

Using type I interferon receptor–deficient mice and immunocompetent mice, this study evaluates clinical manifestations, tissue tropism, viral replication kinetics, and histopathological changes to clarify the virulence and disease mechanisms of IWVV.

By addressing these aims, this thesis seeks to provide comprehensive insights into the ecological background and potential public health risks of emerging tick-borne orthonairoviruses circulating in East Asia.

1. 4. Figure legend

Figure 1-1. Locations and tick species of YEZV-positive ticks. Red circles indicate collection sites of *Ixodes persulcatus*; green circles, *I. ovatus*; yellow circles, *Dermacentor nuttalli*; brown circles, *Rhipicephalus microplus*; and blue circles, *I. pavlovskyi*. Sampling locations in China are shown at the provincial level, those in the Russian Far East are indicated by latitude and longitude coordinates, and all other sampling locations are shown at the municipality level.

1. 5. Tables and figure

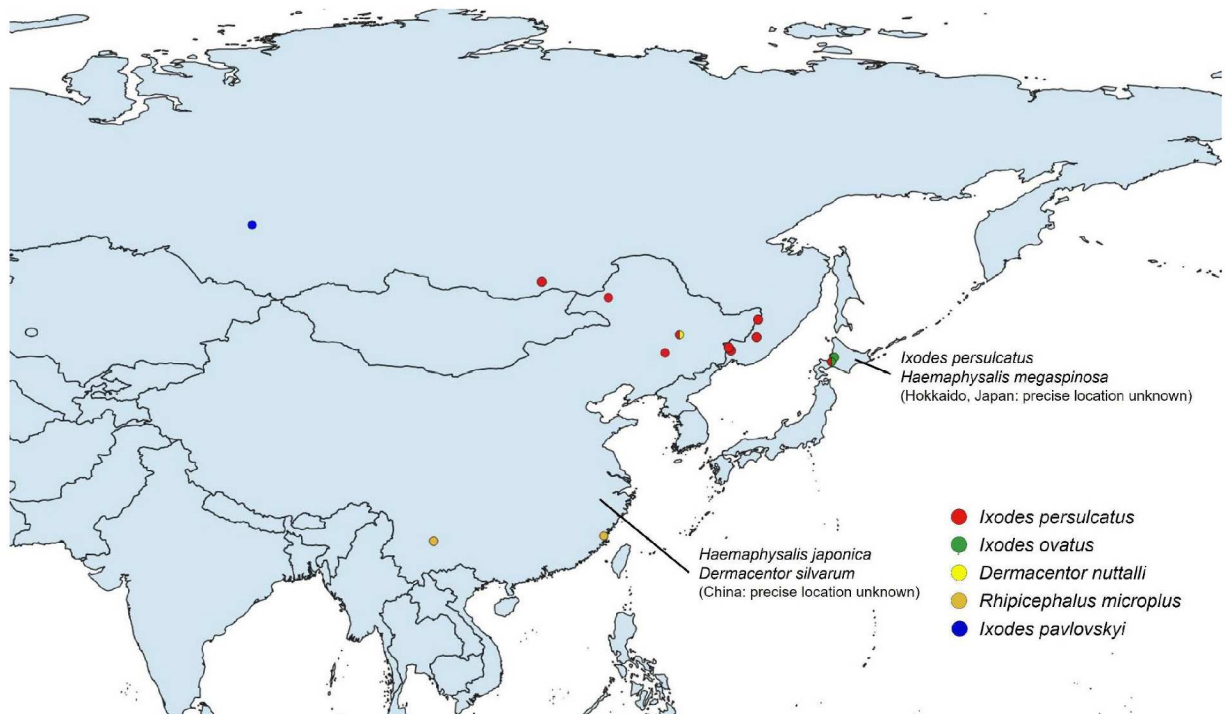
Table 1-1. Locations and tick species of Yezo virus-positive ticks

Country	City/Area	Tick species
Japan	Hokkaido	<i>Ixodes persulcatus</i> , <i>Ixodes ovatus</i> , <i>Haemaphysalis megaspinosa</i>
	Heilongjiang	<i>Ixodes persulcatus</i> , <i>Dermacentor nuttalli</i>
	Jilin	<i>Ixodes persulcatus</i>
China	Fujian	<i>Rhipicephalus microplus</i>
	Yunnan	<i>Rhipicephalus microplus</i>
	Hulunbuir, Inner mongolia	<i>Ixodes persulcatus</i>
	Unknown	<i>Haemaphysalis japonica</i> , <i>Dermacentor silvarum</i>
	Primorsky territory	<i>Ixodes persulcatus</i>
Russia	Khabarovsk territory	<i>Ixodes persulcatus</i>
	Zabaikalsky territory	<i>Ixodes persulcatus</i>
	Tomsk	<i>Ixodes pavlovskyi</i>

Table 1-2. Tick-borne viruses detected in ticks collected from migratory birds: a summary of species, hosts, and flyways

Virus (genus / species)	Virus-positive tick species (stage)	Avian host (representative species)	Region / migratory flyway	Key findings / significance	Reference
Crimean–Congo hemorrhagic fever virus (CCHFV)	<i>Hyalomma rufipes</i> (nymphs)	<i>Lanius senator senator</i>	Antikythira Island, Greece, and Capri Island, Italy / African–Western Palearctic migratory flyway	First molecular evidence that migratory birds can carry CCHFV-infected ticks across continents.	Lindeborg M. et al., 2012.
	<i>Hyalomma marginatum</i> (larvae, nymphs)	<i>Phoenicurus phoenicurus</i> <i>Erythropygia galactotes</i> <i>Iduna opaca</i> <i>Acrocephalus scirpaceus</i> <i>Iduna pallida</i>	Morocco / African–Western Palearctic migratory flyway	First CCHFV RNA detection in bird-derived ticks from Africa–Europe flyway.	Palomar et al., 2013.
	<i>Hyalomma rufipes</i> (nymphs)	<i>Saxicola rubetra</i>	Ventotene Island, Italy / African–Western Palearctic migratory flyway	Bird-borne tick positive for CCHFV RNA; Western Palearctic migration route confirmed.	Mancuso et al., 2019.
	<i>Hyalomma</i> spp. (nymphs)	<i>Acrocephalus arundinaceus</i> <i>Erithacus rubecula</i>	Kızılırmak Delta, Turkey / African–Western Palearctic migratory flyway (Mediterranean migratory corridor)	Bird-associated ticks positive for CCHFV RNA; supports intercontinental spread.	Leblebicioglu et al., 2014.
Alkhurma hemorrhagic fever virus (AHFV)	<i>Hyalomma rufipes</i> (Unknown)	<i>Motacilla flava</i> <i>Lanius senator niloticus</i> <i>Phoenicurus phoenicurus</i> <i>Acrocephalus schoenobaenus</i>	Greece / Turkey (Africa → Europe)	AHFV RNA detected in ticks from migratory birds; northward introduction to Europe suggested.	Hoffmann et al., 2018.
Tick-borne encephalitis virus (TBEV)	<i>Ixodes ricinus</i> (larvae, nymphs)	<i>Anthus trivialis</i> <i>Erithacus rubecula</i> <i>Phoenicurus phoenicurus</i> <i>Turdus philomelos</i>	Ottenby Bird Observatory, Öland Island, Sweden / African–Western Palearctic flyway	TBEV RNA detected in bird-derived ticks; indicates migratory birds can transport infected <i>I. ricinus</i> .	Waldenström et al., 2007.
	<i>Ixodes persulcatus</i> <i>Ixodes pavlovskyi</i> <i>Ixodes plumbeus</i> (larvae, nymphs)	<i>Turdus pilaris</i> <i>Turdus iliacus</i> <i>Anthus trivialis</i> <i>Passer montanus</i> <i>Phoenicurus phoenicurus</i> <i>Acrocephalus dumetorum</i>	Tomsk City and suburbs, Western Siberia / Eurasian inland migration corridor	TBEV RNA and antigen detected in birds and their ticks; two strains (Far Eastern genotype) isolated from birds.	Chausov et al., 2013.

Figure 1-1.



2. Chapter 1

Transboundary movement of Yezo virus via ticks on migratory birds

2. 1. Abstract

YEZV, an emerging tick-borne orthonairovirus first identified in Hokkaido, Japan, causes febrile illness in humans, with cases reported in both Hokkaido and northeastern China to date. The virus has also been detected in questing ticks collected in Hokkaido, northeastern China, and Russia. Although migratory birds are known to transport ticks harboring various pathogens across borders, their role in YEZV dissemination remains unclear.

We collected 2,534 ticks parasitizing 15 migratory bird species during the 2020 and 2021 autumn migration seasons at Lake Kutcharo, Hamatonbetsu Town, and Lake Furen, Nemuro City, in northern Hokkaido. Ticks were morphologically identified, pooled by species and life stage, and tested for YEZV RNA using qRT-PCR. Positive pools underwent complete genome sequencing with phylogenetic and recombination analyses.

Eight pools of *I. persulcatus* (three larval, five nymphal pools) were YEZV-positive (minimum infection rate: larvae 0.2%, nymphs 0.8%), all collected from black-faced buntings (*Emberiza spodocephala*). Seven low-Ct pools yielded complete genomes, which clustered with patient-derived strains from Hokkaido. YEZV/tick/BT-2135/Japan/2021 was identified as a reassortant strain with a replaced M segment. In addition, Japanese, Chinese, and Russian strains did not form geographically distinct clusters, but instead showed close genetic relatedness across regions.

These findings provide the first direct evidence that migratory birds transport YEZV-infected ticks into Japan and support the transboundary circulation of YEZV across East Asia. Expanded surveillance of ticks, birds, and wildlife is needed to clarify the ecology and public health risks of this emerging virus.

2. 2. Introduction

As summarized in the General introduction, YEZV is an orthonairovirus associated with febrile illness following tick bites, with human infections reported in Hokkaido, Japan, and northeastern China. YEZV has been detected in several tick species across Japan, China, and Russia, suggesting broad ecological connectivity.

Migratory birds carry ticks harboring various pathogens, such as CCHFV, belonging to the family *Nairoviridae* (Ishiguro et al. 2000; Lindeborg et al. 2012; Waldenström et al. 2007). Phylogenetic analysis of tick-borne severe fever with thrombocytopenia syndrome virus indicated that this virus might be carried by ticks found on migratory birds that fly between China, South Korea, and Japan (Yoshikawa et al. 2015; Yun et al. 2015). Increasing evidence suggests that migratory birds play a critical role in spreading tick-borne pathogens. In archipelagos, such as Japan, understanding transmission of pathogens by migratory birds is critical; however, information on tick-borne pathogens carried by migratory birds is lacking.

In this chapter, we investigated the prevalence of YEZV in ticks found on migratory birds in Japan to determine virus transmission pathways.

2. 3. Materials and methods

2. 3. 1. Ethical statement

We conducted this research under approval by the animal research review board of Yamashina Institute for Ornithology, Chiba, Japan (approval nos. 2020-004 and 2021-002).

2. 3. 2. Tick collection

We participated in bird-banding activities conducted by the Yamashina Institute for Ornithology. The surveys were carried out at two first-class birding stations: Hamatonbetsu Station (Lake Kutcharo, Hamatonbetsu Town, Hokkaido) and Furenko Station (Lake Furen, Nemuro City, Hokkaido) (Figure 2-1). The sampling periods were October 2–12, 2020, and October 2–13, 2021. Migratory birds were captured using Japanese mist nets set at ground level.

All bird species were identified by banders, and their sex and age were determined whenever possible. Before banding and release, we carefully examined each bird, especially around the head and neck, where ticks are commonly attached. Ticks were removed with forceps and placed in separate glass vials containing moist sterile filter paper, according to the individual bird from which they were attached. The developmental stage and species of each tick were identified morphologically.

2. 3. 3. Homogenization of ticks

The pooled ticks were homogenized in 500 µl of PBS using BioMasher II (Nippi. Inc., Tokyo, Japan). The homogenates were centrifuged at 6,300 g at 4°C for 10 min and supernatants were filtrated and stored at –80°C until use.

2. 3. 4. RNA extraction and Real-time RT-PCR

RNA was extracted from each supernatant using the MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit (Thermo Fisher Scientific, Rockford, IL, USA). YEZV S segment RNAs were amplified using the QuantiTect Probe RT-PCR Kit (primer 1: 5'-AGCCCTTGACAC TGCATTT-3', primer 2: 5'-CATACAGGAAGGCCATCTCATT-3', and probe: 6-FAM-ACCTAC TACTGGATGTGGAAGGCAGA-3IABkFQ) (QIAGEN, Hilden, Germany) under the following thermal conditions: 50°C for 30 min, 95°C for 15 min, and 45 cycles of 94°C for 15 s and 60°C for 60 s.

2. 3. 5. Determination of Yezo virus complete genome sequences

To determine the complete genome sequences of the Yezo viruses, we performed RT-PCR using a series of primer sets designed based on deposited nucleotide sequences of YEZV strains. RT-PCR was performed using the QIAGEN One Step RT-PCR Kit (QIAGEN) or cDNA was synthesized from RNA using SuperScript™ III First-Strand Synthesis SuperMix (Thermo Fisher Scientific) and PCR was performed using Tks Gflex™ DNA Polymerase (Takara Bio,

Kusatsu, Japan). Using the purified PCR products of four viruses, Sanger sequencing was performed by Eurofins Genomics, Japan. The other three viruses were sequenced using next-generation sequencing, and libraries of the purified PCR products were constructed using the QIAseq FX DNA Library Kit (QIAGEN). The libraries were pooled and purified using Agencourt AMPure XP (Beckman Coulter, Inc., Brea, CA, USA). Sequencing was performed using MiSeq (Illumina, Inc., San Diego, CA, USA) and MiSeq Reagent Nano Kit v2 (Illumina, Inc.). All reads were mapped to the YEZV HH003-2020 strain, and consensus sequences were obtained using CLC Genomics Workbench software (QIAGEN).

2. 3. 6. Phylogenetic analysis

Coding sequences of the YEZV genome and publicly available sequences retrieved from GenBank were extracted, aligned, and analyzed using MEGA X (Kumar et al., 2018). The most appropriate amino acid substitution models were selected, and phylogenetic trees were constructed using the maximum-likelihood method with 1,000 bootstrap replicates. Consensus trees with branch support values were subsequently visualized within the same software.

2. 4. Results

2. 4. 1. Tick collection from migratory birds

We collected ticks infesting birds that mostly fly from Sakhalin and the Kuril Islands to Hokkaido, Japan. We collected the ticks during October 2–12, 2020, and October 2–13, 2021, in Lake Kutcharo, Hamatonbetsu Town, and Lake Furen, Nemuro City, Hokkaido (Figure 2-1). We analyzed YEZV genes in the ticks to clarify virus spread. Because migratory birds at these locations in autumn are considered to have just arrived from Sakhalin (Sakhalin route) and the Kuril Islands (Kuril Islands route) (Figure 2-1), it is likely that the ticks crossed the sea attached to the birds.

We collected 2,534 ticks from 15 species of birds in October 2020 and 2021 (Tables 2-1, 2-2). All ticks were morphologically identified and pooled according to their species, life stage, and host species. Each pool consisted of <10 larval or 5 nymphal ticks.

2. 4. 2. Detection of YEZV in tick samples

We examined 2,323 *I. persulcatus* ticks (1,727 larvae and 596 nymphs), 203 *I. pavlovskyi* ticks (169 larvae and 34 nymphs), 1 *I. turdus* tick (nymph), 3 *Haemaphysalis megaspinosa* ticks (larvae), and 4 *H. concinna* ticks (3 larvae and 1 nymph) by using quantitative reverse transcription PCR. Of those, 8 pools of *I. persulcatus* ticks were positive for YEZV RNA; no pools for the other tick species were YEZV positive (overall minimum infection rate 0.3%) (Table 2-3). Seven of 8 positive pools showed low cycle threshold (Ct) values of 22.08–30.08; 1 pool had a high Ct value of 39.19. Seven pools with low Ct values were used for further analysis. Ticks in all positive pools were *I. persulcatus* collected from black-faced buntings (*E. spodocephala*) (Table 2-4). Five pools were collected in Lake Furen, Nemuro City, Hokkaido, and the other pools were collected in Lake Kutcharo, Hamatonbetsu Town, Hokkaido.

In *I. persulcatus* ticks, the minimum infection rate of YEZV was 0.2% in larvae and 0.8% in nymphs (Table 2-3). YEZV has been reported in 3 adult tick species, *H. megaspinosa*, *I. ovatus*, and *I. persulcatus*, found on vegetation in Hokkaido (Kodama et al. 2021). In addition, 4 YEZV-positive pools consisted of only unfed ticks (Table 2-5), indicating that *I. persulcatus* might transmit YEZV.

2. 4. 3. Genome sequencing of tick-derived YEZV strains

We sequenced 7 complete genomes of YEZV: YEZV/tick/BT-1821/Japan/2020, YEZV/tick/BT-1826/Japan/2020, YEZV/tick/BT-1844/Japan/2020, YEZV/tick/BT-1864/Japan/2020, YEZV/tick/BT-1968/Japan/2020, YEZV/tick/BT-2135/Japan/2021, and YEZV/tick/BT-2155/Japan/2021 and deposited those sequences into the DNA Data Bank of Japan (<https://www.ddbj.nig.ac.jp>) (Table 2-4). For 1 pool that had a high Ct value, YEZV/tick/BT-1822/Japan/2020, we could not

determine the complete genome sequence; however, we amplified a short fragment of the S segment by nested reverse transcription PCR and deposited that sequence in the DNA Data Bank of Japan (Table 2-4).

2. 4. 4. Genetic diversity and phylogenetic relationships

We analyzed the nucleotide and amino acid sequences of the RNA-dependent RNA polymerase, glycoprotein precursor, and nucleoprotein among YEZV strains and compared those sequences to Sulina virus IxriSL16–01 sequences (Tomazatos et al. 2021) (Tables 2-6–2-8). Sequence identity between YEZV strains was 93.0%–100.0% at the nucleotide level and 99.2%–100.0% at the amino acid level.

We also performed phylogenetic analysis of all 64 previously registered YEZV strains by using the nucleotide sequences of the open reading frames of the L, M, and S segments (Figure 2-2). All tick-derived strains detected in this chapter belonged to the same cluster as the strains obtained from patients in Hokkaido (Kodama et al. 2021; Yamaguchi et al. 2026).

YEZV/tick/BT-2135/Japan/2021 was a reassortant strain in which the M segment had been replaced through genetic reassortment. The Japanese, Chinese, and Russian strains did not cluster according to geographic origin, but instead showed close genetic relatedness across regions.

2. 5. Discussion

Previous studies have demonstrated that migratory birds can carry various tick-borne pathogens, including viruses, bacteria, *Anaplasma*, *Rickettsia*/*Coxiella*, together with Ixodid ticks (Hasle 2013). Among viruses, CCHFV and Alkhurma hemorrhagic fever virus have been reported to be transported by migratory birds and their attached ticks between Africa and Europe (Hoffman et al. 2018; Leblebicioglu et al. 2014; Lindeborg et al. 2012; Mancuso et al. 2019; Palomar et al. 2012; Table 1-1). In addition, tick-borne encephalitis virus (TBEV) has been reported to be carried by migratory birds and ticks both along the Africa–Europe route and within the Eurasian inland

migration corridor (Mikryukova et al. 2014; Waldenström et al. 2007; Table 1-1). In Asia, there is also evidence suggesting that Severe fever with thrombocytopenia syndrome virus (SFTSV) may be transported by migratory birds carrying ticks (Yun et al. 2015). In Japan, *Borrelia* spp. causing Lyme disease were detected in ticks collected from migratory birds in Nemuro city, Hokkaido, and Fukui Prefecture (Ishiguro et al. 2000; Miyamoto et al. 1997).

Based on these findings, we hypothesized that YEZV might also be transported by migratory birds and ticks. To test this hypothesis, we collected ticks from migratory birds arriving in Hokkaido and examined them for the presence of YEZV.

We successfully detected YEZV RNA in ticks collected from migratory birds that had arrived in Hokkaido. YEZV RNA–positive tick pools were identified at both Lake Furen, Nemuro City, and Lake Kutcharo, Hamatonbetsu Town, Hokkaido (Table 2-2). These findings indicate that YEZV-infected ticks were likely transported by migratory birds along both the Sakhalin and Kuril Islands flyways.

All YEZV RNA–positive tick pools consisted of *I. persulcatus* collected from *E. spodocephala* (Black-faced Bunting) (Table 2-2). A previous study in the Nemuro region of Hokkaido reported that *I. persulcatus* larvae and nymphs collected from *E. spodocephala* were infected with *B. garinii* (Miyamoto et al. 1997).

E. spodocephala is a ground-foraging passerine species widely distributed across northern Eurasia, breeding from central Siberia and the Russian Far East (including northern Sakhalin and the Sea of Japan coast) to northeastern China and North Korea, and wintering in southern China, Taiwan, and parts of South and Southeast Asia such as Nepal, India, and Myanmar (BirdLife International. n.d.c.). In Japan, this species migrates from Sakhalin and the Kuril Islands to northern Hokkaido, including Lake Kutcharo, Hamatonbetsu Town, and Lake Furen, Nemuro City—the same areas surveyed in this chapter—during autumn, and then continues southward across the Japanese Archipelago (Yamashina Institute for Ornithology. 2002).

I. persulcatus is distributed from eastern Europe through Russia to northern China (Takada et al. 2019). In Japan, this species occurs throughout Hokkaido and in the cool highlands

of Honshu, Shikoku, and Kyushu (approximately above 800 m in altitude) (Takada et al. 2019). It belongs to the *I. ricinus* species complex, a group known for its broad capacity to transmit various pathogens like *Borrelia* spp. causing Lyme disease and TBEV (Takada et al. 2019).

Consequently, the distribution ranges of *E. spodocephala* and *I. persulcatus* overlap not only in Hokkaido but also across northern and central Honshu, Japan (Figure 2-1). Therefore, when *E. spodocephala* migrate southward from Hokkaido to Honshu, *I. persulcatus* ticks carrying YEZV may detach from the birds and drop into new environments along the migration route. Such events could facilitate the southward spread of YEZV or its tick vectors to regions where the virus has not yet been detected. To verify this possibility, extensive field-based epidemiological investigations targeting both questing ticks and wild mammals in Honshu are needed. These studies would clarify whether YEZV is being introduced or established beyond Hokkaido and help to assess the risk of its geographic expansion within Japan.

In this chapter, most of the tick pools collected from migratory birds were *I. persulcatus* derived from *E. spodocephala*. Considering that YEZV has been detected from several tick species collected from vegetation (Kodama et al. 2021), the reason all YEZV RNA-positive tick pools in this chapter were *I. persulcatus* from *E. spodocephala* may reflect sampling bias. Further surveys at different sites and with other bird-tick combinations are needed to clarify whether YEZV can also be transported by other avian hosts and tick species.

All ticks collected from migratory birds were larvae and nymphs (Table 2-5). It is known that in the life cycle of ixodid ticks, the larval and nymphal stages commonly parasitize small birds and small mammals (Hasle 2013). Consistent with this, most previous studies collecting ticks from migratory birds have reported mainly larvae and nymphs, which agrees with the present results (Hoffman et al. 2018; Leblebicioglu et al. 2014; Lindeborg et al. 2012; Mancuso et al. 2019; Mikryukova et al. 2014; Palomar et al. 2012; Waldenström et al. 2007).

In addition, some YEZV RNA-positive tick pools consisted only of unfed ticks, suggesting that YEZV may be maintained through transstadial transmission within *I. persulcatus*.

This possibility should be further investigated through laboratory studies using ticks to clarify the mechanisms of virus maintenance and transmission.

The tick-derived strain YEZV/tick/BT-2135/Japan/2021 was inferred to have arisen through genetic reassortment involving Japanese human-derived strains. This suggests that YEZV has the potential to undergo genetic reassortment, which may contribute to the emergence of strains with altered pathogenicity or host range in the future.

The Japanese, Chinese, and Russian strains were phylogenetically closely related, suggesting that YEZV may circulate widely across Japan, China, and Russia. These regions are all located along the northern part of the East Asian–Australasian Flyway, which connects the breeding, stopover, and wintering sites of migratory birds across East Asia (BirdLife International, n.d.a.). Therefore, it is plausible that YEZV circulation among these areas is facilitated by migratory birds carrying infected ticks along this flyway.

Overall, our results provide important evidence that migratory birds play a role in the long-distance dispersal and genetic connectivity of YEZV populations across East Asia. However, several limitations should be considered when interpreting these findings.

The spatial and temporal coverage of sampling was limited. Field collections were conducted only at several migratory stopover sites in northern Hokkaido during the autumn migration seasons of 2020–2021. Therefore, the obtained ticks and viral sequences may not fully represent the genetic diversity of populations along the entire East Asian–Australasian flyway. Year-to-year variations in tick infestation and virus prevalence were not comprehensively assessed.

Infectious YEZV has not yet been isolated from bird-derived samples, and therefore the infectivity and pathogenicity of the reassortant strain could not be experimentally evaluated. We plan to attempt virus isolation from these samples in future work and to perform comparative studies using recovered isolates to assess their replication properties and pathogenic potential.

The inference of migratory routes was based on existing bird-banding and observational data rather than direct satellite-tracking evidence (Yamashina Institute for Ornithology, 2002).

Consequently, the connection between Hokkaido and the Russian Far East along the flyway remains inferential. Nevertheless, the study sites in Hamatonbetsu Town and Lake Furen in Nemuro City are known as the first stopover points for migratory birds arriving from Sakhalin and the Kuril Islands, supporting their suitability for investigating avian-mediated virus dispersal.

The reference sequences used for phylogenetic analysis were unevenly distributed geographically, which may have influenced the inference of genetic relationships and reassortment patterns. Continued genomic surveillance of YEZV and related orthonairoviruses across East Asia and Russia, particularly at migratory stopover sites, will be essential to validate and expand the findings of this chapter.

This chapter provides evidence that migratory birds can mediate the interregional movement of ticks carrying orthonairoviruses and may facilitate genetic reassortment of YEZV. To further expand upon these findings, several research directions should be pursued. Continuous surveillance of ticks and migratory birds across a broader geographic range—including East Asia and Russia—will be essential to elucidate the spatial continuity of viral distribution and genetic diversity. Integrated sampling of ticks, birds, and wild animals along migratory flyways will allow a more precise reconstruction of the ecological network of virus circulation.

Attempts should also be made to isolate YEZV from bird-derived samples, followed by experimental, *in vitro*, and *in vivo* studies using the obtained isolates to assess replication kinetics, pathogenicity, and potential changes in host range associated with reassortment. Such work will provide direct biological insight into the evolutionary and ecological consequences of genome segment exchange.

By applying full-genome sequencing combined with molecular clock and phylogeographic analyses, it will become possible to estimate the frequency and timing of reassortment events and to reconstruct the evolutionary history of YEZV in a temporal framework.

Finally, future studies should evaluate the potential effects of climate change and shifting migratory patterns on tick distribution and virus transmission dynamics.

Through these approaches, we aim to deepen our understanding of virus circulation mediated by migratory birds and ticks and to contribute to the development of regional and global strategies for the prediction and prevention of emerging tick-borne viral diseases in East Asia.

2. 6. Figure legends

Figure 2-1. Migratory bird flyways and distribution of *Ixodes persulcatus* ticks in the study of transboundary movement of YEZV via ticks on birds, Japan, 2020–2021. Distribution of *I. persulcatus* ticks (red rhombus), prefectures of Japan where black-faced buntings (*Emberiza spodocephala*) banded in Hokkaido were recaptured (light blue), and flyway of *E. spodocephala* passing through Hokkaido during autumn (orange dotted line). Distribution outside Japan is indicated at the country level. The black circle shows Lake Furen, Nemuro City, Hokkaido; the white circle shows Lake Kutcharo, Hamatonbetsu Town, Hokkaido.

Figure 2-2. Phylogenetic analysis of YEZV strains in study of transboundary movement of YEZV via ticks on migratory birds, Japan, 2020–2021. Trees were constructed by using the maximum-likelihood method in MEGA X (<https://www.megasoftware.net>) and 1,000 bootstrap replicates for nucleotide sequences. A) Large segment; B) medium segment; C) small segment. Black circles indicate sequences from this chapter. The number at each branch indicates the bootstrap value. GenBank accession numbers for nucleotide sequences are shown in parentheses. YEZV/gray seal/20-VLT000893/Sweden/2020 was used as the outgroup to determine the root of YEZV trees but is not shown. Scale bars indicate nucleotide substitutions per site.

2. 7. Tables and figure

Table 2-1. Information on migratory birds captured in 2020 and the infesting ticks

Species of migratory bird	No. of captured birds			No. of birds infested by ticks*			No. of ticks*		
	Hamatonb etsu (Lake Kutcharo)	Nemuro (Lake Furen)	Total	Hamatonb etsu (Lake Kutcharo)	Nemuro (Lake Furen)	Total	Hamatonb etsu (Lake Kutcharo)	Nemuro (Lake Furen)	Total
<i>Emberiza spodocephala</i>	693	1142	1835	129	519	648	185	991	1176
<i>Horornis diphone</i>	100	36	136	15	2	17	16	2	18
<i>Turdus chrysolaus</i>	4	35	39	2	17	19	2	26	28
<i>Tarsiger cyanurus</i>	0	17	17	0	5	5	0	5	5
<i>Emberiza variabilis</i>	4	10	14	1	4	5	4	9	13
<i>Luscinia calliope</i>	6	2	8	3	1	4	6	2	8
<i>Sitta europaea</i>	3	0	3	1	0	1	1	0	1
<i>Troglodytes troglodytes</i>	0	3	3	0	2	2	0	4	4
<i>Erithacus akahige</i>	0	1	1	0	1	1	0	3	3
<i>Phylloscopus schwarzi</i>	1	0	1	1	0	1	2	0	2
<i>Turdus obscurus</i>	1	0	1	1	0	1	1	0	1
Total	812	1246	2058	153	551	704	217	1042	1259

*Only live ticks on migratory birds were counted.

Table 2-2. Information on migratory birds captured in 2021 and the infesting ticks

Species of migratory bird	No. of captured birds			No. of birds infested by ticks*			No. of ticks*		
	Hamatonb etsu (Lake Kutcharo)	Nemuro (Lake Furen)	Total	Hamatonb etsu (Lake Kutcharo)	Nemuro (Lake Furen)	Total	Hamatonb etsu (Lake Kutcharo)	Nemuro (Lake Furen)	Total
<i>Emberiza spodocephala</i>	651	1098	1749	157	421	578	273	896	1169
<i>Uragus sibiricus</i>	0	61	61	0	1	1	0	1	1
<i>Horomis diphone</i>	51	8	59	9	1	10	9	1	10
<i>Turdus chrysolaus</i>	8	24	32	4	20	24	7	37	44
<i>Emberiza variabilis</i>	7	9	16	4	4	8	13	17	30
<i>Tarsiger cyanurus</i>	0	8	8	0	4	4	0	6	6
<i>Certhia familiaris</i>	2	4	6	1	1	2	1	1	2
<i>Parus minor</i>	6	0	6	1	0	1	1	0	1
<i>Troglodytes troglodytes</i>	0	6	6	0	4	4	0	7	7
<i>Parus palustris</i>	0	5	5	0	1	1	0	1	1
<i>Luscinia calliope</i>	4	0	4	1	0	1	2	0	2
<i>Sitta europaea</i>	0	2	2	0	1	1	0	1	1
<i>Turdus obscurus</i>	1	0	1	1	0	1	1	0	1
Total	730	1225	1955	178	458	636	307	968	1275

*Only live ticks on migratory birds were counted.

Table 2-3. Prevalence of Yezo virus in ticks collected from migratory birds

Species	Stage	No. of examined ticks	No. of examined pools	No. of positive pools	Minimum infection rate (%)
<i>Ixodes persulcatus</i>	Larva	1727	193	3	0.2
	Nymph	596	137	5	0.8
<i>Ixodes pavlovskyi</i>	Larva	169	30	0	0
	Nymph	34	19	0	0
<i>Ixodes turdus</i>	Larva	0	0	0	0
	Nymph	1	1	0	0
<i>Haemaphysalis megaspinosa</i>	Larva	3	2	0	0
	Nymph	0	0	0	0
<i>Haemaphysalis concinna</i>	Larva	3	2	0	0
	Nymph	1	1	0	0
Total		2534	385	8	0.3

Table 2-4. Information on Yezo viruses detected from ticks on migratory birds

Strain	Tick species	Stage	No. of ticks in the pool	Collection site	Collection date (y/m/d)	Bird species	DDBJ accession no.
YEZV/tick/BT-1821/Japan/2020	<i>Ixodes persulcatus</i>	Nymph	5	Hamatonbetsu (Lake Kutcharo)	2020/10/6,7	<i>Emberiza spodocephala</i>	L: LC735725 M: LC735726 S: LC735727
YEZV/tick/BT-1822/Japan/2020	<i>Ixodes persulcatus</i>	Nymph	5	Hamatonbetsu (Lake Kutcharo)	2020/10/6,7	<i>Emberiza spodocephala</i>	S (partial sequence) : LC737964
YEZV/tick/BT-1826/Japan/2020	<i>Ixodes persulcatus</i>	Nymph	5	Hamatonbetsu (Lake Kutcharo)	2020/10/7	<i>Emberiza spodocephala</i>	L: LC735728 M: LC735729 S: LC735730
YEZV/tick/BT-1844/Japan/2020	<i>Ixodes persulcatus</i>	Larva	10	Nemuro (Lake Furen)	2020/10/8	<i>Emberiza spodocephala</i>	L: LC735731 M: LC735732 S: LC735733
YEZV/tick/BT-1864/Japan/2020	<i>Ixodes persulcatus</i>	Larva	10	Nemuro (Lake Furen)	2020/10/8	<i>Emberiza spodocephala</i>	L: LC735734 M: LC735735 S: LC735736
YEZV/tick/BT-1968/Japan/2020	<i>Ixodes persulcatus</i>	Larva	10	Nemuro (Lake Furen)	2020/10/11	<i>Emberiza spodocephala</i>	L: LC790674 M: LC790675 S: LC790676
YEZV/tick/BT-2135/Japan/2021	<i>Ixodes persulcatus</i>	Nymph	5	Nemuro (Lake Furen)	2021/10/13	<i>Emberiza spodocephala</i>	L: LC790677 M: LC790678 S: LC790679
YEZV/tick/BT-2155/Japan/2021	<i>Ixodes persulcatus</i>	Nymph	5	Nemuro (Lake Furen)	2021/10/13	<i>Emberiza spodocephala</i>	L: LC790680 M: LC790681 S: LC790682

Table 2-5. Information on ticks from which Yezo virus was detected

Strain	Tick species	Stage	No. of ticks in pool	Status of bloodfeeding		
				Engorged	Unfed	Engorged or unfed
YEZV/tick/BT-1821/Japan/2020	<i>Ixodes persulcatus</i>	Nymph	5	1	4	0
YEZV/tick/BT-1822/Japan/2020	<i>Ixodes persulcatus</i>	Nymph	5	1	4	0
YEZV/tick/BT-1826/Japan/2020	<i>Ixodes persulcatus</i>	Nymph	5	0	5	0
YEZV/tick/BT-1844/Japan/2020	<i>Ixodes persulcatus</i>	Larva	10	3	6	1
YEZV/tick/BT-1864/Japan/2020	<i>Ixodes persulcatus</i>	Larva	10	0	10	0
YEZV/tick/BT-1968/Japan/2020	<i>Ixodes persulcatus</i>	Larva	10	3	1	6
YEZV/tick/BT-2135/Japan/2021	<i>Ixodes persulcatus</i>	Nymph	5	0	5	0
YEZV/tick/BT-2155/Japan/2021	<i>Ixodes persulcatus</i>	Nymph	5	0	5	0

Table 2-6. Pairwise comparison of the nucleotide and amino acid sequences of the RNA-dependent RNA polymerase of Yezo virus strains and Sulina virus IxriSL16-01

Virus	YEZV /ick /BT-1821 /Japan /2020	YEZV /ick /BT-1826 /Japan /2020	YEZV /ick /BT-1844 /Japan /2020	YEZV /ick /BT-1864 /Japan /2020	YEZV /ick /BT-1968 /Japan /2020	YEZV /ick /BT-2135 /Japan /2021	YEZV /ick /BT-2155 /Japan /2021	YEZV /human /H-4001- 2019 /Japan /2019	YEZV /human /H-4003- 2020 /Japan /2020	YEZV /human /H-4007- 2016 /Japan /2016	YEZV /human /H-4008- 2017 /Japan /2017	YEZV /human /H-4020- 2017 /Japan /2017	YEZV /human /H-4011- 2020 /Japan /2020	YEZV /human /H-IM01 /China /2018	YEZV /ick /T-HLJ01 /China /2021	YEZV /ick /T-HLJ02 /China /2021	YEZV /ick /T-HLJ03 /China /2021	YEZV /ick /TGMC_1 /China /2019	YEZV /ick /TGMC_2 /China /2019	YEZV /ick /TGMC_3 /China /2019	YEZV /ick /T-IM01 /China /2021	YEZV /ick /T-JL01 /China /2020	Sulina virus IxriSL 16-01
YEZV/ick/BT-1821 /Japan/2020	—	99.8	95.6	98.8	96.1	99.1	99.6	97.3	99.1	97.4	98.8	97.2	97.4	98.4	98.7	98.4	98.8	98.7	98.4	98.5	98.4	98.8	72.8
YEZV/ick/BT-1826 /Japan/2020	99.9	—	95.6	98.8	96.1	99.1	99.6	97.2	99.1	97.4	98.8	97.2	97.4	98.3	98.6	98.4	98.8	98.7	98.4	98.5	98.3	98.7	72.8
YEZV/ick/BT-1844 /Japan/2020	99.9	99.9	—	98.8	96.1	99.1	99.9	97.2	99.1	97.4	98.8	97.2	97.4	98.3	98.6	98.4	98.8	98.7	98.4	98.5	98.3	98.7	72.8
YEZV/ick/BT-1864 /Japan/2020	99.4	99.4	95.5	—	98.9	98.9	98.8	97.3	98.9	97.3	99.7	97.2	97.3	98.1	98.6	98.3	98.7	98.6	98.3	98.4	98.1	98.7	72.8
YEZV/ick/BT-1968 /Japan/2020	99.8	99.8	95.9	99.6	—	99.7	99.2	97.5	99.5	97.6	99.0	97.4	97.6	98.4	98.8	98.5	98.9	98.6	98.5	98.0	98.4	98.9	72.8
YEZV/ick/BT-2135 /Japan/2021	99.7	99.7	99.8	99.6	99.9	—	99.1	97.4	99.4	97.5	98.9	97.4	97.5	98.4	98.7	98.5	98.9	98.8	98.5	98.6	98.3	98.9	72.8
YEZV/ick/BT-2155 /Japan/2021	99.9	99.9	100.0	99.5	99.0	99.0	—	97.3	99.1	97.4	98.0	97.2	97.4	98.4	98.0	98.4	98.0	98.0	98.5	98.5	98.3	98.0	72.8
YEZV/human/H4001- 2019/Japan/2019	99.5	99.5	99.6	99.5	99.8	99.7	99.6	—	97.4	98.4	97.2	98.3	98.4	97.4	97.2	97.4	97.4	97.2	97.4	97.5	97.3	97.3	72.9
YEZV/human/H4003- 2020/Japan/2020	99.7	99.7	99.8	99.6	99.9	99.9	99.8	99.7	—	97.5	98.9	97.4	97.5	98.4	98.7	98.5	98.9	98.6	98.5	98.6	98.3	98.9	72.8
YEZV/human/H4007- 2016/Japan/2016	99.5	99.5	99.6	99.4	99.7	99.7	99.6	99.8	99.6	—	97.3	98.2	100.0	97.5	97.3	97.5	97.5	97.4	97.6	97.6	97.4	97.4	72.9
YEZV/human/H4008- 2017/Japan/2017	99.5	99.5	99.6	99.9	99.8	99.7	99.6	99.6	99.7	99.5	—	97.2	97.3	98.2	98.6	98.3	98.7	98.7	98.3	98.4	98.1	98.7	72.8
YEZV/human/H4020- 2017/Japan/2017	99.4	99.4	99.5	99.3	99.6	99.5	99.4	99.7	99.5	99.7	99.4	—	99.2	97.3	97.2	97.4	97.4	97.2	97.4	97.4	97.3	97.3	72.8
YEZV/human/H4011- 2020/Japan/2020	99.5	99.5	99.6	99.4	99.7	99.7	99.6	99.8	99.6	100.0	99.5	99.7	—	97.5	97.3	97.5	97.5	97.4	97.6	97.6	97.4	97.4	72.9
YEZV/human/H- IM01/China/2018	99.5	99.5	99.6	99.5	99.7	99.7	99.6	99.7	99.6	99.6	99.6	99.5	99.6	—	98.2	98.5	98.4	98.3	98.5	98.6	100.0	98.3	72.1
YEZV/ick/T- HLJ01/China/2021	99.8	99.6	99.8	99.5	99.9	99.8	99.7	99.7	99.8	99.6	99.6	99.5	99.6	99.7	—	98.5	98.9	98.8	98.4	98.4	98.2	98.8	72.6
YEZV/ick/T- HLJ02/China/2021	99.8	99.6	99.7	99.5	99.8	99.8	99.7	99.8	99.7	99.7	99.6	99.6	99.7	99.7	99.8	—	98.4	98.4	98.6	98.7	98.5	98.3	72.5
YEZV/ick/T- HLJ03/China/2021	99.6	99.6	99.7	99.5	99.8	99.8	99.8	99.6	99.7	99.6	99.6	99.4	99.6	99.6	99.8	99.7	—	98.0	98.5	98.5	98.3	99.0	72.6
YEZV/ick/TGMC_1 /China/2019	99.7	99.7	99.8	99.5	99.8	99.8	99.8	99.7	99.8	99.7	99.6	99.5	99.7	99.7	99.8	99.8	99.8	—	98.4	98.5	98.2	98.9	72.8
YEZV/ick/TGMC_2 /China/2019	99.8	99.6	99.7	99.5	99.8	99.8	99.7	99.8	99.6	99.7	99.6	99.6	99.7	99.8	99.8	99.8	99.7	99.8	—	99.0	98.5	98.4	72.9
YEZV/ick/TGMC_3 /China/2019	99.7	99.7	99.8	99.6	99.9	99.9	99.8	99.8	99.8	99.7	99.6	99.8	99.8	99.8	99.9	99.8	99.9	100.0	—	98.5	98.5	72.9	
YEZV/ick/T-IM01 /China/2021	99.5	99.5	99.6	99.5	99.7	99.7	99.6	99.7	99.6	99.6	99.5	99.6	100.0	99.7	99.7	99.7	99.6	99.7	99.8	99.8	—	98.3	72.4
YEZV/ick/T-JL01 /China/2020	99.5	99.5	99.6	99.4	99.8	99.7	99.8	99.6	99.7	99.5	99.4	99.5	99.6	99.8	99.6	99.8	99.7	99.7	99.7	99.7	99.5	—	72.8
Sulina virus IxriSL16-01	82.1	82.2	82.2	82.0	82.1	82.1	82.1	82.2	82.1	82.2	82.1	82.1	82.2	82.0	82.2	82.1	82.1	82.1	82.1	82.1	82.0	82.1	—

*Nucleotide sequence identity (%) is shown above the diagonal, and amino acid sequence identity (%) is shown below the diagonal.

Table 2-7. Pairwise comparison of the nucleotide and amino acid sequences of the glycoprotein precursor of Yezo virus strains and Sulina virus IxriSL16-01

Virus	YEZV /tick /BT-1821 /Japan /2020	YEZV /tick /BT-1826 /Japan /2020	YEZV /tick /BT-1844 /Japan /2020	YEZV /tick /BT-1864 /Japan /2020	YEZV /tick /BT-1968 /Japan /2020	YEZV /tick /BT-2135 /Japan /2021	YEZV /tick /BT-2155 /Japan /2021	YEZV /human /HH001- 2019 /Japan /2019	YEZV /human /HH003- 2020 /Japan /2020	YEZV /human /HH007- 2016 /Japan /2016	YEZV /human /HH008- 2017 /Japan /2017	YEZV /human /HH009- 2017 /Japan /2017	YEZV /human /HH011- 2020 /Japan /2020	YEZV /human /H-IM01 /China /2018	YEZV /tick /T-HLJ01 /China /2021	YEZV /tick /T-HLJ02 /China /2021	YEZV /tick /T-HLJ03 /China /2021	YEZV /tick /T-IM01 /China /2021	YEZV /tick /T-JL01 /China /2020	Sulina virus IxriSL 16-01
YEZV/tick/BT-1821/Japan/2020	—	99.8	99.6	99.3	99.3	97.5	99.8	97.8	99.1	97.5	99.2	97.1	97.5	99.3	98.6	98.5	98.6	98.3	98.5	68.7
YEZV/tick/BT-1826/Japan/2020	99.8	—	99.7	99.4	99.4	97.5	99.7	97.8	99.2	97.5	99.3	97.1	97.5	98.3	98.7	98.6	98.7	98.3	98.6	68.8
YEZV/tick/BT-1844/Japan/2020	99.6	99.8	—	99.4	99.5	97.5	100.0	97.8	99.2	97.5	99.3	97.2	97.5	98.3	98.7	98.6	98.7	98.3	98.7	68.9
YEZV/tick/BT-1864/Japan/2020	99.0	99.9	99.0	—	99.3	97.0	99.4	98.0	99.3	97.0	99.8	97.3	97.0	98.5	98.9	98.7	98.8	98.5	98.8	69.1
YEZV/tick/BT-1968/Japan/2020	99.5	99.7	99.6	99.6	—	97.5	99.5	97.9	99.2	97.5	99.3	97.2	97.5	98.3	98.7	98.5	98.6	98.3	98.6	69.6
YEZV/tick/BT-2135/Japan/2021	99.2	99.4	99.3	99.3	99.2	—	97.5	99.0	97.5	98.3	97.6	98.0	98.3	97.5	97.5	97.7	97.4	97.5	97.4	69.0
YEZV/tick/BT-2155/Japan/2021	99.6	99.9	99.9	99.7	99.7	99.3	—	97.8	99.2	97.5	99.3	97.2	97.5	98.3	98.7	98.6	98.7	98.3	98.7	68.9
YEZV/human/HH001-2019/Japan/2019	99.4	99.6	99.5	99.5	99.4	99.5	99.6	—	97.9	98.7	97.9	98.3	98.7	97.8	97.8	98.0	97.7	97.8	97.7	69.7
YEZV/human/HH003-2020/Japan/2020	99.6	99.7	99.6	99.6	99.5	99.3	99.6	99.5	—	97.4	99.2	97.1	97.4	98.2	98.7	98.5	98.6	98.2	98.6	69.1
YEZV/human/HH007-2016/Japan/2016	99.4	99.6	99.5	99.5	99.4	99.5	99.6	99.7	99.5	—	97.5	98.8	100.0	97.4	97.4	97.6	97.3	97.4	97.4	69.8
YEZV/human/HH008-2017/Japan/2017	99.5	99.7	99.6	99.9	99.5	99.3	99.6	99.5	99.6	99.5	—	97.2	97.5	98.5	98.9	98.6	98.7	98.5	98.7	69.1
YEZV/human/HH009-2017/Japan/2017	99.3	99.0	99.4	99.4	99.3	99.4	99.5	99.0	99.4	99.0	99.4	—	98.0	97.2	97.2	97.4	97.0	97.2	97.1	69.7
YEZV/human/HH011-2020/Japan/2020	99.4	99.6	99.5	99.5	99.4	99.5	99.6	99.7	99.5	100.0	99.5	99.6	—	97.4	97.4	97.6	97.3	97.4	97.4	69.8
YEZV/human/H-IM01/China/2018	99.4	99.6	99.5	99.5	99.4	99.3	99.6	99.6	99.5	99.6	99.5	99.5	99.6	—	98.3	98.6	98.2	100.0	98.2	69.2
YEZV/tick/T-HLJ01/China/2021	99.5	99.7	99.6	99.6	99.5	99.3	99.6	99.5	99.6	99.5	99.6	99.4	99.5	99.5	—	98.5	98.8	98.3	98.9	68.5
YEZV/tick/T-HLJ02/China/2021	99.6	99.8	99.6	99.6	99.6	99.5	99.7	99.6	99.7	99.6	99.6	99.7	99.7	99.6	98.5	—	98.3	98.6	98.4	69.1
YEZV/tick/T-HLJ03/China/2021	99.6	99.8	99.6	99.6	99.6	99.3	99.7	99.6	99.6	99.6	99.6	99.5	99.6	99.6	99.6	99.7	—	98.2	98.8	69.3
YEZV/tick/T-IM01/China/2021	99.4	99.6	99.5	99.5	99.4	99.3	99.6	99.6	99.5	99.6	99.5	99.5	99.6	100.0	99.5	99.7	99.6	—	98.2	69.2
YEZV/tick/T-JL01/China/2020	99.5	99.7	99.0	99.6	99.5	99.3	99.6	99.5	99.0	99.5	99.0	99.4	99.5	99.6	99.6	99.6	99.6	99.6	—	69.1
Sulina virus IxriSL16-01	57.0	57.1	57.1	57.0	57.2	57.0	57.1	57.1	57.1	57.1	57.0	57.3	57.1	57.3	57.1	57.1	57.0	57.3	57.1	—

*Nucleotide sequence identity (%) is shown above the diagonal, and amino acid sequence identity (%) is shown below the diagonal.

Table 2-8. Pairwise comparison of the nucleotide and amino acid sequences of the nucleoprotein of Yezo virus strains and Sulina virus IxriSL16-01

Virus	YEZV /tick /BT-1821 /Japan /2020	YEZV /tick /BT-1826 /Japan /2020	YEZV /tick /BT-1844 /Japan /2020	YEZV /tick /BT-1864 /Japan /2020	YEZV /tick /BT-1968 /Japan /2020	YEZV /tick /BT-2135 /Japan /2021	YEZV /tick /BT-2155 /Japan /2021	YEZV /human /HH001- 2019 /Japan /2019	YEZV /human /HH003- 2020 /Japan /2020	YEZV /human /HH007- 2016 /Japan /2016	YEZV /human /HH008- 2017 /Japan /2017	YEZV /human /HH009- 2017 /Japan /2017	YEZV /human /HH011- 2020 /Japan /2020	YEZV /human /H-IM01 /China /2018	YEZV /tick /T-HLJ01 /China /2021	YEZV /tick /T-HLJ02 /China /2021	YEZV /tick /T-HLJ03 /China /2021	YEZV /tick /T-IM01 /China /2021	YEZV /tick /T-JLD1 /China /2020	Sulina virus IxriSL 16-01
YEZV/tick/BT-1821 /Japan/2020	—	99.8	99.3	98.8	99.4	99.4	99.4	93.5	98.9	93.4	98.8	93.1	93.4	98.5	98.6	93.1	98.4	98.6	98.9	75.6
YEZV/tick/BT-1826 /Japan/2020	99.8	—	99.5	99.0	99.6	99.6	99.6	93.6	99.1	93.5	99.0	93.2	93.5	98.8	98.8	93.2	98.6	98.8	99.1	75.9
YEZV/tick/BT-1844 /Japan/2020	99.8	100.0	—	99.0	99.6	99.6	99.6	93.7	99.2	93.7	99.0	93.3	93.7	98.8	98.8	93.3	98.6	98.8	99.1	88.8
YEZV/tick/BT-1864 /Japan/2020	99.8	100.0	100.0	—	99.3	99.3	99.1	93.0	99.1	93.4	99.9	93.2	93.4	98.9	98.0	93.2	98.8	98.9	99.3	60.0
YEZV/tick/BT-1968 /Japan/2020	99.8	100.0	100.0	100.0	—	99.9	99.7	93.7	98.3	93.0	99.3	93.3	93.0	99.1	98.1	93.3	98.9	99.1	99.3	60.5
YEZV/tick/BT-2135 /Japan/2021	99.8	100.0	100.0	100.0	100.0	—	99.7	93.7	96.3	93.6	99.3	93.3	93.6	99.1	96.1	93.3	98.9	99.1	99.3	68.4
YEZV/tick/BT-2155 /Japan/2021	99.8	100.0	100.0	100.0	100.0	100.0	—	93.7	98.3	93.8	99.1	93.3	93.8	98.9	98.8	93.3	98.7	98.9	98.2	68.6
YEZV/human/HH001- 2019/Japan/2019	99.6	99.8	99.8	99.8	99.6	99.8	99.8	—	93.7	97.5	93.6	99.2	97.8	94.0	93.6	96.1	93.8	93.8	93.7	76.1
YEZV/human/HH003- 2020/Japan/2020	99.6	100.0	100.0	100.0	100.0	100.0	100.0	99.8	—	93.6	99.1	93.3	93.6	98.8	98.8	93.4	96.7	98.6	99.2	66.8
YEZV/human/HH007- 2016/Japan/2016	99.4	99.6	99.6	99.6	99.6	99.6	99.6	99.8	99.6	—	93.9	97.2	99.9	91.0	93.6	97.2	93.5	93.9	93.7	76.6
YEZV/human/HH008- 2017/Japan/2017	99.6	99.8	99.8	99.8	99.8	99.8	99.8	99.6	99.8	99.4	—	93.2	93.9	98.9	99.0	93.2	98.8	98.9	99.3	68.6
YEZV/human/HH009- 2017/Japan/2017	99.4	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.4	—	97.3	93.8	93.2	98.0	93.4	93.4	93.3	76.1
YEZV/human/HH011- 2020/Japan/2020	99.6	99.8	99.8	99.8	99.8	99.8	99.8	100.0	99.8	99.8	99.6	99.8	—	94.0	93.6	97.2	93.5	93.9	93.7	76.6
YEZV/human/H-IM01 /China/2018	99.6	99.8	99.8	99.8	99.8	99.8	99.8	99.6	99.8	99.4	99.6	99.4	99.6	—	98.8	93.6	98.6	99.9	99.1	68.4
YEZV/tick/T-HLJ01 /China/2021	99.6	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.6	99.6	99.6	99.8	99.8	—	93.0	98.9	98.8	99.3	75.8
YEZV/tick/T-HLJ02 /China/2021	99.6	99.8	99.8	99.8	99.8	99.8	99.8	100.0	99.8	99.8	99.6	99.8	100.0	99.8	99.8	—	93.5	93.4	93.3	75.7
YEZV/tick/T-HLJ03 /China/2021	99.4	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.4	99.4	99.4	99.6	99.4	96.8	99.6	—	98.6	99.1	66.2
YEZV/tick/T-IM01 /China/2021	99.6	99.8	99.8	99.8	99.8	99.8	99.8	99.6	99.8	99.4	99.6	99.4	99.6	100.0	96.8	99.6	99.4	—	99.1	66.5
YEZV/tick/T-JLD1 /China/2020	99.6	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.6	99.6	99.6	99.8	99.8	100.0	99.8	99.8	99.6	—	68.4
Sulina virus IxriSL16-01	60.5	60.5	60.5	60.5	60.5	60.5	60.5	60.5	60.5	60.3	60.5	60.3	60.5	60.5	60.5	60.5	60.7	60.5	60.5	—

*Nucleotide sequence identity (%) is shown above the diagonal, and amino acid sequence identity (%) is shown below the diagonal.

Figure 2-1.

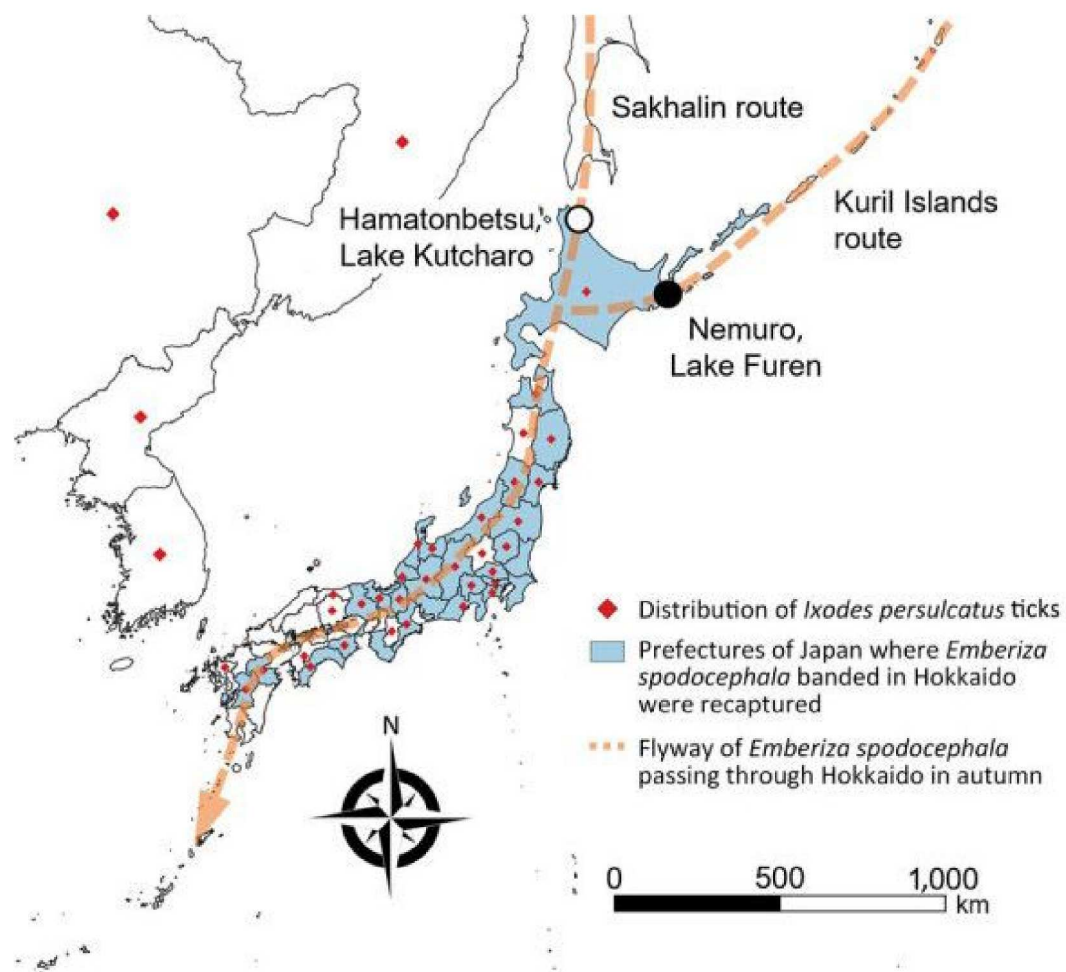
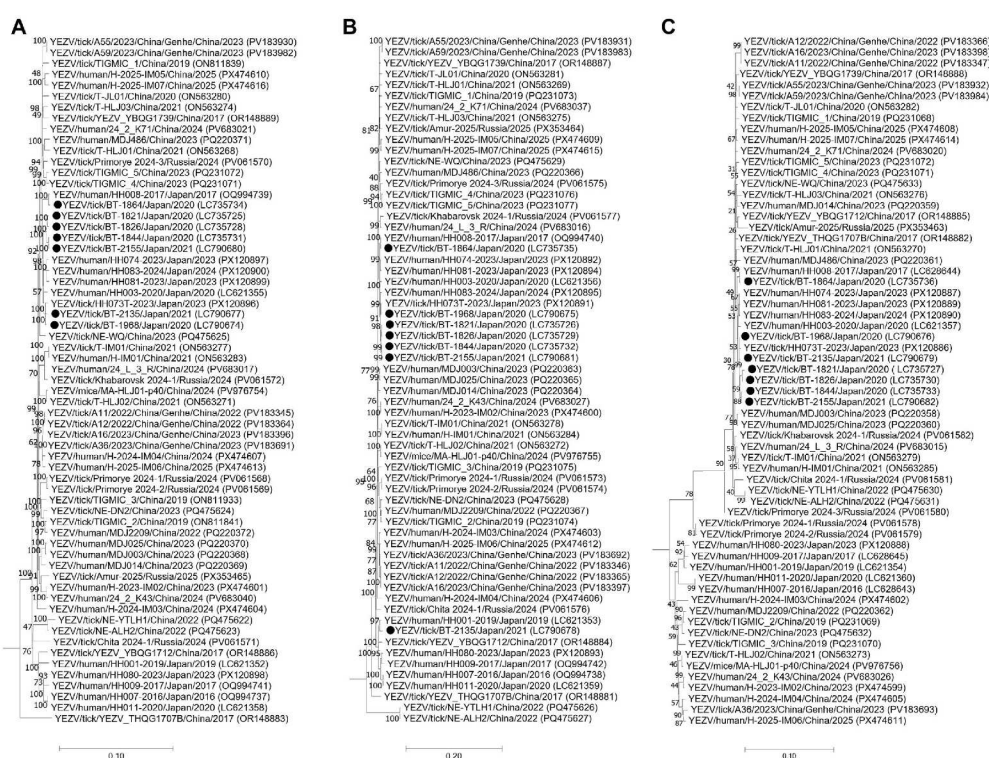


Figure 2-2.



3. Chapter 2

Pathogenicity of a novel Orthonairovirus, Iwanai Valley virus, in mice

3. 1. Abstract

IWVV is a novel tick-borne orthonairovirus classified into the Tamdy genogroup. Since several orthonairoviruses in the Tamdy and closely related Sulina genogroups have been associated with human diseases, evaluating the pathogenic potential of IWVV is an important public health priority. Despite the increase in reports of orthonairoviruses belonging to the Tamdy and Sulina genogroups in East Asia, studies on their pathogenicity remain limited. In this chapter, we established a mouse model of IWVV infection using type I interferon receptor knockout (IFNAR1^{-/-}) mice and analyzed the pathogenicity of IWVV infection. IWVV infection was lethal in IFNAR1^{-/-} mice but did not cause any significant symptoms in BALB/c mice. IWVV replicated well in the liver and spleen of IFNAR1^{-/-} mice, leading to acute necrotizing hepatitis and damage to the spleen, characterized by lymphocyte depletion. These pathological features are consistent with those observed in immunocompromised mice infected with other human-pathogenic orthonairoviruses, such as CCHFV and YEZV. Although human infections with IWVV have not been reported, the observed virulence in animal models suggests that further evaluation of its zoonotic potential is warranted. Our findings highlight the importance of continued surveillance of emerging tick-borne IWVV to assess potential risks to public health.

3. 2. Introduction

The genus *Orthonairovirus*, belonging to the family *Nairoviridae*, comprises 52 recognized virus species (International Committee on Taxonomy of Viruses 2024; Kuhn et al. 2022). Several members of this genus are known to cause disease in humans and/or animals, including CCHFV (Hawman and Feldmann 2023), NSDV (Krasteva et al. 2020), DUGV (Burt et al. 1996), KASV (Kalunda et al. 1986), ISKV (L'vov et al. 1984), ERVEV (Treib et al. 1998; Woessner et al. 2000), and WELV (X.A. Zhang et al. 2024).

In recent years, some emerging orthonairoviruses belonging to the Tamdy and Sulina genogroups have been identified as human pathogens in China and Japan, such as TAMV (Moming et al. 2021), TcTV-1 (Liu et al. 2020; Y. Zhang et al. 2021), SGLV (Ma et al. 2021), and

YEZV (Chen et al. 2024; Kodama et al. 2021; Lv et al. 2023; Ogata et al. 2024; Suzuki et al. 2024; M.Z. Zhang et al. 2025). TAMV has been associated with febrile illness in western China, while TcTV-1 and SGLV have been detected in patients presenting with acute febrile illness following tick bites in China (Liu et al. 2020; Ma et al. 2021; Moming et al. 2021; Y. Zhang et al. 2021). In Japan and northeastern China, YEZV has been shown to cause febrile illness with thrombocytopenia, leukopenia, and elevated liver enzymes (Chen et al. 2024; Kodama et al. 2021; Lv et al. 2023; Ogata et al. 2024; Suzuki et al. 2024; M.Z. Zhang et al. 2025). Although the pathogenicity of YEZV has been examined using AG129 mice (Ariizumi et al. 2024), which developed lethal disease with weight loss and liver injury, the pathogenic mechanisms and virulence determinants of other Tamdy- and Sulina-related orthonairoviruses remain largely unknown, and well-established animal models for this virus group are still lacking.

IWVV was recently isolated from *I. ovatus* ticks in Hokkaido, Japan (Matsumura et al. 2024). Phylogenetic analysis showed that IWVV is closely related to the Tamdy genogroup and is positioned on the outer branch of the Sulina genogroup (Matsumura et al. 2024). IWVV exhibited robust replication, particularly in the human adenocarcinoma cell line SW-13 and the human hepatocellular carcinoma cell line HuH-7 (Matsumura et al. 2024).

To date, orthonairovirus infection models in small animals have primarily utilized immunocompromised mice (e.g., interferon receptor knockout mice) infected with CCHFV and other viruses (Bente et al. 2010; Bereczky et al. 2010; Coates and Sweet 1990; Dowall et al. 2012; Garrison, Smith, and Golden 2019; Lindquist et al. 2018; Shimada et al. 2016; Sugimoto et al. 2022; Zivcec et al. 2013). Type I interferon receptor knockout (IFNAR1^{-/-}) mice infected with orthonairoviruses have been reported to exhibit hepatic, gastrointestinal, and neurotoxic lesions (Bereczky et al. 2010; Boyd, Fazakerley, and Bridgen 2006; Dowall et al. 2012; Shimada et al. 2016). In particular, liver injury is a typical pathological feature commonly observed in mice infected with various orthonairoviruses, such as CCHFV (Bente et al. 2010, Bereczky et al. 2010, Zivcec et al. 2013), Hazara virus (HAZV) (Dowall et al. 2012), KASV (Sugimoto et al. 2022), Leopards Hill virus (LPHV) (Ishii et al. 2014), as well as in CCHF patients (Cevik et al. 2008;

Hawman and Feldmann 2023; Karakecili et al. 2018; Rathore et al. 2021) and AG129 mice infected with YEZV (Ariizumi et al. 2024).

In this chapter, to elucidate the pathogenicity of IWVV infection, we developed a lethal mouse model of IWVV infection using 7-week-old IFNAR1^{-/-} mice and analyzed pathological changes in the mouse model.

3. 3. Materials and methods

3. 3. 1. Ethical statement

The experiments with animals were performed in strict accordance with the Animal Experimentation Guidelines of the National Institute of Infectious Diseases (NIID). The protocol was approved under Permission Number 121171-II by the Institutional Animal Care and Use Committee (IACUC) of NIID. We complied with the ARRIVE guidelines.

3. 3. 2. Cells

Vero E6 cells (derived from African green monkey; Department of Veterinary Science, NIID, Tokyo, Japan) were cultured in Eagle's Minimum Essential Medium (EMEM) containing 5% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin and incubated at 37 °C in a 5% CO₂ atmosphere. SW-13 cells (derived from human adenocarcinoma; JCRB9069, JCRB Cell Bank, National Institutes of Biomedical Innovation, Health and Nutrition, Osaka, Japan) and Vero cells (ATCC # CCL-81, derived from African green monkey; Department of Veterinary Science, NIID) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 2% heat-inactivated FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin, incubated at 37 °C in a 5% CO₂ atmosphere.

3. 3. 3. Virus

IWVV strain 20OH29 was used for animal infections and indirect immunofluorescence assay (IFA) in the present study. As reported previously, this was isolated from ticks collected in Obihiro City, Hokkaido, Japan (Matsumura et al. 2024) and propagated in the SW-13 cell line.

3. 3. 4. Rabbit infection and preparation of anti-rabbit serum against IWVV

All animal experiments were conducted in a BSL-2 laboratory at NIID with the above-described ethical approval. Antiserum against IWVV was generated in rabbits infected with the virus. Two Japanese white rabbits (female, eight weeks old; KITAYAMA LABES, Nagano, Japan) were subcutaneously inoculated with IWVV strain 20OH29 at a dose of 5.5×10^7 TCID₅₀ three times at 28-day intervals. Blood samples were collected via cardiac puncture under deep anesthesia induced by intravenous injection of Pentobarbital Sodium Salt (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan; 45 mg/kg). The sera were thermally inactivated at 56 °C for 30 minutes and used as anti-IWVV antibody.

3. 3. 5. Virus titration

The viral titer was determined by IFA. Vero cells (ATCC # CCL-81) were seeded into 96-well microplates one day prior to infection to establish a subconfluent monolayer by incubation at 37 °C with 5% CO₂ overnight. Briefly, IWVV was subjected to 10-fold serial dilutions starting from a 1:10 dilution and inoculated into Vero cells, followed by incubation at 37 °C in 5% CO₂. At 4 days post-infection (dpi), the cells were fixed and permeabilized with 10% Formalin Neutral Buffer Solution (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) containing 0.4% Triton X-100 for more than 4 hours. The cells were then washed three times with PBS and stored at 4 °C. Anti-rabbit sera against IWVV were diluted to 1:200 in PBS containing 5% skim milk (MEGMILK SNOW BRAND Co., Ltd., Hokkaido, Japan), added to the wells, and incubated at 37 °C for 30 minutes. After washing three times with PBS, Goat anti-rabbit IgG (H+L) secondary antibody conjugated with FITC (65-6111; Thermo Fisher Scientific, Inc., Ward

Hill, MA, USA) was added and incubated for 1 hour at room temperature. After a final series of three PBS washes, fluorescence staining patterns were examined under a fluorescence microscope (Nikon, Tokyo, Japan), and the 50% tissue culture infectious dose (TCID₅₀) was calculated.

3. 3. 6. Mouse infection

All animal experiments were conducted in a BSL-2 laboratory at NIID according to the above-described ethical approval. 11-week-old and over 30-week-old BALB/c mice were obtained from Japan SLC (Shizuoka, Japan) and used for animal experiments. IFNAR1^{-/-} mice (Tani et al. 2016) were bred and housed at NIID, following the policies and guidelines of the IACUC.

To assess the pathogenic potential of IWVV, 7-week-old IFNAR1^{-/-} mice were intraperitoneally inoculated with 100 µL of DMEM with 2% heat-inactivated FBS either with (n = 9) or without (n = 3) 10⁶ TCID₅₀/100 µL of IWVV strain 20OH29 under anesthesia. All mice were monitored for body weight changes and survival for up to 3 dpi. Blood and tissue samples were collected from four mice that had died on 3 dpi.

To evaluate the pathogenicity of wild-type mice, 11-week-old and over 30-week-old BALB/c mice were intraperitoneally inoculated with 100 µL of DMEM with 2% heat-inactivated FBS (2F-DMEM) with or without 10⁶ TCID₅₀/100 µL of IWVV strain 20OH29 under anesthesia. All mice were monitored for body weight changes and survival for 21 dpi.

To determine the LD₅₀ of IWVV, 7-week-old IFNAR1^{-/-} mice were intraperitoneally inoculated under anesthesia with 100 µL of 2F-DMEM containing 10-fold serial dilutions of IWVV strain 20OH29 (10⁶ to 10¹ TCID₅₀/100 µL), or 2F-DMEM alone for the mock group. Five mice were included in the mock group, and five mice were assigned to each virus-infected group per dose. Body weight and survival were monitored daily for up to 21 dpi. The LD₅₀ was estimated using the Spearman–Kärber method (Spearman 1908; Kärber 1931).

To explore the pathogenesis of lethal IWVV infection by time-course sampling, 7-week-old IFNAR1^{-/-} mice were intraperitoneally inoculated with 100 µL of 2F-DMEM with or without

10 LD₅₀ (1.3×10^5 TCID₅₀) of IWVV strain 20OH29 under anesthesia and daily observed body weight changes and survival for up to 6 dpi. Blood and tissue samples of mice were collected on 2, 4, and 6 dpi. Mice were planned to be monitored and sampled up to 6 dpi; however, in this experiment, all IWVV-infected IFNAR1^{-/-} mice had either died from infection or had been euthanized for scheduled necropsy by 4 dpi. Therefore, no animals remained for observation at 6 dpi.

Mice were anesthetized with a combination of medetomidine hydrochloride (Domitor, Nippon Zenyaku Kogyo Co., LTD., Fukushima, Japan; 0.3 mg/kg), midazolam (Dormicum Injection Solution 10mg, Maruishi Pharmaceutical Co., Ltd., Osaka, Japan; 4.0 mg/kg), and butorphanol tartrate (Betorfol 5mg, Meiji Animal Health Co., Ltd., Kumamoto, Japan; 5.0 mg/kg), and euthanized by cardiac puncture..

3. 3. 7. Detection of IWVV RNA by qRT-PCR

RNA was extracted from the brain, lungs, heart, liver, spleen, kidneys, small intestine, large intestine, and serum using ISOGEN (Nippon Gene, Tokyo, Japan). RNA extraction followed the manufacturer's protocol. IWVV L segment RNA was detected by qRT-PCR targeting a partial region of the L segment with the QuantiTect Probe RT-PCR Kit (QIAGEN, Hilden, Germany) using primer 1: 5'-ATGGTATATAGAGGGAGGGACAG-3', primer 2: 5'-GATTACGAGGCAGAGGTTGATAG-3', and probe: 5'-6-FAM-TGCCAGTCC-ZEN-GTGTATAATGTTCCGG-3IABkFQ-3'. The thermal cycling conditions were as follows: 50 °C for 30 minutes, 95 °C for 15 minutes, and 45 cycles of 94 °C for 15 seconds and 60 °C for 60 seconds. A real-time RT-PCR test was performed using LightCycler 480 (Roche Diagnostics, Indianapolis, IN, USA) (Matsumura et al. 2024).

For absolute quantification of viral RNA, a plasmid containing the IWVV L gene amplicon was used, 10-fold serial dilutions (10^0 - 10^8 copies per reaction) were prepared. Viral RNA copy numbers in tissue samples were calculated from threshold cycle values and expressed

as copies per 1 µg of extracted RNA. For serum samples, viral RNA levels were expressed as copies per 1 µL of extracted RNA.

3. 3. 8. Titration of live viruses

The viral titers of the samples were quantified as focus-forming units (FFU) per milliliter. Tissue specimens were homogenized in EMEM with 2% heat-inactivated FBS (2F-EMEM). The homogenates were centrifuged at 12,000×g for 3 minutes to obtain the supernatants. Organ homogenate supernatants were first diluted 1:10, and serum samples 1:100, followed by 10-fold serial dilutions for both sample types in 2F-EMEM. VeroE6 cells were seeded in 6-well plates the day before virus isolation to establish a subconfluent monolayer by incubation at 37 °C in 5% CO₂ overnight. Diluted specimens (100 µL) were applied to each well, in duplicate, and incubated at 37°C for 1 hour with gentle rocking every 15 minutes. After incubation, the supernatants were removed, and the cells were washed with EMEM without FBS. The treated cells were then overlaid with EMEM containing 5% heat-inactivated FBS (5F-EMEM) and PBS with 2.4% SeaPlaque™ agarose (Lonza K.K., Tokyo, Japan) at a 2:1 ratio, followed by incubation at 37 °C in 5% CO₂ for 7 days. After 7 days, the cells were fixed with 10% Formalin Neutral Buffer Solution (FUJIFILM Wako Pure Chemical Corporation) for over 4 hours and then further incubated with PBS containing 0.2% Triton X-100 for over 2 hours for membrane permeabilization. Following fixation, the cells were washed three times with PBS and incubated overnight at 4 °C with an anti-rabbit serum against IWVV (1: 400). After washing three times with PBS, the cells were incubated with protein A/G conjugated with HRP (Thermo Fisher Scientific, Inc.) (dilution 1:2,000) for 2–3 hours at room temperature. The cells were washed three times with PBS and treated with 3,3'-diaminobenzidine (DAB) tetrahydrochloride for 10–20 minutes for staining. Stained foci were counted, and the viral titers were calculated. All fixing and staining procedures were conducted at room temperature unless otherwise specified.

3. 3. 9. Histopathological analysis

Macroscopic observation was initially performed in mice, followed by the collection of the brain, heart, lungs, liver, spleen, kidneys, small intestine, and large intestine by time-course sampling at 2 and 4 dpi for histopathological analysis. The tissues were fixed in 10% Formalin Neutral Buffer Solution (FUJIFILM Wako Pure Chemical Corporation). After processing, the samples were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) at the Sapporo General Pathology Laboratory (Sapporo, Japan).

IHC was performed to detect viral antigens, macrophages, B cells, and T cells in the paraffin-embedded sections. The reagents used are listed in Supplementary Table 3-1. Antigen retrieval was achieved by hydrolytic autoclaving in antigen retrieval solutions for 15 minutes at 121 °C. Endogenous peroxidase activity was quenched with 0.3% H₂O₂ solution in methanol, followed by blocking with Tris-buffered saline (TBS) containing 5% skim milk (MEGMILK SNOW BRAND Co., Ltd.). The primary antibody was applied at 4 °C overnight. After washing three times with TBS, the secondary antibody was applied at room temperature for 1 hour. After washing three times with TBS, visualization of the antigens was achieved using DAB as a chromogen (ImmPACT® DAB Substrate Kit, Peroxidase; Vector Laboratories Inc., Newark, CA, USA), and nuclei were counterstained with hematoxylin.

To identify cells infected with IWVV, double-label immunofluorescent staining was conducted. The reagents used are listed in Supplementary Table 3-1. Antigen retrieval was performed by hydrolytic autoclaving in Antigen Unmasking Solution, Citric Acid-Based (pH 6.0; Vector Laboratories, Inc.) for 15 minutes at 121 °C, followed by blocking with TBS containing 5% skim milk (MEGMILK SNOW BRAND Co., Ltd.). The first primary antibody was applied at 4 °C overnight. After washing with TBS three times, the second primary antibody was applied at 37 °C for 1 hour. After washing three times with TBS, the secondary antibodies were applied at room temperature for 1 hour. After washing three times with TBS, autofluorescence was quenched using the Vector TrueVIEW Autofluorescence Quenching Kit (Vector Laboratories, Inc.), and sections were mounted with VECTASHIELD Vibrance Antifade Mounting Medium

containing DAPI (Vector Laboratories, Inc.). All microscopic observations were confirmed in multiple independent fields per section (at least three fields per sample).

3.3.10. IFA

For the detection of IgG antibodies, IF plates containing IWVV-infected cells were prepared. VeroE6 cells were seeded in 96-well microplates the day before establishing a subconfluent monolayer by incubation at 37 °C in 5% CO₂ overnight. Briefly, IWVV was inoculated into VeroE6 and incubated at 37 °C in 5% CO₂ (the multiplicity of infection (MOI) of 0.7). At 7 dpi, the cells were fixed and permeabilized with 10% Formalin Neutral Buffer Solution (FUJIFILM Wako Pure Chemical Corporation) with 0.4% Triton X-100 for over 4 hours. The cells were washed three times with PBS and stored at 4 °C. Mouse sera were serially diluted in PBS containing 5% skim milk (MEGMILK SNOW BRAND Co., Ltd.) at a dilution of 1:10 to 1:10,240. They were applied to the wells and reacted for 30 minutes at 37 °C. After washing three times with PBS, goat anti-mouse-IgG (H + L) conjugated with fluorescein (FITC) (Vector Laboratories, Inc.) was applied and incubated for 1 hour at room temperature. Anti-rabbit serum against IWVV and goat anti-rabbit-IgG (H + L) conjugated with FITC (Thermo Fisher Scientific, Inc.) were used as positive controls. After washing three times with PBS, the plates were examined for staining patterns under a fluorescence microscope (Nikon, Tokyo, Japan). The IgG antibody titers of the tested sera were recorded as the reciprocals of the highest dilutions producing positive staining.

3.3.11. Statistical analysis

Survival rates were analyzed using Kaplan–Meier survival analysis. Data were analyzed using a two-way ANOVA with Sidak’s multiple comparisons test. All statistical analyses were performed using GraphPad Prism 9.2 for Windows (GraphPad Software, San Diego, California, U.S.A.).

3. 4. Results

3. 4. 1. Pathogenicity of IWVV in IFNAR1^{-/-} mice, wild-type mice, and rabbits

To assess the pathogenic potential of IWVV in a mouse model, IFNAR1^{-/-} mice were intraperitoneally inoculated with 10⁶ TCID₅₀/100 µL of IWVV per mouse. Body weight and survival were monitored every day during the observation period. The infected IFNAR1^{-/-} mice exhibited significant weight loss and 100% mortality by 3 dpi (Figure 3-1A). To evaluate the pathogenicity in wild-type mice, 11-week-old and over 30-week-old BALB/c mice were inoculated with 10⁶ TCID₅₀/100 µL of IWVV per mouse. To assess whether age-related differences in immune function influence susceptibility to IWVV infection, two age groups of wild-type mice were used: 11-week-old BALB/c mice as a standard adult cohort and over 30-week-old BALB/c mice as an aged cohort. In both 11-week-old and over 30-week-old BALB/c mice, body weight changes were similar to those observed in a mock-infected group, with no mortality up to 21 dpi (Figure 3-1B).

In addition, to assess the pathogenicity of IWVV in rabbits and to prepare anti-rabbit serum against IWVV, two Japanese white rabbits were inoculated with IWVV at 10⁶ TCID₅₀ subcutaneously three times. Similar to wild-type mice, the rabbits showed no clinical symptoms, including signs such as reduced appetite or decreased activity. The induction of antibodies against IWVV was confirmed by indirect IFA. The titer of IgG against IWVV increased after the first inoculation, and reached 1:25,600 to 1:51,200 after the third inoculation. After the third inoculation, rabbit sera were collected and used in immunohistochemistry (IHC) as the primary antibody against IWVV.

3. 4. 2. Determination of LD₅₀ of IWVV in IFNAR1^{-/-} mice

To determine the median lethal dose (LD₅₀), IFNAR1^{-/-} mice were intraperitoneally inoculated with serially diluted IWVV. The 21-day survival rates for the infected IFNAR1^{-/-} mice were 20% in the high-titer group inoculated with 10⁶ and 10⁵ TCID₅₀/100 µL of IWVV (Figure 3-1C). In the group inoculated with 10⁶ TCID₅₀/100 µL of IWVV, body weight loss began at 4

dpi, and mortality was first observed at 5 dpi. Based on these findings, the LD₅₀ was calculated to be 1.3×10⁴ TCID₅₀.

3. 4. 3. Time-course sampling of IWVV-infected IFNAR1^{-/-} mice

To explore the pathogenesis of lethal IWVV infection by time-course sampling, IFNAR1^{-/-} mice were intraperitoneally inoculated with 10 LD₅₀ of IWVV, and mock-infected mice inoculated with medium only served as negative controls. Blood and organ samples were collected at 2 and 4 dpi. All IWVV-inoculated mice either succumbed by 4 dpi or were euthanized for sample collection. Following the sampling of three mice at 2 dpi, several of the remaining mice exhibited over 10% weight loss, with six mice dying at 3 dpi. On 4 dpi, three additional mice were found dead and were subsequently sampled (Figure 3-1D).

3. 4. 4. Detection of IWVV RNA by quantitative one-step reverse transcription PCR (qRT-PCR) and isolation of IWVV

To compare viral replication in tissues and serum samples, viral RNA and infectious virus were quantified by qRT-PCR and virus titration. In samples collected from IWVV-infected mice, both viral RNA and viral titers increased at 4 dpi compared to 2 dpi (Figure 3-2A, B). Among the tissues, the liver and spleen exhibited high mean viral RNA levels at both 2 and 4 dpi (Figure 3-2A). Additionally, viral titers in the liver and spleen were elevated from 2 dpi compared to other organs (Figure 3-2B). The presence of RNA in the serum indicates the occurrence of viremia (Figure 3-2A). Viral RNA levels and viral titers were under the limit of detection in samples from the negative-control mice collected at 2 and 4 dpi.

3. 4. 5. Pathological examinations

To elucidate the mechanism of lethal infection with IWVV, histopathological analysis using time-course samples collected at 2 and 4 dpi was performed. Macroscopically, no significant findings were observed. Microscopically, hepatocyte degeneration and infiltration of inflammatory

cells were observed in the liver, and the severity increased over time, indicating acute necrotizing hepatitis (Figure 3-3A). Furthermore, on day 4, the splenic white pulp was depleted, including necrosis with nuclear condensation and loss of lymphocytes (Figure 3-3B). No clear histological changes were observed in the other tissues. To characterize the distribution of the virus, IWVV antigens in the tissues were examined by IHC using anti-rabbit serum against IWVV. Positive signals of IWVV antigen were detected in the liver at both 2 and 4 dpi, with a more extensive distribution at 4 dpi (Figure 3-4A). Iba1-positive macrophages, including Kupffer cells, accumulated around the inflammatory lesions, and their numbers increased as the inflammatory response progressed (Figure 3-4B). To identify IWVV-infected cells, double-label immunofluorescent staining was performed. Viral antigens were detected in HepPar1-positive hepatocytes in the liver (Figure 3-5A). However, the Iba1-positive Kupffer cells appeared to adhere to viral antigen-positive cells, but not to colocalize with them (Figure 3-5A). In the spleen, viral antigen-positive cells colocalized with Iba1-positive macrophages (Figure 3-5B). In spleen sections analyzed by IHC, positive IWVV antigen signals were observed in the necrotic area at 4 dpi (Figure 3-6). A reduction in CD45-positive B cells and CD3-positive T cells, along with an increase in Iba1-positive macrophages, was noted in the spleen at 4 dpi (Figure 3-6). These findings were consistently observed in multiple independent microscopic fields.

3. 4. 6. IgG antibody response in IWVV-inoculated mice

IgG antibodies against IWVV were measured by IFA. In IWVV-inoculated BALB/c mice, IgG antibodies were detected at 28 dpi in both 11-week-old and over-30-week-old groups (Table 3-1). However, IgG antibodies were not detected at 28 dpi in surviving IFNAR1^{-/-} mice inoculated with any dose used for LD₅₀ determination (Table 3-2).

3. 5. Discussion

IWVV is a novel tick-borne orthonairovirus classified into the Tamdy genogroup (Matsumura et al. 2024). Since some orthonairoviruses belonging to the Tamdy genogroup and

the closely related Sulina genogroup have demonstrated pathogenicity in humans, evaluation of the pathogenicity of IWVV is an important public health priority.

In previous studies, experimental mouse models have been established for several human-pathogenic orthonairovirus species, including CCHFV, DUGV, and ISKV, utilizing type I interferon receptor-knockout mice (C57BL/6, 129 background), STAT-1 knockout mice, and cyclophosphamide-treated mice (Bente et al. 2010; Bereczky et al. 2010; Coates and Sweet 1990; Lindquist et al. 2018; Sugimoto et al. 2022). Additionally, AG129 mice have been utilized to establish infected models for YEZV, an emerging orthonairovirus in the Sulina genogroup (Ariizumi et al. 2024). However, comprehensive pathogenetic analysis of orthonairoviruses belonging to the Tamdy and Sulina genogroups is still limited. This chapter demonstrates that challenge experiments using IFNAR1^{-/-} mice are effective in elucidating the pathogenicity of orthonairoviruses classified into the Tamdy and Sulina genogroups.

Type I interferon responses have strong inhibitory effects on nairovirus replication (Lasecka and Baron 2014). IFN- α treatment markedly reduces CCHFV yields in human target cells, mainly through induction of the interferon-induced dynamin-like GTPase MxA, which interacts with the viral nucleocapsid protein and sequesters it in perinuclear compartments, thereby blocking genome replication (Andersson et al. 2004; Andersson et al. 2006). Consistently, DUGV replication is also suppressed in cells stably expressing human MxA (Bridgen et al. 2004). As a consequence, immunocompetent mice generally show limited clinical disease following infection with orthonairoviruses, whereas lethal disease has predominantly been observed in mice lacking type I interferon signaling. Consistent with this, lethality in our experiments was restricted to IFNAR1^{-/-} mice, whereas wild-type mice remained asymptomatic despite evidence of infection.

Inclusion of these two age groups therefore enabled us to assess whether aging alters the outcome of IWVV infection in immunocompetent hosts. However, neither age group showed clinical signs or mortality following inoculation, indicating that wild-type mice are resistant to IWVV infection under our experimental conditions.

We selected intraperitoneal inoculation in this chapter because lethal orthonairovirus infection has been successfully established using this route in IFNAR^{-/-} mice, as demonstrated for both CCHFV and HAZV (Dowall et al. 2012; Zivcec et al. 2013). Thus, the intraperitoneal route provides a practical and well-validated model for evaluating the pathogenicity of emerging orthonairoviruses such as IWVV. However, studies using mouse models of tick-borne viruses have suggested that infection dynamics and host immune responses can vary depending on the inoculation route, particularly when the natural transmission pathway involves exposure through the skin and tick salivary immunomodulators (Hermance and Thangamani 2017; Šimo et al. 2017; Zivcec et al. 2013). Similar observations have also been reported for tick-borne flaviviruses such as Powassan virus (Hermance and Thangamani 2015). Therefore, the infection kinetics and host responses to IWVV may vary when a more physiologically relevant transmission route—such as subcutaneous inoculation or a tick-bite model—is used, and further investigation will be required to clarify such differences.

This chapter demonstrated that IWVV exhibits pathogenicity in IFNAR1^{-/-} mice. Like other human pathogenic orthonairoviruses, IWVV infection was lethal in immunocompromised mice but not in wild-type mice. Although wild-type mice remained asymptomatic, the increase in serum IgG antibody titers indicated that they had been infected with the virus. Furthermore, among IFNAR1^{-/-} mice that survived the LD₅₀ determination experiment, none exhibited an increase in serum IgG antibody titers, including those inoculated with a high viral dose. This suggests that the surviving mice may not have developed productive infection, although we cannot fully exclude the possibility of low-level or abortive infection. IWVV was lethal to all mice that developed detectable infection. Interestingly, the 10³ TCID₅₀/100 µL group showed a slightly higher mortality than the 10⁴ TCID₅₀/100 µL group. Although a dose–mortality inversion has not been reported for nairovirus animal models, several mechanisms may underlie this observation. High viral titers can promote viral particle aggregation, which reduces effective infectivity and limits the early dispersal of viral particles (Pradhan et al. 2022). Although this phenomenon has not been reported for nairoviruses, studies of influenza virus infection have

shown that lower inocula delay the induction of innate antiviral responses (Marois et al. 2012), and modeling studies of Zika virus infection have suggested that inoculum dose influences the timing of immune activation and subsequent viral dynamics (Best et al. 2021). Together, these findings raise the possibility that delayed innate immune activation at lower doses may allow more extensive viral replication before host defenses become effective. Thus, the 10^3 TCID₅₀/100 μ L inoculum might have created a more favorable environment for rapid viral dissemination than the higher dose. Further experiments with finer dose spacing and larger sample sizes will be required to confirm the reproducibility and biological significance of this observation.

A slight difference in mortality was observed between two experiments using the same inoculation dose of 10^6 TCID₅₀/100 μ L (Figure 3-1A, C). Although the precise cause of this discrepancy is unclear, variability in host responses among individual IFNAR1^{-/-} mice, as well as subtle experimental factors such as body weight or handling stress, may have contributed to the different survival outcomes. Nevertheless, both experiments consistently demonstrated that IWVV is highly lethal in IFNAR1^{-/-} mice at this dose, and this variation does not alter the overall interpretation of the pathogenicity results.

IWVV primarily replicates in the liver, inducing severe acute necrotizing hepatitis in IFNAR1^{-/-} mice. Additionally, spleens from IWVV-infected IFNAR1^{-/-} mice exhibited a loss of follicular structure and necrotic changes. The depletion of splenic white pulp was composed of CD45-positive B cells and CD3-positive T cells, especially. These pathological findings align with observations in immunocompromised mice infected with CCHFV, ISKV, STBV, LPHV, and YEZV (Ariizumi et al. 2024; Hawman et al. 2019; Ishii et al. 2014; Sugimoto et al. 2022; Welch et al. 2019; Zivcec et al. 2013). Furthermore, our results, which demonstrate evident pathological alterations in the liver together with high viral RNA levels and infectious titers in hepatic tissues, suggest that IWVV has a hepatic tropism.

Previous studies have identified Kupffer cells, hepatocytes, and endothelial cells as the primary target cells of CCHFV in infection models involving mice, macaques, as well as in human patients (Bente et al. 2010; Bereczky et al. 2010; Burt et al. 1997; Garrison, Smith, and Golden

2019; Haddock et al. 2018; Hawman and Feldmann 2023; Lindquist et al. 2018; Welch et al. 2019). Kupffer cells have been proposed to play a critical role in viral dissemination and replication in the liver during the early stages of infection in CCHFV-infected IFNAR1^{-/-} mice and mice inoculated with anti-IFNAR1 (Lindquist et al. 2018; Welch et al. 2019; Zivcec et al. 2013;). However, recent studies on YEZV-infected AG129 mice have demonstrated that the majority of virus antigen-positive cells in the liver are hepatocytes, with only a few virus antigen-positive Kupffer cells detected (Ariizumi et al. 2024). In IWVV-infected IFNAR1^{-/-} mice, the virus antigen-positive cells in the liver were hepatocytes, with no detectable virus antigen-positive Kupffer cells. As the pathogenicity of IWVV in humans is still unknown, it remains unclear whether the absence of IWVV antigen-positive Kupffer cells is a virus-specific feature or the result of species-specific differences in mice. In the spleen, IWVV antigen-positive cells were identified as macrophages. However, it remains uncertain whether these macrophages were directly infected with IWVV or had phagocytosed IWVV-infected cells. In addition, virus isolation observed in the spleen could potentially be explained by residual serum. While infectious viral particles and/or viral RNA were detected in multiple organs, including the brain, lungs, heart, kidneys, stomach, small intestine, and large intestine, no histopathological evidence was observed. Since mice infected with IWVV developed viremia, infectious viral particles and viral RNA detected in these organs were likely derived from the blood. In the small intestine, viral RNA was detectable despite the absence of infectious virus. This likely reflects rapid inactivation of viral particles by intestinal antiviral factors and digestive enzymes, leaving behind non-infectious RNA detectable by qRT-PCR, or passive presence of viral RNA rather than productive replication. Unlike the gastrointestinal disturbances reported in mouse models of LPHV and TFLV (Ishii et al. 2014; Shimada et al. 2016), such a change was not observed in IWVV-infected mice.

In this chapter, we established a mouse model of IWVV infection and characterized its pathogenicity. As reports on the pathogenicity of tick-borne orthonairovirus infections associated with the Tamdy and Sulina genogroups in humans and mice remain limited (Ariizumi et al. 2024), analyses using the IFNAR1^{-/-} mouse model may contribute to a better understanding of the

pathogenesis of these viruses. Furthermore, we found that IWVV caused severe liver and spleen lesions, leading to mortality in IFNAR1^{-/-} mice, similar to other human-pathogenic orthonairoviruses such as CCHFV and YEZV. Our data suggest that IWVV may pose a risk of infection and pathogenicity in humans. To determine whether IWVV can infect humans and animals, further research, including seroepidemiological studies utilizing human and animal samples, is necessary. Moreover, future work should address vector competence of tick species, their geographic distribution, and ecological interactions. These vector- and ecology-focused approaches will be essential for fully assessing the transmission potential and public health risk of IWVV.

3. 6. Figure legends

Figure 3-1. IWVV infection in mice. A) Seven-week-old IFNAR1^{-/-} mice were intraperitoneally inoculated with 10⁶ TCID₅₀/100 µL/mouse of IWVV. Three-day survival rates and body weight changes were compared between the mock-infected mice (n = 3) and IWVV-infected mice (n = 9). Relative body weight is presented as the mean and standard deviation. ns: not significant, ** $p < 0.01$, **** $p < 0.0001$. B) Eleven-week-old and over 30-week-old BALB/c mice were intraperitoneally inoculated with 10⁶ TCID₅₀/100 µL/mouse of IWVV. Survival rates over 21 days and body weight changes were compared between the mock- and IWVV-infected groups. Relative body weight is presented as the mean and standard deviation. C) IFNAR1^{-/-} mice were intraperitoneally inoculated with 10¹ to 10⁶ TCID₅₀/100 µL/mouse of IWVV (n = 5 per dose). Survival rates over 21 days and body weight changes were monitored. Relative body weight is presented as the mean and standard deviation. * $p < 0.05$. D) IFNAR1^{-/-} mice were inoculated intraperitoneally with 10 LD₅₀ (1.3×10⁵ TCID₅₀) /100 µL/mouse of IWVV. To collect samples at 2 and 4 dpi, three groups of mice (three mice per group) were prepared.

Figure 3-2. Viral RNA levels and virus titers in IWVV-infected IFNAR1^{-/-} mice. A) Viral RNA levels. Brain, lung, heart, liver, spleen, kidney, stomach, small intestine, large intestine, and serum

from IWVV-inoculated IFNAR1^{-/-} mice were collected at 2 dpi and 4 dpi (2 dpi: n = 3, 4 dpi: n = 3) for RNA extraction. Viral RNA levels were quantified by RT-qPCR. Black circles, thick bars, and thin lines indicate individual values, mean, and standard deviation, respectively. B) Virus titers. Brain, lung, heart, liver, spleen, kidney, stomach, small intestine, large intestine, and serum from IWVV-inoculated IFNAR1^{-/-} mice were collected at 2 dpi and 4 dpi (2 dpi: n = 3, 4 dpi: n = 3) for virus titration. Viral titers were quantified by focus formation assay. Black circles, thick bars, and thin lines indicate individual values, mean, and standard deviation, respectively. Values below the detection limit (0.5 FFU/mg for tissues and 50 FFU/mL for serum) were plotted at the respective detection limit.

Figure 3-3. Histochemistry with hematoxylin and eosin (H&E) staining. Microscopic lesions of liver (**A**) and spleen (**B**) from IWVV- and mock-infected mice collected at 2 and 4 dpi. The higher magnification images of the dotted box areas are shown to the right. Magnification: (A) IWVV: x100 and x200; Mock: x200. (B) IWVV: x40, x100, and x200 (4 dpi); Mock: x40 and x100.

Figure 3-4. Immunohistochemistry for the distribution of IWVV- or Iba1-positive cells in liver sections. (**A**) IHC of IWVV-specific staining in liver from IWVV-infected mice and mock-infected mice collected at 2 and 4 dpi. (**B**) IHC of Iba1-specific staining in liver from IWVV-infected mice collected at 2 and 4 dpi. Magnification: (A) IWVV: x200, Mock: x100, (B) x100.

Figure 3-5. Double-label immunofluorescent staining for the distribution of HepPar1/Iba1 and IWVV-positive cells in liver sections, and Iba1 and IWVV-positive cells in spleen sections. (A) Double-labeled immunofluorescence staining of HepPar1/Iba1 and IWVV-positive cells in liver from IWVV-infected mice collected at 2 dpi. HepPar1- and Iba1-positive signals were represented with red signals. IWVV-positive signals were represented with apple green signals. (B) Double-labeled immunofluorescence staining of Iba1 and IWVV-positive cells in the spleen from IWVV-infected mice collected at 4 dpi. Iba1-positive signals were represented with red

signals. IWVV-positive signals were represented with apple green signals. Representative images are shown. Similar results were observed in multiple microscopic fields. Magnification: (A) x400, (B) x400.

Figure 3-6. H&E staining and immunohistochemistry in spleen sections. Spleen sections from IWVV- and mock-infected mice were collected at 4 dpi. IWVV-positive signals were observed around the depleted white pulp. CD45-positive B cells and CD3-positive T cells were depleted in the splenic white pulp. Representative images are shown. Similar results were observed in multiple microscopic fields. Magnification of x200.

3. 7. Tables and figures

Table 3-1. IFA titers of IgG antibodies against IWVV in the sera of surviving BALB/c mice.

Mouse ID	11-week-old	Over-30-week-old
1	1:640	1:640
2	1:640	1:640
3	1:640	<1:10
4	1:640	1:640
5	1:640	1:640

The IgG antibodies were measured by indirect immunofluorescence assay using serum samples collected from 11-week-old or over-30-week-old BALB/c mice at 28 dpi with IWVV.

Table 3-2. IFA titers of IgG antibodies against IWVV in the sera of surviving IFNAR1^{-/-} mice.

Mouse ID	IWVV titer for challenge (TCID ₅₀ /100 µL)						
	10 ⁰	10 ¹	10 ²	10 ³	10 ⁴	10 ⁵	10 ⁶
1	<1:10	<1:10	<1:10	NT	<1:10	NT	NT
2	<1:10	<1:10	<1:10	<1:10	<1:10	NT	NT
3	<1:10	<1:10	<1:10	NT	<1:10	<1:10	<1:10
4	<1:10	<1:10	<1:10	<1:10	<1:10	NT	NT
5	<1:10	<1:10	<1:10	NT	NT	NT	NT

The IgG antibodies were measured by indirect immunofluorescence assay using sera collected from surviving IFNAR1^{-/-} mice at 28 dpi with IWVV. NT: Not tested due to the death of the mice.

Figure 3-1.

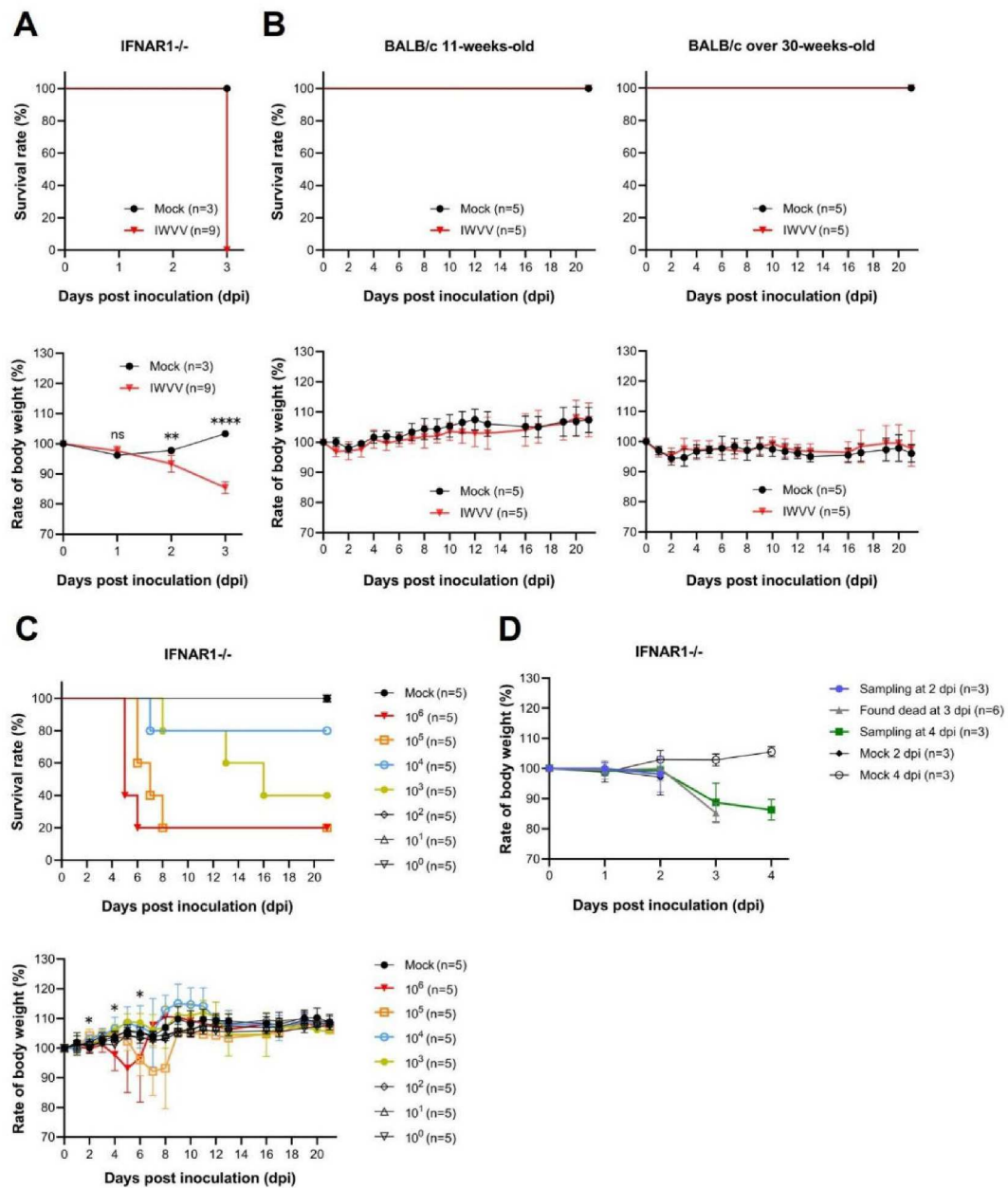


Figure 3-2.

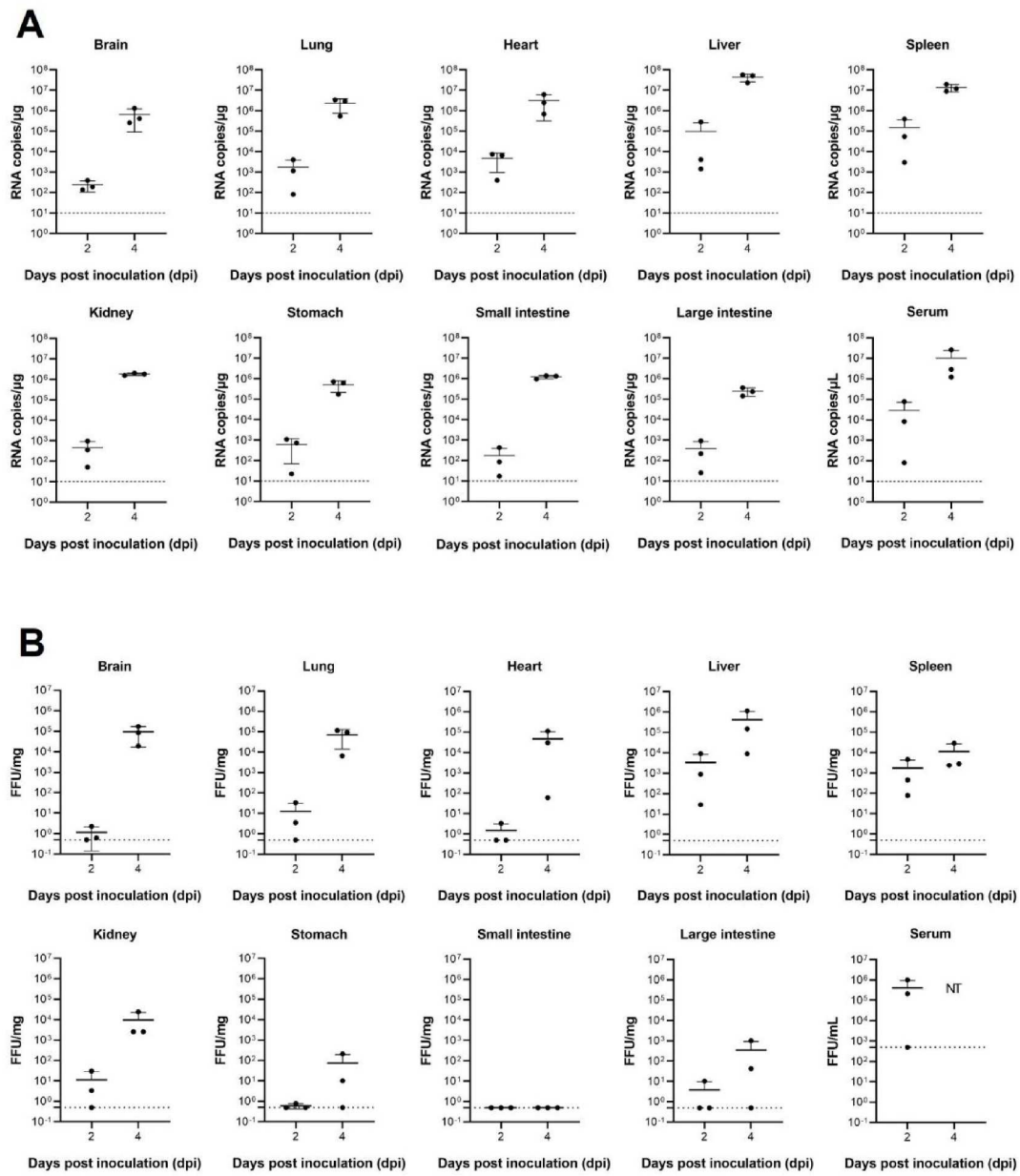
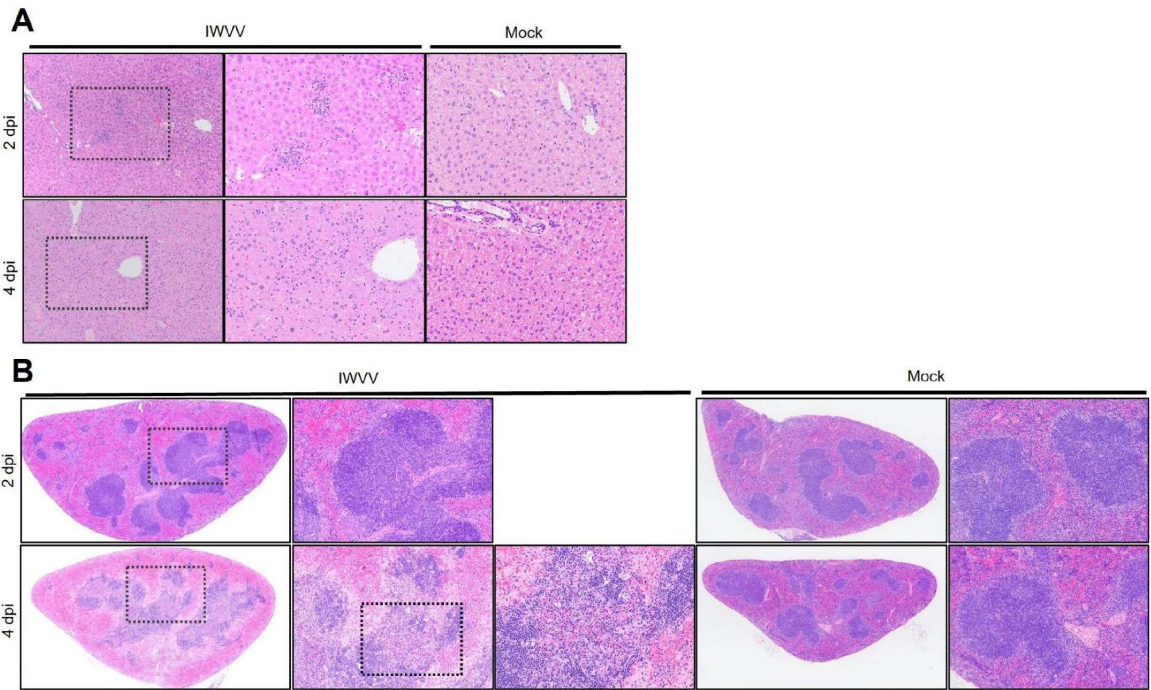
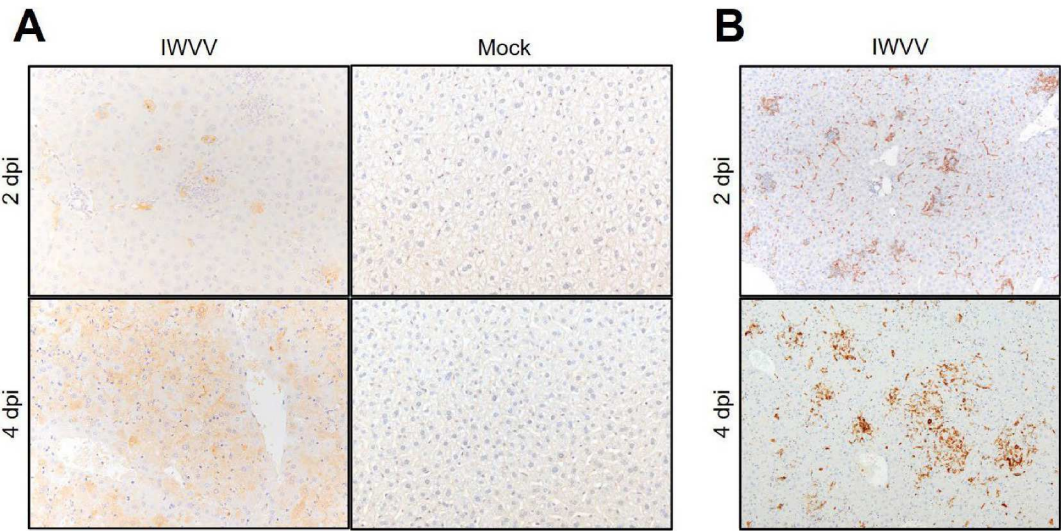


Figure 3-3.

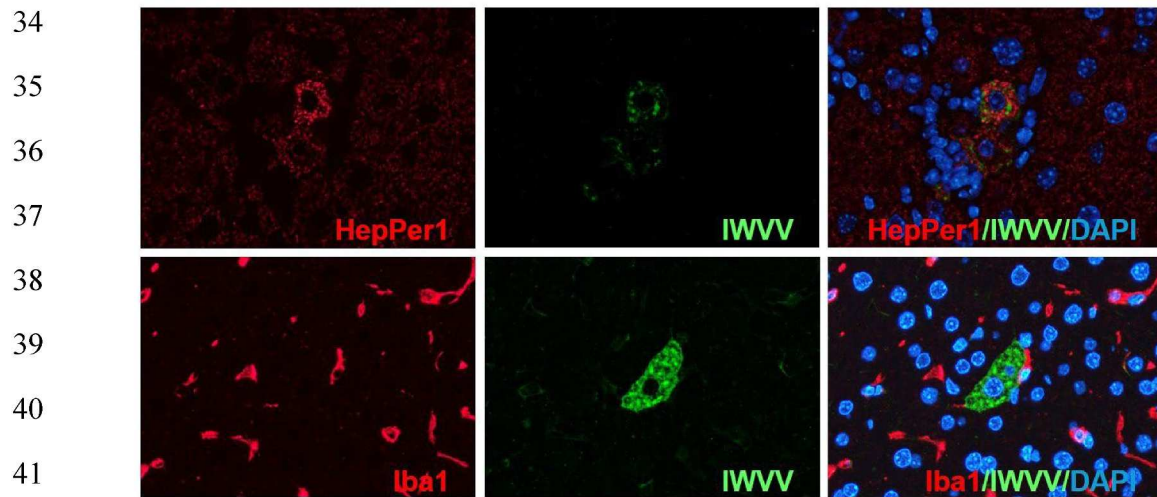


16 Figure 3-4.



32 Figure 3-5.

33 **A**



42 **B**

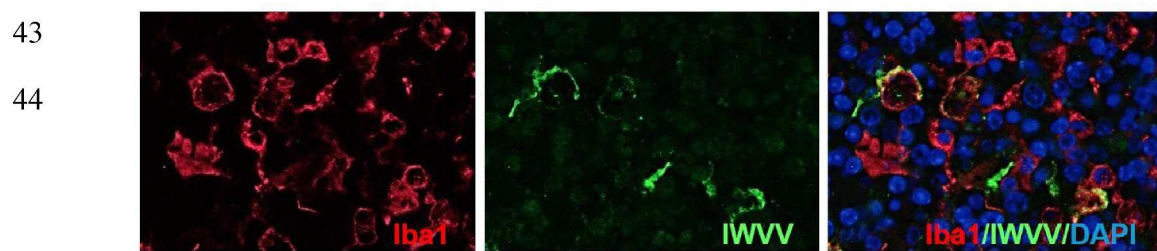
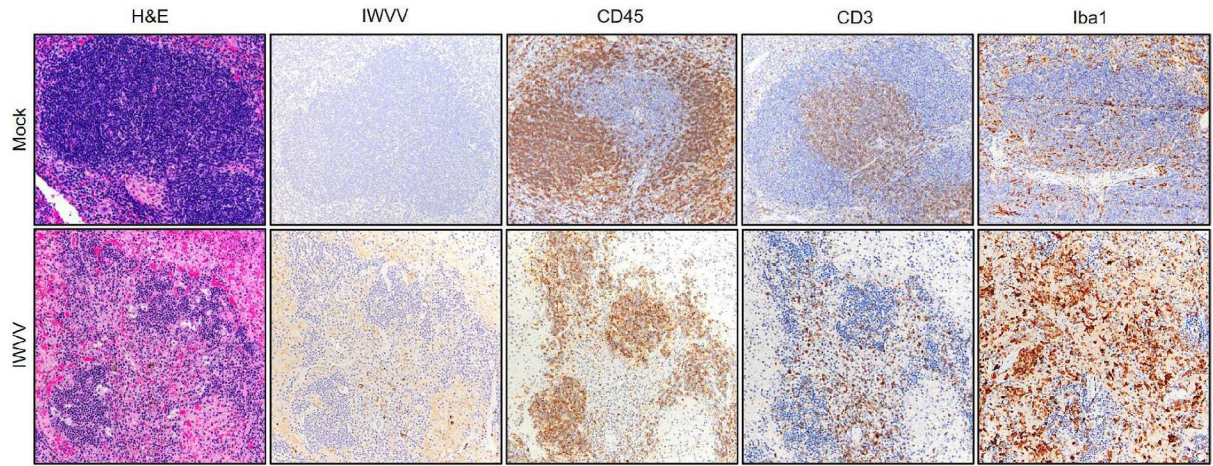


Figure 3-6.



4. General conclusion

Emerging orthonairovirus YEZV has been reported in human patients in Hokkaido, Japan, and northeastern China, and is recognized as a tick-borne infectious disease of growing concern in East Asia. Because YEZV has been detected in ticks in Hokkaido, China, and Russia, its maintenance and dispersal are considered to involve extensive ecological networks. In particular, understanding the role of migratory birds in transporting YEZV-infected ticks is essential for assessing infection risks in nature and predicting its potential spread.

IWVV is a novel orthonairovirus recently isolated from vegetation-associated ticks in Hokkaido. Although IWVV has not yet been detected in humans or animals, it belongs to the Tamdy genogroup, which is phylogenetically close to the Sulina genogroup that includes YEZV, indicating the need to evaluate its potential pathogenicity.

In this thesis, I investigated two aspects of orthonairovirus ecology and pathogenicity in Japan: (1) the potential transboundary movement of YEZV via ticks attached to migratory birds, and (2) the pathogenicity of IWVV in a mouse model.

In Chapter 1, ticks parasitizing migratory birds at major stopover sites in northeastern Hokkaido were screened for YEZV infection. YEZV RNA was detected exclusively in larval and nymphal *I. persulcatus* collected from black-faced buntings (*E. spodocephala*). Because this species migrates from Sakhalin and the Kuril Islands to Hokkaido, the findings strongly suggest that YEZV-positive ticks may have crossed international boundaries along these flyways. Furthermore, whole-genome sequencing and reassortment analyses indicated genetic exchange among strains from Japan and China (Inner Mongolia and Heilongjiang), suggesting that YEZV may maintain genetic continuity along the East Asian–Australasian Flyway.

In Chapter 2, IWVV pathogenicity was evaluated using IFNAR1-deficient mice. IWVV infection caused pathological lesions in the liver and spleen and resulted in high mortality. These pathological features were consistent with those observed in infections with other orthonairoviruses, including CCHFV, HAZV, and YEZV, suggesting that IWVV may also have pathogenic potential in natural vertebrate hosts.

Taken together, these findings indicate that migratory birds may play an important role in the ecological network of YEZV, and that analysis of IWVV pathogenicity contributes to a deeper understanding of both conserved and virus-specific pathogenic features among orthonairoviruses. To further elucidate the transmission dynamics, pathogenic mechanisms, and public health risks of orthonairoviruses, long-term and wide-ranging surveillance of ticks, birds, and wild mammals, along with experimental studies using isolated virus strains, will be essential.

Although tick-borne viral diseases are unlikely to cause large-scale pandemics, orthonairovirus infections occur annually in East Asia, and some patients require hospitalization. Therefore, continuous public health surveillance and preventive measures are indispensable for reducing the burden and potential impact of these infections. This chapter provides fundamental knowledge on the transboundary transmission of orthonairoviruses mediated by migratory birds and ticks, as well as their pathogenicity in mice, contributing to improved prevention and risk assessment of tick-borne viral diseases in Japan and the broader East Asian region.

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7. Abstract (in Japanese)

Integrative studies on emerging orthonairoviruses:

viral dissemination via ticks on migratory birds, and pathogenicity in mouse models

新興オルソナイロウイルスの統合研究：

渡り鳥由来マダニを介したウイルスの伝播とマウスにおける病原性の評価

新興オルソナイロウイルスである Yezo virus (YEZV) は、日本の北海道および中国北東部で患者が報告されており、東アジアにおける新興ダニ媒介感染症として注目されている。マダニでは北海道、中国、ロシアで検出されていることから、その維持・拡散には広域の生態系が関与していると考えられる。特に、渡り鳥が YEZV 保有マダニの移動に果たす役割を理解することは、自然界における感染リスクの評価や感染症の地理的拡散の予測に不可欠である。

Iwanai Valley virus (IWVV) は、近年北海道の植生マダニから分離された新規オルソナイロウイルスであり、現在のところヒトや動物からの検出報告はない。しかし、YEZV が属する *Sulina* genogroup と近縁な *Tamdy* genogroup に分類されることから、その潜在的な病原性を評価する必要がある。

本研究では、(1) 渡り鳥に咬着したマダニを介した YEZV の越境移動の可能性、(2) IWVV のマウスでの病原性の評価、という 2 つの側面から、日本におけるオルソナイロウイルスの生態および病原性を検討した。

第 1 章では、北海道北部・東部の主要な渡り鳥飛来地において渡り鳥に寄生したマダニを捕集し、YEZV 保有状況を解析した。その結果、YEZV RNA はアオジ (*Emberiza spodocephala*) に寄生していた *Ixodes persulcatus* の幼ダニおよび若ダニからのみ検出さ

れた。アオジはサハリンおよび千島列島から北海道へ渡来することが知られており、本研究の結果は、これらの飛行ルートに沿って YEZV 陽性マダニが越境移動した可能性を強く示唆した。さらに、全ゲノム解析により、日本、中国、ロシアの間で遺伝的交流が生じていることが示唆され、東アジア・オーストララシアフライウェイに沿って YEZV の遺伝的連続性が維持され得ることが明らかとなった。

第2章では、I型インターフェロン受容体欠損マウスを用いて IWVV の病原性を評価した。IWVV 感染マウスでは、肝臓および脾臓に病変が認められ、高い致死性を示した。これらの病理学的特徴は、Crimean–Congo hemorrhagic fever virus、Hazara virus、YEZV など他のオルソナイロウイルス感染モデルと共通する点が多く、IWVV が自然宿主においても病原性を示す可能性があることが示された。

以上の結果から、渡り鳥は YEZV の生態学的ネットワークにおいて重要な役割を果たしている可能性が示され、IWVV の病原性解析はオルソナイロウイルス属ウイルスの共通病態およびウイルス固有の特徴の理解に寄与するものである。ダニ媒介ウイルス感染症は大規模なパンデミックを引き起こす可能性は低いものの、オルソナイロウイルスによる感染症は東アジアにおいて毎年発生しており、患者が入院を要する場合もある。そのため、継続的な公衆衛生監視と予防措置は、これらの感染症の負担および潜在的影響を軽減する上で不可欠である。本研究は、日本におけるオルソナイロウイルスの渡り鳥およびマダニを介した越境的伝播とマウスでの病原性を明らかにし、日本および東アジア地域におけるダニ媒介ウイルス感染症の予防とリスク評価に資する基盤的知見を提供するものである。今後は、より広域かつ長期的なマダニ・鳥類・野生動物のサーベイランスと、分離株を用いた実験的研究を通じて、オルソナイロウイルスの伝播動態、病態機序、公衆衛生リスクを包括的に解明する必要がある。