Studies on zoonotic infection of novel tick-borne viruses in Japan

日本における新規マダニ媒介性ウイルスのヒトへの感染に関する研究

The United Graduate School of Veterinary Science

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GENERAL INTRODUCTION

1.1. General information of tick-borne viral diseases

1.1.1. Tick-borne viral diseases

Ticks are one of the most important vectors of pathogens in human and domestic and wild animals and considered to be the second major vector of human diseases after mosquitoes (De la Fuente et al., 2008). Tick-borne viruses are transmitted by ticks and a significant public health problem in the world (Parola and Raoult**,** 2001). Tick-borne viruses belong to two orders, nine families, and at least 12 genera. Some of them are known to cause severe diseases with high mortality rates in humans and livestock, while the other unclarified viruses may pose risks to public health (Shi et al., 2018).

1.1.2. Epidemilogy of tick-borne viruses

Many of the tick-borne viruses are known to cause diseases in animals and humans and have been frequently reported to be associated with large epidemics (Shi et al., 2018). Louping ill virus (LIV), one of the first identified tick-borne viruses, was identified as being responsible for severe encephalitis in sheep and other livestock for hundreds of years (Mansfield et al., 2017). Over the past few decades, the global spread of tick-borne viral diseases have been posed an emerging threat to public health worldwide, and the economic impact of tick-borne diseases is significantly severe (Rochlin and Toledo, 2020). Tick-borne encephalitis virus (TBEV) (Charrel et al., 2004), Crimean–Congo haemorrhagic fever virus (CCHFV) (Bente et al., 2013), Omsk haemorrhagic fever virus (OHFV) (Karan et al., 2014), Kyasanur Forest disease virus (KFD) (Chakraborty et al., 2019), Louping ill virus (LI) (Mansfield et al., 2017), severe

fever with thrombocytopenia syndrome virus (SFTSV) (Silvas and Aguilar, 2017), Heartland virus (HRTV) (Brault et al., 2018), Powassan virus (POWV) (Hermance and Thangamani, 2017), and Alkhurma hemorrhagic fever virus (AHFV) (Memish et al., 2014) caused severe diseases in human. African swine fever virus (Dixon and Chapman, 2008), Nairobi sheep disease virus (Marczinke and Nichol, 2002) and Louping ill virus (Jeffries et al., 2014) cause severe diseases in livestock.

1.1.3.Transmission cycle of tick-borne viruses

Tick-borne viruses are transmitted either by hard ticks or soft ticks, but rarely by both (Kazimírová et al., 2017). Vectors of tick-borne viruses are maintained in ticks during all four developmental stages or during metamorphosis from larva to nymph and from nymph to adult (Brites-Neto et al., 2015). Transmission of tick-borne pathogens can occur transstadially from one stage to the next, vertically from adult to offspring, and by cofeeding infection. SFTSV can also be transmitted from person to person or from infected animal to person through infected blood (Madison-Antenucci et al., 2020).

1.1.4. Pathogenesis of tick-borne viruses

The diseases caused by tick-borne viruses range from asymptomatic or mild febrile illness to hemorrhagic fever or severe encephalitis with significant morbidity and mortality (Lehrer and Holbroo, 2011). The clinical diseases caused by tick-borne viruses are various and can be separated into two categories including neurologic disease and visceral disease often associated with hemorrhagic manifestations (Zivcec et

al., 2013). Neurologic diseases are caused by tick-borne encephalitis virus (Bogovic and Strle, 2015), Powassan virus (Birge and Sonnesyn, 2012) and Louping ill virus (Davidson et al., 1991). Hemorrhagic fevers are caused by Crimean–Congo haemorrhagic fever virus (Ergönül, 2006), Omsk hemorrhagic fever virus (Karan et al., 2014), Kyasanur Forest disease virus (Work et al., 1959), Alkhurma virus (Bhatia et al., 2020), severe fever with thrombocytopenia syndrome virus (Liu et al., 2014). The predominant clinical syndrome associated with Kyasanur Forest disease is hemorrhagic fever, but a few cases are complicated by severe neurologic illness (Holbrook, 2012).

1.1.5. Diagnosis

Detection and diagnosis of many tick-borne infections in humans are also challenging, because of the broad clinical presentation of many tick-borne diseases. Most reliable diagnostic tests needed to confirm pathogens (Lippi et al., 2021). Isolation of viruses can be used for detection, but it requires special biosafety level and it is time consuming. In addition, many tick-borne pathogens grow slowly and require special media or cell cultures (Springer et al., 2021). Molecular diagnostic tools (PCR, RT-PCR) (Madison-Antenucci et al., 2020) are used for detecting the presence of the pathogen. Serological tests are commonly employed in tick-borne disease diagnosis and the serologic methods include virus-neutalization (VN) test, enzyme-linked immunosorbent assay (ELISA), immunofluorescence antibody test (IFA) and immunoblotting (Springer et al., 2021). Since many serological assays can detect antibodies cross-reactive with similar viruses, the diagnosis should be judged carefully (Madison-Antenucci et al.,

2020). However, VN test is gold standard and the most reliable for serological diagnosis (Lv et al., 2019)

1.1.6. Prevention and control of tick-borne viral diseases

Prevention and treatment in clinical settings are similarly limited. Despite of their severe prognosis and high case fatality rate, few vaccines are available for tick-borne pathogens (Lippi et al., 2021). According to Madison-Antenucci et al. (2020), personal protective strategies can help the prevention of tick-borne disease; (1) avoid spending time in high-risk areas as places with high grass, dense woods, and low-lying bush and stay to the center of cleared walking paths; (2) prevention of tick attachment by wearing long-sleeved shirts and long pants, closed-toed shoes or boots, tucking the shirt into the pants and the pants into socks, wearing light color clothing; (3) tick prevention in pets by using repellents and tick checks after domestic animal exposure; (4) environmental controls such as keeping grass mowed short, keeping bush and leaf litter away from dangerous areas, keeping children's swing sets in sunny areas and away from forested areas, and keeping stocks of wood away from houses and off the ground.

1.2. Tick-borne viruses in Japan

1.2.1. History

In 1948, a virus, Negishi virus, was isolated in Tokyo City from a patient who was clinically diagnosed as Japanese B encephalitis and an autopsied patient's brain. The isolated virus was retrospectively identified as a member of the Louping ill virus

through phylogenetic analyses performed decades later (Okuno et al., 1961; Venugopal et al., 1992). In 1993, the first case of TBEV infection in Japan was diagnosed in the southern area of Hokkaido (Takashima et al., 1997), four additional cases of TBE were reported in Hokkaido between 2016 and 2018 (Yoshii et al., 2017b; Jamsransuren et al., 2019). TBEV infection was also reported in domestic and wild animals in Hokkaido (Yamaji et al., 2018). In 2012, the first case of infection with severe fever with thrombocytopenia syndrome virus (SFTSV) was observed in Japan (Takahashi et al., 2014), since then SFTSV infection has been found in 23 provinces of Japan and 573 cases of SFTS were reported until 2020 (Kirino et al., 2021). A novel tick-borne orthonairovirus, Yezo virus (YEZV), was identified from two patients in Hokkaido who showed acute febrile illness with thrombocytopenia and leukopenia after tick bite in 2019 and 2020, respectively (Kodama et al., 2021).

In recent years, several novel tick-borne viruses have been isolated in Japan as Tofla virus (TFLV) - a novel member of the genus *Orthonairovirus* (Shimada et al., 2016), Tarumizu tick virus (TarTV) - a novel member of the genus *Coltivirus* (Fujita et al., 2017), Kabuto Mountain virus (KAMV) - a novel member of the genus *Uukuvirus* (Ejiri et al., 2018a), Oz virus - a novel member of the genus *Thogotovirus* (Ejiri et al., 2018b), Mukawa virus (MKWV) - a novel member of the genus *Phlebovirus* (Matsuno et al., 2018), Yamaguchi virus - a novel member of the genus *Flavivirus* (Shimoda et al., 2019). However, the information on their pathogenesis and transmission cycle has been still limited.

1.2.2. Human pathogenic tick-borne viruses in Japan

1.2.2.1. Tick-borne encephalitis virus (TBEV)

TBEV is a single-stranded positive-sense RNA, belongs to the genus *Flavivirus* within the family *Flaviviridae*, and is mainly transmitted by *Ixodes* ticks (Kuivanen et al., 2014). Based on phylogenetic analysis, the subtypes of TBEV include gthe Far-Eastern subtype (known as the Russian spring-summer encephalitis virus), the European subtype, the Siberian subtype (Ecke et al., 1999), the Baikalian subtype (Kovalev and Mukhacheva, 2018) and the Himalayan subtype (Dai et al., 2018).

In Japan, the first case of TBEV infection was diagnosed in the southern area of Hokkaido in 1993 (Takashima et al., 1997). Three other cases of TBE were confirmed in Hokkaido from 2016 to 2017 (Yoshii et al., 2017b, Yamaguchi, 2018), of which two cases were associated with fatal outcomes (Yamaji et al., 2018). In 2018, the fifth case of TBEV infection was reported in northern Hokkaido (Asahikawa City Public Health Center, 2021). TBEV infection was also reported in domestic animals, livestock, and wild animals (Takeda et al., 1999; Uchida et al., 2018; Yoshii, 2019; Jamsransuren et al., 2019). In addition, anti-TBEV-seropositive rodents were also detected in Shimane prefecture (Yoshii et al., 2017a) and deer sampled in Tochigi Prefecture (Jamsransuren et al., 2019), suggesting that TBEV may be distributed in other regions of Japan than Hokkaido.

1.2.2.2. Severe fever with thrombocytopenia syndrome virus (SFTSV)

SFTSV has been officially named dabie bandavirus, which belongs to the genus *Bandavirus* in the family *Phenuiviridae*, order *Bunyavirales* (ICTV, 2020). SFTSV comprises of three segmented, negative-strand RNA (Yu et al., 2011). The

clinical symptoms of SFTS are nonspecific and include severe fever, vomiting, thrombocytopenia, leukocytopenia, multi-organ failure, and hemorrhagic tendency (Wormser et al., 2020). *Haemaphysalis longicornis* acts as the main transmission vector of SFTSV (Yu et al., 2011). The SFTSV RNAs were detected in several tick species, including *Haemaphysalis flava, Rhipicephalus microplus, Amblyomma testudinarium, Dermacentor nuttalli, Hyalomma asiaticum, and Ixodes nipponensis* (Jo et al., 2019; Yun et al., 2013; Zhang et al., 2019; Zhu et al., 2019). Besides tick bite, human-to-human transmission of SFTSV was also reported to occur through close contact with blood or bodily secretions of patients (Huang et al., 2017). In Japan, the first case of SFTSV infection was reported in 2012 (Takahashi et al., 2014). Since then, SFTSV has been found in 23 provinces of Japan and 573 confirmed cases of SFTS had been reported by 2020 (Kirino et al., 2021). Recently, many cases of SFTS in domestic cats were reported in western Japan (Matsuu et al., 2019) and animal-to-human transmission was also reported (IASR, 2019).

1.2.2.3. Yezo virus

According to Kodama et al. (2021), YEZV, a novel orthonairovirus, were identified from two patients showing acute febrile illness with thrombocytopenia and leukopenia after tick bite in Hokkaido, Japan, in 2019 and 2020, respectively. This virus is phylogenetically grouped with Sulina virus detected in *Ixodes ricinus* ticks in Romania. Antibodies to YEZV are found in wild deer and raccoons, and YEZV RNAs were detected from ticks in Hokkaido (Kodama et al., 2021).

1.2.3. Other tick-borne viruses in Japan

Tofla virus (TFLV) belongs to the genus *Orthonairovirus* of family *Nairoviridae* in order *Bunyavirales*, was isolated from *Heamaphysalis* species in Tokushima and Nagasaki, Japan (Shimada et al., 2016). TFLV has been associated with very high pathogenicity and fatality rates in type-I interferon receptor knockout (IFNAR KO) mouse model, and this virus exhibited efficient infectivity in mammalian-derived cells, including human cells (Shimada et al., 2016).

KAMV which belongs to the genus *Uukuvirus* of family *Phenuiviridae*, order *Bunyavirales* was isolated from *Haemaphysalis flava* in Hyogo, Japan (Ejiri et al., 2018a). Suckling mice inoculated intracerebrally with KAMV showed severe symptoms; the virulence of this isolate increased after passage in mice, demonstrating 100% mortality rate in this mouse model (Ejiri et al., 2018a).

Oz virus, a new member of the genus *Thogotovirus* of the family *Orthomyxoviridae*, was first isolated from a pool of three *Amblyomma testudinarium* tick nymphs collected in Ehime prefecture, Japan (Ejiri et al., 2018b). In addition, Oz virus caused lethal infection in experimentally challenged suckling mice (Ejiri et al., 2018b).

Muko virus (MUV), a new member of the genus *Orbivirus*, family *Reoviridae* (Ejiri et al., 2015), was isolated from *Ixodes turdus* ticks in Hyogo, Tokushima, and Nagasaki prefectures. This virus possessed pathogenicity in suckling mice and A129 mice, and exhibited infectivity in mammalian-derived cells (Ejiri et al., 2015; Ulanday et al., 2016).

Tarumizu tick virus (TarTV) is considered the third member of the genus *Coltivirus* in the family *Reoviridae*, was isolated from *Haemaphysalis flava* tick collected in Tarumizu City, Kagoshima prefecture. TarTV could infect and replicate in several mammalian cell lines, but no clinical symptoms was observed in intracerebrally inoculated mice (Fujita et al., 2017). Recently, TarTV was isolated from the brain of dead raccoon dogs (Nouda et al., 2021).

Mukawa virus (MKWV), a novel member of the genus *Phlebovirus*, was isolated from host-questing *Ixodes persulcatus* females captured in Mukawa, Hokkaido, Japan (Matsuno et al., 2018). Although this virus was isolated from *Ixodes persulcatus* tick and molecular and biological footprints confirmed this virus is a tick-borne virus, its viral RNA genome sequences were similar to those of mosquito/sandfly-borne viruses (Matsuno et al., 2018).

Yamaguchi virus (YGV), a novel tick-borne flavivirus, was detected from liver and serum samples obtained from a wild boar in Yamaguchi prefecture, Japan. Phylogenetic analysis revealed that YGV belongs to the TBEV complex and is closely related to Langat virus (LGTV) (Shimoda et al., 2019).

1.3. Tick-borne viral disease in Vietnam

Eighty-one tick species have been reported in Vietnam (IMPE, 2019). The tick species of *Argas sp., Amblyomma sp., Amblyomma varanensis, Aponommacrassipes, Aponommagervaisi, Dermacentor auratus, Dermacentor compactus, Haemaphysalis yeni, Ixodes (Ixodes) granulatus, Rhipicephalus (Boophylus) microplus, Rhipicephalus*

(Rhipicephalus) haemaphysaloides, Rhipicephalus sanguineus were mainly parasitic on cows, goats, dogs, and wild animals as pythons, pangolins and wild pigs (IMPE, 2019; Nguyen, et al., 2019; Huynh et al., 2021). Among those ticks, *Rhipicephalus sanguineus* and *Rhipicephalus (Boophylus) microplus* were dominant species and distributed widely (IMPE, 2019; Nguyen et al., 2019). The surveillance of microorganism in ticks by PCR and RT-PCR showed that ticks were positive with rickettsia (IMPE, 2019) or bacteria (Huynh et al., 2021), but no tick samples were positive for tick-borne viruses. However, there were a few reports suggesting endemic of tick-borne viruses in human, 116 human sera collected in Dong Thap province of the Mekong Detla were positive with TBEV (Cuong et al., 2015), and two cases of SFTSV infection were confirmed in Hue - the central part of Vietnam, in 2017 (Tran et al., 2019).

Although ticks can carry and transmit a remarkable array of pathogens and are widely distributed throughout the world, particularly in the tropics and subtropics (De Castro, 1997), there is still a lack of information regarding the prevalence of ticks and tick-borne pathogens in Vietnam (Nguyen et al., 2019; Huynh et al, 2021). Therefore identification of ticks and tick-associated diseases in Vietnam should be conducted and epidemiological investigation and risk assessment should be performed.

2. CHAPTER 1

Zoonotic infection with Oz virus, a novel thogotovirus

2.1. Abstract

Oz virus (OZV), the new member of the genus *Thogotovirus*, was first isolated from the tick *Amblyomma testudinarium* in 2018 in Japan. Until now, there is no information on human and animal infections of Oz virus. In this chapter, serological surveillance of Oz virus among human and wild mammalians was conducted by virus-neutralization (VN) test and enzyme-linked immunosorbent assay (ELISA). Sera of 24 humans, 40 monkeys, 124 wild boars and 76 Sika deer in Yamaguchi were analyzed by VN test resulting with prevalence of 8.3%, 47.5%, 60.5% and 74%, respectively. To clarify the seroprevalence in the other regions, ELISA using extract from the infected cells as an antigen was established. The results showed that many wildlife were infected with Oz virus, especially in the western part of Japan. In conclusion, humans and many other mammalians including non-human primate may be naturally infected by Oz virus, suggesting that Oz virus may be the tick-borne zoonotic virus.

2.2. Introduction

The genus *Thogotovirus*, which belongs to the family *Orthomyxoviridae,* comprises viruses that are most frequently transmitted by a variety of hard and soft tick species (Savage et al., 2017; Peng et al., 2017). Although most thogotoviruses are associated with tick species, there have been several exceptions, such as Sinu virus, which was isolated from mosquitoes (Lvov et al., 1974; Contreras-Gutiérrezb et al., 2017), *Dielmo orthomyxovirus*, which was isolated from *Culicoides* midges (Temmam et al., 2016), and Araguari virus, which was isolated only from vertebrates (Savage et al., 2017). Until now, viruses that belong to the genus *Thogotovirus* include Upolu virus (Doherty et al., 1968), Dhori virus (Rosa et al., 2007), Thogotovirus (Staunton et al., 1989), Batken virus (Frese et al., 1997), Araguari virus (Pinto da Silva et al., 2005), Jos virus (Bussetti et al., 2012), Aransas Bay virus (Briese et al., 2014), Dielmo orthomyxovirus (Temmam et al., 2016), Bourbon virus (Savage et al., 2017), Sinu virus (Contreras-Gutiérrezb et al., 2017) and Oz virus (Ejiri et al., 2018b). Among thogotoviruses, Thogotovirus, Dhori virus, and Bourbon virus are associated with human diseases (Contreras-Gutiérrezb et al., 2017; Savage et al., 2017; Peng et al., 2017). Thogotovirus and Dhori virus have been reported to cause encephalitis, febrile illness, and/or death in humans (Siebler et al., 1996; Kosoy et al., 2015), and Bourbon virus has been reported to cause febrile illness and death in humans (Fuchs et al., 2019). In addition, Thogotovirus has also been reported to cause abortions in sheep (Davies et al., 1984), and many wild animals are positive for antibodies against Bourbon virus (Jackson et al., 2019).

Oz virus, a new member of the genus *Thogotovirus*, was first isolated from a pool of three *Amblyomma testudinarium* tick nymphs collected in Ehime Prefecture,

Japan (Ejiri et al., 2018b). Phylogenetic analyses revealed that Oz virus is more closely related to Dhori virus, Batken virus, and Bourbon virus than to other thogotoviruses (Ejiri et al., 2018b). In addition, Oz virus has been shown to cause lethal infection in experimentally challenged suckling mice. In this chapter, serosurveillance of Oz virus infection was performed among mammalians, including humans to understand the potential of Oz virus as a zoonotic pathogen.

2.3. Materials and methods

2.3.1. Cells

Vero cells (JCRB9013) which were derived from a kidney of African green monkey were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco™, Carlsbad, U.S.A) containing 10% heat-inactivated fetal calf serum (FCS; Hyclone Laboratories, UT, USA), 100 U/ml of penicillin and 100 μg/ml of streptomycin. The cells were maintained at 37°C in 5% CO_2.

2.3.2. Virus

Oz virus strain EH8 was isolated from the hard ticks *A. testudinarium* in Ehime, Japan (Ejiri et al., 2018b). The virus was propagated in Vero cells in DMEM containing 2% FCS at 37°C in 5% CO_2 and stored at -80°C until use. Virus titer was determined by plaque assay.

2.3.3. Plaque assay

Vero cells were seeded at 5 x 10^5 cells per well in two ml of growth medium with 10% FBS per well in 6 well plates. Cells were incubated at 37°C with 5% CO_2 for 24 hours. Virus stock was diluted (10^{-1} to 10^{-6}) and 200 μl of each diluted virus was inoculated onto Vero cells after one wash with DMEM. After incubation for 60 min at 37°C with 5% CO_2, the cells were washed twice with DMEM and overlaid with 0.8% agarose (Seaplaque GTG Agarose, Lonza, Rockland, ME USA) in DMEM containing 6.7% FCS. The plates were then incubated at 37°C until plaques appeared. The cells were fixed with 10% buffered formaldehyde for one hour at room temperature, the

agarose layers were removed, and then stained with crystal violet (Wako Pure Chemical Industries, Osaka, Japan). Plaques were counted, and the viral titer was calculated as the plaque-forming units (PFU) per milliliter.

2.3.4. Serum samples

Serum samples were collected from various species of mammalians, wild boar (*Sus scrofa*), Sika deer (*Cervus nippon*), and monkey (*Macaca fuscata*) from 2007 to 2019. Serum samples from a total of 879 from wild boars captured in Oita (n=58), Yamaguchi (n=344), Wakayama (n=89), Gifu (n=19), Toyama (n=20) and Tochigi (n=349) prefectures, a total of 450 Sika deer captured in Yamaguchi (n=407), Wakayama (n=9), Gifu (n=24) and Chiba (n=10) prefectures, and a total of 262 monkeys captured in Yamaguchi (n=40), Wakayama (n=80) and Mie (n=142) prefectures were collected (Figure 2.1.). Wild boars, Sika deer and monkeys were hunter-harvested or culled for nuisance control under the Program of Prevention from the Bird and Animal Damages. Blood samples were directly collected from their hearts using a sterile needle. No animals were killed specifically for this study. Serum samples were also collected from 24 hunters who hunt wild animals in Yamaguchi prefecture, Japan under permission from the Center for Clinical Research, Yamaguchi University Hospital (Control Number: H26-116). The collected sera were stored at -20°C until use.

2.3.5. Virus-neutralization (VN) test

An 80% plaque-reduction neutralizing test ($PRNT_{80}$) using Oz virus was performed. Vero cells were seeded in 24-well plates and incubated at 37°C with 5%

CO_2 for 24 hours. Serum samples were heat-inactivated at 56°C for 30 min and subjected to serial two-fold dilutions (ranging from 1:5 to 1:160) in DMEM containing 2% FCS. The diluted sera or medium alone (control) were mixed with equal volumes of virus solution containing 50 PFU of Oz virus for 60 min at 37°C. A total of 50 µl of the mixture was inoculated in duplicate onto Vero cells in 24-well plates. In each plate, four wells were inoculated with 50 µl of the mixture of virus solution and DMEM containing 2% FCS as a control to determine the average plaque count per well. After incubation for 60 min at 37°C with 5% CO_2, the cells were washed twice with DMEM and overlaid with 0.8% agarose (Seaplaque GTG Agarose, Lonza, Rockland, ME USA) in DMEM containing 6.7% FCS. The plates were then incubated at 37°C until plaques appeared. The cells were fixed with 10% buffered formaldehyde for one hour at room temperature, the agarose layers were removed, and then stained with crystal violet (Wako Pure Chemical Industries, Osaka, Japan). The $PRNT_{80}$ titer was defined as the highest dilution of serum with a plaque counts less than 20% in comparison with control wells without serum.

2.3.6. Enzyme-linked immune sorbent assay (ELISA)

ELISA protocol used for the serosurveillance of various infectious diseases in the previous studies (Suzuki et al., 2015; Shimoda et al., 2019; Yonemitsu et al., 2019) was applied for detection of antibody against Oz virus among wild animals. Oz virus- or mock-infected Vero cells were lysed with lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 0.5 mM ethylenediaminetetraacetic acid, 1% sodium deoxycholate, and 1% Triton X-100) and used as antigens. The concentration of

antigens was determined using protein assay reagent (Bio-Rad Laboratories, Inc., USA) according to manufacturer's instructions. 96-well microplates (Maxisorp; Nunc, Roskilde, Denmark) were coated with 5 μg/ml of antigens at 100 μl/well in adsorption buffer (0.05 M carbonate bicarbonate buffer, pH 9.6) and incubated at 37°C for 2 hours and replaced in 4°C overnight. Plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T) and incubated with 200 μl of 1% Block Ace (Dainippon Pharmaceutical, Osaka, Japan) at 37°C for 30 min. After washing the wells three times with PBS-T, 100 μl of 100-fold diluted sera in PBS-T containing 0.4% Block Ace was added as the primary antibody and the plates were incubated at 37°C for 30 min. Subsequently, wells were washed three times with PBS-T before addition of 100 μl of diluted Peroxidase Conjugated Purified Recomb® Protein A/G (Thermo Fisher Scientific, Waltham, MA, U.S.A.) in PBS-T containing 0.4% Block Ace. The plates were incubated at 37°C for 30 min. After three time washed with PBS-T, 100 μl of ABTS Peroxidase Substrate (KPL, SeraCare life Sciences, Milford, USA) was added to each well as the detection reagent and incubated the plates at room temperature for 30 min. At the end of incubation period, the enzymatic reaction was stopped by adding 100 μl of 1% sodium dodecyl sulfate (SDS) to each well. The absorbance was measured by a spectrophotometer (Bio-Rad, Hercules, CA, USA) with a 405-nm filter. The value of the corresponding control mock-infected cells was subtracted from all values. The optical density values of the ELISA were compared to the results of the VN test by two-graph receiver-operating characteristic (ROC) curve analysis at a 95% confidence interval as described in a previous report (Yoshida et al., 2011).

2.4. Results

2.4.1. Surveillance of Oz virus infection among human and wildlife in Yamaguchi

A total of 24 human sera and 240 wild animals sera captured in Yamaguchi, Japanese monkeys (n=40), wild boars (n=124) and Sika deer (n=76) were tested by VN test to detect antibodies against Oz virus (Table 2.1). The titer lower than 1:10 dilution was judged below the detection limit of the assay or as negative. In humans, 2 out of 24 (8.3%) of the serum samples possessed virus-neutralization (VN) antibodies against Oz virus, and the VN titers were 1:40 and 1:80; in wild animals, 19 out of 40 monkeys (47.5%), 75 out of 124 wild boars (60.5%), and 56 out of 76 Sika deer (74%) captured in Yamaguchi possessed serum with VN antibodies against Oz virus (Table 2.1).

2.4.2. Establishment of ELISA

To determine the cut-off value of ELISA, sera from 40 monkeys, 124 wild boars, 76 Sika deer in Yamaguchi Prefecture were tested by ELISA. The optical density values of the ELISA were compared to the results of the VN test by two-graph receiver-operating characteristic (ROC) curve analysis and the minimal difference between the sensitivity and specificity was used as a cut-off value. In monkey, the correlation coefficient between the ELISA and VN test was 0.9163, and with the ELISA, a cut-off value of 0.2245 had an equal sensitivity and specificity of 100%. In wild boars, the correlation coefficient was 0.8807, and the sensitivity and specificity were 88.0% and 89.8%, respectively, at a cut-off value of 0.1965. In Sika deer, the correlation coefficient was 0.7569, and the sensitivity and specificity were 78.6% and 80.0%,

respectively, at a cut-off value of 0.3165 (Figure 2.2). In further surveillance of Oz virus infection among monkeys, wild boars and Sika deer, these cut-off values were applied.

2.4.3. Application of ELISA to the serological survey in wild animals in Japan

The prevalence of Oz virus infection among wild animals was surveyed in various prefectures in Japan using the established ELISA (Table 2.2). Among 197 monkeys captured in Yamaguchi, Wakayama and Mie prefectures from 2007 to 2019, the seropositivity rates were 47.5% (19 of 40), 33.3% (5 of 15), and 6.3% (9 of 142), respectively. Among 879 wild boars captured in Oita, Yamaguchi, Wakayama, Gifu, Toyama, and Tochigi prefectures from 2007 to 2014, the seropositivity rates were 10.3% (6 of 58), 55.8% (192 of 344), 34.8% (31 of 89), 10.5% (2 of 19), 0% (0 of 20), and 0% (0 of 349), respectively. Among 450 Sika deer captured in Yamaguchi, Wakayama, Gifu, and Chiba prefectures, the seropositivity rates were 37.8% (154 of 407), 11.1% (1 of 9), 8.3% (2 of 24), and 30% (3 of 10), respectively.

2.5. Discussion

There was little information on Oz virus, a new member of tick-borne *Thogotovirus*. This virus was isolated from *Amblyomma testudinarium* in Ehime, Japan and caused lethal infection to suckling mice after intracerebral challenge. Therefore, it is hypothesized that the virus might be a potential tick-borne zoonotic pathogen. In this study, Oz virus infection to mammal species including human and non-human primates was examined.

At first, the $PRNT_{80}$ was applied to detect anti-Oz virus antibodies in humans and wild animals in Yamaguchi Prefecture, which is located in the western part of the main island of Japan, and close to Ehime Prefecture (Figure 2.1). Wild boar is a major host of *A. testudinarium*, from which Oz virus was first isolated. The results showed that many wild boars (60.5%) and Sika deer (74%) in Yamaguchi Prefecture during 2013 and 2015 had VN antibodies against Oz virus, indicating that Oz virus was infecting wild animals in the western part of Japan. Next, wild monkeys (*Macaca fuscata*) were examined for Oz virus infection; 47.5% of monkeys were infected with Oz virus, and the antibody titers were high. Additionally, two hunters (*Homo sapiens)*, who hunted wild boars and Sika deer in Yamaguchi Prefecture, also possessed antibodies against Oz virus. These results showed that primates, i.e., humans and monkeys, are also hosts of Oz virus.

ELISA using an Oz virus-infected cell extract was established for the surveillance of Oz virus infection among many mammalians. The results were compared to those of the VN test to determine the correlation between the ELISA and the VN test; in monkeys, wild boars, and Sika deer, the correlation coefficients were 0.9163, 0.8807, and 0.7569, respectively. These results suggested that the ELISA is

applicable for the serosurveillance of Oz virus infection in many samples. However, since its sensitivity and specificity differed among animal species, cut-off value should be determined for each animal species. In particular, since the ELISA was less specific and sensitive for Sika deer, the VN test should also be performed to confirm the presence of antibodies against Oz virus.

Our nationwide surveillance of Oz virus infection indicated that many wild animals were positive for antibodies against Oz virus. However, wild boars in Toyama and Tochigi Prefectures did not possess antibodies against Oz virus, suggesting that Oz virus might not be distributed in the northern and eastern parts of Japan. *A. testudinarium* is the major tick species that infests humans in the southern and western parts of Japan (Okino et al., 2007; Liyanaarachchi et al., 2015; Ejiri et al., 2018b), and since Oz virus was distributed mainly in the southern and western parts of Japan, it appears that the habitat of the tick correlates with the distribution of Oz virus-infected animals. In addition, since hunters in Yamaguchi Prefecture were positive for antibodies against Oz virus, and since intracerebral inoculation of the virus causes lethal disease in suckling mice (Ejiri et al., 2018b), Oz virus must be a zoonotic pathogen.

In conclusion, this study is the first to report Oz virus infection in humans and wild animals. It will be required to clarify the infection of Oz virus can cause diseases in these sensitive hosts is an important issue.

2.6. Figure legends

Fig.2.1. Map showing the collection sites of serum samples in this study. Black color indicates where Oz virus was originally isolated. Gray color indicates prefectures in which serum samples were collected.

Fig.2.2. Comparison between VN test and ELISA against Oz virus. The correlation between VN test and ELISA using monkey (n=40) (A), wild boar (n=124) (B) and Sika deer (n=76) (C) sera collected in Yamaguchi Prefecture was analyzed. Dot plot in X- and Y-axes indicated VN titer and ELISA absorbance, respectively. The correlation coefficient between VN test and ELISA in monkeys, wild boars and sika deer were 0.9163, 0.8807 and 0.7569, respectively. The optimal cut-off value of ELISA was calculated by two-graph ROC curve. The optimal cut-off values of monkey, wild boar and Sika deer sera were set at 0.225, 0.197 and 0.317, respectively.

2.7. Figures and Tables

Fig.2.1.

Fig.2.2.

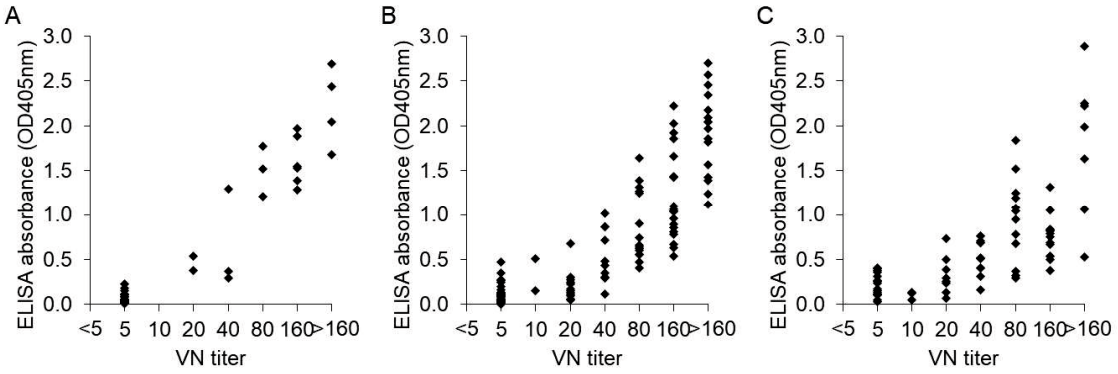
A
B
C
ELISA absorbance (OD405nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
<5 5 10 20 40 80 160 >160
VN titer

Table 2-1. Serosurveillance of Oz virus infection among mammalians in Yamaguchi by VN test

Species	Scientific name	Year	Total	Virus-neutralization titer						
				<1:10	1:10	1:20	1:40	1:80	1:160	>1:160
Human	*Homo sapiens*	2015	24	22	0	0	1	1	0	0
Monkey	*Macaca fuscata*	2018-2019	40	21	0	2	3	3	6	5
Wild boar	*Sus scrofa leucomystax*	2013-2014	124	49	2	12	10	15	20	16
Sika deer	*Cervus nippon*	2014-2015	76	20	5	8	11	12	13	7

Table 2-2. Serosurveillance of OZV infection among wild animals in Japan by ELISA

Species	Scientific name	Prefecture	Year	Cut-off value	No. of examined sera	No. of positive sera	% of positive sera
Monkey	*Macaca fuscata*	Yamaguchi	2018-2019	0.225	40	19	47.5
		Wakayama	2012-2013		15	5	33.3
		Mie	2007		142	9	6.3
Wild boar	*Sus scrofa*	Oita	2012	0.197	58	6	10.3
		Yamaguchi	2010-2014		344	192	55.8
		Wakayama	2007-2013		89	31	34.8
		Gifu	2014		19	2	10.5
		Toyama	2014		20	0	0
		Tochigi	2010-2012		349	0	0
Skia deer	*Cervus nippon*	Yamaguchi	2010-2014	0.317	407	154	37.8
		Wakayama	2010-2014		9	1	11.1
		Gifu	2014		24	2	8.3
		Chiba	2014		10	3	30

3. CHAPTER 2

Epidemiological study of Kabuto Mountain virus, a novel Uukuvirus, in Japan

3.1. Abstract

Kabuto Mountain virus (KAMV), the new member of the genus *Uukuvirus*, was isolated from the tick *Haemaphysalis flava* in 2018 in Japan. To date, there is no information on KAMV infection in human and animals. Therefore, serological surveillance of the infection among humans and wild mammals was conducted by virus-neutralization (VN) test and indirect immunofluorescence assay (IFA). Sera of 24 humans, 59 monkeys, 171 wild boars, 233 Sika deer, 7 bears, and 27 nutria in Yamaguchi Prefecture were analyzed by VN test. The positive ratio of humans, monkeys, wild boars, and Sika deer were 20.8%, 3.4%, 33.9% and 4.7%, respectively. No positive samples were detected in bears and nutria. The correlation coefficients between VN test and IFA in human, monkey, wild boar, and Sika deer sera were 0.5745, 0.7198, 0.9967 and 0.9525, respectively. In addition, KAMV was detected in one pool of *Haemaphysalis formosensis* ticks in Wakayama Prefecture. These results indicated that KAMV or KAMV-like virus is circulating among many wildlife and ticks, and that this virus incidentally infects humans.

3.2. Introduction

Ticks are the primary vectors for pathogens of domesticated and wild animals and the secondary vectors for pathogens of humans (Mansfield et al, 2017). Tick-bites are considered an occupational health issue for forestry personnel and others working (e.g., agricultural industry, military) and/or participating in recreational activities (e.g., hunting, hiking) in forested areas (Richards et al., 2017). Since the beginning of this century, an increasing number of tick-borne viral diseases have been reported in many parts of the world, with examples including Alkhurma haemorrhagic fever (Alzahrani et al., 2010), African swine fever (Rahimi, 2010), Crimean-Congo haemorrhagic fever (Leblebiciogluet al., 2015), severe fever with thrombocytopenia syndrome virus infection (Silvas and Aguilar, 2017), Heartland virus infection (Mansfield et al, 2017), Powassan virus infection (Hermance and Thangamani, 2017), Kyasasur forest disease (Ajesh et al., 2017), Yezo virus infection (Kodama et al., 2021), Oz virus infection (Ejiri et al., 2018b; Tran et al., 2021) and so on.

Tick-borne viruses (TBVs) belong to two orders (*Bunyavirales* and *Mononegavirales*), as well as another nine families and at least 12 genera that remain unassigned to any order (Kazimírová et al., 2017; Shi et al., 2018). The order *Bunyavirales* was established in 2017 and comprises more than 500 viruses classified into 12 families, including several viruses that are highly pathogenic to mammalian hosts (Lerolle et al., 2021). The genus *Uukuvirus* (Kuhn et al., 2020), which belongs to the family *Phenuiviridae* of the order *Bunyavirales*, includes KAMV (Ejiri et al., 2018a), Zaliv Terpeniya virus (Lvov et al., 1973), Uukuvirus (Eley and Nuttall, 1984), American dog tick virus (Tokarz et al., 2014), Sunday Canyon virus (Yunker et al., 1977), Grand Arbaud virus (Hubále and Rudolf, 2012), Kaisodi virus (Bhatt et al., 1966; Yadav et al., 2018), Dabieshan virus (Li et al., 2015), EgAn 1825-61 virus (also known as Nile warbler virus) (Palacios et al., 2013), Precarious Point virus (St George et al., 1985), Murre virus (Palacios et al., 2013), Lihan virus (Li et al., 2015),

Pacific coast tick virus (Bouquet et al., 2017), Tacheng tick virus 2 (Li et al., 2015), Rukutama virus (Lvov et al., 1990), Silverwater virus (McLean and Bryce Larke, 1963), Yongjia virus 1 (Li et al., 2015), and Toyo virus, a new member of this genus (Kobayashi et al., 2021). Some species of the genus can infect humans and animals, since anti-Uukuvirus antibodies have been detected in sera from humans (Hubále and Rudolf, 2012; Lvov et al., 2015), cows, and reptiles (Hubále and Rudolf, 2012). The first case of human Tacheng tick virus 2 infection in China was reported to show symptoms of fever, headache, anorexia, nausea, vomiting, and erythema (Dong et al., 2019). However, the epidemiology and pathology of many uukuviruses remain unclear.

KAMV, strain T32, was first isolated from a pooled sample of two larvae, 20 nymphs, and one adult male of *Haemaphysalis flava* ticks collected by flagging from September to November 2009 in Hyogo, Japan (Ejiri et al., 2018a). Suckling mice inoculated intracerebrally with KAMV showed severe symptoms; the virulence of this isolate increased after passage, demonstrating 100% mortality rate in this mouse model (Ejiri et al., 2018a). However, there exists (to our knowledge) no further information on KAMV.

In this chapter, seroserveillance was performed for KAMV infection among mammals, including humans, along with molecular screening for tick-borne KAMV.

3.3. Materials and methods

3.3.1. Cells

Vero cells (JCRB9013), which are derived from the kidney of an African green monkey, and BHK-21 cells (JCRB9020), which are derived from baby hamster kidneys, were used in this study. The culture media and incubation conditions are the same as described in CHAPTER 1.

3.3.2. Virus

KAMV strain T32 was isolated from the *Haemaphysalis flava* hard ticks in Hyogo, Japan, as described previously (Ejiri et al., 2018a). The virus was propagated in BHK-21 cells in DMEM containing 2% FCS at 37°C in 5% CO_2 and stored at −80°C until use.

3.3.3. Virus titration

The virus titer of KAMV was determined by 50% Tissue Culture Infectious Dose ($TCID_{50}$) assay using 96-well flat-bottom microplates (Sumitomo Bakelite, Inc., Tokyo, Japan). Briefly, 100 µL of serial 10-fold dilutions of virus suspension (10^{-1} to 10^{-10}) and 100 µL of BHK-21 cells (1 x 10^5/mL) were mixed in each well and incubated at 37°C with 5% CO_2. After 7 days of incubation, cells were fixed with 10% phosphate-buffered formalin and stained with crystal violet (Wako Pure Chemical Industries, Osaka, Japan). After visual inspection of wells, viral titer was calculated by using the Reed-Muench calculation (Lei et al., 2021).

3.3.4. Serum samples

Serum samples were collected from multiple species of wild mammals captured in Yamaguchi Prefecture from 2013 to 2019, including wild boars (*Sus scrofa*, n=171), Sika deer

(*Cervus nippon*, n=233), monkeys (*Macaca fuscata*, n=59), Asian black bears (*Ursus thibetanus*, n=7) and nutria (*Myocastor coypus*, n=27). Monkeys, wild boars, Sika deer, bears, and nutria were hunter-harvested or culled, and blood were collected as described in CHAPTER 1. Human sera were collected from 24 hunters who hunt wild animals in Yamaguchi Prefecture, Japan as described in CHAPTER 1. The collected sera were stored at -20°C until use.

3.3.5. VN test

Heat-inactivated sera (56°C, 30 min) were subjected to serial 2-fold dilutions from 1:5 to 1:160 in DMEM containing 10% FCS; the dilutions were distributed at 50 µL/well to duplicate wells in 96-well culture plates (Sumitomo Bakelite, Inc., Tokyo, Japan). An equal volume of 50 $TCID_{50}$ of KMAV diluted in DMEM containing 10% FCS was added to each well, and the plates were incubated for one hour at 37°C. BHK-21 cell suspension (100 µL/well; 1×10^5 cells/mL) then was added to each well, and the plates were incubated at 37°C for 7 days. The cells were then fixed with 10% phosphate-buffered formalin at room temperature for 30 min and stained with crystal violet. Cytopathic effect (CPE) was confirmed visually and the CPE-positive wells were recorded. Titers lower than the 1:10 dilution were judged negative and below the detection limit of the assay. The neutralizing antibody titer was defined as the reciprocal of the highest serum dilution that inhibited the CPE in duplicate wells.

3.3.6. Immunofluorescence assay (IFA)

Vero cells were infected with KAMV at 0.01 multiplicity of infection (or mock-infected) and collected at 7 days post infection. After washing twice with phosphate-buffered saline (PBS), the cells were resuspended in a small volume of PBS and 10 µL of cells were

transferred to each well of a glass micro-slide (Matsunami Glass IND, Osaka, Japan). After fixation in chilled acetone (Fujifilm, Osaka, Japan) at -20°C for 30 min, the cells were incubated with serum samples diluted in PBS containing 0.05% sodium azide and 2% FCS in a moist chamber for 30 min at 37°C. Following three washes with PBS, the cells were incubated with Protein A/G-FITC (BioVision, Mountain View, USA) diluted in PBS containing 0.05% sodium azide and 2% FCS in a moist chamber at 37°C for 30 min. After three washes with PBS, the slides were covered with buffered glycerin and a cover-glass and then observed using a fluorescence microscope (Olympus BX53 Digital Fluorescence Microscope). IFA titers were recorded as the highest serum dilution that showed specific fluorescence. Mock-infected cells were used as a negative control.

3.3.7. Collection of ticks

The ticks were collected by flagging on vegetation from sites in four prefectures in Japan, including Yamaguchi (N34°10'59.99", E131°28'0.01"), Ehime (N33°50'20.98", E132°45'56.66"), Wakayama (N33°55'0.01", E135°25'0.01"), and Fukushima (N37°45'0.00", E140°28'0.01) (Figure 3.2). All tick specimens were morphologically identified and pooled by species, stage, sex, and sampling location such that each pool contained fewer than 5 adults, 20 nymphal, or 50 larval ticks. The pooled tick samples were homogenized in 600 µL of PBS using a Multi-beads shocker (Yasui Kikai Co., Osaka, Japan). Each homogenate was transferred to a 1.5-mL tube and centrifuged (1,500 x g, 5 min, 4°C); and the resulting supernatant was passed through a sterile 0.45-µm filter (Costar® Spin-X®, New York, USA). The filtrates were stored at -80°C until use.

3.3.8. Viral RNA detection

RNA was extracted from tick homogenates using a QIAamp Viral RNA Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommended protocol. The extracted RNA was reverse transcribed using a One Step RT-PCR Kit (QIAGEN) with a specific primer pairs consisting of forward primer T32-SegL-6.5kF (5′-CTTGAGAGCTCGCTTAGGAGGAGG-3′) and reverse primer T32-SegL-6.5kR (5′-AGAGGACTAAGTGCTCCTCATTCA-3′). Reverse transcription-polymerase chain reaction (RT-PCR) was performed using the following program: reverse transcription at 50°C for 30 min; denaturation at 95°C for 15 min; 40 cycles of denaturation at 94°C for 0.5 min, annealing at 53°C for 0.5 min, and extension at 72°C for 1 min; and final extension at 72°C for 10 min. The resulting PCR products were electrophoresed on 2% agarose gels, stained with ethidium bromide, and visualized under ultraviolet (UV) illumination. The estimation of infection rate from pooled samples was calculated by the maximum likelihood estimation (MLE) method of Chiang and Reeves (Gu et al., 2003), using the equation where Y is the number of positive pools, X is the number of pools, and m is the pool size.

$$\mathrm{MLE} = 1 - \left(1 - \frac{\mathrm{Y}}{\mathrm{X}}\right)^{1/m}$$

3.3.9. Sequence analysis

The PCR products were purified by using a MinElute® PCR Purification Kit (QIAGEN) and directly sequenced using a BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Austin, TX, USA) according to the manufacturer's instructions.

3.3.10. Statistical analysis

For statistical analysis, McNemar's chi-squared tests were performed. P values of <0.05 were considered statistically significant.

3.4. Results

3.4.1. Serosurveillance of KAMV infection in humans and wild animals in Yamaguchi Prefecture

A total of 24 human sera and 497 sera of wild animals including Japanese monkeys (n=59), wild boars (n=171), Sika deer (n=233), Asian black bears (n=7), and nutria (n=27) captured in Yamaguchi from 2013 to 2019 were analyzed by the VN test against KAMV (Table 3.1). Five human sera (20.8%) possessed VN antibodies, exhibiting VN titers of 1:10 (n=3) and 1:20 (n=2). Among the wild animals, 3.4% of monkeys (2 of 59 sera), 33.9% of wild boars (58 of 171) and 4.7% of Sika deer (11 of 233) in Yamaguchi were positive for VN antibody against KAMV. None of the bear and nutria samples were seropositive (Table 3.1). Although VN titers in humans and monkeys were 1:20 or less, the highest VN titers in wild boars and Sika deer were 1:160 and 1:80, respectively.

3.4.2. Comparison of seroprevalence in wild boar by sex and body weight

Seropositivities of wild boars were compared by sex and body weight. The results showed that there was no significant difference either in positivity between males (31.3%) and females (37.6%), or in positivity between wild boars of 30 kg or less (25.5%) and over 30 kg (40.9%) (Table 3.2)

3.4.3. Comparison of the results between VN test and IFA

To confirm the results of the VN test, sera of humans (n=24) and wild animals (n=497) in Yamaguchi Prefecture were examined by IFA. All VN-positive sera were positive for IFA using sera diluted to 1:10 or more, but one VN-negative human serum and six VN-

negative monkey sera tested positive by IFA. Using IFA, the seropositivity of human sera was 25% (6 of 24 sera). Among 497 wild animals, 13.6% of monkeys (8 of 59 sera), 35.1% of wild boars (60 of 171) and 6.4% of Sika deer (15 of 233) possessed antibodies against KAMV. The correlation coefficients between the results of the VN test and IFA in human, monkey, wild boar, and Sika deer were 0.5745, 0.7198, 0.9967 and 0.9525, respectively (Figure 3.1).

3.4.4. Detection of KAMV from collected ticks

A total of 1042 larval, nymphal, and adult ticks collected by flagging on vegetation in Yamaguchi, Ehime, Wakayama, and Fukushima Prefectures were analyzed for KAMV. RNA was extracted from homogenates of ticks pooled by species (and other parameters) and analyzed by RT-PCR. Only one pool of *H. formosensis* nymphs collected in Wakayama on May 13, 2015, was positive for KAMV RNA (Table 3.3). When compared with sequences in the National Center for Biotechnology Information (NCBI) database, the obtained sequence (398bp) was 100% identical with KAMV strain T32 (LC153711.1).

3.5. Discussion

KAMV, a new member of the tick-borne Uukuvirus, was isolated from *Haemaphysalis flava* in Hyogo Prefecture, this virus was shown to replicate in many mammalian cell lines and to cause lethal infection in suckling mice after intracerebral challenge (Ejiri et al., 2018a). Therefore, it was hypothesized that this virus may be a potential tick-borne zoonotic pathogen. Therefore, serological survey of the prevalence of KAMV infection in humans and wild animals in Yamaguchi Prefecture, in the western part of Japan, was conducted in the present study.

By the VN test, wild boars showed the highest positive rate (33.9%), but the positivities of monkeys and Sika deer were relatively low, 3.4 % and 4.7 %, respectively. Furthermore, all bears and nutria were seronegative (Table 3.1). All VN-positive sera were positive by IFA using infected cells, confirming that VN-positive animals indeed were infected with KAMV or a KAMV-like virus. In Japan, novel uukuviruses, Toyo virus (Kobayashi et al., 2021) and Okutama tick virus (Kobayashi et al, 2020) were recently discovered. In addition, fifteen wild boars possessed relatively high VN titers of 1:80 or more. In Sika deer, the frequency of seropositivity was low, but three deer possessed high titers of 1:40 or more. In monkeys, the seropositivity and VN titers were very low, but six VN-negative monkeys were positive by IFA, indicating that the VN test was less sensitive than IFA in monkeys. The partial incompatibility between the results of VN test and IFA may be explained by the general fact that the VN test only detects antibody that can block virus replication (Payne, 2017), while IFA detects all antibodies reactive with the fixed antigens in virus-infected cells (Niedrig et al., 2008). Our serosurveillance showed that some (but not all) species of wild animals are naturally infected with KAMV (or a related virus), and that wild boars may be a natural host of KAMV. Although the different seroprevalence between body weight groups in wild boars was not significant (Table 3.2), the bigger wild boars might have

the more opportunities to be exposed by virus-infected ticks. Interestingly, five and six hunters were seropositive as assessed by VN tests and IFA, respectively. All 24 hunters were negative (by VN test) for SFTS virus belonging to the Family *Phenuiviridae* (data not shown). Therefore, at least five hunters appeared to be infected with KAMV or a related viruses.

KAMV strain T32 was first isolated from a pooled sample of *H. flava* ticks comprising two larvae, 20 nymphs, and one adult male collected by flagging from vegetation in Hyogo (Ejiri et al., 2018a). Other strains of KAMV also were isolated from blood-feeding *H. flava* recovered from wild boars collected in Hyogo (Ejiri et al., 2018a), and KAMV strain 17ISK-T11 was isolated from a pool of 26 nymphal *H. flava* ticks collected in Ishikawa Prefecture (Kobayashi et al., 2020). In the present study, KAMV was detected in a nymphal pool of *H. formosensis* ticks collected from vegetation in Wakayama by flagging (Table 3.3). Detection of KAMV previously was reported only from *H. flava* (Ejiri et al., 2018a, Kobayashi et al., 2020). *H. flava* must be a major vector tick for KAMV, but *H. formosensis* tick should be considered a candidate vector, given that RNA-positive ticks were collected from vegetation by flagging. Since KAMVs were detected from ticks in Ishikawa, Hyogo and Wakayama Prefectures and the positive sera were detected in humans and wild animals in Yamaguchi Prefecture (Figure 3.2), KAMV has been circulating in the western part of Japan.

The *H. flava* tick is distributed widely in Japan (Fujimoto, 2001; Yamauchi and Takeno, 2000), mainly in woodlands in urban/suburban areas (Fujita and Tsuboi, 2000). In addition, *H. flava* ticks are active in all seasons (Furuno et al., 2017), exhibiting a very broad host range that includes birds, small rodents, large animals, and humans (Inokuma et al., 2004; Kakuda et al., 1989). *H. formosensis* is distributed widely in tropical and subtropical areas of Japan (Iwakami et al., 2014) and seems to be the predominant species associated with wild boar (Fujita and Tsuboi, 2000). This evidence suggests that KAMV transmitted by these two ticks might infect many mammalian species in Japan.

In conclusion, KAMV or a KAMV-like virus is circulating in Japan among mammals, including humans, and *Haemophysalis* ticks. Further investigation will be required to clarify whether KAMV is a human pathogen.

3.6. Figure legends

Fig 3.1. Comparison between results of the virus-neutralization (VN) test and the indirect immunofluorescence assay (IFA) against Kabuto Mountain virus. The correlation between VN test and IFA using human (n=24) (A), monkey (n=59) (B), wild boar (n=171) (C), and Sika deer (n=233) (D) sera collected in Yamaguchi Prefecture was analyzed. The X- and Y-axes indicate VN titer and IFA titer, respectively. The correlation coefficient between the VN test and IFA data in humans, monkeys, wild boars, and sika deer were 0.5745, 0.7198, 0.9967, and 0.9525, respectively.

Figure 3.2. A map of KAMV endemic areas. Black circle and square indicate the prefecture in which KAMV gene and antibody were detected in this study, respectively. Dotted circles indicate the prefecture in which KAMV was isolated in the previous studies. Each strain name was indicated in the parenthesis.

3.7. Figures and Tables

Fig.3.1.

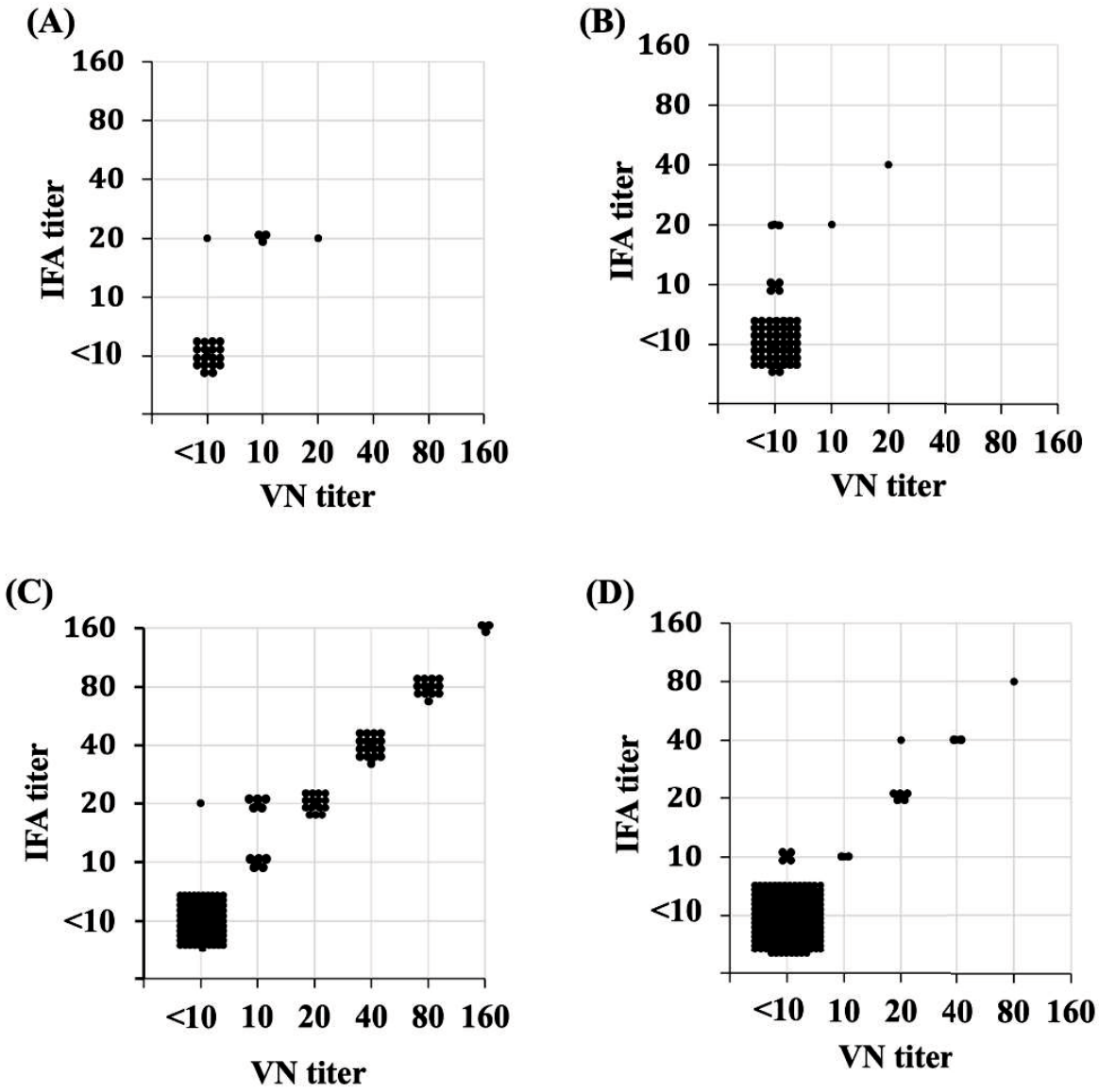

Fig.3.2

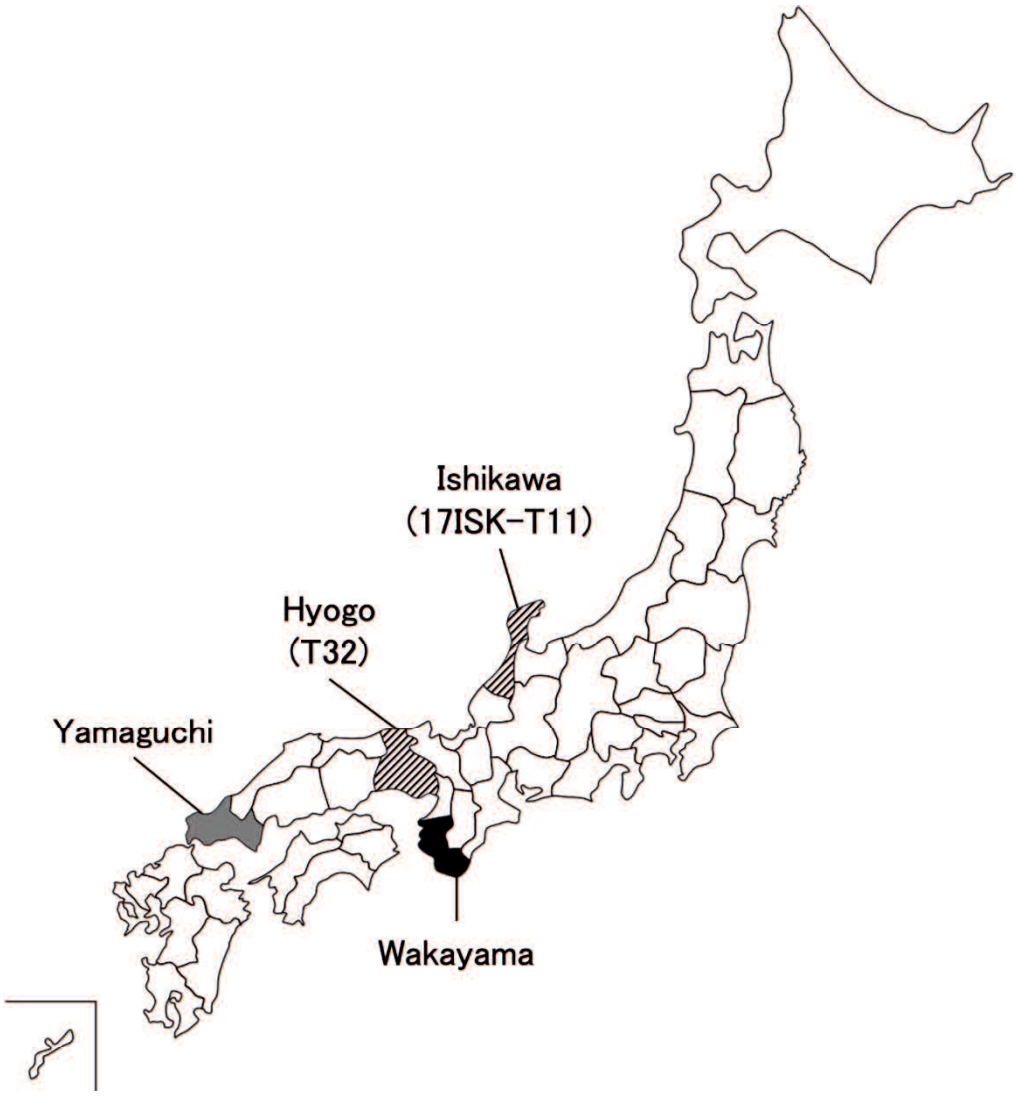

Table 3-1. VN test against Kabuto Mountain virus using sera from mammals in Yamaguchi Prefecture

Species	Scientific name	Year	Total	Virus neutralization titer						% of positive rate
				<1:10	1:10	1:20	1:40	1:80	1:160	
Human	*Homo sapiens*	2015	24	19	3	2	0	0	0	20.8
Monkey	*Macaca fuscata*	2018-2019	59	57	1	1	0	0	0	3.4
Wild boar	*Sus scrofa leucomystax*	2013-2016	171	113	10	16	17	12	3	33.9
Sika deer	*Cervus nippon*	2013-2015	233	222	2	6	2	1	0	4.7
Bear	*Ursus thibetanus*	2013-2017	7	7	0	0	0	0	0	0
Nutria	*Myocastor coypus*	2015-2017	27	27	0	0	0	0	0	0

Table 3.2. Comparison of seroprevalence of Kabuto Mountain virus infections in wild boars by sexs and body weights

		No. of examined sera	No. of positive sera	% of positive sera
Sex	Male	67	21	31.3
	Female	93	35	37.6
	No record	11	2	18.2
Body weight	⩽30 kg	55	14	25.5
	>30 kg	93	38	40.9
	No record	23	6	26.1
Total		171	58	33.9

Table 3-3. Detection of Kabuto Mountain virus in ticks by RT-PCR

Prefecture	Species	No. of ticks	No. of pools	No. positive pools	Estimated infection rate (%)
Yamaguchi	*Haemaphysalis flava*	42 (40N, 2F)	4	0	0
	Haemaphysalis formosensis	3 (3M)	1	0	0
	Haemaphysalis hystricis	1 (1N)	1	0	0
	Haemaphysalis longicornis	212 (2L, 198N, 11F, 1M)	17	0	0
	Ixodes turdus	4 (2L, 2N)	2	0	0
	Total	262	25	0	0
Ehime	*Amblyomma testidunarium*	7 (7N)	2	0	0
	Haemaphysalis flava	5 (5N)	2	0	0
	Haemaphysalis formosensis	10 (7N, 1F, 2M)	4	0	0
	Haemaphysalis hystricis	10 (5N, 3F, 2M)	6	0	0
	Haemaphysalis longicornis	24 (24N)	5	0	0
	Ixodes ovatus	1 (1M)	1	0	0
	Total	57	20	0	0
Wakayama	*Amblyomma testidunarium*	53 (52N, 1M)	21	0	0
	Dermacentor taiwanensis	5 (2F, 3M)	5	0	0
	Haemaphysalis cornigera	36 (28N, 2F, 6M)	14	0	0
	Haemaphysalis flava	171 h (113N, 19F, 39M)	27	0	0
	Haemaphysalis formosensis	76 (53N, 8F, 15M)	17	1 (Nymph)	1
	Haemaphysalis hystricis	15 (8F, 7M)	9	0	0
	Haemaphysalis kitaokaii	39 (21F, 18M)	17	0	0
	Haemaphysalis longicornis	169 (148N, 5F, 16M)	16	0	0
	Haemaphysalis megaspinosa	106 (87N, 10F, 9M)	13	0	0
	Ixodes ovatus	7 (3F, 4M)	7	0	0
	Ixodes turdus	2 (2N)	1	0	0
	Total	679	147	1	0.1
Fukushima	*Haemaphysalis flava*	21 (20N, 1M)	5	0	0
	Haemaphysalis longicornis	1 (1F)	1	0	0
	Haempahysalis megaspinosa	6 (1L, 4N, 1F)	4	0	0
	Ixodes ovatus	6 (3F, 3M)	6	0	0
	Ixodes persulcatus	10 (2N, 5M, 3F)	10	0	0
	Total	44	26	0	0

4. GENERAL CONCLUSION

In this thesis, serosurveillance of novel tick-borne viruses which were isolated in Japan in 2018, Oz virus and KAMV, were performed among mammalians including humans to understand the potential as zoonotic pathogens.

In CHAPTER 1, sera of 24 humans, 40 monkeys, 124 wild boars and 76 Sika deer in Yamaguchi were analyzed by VN test against Oz virus, resulting in positive ratio of 8.3%, 47.5%, 60.5% and 74%, respectively. An ELISA using an Oz virus-infected cell extract was established for the surveillance of Oz virus infection among various mammalians in Japan. The results were compared to those of the VN test to determine the correlation between the ELISA and the VN test, resulting the correlation coefficient between established ELISA and VN test in monkeys, wild boars, and Sika deer were 0.9163, 0.8807, and 0.7569, respectively. Since the results from ELISA and VN test are correlated significantly, the established ELISA was applied for the serosurveillance of Oz virus infection in various samples in Japan. The nationwide surveillance of Oz virus infection indicated that Oz virus was distributed mainly in the southern and western parts of Japan, but not in the northern and eastern parts of Japan. In addition, two hunters were positive for antibodies against Oz virus, suggesting Oz virus might be a zoonotic pathogen.

In CHAPTER 2, serological surveillance of KAMV infection among humans and wild mammalians including monkey, wild boar, Sika deer, bear and nutria in Yamaguchi Prefecture was conducted by VN test and indirect immunofluorescence assay (IFA). Sera of 24 humans, 59 monkeys, 171 wild boars, 233 Sika deer, 7 bears, and 27 nutria in Yamaguchi Prefecture were analyzed by VN test. The positive ratio of humans, monkeys, wild boars, and Sika deer were 20.8%, 3.4%, 33.9% and 4.7%, respectively. No positive samples were detected in bears and nutria. Though VN test was less sensitive than IFA especially in

monkey sera, all VN-positive sera were confirmed to be positive by IFA using infected cells. The correlation coefficients between VN test and IFA in human, monkey, wild boar, and Sika deer sera were 0.5745, 0.7198, 0.9967 and 0.9525, respectively. Since several hunters were seropositive against KAMV, KAMV might be a potential tick-borne zoonotic pathogen. In addition, KAMV was detected in a nymphal pool of *Haemaphysalis formosensis* ticks collected from vegetation by flagging in Wakayama Prefecture. *H. flava* seems to be a vector tick for KAMV, but also *H. formosensis* tick might be considered a candidate vector.

In conclusion, our results showed that Oz virus and Kabuto Mountain virus are circulating among wild mammalians and ticks in Japan and sometimes infected human, suggesting that these two viruses might be the tick-borne zoonotic viruses. Further investigation is required to clarify whether Oz virus and Kabuto Mountain virus can cause diseases in human.

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