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emerging orthonairovirus, Yezo virus,
and effects of antiviral agents against Yezo virus
using a mouse model**

(感染マウスモデルを用いた
新興オルソナイロウイルスであるエゾウイルスの
病原性と抗ウイルス薬の効果に関する研究)

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Abbreviations

ALB	albumin
ALT	alanine transaminase
AST	aspartate transaminase
BSL	biosafety level
BUN	blood urea nitrogen
CCHF	Crimean-Congo hemorrhagic fever
CCHFV	Crimean-Congo hemorrhagic fever virus
DAB	3,3'-Diaminobenzidine
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
dpi	days post infection
FBS	fetal bovine serum
FFA	focus-formation assay
FFU	focus-formation unit
Fig	figure
GLOB	globulin
Glu	glucose
GPC	glycoprotein precursor
H&E	hematoxylin-eosin
HRP	horse radish peroxidase
Iba1	ionized calcium binding adapter protein 1
IC ₅₀	50% inhibitory concentration
IC ₉₀	90% inhibitory concentration
IFN	interferon
IFNR	interferon receptor
IgG	immunoglobulin G
IHC	immunohistochemical staining
ip	intraperitoneal injection
KO	knockout
L	large protein
LD ₅₀	50% lethal dose
LOD	limit of detection
LPHV	Leopards Hill virus
MEM	minimum essential medium
MOI	multiplicity of infection
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium Bromide
N	nucleoprotein
NSD	Nairobi sheep disease
PBS	phosphate buffered saline
PLT	platelet counts

qPCR	quantitative polymerase chain reaction
RdRP	RNA-dependent RNA polymerase
RMP	ribavirin monophosphate
RT-qPCR	reverse transcription-quantitative polymerase chain reaction
RTP	ribavirin triphosphate
sc	subcutaneous injection
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SFTSV	severe fever with thrombocytopenia syndrome virus
STAT	signal transducer and activator of transcription
T-705	favipiravir
TBIL	total bilirubin
TBST	0.05% Tween 20 in tris-buffered saline
TP	total protein
WBC	white blood cell counts
WHO	World Health Organization
YEZV	Yezo virus

Notes

Chapter I and II:

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General Introduction

The *Orthonairovirus* is a genus of viruses belonging to the family *Nairoviridae* within the class *Bunyaviricetes* (1). An orthonairovirus genome is characterized by three single-stranded, negative-sense RNAs called S, M, and L segments, which encode nucleoprotein (N), glycoprotein precursor (GPC), and large protein (L, RNA-dependent RNA polymerase), respectively (Fig 1A). Orthonairoviruses are transmitted primarily by ticks and infect vertebrate hosts, including birds, rodents, ruminants, bats, and humans (2). The orthonairoviruses are phylogenetically divided into nine genogroups (Fig 1B) (2, 3). Most of human pathogenic orthonairoviruses belong to the Nairobi sheep disease (NSD) genogroup, such as Crimean-Congo hemorrhagic fever virus (CCHFV), Nairobi sheep disease virus, and Dugbe virus (4–6), distributing a wide range of African and Middle Eastern countries. Sporadic human cases of infection with orthonairoviruses in other genogroups, including Kasokero virus in Kasokero genogroup, Issyk-Kul virus in Katerah genogroup, and Tamdy virus in Tamdy genogroup, have also been reported in Uganda, Central Asian countries, and Central and Kyrgyzstan, respectively (7–11). Recently, human infection cases of emerging and reemerging orthonairoviruses in far eastern Asian countries, i.e. Tamdy virus, Táchéng tick virus-1, Songling virus, Yezo virus (YEZV), and Wetland virus, have been attracting attention (3, 10, 12–15). These reports suggest that the increasing number of human pathogenic orthonairoviruses has been becoming greater public health concern. However, genetic divergence of orthonairoviruses and sporadic outbreaks of their infection hampers diagnosis and development of therapeutic agents or vaccines.

Human pathogenic orthonairovirus infections are associated with acute febrile illness. Crimean-Congo hemorrhagic fever (CCHF) caused by CCHFV is one of the most serious tick-borne infectious diseases and well documented. CCHF has a range of outcomes, from asymptomatic or mild symptoms to fatal infection. CCHF presents in four distinct stages: incubation, pre-hemorrhagic, hemorrhagic, and convalescence (5). The incubation period usually lasts less than one week, depending on the viral load and the transmission route. In the pre-hemorrhagic stage, patients present with nonspecific symptoms such as fever, headache, myalgia, and dizziness. In the hemorrhagic stage, hemorrhagic manifestations including ecchymoses on the skin, epistaxis, melena, hemoptysis, hematuria, and bleeding from injection sites are common. Hematology and blood biochemistry tests show thrombocytopenia, leukopenia, and elevated alanine transaminase (ALT), aspartate transaminase (AST), lactate dehydrogenase, and creatine phosphokinase levels (5, 16, 17). In severe cases, the disease progresses rapidly, causing disseminated intravascular coagulation, multiple organ failure, shock, resulting in fatality (5, 18–21). In survivors, the convalescence phase begins approximately 10 days after onset, with

returning of hematologic parameters to normal and development of humoral and cellular immunity. Comparative studies suggest that high levels of viremia, thrombocytopenia, elevated AST, ALT, and inflammatory cytokine levels, hematemesis, and melena are associated with fatal cases (17, 18, 22, 23). However, the viral factors involved in the disease severity are unknown. In other cases of orthonairovirus infection, nonspecific symptoms such as fever, headache, dizziness, and myalgia are reported primarily, with some cases showing elevated AST and ALT levels, thrombocytopenia, and leukopenia (3, 6–8, 10, 12, 14, 15). However, due to the limited number of available case reports and sporadic outbreaks, these diseases are not well characterized, hampering their treatment, prevention, and diagnosis. These facts emphasize the public health importance of controlling orthonairovirus infections. A major difficulty in controlling orthonairovirus infections is the lack of available specific treatments. Therefore, the development of therapeutic options and antiviral drugs is urgently required.

The establishment of infection models that mimic the pathogenesis of the disease would provide valuable insight into the discovery of new therapeutic targets and the validation of therapeutic agents. Among the human pathogenic orthonairoviruses, animal infection models have been reported for CCHFV, Dugbe virus, Issyk-Kul virus, and Wetland virus to understand their pathogenicity in mammals. CCHFV is the most pathologically studied orthonairovirus using animal infection models, including mouse and macaque infection models (5, 24). Mice deficient in interferon (IFN) signaling by genetic deficiency in the type I interferon receptor (IFNR) or signal transducer and activator of transcription 1 (STAT1), or by administration of anti-type I IFNR antibodies have been widely used as models for lethal CCHFV infection (25–28). In these mouse models, uncontrolled viral replication, inflammatory immune response, and liver damage were associated with lethality and correlated with findings in human fatal cases. These findings suggest that mouse infection models may partially reflect the pathogenesis of human lethal infection and are useful for the evaluation of antiviral agents, monoclonal antibodies, and vaccines. Neuroinvasive property of Dugbe virus infection has been demonstrated using newborn mice and type I IFNR-knockout (KO) mice (29, 30). A study of Issyk-Kul virus infection in type I IFNR-KO mice showed that Issyk-Kul virus replicated in the liver and spleen, resulting in liver dysfunction and increased cytokine and chemokine levels in the blood (31). Wetland virus was lethal to wild-type C57BL/6, BALB/c, and Kunming mice (15). In Wetland virus-infected BALB/c mice, viral antigens and viral RNA were detected in liver, spleen, kidney, lung, and brain tissues. Histopathological analysis revealed hepatocyte necrosis in the liver, disappearance of white pulp in the spleen, alteration of bronchiolar structures in the lungs, and inflammation of the cerebral cortex in the brain.

In addition to human pathogenic orthonairoviruses, animal infection models of several orthonairoviruses isolated from ticks or bats, including Hazara virus, Tofla virus, and Leopards Hill virus (LPHV), have also been established (31–33). Although no human cases of infection with these viruses have been reported, these animal infection models are valuable to understand their potential infectivity in mammals and comprehensive infection mechanisms of orthonairoviruses. In addition, they can be useful surrogates for studying CCHFV infection and testing antiviral agent candidates against CCHFV without requiring biosafety level (BSL) 4 facilities. Hazara virus and Tofla virus belong to the NSD genogroup, which includes CCHFV (Fig 1B). Inoculation with Hazara virus caused lethal infection in type I IFNR-KO mice (32). Pathological changes in the liver, spleen, and lymph nodes observed in this mouse model were similar to those in the CCHFV-infected mouse models. Tofla virus caused lethal infection with gastrointestinal disorders in type I IFNR-KO mice (33), and *in situ* hybridization analysis detected viral RNA in the epithelium of the stomach, duodenum, and small intestine with erosive changes. LPHV belongs to the Kasokero genogroup (Fig 1B) and was isolated from bats in Zambia (34). LPHV caused lethal infection in wild-type C57BL/6 mice and hepatic necrosis and small intestinal hemorrhage were observed. Blood biochemistry tests showed elevated liver enzymes and serum total bilirubin levels and decreased blood glucose levels. Complete blood count revealed severe leucopenia and thrombocytopenia. These pathological changes, biochemical changes and blood cell counts closely resemble those seen with CCHFV in humans.

Among the emerging and reemerging orthonairoviruses reported in East Asia, Tamdy virus, Táchéng tick virus-1, and Songling virus belong to the Tamdy genogroup, and YEZV belongs to the Sulina genogroup, respectively (Fig 1B). These viruses are phylogenetically distinct from other orthonairoviruses that have been analyzed for their pathogenicity in human cases and/or animal infection models. Therefore, the characterization of these emerging and re-emerging orthonairoviruses is important for a comprehensive understanding leading to prevention and treatment of orthonairovirus infections. However, the pathogenicity of those orthonairoviruses, belonging to the Tamdy or Sulina genogroup, in humans and animals remains uncharacterized, as there are currently few case reports and the lack of established animal infection models.

YEZV (*Orthonairovirus yezoense*) is an emerging orthonairovirus isolated from human patients who had bitten by ticks in Hokkaido, Japan (3). YEZV genome was detected in field ticks, including *Ixodes persulcatus*, *Ixodes ovatus*, and *Haemaphysalis megaspinosa*, in Japan and China (3, 35–37). Furthermore, YEZV RNA was detected in ticks infesting raccoons and migratory birds in Japan, suggesting that the virus circulates between ticks and wildlife and wild birds (36, 37). In serological studies, anti-YEZV antibodies were detected in serum samples from

wild raccoons, deer, and raccoon dogs in Hokkaido, Japan (3, 36). Currently, at least 28 infected patients have been reported in Japan and China (3, 35, 38–40). In clinical cases, acute fever, thrombocytopenia, leukopenia, and elevation of liver enzymes, creatine kinase, and ferritin levels were reported. Patients can only be treated symptomatically because of no available specific treatment. The pathogenicity of YEZV is still unclear due to the small number of clinical cases and the lack of animal models of YEZV infection. Therefore, the establishment of animal models is necessary to elucidate the pathogenicity of YEZV and to evaluate antiviral agents.

Ribavirin is a guanosine analog with a broad-spectrum of antiviral activity (41). The mechanism of action of ribavirin is still under investigation, but it has been reported to inhibit viral RNA synthesis, mRNA capping, and inosine monophosphate dehydrogenase activity, to induce lethal mutations in the virus, and to have immunomodulating effects in animals (41, 42). Ribavirin is an antiviral agent widely used for CCHF patients with the recommendation by the World Health Organization (WHO) as a treatment for CCHF. However, the data on its efficacy in humans and animal models remain controversial. Although some clinical reports propose its therapeutic efficacy, meta-analyses suggest that there are insufficient data to support its efficacy due to confounding factors in the data set (22, 23, 43–45). The efficacy of ribavirin against CCHFV infection is inconsistent in animal experiments. Ribavirin showed anti-CCHFV effects in STAT-1-KO mouse and STAT-2-KO hamster models, but no efficacy in a type I IFNR-KO mouse model (25, 46–48). In contrast to ribavirin, favipiravir (T-705) showed significant anti-CCHFV effects in animal infection models, including mouse and macaque models (46, 47, 49, 50). T-705 is a pyrazine analog with a broad-spectrum of antiviral activity and converted to favipiravir ribofuranosyl-5'-triphosphate by host intracellular kinases, which inhibit viral replication by acting on viral polymerases (51, 52). T-705 is approved for treatment of novel influenza and COVID-19 in several countries. In addition, clinical trials conducted in China and Japan have suggested that T-705 is effective in the treatment of severe fever with thrombocytopenia syndrome (SFTS), a tick-borne viral infectious disease in East Asia, and it was approved as the first treatment for SFTS in Japan in 2024 (53–55). These findings suggest that ribavirin and T-705 have potential for the treatment of a broad range of orthonairovirus infections.

In this dissertation, a mouse model of YEZV infection was established for the analysis of the pathogenicity of YEZV and validation of antiviral agents. In Chapter I, histopathological and virological analysis using type-I/II IFNRs-KO mouse (AG129) model revealed that YEZV replicates primarily in the liver and spleen resulting in fatal hepatitis. In Chapter II, the effect of antiviral agents against YEZV was examined. Both ribavirin and T-705 showed anti-YEZV effects *in vitro*. AG129 mice treated with T-705 were rescued from lethal YEZV infection thereby

successfully demonstrating the antiviral effect of T-705 in this mouse model. These studies will provide further understanding of the pathogenesis of YEZV and allow for the evaluation of new antiviral agents.

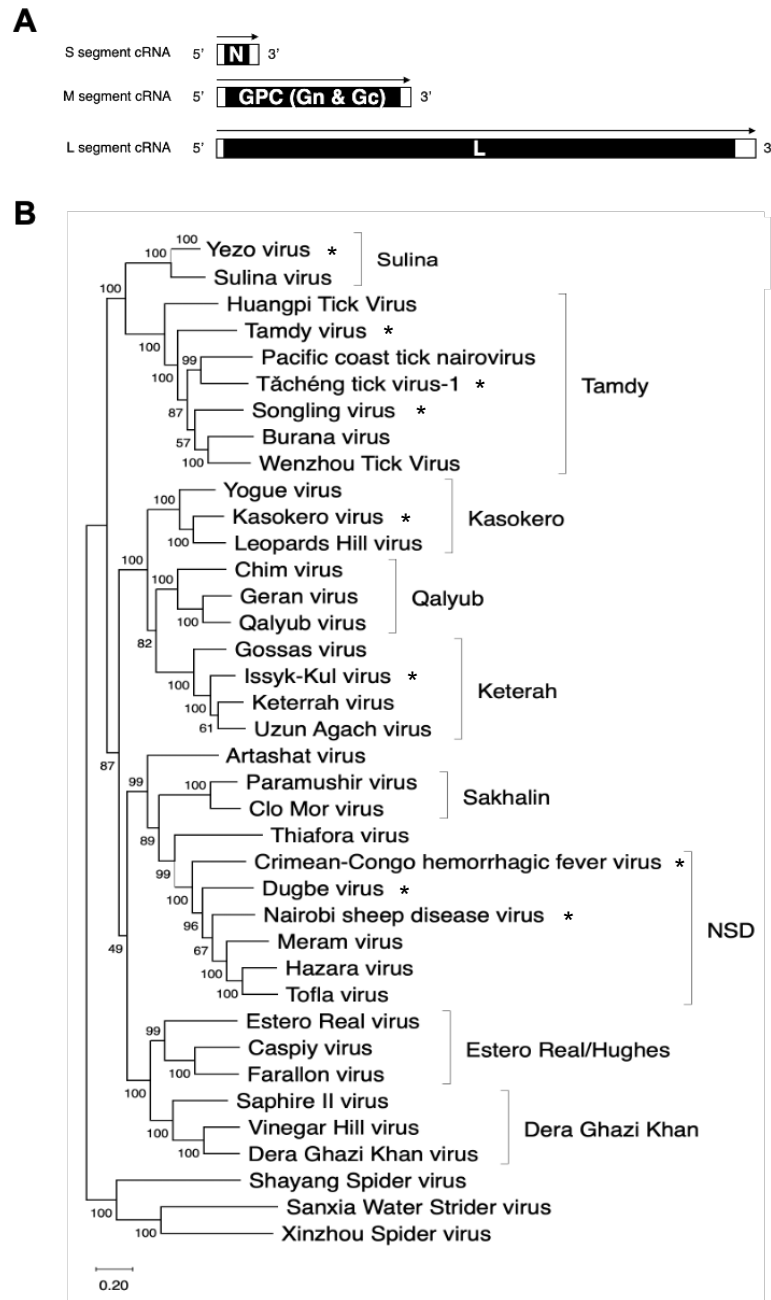


Fig 1. Schematic diagram of YEZV cRNA segments and phylogenetic relationship of representative orthonairoviruses.

(A) A schematic diagram of the cRNA segments encoding the proteins (N, GPC and L) of YEZV is shown. Arrows and black boxes indicate the direction of translation and open reading frames, respectively. (B) To illustrate the phylogenetic relationships among orthonairoviruses, a phylogenetic tree of the L protein coding sequence is shown as a representative example. Orthonairovirus genogroups are indicated to the right of the trees. Asterisks indicate human pathogenic viruses. The trees were constructed using the maximum-likelihood method with 1,000 bootstraps. The percentage of trees in which associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA11.

Chapter I: Establishment of a lethal mouse model of YEZV infection

Summary

Emerging and reemerging tick-borne orthonairovirus infections (family *Nairoviridae*), which are genetically distinct from Crimean-Congo hemorrhagic fever virus, have been recently reported in East Asia. Here, to better understand the pathogenesis of these infections, a mouse model of YEZV infection was established using AG129. YEZV-inoculated AG129 mice showed body weight loss and died by 6 days post infection (dpi). Blood biochemistry tests showed elevated liver enzyme levels, similar to YEZV-infected human patients. Histopathological analysis revealed degeneration of hepatocytes and disruption of follicular structures in the spleen. The viral antigens were detected diffusely in the liver and spleen at 2 and 4 dpi by immunohistochemical staining, and these signals were decreased at 6 dpi. Viral titers in organs and serum peaked at 2 dpi and decreased in a time-dependent manner. The present mouse model will help us to comprehend the pathogenicity of the emerging tick-borne orthonairoviruses.

Introduction

The genus *Orthonairovirus* in the family *Nairoviridae* consists of 61 virus species (1). The genome of orthonairovirus consists of three single stranded negative-sense RNAs called the S, M, and L segments, which encode N, GPC, and L, respectively. (Fig 1A). Certain orthonairoviruses are well known as human and/or animal pathogens. These include CCHFV (5), Nairobi sheep disease virus (4), Dugbe virus (6), Kasokero virus (7), Issyk-Kul virus (8), and Erve virus (9). Recently, emerging and reemerging human infections of tick-borne orthonairoviruses belonging to Tamdy or Sulina genogroups, which are phylogenetically distinct from previously reported human pathogenic orthonairoviruses have been identified (Fig 1B). These include Tamdy virus (10), Tǎchéng tick virus-1 (12, 13), Songling virus (14), and YEZV (3, 35) and have been reported to occur in China and Japan. These emerging and reemerging tick-borne orthonairoviruses highlight the potential public health importance associated with the Tamdy and Sulina genogroups.

YEZV, an emerging orthonairovirus in the Sulina group was originally isolated from febrile patients who had a history of tick bites in Hokkaido, Japan. To date 28 infected patients have been reported which include retrospectively identified patients both in Japan and China (3, 35, 38–40). In clinical cases, acute fever with thrombocytopenia, leukopenia, and elevation of liver enzymes, creatine kinase, and ferritin levels were reported. At this time, no pathological studies have been conducted in human patients infected with YEZV or those with the other emerging orthonairoviruses including Tamdy virus, Tǎchéng tick virus-1, and Songling virus.

Previously reported small animal infection models of orthonairoviruses, such as CCHFV have been established using immunodeficient mice, such as IFNR-KO mice (25–28, 30–33). In these models, the mice exhibit a variety of pathogenic features including liver damage, gastrointestinal disorders, and neurovirulence. Notably liver damage is a representative pathological feature commonly found in variety of orthonairovirus-infected mice (25–28, 31, 32, 34) and also human CCHF patients (5, 21, 56–58). Elevated levels of liver enzymes in YEZV patients suggest that YEZV infection may also potentially cause liver damage in humans. To investigate the pathology of YEZV infection, a lethal mouse model of YEZV infection using AG129 mice (59) was established. Histopathological and virological analysis using this model revealed that YEZV replicates primarily in the liver and spleen resulting in a fatal hepatitis. The present study is the first small animal model for YEZV infection and will help our understanding of the pathogenetic features of these emerging infections.

Material and Methods

Ethics statement

All animal experiments were approved by the Animal Care and Use Committee of Hokkaido University (Approval numbers: 18-0149 and 23-0063) and were performed in accordance with the committee's guidelines.

Cells and viruses

Vero (JCRB9013) and Vero E6 (JCRB9007) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 unit/mL penicillin, and 100 µg/mL streptomycin at 37°C with 5% CO₂.

YEZV strain HH003-2019 was isolated from infected patients as previously reported (3). Briefly, the virus was isolated from the supernatant of Vero E6 cells inoculated with the AG129 mouse serum collected at 5 days after intraperitoneally inoculation of infected patient's plasma. The virus was stored at -80°C until use.

Mouse infection experiments

All animal experiments were conducted in a biosafety level (BSL)-2 laboratory at the International Institute for Zoonosis Control, Hokkaido University with the above-described ethical approval. Female 5-week-old BALB/cCrSlc and C57BL/6NCrSlc mice were obtained from Japan SLC (Shizuoka, Japan) and used for animal experiments. AG129 mice, double KO immunocompromised mice lacking both type I and type II INFRs (S129 background), were obtained from Marshall BioResources (North Rose, USA) and maintained in our laboratory. Sex-mixed 5-weeks-old AG129 mice were used for the animal infection experiments.

BALB/cCrSlc, C57BL/6NCrSlc and AG129 mice were intraperitoneally inoculated with 100 µL of DMEM with or without 10⁴ focus formation units (FFUs) of YEZV under anesthesia. All mice were monitored for body weight changes and survival for 14 days, and in order to collect blood and tissue samples, five mice were mock-infected or infected with YEZV and sacrificed at 2, 4, and 6 dpi, respectively. A portion of the harvested tissues was homogenized in DMEM to prepare a 10% tissue emulsion for virus titration and RNA extraction. The other tissues were stored in phosphate-buffer 10% formalin for histopathological analyses. Blood biochemistry tests were conducted using VetScan VS2 with Multi Roter I Preventive Care Panel (Zoetis Japan, Tokyo, Japan). For the determination of the 50 % lethal dose (LD₅₀), AG129 mice were inoculated by intraperitoneal (ip) or subcutaneous (sc) injection with DMEM (100 µL) with or without 10⁴, 10³, 10², 10 or 1 FFU of YEZV under anesthesia and observed daily for body weight changes and

survival for 14 days.

Virus titration

The titers of YEZV used in this study were defined as FFUs. The samples diluted in DMEM were inoculated into Vero cells on 24-well plates and incubated at 37°C for 1 hour. The supernatant was removed, and the treated cells were cultured in minimum essential medium (MEM) containing 1% methylcellulose and 2% FBS for 6 days. The cells were then fixed with methanol for 20 minutes at -30°C. After blocking with phosphate buffered saline (PBS, pH7.5) containing 1% bovine serum albumin for 30 minutes, the cells were incubated with 1,000-time-diluted YEZV-infected mouse serum for 1 hour. After washing three times with PBS, the cells were incubated with Goat Anti-Mouse immunoglobulin G (IgG) H&L-horse radish peroxidase (HRP) (1:1,000, Abcam, Cambridge, UK) for 1 hour. After washing in the same manner, the cells were incubated with 3,3'-Diaminobenzidine (DAB), tetrahydrochloride (Nacalai twsque, Kyoto, Japan) for 10 to 20 minutes. The staining procedure was performed at room temperature. Stained foci were counted, and the titers were determined.

RNA extraction and Quantitative real-time PCR

Total RNA was extracted from the 10% tissue emulsions, mice serum or cell culture supernatant using TRIzol LS Reagent (Thermo Fisher Scientific, USA) and used to quantify YEZV viral RNA expression. Quantities of YEZV viral RNA were measured using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) with One Step PrimeScript III RT-qPCR Mix (Takara Bio, Shiga, Japan) and the specific primers (5'-GGTGTAAGCCCAACATCCT-3' and 5'-CTCAACCTGCTTCCAACCTATC-3') and probe (5'-/5Cy5/CCAAGGAAG/TAO/CACACAGATGGGTACA/3IAbRQSp/-3') for the L gene. The RT-qPCR reaction protocol included 5 minutes at 52°C, 10 seconds at 95°C and then 40 cycles of 5 seconds at 95°C and 30 seconds at 60°C. The signals were measured at the elongation step. A cloned plasmid was used for the standard to estimate the copy number. RT-qPCR was performed using qTower³ G (Analytik Jena, Jena, Germany), and the obtained data were analyzed by qPCR soft (Analytik).

Histopathological analysis

Formalin-fixed, paraffin embedded tissues were sectioned into 3 µm and stained with hematoxylin-eosin (H&E) or used for immunohistochemical staining (IHC). IHC was performed using YEZV-N peptide (POS: 165-182) immunized rabbit serum (1:20,000) (Cosmo Bio, Tokyo,

Japan) or anti ionized calcium binding adapter protein 1 (Iba1) goat polyclonal antibody (1:500, #001-27991, Wako, Osaka, Japan) as primary antibodies. For YEZV antigen detection, peroxidase-labeled goat anti-rabbit IgG polyclonal antibody (Nichirei Bioscience, Tokyo, Japan) and DAB were used as secondary antibodies and substrates, respectively. In the Iba1 and YEZV antigen double staining experiments, ImmPRESS (Peroxidase) Polymer Anti-Goat IgG Reagent (Vector laboratories, California, USA) and alkaline phosphatase-labeled goat anti-rabbit IgG polyclonal antibody (Nichirei Bioscience, Tokyo, Japan) were used as secondary antibodies and ImmPACT AMEC Red Peroxidase Substrate (Vector laboratories, CA, USA) and Vector Blue (Vector laboratories) as substrates. All images were acquired using Nikon ECLIPSE 80i light microscopy (Nikon, Tokyo, Japan).

Western Blotting

Liver emulsions, YEZV-N (Fig 1A)-transfected 293T cells, and YEZV-GPC (Fig 1A)-transfected HEK293 cells were treated with an EzRipa Lysis Kit (ATTO, Tokyo, Japan). The treated samples were separated by SDS-PAGE and subsequently transferred onto polyvinylidene difluoride membranes (Merck Millipore, Burlington, MA, USA). The membranes were blocked for 1 hour with 0.05% Tween 20 in Tris-buffered saline (TBST) that contained 5% skim milk at 25°C. The membranes were incubated overnight at 4°C with YEZV-N peptide immunized rabbit serum (1:1,000) or YEZV-G peptide (POS: 349-369) immunized rabbit serum (1:1,000) (Cosmo Bio, Tokyo, Japan) in TBST with 0.05% skim milk. After washing with TBST, the membranes were incubated 1 hour at 25°C with Goat Anti-Rabbit IgG H&L-HRP (1:10,000, Abcam) in TBST with 0.05% skim milk. The membranes were washed again with TBST and subsequently incubated with Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore) to visualize the peroxidase signal. Beta-actin was detected by the same protocol using Anti- β -Actin pAb-HRP-Direct (Medical & Biological Laboratories company limited, Tokyo, Japan) and HRP substrates. Chemical luminescent signals were detected by Amersham imageQuant 800 (Cytiva, MA, USA) and signal intensities were quantified using ImageJ software (60).

Statistical analysis

Survival rates were compared using log-rank test. Blood biochemistry levels were compared between uninfected and infected mice by the steel test. All statistical analyses were performed using Easy R (61). Statistical significance was set at $p < 0.05$.

Results

Clinical findings in YEZV-infected mice

To confirm the pathogenicity of YEZV in mice, 10^4 FFUs of YEZV isolated from a human patient were inoculated into BALB/c, C57BL/6, and AG129 (type-I/II IFNRs-KO) mice by ip (59). Body weight and survival of the mice were monitored for 14 days after the viral challenge. The body weights of BALB/c and C57BL/6 mice inoculated with YEZV gradually increased, similar to mock-infected mice, for 14 days without any fatalities, but the YEZV-inoculated AG129 mice showed body weight loss and 100% mortality by 6 dpi (Fig 2A). Subsequently, AG129 mice were inoculated with serially diluted YEZV by ip or sc. The 14-day fatality rates for ip of AG129 mice with 10^4 , 10^3 , 10^2 , 10, and 1 FFU of YEZV were 100%, 100%, 83%, 71%, and 71%, respectively, with respective median survival rates of 6, 7, 8, 8, and 9 days (Fig 2B). AG129 mice inoculated by sc with 10^4 , 10^3 , and 10^2 FFU of YEZV resulted in 14-day fatality rates of 100% and 71%, and 71%, respectively, with respective median survival rates of 8, 9, and 9 days (Fig 2C). Based on these results, AG129 mice were used in all subsequent experiments.

To investigate the pathology of lethal YEZV infection in AG129 mice, AG129 mice were inoculated with 10^4 FFU of YEZV by ip and sacrificed for blood and organ collection at 2, 4, and 6 dpi. Blood biochemistry tests showed elevated levels of alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin (TBIL) and decreased levels of total protein (TP), albumin (ALB), and glucose (Glu) in the YEZV-infected mice (Fig 3A). Marked elevation of ALT and AST levels were observed at 2 and 6 dpi, and interestingly, the increase in ALT and AST at 4 dpi was moderate. Serum TP and ALB levels decreased significantly at 4 and 6 dpi, and blood glucose levels decreased remarkably at 2, 4, and 6 dpi (Fig 3A). TBIL levels were found to be elevated only at 6 dpi (Fig 3A). Blood cell counts revealed increased white blood cell counts (WBC) after 4 dpi (Fig 3B). WBC and platelet counts (PLT) decreased slightly at 2 dpi, but there was no obvious leukopenia or thrombocytopenia as has been reported in human patients (Fig 3B and 3C). To examine the possibility of viremia, viral titers and viral RNA levels of YEZV in serum were quantified by focus-formation assay (FFA) and RT-qPCR, respectively. Viral titers in serum peaked at 2 dpi and remarkably decreased after 4 dpi (Fig 3D). In contrast YEZV viral RNA in serum was detected at similar levels at 2, 4, and 6 dpi (Fig 3E).

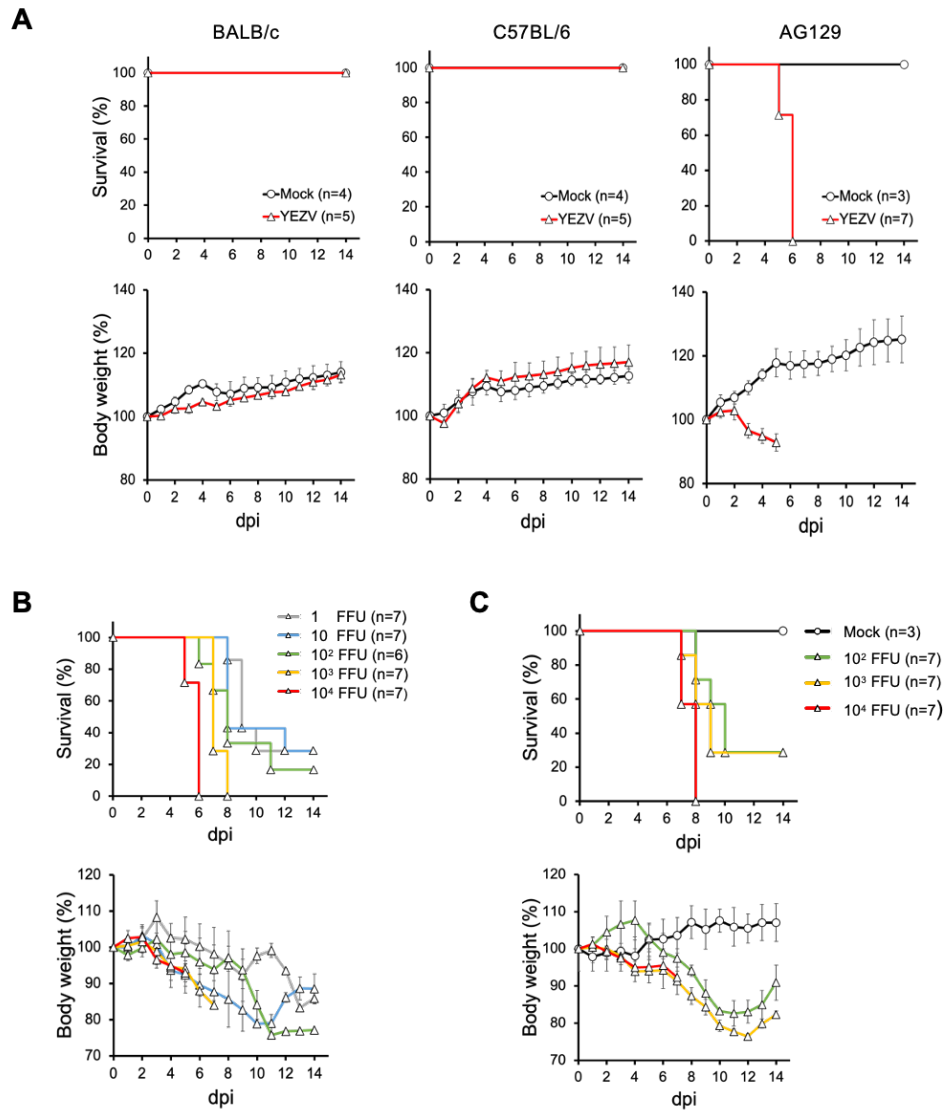


Fig 2. Body weight changes and survival of YEZV-infected mice.

(A) Female 5-week-old C57BL/6 mice and BALB/c mice ($n = 5$) and sex-mixed 5-week-old AG129 mice (male: $n = 4$, female: $n = 3$) were inoculated intraperitoneally with 10^4 FFU of YEZV. Fourteen-day survival rates and body weight changes were compared between mock and YEZV infected groups. Relative body weight is shown as means with standard deviations. (B) AG129 mice were inoculated intraperitoneally with 10^4 FFU (male: $n = 3$, female: $n = 3$, red line), 10^3 FFU (male: $n = 4$, female: $n = 3$, yellow line), 10^2 FFU (male: $n = 3$, female: $n = 3$, green line), 10 FFU (male: $n = 4$, female: $n = 3$, blue line), and 1 FFU (male: $n = 4$, female: $n = 3$, gray line) of YEZV and monitored for 14-day survival rate and body weight change. Relative body weight was calculated and is shown as means with standard deviations. (C) AG129 mice were inoculated subcutaneously with 10^4 FFU (male: $n = 3$, female: $n = 4$, red line), 10^3 FFU (male: $n = 3$, female: $n = 4$, yellow line), and 10^2 FFU (male: $n = 4$, female: $n = 3$, green line) of YEZV and monitored the 14-day survival and body weight change. Relative body weights are shown as means with standard deviations.

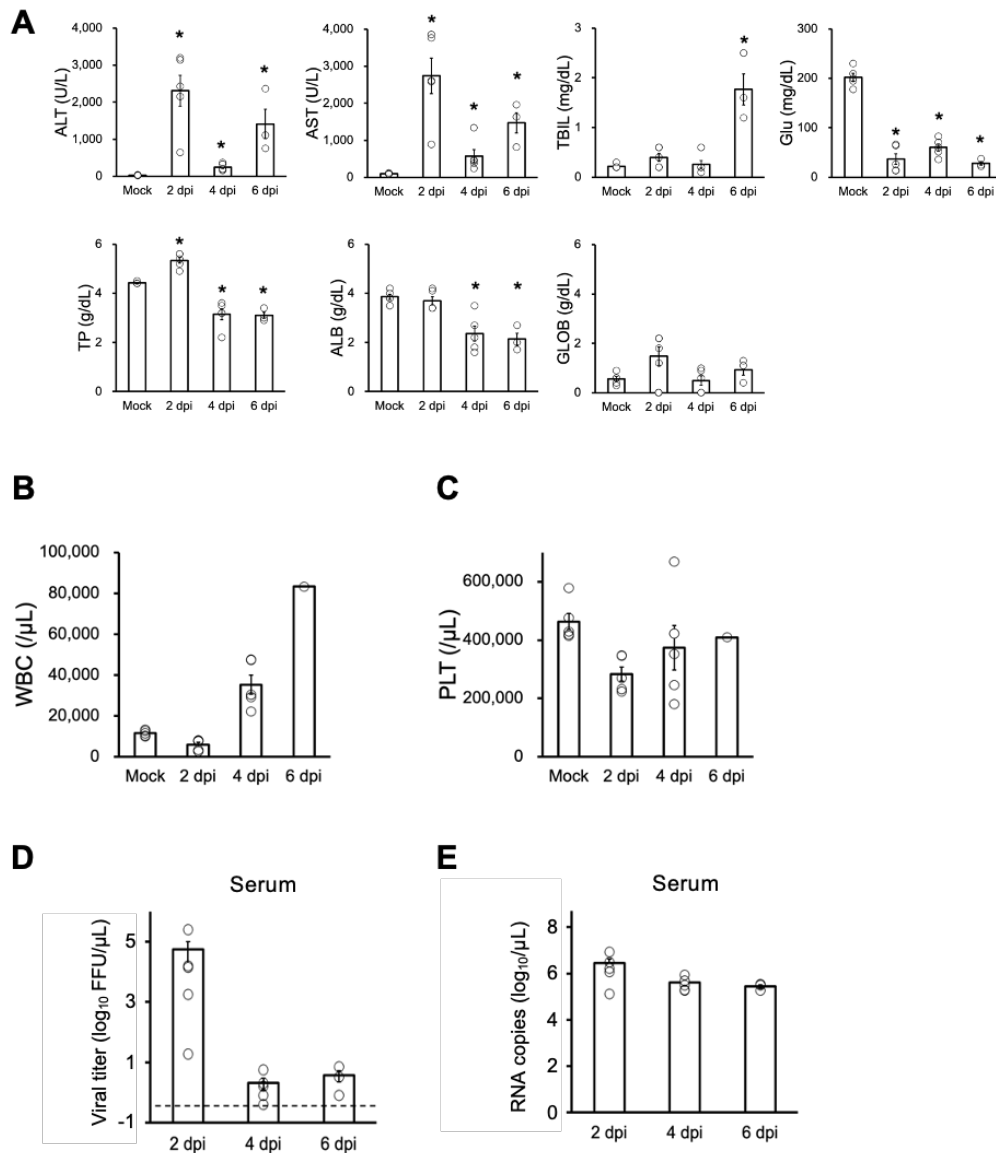


Fig 3. Hematological testing and validation of viremia in YEZV-infected AG129 mice.

(A-E) AG129 mice were inoculated with 10^4 FFUs of YEZV by ip and sacrificed for serum sampling at 2, 4, and 6 dpi (2 dpi: n = 5, 4 dpi: n = 5, 6 dpi: n = 3) for (A) blood biochemistry tests, (B) counting white blood cells, (C) counting platelets, and quantification of (D) viral titer and (E) viral RNA in serum. Means with standard errors were shown with individual values as white circles; (A) ALT, AST, TBIL, TP, ALB, globlin (GLOB) and Glu values in serum, (B) WBC, (C) PLT, (D) virus titers in serum quantified byFFA, and (E) copy numbers of viral RNA quantified by RT-qPCR. Statistical analyses were performed using the Steel test, and the significant difference ($p < 0.05$) is indicated as an asterisk. (D) Dotted line indicated the lower limit of detection (LOD) for the assay (0.4 FFU/μL). Plots lower than the LOD are indicated by the half value of LOD on the graph.

Pathological findings in YEZV-infected AG129 mice

To identify the mechanism underlying fatal infection of YEZV in AG129 mice, histopathological analysis was conducted at 2, 4, and 6 dpi. Macroscopically, the livers and spleens of YEZV-infected AG129 mice were enlarged and whitish at 4 and 6 dpi compared to those of mock-infected mice (Fig 4A). Microscopically, the liver tissue structure was disrupted with hepatocyte degeneration and infiltration of inflammatory cells at 4 and 6 dpi (Fig 4A). The splenic follicular structures were obscured at 4 and 6 dpi (Fig 4A). No clear histological changes were observed in the kidney, heart or small intestine (Fig 4B). To characterize the distribution of virus, YEZV antigens in the tissues were examined by IHC. Positive signals of YEZV antigen were diffusely observed in the liver and spleen at 2 and 4 dpi, and decreased at 6 dpi (Fig 5A). Since macrophages in the tissues, such as Kupffer cells are known as one of the target cells of CCHFV, YEZV infection in macrophages was analyzed by co-staining of Iba1, a marker protein constantly expressed on macrophages, with YEZV N. Viral antigens were detected mainly in hepatocytes in the liver, whereas Iba1(+) macrophages were positive for viral antigen in the spleen (Fig 5A and 5B). YEZV antigens were also detected in the kidney and heart, but at much lower levels than observed in the liver and spleen (Fig 5C). In contrast to AG129 mice, in C57BL/6 and BALB/c mice inoculated by ip with 10^4 FFU of YEZV, slight inflammatory cell infiltration was observed in the livers of these mice, but no clear pathological changes were observed in any of the organs including the liver (Fig 6). And very few viral antigens were detected only in hepatocytes of these mice at 2 and 4 dpi (Fig 6). Next the relationships between the observed pathological changes and inoculation route was examined. Similar pathological observations were also found in AG129 mice inoculated by sc with YEZV (Fig 7). These results suggest that YEZV replicates primarily in the liver and spleen and causes an acute hepatitis in AG129 mice.

To further confirm viral replication in the tissues, infectious virus and viral RNA loads were quantified. Viral titers in the liver, spleen, kidney, heart peaked at 2 dpi and decreased in a time-dependent manner (Fig 8A). Similarly copy numbers of viral RNA were also detected in the liver, spleen, kidney, and heart through 2 to 6 dpi (Fig 8B). In contrast to the AG129 model, infectious virus particles were not detected in the serum, liver, and spleen of either BALB/c and C57BL/6 mice at 2 dpi and 4 dpi, but viral RNA was detected in those samples (Fig 9).

To rationalize the gap between the viral titer and the viral RNA copy number in the AG129 model, viral protein production in the liver was measured by Western Blotting. Consistent with the results of the IHC, the N signal in the liver was not different between 2 dpi and 4 dpi (Figs 10A and 10B). On the other hand, at 4 dpi, the production of the envelope glycoprotein (Gn) was

reduced, which may have resulted in an inability to form complete viral particles and a concomitant reduction of viral titer (Figs 10A and 10B).

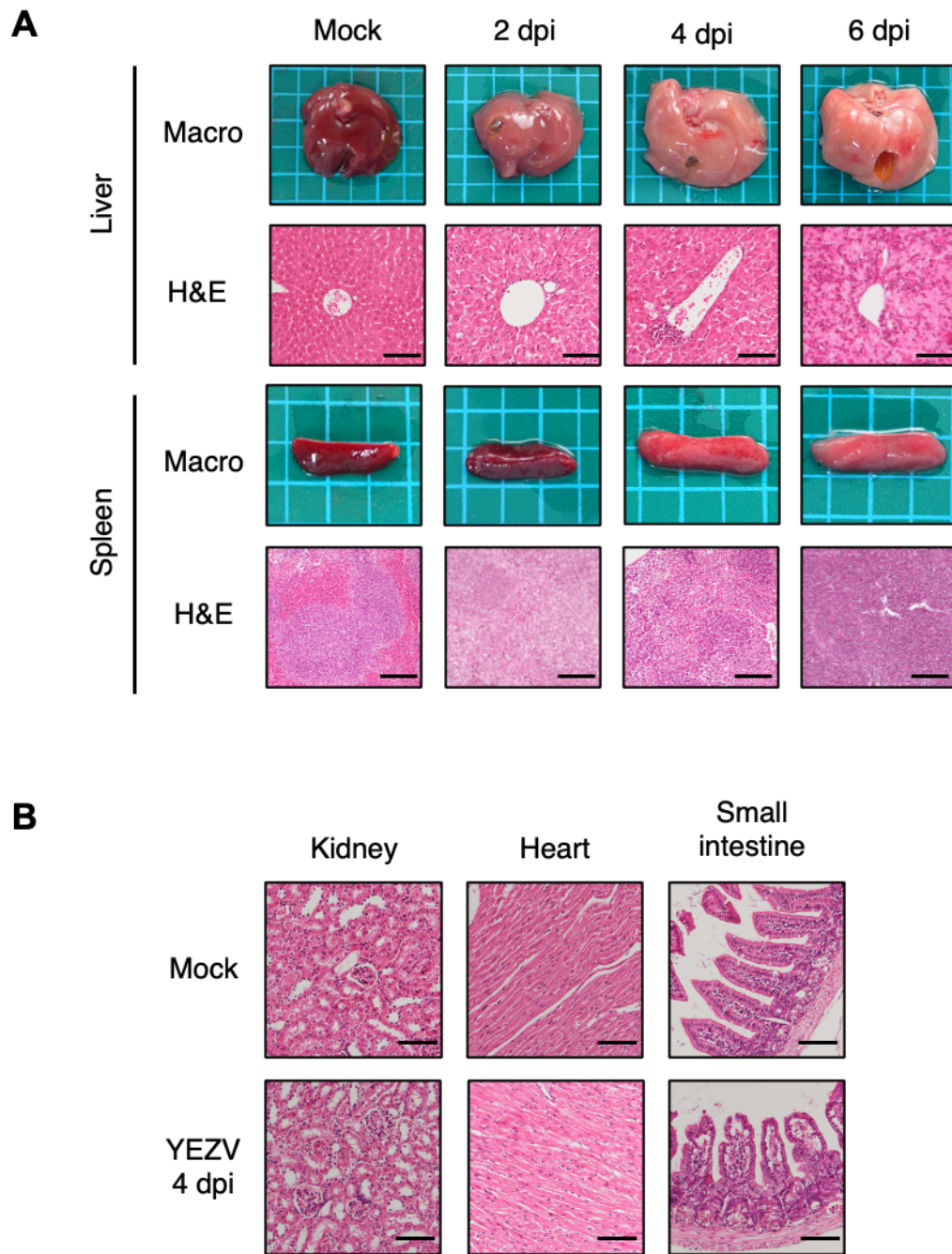


Fig 4. Histopathological findings in YEZV-infected AG129 mice.

AG129 mice inoculated by ip with 10^4 FFUs of YEZV were sacrificed at 2, 4, and 6 dpi to collect organ samples. Mock-infected mice sacrificed at 6 dpi are shown as controls. Mouse organs were fixed in 10% phosphate-buffered formalin and stained with hematoxylin-eosin (H&E). (A) Representative gross images and H&E-stained tissue images of the liver and spleen are shown. Each side of a square in the gross images is 5 mm. The scale bars on the histological images of the liver and spleen are 100 μ m and 200 μ m, respectively. (B) Representative H&E-stained histological images of the kidney, heart and small intestine of mock and YEZV-inoculated mice at 4dpi are shown. The scale bars on the histological images are 100 μ m.

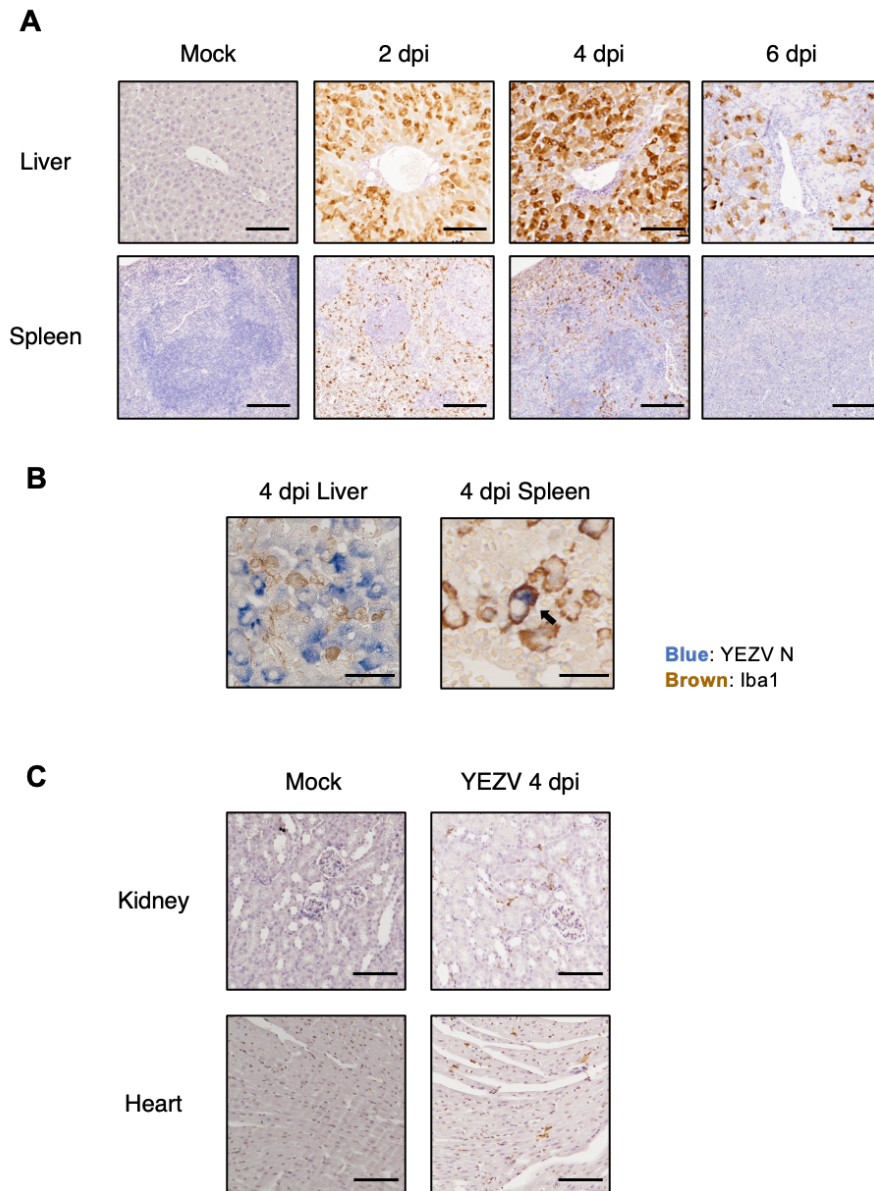


Fig 5. The distribution of YEZV antigens in the tissues of YEZV-infected AG129 mice.

AG129 mice inoculated by ip with 10^4 FFUs of YEZV were sacrificed at 2, 4, and 6 dpi to collect organ samples. Mock-infected mice sacrificed at 6 dpi are shown as controls. Mouse organs were fixed in 10% phosphate-buffered formalin and immunohistochemically stained for YEZV N protein. (A) Representative IHC images of the liver and spleen stained for YEZV N proteins are shown with viral antigens in brown. The scale bars on IHC images are 100 μ m. (B) YEZV N protein (blue) and macrophage marker, Iba1 (brown) were stained by IHC double staining in the liver and spleen of YEZV-infected mice. The scale bars on IHC images of liver and spleen tissues are 50 μ m and 20 μ m, respectively. Arrows indicate a cell in which both YEZV antigen and Iba1 were detected. (C) Representative IHC images of the kidney and heart of mock and YEZV-inoculated mice at 4 dpi are shown. Viral antigens were detected using anti-YEZV N protein rabbit antibody. The scale bars on IHC images are 100 μ m.

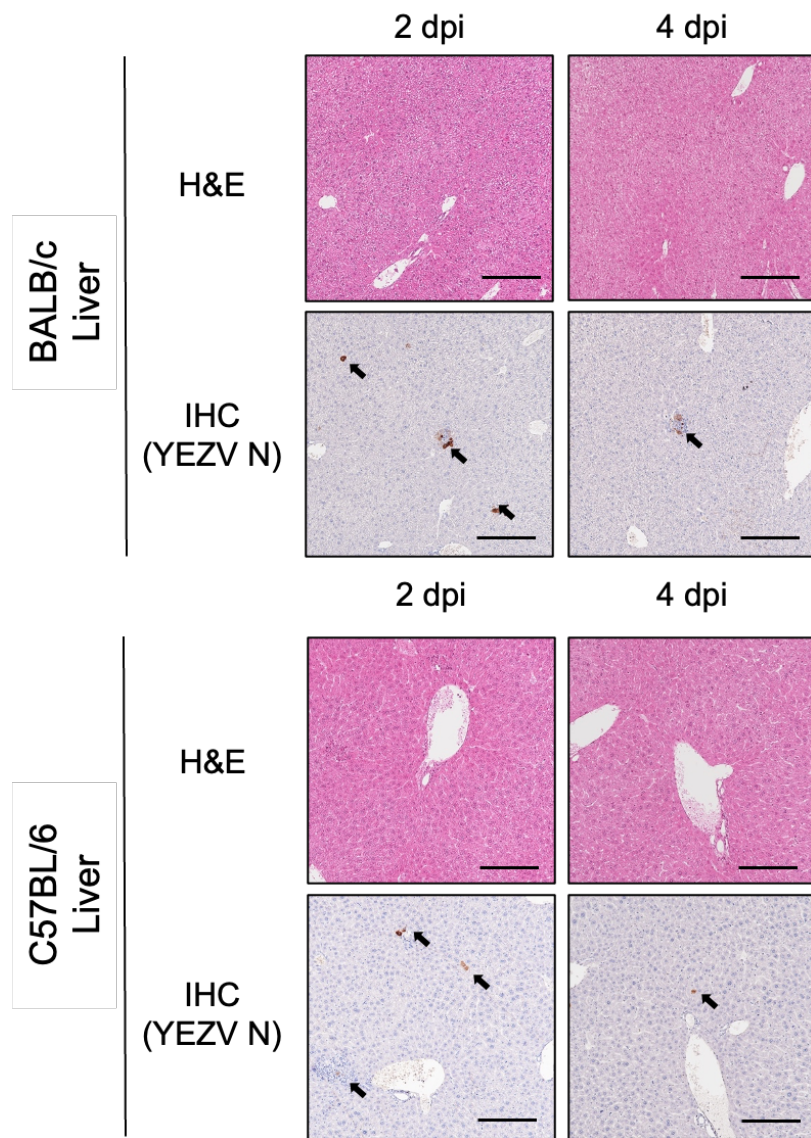


Fig 6. Representative H&E-stained tissue images and IHC images of the liver of YEZV-inoculated BALB/c mice and C57BL/6 mice.

BALB/c and C57BL/6 mice inoculated by ip with 10^4 FFUs of YEZV were sacrificed at 2 and 4 dpi to collect liver samples. Mouse livers were fixed in 10% phosphate-buffered formalin and stained with H&E or immunohistochemically stained for YEZV N protein. Representative H&E-stained histological images and IHC images of the liver of YEZV-inoculated BALB/c mice and C57BL/6 mice at 2 and 4 dpi are shown. The scale bars on the histological images are 200 μ m.

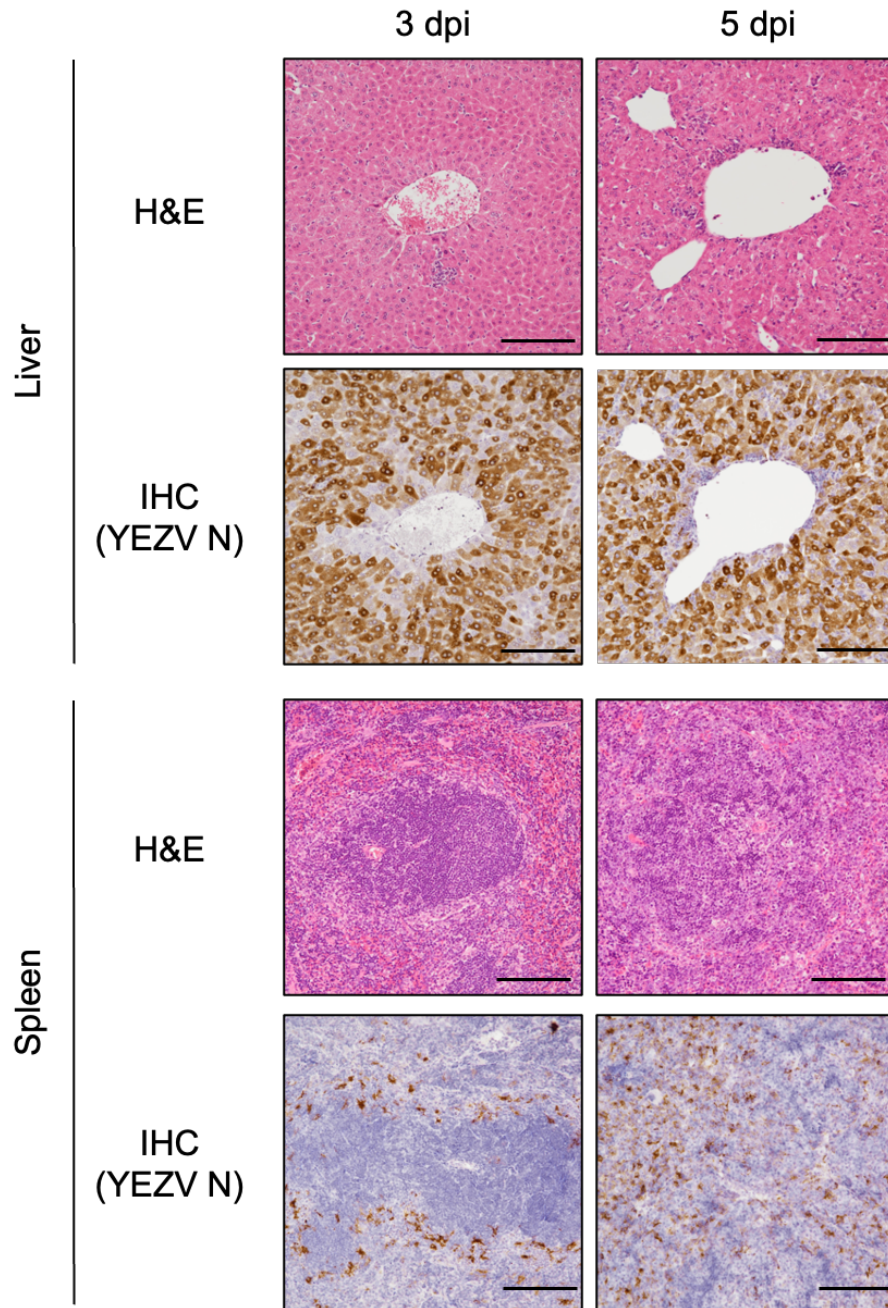


Fig 7. Representative hematoxylin-eosin-stained tissue images and IHC images of the liver and spleen of AG129 mice inoculated by sc with YEZV.

AG129 mice inoculated by sc with 10^4 FFUs of YEZV were sacrificed at 3 and 5 dpi to collect liver and spleen samples. Mouse organs were fixed in 10% phosphate-buffered formalin and stained with H&E or immunohistochemically stained for YEZV N protein. Representative H&E-stained histological images and IHC images of the livers and spleens of sc inoculated AG129 mice at 2 and 4 dpi are shown. The scale bars on the histological images are 200 μ m.

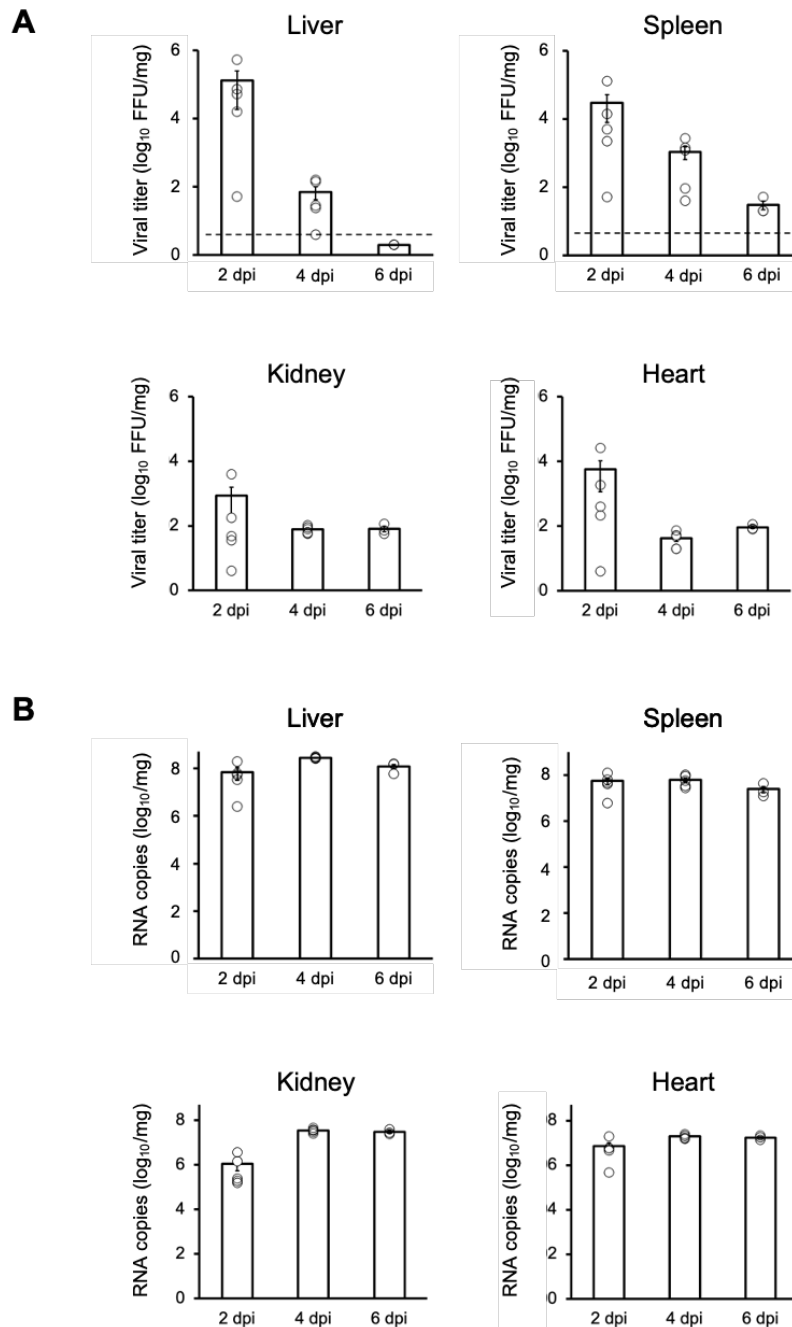


Fig 8. Viral titers and viral RNA in the liver and spleen of YEZV-infected AG129 mice.

Livers, spleens, kidneys, and hearts of YEZV-inoculated AG129 mice collected at 2, 4, and 6 dpi were used (2 dpi: $n = 5$, 4 dpi: $n = 5$, 6 dpi: $n = 3$) for analyzing (A) viral titers quantified by focus-formation assay and (B) the copy numbers of viral RNA (YEZV L-segment) quantified by RT-qPCR. Dotted lines indicated the LOD for the assay (4 FFU/mg). Plots lower than the LOD are indicated by the half value of LOD on the graph. White circles, thick bars, and thin lines indicate individual values, mean values and standard errors, respectively.

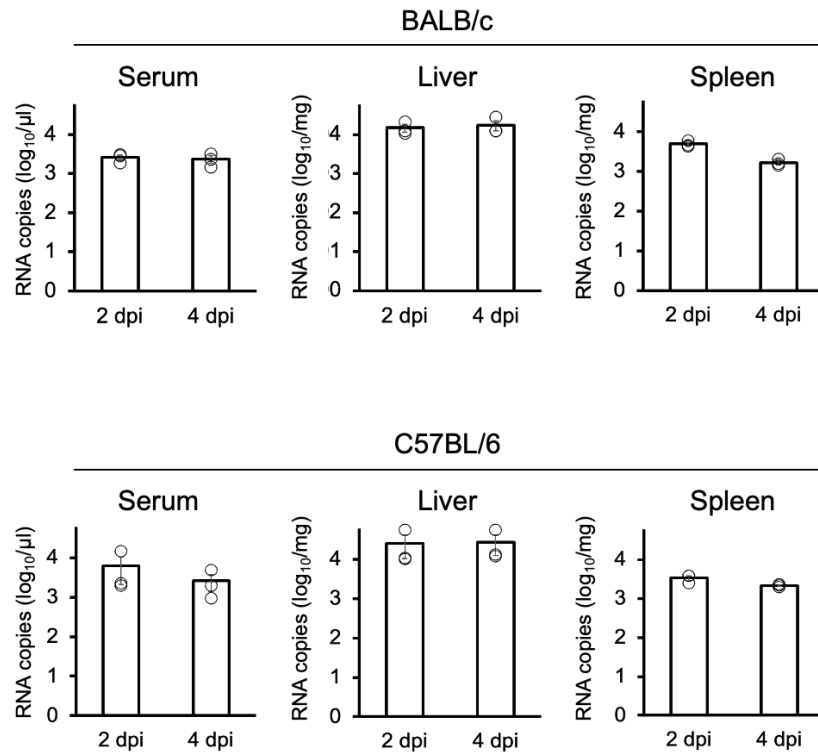


Fig 9. Viral RNA load of YEZV-infected BALB/c and C57BL/6 mice.

Serum, livers, and spleens of YEZV-inoculated BALB/c and C57BL/6 mice were collected at 2 and 4 dpi (2 dpi: n = 3, 4 dpi: n = 3) and subjected to RNA extraction. The amount of viral RNA (YEZV L segment) was quantified by RT-qPCR. White circles, thick bars, and thin lines indicate individual values, mean values, and standard errors, respectively.

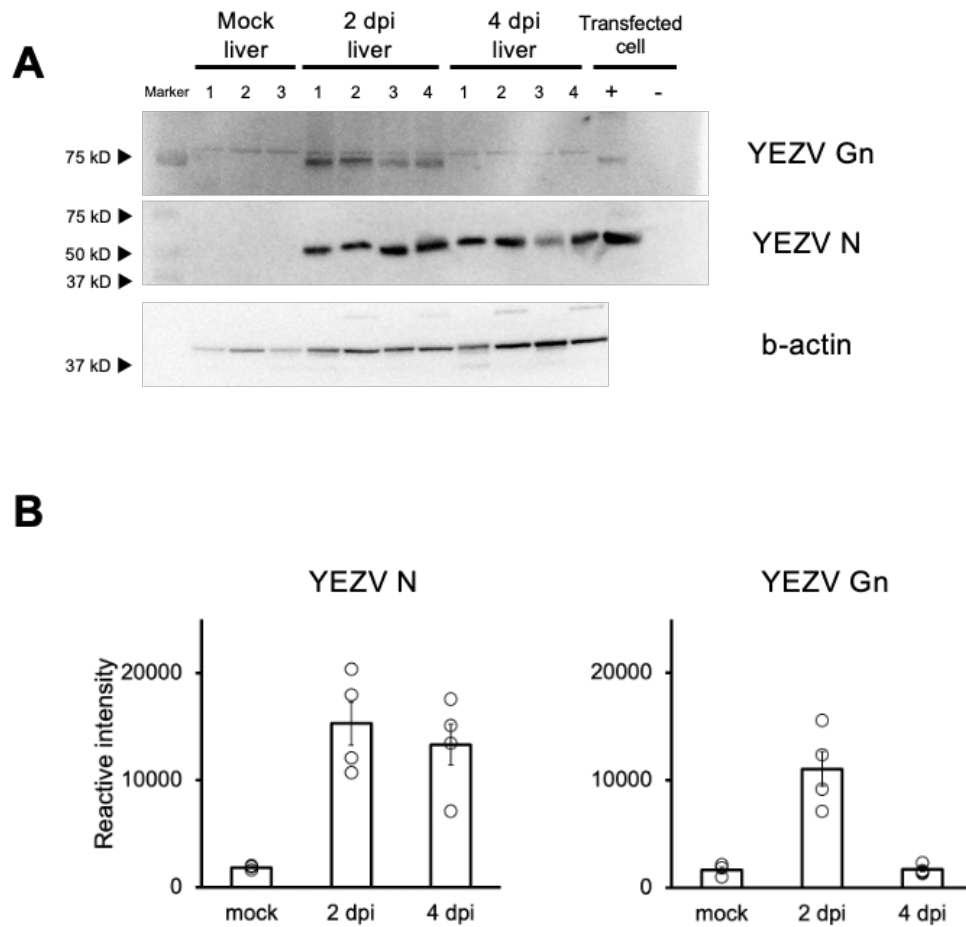


Fig 10. Quantification of YEZV-G and N protein expression in the liver of YEZV-infected AG129 mice.

(A and B) Equal amounts (4 μ g) of mock-infected and YEZV-infected mice liver emulsions were separated by SDS -PAGE. Immunoblotting analyses were conducted using anti-YEZV-G-protein, anti-YEZC-N-protein, and anti-beta-actin antibodies. HEK 293 cells transfected with YEZV-Gn or YEZV-N protein were used as positive controls. (B) Signal intensities were quantified using ImageJ software. White circles, thick bars, and thin lines indicate individual values, mean values and standard errors, respectively.

Discussion

YEZV is an emerging tick-borne orthonairovirus belonging to the Sulina genogroup and the first human pathogenic orthonairovirus in the Sulina genogroup. Together with the Tamdy genogroup viruses, which are phylogenetically related to the Sulina genogroup, YEZV can be considered as an etiological agent of emerging orthonairovirus infections in East Asia and thus, has the potential to be a burden on public health (10, 12–14). However, no pathological analyses of the orthonairoviruses belonging to the Sulina and Tamdy genogroups have been conducted. This mouse model for YEZV infection is the first for emerging tick-borne orthonairoviruses involving the Sulina and Tamdy genogroup.

Previous studies have used type-I IFNR-KO mice with either C57BL/6 or 129 backbone, STAT-1-KO mice and mice treated with cyclophosphamide to establish infection models of human pathogenic orthonairoviruses such as CCHFV and Dugbe virus (25, 26, 30). Here, this study demonstrated that AG129 mice, which are widely utilized in the field of flavivirus research as lethal infection models (62–66), can also be used to establish a model of tick-borne orthonairovirus infection. Because YEZV was isolated from a human patient using AG129 mice and showed swelling of the spleen (3), AG129 was the first choice for establishing a mouse model of YEZV infection.

The pathogenesis of lethal YEZV infection in AG129 was characterized in the present study. Consistent with previous reports of the other human pathogenic orthonairoviruses, YEZV was lethal in immunodeficient but not in wild-type mice. Wild-type mice showed no clinical signs but may have developed viremia, as viral RNA was detected in their serum. Therefore, it may be important to utilize the immunocompetent mouse as a relatively mild disease model in future studies. In comparison to the immunodeficient mouse models of orthonairoviruses, such as CCHFV, Hazara virus, and Tofla virus models, whose LD₅₀ were usually below 10 FFU or plaque forming unit regardless of the inoculation route, the LD₅₀ of YEZV infection in AG129 was compatible (26, 31–33). YEZV replicated mainly in the liver and spleen in AG129 mice, resulting in severe liver inflammation. Infectious virus was rapidly eliminated before the AG129 mice died, but the severe hepatitis remained even after virus clearance and could have contributed to the observed mortality. Elevated liver enzyme levels, including AST and ALT, consistent with reported human cases (3, 35), also suggests serious liver damage as found in mouse models of other orthonairovirus infection, including CCHFV, Hazara virus, Leopards Hill virus, and Keterah virus (25–28, 31, 32, 34, 67). Interestingly, the present mouse model showed a biphasic elevation of AST and ALT value consistent with biphasic-like increase of AST in a human patient [12], and which has not been reported in the other orthonairovirus infections in humans and also animal

models. In the studies of CCHFV, Kupffer cells, hepatocytes, and endothelial cells have been identified as the primary target cells in mouse and macaque infection models, and in human patients. (5, 24, 25, 28, 68–71). Kupffer cells have been proposed to be important for viral spread and replication in the liver during the early stages of CCHFV infection (28, 68, 69). However, in YEZV-infected mice, most of the virus antigen-positive cells in the liver were hepatocytes and only a small number of virus antigen-positive Kupffer cells were detected, even though virus antigen-positive signals were detected in Iba1 (+) macrophages in the spleen. While infectious viral particles and/or virus RNAs were detected in the kidneys and heart, no tissue damage or severe inflammation was observed histopathologically. In addition, blood urea nitrogen (BUN) and creatinine levels, indicators of renal function, were normal. Gastrointestinal disturbances as reported in mouse models of Leopards Hill virus and Tofla virus (33, 34) were not observed. These results suggest that YEZV is a hepatotropic virus like the other orthonairoviruses, although the less tissue damage other than in hepatocytes might be one of the unique features of YEZV that distinguishes it from the other orthonairoviruses, such as CCHFV.

In this model, even though the viral N protein was detected in the liver at 2 dpi and 4 dpi by both IHC and immunoblot analysis, the viral titer at 4 dpi was significantly lower than that at 2 dpi. On the other hand, the expression of viral G protein was significantly reduced at 4 dpi compared to 2 dpi. This result may be related with decreased viral titer, though further studies are needed to clarify the mechanism of decreased G protein expression.

Furthermore, although viral titers in tissue and serum declined after 4 dpi, the copy numbers of viral RNA in tissue and serum remained stable until 6 dpi. Together with the temporal reduction of AST and ALT levels at 4 dpi, later liver injury could be caused without the production of infectious virus particles but may still be associated with RNA replication.

Recently reported novel pathogenic tick-borne orthonairovirus infections associated with the Tamdy and Sulina genogroups (3, 10, 12–14, 35) have not been fully analyzed for their pathogenesis in humans as patients have been mainly identified by retrospective studies. Analysis using the present mouse model will contribute to our understanding of viral pathogenesis. In the future, it will be important to conduct detailed analyses in both human-infected patients and animal models to identify common pathological features. It will also be important to consider co-infections with other pathogens with tick-borne virus, which has been recently highlighted (72). In fact, four of the seven YEZV-infected patients reported in Japan were infected with *Borrelia* spp. (3). Furthermore, symptoms such as fever and malaise seen in YEZV-infected patients are atypical and might be difficult to distinguish from other infections. A co-infection mouse model of tick-borne pathogens may facilitate the development of broad-spectrum pan-tick-borne disease

treatment.

Chapter II: Verification of the effects of antiviral agents against YEZV

Summary

As an urgent measure against the newly emerging infectious disease, YEZV infection, it is important to validate the efficacy of existing antiviral drugs. In this chapter, the effects of antiviral agents against YEZV were examined using the YEZV-infected AG129 mouse model established in Chapter I. Favipiravir (T-705) and ribavirin were selected as candidates for therapeutic agents and both showed anti-YEZV activity *in vitro*. Since T-705 had a stronger anti-YEZV effect than ribavirin, the anti-YEZV effect of T-705 *in vivo* was subsequently verified using the mouse model of YEZV infection. AG129 mice treated with T-705 were rescued from lethal YEZV infection. In addition, viral titers and viral RNA copy numbers in tissue and serum of mice in the T-705-treated group were significantly decreased compared to those in the vehicle-treated group. This study demonstrates the anti-YEZV effect of T-705 in the mouse model of YEZV infection and provides a useful model for evaluating drug efficacy to develop therapeutic agents.

Introduction

Recently, emerging human pathogenic orthonaviruses have been reported in East Asia in succession (3, 10, 12–15). There is concern that the number of patients with newly emerging orthonairovirus infections will continue to increase, but there is no currently available treatment and relies on symptomatic therapy. The number of reported patients infected with YEZV, one of the emerging orthonaviruses, has increased to 28 in these three years since the first case was reported in 2021 to the present (3, 35, 38–40). Therefore, the development of antiviral agents against emerging orthonaviruses, including YEZV, is important to improve the public health response.

The nucleic acid analogs, favipiravir (T-705) and ribavirin have shown antiviral effects against a broad spectrum of RNA viruses (41, 51), including human pathogenic tick-borne bunyaviruses, severe fever with thrombocytopenia syndrome virus (SFTSV) and CCHFV (46, 47, 50, 73, 74). T-705 is converted to favipiravir ribofuranosyl-5'-triphosphate by host intracellular kinases, which inhibit viral replication by acting on viral polymerases (52). Previous studies have demonstrated the antiviral effects of T-705 in SFTSV-infected mouse models, CCHFV-infected mouse models and macaque models (46, 47, 49, 50, 73). Ribavirin is metabolized into ribavirin monophosphate, and ribavirin triphosphate. Several antiviral mechanisms are known for ribavirin, including inhibition of viral RNA-dependent RNA polymerase (RdRP) activity and the induction of viral mutation by substitution of GTP for ribavirin triphosphate (41). Ribavirin is recommended by the WHO as a treatment for CCHFV infection, but data on its efficacy in animal models and in humans remain controversial (23, 44, 47). Ribavirin showed anti-CCHFV effects in STAT-1-KO mouse and STAT-2-KO hamster models, but no efficacy in a type I IFNR-KO mouse model (25, 46–48).

In this study, the anti-YEZV activity of T-705 and ribavirin was demonstrated *in vitro*. Furthermore, AG129 mice treated with T-705 were rescued from lethal YEZV infection thereby successfully demonstrating the antiviral effect of T-705 in the established mouse model.

Material and Methods

Ethics statement

All animal experiments were approved by the Animal Care and Use Committee of Hokkaido University (Approval numbers: 18-0149 and 23-0063) and were performed in accordance with the committee's guidelines.

Cells and viruses

Vero (JCRB9013) and Vero E6 (JCRB9007) cells were maintained in DMEM supplemented with 10% FBS, 100 unit/mL penicillin, and 100 µg/mL streptomycin at 37°C with 5% CO₂.

YEZV strain HH003-2019 was isolated from infected patients as previously reported (3). Briefly, the virus was isolated from the supernatant of Vero E6 cells inoculated with the AG129 mouse serum collected at 5 days after intraperitoneally inoculation of infected patient's plasma. The virus was stored at -80°C until use.

Mice

All animal experiments were conducted in a BSL-2 laboratory at the International Institute for Zoonosis Control, Hokkaido University with the above-described ethical approval. AG129 mice, double KO immunocompromised mice lacking both type I and type II IFNRs (S129 background), were obtained from Marshall BioResources and maintained in our laboratory. Sex-mixed 5-weeks-old AG129 mice were used for the animal infection experiments.

Verification of the efficacy of antiviral agents

Favipiravir (T-705) (Angene International, Nanjing, China) and ribavirin (Fujifilm Wako Pure Chemical, Osaka, Japan) was purchased from Namiki Shoji (Tokyo, Japan) and Fujifilm Wako Pure Chemical Industries, respectively. T-705 was dissolved in dimethyl sulfoxide (DMSO) for *in vitro* experiments and in 0.5% methyl cellulose 400 solution for *in vivo* experiments. Ribavirin was dissolved in DMSO for *in vitro* experiments.

In the experimental infection *in vitro*, Vero cells were infected with YEZV at a multiplicity of infection (MOI: FFUs of inoculated virus/ number of seeded cells) of 0.001 per cell in the presence of various concentration of T-705 (0.01, 0.1, 1, 2, 4, 8, 16, 32, 64, and 128 µM) or ribavirin (0.01, 0.1, 2, 4, 8, 16, 32, 64, 128, and 256 µM). Supernatants of cell culture were then collected at 6 dpi and used for virus titration. The 50% inhibitory concentration (IC₅₀) and the 90% inhibitory concentration (IC₉₀) were calculated by regression analysis using ImageJ software (60). The 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium Bromide (MTT) assay was

performed to evaluate cell viability according to previously reported methods (75). Vero cells were cultured for 6 days in the presence of the designated concentration of agents without viral infection. Cell viability was calculated as follows: $[(\text{absorbance of cell culture supernatant in the presence of the agent} - \text{absorbance of culture medium without cells in the absence of the agent}) / (\text{absorbance of cell culture supernatant in the absence of the agent} - \text{absorbance of culture medium without cells in the absence of the agent})] \times 100$. Viral replication reduction assays and cell viability assays were performed in triplicate.

In the animal experiments, each AG129 mouse was inoculated by ip with 100 μL of YEZV (10^4 FFUs). The infected mice were orally dosed with T-705 (100 or 300 mg/kg/day) or vehicle using a stomach probe at 1 hour, 1 day, 2 days, and 3 days post-infection. The body weight change and survival of 6 vehicle-treated-mice, 7 mice treated with 100 mg/kg/day of T-705, and 6 mice treated with 300 mg/kg/day of T-705 were monitored for 14 days. In order to collect blood and tissue samples, 5 mice treated with 300 mg/kg/day T-705 and 5 mice treated with vehicle were sacrificed at 2 and 4 dpi, respectively.

Virus titration

The titers of YEZV used in this study were defined as FFUs. The samples diluted in DMEM were inoculated into Vero cells on 24-well plates and incubated at 37°C for 1 hour. The supernatant was removed, and the treated cells were cultured in MEM containing 1% methylcellulose and 2% FBS for 6 days. The cells were then fixed with methanol for 20 minutes at -30°C. After blocking with PBS containing 1% bovine serum albumin for 30 minutes, the cells were incubated with 1,000-time-diluted YEZV-infected mouse serum for 1 hour. After washing three times with PBS, the cells were incubated with Goat Anti-Mouse IgG H&L- HRP (1:1,000, Abcam) for 1 hour. After washing in the same manner, the cells were incubated with DAB (Nacalai twsque) for 10 to 20 minutes. The staining procedure was performed at room temperature. Stained foci were counted, and the titers were determined.

RNA extraction and Quantitative real-time PCR

Total RNA was extracted from the 10% tissue emulsions, mice serum using TRIzol LS Reagent (Thermo) and used to quantify YEZV viral RNA expression. Quantities of YEZV viral RNA were measured using RT-qPCR with One Step PrimeScript III RT-qPCR Mix (Takara Bio) and the specific primers

(5'-GGTGTAAGCCCAACATCCT-3' and 5'-CTCAACCTGCTTCCAACCTATC-3') and probe (5'-/5Cy5/CCAAGGAAG/TAO/CACACAGATGGGTACA/3IAbRQSp/-3') for the L

gene. The RT-qPCR reaction protocol included 5 minutes at 52°C, 10 seconds at 95°C and then 40 cycles of 5 seconds at 95°C and 30 seconds at 60°C. The signals were measured at the elongation step. A cloned plasmid was used for the standard to estimate the copy number. RT-qPCR was performed using qTower³ G (Analytik Jena), and the obtained data were analyzed by qPCR soft (Analytik Jena).

Histopathological analysis

Formalin-fixed, paraffin embedded tissues were sectioned into 3 µm and stained with hematoxylin-eosin or used for IHC. IHC was performed using YEZV-N peptide (POS: 165-182) immunized rabbit serum (1:20,000) (Cosmo Bio) or anti Iba1 goat polyclonal antibody (1:500, #001-27991, Wako) as primary antibodies. For YEZV antigen detection, peroxidase-labeled goat anti-rabbit IgG polyclonal antibody (Nichirei Bioscience) and DAB were used as secondary antibodies and substrates, respectively. All images were acquired using Nikon ECLIPSE 80i light microscopy (Nikon).

Statistical analysis

Survival rates were compared using log-rank test. Blood biochemistry levels were compared between uninfected and infected mice by the steel test. Statistical differences in viral RNA copy number between the two groups were examined by the Mann-Whitney U test. All statistical analyses were performed using EZR (61). Statistical significance was set at $p < 0.05$.

Results

Evaluation of the effects of antiviral agents on YEZV *in vitro*

The anti-YEZV effects of T-705 and ribavirin were evaluated using Vero cells *in vitro*. Both T-705 and ribavirin inhibited YEZV replication in Vero cells (Fig 10). The IC₅₀ and IC₉₀ of T-705 were 3.51 μ M and 8.79 μ M, respectively, and those of ribavirin were 12.32 μ M and 25.47 μ M, respectively. Since T-705 showed a stronger antiviral effect than ribavirin *in vitro*, the antiviral effect of T-705 was further analyzed *in vivo* using YEZV-infected AG129 mice model.

Evaluation of the effects of antiviral agents on YEZV *in vivo*

To investigate the effects of T-705 on YEZV *in vivo*, YEZV-infected AG129 mice were treated with 100 mg/kg or 300 mg/kg of T-705 immediately after the viral challenge. In these experiments, T-705 was orally administered once a day from 1 hour post-infection to 3 dpi. All mice in the vehicle-treated group died up to 7 dpi with body weight loss (Fig 11). In contrast, 100 mg/kg/day and 300 mg/kg/day of T-705-treated groups showed 57% and 100% survival rates, respectively (Fig 11). Mice in the T-705-treated group which survived during the monitoring period showed milder body weight loss than those in the vehicle-treated group (Fig 11).

To investigate the effects of T-705 treatment on tissue damage and viral replication, liver, spleen, and serum were collected at 2 and 4 dpi for pathological and virological analysis. Hepatocyte degeneration and infiltration of inflammatory cells in the liver was milder in T-705-treated mice than vehicle-treated mice (Fig 12). The follicular structure of the spleen was preserved at 4 dpi in T-705-treated mice (Fig 12). Positive signals for YEZV N proteins in the liver and spleen were markedly less in T-705-treated mice than in vehicle-treated mice (Fig 12). Viral titers in the liver, spleen, and serum of T-705-treated mice were under the detection limits (Fig 13A). Consistent with the virus antigen expression and titer, the copy number of YEZV viral RNA in the liver, spleen, and serum of T-705-treated mice was significantly lower than that of vehicle-treated mice (Fig 13B). In summary, the mouse model of YEZV infection clearly demonstrated the anti-YEZV effect of T-705 *in vivo*.

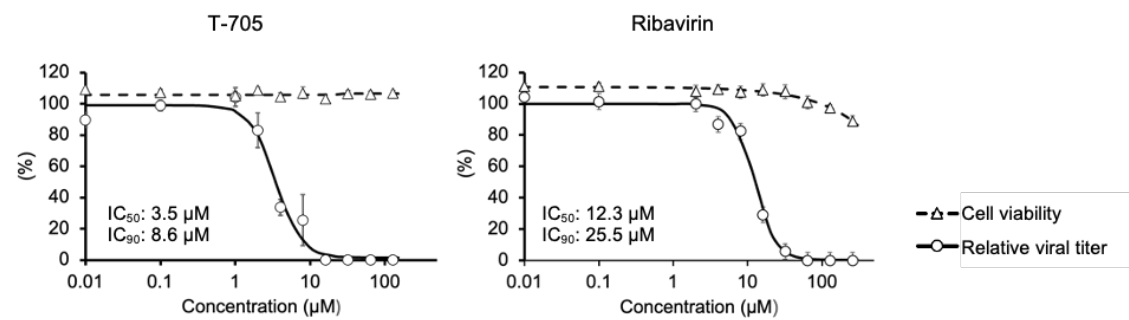


Fig 11. The effects of antivirals on YezV infection *in vitro*.

Inhibitory effects of T-705 and ribavirin on YezV replication in Vero cells were verified. Viral titers (white circles and solid lines) in the supernatant and cell viability (white triangles and dotted lines) at 6 dpi were compared with those in the absence of the chemical compounds.

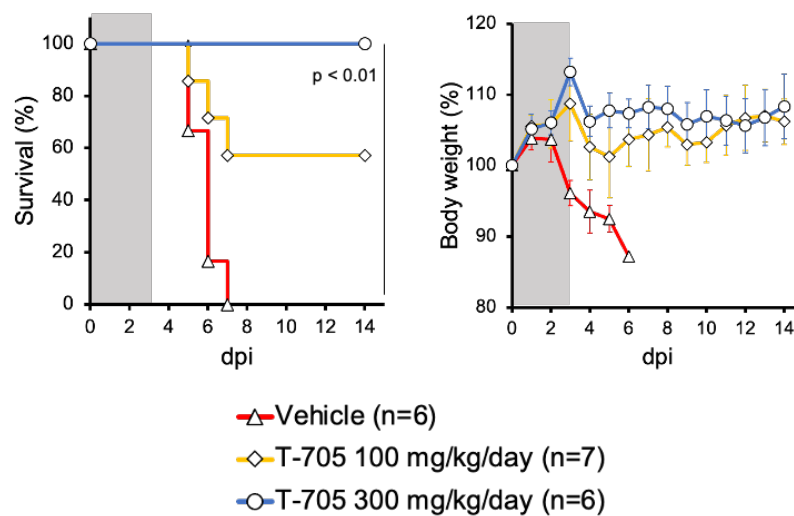


Fig 12. Survival and body weight change for 14 days of YEZV-infected AG129 mice treated with T-705 or vehicle

AG129 mice inoculated by ip with 10^4 FFU of YEZV were orally dosed with T-705 (100 or 300 mg/kg/day) or vehicle using a stomach probe at 1 hour, 1, 2 and 3 dpi (represented by gray color on the figure). All mice were monitored for survival (left panel) and body weight changes (right panel) for 14 days. Fourteen-day survival rates and body weight changes were compared with the vehicle-treated group, 100 mg/kg/day of T-705-treated group and 300 mg/kg/day of T-705-treated group using a log-rank test.

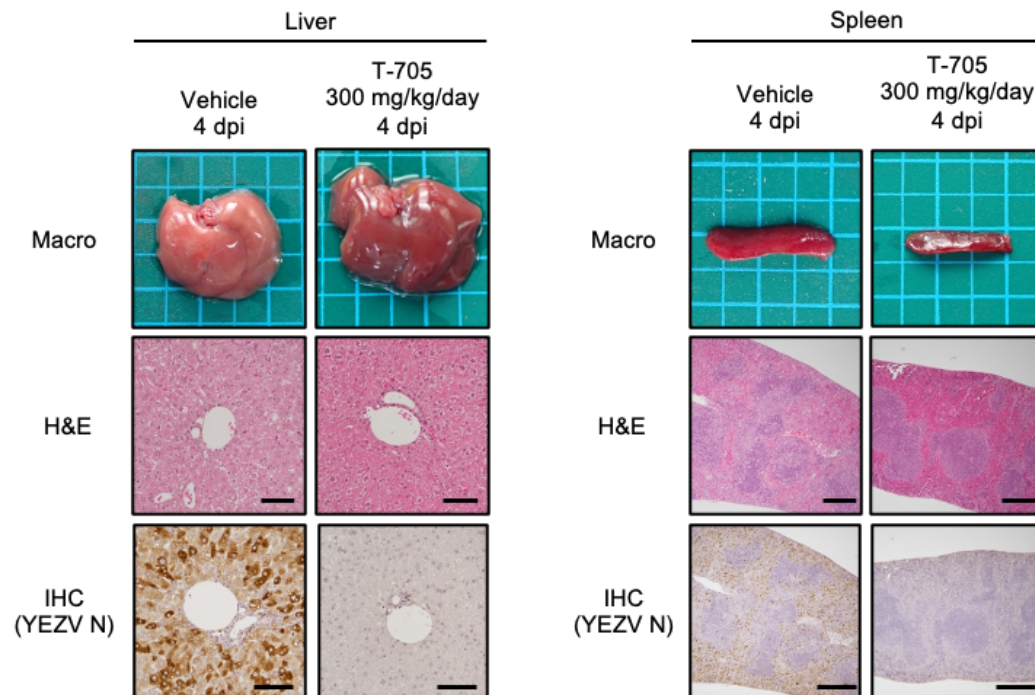


Fig 13. Histopathological findings in YEZV-infected AG129 mice treated with T-705 or vehicle

Vehicle- or 300 mg/kg/day of T705-treated YEZV-infected AG129 mice were sacrificed at 2 and 4 dpi to collect blood and tissue samples. Representative gross images and H&E-stained tissue images and IHC images of the liver and spleen of mice are shown. YEZV N proteins were detected by IHC in the liver and spleen of vehicle-treated and T-705-treated mice. Each side of a square in the gross images is 5 mm. The scale bars on the histological images of the liver and spleen are 100 μ m and 500 μ m, respectively.

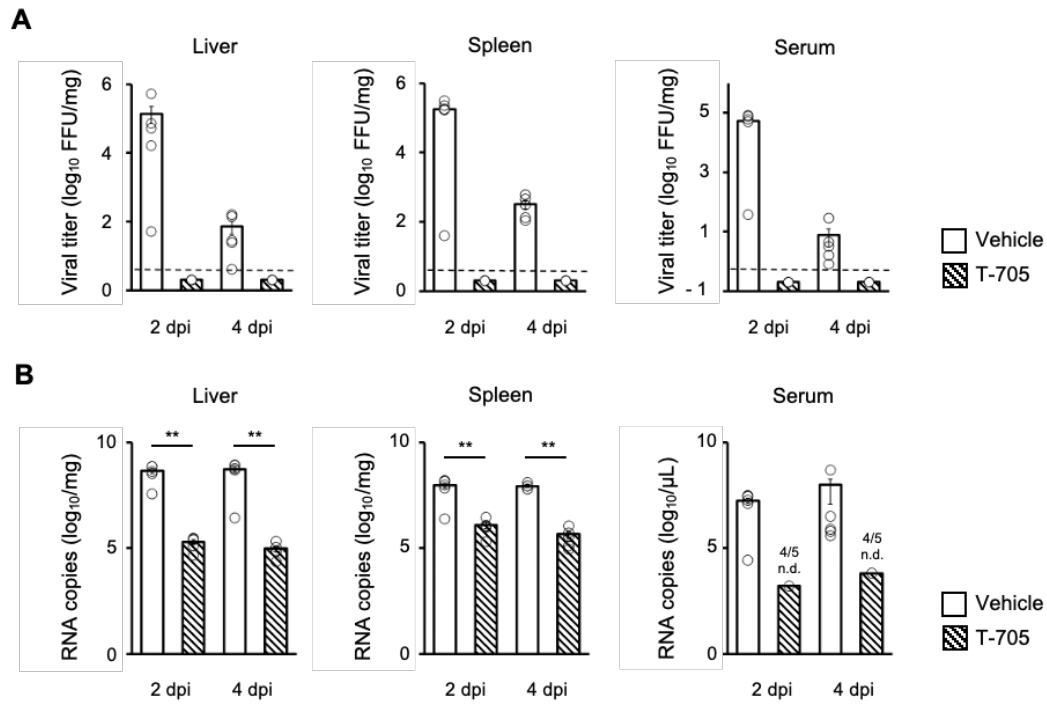


Fig 14. Viral titers and viral RNA in the liver, spleen and serum of YEZV-infected AG129 mice treated with T-705 or vehicle.

(A) Viral titers and (B) the copy number of viral RNA (YEZV L-segment) in the liver and spleen of vehicle-treated and T-705-treated mice were quantified. Dotted lines indicated the LOD for the assay (4 FFU/mg of liver and spleen and 0.4 FFU/μL of serum). Plots lower than the LOD are indicated by the half value of LOD on the graph. Statistical differences in viral RNA copy number between the vehicle-treated group and T-705-group were examined by the Mann-Whitney U test, and the significant difference ($p < 0.01$) is indicated as double asterisks.

Discussion

Recently, the emergence of new human pathogenic orthonairoviruses, including YEZV, has been reported in East Asia. There is concern that the number of those infection cases will increase, but there are no currently active therapeutic agents for the treatment of these newly emerging tick-borne orthonairovirus infections. One of the options for an immediate response to emerging infectious diseases is the validation of existing antiviral drugs. In this study, the anti-YEZV effects of T-705 and ribavirin were evaluated, and both agents inhibited YEZV replication *in vitro*. Consistent with other human pathogenic tick-borne bunyaviruses including CCHFV and SFTSV, T-705 showed smaller IC₅₀ and IC₉₀ than ribavirin against YEZV *in vitro* (46, 52, 73). In addition to *in vitro* results in this chapter, since previous studies have reported that T-705 showed better therapeutic effects than ribavirin in mouse infection models of CCHFV, SFTSV, and Heartland virus (47, 73, 76), the anti-viral effect of T-705 was evaluated using the YEZV infection mouse model. Oral administration of T-705 rescued YEZV-infected AG129 mice from lethal infection in a dose-dependent manner, and viral titers in tissues of T-705-treated mice were lower than the limit of detection (LOD). In addition, severe inflammation was not observed in tissues of mice treated with T-705. These results suggest that T-705 inhibited YEZV replication in the early stages of infection, thus preventing lethal inflammation.

In this study, the mice were treated with T-705 immediately after YEZV inoculation, so the therapeutic effect of T-705 after the onset of disease is unknown. Considering that treatment is usually initiated after the onset of disease, further experiments are needed to verify the efficacy of post-infection treatment. Furthermore, as shown in Chapter I, in the YEZV-infected AG129 mice model, severe inflammation persists even after the virus has been eliminated. Therefore, control of inflammation may be important in addition to administration of antiviral agents in the treatment of YEZV infection.

Clinical studies evaluating the therapeutic effects of T-705 on SFTSV-infected patients have been reported in China and Japan (53–55). These studies showed improvement in clinical symptoms and decreased mortality in SFTSV-infected patients treated with T-705, suggesting that T-705 is effective in the treatment of SFTS. Based on these results, T-705 was approved in Japan as the first antiviral treatment for SFTS in June 2024. Importantly, SFTSV and YEZV coexist in China and Japan. In addition, clinical findings such as fever after tick bites, thrombocytopenia, leukopenia, and elevated liver enzyme levels are consistent between SFTSV- and YEZV-infected cases. T-705 has the potential to be an antiviral agent applicable to both SFTSV-infected cases and YEZV-infected cases. Therefore, the early administration of T-705 may be an important option for febrile patients with thrombocytopenia after tick bites, even before diagnosis.

General Conclusion

To control emerging infectious diseases, establishment of animal infection models is necessary to analyze pathogenicity of the causative agents and to develop prevention and treatment measures. In this dissertation, a lethal mouse model of YEZV, an emerging orthonairovirus, infection was established for pathological and virological analysis. In addition, as an immediate countermeasure against the emerging infectious disease, YEZV infection, anti-YEZV effects of existing antiviral agents were verified.

YEZV is an emerging tick-borne orthonairovirus isolated from febrile patients in Hokkaido, Japan in 2019 and 2020. YEZV belongs to the Sulina genogroup, which is phylogenetically distinct from the CCHFV, known as a highly pathogenic orthonairovirus in humans, and is closely related to other emerging orthonairoviruses belonging to Tamdy genogroup, which has been recently reported in East Asia. In clinical cases, patients showed fever, thrombocytopenia, leukopenia, and elevated liver enzyme levels. However, due to the small number of cases and lack of animal infection models, the pathogenicity of YEZV remains unclear.

In Chapter I, the pathogenicity of YEZV in mice was examined. YEZV infection did not cause any clinical symptoms in wild-type mice but was lethal to AG129. Therefore, a mouse model of lethal YEZV infection was established using AG129 mice, and pathological and virological analyses were conducted. Blood biochemical tests showed elevated levels of liver enzymes in the serum, consistent with YEZV-infected cases. Histopathological analysis revealed hepatocyte degeneration and infiltration of inflammatory cells in the liver and disruption of follicular structures in the spleen of YEZV-infected mice. Positive signals of YEZV antigen were detected diffusely in the liver and spleen of YEZV infected-AG129 mice by IHC. Viral titers in the tissues peaked at 2 dpi and decreased after 4 dpi. These results suggested that YEZV replicates primarily in the liver and spleen, and causes acute hepatitis in AG129 mice.

In Chapter II, the effect of antiviral agents against YEZV was examined. Ribavirin and T-705 showed anti-YEZV effects *in vitro*. In addition, T-705 showed anti-YEZV effects in the mouse model of YEZV infection. AG129 mice treated with T-705 were rescued from lethal infection with significantly decreased viral titers in tissues and serum. These results suggest that this AG129 mice model can be useful for the validation of antiviral agents against YEZV infection.

The present mouse model is the first mouse infection model of emerging human pathogenic orthonairoviruses involving the Tamdy and Sulina genogroups. Infected patients with emerging orthonaviruses have been found mainly through retrospective cohort studies, so pathogenicity in humans has not well analyzed. This study will provide a better understanding of the pathogenicity of emerging orthonairoviruses, including YEZV, and provide a basis for the development of

therapeutics that will help to control the infection. In addition, this study demonstrated the anti-YEZV effect of T-705 in the mouse model of YEZV infection. T-705 is approved in Japan as a treatment for severe fever with thrombocytopenia syndrome (SFTS). YEZV infection and SFTS coexist in Japan and China. In addition, symptoms and laboratory findings are similar in both cases, such as fever after tick bite, thrombocytopenia, and leukopenia. Therefore, T-705 has the potential to be an antiviral agent applicable to both YEZV infection and SFTS cases, even before diagnosis.

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Summary in Japanese

新興感染症の制御を目指してその病原体の持つ病原性を解析し、予防・治療法を開発するためには、動物感染モデルの樹立が望まれる。本研究では新興オルソナイロウイルスであるエゾウイルス (*Orthonairovirus yezoense*, YEZV) の致死感染マウスモデルを確立し、病理学およびウイルス学的解析を実施した。また、新興感染症である YEZV 感染症への早急な対策として、既存の抗ウイルス薬の抗 YEZV 効果を検証した。

YEZV は 2019 年および 2020 年に北海道でマダニ刺咬後に発熱を呈した患者から分離された新規オルソナイロウイルス (ブニヤウイルス綱、ナイロウイルス科) であり、日本と中国で感染者が報告されている。ヒトへの病原性が極めて高いオルソナイロウイルスとして知られているクリミア・コンゴ出血熱ウイルス (*Orthonairovirus haemorrhagiae*) とは系統学的に異なる Sulina 遺伝子群に属しており、近年東アジアで報告されている Tamdy 遺伝子群に属する新興オルソナイロウイルスに近縁である。YEZV 感染症の臨床症例では、発熱、倦怠感、下肢痛などの症状、および血小板数減少、白血球数減少、肝逸脱酵素値の上昇、血清フェリチン値の上昇などの臨床検査結果が報告されている。しかし、未だ症例数は少なく、また、動物感染モデルが樹立されていないため YEZV 感染症の病態の全容は不明である。

第 1 章では、YEZV のマウスへの病原性を解析した。YEZV は野生型マウスには臨床症状を起こさなかったが、I 型および II 型インターフェロン受容体ノックアウトマウスである AG129 マウスでは致死的病態を惹起した。YEZV を接種した AG129 マウスにおける血液生化学検査は、YEZV 感染症例と一致する血清中の肝逸脱酵素値の上昇を示した。病理組織学的解析の結果、YEZV 感染マウスの肝臓では肝細胞の変性および炎症性細胞の浸潤、脾臓では濾胞構造の消失が認められた。免疫組織化学染色法により組織中の YEZV 抗原を検出した結果、マウスの肝臓およ

び脾臓で YEZV 抗原がびまん性に認められた。また、組織中のウイルス力価は感染後 2 日で最大となり、4 日以降で減少していた。以上の結果から、YEZV は AG129 マウスに感染後、主に肝臓と脾臓で増殖し、致死的な急性肝炎を起こすことが明らかとなった。

第 2 章では、既存の抗ウイルス薬であるリバビリンとファビピラビル (T-705) の抗 YEZV 効果を検証した。Vero 細胞を用いて *in vitro* での抗ウイルス効果を検証した結果、リバビリンおよび T-705 は YEZV の複製を阻害した。YEZV の複製に対する T-705 の 50%阻害濃度および 90%阻害濃度はリバビリンよりも低濃度であり、T-705 がリバビリンよりも強い抗 YEZV 効果を示すことが明らかとなった。第 1 章で樹立した YEZV 感染 AG129 マウスモデルを用いて T-705 の抗 YEZV 効果を検証した結果、300 mg/kg/日の T-705 を 3 日間投与された AG129 マウスでは、肝臓および脾臓でのウイルス増殖量が抑制され、致死的な病態に至らなかった。以上の結果から、本研究で樹立した YEZV 感染 AG129 マウスモデルが抗ウイルス薬の評価に有用であることが示された。

本研究で樹立したマウス感染モデルは、Tamdy および Sulina 遺伝子群に関わるヒトに病原性を有する新興オルソナイロウイルス群における最初のマウス感染モデルである。新興オルソナイロウイルスの感染患者は主に後ろ向きコホート研究によって発見されており、ヒトへの病原性の解析は進んでいない。そのため、本研究結果は YEZV を含む新興オルソナイロウイルスの病原性をより深く理解し、感染症の制御へとつながる治療法開発の基盤となることが期待される。また、本研究では YEZV 感染マウスモデルにおいて T-705 の抗 YEZV 効果が示された。T-705 は日本において重症熱性血小板減少症候群 (SFTS) の治療薬として認可されている。日本と中国では YEZV 感染症と SFTS が共存しており、さらにダニ刺咬後の発熱、血小板数減少および白血球数減少など両症例で認められる症状や検査所見は類似している。そのため、T-705 は検査前であっても

YEZV 感染症または SFTS が疑われる症例に適応可能な抗ウイルス薬となる可能性がある。
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