Epidemiological studies on hepatitis E virus among wildlife and discovery of a novel rabbit HEV strain in Japan

(国内の野生動物における E 型肝炎ウイルスの疫学調査と新規ウサギ E 型肝炎ウイルスの発見)

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1. General Introduction

1.1 History of hepatitis E virus

The existence of viral hepatitis was recognized as early as the 8th century AD, as a disease usually associated or followed by wars, famines and natural disasters (Schmid, 2001). By the mid of the 20th century, hepatitis A and hepatitis B viruses were recognized as agents to be enterically or parentally transmitted (MacCallum, 1972). However, in the following years, outbreaks of non-A, non-B hepatitis were identified in different regions worldwide and associated to blood transfusion or related to water borne epidemics. These two different serological agents were later described as hepatitis C virus (HCV) and hepatitis E virus (HEV) (Feinstone et al., 1975, Schmid, 2001, Villarejos et al., 1975).

The first suspected HEV outbreak occurred on the island of Martinique in 1858, but definite recognition of HEV as a novel hepatitis agent occurred after a water-borne epidemic in Kashmir Valley, India in 1978 (Khuroo, 1980, Lemon and Walker, 2019). The serological data supported the claim of that this novel etiological agent was different from hepatitis A and B viruses. Following reports from India, Nepal, Africa and Mexico, showed that HEV infection did not progress into chronic disease but had high mortality in pregnant women, especially in the third trimester (Belabbes et al., 1985, Kane et al., 1984, Khuroo et al., 1983, Velazquez et al., 1990, Wong et al., 1980).

In 1983, HEV characterization was achieved by the oral administration of pooled stool extracts to a human volunteer (Balayan et al., 1983). HEV virions were detected in stool samples and described as a 27-to 30-nm non enveloped spherical particles. The acute symptoms of hepatitis were observed after 36 days post infection and included jaundice, liver enlargement, fever, vomiting and alteration of serum transaminase levels. The successful infection in a human volunteer possessing antibodies against HAV and negative to HBV markers, as well as the oral transmission to two cynomolgus monkeys, were sufficient to confirm the existence of a new hepatitis agent (Balayan et al., 1983).

The first cDNA library was reported in 1990 and constructed from bile samples of experimentally infected cynomolgus macaques (Reyes et al., 1990). One year later, the full-length genome of the Burma strain was described as a single stranded, positive sense RNA virus with a

genome of approximately 7.5 kb (Reyes et al., 1991, Tam et al., 1991). Molecular analysis indicated that HEV genome organization and characteristics were not compatible to the family *Caliciviridae*, and might belong to a new class of RNA virus (Tam et al., 1991).

Further reports of HEV infection in humans confirmed the fecal-oral transmission of the virus and outbreaks were predominant in non-developed countries (Krawczynski, 1993). However, in 1997, an HEV-like virus was reported to be prevalent in pigs' farms in the United States (Meng et al., 1997). This HEV strain proved to be zoonotic and was later classified as genotype 3 (Erker et al., 1999, Kwo et al., 1997, Schlauder et al., 1998). This discovery showed that HEV is different from other viral hepatitis agents and is the only one to have animal reservoirs. Since then, HEV food-borne transmission reports have been increasing in industrialized nations and have been linked to the consumption of swine meat (Dalton et al., 2008, Takahashi and Okamoto, 2014).

In 2003, the first case of HEV zoonosis caused by the ingestion of game meat (Sika deer) was reported in Japan (Tei et al., 2003). Soon afterward, several surveys showed HEV circulation in wild boar populations and suggested association of undercooked wild boar meat ingestion with food-borne transmission in humans (Kukielka et al., 2016, Li et al., 2005, Sato et al., 2011, Okita, 2012). Moreover, worldwide reports of novel HEV genotypes and animal reservoirs, including chicken, rabbits and camels, have been increasing during the last decade (Okamoto, 2007, Primadharsini et al., 2019). Historical discoveries and continuous research on HEV have shown that HEV is no longer a disease only associated to water-borne epidemics, but also a disease that causes zoonosis and represents a risk for public health.

1.2 Virus properties

HEV is a single stranded, capped positive sense RNA virus with a length of approximately 7.2 kb, containing three partially overlapping open reading frames (ORFs) flanked by 5' and 3'

untranslated regions (UTRs) (Tam et al., 1991, Zhang et al., 2001). HEV virions size range from 20-40 nm in diameter and exists in two different forms in the host, as quasi-enveloped particles circulating in the blood-stream (eHEV) and as a non-enveloped virus (neHEV) in feces (Balayan et al., 1983, Nagashima et al., 2017, Takahashi et al., 2010).

HEV genome mapping showed that a 76-nt region at the 5' end of the HEV was responsible for the ORF2 protein binding, suggesting that the 5' UTR plays a role in viral encapsidation (Surjit et al., 2004). The 3' UTR consists of a poly A tail of approximately 150 -200 nt and interacts with the RNA-dependent RNA polymerase region for HEV transcription during viral replication (Agrawal et al., 2001). The translated negative-sense genome synthesizes two different positive-sense RNAs: the full-length transcript, that is packaged into progeny virus and a 2.2 kb subgenomic RNA, which encodes ORF2 and ORF3(Varma et al., 2011, LeDesma et al., 2019, Graff et al., 2006).

ORF1 is the longest ORF in the HEV genome (~ 5 kb) and encodes the nonstructural proteins which are involved in the viral replication (Panda and Varma, 2013). The ORF1 functional domains are: methyltransferase (Met), Y domain, papain-like cystine protease (PCP), hypervariable region (HVR) or proline-rich region (PRR), X domain, helicase (Hel) and the RNA-dependent RNA polymerase (RdRp) (Kenney and Meng, 2019). It remains unclear if the ORF1 is expressed a single polyprotein or as cleaved into individual proteins (Debing et al., 2016a).

The RdRp, Hel and Y domain have been associated with virus replication while the PCP and X domain might be involved with the immune evasion strategies of HEV (Kenney and Meng, 2019, LeDesma et al., 2019). The HVR region sequences of human HEV (genotypes 1 and 2) are more conserved than zoonotic HEV strains (genotypes 3 and 4), suggesting that HVR might modulate HEV infectivity in a host-depended manner. Recombination event in the HVR region was observed in a HEV strain recovered from a HIV-1 and HEV chronically infected patient (Kernow-C1 gt3) (Johne et al., 2014). The nucleotide insertion of a human S17 ribosomal protein gene in the HVR domain resulted in a highly replicating virus in human hepatoma cells, that could also infect animal-derived cell lines.

This and other reports have shown evidence that insertions or rearrangements in the ORF1 protein could enhance viral replication of wild-type HEV strains (Debing et al., 2014, Drave et al., 2016, Fu et al., 2019, Shukla et al., 2012).

ORF2 translates the capsid protein which is the major structural component of the HEV virion. The capsid protein is highly immunogenic, elicits neutralizing antibodies and is essential for the binding and attachment to cell receptor for initiate HEV viral entry (Ankavay et al., 2019, Wang and Meng, 2021) . The ORF2 genome have a length of approximately 1.9 kb and encodes a predicted protein of 660 amino acids (aa), displaying a signal peptide at its *N*-terminus. The capsid protein is structurally divided into 3 domains: the shell (S), middle (M) and protruding (P) domains, possessing 3 potential *N*-glycosylation sites (Yamashita et al., 2009). The P domain is considered the main antigenic site, containing virus-neutralizing epitopes and have been associated to the binding process to cell receptors (Lin and Zhang, 2021, Yamashita et al., 2009).

Two different translation products of ORF2 have been described. One ORF2 (capsid protein) associate to infective HEV particles and a free ORF2 glycosylated form, which is abundant in serum of HEV-infected patients and in supernatant of infected cells (Montpellier et al., 2018, Yin et al., 2018). The secreted glycosylated ORF2 (ORF2s) have lost the epitopes involved in cell binding, is not associated to HEV virions but does not block cell entry. Furthermore, ORF2s inhibits antibody mediated neutralization of HEV, suggesting that it functions as a decoy against the humoral immune system rather than playing a role in HEV infection cycle (Ankavay et al., 2019, Yin et al., 2018).

ORF3 encodes a small phosphorylated protein of 113–114 aa involved in virion morphogenesis and viral egress (Huang et al., 2007, Tam et al., 1991). ORF3 has been categorized as a viroporin and is associate to the biogenesis of the lipid membrane found in eHEV particles (Ding et al., 2017). Moreover, ORF3 have shown to be incorporated into eHEV but was not found in naked virions. The lipid membrane protects the HEV particles from neutralizing antibodies and leads to a distinct entry pathway depending of the endosomal trafficking, which involves the degradation of the quasi-envelope

of eHEV in the cell lysosome (Aggarwal, 2011, Nagashima et al., 2017, Takahashi et al., 2010, Takahashi et al., 2008, Yin et al., 2016) .

A novel ORF4 was first described in human HEV genotype 1 and activated in response to endoplasmic reticulum (ER) stress (Nair et al., 2016). The genotype 1 ORF4 protein enhance viral replication by stimulating the RdRp activity, however, the recently proposed ORF4 found in rat HEV was unnecessary for viral replication (Tanggis et al., 2018, Yadav et al., 2021). Further studies are need to elucidate ORF4 function.

1.3 Classification

HEV belongs to the family Hepeviridae and is classified in two genera: Othohepevirus with four species (A to D) and Piscihepevirus with a single species (cutthroat trout virus). Othohepevirus A has been isolated from a large variety of mammals, including humans, and classified into 8 genotypes (Primadharsini et al., 2019). Genotypes 1 and 2 are obligate human pathogens and are transmitted by the fecal-oral route. Genotypes 3 and 4 are zoonotic and have been identified in a wide range of species, but domestic pigs are considered the main animal reservoir (Okamoto, 2007, Thiry et al., 2017a) .Genotypes 5 and 6 were detected in Japanese wild boar, while genotypes 7 and 8 were described in dromedary and bactrian camels, respectively (Sato et al., 2011, Woo et al., 2016).

Othohepevirus B is restricted to birds and is also known as Avian HEV (Su et al., 2020a, Sun et al., 2019). Until now, Othohepevirus B has been designated into 4 major genotypes (1-4). The avian strains are usually segregated according its geographical distribution and have been associated with hepatitis-splenomegaly syndrome in chickens (Iqbal et al., 2019, Matczuk et al., 2019, Su et al., 2020b).

Othohepevirus C divides into 2 groups. The HEV-C1 group includes strains recovered from rat, greater bandicoot and Asian musk shrew, while HEV-C2 group includes ferret and mink strains (Guan et al., 2013, Johne et al., 2010a, Johne et al., 2010b, Li et al., 2017, Li et al., 2013, Wang et al.,

2018). Despite to not be considered zoonotic, recent reports have shown that rat HEV caused persistent hepatitis in immunocompromised patients (Andonov et al., 2019, Sridhar et al., 2018). However, the exact route of transmission to humans is still unclear.

Othohepevirus D contains strains originated from different bats species and highly heterogenic according region. Strains have been detected in macrobats and microbats from different countries and there is no evidence of zoonosis transmission (Drexler et al., 2012, Kobayashi et al., 2018, Wu et al., 2016). Furthermore, several other HEV-like virus are yet to be classified, including the partial HEV genomes detected in moose, red foxes, little egret, frogs and others animals (Bodewes et al., 2013, Lin et al., 2014, Reuter et al., 2016, Reuter et al., 2018) .

1.4 Epidemiology

1.4.1 Epidemiology in humans

HEV infection spreads worldwide. In non-developed countries HEV causes water-borne epidemics while in industrialized nations sporadic HEV cases are related to consumption of infected meat, blood transfusion and organ-transplantation (Kamar et al., 2008a, Khuroo et al., 1983, Takeda et al., 2010).

Genotype 1 and 2 are restricted to humans and have caused outbreaks due to the consumption of contaminated drinking-water (Primadharsini et al., 2019). Both genotypes are transmitted by the fecal-oral route and are prevalent in the regions of Asia, Africa, Mexico and the Middle east (Aggarwal, 2011, Belabbes et al., 1985, Khuroo et al., 1983, Velazquez et al., 1990). Epidemics caused by genotype 1 and 2, still represents a burden for low-income countries and causes mortality in pregnant women (Kirkwood et al., 2020) .

HEV genotypes 3 and 4 are generally widespread in developed countries. In European nations, genotype 3 strains are the most prevalent, but increasing cases of genotype 4 have been observed (Bouamra et al., 2014, Casares-Jimenez et al., 2021). In Asia, autochthonous cases of genotypes 3 and 4 are the most common in human patients (Sato et al., 2020, Smith et al., 2020). Despite having more than one transmission route, consumption of infected meat products or raw meat are the most important transmission pathway for both genotypes. (Okamoto, 2007)

Several studies have suggested that genotype 3 and 4, are most likely to develop in clinically apparent acute hepatitis in older men than in women (EASL., 2018, Takahashi and Okamoto, 2014).The predisposition of older men patients might be related to host factors, such as the presence of subclinical hepatic steatosis or fibrosis. However, the underlying reason is still unknown (EASL., 2018) .

1.4.2 Epidemiology in animals

Pigs are considered as the primary host of HEV infection (Thiry et al., 2017a). In addition, HEV have shown to be highly infectious and prevalent in swine farms, but asymptomatic in pigs (Meng et al., 1997). Fecal-oral transmission is most predominant in pigs, since vertical transmission importance is negligible in HEV cycle (Kasorndorkbua et al., 2004). Viremia is observed after the depletion of the maternal antibodies, around the 11-18 weeks of age. Consequently, IgG level increases resulting in seroprevalences rates up to 100% in pigs above 25 weeks of age (Meester et al., 2021). In addition, HEV RNA have been found in every step of the food chain of pig meat or pork-derived products (Pavio et al., 2014).

Infected pigs release large amounts of HEV particles in the stool and it has led to environmental contamination of natural water sources (Andraud et al., 2013, EASL., 2018). As a consequence, HEV have been detected in shellfish and dolphin, as well as in fruits and vegetables irrigated with infected

water (Maunula et al., 2013, Montalvo Villalba et al., 2017, Thiry et al., 2017a). These HEV spill over infection have been observed in an increasing number and variety of animals.

HEV genotypes 3 and 4 foodborne zoonotic cases are also associated to the ingestion of game meat, mostly from wild boar origin, and sporadically from deer meat (Choi et al., 2013, Li et al., 2005, Rivero-Juarez et al., 2017). Wild boars are highly susceptible to HEV infection and HEV dynamics in these populations have been documented worldwide (Anheyer-Behmenburg et al., 2017, Thiry et al., 2017c). Moreover, genotype 5 and 6 have been detected from Japanese wild boar only (Sato et al., 2011, Takahashi et al., 2020).

1.5 Disease and treatment

HEV infected patients usually experience a self-limited acute hepatitis (Webb and Dalton, 2019). Patients are mostly asymptomatic or show mild clinical signs, such as fever, jaundice, malaise, nausea, vomiting and liver enzyme elevation (Lin and Zhang, 2021). Immunocompetent patients are able to clear the disease spontaneously, therefore, no specific treatment is recommended apart of symptomatic care (Ahmad et al., 2021). Nonetheless, HEV infection have shown higher mortality rates in pregnant women specially during the third trimester (Patra et al., 2007, Perez-Gracia et al., 2017).

Pregnant women infected with genotype 1 can develop fulminant hepatic failure (FHF), different of genotype 3 infection (Anty et al., 2012, Lachish et al., 2015). The symptomatology leading to FHF includes eclampsia, hemorrhagic complications and liver failure (Lin and Zhang, 2021) . The exact mechanism of FHF is not elucidated yet, but it might have relation to the levels of estrogen/progesterone or with the alteration of the immune response during pregnancy (Todt et al., 2020, Yang et al., 2018).

Chronic HEV infection is defined as the persistence of HEV replication over a period of 6 months, but the criteria have been reconsidered in solid organ transplant recipients, in which viremia over 3 months can be regarded as chronic infection (EASL., 2018). Until now, only genotype 3 and 4 have shown progression to chronic hepatitis, being HIV-positive patients, organ transplant receivers and immunocompromised individuals the ones at most risk of developing this form of infection. (Kamar et al., 2008a, Webb and Dalton, 2019).

Extrahepatic manifestations of HEV infection have been recognized in both acute and chronic cases including neurological and renal injury, pancreatitis and hematological disorders (EASL., 2018). Particularly, Guillian-Barre syndrome (GBS) have been reported during or preceding HEV infection (Aslan and Balaban, 2020, Fousekis et al., 2020) . HEV viral genome have been detected in the cerebrospinal fluid in human and HEV-infected gerbils, suggesting that HEV is capable of crossing the blood-brain barrier (Shi et al., 2016, Stevens et al., 2017).

Ribavirin and Pegylated interferon alfa (Peg-IFNα) therapy have been used successfully to treat HEV chronic infection. Both drugs have shown to inhibit HEV replication by different mechanisms. Ribavirin leads to the depletion of intracellular guanosine triphosphate (GTP) pools, while Peg-IFNα activates the immune system (Debing et al., 2016a, EASL., 2018). Nonetheless, ribavirin is contraindicated in pregnant women due to the risk of teratogenesis and Peg-IFN is not recommended in patients receiving immunosuppressive therapy since it increases the risk of organ rejection (Aslan and Balaban, 2020). In addition, ribavirin resistance has been observed in chronic infected patients, impairing the need of more efficient, high spectrum drugs for HEV therapy (Debing et al., 2016b).

Promising treatment options includes sofosbuvir, an antiviral drug for HCV. Sofosbuvir is capable to reduce HEV replication *in vitro*, however, its efficacy *in vivo* is still unknow (Debing et al., 2016a, EASL., 2018). Other compounds to have shown inhibitory effect against HEV are 2'-C-

methylguanosine, silvestrol and NITD008 (Aslan and Balaban, 2020). However, these compounds antiviral efficacy and safety are still unknown in humans.

1.6 Diagnosis

HEV infection incubation period last for 2 to 6 weeks (Colson et al., 2010). HEV genome is detected in blood and stool samples around 3 weeks post infection, usually shortly before the onset of the symptoms. Viremia continues for approximately 3 to 6 weeks, while viral shedding in feces can last for 4 to 6 weeks in average (Ahmad et al., 2021, EASL., 2018).

Detection of HEV RNA in blood serum, plasma or stool is indicative of HEV infection (EASL., 2018). Several reliable nucleic acid amplification techniques (NATs) have been described, including conventional reverse transcription PCR (RT-PCR) and nested protocols, real time RT-PCR and transcription-mediated amplification methods. Primers targeting the overlapping region of ORF2/ORF3 are able to detect genotypes 1 to 4, however polymorphisms have resulted in false negative results (Garson et al., 2012, Jothikumar et al., 2006). In 2013, the World Health Organization developed the international standard and reference panel for comparing the results obtained from different NATs, which adjusted the results to a common international unit (Baylis et al., 2019).

Biochemical markers and IgM elevations appears near the start of clinical onset, followed by the IgG response. The IgM antibodies are detectable during a short period (3 to 4 months on average) and are indicative of recent infection (Huang et al., 2010, Kamar et al., 2017). On the other hand, IgG antibodies are long lasting but do not necessary provide lifelong immunity, since antibodies titters decrease over time (Aslan and Balaban, 2020).

Serological methods for determine HEV acute infection are based in the detection of anti IgM and increasing levels of IgG (Al-Sadeq et al., 2018). Commercially available assays have shown a

wide range of sensitivity and specificality, and parallel NAT testing is usually recommended (Abravanel et al., 2013, Webb and Dalton, 2019). Occasionally, IgA antibodies are also used for acute HEV diagnosis along with the detection of IgM, but commercial assays are not widely available (Al-Sadeq et al., 2018, Takahashi et al., 2005). In Japan, nationwide cases of acute HEV infection started increasing after the incorporation of IgA screening into the national insurance program (Owada et al., 2020).

Other less common diagnosis methods are antigen assays and immunohistochemistry. Several studies have showed that HEV antigen in patient serum is not necessarily related with infectious virions, therefore the role of antigen diagnosis tools remains unclear (EASL., 2018, Yin et al., 2018). Recently, an electrical pulse-induced electrochemical biosensor for HEV was proposed as a robust and sensitive detection method (Chowdhury et al., 2019). However, further studies are need to evaluate efficacy and performance of new technologies for HEV diagnosis.

1.7 Prevention and control

HEV prevention and control measures should take into consideration the HEV transmission pathways and risk-groups. In genotype 1 and 2 endemic areas, proper hygiene and sanitation can prevent HEV water-borne transmission. These measures mainly consist of the chloride treatment of drinking water, the periodical quality assessment of public water supplies and the disinfection of fruits and vegetables using chlorine solutions (Ahmad et al., 2021, Melgaco et al., 2018). However, these measures might not be doable in low-income areas

Several studies have showed that short term heating of genotypes 3 and 4 (zoonotic HEV) cannot inactivate HEV virions in supernatant nor in infected meat (Kirkwood et al., 2020). HEV i*n vitro* inactivation was achieved using temperature above 90 °C, while internal temperatures above 70 °C were able to inactivate virions in meat (Imagawa et al., 2018, Johne et al., 2016). Considering

HEV heat stability, proper cooking of meat and the avoidance of raw pig liver or derivates can prevent food-borne zoonotic infection.

HEV have shown to be highly resistant to alcohol-based disinfectants (Behrendt et al., 2022). Therefore, biosafety practices should be followed in order to reduce the risk of infection in hospital environments, including the correct disposal of needles and medical waste from infectious hospitalized patients. Health care professional should be aware of the correct use of the personal protective equipment (Thiry et al., 2017a), such as gloves, gowns, mask, eye protection, or face shields. (Kirkwood et al., 2020, Melgaco et al., 2018)

Despite reports of several HEV vaccine candidates showing efficacy in preclinical studies, Hecolin® vaccine is the sole licensed vaccine in the world (Cao et al., 2018). Hecolin® or HEV 239 vaccine, is a bacterially expressed recombinant peptide, manufactured by Xiamen Innovax Biotech and launched into the market in 2012. This vaccine targets amino acids residues 368-660 of genotype 1 ORF2, and the truncated ORF2 recombinant self-assembled into 23nm virus-liked particles (VLP) (Li et al., 2015, Liu et al., 2014, Wu et al., 2012). Hecolin® is approved for use in people older than 16 years and its inoculation have induced antibodies against HEV for at least 4.5 years (Zhang et al., 2015a). Nonetheless, HEV 239 has not received WHO prequalification for use in endemic areas nor approved in any other countries (Cao et al., 2018, EASL., 2018). Further clinical trials are ongoing in Bangladesh for evaluating the safety and protection of Hecolin® in pregnant women (Aslan and Balaban, 2020).

2. CHAPTER 1

Nationwide survey of hepatitis E virus infection among wildlife in Japan

2.1 Abstract

In Japan, hepatitis E virus (HEV) causes hepatitis in humans through the consumption of raw or undercooked meat, including game meat. In the present study, nationwide surveillance of HEV infection among a total of 5,557 wild animals, including 15 species, was conducted in Japan. The prevalence of anti-HEV antibodies in wild boar was 12.4%, with higher positive rates in big boars (over 50 kg, 18.4%) than in small individuals (less than 30 kg, 5.3%) Furthermore, HEV RNA was more frequently detected in piglets than in older boars. Interestingly, the detection of HEV among wildlife by ELISA and RT-PCR suggested that HEV infection in Sika deer was a very rare event, and that there was no HEV infection among wild animals except for wild boar, Sika deer and Japanese monkeys. In conclusion, wild boar, especially piglets, are at high risk of HEV infection, while other wild animals showed less risk or no risk of HEV transmission.

Keywords: hepatitis E virus, seroprevalence, Sika deer, wild animals, wild boar

2.2 Introduction

Worldwide, hepatitis E virus (HEV) is the causative agent of acute viral hepatitis. HEV outbreaks have been described in developing countries, with mortality rates reaching 20–30% in pregnant women (Patra et al., 2007, Perez-Gracia et al., 2017). In industrialized countries, zoonotic food-borne transmission due to the ingestion of infected animal meat is considered to be the main route of infection, but solid organ transplantation and blood transfusion routes have also been described (Kamar et al., 2008b, Takeda et al., 2010). Although HEV infection is generally self-limited, it can cause chronic hepatitis in immunocompromised patients(Kamar et al., 2008b).

HEV is a non-enveloped, single stranded positive sense RNA virus with a genome length of approximately 7.2 kb, which encodes 3 opening reading frames (ORFs). HEV belongs to the family *Hepeviridae* with two assigned genera: *Othohepevirus* with four species (A to D) and *Piscihepevirus* with a single species (Purdy et al., 2017, Smith et al., 2020). Until now, *Othohepevirus A* has been classified into 8 genotypes. Genotypes 1 and 2 are found exclusively in humans, genotypes 3 and 4 are zoonotic and have been reported from various mammals (Casares-Jimenez et al., 2021, Primadharsini et al., 2019, Thiry et al., 2017a). Genotypes 5 and 6 were detected in Japanese wild boar, while genotypes 7 and 8 were described in dromedary and bactrian camels, respectively (Sato et al., 2011, Woo et al., 2016).

In Japan, HEV genotypes 3 and 4 are predominant in human and animal populations, and domestic pigs are the main reservoir (Okamoto et al., 2003). Food-borne transmission from game meat was first reported by the consumption of Sika deer meat (*Cervus nippon*) in 2003 (Tei et al., 2003). Since then, HEV infections in humans were mainly linked to the consumption of pork and wild boar meat and several epidemiological surveys suggested that wild boar are highly susceptible to HEV infection (Li et al., 2005, Sato et al., 2011, Sonoda et al., 2004, Takahashi et al., 2004). Furthermore, the HEV genome and antibodies have been found in companion, feral and wild animals, showing that

the other species are also exposed to HEV infection by unknown route(s) (Hirano et al., 2003, Mendoza et al., 2021, Nakamura et al., 2006, Yonemitsu et al., 2016).

Despite increasing reports of zoonotic transmission from game meat, the distribution, and characteristics of HEV strains circulating among wild animals, including wild boar and Sika deer populations have not been fully understood. In addition, the shedding of the virus in wild boar feces has been observed (Schlosser et al., 2015), indicating that cohabiting wild animals are at risk of HEV infection.

In this study, a nationwide survey was conducted in order to assess the risk of zoonotic HEV infection among wild animals as well as to characterize the genotypes of the HEV strains in Japan.

2.3 Materials and methods

Serum samples

A total of 5,557 serum samples were collected from wild boar (*Sus scrofa*), Sika deer (*Cervus nippon*), raccoons (*Procyon lotor*), mice (*Apodemus speciosus*, *Apodemus argenteus* and *Myodes smithii*), Japanese monkeys (*Macaca fuscata*), raccoon dogs (*Nyctereutes procyonoides*), Japanese badgers (*Meles anakuma*), mask palm civets (*Paguma larvata*), nutrias (*Myocastor coypus*), weasels (*Mustela itatsi* and *Mustela sibirica*), Japanese black bears (*Ursus thibetanus*), Japanese martens (*Martes melampus*), Japanese hares (*Lepus brachyurus*), red foxes (*Vulpes vulpes*) and Reeve's muntjac (*Muntiacus reevesi*).

A total of 2,375 serum samples from wild boar were collected in Aomori (n=4), Chiba (n=91), Ehime (n=311), Gifu (n=144), Gunma (n=48), Hyogo (n=44), Kagawa (n=116), Kagoshima (n=5), Kumamoto (n=182), Oita (n=92), Okinawa (n=97), Tochigi (n=163), Toyama (n=183), Wakayama (n=457), and Yamaguchi (n=438) prefectures (Fig. 1). From Sika deer, 2,250 serum samples were collected from Aomori (n=39), Chiba (n=107), Ehime (n=45), Gifu (n=339), Gunma (n=106), Hokkaido (n=49), Kagawa (n=65), Kagoshima (n=29), Nagano (n=47), Oita (n=12), Wakayama

(n=347), Yamaguchi (n=1000) and Yamanashi (n=65) prefectures. In addition, 275, 160, 149, 115, 110, 36, 24, 22, 13, 13, 8, 6 and 1 serum samples were collected from raccoons, mice, monkeys, racoon dogs, badgers, mask palm civets, nutrias, weasels, bears, martens, hares, foxes and muntjac, respectively. The collection and location are described in Fig. 1 and Table 1. These wild animals were found dead (roadkill or unknown reasons) or were mainly captured as countermeasures under the official population control program. All collected serum samples were stored at -20°C until use. There was no overlap in examined animals between this study and our previous reports (Hara et al., 2014, Yonemitsu et al., 2016).

Detection of anti-HEV antibodies among animal sera

Anti-HEV antibodies in wild animal sera were detected using our established ELISA (Yonemitsu et al., 2016), with a minor modification. ELISA antigen was prepared from HEV capsid protein expressing cells by transfection with the expression plasmid (Yonemitsu et al., 2016). Peroxidase Conjugated Purified Recomb® Protein AG (Thermo Fisher Scientific, Waltham, MA, USA) was added as a secondary antibody. Following three washes with PBS-T, 100 µl of substrate agent (ABTS Microwell Peroxidase Substrate, Sera Care Life Sciences, Milford, MA, USA) was added to each well and the plates were gently shaken for 30 min at room temperature. Finally, the enzymatic reaction was stopped by adding 100 µl per well of 1% sodium dodecyl sulfate and the absorbance at a wavelength of 405 nm was measured using a spectrophotometer (Bio-Rad, Hercules, CA, USA). According to our previous report, the cut-off value was set at 0.437 for wild boar sera. For the other mammals, the cut-off value was tentatively set at 0.500.

Detection of HEV RNA in wild animals

A total of 3,489 samples were screened for the presence of HEV genomes. RNA was extracted from 140 µl of each serum sample using the QIAamp Viral RNA Mini kit (QIAGEN, Hilden,

Germany) in accordance with the manufacturer's instructions. Nested reverse transcription (RT)-polymerase chain reaction (PCR) was performed for the detection of HEV RNA using OneStep RT-PCR (QIAGEN) and KOD-Plus-NEO (Toyobo, Osaka, Japan) according to the kit protocols. Primers to detect ORF 2 gene of HEV genotypes 1, 3 and 4 (Li et al., 2005) were used for HEV detection (Mendoza et al., 2021). The 378 bp amplicon was purified using a QIAquick Gel Extraction Kit (QIAGEN) and the sequence was determined using BigDye Terminator v.3.1 technology (FASMAC, Atsugi, Japan). The obtained sequences were deposited in the DNA Data Bank of Japan (DDBJ accession number: LC706485-LC706506).

Sequence analysis of mitochondrial DNA from sera

DNA extraction was performed using 100 µl of deer serum samples that were found to be positive for HEV RNA or anti-HEV antibodies, using the DNeasy Blood & Tissue Kit Viral RNA Mini kit (QIAGEN). To determine the host genome, we used a set of primers (Mammalian-1 and Mammalian-2) targeting the cytochrome b gene region of the mitochondrial DNA, as described previously (Kim et al., 2009).

Phylogenetic analysis

The phylogenetic analysis was performed by using the MEGA7 software program (Kumar et al., 2016) based on the partial ORF2 sequences (338 bp) and the phylogenetic tree was generated by the neighbor-joining method based on 1,000 replicates, using the Kimura's two-parameter model. Updated HEV subtype reference strains were included for comparison (Smith et al., 2020).

Statistical analysis

Pearson's chi-squared analysis was performed to evaluate the associations among HEV seroprevalence, HEV genome detection, and the variables of sex and body weight. P values of <0.05 were considered to be statistically significant.

2.4 Results

Prevalence of anti-HEV antibodies among wild boar

Sera were obtained from 2,375 wild boar captured in 15 prefectures in Japan between 2012 and 2021 and were tested for the presence of anti-HEV antibodies (Fig. 1). The overall seroprevalence of anti-HEV antibodies in the wild boar population was 12.4% (294/2,375) and the prevalence by prefecture ranged from 0% to 49.5% (Table 1). The seroprevalence in wild board of >50 kg in body weight (18.4%) was significantly higher in comparison to that among wild boars of <30 kg in body weight (5.3%) ($p<0.001$). No significant difference was observed between males (12.1%) and females (13.6%) (Table 2).

Prevalence of anti-HEV antibodies among Sika deer

Serum samples from 2,250 Sika deer captured in 13 prefectures between 2008 and 2021, were screened for antibodies against HEV. The total seroprevalence was 0.04% (1/2250). The seropositive deer was captured in Kagawa Prefecture (1/65) and was negative for HEV RNA (Table 1). The anti-HEV antibody-positive serum was confirmed to have originated from deer by a sequence analysis of mitochondrial DNA in the serum.

Prevalence of anti-HEV antibodies in other wild animals

Sera of various wild animals collected between 2008 and 2020 were tested for the presence of anti-HEV antibodies. Two of 149 Japanese monkeys (1.4%) possessed anti-HEV antibodies and both were captured in Fukuoka Prefecture, resulting in a local prevalence of 6.3% (2/32). Sera collected

from 275 raccoons, 160 mice, 115 raccoon dogs, 110 Japanese badgers, 36 mask palmed civets, 24 nutrias, 22 weasels, 13 Japanese black bears, 13 Japanese martens, 8 Japanese hares, 6 red foxes and 1 Reeve's muntjac were negative for anti-HEV antibodies (Table 1).

HEV RNA detection in sera of wild boar, deer and other wild animals in Japan

HEV RNA was detected from 21 wild boar serum samples (1.2%). The prevalence in the different prefectures ranged from 0% to 5.5% (Table 3), and a significant difference was observed between males (1.8%) and females (0.6%) ($p<0.05$). In addition, the prevalence of HEV RNA in wild boars of <30 kg in body weight (2.2%) was significantly higher than that in wild boars of >50 kg in body weight (0%) ($p<0.001$) (Table 4).

The HEV genome prevalence among Sika deer was 0.06% (1/1688). The HEV-positive deer was captured in Yamaguchi prefecture. This HEV-positive serum was confirmed to have originated from Sika deer by a sequence analysis of mitochondrial DNA.

HEV RNA was not detected in serum samples from 12 Japanese black bears, 7 racoon dogs, 2 red foxes, 1 Japanese badger and 1 Japanese hare.

Phylogenetic analysis of HEV

The phylogenetic analysis of the 338 bp amplicons showed that 9 strains of HEV in wild boar and 1 strain of HEV in Sika deer belonged to genotype 4, while 12 belonged to genotype 3 (Fig. 2, Table 5). The Sika deer strain formed one cluster together with the other wild boar strains collected in the same area, and the cluster was tentatively classified as cluster 4j (Fig. 2).

2.5 Discussion

In this study, the prevalence of HEV infection among wild mammals was compared, indicating that HEV mainly circulated among wild boar populations, while the other mammals showed less or no susceptibility to HEV infection.

In Japan, the prevalence of the anti-HEV antibody-positive wild boar was 12.4% (294/2,375). The prevalence of anti-HEV antibodies in the wild boar population ranged from 4.9% to 57.6% in Europe (Anheyer-Behmenburg et al., 2017, Barroso et al., 2021, Caruso et al., 2015, de Deus et al., 2008, Kozyra et al., 2020, Kukielka et al., 2016, Roth et al., 2016, Spancerniene et al., 2016, Zele et al., 2016), and from 4.5% to 38.1% in Asian countries (Choe et al., 2020, Liang et al., 2019, Sakano et al., 2009). In Japan, HEV seropositivity rates among wild boar varied by prefecture, ranging from 4.5% to 42%(Hara et al., 2014, Michitaka et al., 2007, Motoya et al., 2019, Sakano et al., 2009) .In this study, the prefectures of Chiba and Gunma, which are located in the Kanto region showed higher rates of seropositivity in comparison to other regions (Fig. 1). This HEV geographical distribution was similar to the one observed in human patients in Japan (Okamoto et al., 2003, Sakata et al., 2008, Takeda et al., 2010). Nonetheless, wild boars have not been found in Hokkaido prefecture, which is a highly endemic area for HEV infection (Sakata et al., 2021). Therefore, the relationship between seropositivity in wild boar and the number of HEV patients remains unclear.

In this study, small wild boar (<30 kg) were infected with HEV, while heavy wild boar (>50kg) possessed anti-HEV antibodies. These results indicated that small wild boar were infected with HEV and that big boar developed antibodies after recovering from HEV infection. The previous studies in wild boar also showed an association between body weight and the prevalence of anti-HEV antibodies or with HEV RNA detection (Burri et al., 2014, Nakane et al., 2015). These results are similar to those in domestic pigs (Meng, 2011). On the other hand, there was no significant difference in detection of anti-HEV antibody between male and female, similar to our previous reports (Yonemitsu et al., 2016). However, HEV RNA was detected in male more than in female. In the previous reports, there was significant association between sex and HEV infection in human, but not in pigs and wild boar (EASL.,

2018, Meester et al., 2021, Modiyinji et al., 2020, Pavio et al., 2017, Schotte et al., 2022, Temmam et al., 2013). Further study will be required to resolve this discordance in detection between HEV RNA and anti-HEV antibody.

In Sika deer, the seroprevalence was only 0.04% (1/2,250), which was consistent with previous reports showing a low prevalence of anti-HEV antibodies, ranging from 0% to 6.8% in Sika deer, roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*) and fallow deer (*Dama dama*) populations (Anheyer-Behmenburg et al., 2017, Matsuura et al., 2007, Neumann et al., 2016, Rutjes et al., 2010, Takahashi et al., 2022, Thiry et al., 2017b, Trojnar et al., 2020, Zhang et al., 2015b). We confirmed that this antibody-positive deer serum originated from a Sika deer by sequencing of mitochondrial DNA and reperforming the ELISA. In this study, the HEV gene was also detected in one Sika deer, indicating the risk of HEV infection by consumption of deer meat. Therefore, it seems likely that Sika deer can be infected with HEV, but that such events must be very rare. In addition, other studies reported higher HEV seroprevalence in moose (*Alces alces*), reindeer (*Rangifer tarandus*) and red deer, which showed seroprevalence rates of 9.1–19.5%, 12–23.1% and 10–12.85%, respectively (Boadella et al., 2010, Di Bartolo et al., 2017, Loikkanen et al., 2020, Sacristan et al., 2021, Slukinova et al., 2021). The variation in seroprevalence among the family *Cervidae* may be influenced by animal behavior or cohabiting species.

Genotypes 3 and 4 are predominant in Asia (Casares-Jimenez et al., 2021, Primadharsini et al., 2019). Moreover, subtypes 3a, 3b, 3e, 3f and 3k and subtypes 4c, 4d, 4f and 4i have been detected in humans, pigs, wild boar, and deer in Japan (Okamoto, 2007, Sato et al., 2020, Smith et al., 2020). Our results showed that genotype 3 has a wide distribution among wild boar populations. Ten of our strains formed a cluster (subtype 3b) with Japan-indigenous strains from swine, rat, wild boar and human origin. One strain from Gunma Prefecture and one from Kagawa Prefecture were more closely related to subtypes 3a and 3k, respectively. The genotype 4 strains, detected from 9 wild boar and 1 Sika deer in Yamaguchi Prefecture, formed one cluster with previously reported strains of wild boar and a human

zoonotic case in Yamaguchi prefecture (Hara et al., 2014, Okita, 2012). The continuous circulation of similar strains in this area since 2011 and the formation of a cluster distinct from previously reported genotype 4 subtypes, suggests the presence of an endemic subtype, which was tentatively named 4j.

Until now, only genotype 3 strains have been detected from deer (Anheyer-Behmenburg et al., 2017, Forgach et al., 2010, Okamoto, 2007, Spancerniene et al., 2018). Two genotype 4 human cases linked to consumption of deer meat were reported in South Korea and Japan (Choi et al., 2013, K et al., 2010), but HEV RNA in the meat was not analyzed. Therefore, this study is the first to report genotype 4 infection in deer.

Some wild animals have been shown to be susceptible to HEV infection (Okamoto et al., 2003, Thiry et al., 2017a). In this survey, we found seropositive rates of 1.4% (2/149) in Japanese monkeys, which was in consistent with previous reports in non-human primates that showed HEV circulation among macaques (Hirano et al., 2003, Melegari et al., 2018, Zhang et al., 2019). However, the other wild animals were negative for anti-HEV antibodies or HEV RNA. The primers used in this study could detect strains belonging to the *Orthohepevirus A* species, so we cannot deny the circulation of other species, like *Orthohepevirus C*. Overall, our findings, which were based on a nationwide survey of wild animals, indicated that the wild boar population is the dominant reservoir of HEV in Japan.

In conclusion, the wild boar population is the dominant reservoir for HEV infection in the field in Japan. In addition, young wild boars were more frequently infected with HEV, suggesting the risk of HEV infection from piglets. Sika deer were rarely infected with HEV, indicating that there is a low, but not zero, risk of HEV infection from Sika deer.

2.6 Figure legends

Figure 1. Geographic distribution of wild boar sampling areas in Japan. The number of anti-HEV antibody-positive animals, the number of examined animals and percentage of positive animals are indicated by prefecture. The percentages are indicated by shading.

Figure 2. Phylogenetic analyses based on the partial ORF2 sequences (338bp). HEV from 21 wild boars and one deer (bold) were compared to the reference strains proposed by Smith *et al*.[54] and the closest strains available in GenBank. The phylogenetic tree with 1,000 bootstrap replicates was generated by the neighbor-joining method. Reference sequences were labeled as "host/country/strain/year (GenBank accession number) subtype".

2.7 Figures and tables

Figure 1.

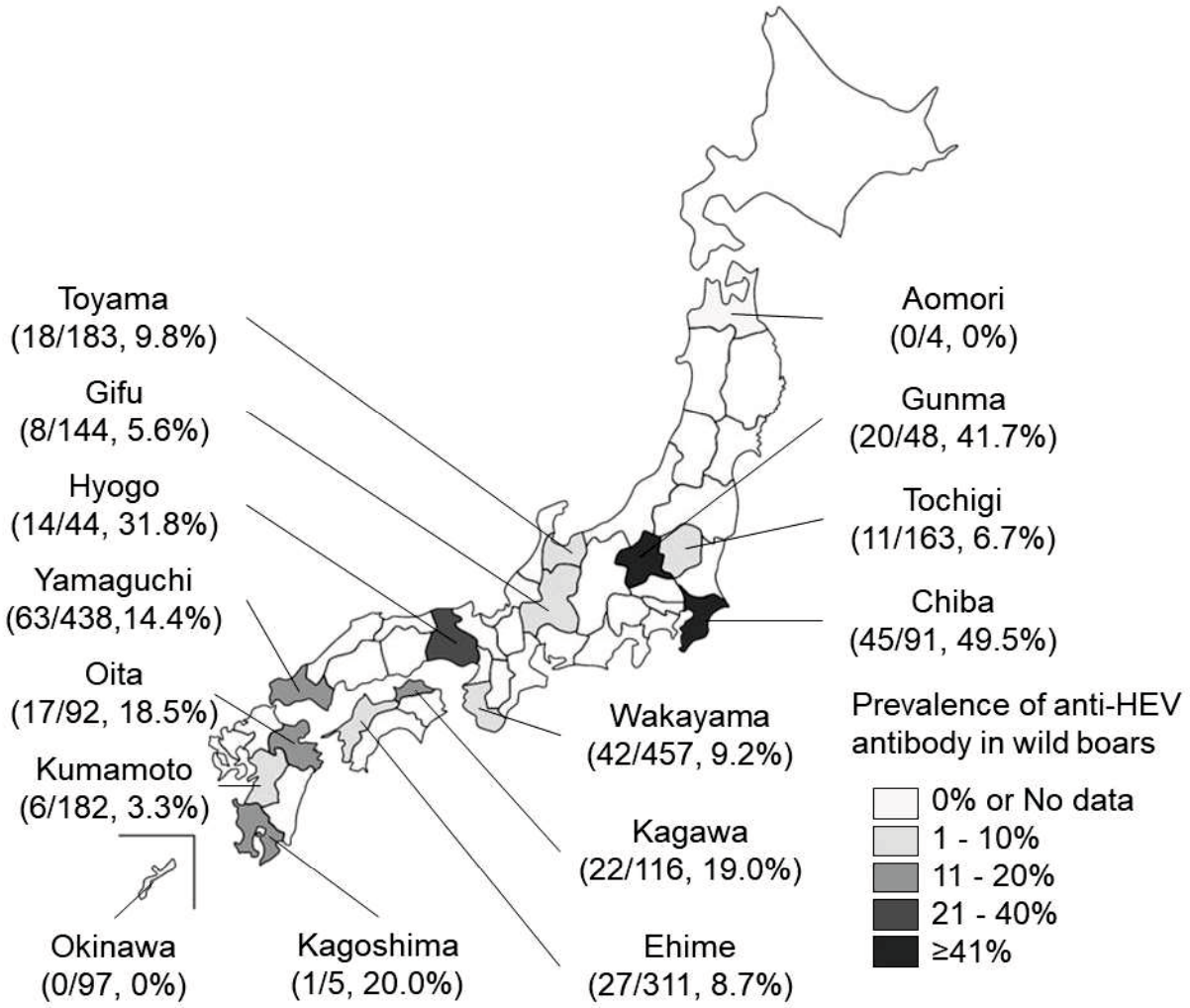

Figure 2.

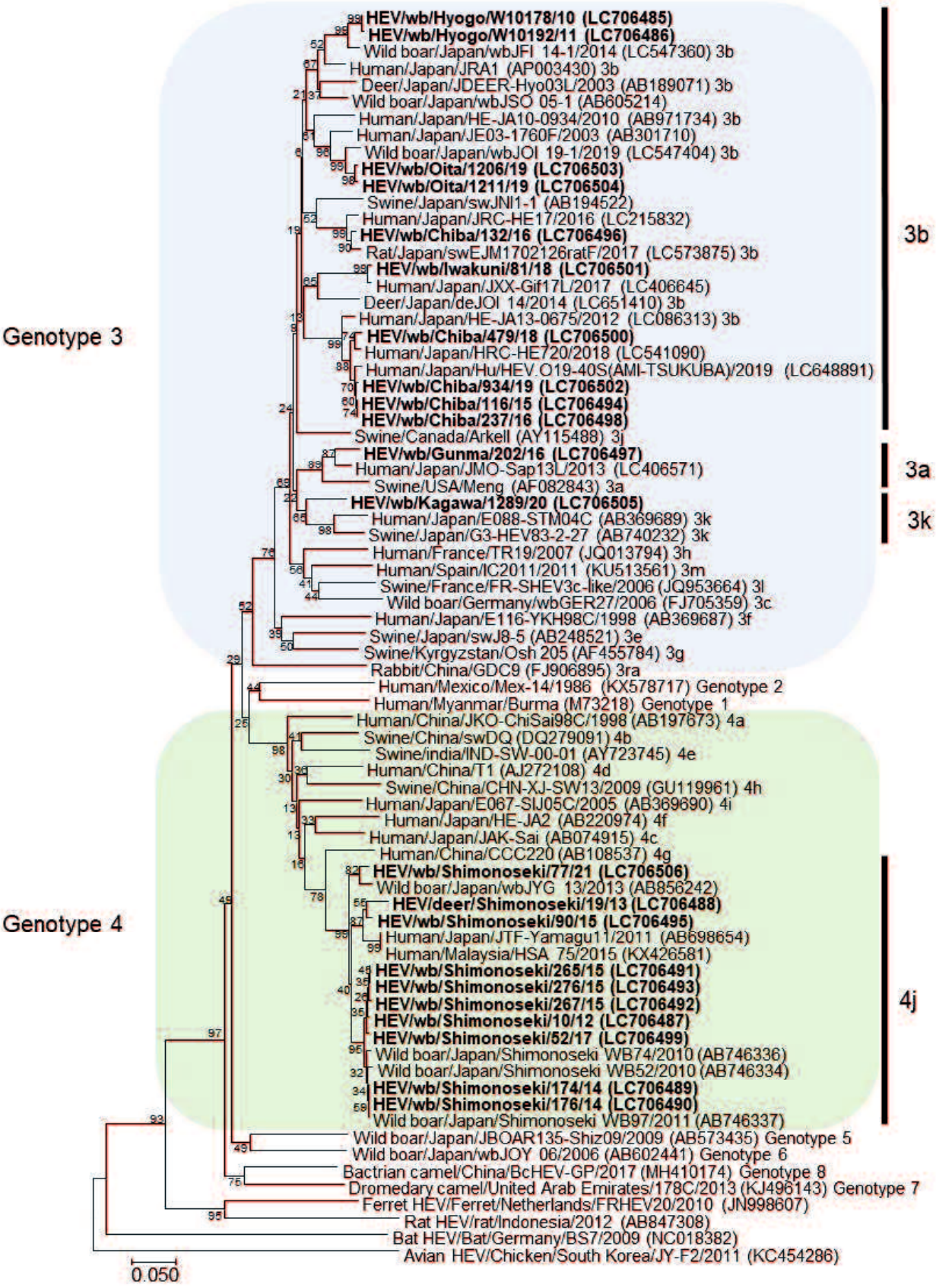
Genotype 3
Genotype 4
HEV/wb/Hyogo/W10178/10 (LC706485)
HEV/wb/Hyogo/W10192/11 (LC706486)
Wild boar/Japan/wbJFI 14-1/2014 (LC547360) 3b
Human/Japan/JRA1 (AP003430) 3b
Deer/Japan/JDEER-Hyo03L/2003 (AB189071) 3b
Wild boar/Japan/wbJSO 05-1 (AB605214)
Human/Japan/HE-JA10-0934/2010 (AB971734) 3b
Human/Japan/JE03-1760F/2003 (AB301710)
Wild boar/Japan/wbJOI 19-1/2019 (LC547404) 3b
HEV/wb/Oita/1206/19 (LC706503)
HEV/wb/Oita/1211/19 (LC706504)
Swine/Japan/swJNI1-1 (AB194522)
Human/Japan/JRC-HE17/2016 (LC215832)
HEV/wb/Chiba/132/16 (LC706496)
Rat/Japan/swEJM1702126ratF/2017 (LC573875) 3b
HEV/wb/Iwakuni/81/18 (LC706501)
Human/Japan/JXX-Gif17L/2017 (LC406645)
Deer/Japan/deJOI 14/2014 (LC651410) 3b
Human/Japan/HE-JA13-0675/2012 (LC086313) 3b
HEV/wb/Chiba/479/18 (LC706500)
Human/Japan/HRC-HE720/2018 (LC541090)
Human/Japan/Hu/HEV.O19-40S(AMI-TSUKUBA)/2019 (LC648891)
HEV/wb/Chiba/934/19 (LC706502)
HEV/wb/Chiba/116/15 (LC706494)
HEV/wb/Chiba/237/16 (LC706498)
Swine/Canada/Arkell (AY115488) 3j
HEV/wb/Gunma/202/16 (LC706497)
Human/Japan/JMO-Sap13L/2013 (LC406571)
Swine/USA/Meng (AF082843) 3a
HEV/wb/Kagawa/1289/20 (LC706505)
Human/Japan/E088-STM04C (AB369689) 3k
Swine/Japan/G3-HEV83-2-27 (AB740232) 3k
Human/France/TR19/2007 (JQ013794) 3h
Human/Spain/IC2011/2011 (KU513561) 3m
Swine/France/FR-SHEV3c-like/2006 (JQ953664) 3l
Wild boar/Germany/wbGER27/2006 (FJ705359) 3c
Human/Japan/E116-YKH98C/1998 (AB369687) 3f
Swine/Japan/swJ8-5 (AB248521) 3e
Swine/Kyrgyzstan/Osh 205 (AF455784) 3g
Rabbit/China/GDC9 (FJ906895) 3ra
Human/Mexico/Mex-14/1986 (KX578717) Genotype 2
Human/Myanmar/Burma (M73218) Genotype 1
Human/China/JKO-ChiSai98C/1998 (AB197673) 4a
Swine/China/swDQ (DQ279091) 4b
Swine/india/IND-SW-00-01 (AY723745) 4e
Human/China/T1 (AJ272108) 4d
Swine/China/CHN-XJ-SW13/2009 (GU119961) 4h
Human/Japan/E067-SIJ05C/2005 (AB369690) 4i
Human/Japan/HE-JA2 (AB220974) 4f
Human/Japan/JAK-Sai (AB074915) 4c
Human/China/CCC220 (AB108537) 4g
HEV/wb/Shimonoseki/77/21 (LC706506)
Wild boar/Japan/wbJYG 13/2013 (AB856242)
HEV/deer/Shimonoseki/19/13 (LC706488)
HEV/wb/Shimonoseki/90/15 (LC706495)
Human/Japan/JTF-Yamagu11/2011 (AB698654)
Human/Malaysia/HSA 75/2015 (KX426581)
HEV/wb/Shimonoseki/265/15 (LC706491)
HEV/wb/Shimonoseki/276/15 (LC706493)
HEV/wb/Shimonoseki/267/15 (LC706492)
HEV/wb/Shimonoseki/10/12 (LC706487)
HEV/wb/Shimonoseki/52/17 (LC706499)
Wild boar/Japan/Shimonoseki WB74/2010 (AB746336)
Wild boar/Japan/Shimonoseki WB52/2010 (AB746334)
HEV/wb/Shimonoseki/174/14 (LC706489)
HEV/wb/Shimonoseki/176/14 (LC706490)
Wild boar/Japan/Shimonoseki WB97/2011 (AB746337)
Wild boar/Japan/JBOAR135-Shiz09/2009 (AB573435) Genotype 5
Wild boar/Japan/wbJOY 06/2006 (AB602441) Genotype 6
Bactrian camel/China/BcHEV-GP/2017 (MH410174) Genotype 8
Dromedary camel/United Arab Emirates/178C/2013 (KJ496143) Genotype 7
Ferret HEV/Ferret/Netherlands/FRHEV20/2010 (JN998607)
Rat HEV/rat/Indonesia/2012 (AB847308)
Bat HEV/Bat/Germany/BS7/2009 (NC018382)
Avian HEV/Chicken/South Korea/JY-F2/2011 (KC454286)
0.050
3b
3a
3k
4j

Table 1. Seroprevalence of hepatitis E virus infection among wild animals in Japan

Species	Prefecture	Year	Percentage of positive animals (Number of HEV-positive animals/ Number of examined animals)
Wild boar	Aomori	2021	0% (0/4)
	Chiba	2015-2019	49.5% (45/91)
	Ehime	2016-2021	8.7% (27/311)
	Gifu	2014-2018	5.6% (8/144)
	Gunma	2015-2019	41.7% (20/48)
	Hyogo	2015-2016	31.8% (14/44)
	Kagawa	2016-2021	19.0% (22/116)
	Kagoshima	2016	20.0% (1/5)
	Kumamoto	2017-2018	3.3% (6/182)
	Oita	2012-2019	18.5% (17/92)
	Okinawa	2019-2020	0% (0/97)
	Tochigi	2011-2012	6.7% (11/163)
	Toyama	2014-2021	9.8% (18/183)
	Wakayama	2015-2021	9.2% (42/457)
	Yamaguchi	2016-2021	14.4% (63/438)
Deer	Aomori	2019-2021	0% (0/39)
	Chiba	2014-2020	0% (0/107)
	Ehime	2016-2019	0% (0/45)
	Gifu	2014-2021	0% (0/339)
	Gunma	2015-2021	0% (0/106)
	Hokkaido	2012	0% (0/49)
	Kagawa	2016-2021	1.5% (1/65)
	Kagoshima	2015-2017	0% (0/29)
	Nagano	2015-2016	0% (0/47)
	Oita	2008-2012	0% (0/12)
	Wakayama	2020-2021	0% (0/347)
	Yamaguchi	2010-2021	0% (0/1000)
	Yamanashi	2014-2015	0% (0/65)
Raccoon	Gunma	2013-2014	0% (0/5)
	Hyogo	2015-2016	0% (0/23)
	Wakayama	2009-2015	0% (0/247)
Mouse	Ehime	2016-2018	0% (0/63)
	Yamaguchi	2015-2019	0% (0/97)
Monkey	Fukuoka	2014-2016	6.3% (2/32)
	Wakayama	2012-2017	0% (0/50)
	Yamaguchi	2018-2019	0% (0/67)
Raccoon dog	Gunma	2013-2014	0% (0/9)
	Wakayama	2014-2015	0% (0/88)

	Yamaguchi	2016-2019	0% (0/18)
Badger	Kagoshima	2016-2017	0% (0/13)
	Wakayama	2008-2020	0% (0/94)
	Yamaguchi	2018	0% (0/3)
Masked palm civet	Gunma	2013-2014	0% (0/3)
	Kagoshima	2017	0% (0/1)
	Wakayama	2012-2015	0% (0/32)
Nutria	Yamaguchi	2015-2016	0% (0/24)
Weasel	Wakayama	2007-2015	0% (0/21)
	Yamaguchi	2018	0% (0/1)
Bear	Akita	2017	0% (0/13)
Marten	Wakayama	2008-2015	0% (0/13)
Hare	Wakayama	2008-2019	0% (0/7)
	Yamaguchi	2018	0% (0/1)
Fox	Wakayama	2008	0% (0/2)
	Yamaguchi	2017-2018	0% (0/4)
Muntjac	Chiba	2015	0% (0/1)

Table 2. Prevalence of anti-HEV antibodies in wild boar in Japan

	Sex			Body weight (kg)				Total
	Male	Female	No record	<30	30-50	>50	No record	
No. of examined animals	1151	1027	197	637	772	538	428	2375
No. of positive animals	139	140	15	34	115	99	46	294
Percentage of anti-HEV antibody-positive animals	12.1%	13.6%	7.6%	5.3%	14.9%	18.4%	10.7%	12.4%

Table 3. Detection of hepatitis E virus genome in wild animals in Japan

Species	Prefecture	Year	Percentage of positive animals (Number of HEV-positive animals/ Number of examined animals)
Wild boar	Aomori	2021	0% (0/4)
	Chiba	2015-2019	5.5% (5/91)
	Ehime	2016-2019	0% (0/115)
	Gifu	2014-2018	0% (0/140)
	Gunma	2015-2019	2.1% (1/48)
	Hyogo	2009-2011	2.6% (2/77)
	Kagawa	2016-2021	0.9% (1/116)
	Oita	2012-2019	2.9% (2/68)
	Toyama	2014-2021	0% (0/183)
	Wakayama	2020-2021	0% (0/354)
	Yamaguchi	2012-2021	1.7% (10/582)
Deer	Aomori	2019-2021	0% (0/39)
	Chiba	2014-2020	0% (0/108)
	Ehime	2016-2019	0% (0/45)
	Gifu	2014-2021	0% (0/339)
	Gunma	2015-2021	0% (0/106)
	Kagawa	2016-2021	0% (0/65)
	Yamaguchi	2010-2021	0.1% (1/986)
Bear	Akita	2017	0% (0/12)
Raccoon dog	Yamaguchi	2018-2019	0% (0/7)
Fox	Yamaguchi	2018	0% (0/2)
Badger	Yamaguchi	2018	0% (0/1)
Hare	Wakayama	2019	0% (0/1)

Table 4. Detection of hepatitis E virus RNA in wild boar in Japan

	Sex			Body weight (kg)				Total
	Male	Female	No record	<30	30-50	>50	No record	
No. of examined animals	873	826	79	460	614	519	185	1778
No. of positive animals	16	5	0	10	8	0	3	21
Percentage of HEV RNA-positive animals	1.8%	0.6%	0.0%	2.2%	1.3%	0.0%	1.6%	1.2%

Table 5. Information on the hepatitis E virus strains detected in the sera of wild animals

Species	Prefecture	Year	Isolate	Accession number	HEV genotype	Sex	Body weight (kg)
Wild boar	Hyogo	2010	HEV/wb/Hyogo/W10178/10	LC706485	3b	♂	32
	Hyogo	2011	HEV/wb/Hyogo/W10192/11	LC706486	3b	♂	16
	Yamaguchi	2012	HEV/wb/Shimonoseki/10/12	LC706487	4j	♂	41
	Yamaguchi	2014	HEV/wb/Shimonoseki/174/14	LC706489	4j	♀	10
	Yamaguchi	2014	HEV/wb/Shimonoseki/176/14	LC706490	4j	♂	10
	Chiba	2015	HEV/wb/Chiba/116/15	LC706494	3b	♂	50
	Yamaguchi	2015	HEV/wb/Shimonoseki/265/15	LC706491	4j	♂	27
	Yamaguchi	2015	HEV/wb/Shimonoseki/267/15	LC706492	4j	♂	23
	Yamaguchi	2015	HEV/wb/Shimonoseki/276/15	LC706493	4j	♂	16
	Yamaguchi	2015	HEV/wb/Shimonoseki/90/15	LC706495	4j	♂	25
	Chiba	2016	HEV/wb/Chiba/132/16	LC706496	3b	♂	30
	Chiba	2016	HEV/wb/Chiba/237/16	LC706498	3b	♀	35
	Gunma	2016	HEV/wb/Gunma/202/16	LC706497	3a	♀	15
	Yamaguchi	2017	HEV/wb/Shimonoseki/52/17	LC706499	4j	♂	40
	Chiba	2018	HEV/wb/Chiba/479/18	LC706500	3b	♂	26
	Yamaguchi	2018	HEV/wb/Iwakuni/81/18	LC706501	3b	♂	31
	Chiba	2019	HEV/wb/Chiba/934/19	LC706502	3b	♂	40
	Oita	2019	HEV/wb/Oita/1206/19	LC706503	3b	♂	No record
	Oita	2019	HEV/wb/Oita/1211/19	LC706504	3b	♀	No record
	Kagawa	2020	HEV/wb/Kagawa/1289/20	LC706505	3k	♀	10
	Yamaguchi	2021	HEV/wb/Shimonoseki/77/21	LC706506	4j	♂	No record
Deer	Yamaguchi	2013	HEV/deer/Shimonoseki/19/13	LC706488	4j	♀	40

3. CHAPTER 2

Characterization of rabbit hepatitis E virus isolated from a feral rabbit

3.1 Abstract

Rabbit hepatitis E virus (HEV) has been detected among rabbits and recently isolated from immunocompromised patients, suggesting zoonotic transmission. In this study, HEV infection among feral rabbits (*Oryctolagus cuniculus*) was assessed by detection of anti-HEV antibodies and HEV RNA. The prevalence of anti-HEV antibodies in sera was of 33% (20/60) and HEV RNA was detected from only one of fecal swabs (1.7%, 1/58). Furthermore, one naïve rabbit was intravenously inoculated with the suspension of the HEV-positive fecal specimen, exhibiting persistent HEV shedding in feces, intermittent viremia, seroconversion to anti-HEV IgM and IgG, and high alanine aminotransferase (ALT) values, indicating persistent HEV infection. The isolate JP-59 had a length of 7,282 bp excluding a poly (A) tail and possessed the characteristic 93 bp-insertion in ORF1. Phylogenetic analysis indicated that JP-59 formed a cluster with other rabbit HEV isolates from rabbits and human origin. The JP-59 shared the nucleotide sequence identities less than 87% with other rabbit HEVs, suggesting that a novel rabbit HEV strain was circulating in Japan.

Key words: rabbit Hepatitis E virus, isolation, seroprevalence

3.2 Introduction

Hepatitis E virus (HEV) has been responsible of outbreaks in developing nations as well as sporadic cases in industrialized countries, causing a self-limited acute infection (Primadharsini et al., 2019). Nonetheless, fulminant hepatitis in pregnant women and HEV chronic infection in immunocompromised patients have raised concern worldwide (Kamar et al., 2008b, Patra et al., 2007).

HEV is a single positive stranded RNA virus with a genome length of 7.2 kb, encoding 3 opening reading frames (ORFs) (Purdy et al., 2017). HEV exists in two distinct forms, as non-enveloped (neHEV) particles shed in feces, or as quasi-enveloped (eHEV) in circulating blood and infected culture supernatant (Primadharsini et al., 2019). HEV belongs to the family *Hepeviridae* and is divided into two genera: *Orthohepevirus* and *Piscihepevirus*. Orthohepevirus A has been assigned into 8 genotypes (Purdy et al., 2017, Woo et al., 2016). Genotype 3 infects a high variety of mammalian species and is closely related with rabbit HEV (rHEV). However, rHEV strains possess a characteristic insertion in the X domain of ORF1 (Izopet et al., 2012, Takahashi and Okamoto, 2014).

Rabbit HEV infection has been found in farmed, feral and wild rabbit populations; and recently in immunocompromised patients in France and Switzerland (Abravanel et al., 2017, Ryll et al., 2018, Sahli et al., 2019, Zhao et al., 2009). Rabbit HEV is capable of infecting rhesus macaques, pigs and mice, as well as replication in human-derived cells such as PLC/PRF/5 and A549 (Cossaboom et al., 2012, Jirintai et al., 2012, Liu et al., 2013, Sun et al., 2018). Experimental infection in rabbits caused persistent HEV infection and also induced fetal mortality in pregnant rabbits (Ahn et al., 2017, Han et al., 2014).

In Japan, HEV genotypes 3 and 4 infections are the most dominant (Li et al., 2005, Takahashi and Okamoto, 2014). Although there have been many reports on HEV infection among wild animals in Japan, rabbit HEV has not been reported. Thus, the purpose of this study was to evaluate the prevalence of rabbit HEV infection among feral rabbits.

3.3 Materials and methods

Sample collection

Sera and fecal swabs were collected from feral European rabbits (*Oryctolagus cuniculus)* in October 2018, in an island located in south west Japan. Adult rabbits were immobilized using a net, 60 serum samples were obtained by venipuncture and 58 fecal swabs were collected by cotton swabs. Sera and fecal swabs were stored at −20 °C and −80 °C, respectively. All rabbits were apparently healthy and their body weights were over 1.5 kg. Since these rabbits were protected under many regulations, only 60 rabbits were permitted.

Detection of anti-HEV antibodies for surveillance

ELISA to detect anti-HEV antibody from many mammalian species (Yonemitsu et al., 2016) was used for screening anti-HEV antibodies from rabbit sera with a minor modification, same as CHAPTER 1. Peroxidase Conjugated Purified Recomb® Protein AG (Thermo fisher scientific) was used as a second antibody. After 3 washes with PBS-T, 100 µL per well of substrate agent (ABTS Microwell Peroxidase Substrate, Sera Care) was added and plates were gently shaken during 30 min at room temperature. Finally, 100 µL of 1% sodium dodecyl sulfate (SDS) was added to each well for stopping the enzymatic reaction and the absorbance (OD_{405nm}) was measured using a spectrophotometer (Bio-Rad). ELISA cut-off value of 0.50 was tentatively applied for judgement.

RNA extraction

Fecal swabs were dissolved in 1 mL of phosphate-buffered saline and centrifuged at 10,000 x *g* for 1 min at 4 °C. The supernatant was collected and 140 µL was used for RNA extraction (QIAamp Viral RNA Mini Kit, Hilden Germany) according to the manufacturer's instructions.

Reverse transcription (RT)-polymerase chain reaction (PCR)

Nested RT-PCR was performed using One-Step RT-PCR Kit (QIAGEN) and KOD-Plus-NEO (Toyobo, Japan) according to the kit protocols. Since rabbit HEV has high heterogeneity, we used two sets of nested primers for detection. The first set of primers amplified the ORF2 region of HEV genotypes 1, 3 and 4 (Li et al., 2005), while the second set of primers targeted the X domain region of rabbit HEV (Abravanel et al., 2017). After RT, 40 cycles of PCR reaction started at 94 °C for 30 s, followed by 30 s at the respective annealing temperature and an amplification at 72 °C for 1 min. Then, a final extension at 72 °C for 10 min was performed. The second PCR reaction started at 94 °C for 2 min, followed by 40 cycles of denaturation at 98°C for 10 s, annealing for 30 s and extension for 1 min at 68 °C. The amplified PCR product was extracted using QIAquick Gel Extraction Kit (QIAGEN) and the sequence was determined using BigDye Terminator v.3.1 technology (FASMAC, Japan).

Experimental infection

The suspension of rabbit fecal specimen was clarified by centrifugation at 10,000 x g for 30 minutes and then passed through a 0.45-μm membrane filter (Millipore, Bedford, MA). One female six-month old Japanese white rabbit (SLC, Hamamatsu, Japan) was negative for anti-HEV antibodies and negative for HEV RNA and inoculated with 1 mL of the fecal suspension through the ear vein. The serum and fecal samples were collected weekly post inoculation (p.i.), and used for the detection of rabbit HEV RNA, anti-rabbit HEV IgG and IgM antibody, or alanine aminotransferase (ALT) values. The experiment under the code 119149 was reviewed and approved by the institutional ethics committee of the National Institute of Infectious Diseases (NIID), and the animal experiment was carried out according to the “Guides for Animal Experiments Performed at NIID”. The rabbit was housed in Biosafety Level-2 facilities.

Liver enzyme level

ALT values in the sera were monitored weekly using a Fuji Dri-Chem Slide GPT/ALT-PIII kit (Fujifilm, Saitama, Japan). The geometric mean titers of the ALT values over the preinoculation period were defined as the normal ALT value, and a 2-fold or greater increase at the peak was considered as a sign of hepatitis (Zhang *et al*., 2020).

Quantitative real-time RT-PCR (RT-qPCR) for detection of rabbit HEV

Viral RNA was extracted from 200 μL of samples using a MagNA Pure LC system with the Total Nucleic Acid isolation kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's recommendations. A one-step RT-qPCR was carried out with a 7500 FAST Real-Time PCR System (Applied Biosystems, Foster City, CA) using TaqMan Fast Virus 1-step Master Mix (Applied Biosystems). The RT-qPCR was performed under the conditions: 5 min at 50°C, 20 s incubation at 95°C, followed by 40 cycles of 3 s at 95°C and 30 s at 60°C, with a primer pair, forward primer 5'-GGTGGTTTCTGGGGTGAC-3' and reverse primer 5'-AGGGGTTGGTTGGATGAA-3', and a probe 5'-FAM- TGATTCTCAGCCCTTCGC-TAMRA-3' (Jothikumar et al., 2006). A 10-fold serial dilution of the capped rabbit HEV RNA (10^7 to 10^1 copies) was used as the standard for the quantitation of viral genome copy numbers. Amplification data were collected and analyzed with Sequence Detector software ver. 1.3 (Applied Biosystems).

Detection of anti-rabbit HEV IgG and IgM antibodies

Anti-rabbit HEV IgG and IgM antibodies were detected by ELISA, as described with a slight modification (Li et al., 2000). In this ELISA, the rabbit HEV-LP was used as ELISA antigen (Zhang et al., 2021). The horseradish peroxidase (HRP)–conjugated goat anti-rabbit IgG-heavy and light-chain antibody (Cappel, Westchester, PA, USA), and HRP-conjugated goat anti-rabbit IgM antibody (Abcam, Tokyo, Japan) were used to detect the rabbit IgG and IgM antibodies, respectively.

Full genome sequencing of rabbit HEV

10% fecal suspensions (w/v) from the infected laboratory rabbit at 12 weeks p.i. were dissolved at 4 °C for 1 h and clarified by centrifugation at 10,000 x g for 30 min and the collected supernatant was passed through a 0.45-µm membrane filter before RNA extraction (QIAamp Viral RNA Mini Kit, Hilden Germany). Full-length rabbit HEV sequences previously deposited on GenBank were used to design primers covering the complete genome. Overlapping fragments were amplified by nested RT-PCR, following One-Step RT-PCR Kit (QIAGEN) and KOD-Plus-NEO (Toyobo, Japan) protocols as previously stated. The 5 prime and 3 prime untranslated regions were obtained after cDNA synthesis using SMARTer® RACE 5'/3' kit (Takara Bio, USA) and RNA LA PCR Kit (AMV) Ver.1.1 (Takara Bio, Japan), respectively.

Phylogenetic analysis of rabbit HEV

The full genome was assembled using MEGA 7.0 software and the phylogenetic tree was constructed using the Maximum Likelihood method based on 1000 replicates. Comparison of genome nucleotide identity with major representative sequences from genotypes 1 to 8 was performed using GENETYX Ver. 14. Simplot and bootscan analysis were performed using SimPlot 3.5.1 software.

3.4 Results and discussion

Anti-HEV antibodies and RNA detection in feral rabbits

Prevalence of anti-HEV antibodies among rabbits was 33% (20/60). No significant difference was observed among sex, 32% female (9/28) and 30% male (9/30) (Table 1). The prevalence of anti-HEV antibodies in feral rabbits was similar to the previous reports in wild and feral rabbit populations in Germany and farmed rabbits in USA, but lower in comparison with those in China,

suggesting that breeding conditions and housing might influence to HEV infection (Cossaboom et al., 2011, Jirintai et al., 2012, Ryll et al., 2018, Zhao et al., 2009).

HEV RNA was detected from only one female rabbit (1.7%) using both sets of primers (Table 1). The 338 bp amplicon within the ORF2 region clustered with other rabbit HEV detected from human, hare, pet and wild rabbits. Anti-HEV antibodies and HEV RNA were not detected from the serum of the HEV-infected rabbit (data not shown). Considering that the original sample of JP-59 successfully infected an experimental rabbit, the rabbit was shedding infectious virions in the feces.

Experimental infection with rabbit HEV

To examine the infectivity of the rabbit HEV in feces of the HEV-positive rabbit, we inoculated a naïve rabbit with the stool suspension. Due to limitation of original sample of JP-59 remained after several efforts of virus isolation *in vitro*, we could not quantify HEV genome copies prior to the animal experiment. The HEV infection cycle in the experimental rabbit was evaluated for 21 weeks. After inoculation, the rabbit HEV RNA was detected in feces with 1.80×10^4 copies/g at week 2 post inoculation (p.i.), and then increased to 1.79×10^7 copies/g at week 12 p.i. The viral RNA was continually detected until week 21 p.i. with RNA titers of 5.43×10^7 copies/g (Fig. 1). Rabbit HEV RNA was detected in serum samples from week 3-21 p.i. with lower RNA titers (8.60×10^2 to 6.91×10^3 copies/ml), and sometimes under the detection limit. The IgM and IgG (Fig. 1) antibodies were first detected at 9 and 10 week p.i., and reached a peak at week 11 p.i. with OD values of 0.506 and 0.861, respectively. Then, both antibodies decreased, and the IgM antibody became undetectable after week 13 p.i.. However, the IgG antibody increased again from week 17 p.i., and reached another peak at week 19 p.i. with an OD value of 1.117, and then decreased again from week 20 p.i. Any significant elevation of ALT was not observed except at 17 weeks p.i. with a value of 97 IU.

Similar to the previous reports, our rabbit also showed persistent virus shedding in feces, intermittent viremia, increase of hepatic enzymes and seroconversion (Han et al., 2014, Zhang et al., 2021).

Complete genome and phylogenetic analysis of the rabbit HEV

The full genome was amplified from the infected rabbit fecal suspension. The isolate JP-59 (accession no. LC535077) had a length of 7,282 bp excluding a poly (A) tail. The 5′ UTR contained 26 nucleotides, followed by ORF1 (27 nt to 5195 nt), ORF2 (5230 nt to 7212 nt), ORF3 (5192 nt to 5560 nt) and the 3' UTR (7213 nt to 7282 nt). Similar to the previous reports (Izopet et al., 2012, Zhao et al., 2009), the phylogenetic analysis showed that JP-59 was closely related with genotype 3, possessed the characteristic 93 nt-insertion in ORF1 and clustered distinctively with the other rabbit HEV genomes and one human isolate from France (Fig.2). In comparison with viruses detected at 32 weeks p.i., this complete genome at 21 weeks p.i. possessed one non-synonymous substitution in the ORF1 (data not shown).

The full-length isolate JP-59 was 72.92-73.44%, 71.70%, 75.59-78.31%, 73.04-73.41%, 72.62%, 72.24-72.6%, 73.45-73.99% and 73.9% similar with HEV genotypes 1, 2, 3, 4, 5, 6, 7 and 8, respectively. In comparison to the other genotypes, JP-59 shared the highest similarities of amino acid sequences with genotype 3 ORF1 (84.97-88.87%) and ORF2 (91.96-94.09%). Further comparison with other rabbit HEV strains did not show any distinctive feature of the isolate JP-59, neither any evidence of recombination. Interestingly, the Japanese rabbit HEV (JP-59) isolate identity with other rabbit HEV full genomes ranged from 80.59 to 86.95 %, suggesting that a unique rabbit HEV strain was circulating in Japan.

Although rabbit HEV was the first reported from farmed rabbits in China, HEV RNA was detected in stocked wild rabbit serum in Germany, suggesting that rabbit HEV has been circulating in

wild rabbit population as early as 1983 (Eiden et al., 2016, Zhao et al., 2009). Since the reports on zoonotic infection with rabbit HEV have increased, further studies are required to clarify the zoonotic risk or rabbit HEV.

3.5 Conclusions

Rabbit HEV genome and anti-HEV antibodies were detected in feral rabbit population in Japan. In addition, the Japanese rabbit HEV isolate caused a persistent infection in a naïve rabbit exhibiting viremia, seroconversion, fecal shedding and an elevation of liver enzymes. As the other reports, our rabbit HEV isolate also possesses a characteristic insertion in the ORF1 and is more closely related to genotype 3. Further studies are required to determine the zoonotic risk of rabbit HEV.

3.6 Figure legends

Fig. 1. Rabbit HEV infection in Japanese white rabbit.

One naive rabbit was intravenously inoculated with suspension from a fecal swab containing rabbit HEV. The kinetics of the rabbit HEV RNA in fecal specimens (○) and sera (△), anti-rabbit HEV-IgG (□) and IgM (▽) and ALT (◇) were measured.

Fig. 2. Phylogenetic analyses based on full-length sequences of hepatitis E virus (HEV).

Japanese isolate JP-59 (bold) was compared with corresponding sequences of rabbit HEV and to HEV genotype 1 to 8 full genome sequences available in GenBank. The phylogenetic tree with 1,000 bootstrap replicates was generated by the maximum likelihood method based on the General Time Reversible model.

3.7 Figures and tables

Fig. 1.

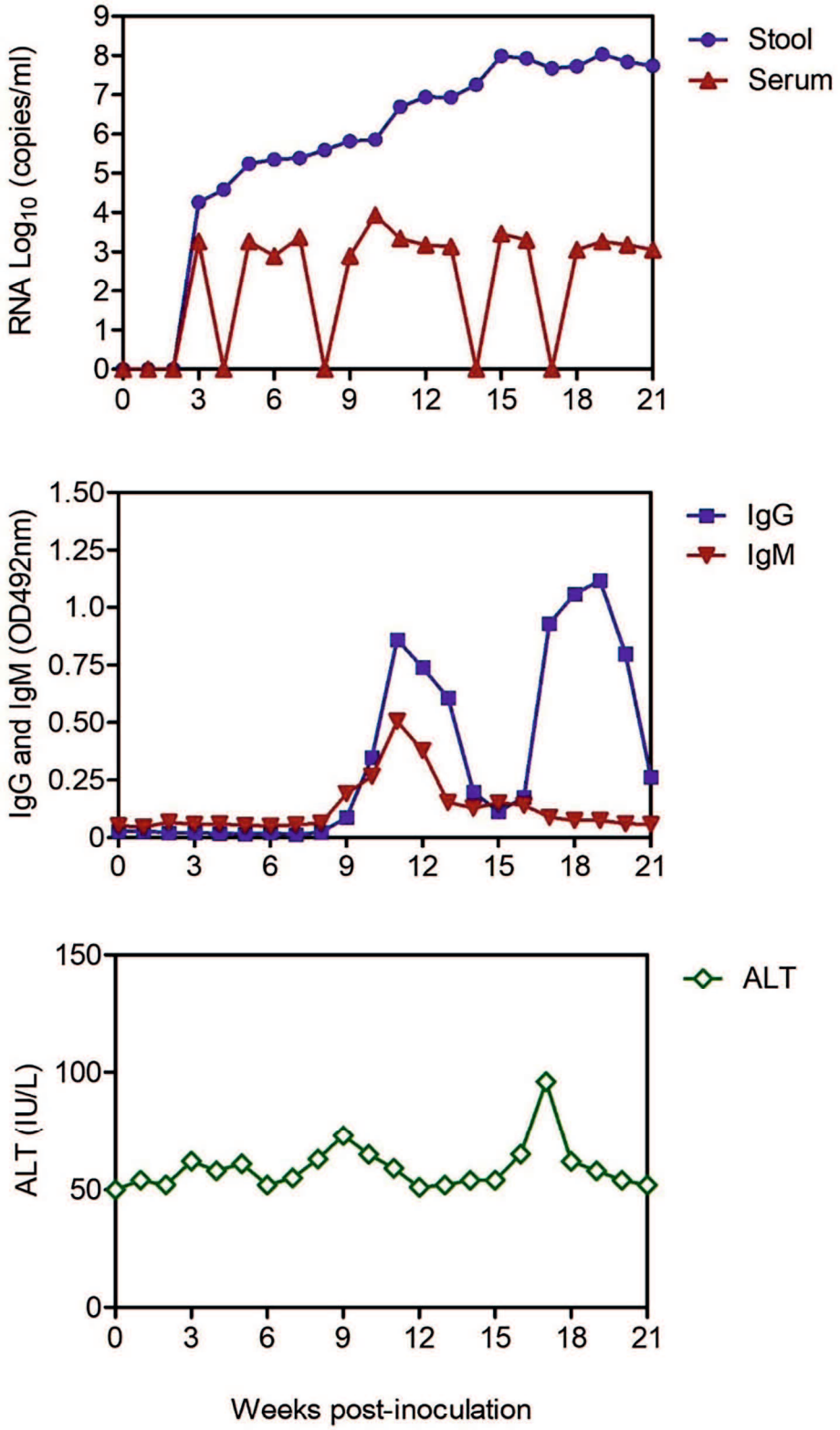

Fig. 2.

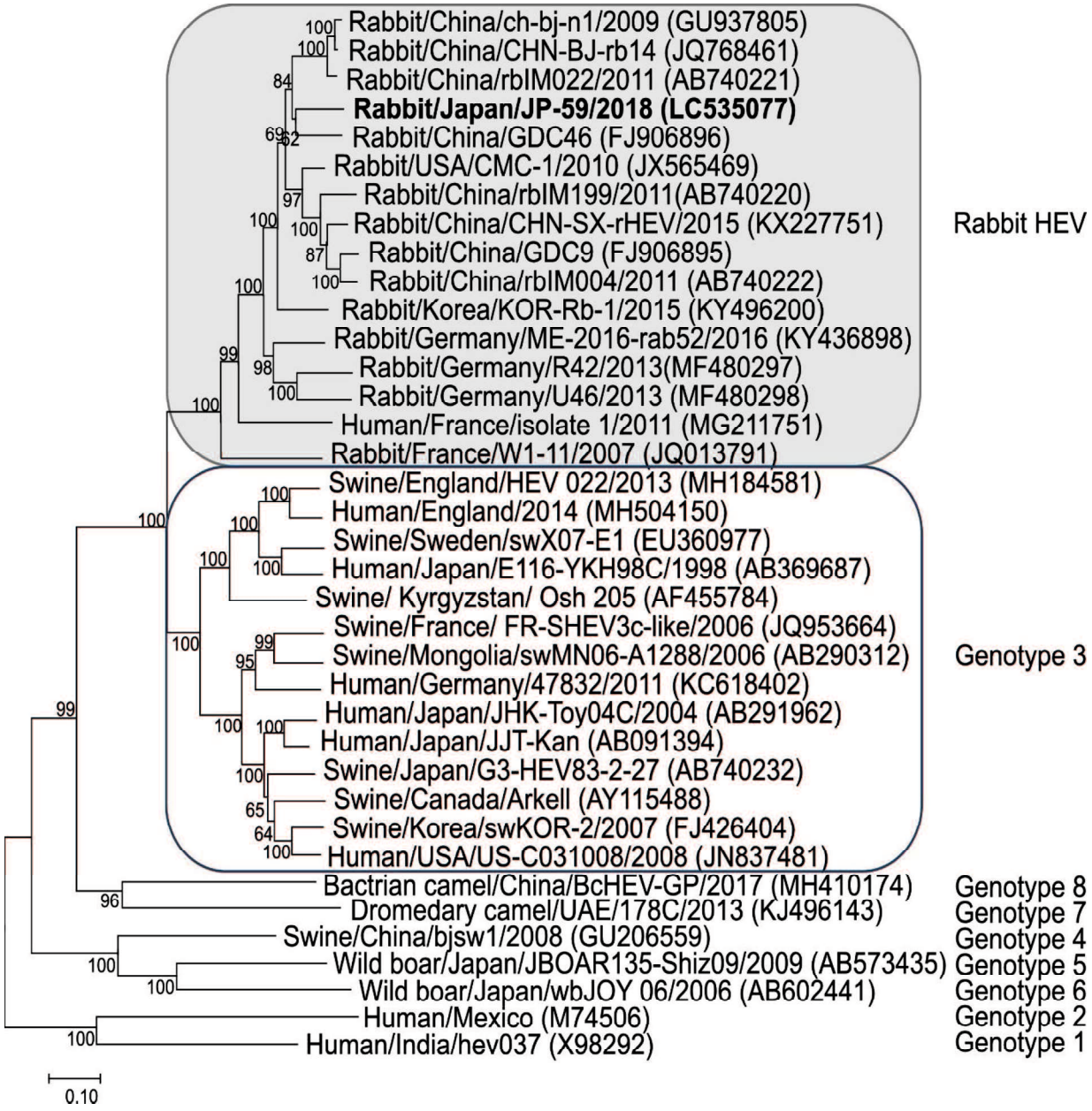
Rabbit/China/ch-bj-n1/2009 (GU937805)
Rabbit/China/CHN-BJ-rb14 (JQ768461)
Rabbit/China/rbIM022/2011 (AB740221)
Rabbit/Japan/JP-59/2018 (LC535077)
Rabbit/China/GDC46 (FJ906896)
Rabbit/USA/CMC-1/2010 (JX565469)
Rabbit/China/rbIM199/2011(AB740220)
Rabbit/China/CHN-SX-rHEV/2015 (KX227751)
Rabbit/China/GDC9 (FJ906895)
Rabbit/China/rbIM004/2011 (AB740222)
Rabbit/Korea/KOR-Rb-1/2015 (KY496200)
Rabbit/Germany/ME-2016-rab52/2016 (KY436898)
Rabbit/Germany/R42/2013(MF480297)
Rabbit/Germany/U46/2013 (MF480298)
Human/France/isolate 1/2011 (MG211751)
Rabbit/France/W1-11/2007 (JQ013791)
Rabbit HEV
Swine/England/HEV 022/2013 (MH184581)
Human/England/2014 (MH504150)
Swine/Sweden/swX07-E1 (EU360977)
Human/Japan/E116-YKH98C/1998 (AB369687)
Swine/ Kyrgyzstan/ Osh 205 (AF455784)
Swine/France/ FR-SHEV3c-like/2006 (JQ953664)
Swine/Mongolia/swMN06-A1288/2006 (AB290312)
Human/Germany/47832/2011 (KC618402)
Human/Japan/JHK-Toy04C/2004 (AB291962)
Human/Japan/JJT-Kan (AB091394)
Swine/Japan/G3-HEV83-2-27 (AB740232)
Swine/Canada/Arkell (AY115488)
Swine/Korea/swKOR-2/2007 (FJ426404)
Human/USA/US-C031008/2008 (JN837481)
Genotype 3
Bactrian camel/China/BcHEV-GP/2017 (MH410174)
Dromedary camel/UAE/178C/2013 (KJ496143)
Swine/China/bjsw1/2008 (GU206559)
Wild boar/Japan/JBOAR135-Shiz09/2009 (AB573435)
Wild boar/Japan/wbJOY 06/2006 (AB602441)
Human/Mexico (M74506)
Human/India/hev037 (X98292)
Genotype 8
Genotype 7
Genotype 4
Genotype 5
Genotype 6
Genotype 2
Genotype 1
0.10

Table 1. Seroprevalence and HEV RNA detection among feral rabbits

Sex	**ELISA**			**Nested RT-PCR**	
	No. of examined animals	**No. of positive animals (%)**		**No. of examined animals**	**No. of positive animals (%)**
Male	30	9(30)		30	0(0)
Female	28	9(32)		26	1(4)
Unknown	2	2(100)		2	0(0)
Total	60	20(33)		58	1(2)

4. General conclusions

Hepatitis E virus (HEV) infection spreads worldwide, causing water-borne outbreaks in developing countries. In high-income nations, sporadic HEV cases are related to consumption of infected meat, blood transfusion and organ-transplantation. In Japan, the first report of food-borne zoonotic transmission of HEV by the ingestion of game meat occurred in 2003. Following surveys revealed that HEV genotypes 3 and 4 are predominant in human patients and mainly linked to the consumption of pork and wild boar meat. Furthermore, HEV genome or antibodies have been found in companion and wild animals, suggesting that other species are also exposed to HEV infection in Japan.

In this thesis, the risk of HEV transmission was assessed by studying the prevalence of HEV infection among wild boar, Sika deer and a wide variety of animals living in the wild. In addition, a novel Japanese rabbit HEV strain was detected and further characterized in an experimental rabbit.

In CHAPTER 1, the prevalence of anti-HEV antibodies and genome characterization of circulating HEV strains were studied, among 15 wild animal species in Japan.

In wild boars, the overall prevalence of anti-HEV antibodies was 12.4% (294/2,375), with higher positive rates in big boars (over 50 kg, 18.4%) than in small individuals (less than 30 kg, 5.3%). In addition, genotypes 3 and 4 were circulating among wild boar populations and HEV RNA was more frequently detected in piglets than in older boars. Interestingly, Sika deer had very low ratios of seroprevalence (0.04%, 1/2,250) and HEV RNA detection (0.06%, 1/1688), with only one individual carrying a genotype 4 strain. This study suggested that HEV infection in Sika deer was a very rare event, and that there was no HEV infection among wild animals except for wild boar, Sika deer and Japanese monkeys.

In CHAPTER 2, we assessed the prevalence of HEV infection among feral rabbits (*Oryctolagus cuniculus*) in Japan and characterized the novel rabbit HEV isolate.

In feral rabbits, the prevalence of anti-HEV antibodies was 33% (20/60) and HEV RNA was detected from only one animal (1.7%, 1/58). The Japanese rabbit HEV isolate, named as JP-59, caused a persistent infection in a naïve rabbit exhibiting viremia, seroconversion, fecal shedding and an elevation of liver enzymes. The Japanese isolate possessed the characteristic 93 bp-insertion in ORF1 and was more closely related to genotype 3, clustering with other rabbit HEV isolates from rabbits and human origin. Moreover, The JP-59 shared the nucleotide sequence identities of less than 87% with other rabbit HEVs, suggesting that a novel rabbit HEV strain was circulating in Japan.

This thesis contributed to clarify HEV dynamics and circulation among various wild animal species in Japan. In CHAPTER 1, a nationwide survey showed that wild boars act as the main reservoir of HEV and piglets might be a major source of HEV infection to humans. In addition, Sika deer and the other wild animal species showed less risk or no risk of HEV transmission. In CHAPTER 2, a novel Japanese rabbit HEV isolate was detected from feral rabbits and caused a persistent infection in a naïve rabbit, indicating that feral rabbits living in the wild were susceptible to HEV infection.

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6.Abstract

Abstract of Doctoral Thesis
Joint Graduate School of Veterinary Medicine,
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Main Supervisor: Ken Maeda

Epidemiological studies on hepatitis E virus among wildlife and discovery of a novel rabbit HEV strain in Japan

(国内の野生動物における E 型肝炎ウイルスの疫学調査と新規ウサギ E 型肝炎ウイルスの発見)

Hepatitis E virus (HEV) is a causative agent of viral hepatitis worldwide. In developing countries, HEV causes water-borne outbreaks with high mortality rates in pregnant women. In industrialized nations, sporadic hepatitis E cases occur by consumption of infected meat, blood transfusion and organ-transplantation. In the last decade, zoonotic food-borne transmission cases have increased in developed countries and novel HEV genotypes have been detected in a large variety of domestic and wild animals, some of which showed zoonotic potential. These findings have showed that hepatitis E is not only a disease limited to water-borne epidemics in low-income countries, but also a zoonotic disease with a high risk for public health.

In 2003, Japan was the first country to report food-borne zoonotic transmission of HEV by the ingestion of game meat (Sika deer). Since then, it has been reported that human is mainly infected with HEV genotypes 3 and 4 and that the main reservoir of HEV in nature was wild boar. Despite of zoonotic transmission from game meat, HEV infection dynamics among wild

or feral animals have not been fully understood in Japan. In CHAPTER 1, evaluation and characterization of HEV strains circulating among a wide variety of wild animals in Japan were performed. In CHAPTER 2, we evaluated the prevalence of rabbit HEV genome or antibodies among feral rabbits in Japan and characterized the novel rabbit HEV isolate.

CHAPTER I Nationwide survey of hepatitis E virus infection among wildlife in Japan

In this chapter, we performed a nationwide surveillance of HEV infection by using a total of 5,557 serum samples collected from 15 wild animal species in many prefectures of Japan. Detection of anti-HEV antibodies and HEV RNA was performed by using ELISA and nested reverse transcription PCR. In addition, Sika deer serum samples that were found to be positive for HEV RNA or anti-HEV antibodies, were further evaluated to confirm host mitochondrial DNA. The overall prevalence of anti-HEV antibodies in wild boars was 12.4% (294/2,375), with higher positive rates in big boars (over 50 kg, 18.4%) than in small individuals (less than 30 kg, 5.3%). In addition, HEV RNA was more frequently detected in piglets than in older boars, suggesting the presence of acute HEV infection in younger population. Interestingly, our survey results indicated that HEV infection in Sika deer was a very rare event, because ratio of RNA detection and seroprevalence were very low, 0.06% (1/1688) and 0.04% (1/2,250), respectively. Japanese monkeys showed seropositive ratio of 1.4% (2/149) while the other examined wild animals were negative to HEV infection. In conclusion, wild boar, especially piglets, are at high risk of HEV infection, while other wild animals showed less risk or no risk of HEV transmission.

CHAPTER II Characterization of rabbit hepatitis E virus isolated from a feral rabbit

Rabbit hepatitis E virus (HEV) has been detected among farmed, wild and pet rabbits worldwide and recently isolated from immunocompromised patients, suggesting zoonotic transmission. Despite reports on HEV infection among domestic and wild animals in Japan, rabbit HEV has not been identified before this survey. In this study, we assessed the prevalence of HEV infection among feral rabbits (*Oryctolagus cuniculus*) by detection of anti-HEV antibodies in sera and HEV RNA in fecal swabs. The prevalence of anti-HEV antibodies was 33% (20/60) and HEV RNA was detected from only one animal (1.7%, 1/58). Furthermore, one naïve rabbit was intravenously inoculated with the suspension of the HEV-positive fecal specimen. During an observation period of 21 weeks, the infected rabbit exhibited persistent HEV shedding in feces, intermittent viremia, seroconversion to anti-HEV IgM and IgG, and high alanine aminotransferase (ALT) values, indicating persistent HEV infection. The rabbit HEV isolate, named as JP-59, had a length of 7,282 bp excluding a poly (A) tail, possessed the characteristic 93 bp-insertion in ORF1 and clustered with other rabbit HEV isolates from rabbits and human origin. The JP-59 shared the nucleotide sequence identities of less than 87% with other rabbit HEVs, suggesting that a novel rabbit HEV strain was circulating in Japan.

Our research contributed to clarify HEV dynamics and circulation among wild animal species in Japan. Wild boar population must act as the main reservoir of HEV in wildlife and piglets might be a major source of HEV infection for human and the other animals. Sika deer were rarely infected with HEV, indicating less risk of HEV transmission. Feral rabbits living in the wild were susceptible to HEV infection, and the Japanese rabbit HEV isolate caused a persistent infection in a naïve rabbit. Finally, the other wild animal species showed less risk or no risk of HEV transmission.