Establishment of ELISA for hepatitis E virus and the epidemiological studies

E 型肝炎ウイルスの ELISA 系の開発と疫学調査

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1. GENERAL INTRODUCTION

1.1. History of hepatitis E virus

Hepatitis E is recently known as one of five major pathogens of viral hepatitis. The outbreak of hepatitis E was firstly reported in 1980s (Khuroo, 1980). In this study, the authors reported the outbreak case of enterically-transmitted Non-A, Non-B hepatitis in India. In the total population of 16,620 inhabitants, 275 cases of viral hepatitis were recorded, and was characterized by a high morbidity and mortality among pregnant women. After this report, some studies about enterically-transmitted Non-A, Non-B hepatitis were conducted and this pathogenic agent was reported in India, Nepal, Africa and Mexico (Belabbes et al., 1985; Kane et al., 1984; Tandon et al., 1982; Velázquez et al., 1990).

In 1983, the confirmation of existence of the new hepatitis agent was reported (Balayan et al., 1983). Stool extracts from presumed case of epidemic non-A, non-B hepatitis were inoculated to human volunteers. The volunteers developed typical acute hepatitis on day 36 and was lasted for about 3 weeks. Spherical 27- to 30-nm virus-like particles detected were reacted with convalescent sera from patients with enteric non-A, non-B hepatitis.

The genome of this virus was first reported in 1990 (Reyes et al., 1990). In this study, nucleic acid clones of a part of the genome were analyzed from bile specimens obtained from experimentally infected animals. Following this report, sequence analysis of the whole genome of isolates in Asia and Mexico was reported (Huang et al., 1992; Tam et al., 1991). A significant difference was found in the genome sequences of these two

isolates, indicating the presence of multiple genotypes. During that time, this virus was named hepatitis E virus.

In 1995, the first HEV identified from animals was reported. HEV RNA and HEV specific antibodies were detected in domestic pigs in Nepal (Giri et al., 1995). Two years later, swine HEV strain in pigs in the United States was identified and genetically characterized (Meng et al., 1997). This virus was genetically distant from the two previously recognized HEVs in human. The existence of indigenous HEV in the developed countries was predicted. Subsequently, novel HEV were recovered from two patients with acute hepatitis E in the United States and demonstrated that these viruses are close to the swine HEV isolate (Schlauder et al., 1998). This discovery has been estimated the risk of zoonotic transmission of HEV and promoted the studies about HEV-related viruses among several animal species around the world.

The first direct evidence of zoonotic transmission of HEV to human was provided in Japan in 2003 (Tei et al., 2003). The families developed the illness after consuming raw deer meat. Identical HEV genome were detected from both clinical specimens of the patients and left-over frozen deer meat. Subsequently, additional direct evidences of HEV transmission to humans by consumption of pork and wild boar meat were reported (Li et al., 2005; Riveiro-Barciela et al., 2015; Yazaki et al., 2003). Further investigations revealed the prevalent of HEV in many animal species and currently recognized as an important public health concern around the world.

1.2. Virus properties

HEV is a non-enveloped virus, 27-30 nm in diameter, with an icosahedral capsid. The virus has a positive-sense, single-stranded, approximately 7.2 kb RNA genome which is capped and polyadenylated (Reyes et al., 1990; Tam et al., 1991). The genome contains three open reading frames (ORFs). ORF1 encodes non-structural proteins, including methyltransferase, cysteine protease, RNA helicase, and RNA-dependent RNA polymerase domains. ORF2 and ORF3 are partially over-lapping and encode a structural protein and a small phosphoprotein, respectively (Mushahwar, 2008). The ORF2 encodes the viral capsid protein of 660 amino acids that is involved in virion assembly, host-virus interaction, and immunogenicity (He et al., 2008; Li et al., 1997; Xing et al., 2011). Three distinct domains have been identified in the capsid protein, the shell domain, middle domain and protruding domain (Guu et al., 2009). ORF3 protein associates with the cytoskeleton and is involved in virion release from infected cells (Yamada et al., 2009; Zafrullah et al., 1997).

The efficient cell culture systems of HEV were developed by using PLC/PRF/5 cells, derived from human hepatocarcinoma, and A549 cells, derived from human lung adenocarcinoma (Tanaka et al., 2007).

1.3. Classification

In recent two decades, HEV and HEV-related viruses have been identified in a large variety of animal species including domestic pigs, wild boars, deer, rabbits, camels, moose, mongooses, ferrets, rodents, chickens, bats and cutthroat trout (Batts et al., 2011;

Cossaboom et al., 2011; Drexler et al., 2012; Lin et al., 2015; Nakamura et al., 2006; Pavio et al., 2010; Raj et al., 2012; Takahashi et al., 2014; Woo et al., 2014). All HEV and HEV-related viruses are classified into the *Hepeviridae* family with two genera: *Orthohepevirus* with four species (A–D) and *Piscihepevirus* with a single species (Doceul et al., 2016). Species within the genus Orthohepevirus are designated *Orthohepevirus A* (strains from human, pig, wild boar, deer, mongoose, rabbit and camel), *Orthohepevirus B* (strains from chicken), *Orthohepevirus C* (strains from rat, greater bandicoot, Asian musk shrew, ferret and mink) and *Orthohepevirus D* (strains from bat) (Smith et al., 2014). Piscihepeviruses have only been isolated from cutthroat trout in North America. The genome structure is similar to those of orthohepeviruses, but piscihepeviruses are phylogenetically distinct (Batts et al., 2011; Smith et al., 2014).

Four main genotypes of HEV, genotypes 1 to 4, which infect to human, belong to the *Orthohepevirus A*. Genotypes 1 and 2 infect only human, mainly transmitted via fecal-oral route in developing countries. Genotype 3 and 4 are recognized as zoonotic virus and have been isolated from both humans and animals including pig, wild boar, deer, and rabbit (Pavio et al., 2010). Sporadic cases of infection are observed all over the world. Genotypes 5 and 6 HEV have been found in wild boar in Japan, and genotype 7 has been found in dromedary camels in United Arab Emirates (Takahashi et al., 2014; Woo et al., 2014). Recently genotype 8 has been reported from Bactrian camels in China (Woo et al., 2016). Rabbit HEV is most closely related to genotype 3 HEV, and shares 77–79 % nucleotide similarity with HEV genotypes 1 to 4 (Geng et al., 2011).

1.4. Epidemiology

The distribution of hepatitis E virus is different between developed and developing countries. HEV genotypes 1 and 2 are mainly distributed in tropical and subtropical regions of Asia, Africa, and Latin America. HEV genotype 4 is distributed in Asia and Europe, and HEV genotype 3 is distributed worldwide (Forni et al., 2018).

The disease is common in developing countries which have poor sanitation. In these areas, HEV infections are reported as both sporadic cases and outbreak associated cases. HEV genotype 1 is most common, and genotype 2 is only reported from Mexico and Africa (Kamar et al., 2017). It is estimated that 20.1 million HEV infections have been occurred, including 3.4 million symptomatic cases, 70,000 deaths, and 3,000 stillbirths (Rein et al., 2012).

HEV infection in developed countries is mainly caused by HEV genotype 3. HEV genotype 4 is mainly reported in eastern Asia, such as Japan, China and Korea (Kamar et al., 2017). In Japan, HEV infection with genotypes 3 and 4 is endemic. The prevalence of anti-HEV IgG of Japanese blood donors was 3.4% and characterized by higher rate in eastern Japan and older people (Takeda et al., 2010).

1.5. Diseases and treatment

In most patients, HEV infection is an acute self-limiting disease which lasts a few weeks. After initial incubation period of 2–6 weeks, symptoms of hepatitis, including fever and nausea, followed by abdominal pain, vomiting, anorexia, malaise and hepatomegaly will be developed. The clinical presentation is quite similar between

developing and developed countries (Aggarwal, 2013a). In rare cases, acute hepatitis E can be severe, and results in fulminant hepatitis. Fulminant hepatitis occurs more frequently when hepatitis E infection occurs during pregnancy. Many studies in developing countries have shown that mortality rate may reach 30% in third trimester of pregnancy (Navaneethan et al., 2008). HEV genotype 3 and 4 infections can become chronic in immunocompromised patients, including solid organ transplant patients and human immunodeficiency virus infected patients (Crum-Cianflone et al., 2012; Kamar et al., 2008).

In many cases, hepatitis E infection is self-limiting, and specific treatment is not required (Kamar et al., 2017). Several antiviral therapies are established for chronic and severe acute HEV infection. In the case of chronic HEV infection in immunosuppressed patient, ribavirin and interferon alpha have been used for treatment (Kamar et al., 2010a, 2010b). Ribavirin has been used in severe acute HEV infection, and seems to be effective (Gerolami et al., 2011; Rahman et al., 2013).

It is still unknown whether hepatitis E virus causes disease in animals. HEV genotype 3 infection does not cause disease in swine and only microscopic evidence of hepatitis is observed (Meng et al., 1997).

1.6. Diagnosis

HEV infection is clinically difficult to distinguish from the other types of acute viral hepatitis (Kumar et al., 2013). HEV infection can be diagnosed by detection of viral genome and specific antibodies against virus (Arends et al., 2014).

HEV infection induces the production of both IgM and IgG antibodies. In the acute phase of hepatitis E, anti-HEV IgM antibody appears in the patient serum and lasts about 8 weeks after onset. Anti-HEV IgG indicates the current or past infection of HEV, since anti-HEV IgG antibody appears to be slightly delayed from IgM and lasts for a long time (Huang et al., 2010). Numerous commercial enzyme immunoassays are available for the detection of anti-HEV IgM and IgG antibodies in serum, though there are considerable differences in their sensitivities and specificities (Kumar et al., 2013). Anti-HEV IgA detection has also been reported to be effective for the diagnosis of recent HEV infection (Takahashi et al., 2005). Since 2011, commercial diagnostic kit for HEV infection based on IgA detection has been used in Japan.

HEV RNA can be detected in both blood and stool samples at the peak of the acute phase. Detection of HEV RNA provides a highly sensitive and specific approach to diagnosis of current HEV infection (Aggarwal, 2013b). Detection rate of HEV RNA is high in 2 weeks before and after onset of symptoms.

1.7. Prevention

HEV genotypes 1 and 2 are transmitted by the fecal-oral route. HEV genotypes 1 and 2 infection can be prevented by providing clean drinking water and improving the sanitary condition in developing countries.

HEV genotypes 3 and 4 cause a zoonotic disease. HEV genotype 3 and 4 are transmitted by consumption of contaminated meat products. Infection may be prevented by avoiding eating raw or undercooked meat. Heating foods to an internal temperature of

71°C for 20 min is necessary to completely inactivate HEV (Barnaud et al., 2012).

Vaccines to prevent hepatitis E virus infection have been developed (Shrestha et al., 2007; Zhu et al., 2010). In 2011, a recombinant subunit vaccine to prevent hepatitis E virus infection was registered in China. However, it is uncertain if this vaccine will be registered in other countries. This vaccine is based on partial ORF2 (aa 368 to aa 606) of HEV genotype 1 (S. W. Li et al., 2005). This vaccine showed equivalent effects to HEV genotype 4, not only HEV genotype 1. Immunization with this vaccine provided protection against hepatitis E for 4.5 years (Zhang et al., 2015).

2. CHAPTER 1

Simple and specific method for detection of antibodies against hepatitis E virus in mammalian species

2.1. ABSTRACT

Hepatitis E virus (HEV) is the causative agent of hepatitis E, a food- and water-borne disease. In developed countries, consumption of meats from pigs, wild boars and deer is a major source of infection. Although HEV and HEV-related viruses have been detected in many animal species, their zoonotic potential and prevalence has not been completely understood. To detect anti-HEV antibody in mammalian species, a simple enzyme-linked immunosorbent assay (ELISA) was established using extract from cells expressing HEV capsid protein and protein A/G as an antigen and a reagent for detection of antibody. Absorbance in the ELISA was compared with those in our previous ELISA using VLPs and anti-swine antibody, suggesting that newly established ELISA was similarly specific and sensitive as the previous ELISA. Seroprevalence of HEV infection among wild boars was examined in Yamaguchi Prefecture, confirming that 111 of 364 wild boars (30.5%) were positive for anti-HEV antibody. Next, this ELISA was applied to humans, dogs, cats, ferrets, raccoons and masked palm civets in Japan, and anti-HEV antibodies were detected in humans, ferrets, dogs and cats. This ELISA is thus useful for serological surveys and comparison of HEV infection among various mammals, including humans.

2.2. INTRODUCTION

HEV genotypes 3 and 4 cause sporadic infections in both developing and developed countries (Aggarwal and Jameel, 2011; Meng, 2010). HEV genotypes 3 and 4 infection is recognized as zoonosis, and the major reservoirs are pigs (*Sus scrofa domesticus*), wild boars (*Sus scrofa leucomystax*) and deer (*Cervus* spp.) (Ruggeri et al., 2013). Genotypes 3 and 4 have been detected in many mammalian species, including pigs, wild boars, deer, rabbits (*Oryctolagus cuniculus*), and mongooses (*Herpestes auropunctatus*) (Cossaboom et al., 2011; Geng et al., 2011; Meng et al., 1997; Nakamura et al., 2006). Recent reports have demonstrated that novel HEV or HEV-related viruses are present in other animals, including wild boars, rats (*Rattus* spp.), rabbits, ferrets (*Mustela putorius furo*), minks (*Neovison* vison), foxes (*Vulpes vulpes*) and bats (*Chiroptera* spp.) (Bodewes et al., 2013; Drexler et al., 2012; Johne et al., 2010b, 2010a; Krog et al., 2013; Lhomme et al., 2013; Raj et al., 2012; Takahashi et al., 2014). However, their zoonotic potential and prevalence in wild animals has not been completely understood (Johne et al., 2014).

In order to investigate antibodies prevalence against HEV and HEV-related viruses in wild animals, enzyme-linked immunosorbent assay (ELISA) using purified virus-like particles (VLPs) and animal-specific anti-immunoglobulins have been carried out. However, there are problems with ELISA using sera from wild animals; (1) Sera from wild animals are sometimes hemolytic, causing non-specific reactions in serological tests; (2) Secondary antibodies specific for animal species are used for detection of the antibodies in each wild animal, but secondary antibodies for some animals are not widely

available; (3) As HEV does not grow efficiently in cell culture, preparation of antigens is difficult (Tanaka et al., 2007). In many laboratories, including our own, VLPs have been used as an ELISA antigen, but impurities in VLPs affect the results of ELISA. Therefore, highly purified VLPs are required for ELISA to detect antibodies specific for HEV.

In this study, we developed an ELISA using extracts from mammalian cells expressing viral protein as an antigen, and peroxidase-conjugated protein A/G as a reagent for detection of antibody. This ELISA was applied to the serosurveillance of HEV infection in humans, wild boars, ferrets, raccoons (*Procyon lotor*), masked palm civets (*Paguma larvata*), dogs (*Canis lupus familiaris*) and cats (*Felis sylvestris Catus*).

2.3. MATERIALS AND METHODS

2.3.1. Serum samples

Serum samples were collected from wild boars, ferrets, raccoons, masked palm civets, dogs and cats. A total of 519 sera from wild boars were collected in Yamaguchi (n = 364), Hyogo (n = 67) and Wakayama (n = 88) Prefectures of Japan; 47 sera from ferrets were collected in animal hospitals throughout Japan (n = 47); 208 sera from raccoons were collected in Hyogo Prefecture, Japan; 65 sera from masked palm civets were collected in Hyogo Prefecture, Japan; 170 sera from dogs were collected in Yamaguchi (n = 135) and Miyagi (n = 35) Prefectures of Japan; and 17 sera from cats were collected in Miyagi Prefecture, Japan. Serum samples were also collected from 24 hunters who hunt wild animals in Yamaguchi Prefecture, Japan under permission from the Center for Clinical Research, Yamaguchi University Hospital (Control Number: H26-116). All collected sera were stored at −20 °C until use.

2.3.2. Construction of plasmids

Viral RNA was extracted from the serum of a patient with fulminant hepatitis E in Yamaguchi Prefecture, Japan in 2011 (Okita et al., 2012) using a QIAamp viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. First-strand cDNA was transcribed using the RNA LA PCR Kit (AMV) Ver.1.1 (TAKARA, Shiga, Japan) with random 9-mer primers at 30 °C for 10 min, 42 °C for 30 min, 70 °C for 15 min and 4 °C for 5 min. Full-length and the N-terminal-truncated fragment of the HEV open reading frame 2 (ORF2) were amplified using two primer pairs,

Yamagu11 ORF2 1F(ClaI) (5′-GT ATC GAT CAC CAT GCG CTC TCG GGC T-3′) and Yamagu11 ORF2 660R-His (5′-GT AGA TCT TCA GTG ATG GTG ATG GTG ATG GTA CTC CCG GGT TTT ACC CA-3′), for the full-length ORF2, and Yamagu11 ORF2 112F(ClaI) (5′-GT ATC GAT CAC CAT GGC TGT GGC TCC GGC CCC T-3′) and Yamagu11 ORF2 660R-His for the N-terminal truncated ORF2. Amplified cDNA was digested with the restriction enzymes ClaI and BglII, and was then cloned into the ClaI-BglII site of pCAGGS plasmid (Niwa et al., 1991). The resultant plasmids were designated pCAGGS-HEVcap (1-660) and pCAGGS-HEVcap (112-660).

2.3.3. Cells

Human embryonic kidney 293T (HEK-293T) cells were grown in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific, Waltham, MA) with 10% heat-inactivated fetal calf serum (FCS; Thermo Fisher Scientific), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in 5% CO2.

2.3.4. Expression in HEK-293T cells

HEK-293T cells were transfected with plasmids, pCAGGS-HEVcap (1-660), pCAGGS-HEVcap (112-660) and pCAGGS using polyethylenimine (PEI; Thermo Fisher Scientific). Briefly, 16 μg of plasmid were mixed with 40 μl of PEI (2 mg/ml) and were transfected into HEK-293T cells in a 90-mm cell culture dish (Sumitomo Bakelite, Tokyo, Japan), as reported previously (Boussif et al., 1995). After 3 days post-transfection, cells were washed twice with phosphate-buffered saline (PBS) and detached from the dishes

with a cell scraper (Sigma-Aldrich, St. Louis, MO). Cells were treated with 0.5 ml of RIPA buffer (1% sodium deoxycholate, 1% Triton X-100, 10 mM Tris-HCl pH 7.4, 150 mM sodium chloride (NaCl), 0.5 mM ethylenediaminetetraacetic acid) for 1 h at 4 °C. After centrifugation at 13,000 × g for 30 min at 4 °C, supernatant was collected and stored at −80 °C until use.

2.3.5. Western blot analysis

Extracts of HEK-293T cells transfected with plasmids were mixed with an equal volume of two-fold concentrated sample buffer (6.25 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 20% glycerol, 0.001% bromophenol blue). These samples were boiled for 3 min, placed on ice for 3 min and centrifuged at 13,000 × g for 3 min at room temperature. Then, cell lysates were electrophoresed on polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membrane (Immobilon-P; Millipore, Billerica, MA). After blocking with Tris-buffered saline (20 mM Tris-HCl and 150 mM NaCl, pH 7.5) (TBS) containing 3% gelatin (EIA Grade Reagent Gelatin; Bio-Rad, Hercules, CA) for 45 min at 37 °C, the membrane was washed three times with TBS containing 0.05% Tween 20 (T-TBS). A mouse anti-His antibody (Tetra-His antibody; QIAGEN) was diluted with T-TBS containing 1% gelatin. After incubation with diluted anti-His antibody for 45 min at 37 °C, the membrane was washed three times with T-TBS. Then, the membrane was reacted with peroxidase-conjugated goat anti-mouse IgG + A + M (Cappel Laboratories, Solon, OH) diluted in T-TBS containing 1% gelatin for 45 min at 37 °C. After washing the membrane with T-TBS and TBS three times each, specific

bands were visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB; Wako, Osaka, Japan).

In order to detect the anti-HEV antibodies in animal sera, the PVDF membrane was prepared using the same method and blocked with TBS containing 1% Blockace (Dainippon Pharmaceutical, Osaka, Japan) for 2 h at room temperature. Sera and Peroxidase Conjugated Purified Recomb® Protein A/G (Thermo Fisher Scientific) were diluted with T-TBS containing 0.4% Blockace.

2.3.6. ELISA

Extracts from HEK-293T cells transfected with the plasmids, pCAGGS-HEVcap (112-660) or pCAGGS, were diluted to 5 μg/ml with adsorption buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6), and were distributed at 100 μl per well into 96-well microplates (Maxisorp; Nunc, Roskilde, Denmark). After incubation at 37 °C for 2 h, plates were placed at 4 °C overnight. Wells were washed three times with PBS containing 0.05% Tween 20 (PBS-T), and were then incubated with 100 μl per well of 1% Blockace in PBS at 37 °C for 30 min. Sera were 100-fold diluted with PBS-T containing 0.4% Blockace. Wells were washed three times with PBS-T, and then diluted sera were added to duplicate wells. After incubation at 37 °C for 30 min, wells were washed three times with PBS-T and incubated with 100 μl per well of Peroxidase Conjugated Purified Recomb® Protein A/G diluted in PBS-T containing 0.4% Blockace at 37 °C for 30 min. Following three washes with PBS-T, 100 μl of substrate reagent (HRP Substrate Kit; Bio-Rad) was added to each well. After gentle shaking at room

temperature for 30 min, the enzymatic reaction was stopped by adding 100 µl of 2% oxalic acid to each well. Absorbance was measured using a spectrophotometer (Bio-Rad) at a wavelength of 415 nm. Absorbance of wells coated with extract from empty plasmid-transfected cells were subtracted from those of antigen-coated wells.

2.3.7. Statistical analysis

For statistical analysis, chi-squared, McNemar's and Wilcoxon signed-rank tests were performed. P values of < 0.05 were considered to be statistically significant. Cohen's kappa coefficient was used to calculate agreement between assays.

2.4. RESULTS

2.4.1 Expression of HEV capsid protein

Expression of full-length and N-terminal truncated capsid proteins in HEK-293T cells was confirmed by Western blot analysis (Fig. 2-1). Specific bands with molecular masses of 74 kiloDaltons (kDa) and 60 kDa in cells transfected with pCAGGS-HEVcap (1-660) and pCAGGS-HEVcap (112-660), respectively, were detected using anti-His antibody, but no bands were detected in pCAGGS-transfected cells. The bands with molecular masses of less than 55 kDa on N-terminal truncated protein likely represented degraded proteins. The amount of expressed N-terminal truncated protein was clearly greater than that of complete capsid protein. Therefore, HEK-293T cells expressing N-terminal truncated HEV capsid protein were extracted by TritonX-100 and sodium deoxycholate and then used as antigens for ELISA and Western blot analysis.

2.4.2. Establishment and evaluation of ELISA for detection of HEV-specific antibodies in wild animals

Sixty-seven sera from wild boars captured in Wakayama Prefecture, Japan, and 104 wild boars captured in Yamaguchi Prefecture, Japan, were compared using two ELISA methods; the new method established in this study and our previous method of VLPs and swine-specific secondary antibody. In Wakayama Prefecture, Japan, anti-HEV antibodies were not detected in wild boars (Hara et al., 2014). Using this new ELISA, the average and standard deviation (S.D.) of OD values were 0.007 and 0.042, respectively. On the other hand, the average and S.D. in our previous method were 0.104 and 0.074,

respectively (Fig. 2-2). Furthermore, samples showing absorbance of over 3.5 in this ELISA were more numerous than in our previous ELISA. The cut-off value of 0.437 was calculated by ROC analysis based on the results of this and the previous ELISA using sera from wild boars in Yamaguchi and Wakayama Prefectures. The area under curve of ROC was 0.986. The sensitivity and the specificity of newly developed ELISA were 0.978 and 0.937, respectively (Table 2-1). By this ELISA, additional eight wild boars were classified as positive, and one previously positive wild boar was classified as negative. The Kappa statistics for agreement between the two methods was 0.871. A conservative higher cut-off value of 0.500 was proposed for the other mammals in order to prevent false positives.

2.4.3. Seroprevalence of HEV infection among wild boars in Yamaguchi Prefecture

Seroprevalence of HEV among wild boars (n = 364) in Yamaguchi Prefecture, located in the western part of Japan, was examined by this ELISA (Table 2-2). In wild boars, 111 of 364 (30.5%) were seropositive for anti-HEV antibody. Seroprevalence in wild boars over 20 kg (35.9%) was significantly higher than that among those less than 20 kg (15%) ($P < 0.05$) and that in wild boars over 50 kg (44.1%) was significantly higher than that in wild boars less than 50 kg (23.9%) ($P < 0.05$). There were no significant differences in seroprevalence between males (30.3%) and females (32.1%).

2.4.4 Detection of antibodies against HEV in humans, wild boars, ferrets, raccoons, masked palm civets, dogs and cats

Sera from 24 hunters who hunt wild animals in Yamaguchi Prefecture, Japan, were tested and nine hunters (38%) were seropositive for anti-HEV antibodies. Sera from wild boars captured in Hyogo Prefecture (n = 67) and Wakayama Prefecture (n = 88) of Japan, were tested, and eight from Hyogo Prefecture (13%) and none from Wakayama Prefecture (0%) were seropositive. Forty-seven sera from ferrets were collected in animal hospitals throughout Japan. Five ferrets (11%) were seropositive for anti-HEV antibodies. Raccoons and masked palm civets were captured in Hyogo Prefecture, Japan, and all raccoons and masked palm civets were seronegative for anti-HEV antibodies. Sera from dogs were collected in Yamaguchi and Miyagi Prefectures in Japan. The results showed that one of 135 dogs in Yamaguchi Prefecture (0.7%) and none of 35 dogs in Miyagi Prefecture (0%) was seropositive for anti-HEV antibodies. In cats, two of 17 (12%) in Miyagi Prefecture, Japan, were seropositive for anti-HEV antibodies (Table 2-3).

2.4.5. Confirmation of antibodies against HEV by western blot analysis

To confirm the specificity of ELISA, western blot analysis was carried out. Selected sera that were positive for anti-HEV antibody by ELISA were used as the first antibody, and a specific protein with a molecular mass of 60 kDa was detected (Fig. 2-3).

2.5. DISCUSSION

In this study, it was demonstrated that the newly established ELISA is useful for detection of HEV infection in various mammalian species, including humans. This ELISA using the new antigen and HRP-labelled Protein A/G was improved when compared with the previous ELISA using VLPs and animal-specific secondary antibodies (Hara et al., 2014). This novel ELISA is expected to be applicable to serological surveys of HEV infection among various wild animals.

In the previous ELISA, VLPs of HEV were expressed in insect cells infected with recombinant baculovirus expressing a truncated HEV capsid protein. VLPs were then purified from culture supernatant by polyethylene glycol precipitation and density-gradient centrifugation (Hara et al., 2014; Yamashita et al., 2009). The preparation of purified VLPs is complex and time-consuming. Our procedure to prepare ELISA antigen comprises only lysis of cells transfected with the plasmid. Transfection using PEI is a recently developed and inexpensive method (Boussif et al., 1995). Furthermore, as control wells were treated with lysates of cells transfected with empty vector, the OD value of non-specific reactions could be correctly subtracted from the OD value in wells using lysates of HEV capsid-expressing cells. In fact, mean absorbance of wild boars in Wakayama Prefecture in our newly established ELISA was 0.007, but that in the previous ELISA was 0.104 (Fig. 2-2), indicating that our newly established ELISA can remove the non-specific reaction. In addition, OD values of HEV-positive individuals by newly established ELISA were significantly higher than those by the previous ELISA ($P < 0.05$, Fig. 2-2, Table 2-1), indicating that it is more sensitive than our previous ELISA.

In order to apply the novel ELISA to numerous mammalian species, HRP-conjugated protein A/G was selected to detect HEV-specific antibodies. Protein A/G has been reported to have a broad binding ability for immunoglobulins of various mammals, including wild boars, monkeys, dogs, raccoons and raccoon dogs (Inoshima et al., 1999; Shimoda et al., 2014, 2013; Suzuki et al., 2015). Moreover, we demonstrated the availability of HRP-conjugated protein A/G as a reagent for detection of antibody for ELISA (Inoshima et al., 1999; Shimoda et al., 2014, 2013; Suzuki et al., 2015; Our unpublished data). Therefore, this novel ELISA could be applied to many animal species and does not require species-specific secondary antibodies. Importantly, the reactivity between antibody and protein A/G should be examined before ELISA using sera from new species.

Previously, we reported a high prevalence of HEV among wild boars in Yamaguchi Prefecture, Japan (Hara et al., 2014). In this study, further investigation was performed using the new ELISA method, indicating that 111 of 364 (30.5%) wild boars in Yamaguchi Prefecture, Japan, were seropositive for HEV. This positive rate was significantly higher than that in the other two prefectures of Japan, Hyogo Prefecture (13%) and Wakayama Prefecture (0%) ($P < 0.05$). This confirms that Yamaguchi Prefecture, Japan, is an endemic area for HEV.

We found that five of 47 (11%) ferrets tested were seropositive for HEV. Recently, it was reported that ferret HEV was detected in Japan (Li et al., 2014) and that it serologically cross-reacted with human HEV (Yang et al., 2013). Therefore, these seropositive ferrets might be infected with ferret HEV, but not human HEV. This suggests

that the present ELISA also works well for investigating the seroprevalence of ferret HEV infection in ferrets.

Sera from raccoons and masked palm civets in Hyogo Prefecture, Japan, where a case of HEV contracted by consumption of deer meat was first reported in 2003 (Tei et al., 2003), were examined. Although wild boars in Hyogo Prefecture, Japan, (13%) were seropositive, all raccoons and masked palm civets were seronegative for HEV. This suggests that raccoons and masked palm civets are not reservoirs of HEV. In sera from dogs and cats, a few samples were positive for HEV. This suggests that dogs and cats in Japan are also at risk for HEV infection. Animal sera positive for anti-HEV antibody by ELISA were examined by Western blotting. A specific band with a molecular mass of 60 kDa was visualized using sera from wild boars, ferrets, dogs and cats (Fig. 2-3). The results confirmed the ELISA data.

In conclusion, this simple and sensitive ELISA was able to detect anti-HEV antibodies in wild boars, ferrets, dogs, cats and humans. This ELISA may therefore become a powerful tool for investigation of HEV prevalence among several mammals.

2.6. LEGENDS FOR FIGURES

Fig. 2-1. Expression of HEV ORF2 protein in HEK-293T cells. The plasmids pCAGGS-HEVcap (1-660), pCAGGS-HEV (112-660) and pCAGGS were transfected into HEK-293T cells and antigens were extracted from transfected cells. Immunoblotting was carried out using anti-His-tag antibody. Molecular masses of protein markers are indicated on the left.

Fig. 2-2. Comparison of absorbance in ELISA using HEV ORF2 protein expressed in cells and VLPs as antigens. ELISA was performed using sera from 67 wild boars captured in Wakayama Prefecture, Japan, and 104 wild boars in Yamaguchi Prefecture, Japan. Black lines show the results for the new ELISA established in this study. Dotted lines show the results for our previous ELISA (Hara et al., 2014).

Fig. 2-3. Detection of anti-HEV antibodies by Western blotting. Extracts from HEK-293T cells were used as antigens for Western blotting. Sera of 12 wild boars, 2 ferrets, 2 cats and 2 dogs were used as the 1st antibody. Peroxidase Conjugated Purified Recomb® Protein A/G was used as a reagent for detection of antibody. Representative results are shown in this figure. Arrow heads show the expressed HEV capsid protein.

Figure 2-1.

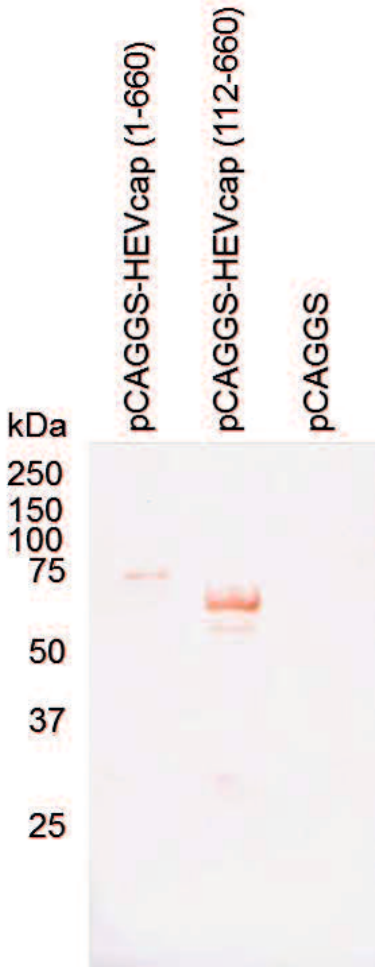

pCAGGS-HEVcap (1-660)
pCAGGS-HEVcap (112-660)
pCAGGS
kDa
250
150
100
75
50
37
25

Figure 2-2.

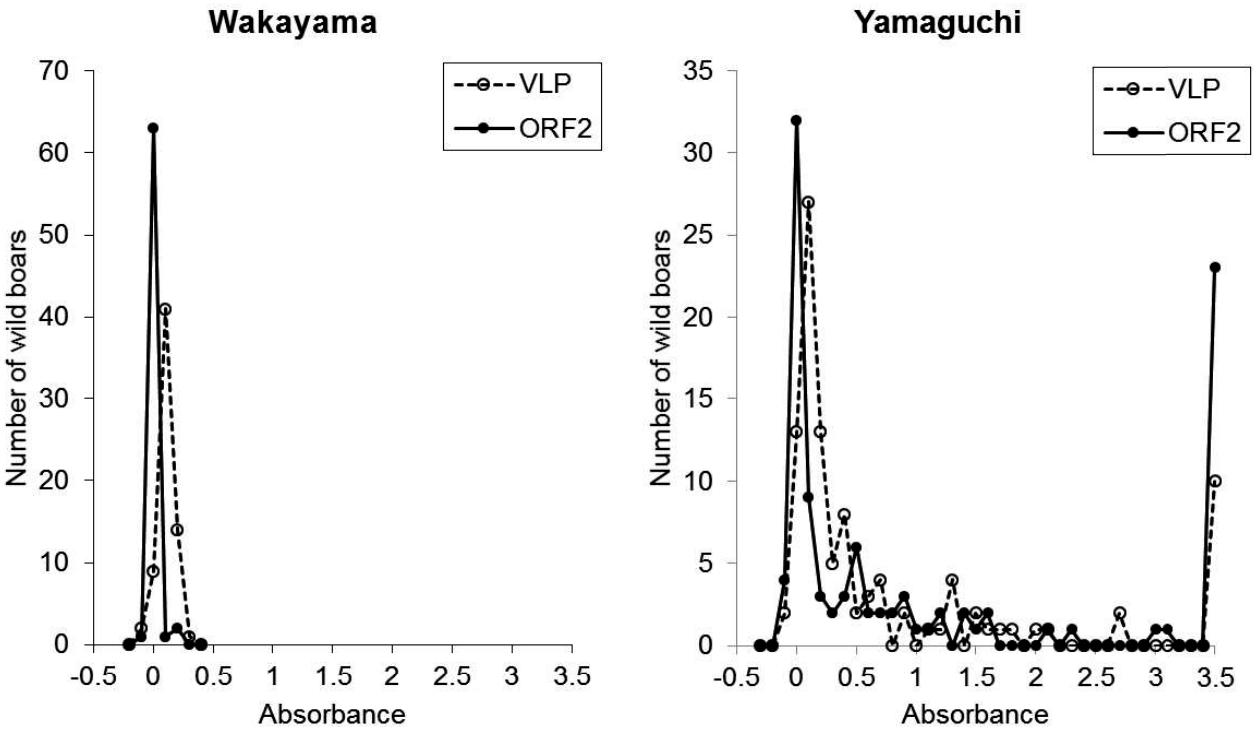

Wakayama
VLP
ORF2
Number of wild boars
Absorbance
70
60
50
40
30
20
10
0
-0.5
0
0.5
1
1.5
2
2.5
3
3.5
Yamaguchi
VLP
ORF2
Number of wild boars
Absorbance
35
30
25
20
15
10
5
0
-0.5
0
0.5
1
1.5
2
2.5
3
3.5

Figure 2-3.

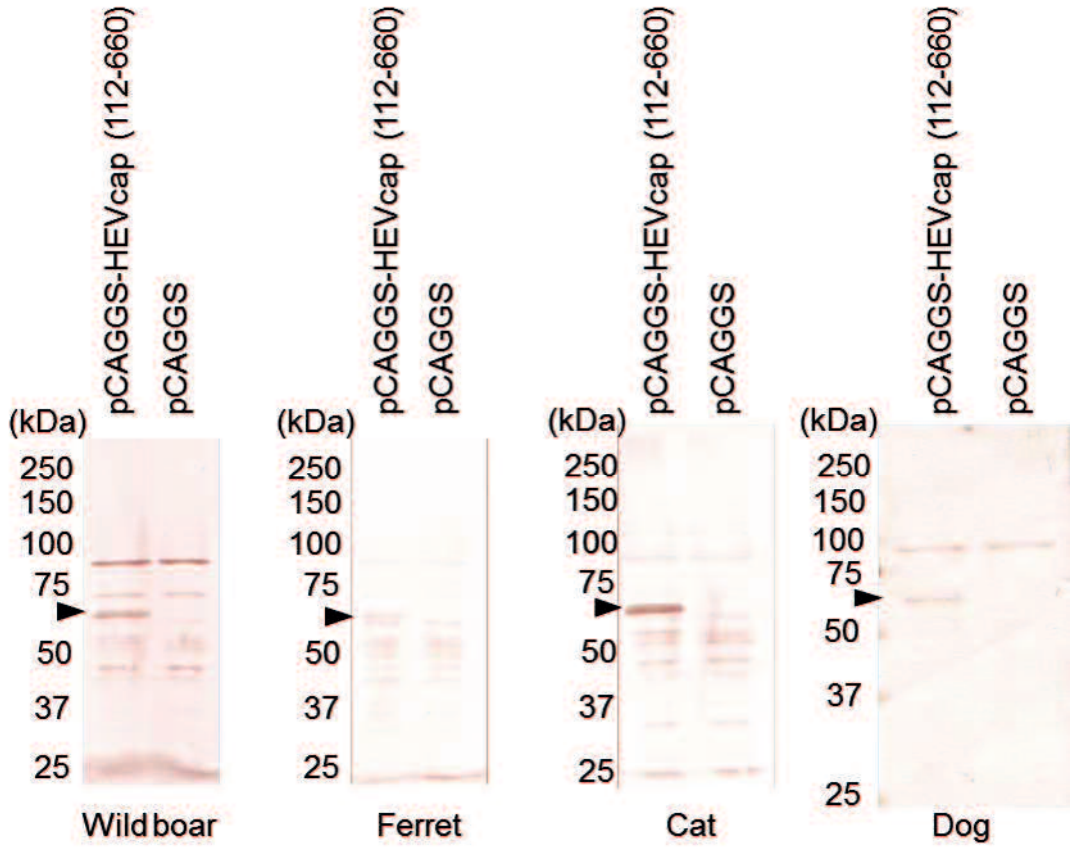

pCAGGS-HEVcap (112-660)
pCAGGS
(kDa)
250
150
100
75
50
37
25
Wild boar
Ferret
Cat
Dog

Table 2-1.Comparison between this and the previous ELISA using sera from wild boars in Yamaguchi and Wakayama prefectures.

ELISA using expressed ORF2	ELISA using VLP (Hara et al., 2014)		
	(+)	(-)	Total
(+)	44	8	52
(-)	1	118	119
Total	45	126	171

Table 2-2. Seroprevalence of HEV in wild boars in Yamaguchi Prefecture.

	Sex			Body weight (kg)				Total
	Male	Female	Unknown	<20	20-50	>50	Unknown	
Number of examined animals	142	209	13	66	135	127	36	364
Number of anti-HEV antibody positive animals	43	67	1	10	38	56	7	111
Percentage of anti-HEV antibody positive animals	30.3	32.1	8	15	28.1	44.1	19	30.5

Table 2-3. Seroprevalence of HEV in mammals.

Species	Place	Year	Percentage of positive animals (number of HEV positive animals / number of examined animals)
Human	Yamaguchi	2015	38 (9/24)
Wild boar	Yamaguchi	2010-2015	30.4 (111/364)
	Hyogo	2011-2014	13 (9/67)
	Wakayama	2007-2013	0 (0/88)
Dog	Yamaguchi	2010-2015	0.7 (1/135)
	Miyagi	2015	0 (0/35)
Cat	Miyagi	2015	12 (2/17)
Ferret	Japan	2012-2014	11 (5/47)
Raccoon	Hyogo	2008-2014	0 (0/208)
Masked palm civet	Hyogo	2011-2014	0 (0/65)

3. CHPATER 2

Detection of anti-viral antibodies from meat juice of wild boars

3.1. ABSTRACT

Wild boars are a reservoir for many zoonotic pathogens and a good sentinel for surveillance of zoonotic viral infections, but collection of serum samples from wild boars in the field is sometimes difficult and requires special equipment and techniques. In this study, ELISA using meat juices extracted from the heart and diaphragm of wild boars, instead of serum samples, was performed to detect antibodies against zoonotic pathogens, Japanese encephalitis virus and hepatitis E virus. The results of ELISA using meat juice samples were significantly correlated with those using serum samples and meat juice contained one-fifth the antibodies of serum samples. As meat juice is easily collected from wild animals in the field without special equipment and techniques, ELISA using meat juice is a simple and superior method for serological survey of zoonosis among wild animals.

3.2. INTRODUCTION

Wild animals play an important role as reservoirs for many zoonoses (Kruse et al., 2004). In particular, wild boars are major reservoirs for zoonotic pathogens, such as Japanese encephalitis virus (JEV) and HEV, in Japan (Hara et al., 2014; Ohno et al., 2009; Yonemitsu et al., 2016).

JEV belongs to the family *Flaviviridae,* genus *Flavivirus* (Mackenzie et al., 2002), and is a causative agent of Japanese encephalitis (JE) in humans. JEV is transmitted by *Culex* species mosquitoes and a leading cause of viral encephalitis in South, Southeast, and East Asia (Liang and Huanyu, 2015). Pigs, wild boars and wild birds are reservoirs of this virus (Mansfield et al., 2017). In Japan, only several cases of human infection have been recently reported, but many domestic pigs, dogs, monkeys, wild boars and wild raccoons are infected with JEV every year (Ohno et al., 2009; Shimoda et al., 2014, 2013). These results indicate that JEV is spreading among livestock, companion, and wild animals, and humans are protected from JE by a vaccination program under recommendation of the Japanese government.

The properties of HEV are already described in previous chapter. In Japan, the number of hepatitis E patients has been increasing, and the main infection sources are pigs and wild boars (Hara et al., 2014; Kanayama et al., 2015).

Surveillance of zoonotic pathogens among reservoirs is one of the best methods to analyze the risk to humans. In Japan, national epidemiological surveillance of JEV infection has been performed using pig sera before and during the active mosquito season in order to alert the nation to the risk of JEV infection. Although domestic pigs are suitable

as sentinels of JEV infection, pig farms are now largely separated from urban areas, and pigs are bred in enclosed spaces where mosquitoes cannot invade. Therefore, surveillance of pigs might not reflect the true risk of JEV infection in urban areas. On the other hand, wild boars have invaded human habitats and are not protected from mosquitoes, suggesting that wild boars are superior sentinels for surveillance of JEV.

Although serum samples are the best specimens for sero-surveillance, isolation of sera in the field is difficult because special equipment and techniques are required for serum collection. Meat juice could be collected by freezing and thawing of heart and diaphragm and contain antibodies against pathogens (Coelho et al., 2015; Meemken et al., 2014; Nielsen et al., 1998; Wacheck et al., 2012). As meat juice contains many other contaminants, virus-neutralization (VN) and hemagglutination-inhibition tests are difficult to perform. On the other hand, ELISA is useful for detection of antibodies against specific pathogens in meat juice.

In this study, our established ELISA for JEV and HEV was evaluated using meat juice samples obtained from the hearts and diaphragms of wild boars, and the results were compared with those using serum samples.

3.3. MATERIALS AND METHODS

3.3.1 Collection of samples

Blood, heart, and diaphragm samples were collected from 46 wild boars from November 2016 to May 2017 in Yamaguchi Prefecture, Japan. These wild boars were hunted by hunters under permission of the local government. Blood was centrifuged at 2000 × g for 10 min at 4°C, and supernatants were collected and stored as serum samples at -20°C until use.

3.3.2. Collection of meat juice

In order to obtain meat juice from heart and diaphragm, approximately 100 grams of meat samples were placed in a plastic bag (Ziploc; Asahi-kasei, Tokyo, Japan) and then frozen at -20°C. Frozen meat samples were thawed at room temperature for 5 hrs and then meat juice were transferred to clean tubes and stored at -20°C until use (Meemken et al., 2014).

3.3.3. Detection of anti-JEV antibody

Indirect ELISA was developed to detect anti-JEV antibodies from wild animals (Shimoda et al., submitted for publication). JEV/sw/Chiba/88/2002 was isolated from a pig in Chiba Prefecture in 2002 and kindly provided by Dr. Takasaki at the National Institute of Infectious Diseases. JEV was inoculated onto Vero cells and infected cells were lysed in RIPA buffer (1% sodium deoxycholate, 1% Triton X-100, 10 mM Tris-HCl pH 7.4, 150 mM sodium chloride, 0.5 mM ethylenediaminetetraacetic acid) after

cytopathic effects were observed. Extract from mock-infected Vero cells was used as a control for the antigen. Protein concentrations were measured by Bradford assay (Bio-Rad, Hercules, CA). Extracts were diluted with adsorption buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6) to 5 μg per ml and then 100 μl of the diluent was added to a 96-well ELISA plate (Maxisorp; Thermo Fischer Scientific, Rockford, IL). Plates were incubated for 2 hr at 37°C and then blocked with 1% Blockace (DS Pharma Biomedical, Osaka, Japan) in PBS containing 0.05% of Tween 20 (PBS-T) for 30 min at 37°C. Serum samples were diluted to 1:100 with 0.4% Blockace in PBS-T. Meat juice samples were diluted to 1:10, 1:20 or 1:40 with 0.4% Blockace in PBS-T. After five washes with PBS-T, 100 μl of diluted sera or meat juice was added to each well in duplicate, followed by incubation for 30 min at 37°C. After five washes with PBS-T, 100 μl of diluted Peroxidase Conjugated Purified Recomb® Protein A/G (Thermo Fischer Scientific) was added, followed by incubation for 30 min at 37°C. After five washes with PBS-T, 100 μl of substrate reagent (HRP substrate kit; Bio-Rad) was added, followed by incubation with gentle shaking at room temperature for 30 min. Enzymatic reaction was stopped by adding 100 μl of 2% oxalic acid. Absorbance was measured at a wavelength of 415 nm. Absorbance of wells coated with extract from mock-infected cells was subtracted from that of JEV-infected cells. Absorbance greater than 0.623 was considered to be positive by comparison between this ELISA and VN test using wild boar sera (Shimoda et al., submitted for publication).

3.3.4. Detection of anti-HEV antibody

Anti-HEV antibodies in serum and meat juice of heart and diaphragm were detected using our established ELISA (Chapter 2, Yonemitsu et al., 2016). Serum samples were used at dilutions of 1:100, and meat juice samples were used at dilutions of 1:10, 1:20 or 1:40. Absorbance greater than 0.437 was considered to be positive according to our previous report (Yonemitsu et al., 2016).

3.4. RESULTS

3.4.1. Collection of meat juice

From 46 wild boars, sera, heart, and diaphragm were collected. Meat juice was collected from all heart samples and from 36 of 46 diaphragm samples. About 10% the volume of meat was collected as meat juice, but only a small amount of meat juice was obtained from some meat samples.

3.4.2. Detection of anti-JEV antibodies in meat juice

Twenty-two of 46 wild boars (48%) were seropositive for JEV using 100-fold diluted serum samples (Fig. 3-1). From meat juice of heart, 22 (48%), 20 (43%) and 19 (41%) of 46 wild boars were positive at 1:10, 1:20 and 1:40 dilution, respectively (Fig. 3-1). From meat juice of diaphragm, 19 (53%), 17 (47%) and 16 (44%) of 36 wild boars were positive at 1:10, 1:20, and 1:40 dilution, respectively (Fig. 3-1). In comparison with the results using serum samples, the sensitivity and specificity of meat juice samples were calculated. The sensitivity of 10-fold, 20-fold and 40-fold diluted heart samples was 100%, 91% and 86%, respectively, and that for the diaphragm samples was 100%, 89% and 84%, respectively. The specificity of all diluted meat juice was 100%. O.D. values of serum and each meat juice sample were plotted and the slopes of the line of best fit were calculated, giving 1.21, 0.91, and 0.63 at 1:10, 1:20, and 1:40 dilution for meat juice of heart, respectively (Fig. 3-3). Slopes of the line of best fit using meat juice of diaphragm samples were 1.13, 0.86, and 0.62 at 1:10, 1:20, and 1:40 dilution, respectively (Fig. 3-3). The correlation coefficient was 0.972, 0.983 and 0.968 at 1:10, 1:20, and 1:40 dilution

of meat juice of heart, respectively. The correlation for diaphragm samples was 0.980, 0.973 and 0.956 at 1:10, 1:20, and 1:40 dilution, respectively.

3.4.3. Detection of anti-HEV antibodies in meat juice

Eight of 46 wild boars (17%) were seropositive for HEV using 100-fold diluted serum samples (Fig. 3-2). From meat juice of heart, 9 (20%), 8 (17%) and 7 (15%) of 46 wild boars were positive at 1:10, 1:20 and 1:40 dilution, respectively (Fig. 3-2). From meat juice of diaphragm, 8 (22%), 8 (22%) and 7 (19%) of 36 wild boars were positive at 1:10, 1:20 and 1:40 dilution, respectively (Fig. 3-2). The sensitivity and specificity of meat juice samples were calculated for each dilution of heart and diaphragm meat juice samples. The specificity of 10-fold diluted heart samples was 97%, the sensitivity of 40-fold diluted heart samples was 88%, the sensitivity of 40-fold diluted diaphragm samples was 88%, and the other sensitivities and specificities were 100%. Slopes of the line of best fit were 1.58, 0.99 and 0.59 at 1:10, 1:20, and 1:40 dilution of meat juice of heart, respectively, and 1.58, 1.01 and 0.61 at 1:10, 1:20, and 1:40 dilution of meat juice of diaphragm, respectively (Fig. 3-4). The correlation coefficient was 0.978, 0.996 and 0.981 at 1:10, 1:20, and 1:40 dilution of meat juice of heart, respectively. The correlation for diaphragm samples was 0.976, 0.985 and 0.958 at 1:10, 1:20, and 1:40 dilution, respectively.

3.5. DISCUSSIONS

In this study, we successfully detected anti-JEV and anti-HEV antibodies in meat juice from wild boars.

Heart and diaphragm samples were collected from 46 wild boars captured in Japan. From all 46 heart samples, we obtained meat juice, but from diaphragm samples, only 36 of 46 yielded meat juice. Thus, heart is a more suitable sample for collection of meat juice than diaphragm.

Meat juice samples were examined by ELISA for detection of anti-JEV and anti-HEV antibodies. The results indicated that the sensitivities and specificities of ELISA using both sources of meat juice were almost 100%. Meat juice is therefore suitable for ELISA.

The slope of the line of best fit for each dilution of meat juice sample in comparison with 100-fold diluted serum samples was calculated. Slopes using 20-fold diluted meat juice samples were almost 1.0. This indicates that meat juice samples contain approximately one-fifth the anti-viral antibodies of serum. For ELISA using meat juice, a 1:20 dilution is recommended, as serum samples are generally diluted to 1:100 in our ELISA system.

In conclusion, meat juice is a good specimen for detection of anti-JEV and anti-HEV antibodies from wild boars. Furthermore, as our established ELISA used protein A/G for detection of immunoglobulins, this ELISA using meat juice must be applicable to detection of anti-viral antibodies from other mammalian species. Our ELISA system using meat juice and protein A/G is expected to be widely suitable for surveillance of

infectious diseases among wild animals.

3.6. LEGENDS FOR FIGURES

Fig. 3-1. ELISA for Japanese encephalitis virus. Serum (A) and meat juice of heart (B) and diaphragm (C) were used as first antibodies for ELISA. Serum was diluted to 1:100 and meat juice samples were diluted to 1:10, 1:20 and 1:40. Absorbance greater than 0.623 was judged to be positive. Red and orange colors indicate samples collected from wild boars positive and negative for anti-JEV antibody, respectively.

Fig. 3-2. ELISA for hepatitis E virus. Serum (A) and meat juice of heart (B) and diaphragm (C) were used as first antibodies for ELISA. Serum was diluted to 1:100 and meat juice samples were diluted to 1:10, 1:20 and 1:40. Absorbance greater than 0.437 was judged to be positive. Red and orange colors indicate samples collected from wild boars positive and negative for anti-HEV antibody, respectively.

Fig. 3-3. Correlation of absorbance values for Japanese encephalitis virus between serum and meat juice of heart (A) and diaphragm (B). Lines of best fit for each dilution of meat juice are also shown.

Fig. 3-4. Correlation of absorbance values for hepatitis E virus between serum and meat juice of heart (A) and diaphragm (B). Lines of best fit for each dilution of meat juice are also shown.

Figure 3-1.

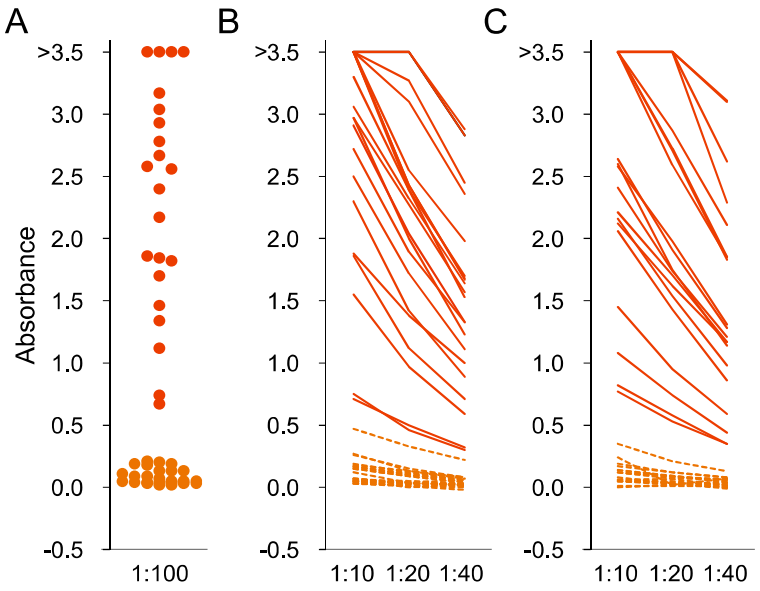

A
B
C
Absorbance
>3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
1:100
1:10 1:20 1:40
1:10 1:20 1:40

Figure 3-2.

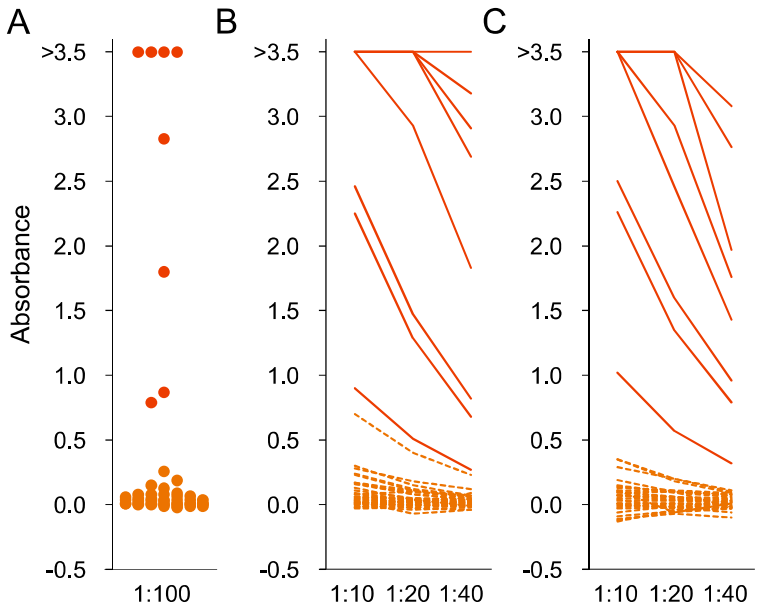

A
B
C
Absorbance
>3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
1:100
1:10 1:20 1:40
1:10 1:20 1:40

Figure 3-3.

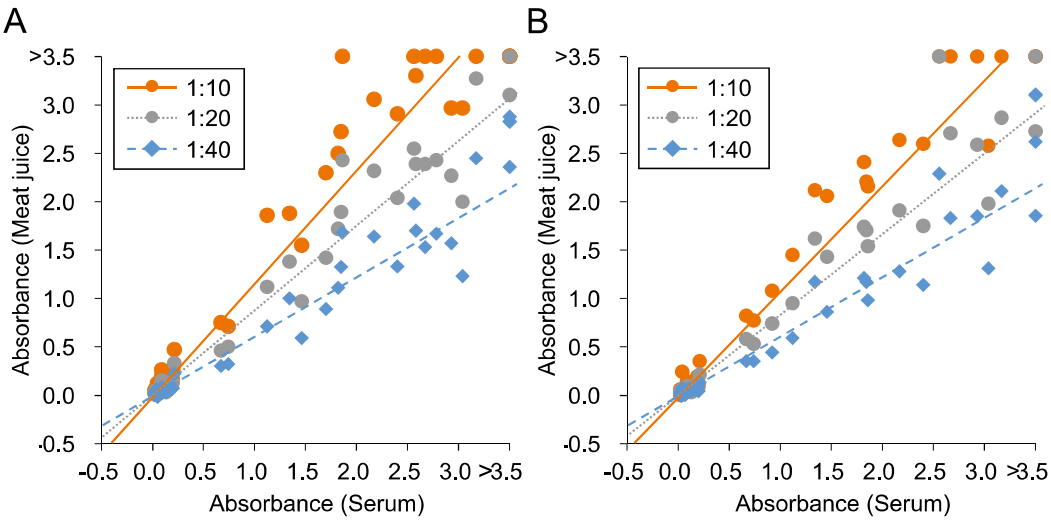
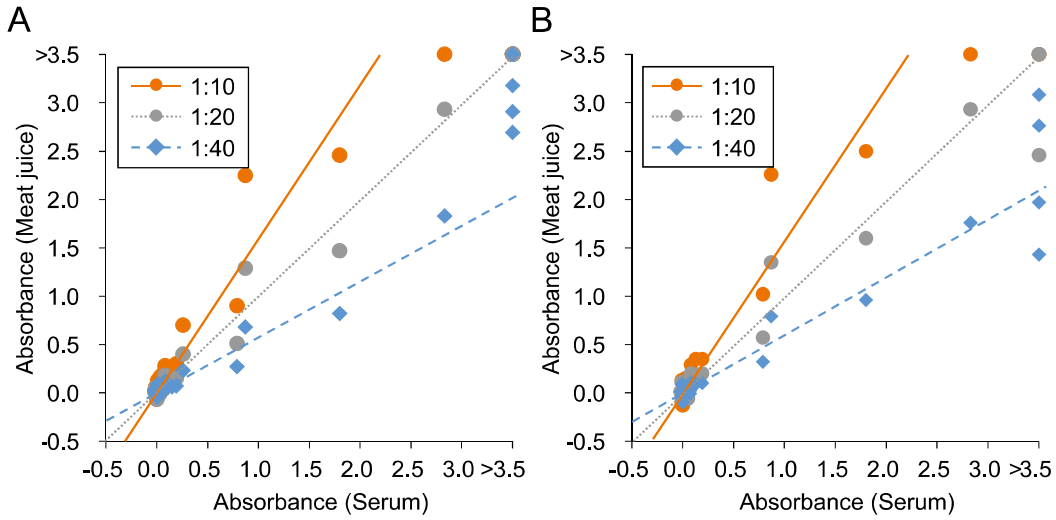

A
B
1:10
1:20
1:40
Absorbance (Meat juice)
Absorbance (Serum)
>3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5

Figure 3-4.


A
B
1:10
1:20
1:40
Absorbance (Meat juice)
Absorbance (Serum)
>3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5

4. CHPATER 3

A nationwide surveillance of HEV infection in wild boars and deer in Japan

4.1. ABSTRACT

In this chapter, nationwide surveys of HEV infection among wild boars and deer in Japan were conducted. Serum samples were collected from wild boars and deer all over Japan and the prevalence of anti-HEV antibodies and HEV RNA were investigated. The results of ELISA showed that, 258 of 1556 wild boars (16.6%) were positive for anti-HEV antibodies. Regional differences were observed, the positive rates of antibodies ranging from 0% to 51%. As the results of RT-PCR, HEV RNA was detected from 20 of 1072 (1.9%) wild boars. On the other hand, only three of 1282 (0.2%) deer were positive for anti-HEV antibodies. Despite of the low antibody positive rate, RNA was detected from one deer. Virus isolation was performed using serum, liver and stool samples of RNA-positive individuals. After inoculation to the PLC/PRF/5 cells, the presence of viral RNA in the culture supernatant was examined by RT-PCR. Twenty-five days after inoculation, one liver sample of wild boar in Chiba Prefecture showed positive result which belongs to genotype 3 HEV.

4.2. INTRODUCTION

In Japan, hepatitis E cases are occurred sporadically (Kanayama et al., 2015; Takahashi and Okamoto, 2014). The major suspected source of infection is food-borne, and the major suspected foods are pig, wild boar and deer meat. The investigation of the current situation of HEV infection in wild animals is important to estimate the risk of infection to humans. We previously reported that the new method for detecting anti-HEV antibodies from many mammalian species including wild boars, ferrets, dogs, cats, raccoons, masked palm civets, and humans (Yonemitsu et al., 2016). In this study, we demonstrated the nationwide survey of HEV in wild boars and deer in Japan.

4.3. MATERIALS AND METHODS

4.3.1. Serum samples

Serum samples were collected from wild boars and deer in Japan. A total of 1556 sera from wild boars were collected in Yamaguchi (n=589), Kagoshima (n=5), Kumamoto (n=90), Oita (n=46), Ehime (n=55), Kagawa (n=36), Hyogo (n=111), Wakayama (n=88), Gifu (n=116), Toyama (n=111), Chiba (n=67), Tochigi (n=220) and Gunma (n=22) Prefectures between 2007 and 2018. Samples of Yamaguchi Prefecture were collected from two cities, Shimonoseki City (n=523) located in the west and Iwakuni City (n=66) located in the east end point. Sera from 1278 deer were collected in Yamaguchi (n=750), Kagoshima (n=29), Oita (n=12), Ehime (n=25), Kagawa (n=25), Gifu (n=122), Nagano (n=47), Yamanashi (n=66), Chiba (n=66), Tochigi (n=24), Gunma (n=33) and Hokkaido (n=79) Prefectures between 2011 and 2018. All sera were stored at -20°C until use.

4.3.2. ELISA

Our established ELISA for detecting antibodies against HEV was performed as described in Chapter 1 (Yonemitsu et al., 2016).

4.3.3. Nested RT-PCR

RNA was extracted from 140 μL of serum samples by using viral RNA mini kit (QIAGEN). Reverse transcription-PCR (RT-PCR) and nested PCR were conducted targeting partial ORF2 gene of HEV. The PCR conditions and primers were previously

described (Hara et al., 2014). Amplicons were confirmed by sequencing using BigDye Terminator v.3.1 Cycle Sequencing kits (Applied Biosystems, USA). The obtained sequences of the partial ORF2 were analyzed using the neighbor-joining method with MEGA 6.06 software. The bootstrap values were calculated from 1,000 replications.

4.3.4. Cells

Human liver hepatoma (PLC/PRF/5) cells were grown in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific, Waltham, MA) with 10% heat-inactivated fetal calf serum (FCS; Thermo Fisher Scientific), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO2.

4.3.5. Virus isolation

HEV RNA positive sera were used for virus isolation of HEV. PLC/PRF/5 cells grown in 6-well plate (Sumitomo Bakelite, Tokyo, Japan) were inoculated with sera. Liver and stool samples of HEV RNA positive wild boars, if available, were also used for virus isolation. Liver samples suspended in PBS homogenated by bio-masher (BioMasher II, Nippi, Tokyo, Japan), then supernatants were inoculated to the cells after filtration with 0.45 μm filter (Corning® Costar® Spin-X® centrifuge tube filters, sigma-aldrich, Tokyo, Japan). After 24 hr cells were washed three times with DMEM, and were maintained with DMEM containing 2% FCS. Half of the culture medium was replaced every three or four days. Supernatant were used for RNA detection by RT-PCR. Isolated virus was sequenced using genotype specific primers (Muñoz-Chimeno et al., 2016).

4.3.6. Statistical analysis

For statistical analysis, chi-squared test was performed. P values of < 0.05 were considered to be statistically significant.

4.4. RESULTS

4.4.1 Detection of anti-HEV antibodies

Totally, 258 of 1556 wild boars (16.6%) were positive for HEV antibody. The seroprevalences of HEV were quite different between prefectures. No wild boars in Wakayama Prefecture were positive for anti-HEV antibodies, while Chiba Prefecture shows highest prevalence of 51%. Wild boars captured in Yamaguchi, Chiba and Gunma Prefectures shows significant high prevalence rate compared with the average prevalence rate of all samples (Table 4-1).

Three of 1282 deer were positive for HEV antibody and prevalence was 0.23 %. HEV positive deer were detected in two of 750 deer (0.3%) in Yamaguchi and one of 25 deer (4%) in Kagawa Prefecture (Table 4-2).

4.4.2 Detection of HEV RNA

Sera were used for HEV RNA detection. HEV RNA was detected in twenty of 1072 wild boars and one of 976 deer. In detail, thirteen of 502 wild boars (2.6%) in Shimonoseki City, two of 77 wild boars (3%) in Hyogo Prefecture, four of 55 wild boars (7%) in Chiba Prefecture and one of 22 wild boars (5%) in Gunma Prefecture were positive for HEV RNA. The other wild boar samples captured in Oita, Ehime, Kagawa, Gifu, Tochigi and Toyama Prefectures were all negative (Table 4-1). For deer, one positive was detected in serum from Shimonoseki City. The other deer samples were negative for HEV RNA (Table 4-2).

The nucleotide sequence of the 338-bp product was determined (Fig. 4-1). HEV genome sequences detected in Shimonoseki City were classified into genotype 4. The other HEV detected in Hyogo, Chiba and Gunma Prefectures belonged to genotype 3. Nucleotide sequences of 14 isolates from Shimonoseki City showed identities of 93.8–100% and formed a single clade with the sequence previously reported from the patient in Shimonoseki (Okita et al., 2012). Four sequences detected in sera from wild boars captured in Chiba Prefecture showed identities of 86.7% to 100%. One isolate from Chiba Prefecture was phylogenically different from the other three isolates.

4.4.3. Isolation of HEV

Virus isolation on PLC/PRF/5 cells was attempted to samples that were positive by RT-PCR. Serum, liver and stool samples were used for virus isolation, if available. The supernatants of cell culture were screened by RT-PCR. Liver sample of wild boar captured in Chiba Prefecture in 2015, Chiba-WB-P27001, showed positive result by RT-PCR at 25 days post inoculation. The nearly complete genome sequence of the isolated virus was determined by genotype 3 HEV specific primers. The obtained sequence was compared with sequences in the NCBI database, resulting that our isolate was the closest to the sequence obtained from Japanese patient with persistent infection, and the similarity was 93 % (Tamura et al., 2007).

4.5. DISCUSSION

In this study, we conducted a nationwide survey of the prevalence of HEV infection in Japanese wild boar and deer. The wild boar showed high prevalence of antibody against HEV by ELISA and HEV RNA was detected by RT-PCR. It was reconfirmed that wild boar plays a important role as reservoir of HEV in the field. However, in some areas, especially in Wakayama Prefecture, wild boars were not infected with HEV. Interestingly, the results of the two areas of Yamaguchi Prefecture, indicated that the prevalencesc were significantly different in the same prefecture. In order to clarify the distribution of endemic and non-endemic areas of HEV and to analyze the risk of infection to humans, it is necessary to perform the surveillance in each region.

The antibody prevalence in deer was less than 1%. It was clear that deer are hardly susceptible to HEV. However, HEV RNA was detected from only one deer in Shimonoseki City. Although several previous studies revealed the low prevalence of HEV in Japanese deer, human hepatitis E cases caused by consumption of deer meat are constantly reported in Japan (Matsuura et al., 2007; Takahashi et al., 2004; Tei et al., 2003). In this current study, HEV RNA was detected from one deer despite antibody prevalence was very low. These data reinforce the importance of deer as source of HEV infection to human in Japan. Although, it is unlikely that Japanese deer might play role as reservoir of HEV, deer could be infected with HEV and be a source of infection to human.

All HEV detected from wild boars and deer in Shimonoseki formed a single clade with that previously reported from the patient in Shimonoseki. These sequence data

from wild animals will be useful for identification of origin and transmission route of HEV, that accumulation of these sequence data is important.

4.6. LEGENDS FOR FIGURE

Fig. 4-1. Phylogenetic tree of HEV. Nucleotide sequences of the partial ORF2 were analyzed using the neighbor-joining method with MEGA 6.06 software. The bootstrap values were calculated from 1,000 replications. The sequences detected from wild boars and deer in this study are shown in red and blue, respectively.

Figure 4-1.

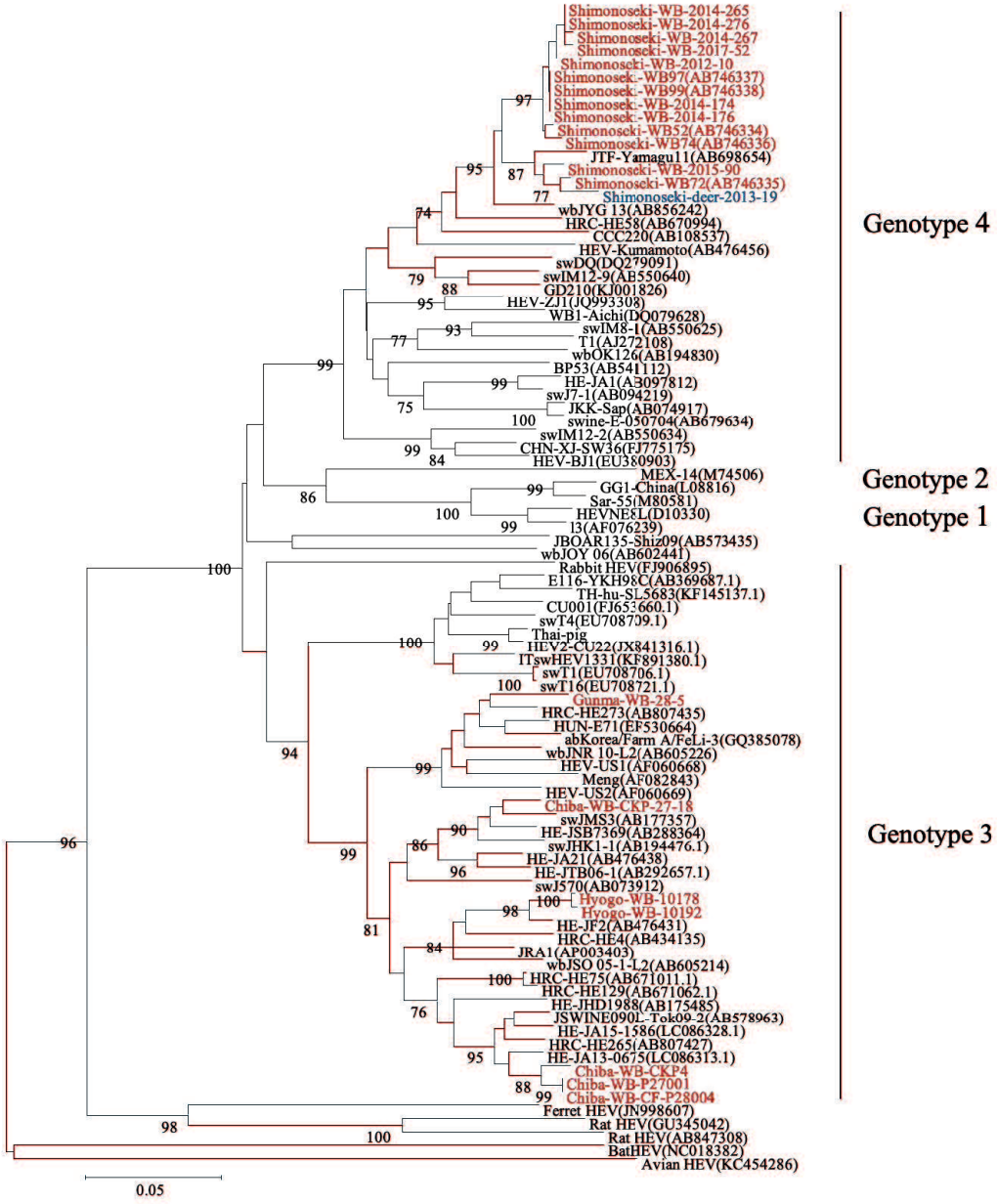

Shimonoseki-WB-2014-265
Shimonoseki-WB-2014-276
Shimonoseki-WB-2014-267
Shimonoseki-WB-2017-52
Shimonoseki-WB-2012-10
Shimonoseki-WB97(AB746337)
Shimonoseki-WB99(AB746338)
Shimonoseki-WB-2014-174
Shimonoseki-WB-2014-176
Shimonoseki-WB52(AB746334)
Shimonoseki-WB74(AB746336)
JTF-Yamagu11(AB698654)
Shimonoseki-WB-2015-90
Shimonoseki-WB72(AB746335)
Shimonoseki-deer-2013-19
wbJYG 13(AB856242)
HRC-HE58(AB670994)
CCC220(AB108537)
HEV-Kumamoto(AB476456)
swDQ(DQ279091)
swIM12-9(AB550640)
GD210(KJ001826)
HEV-ZJ1(JQ993308)
WB1-Aichi(DQ079628)
swIM8-1(AB550625)
T1(AJ272108)
wbOK126(AB194830)
BP53(AB541112)
HE-JA1(AB097812)
swJ7-1(AB094219)
JKK-Sap(AB074917)
swine-E-050704(AB679634)
swIM12-2(AB550634)
CHN-XJ-SW36(FJ775175)
HEV-BJ1(EU380903)
MEX-14(M74506)
GG1-China(L08816)
Sar-55(M80581)
HEVNE5L(D10330)
13(AF076239)
JBOAR135-Shiz09(AB573435)
wbJOY 06(AB602441)
Rabbit HEV(FJ906895)
E116-YKH98C(AB369687.1)
TH-hu-SL5683(KF145137.1)
CU001(FJ653660.1)
swT4(EU708709.1)
Thai-pig
HEV2-CU22(JX841316.1)
ITswHEV1331(KF891380.1)
swT1(EU708706.1)
swT16(EU708721.1)
Gunma-WB-28-5
HRC-HE273(AB807435)
HUN-E71(EF530664)
abKorea/Farm A/FeLi-3(GQ385078)
wbJNR 10-L2(AB605226)
HEV-US1(AF060668)
Meng(AF082843)
HEV-US2(AF060669)
Chiba-WB-CKP-27-18
swJMS3(AB177357)
HE-JSB7369(AB288364)
swJHK1-1(AB194476.1)
HE-JA21(AB476438)
HE-JTB06-1(AB292657.1)
swJ570(AB073912)
Hyogo-WB-10178
Hyogo-WB-10192
HE-JF2(AB476431)
HRC-HE4(AB434135)
JRA1(AP003403)
wbJSO 05-1-L2(AB605214)
HRC-HE75(AB671011.1)
HRC-HE129(AB671062.1)
HE-JHD1988(AB175485)
JSWINE0901-Tok09-2(AB578963)
HE-JA15-1586(LC086328.1)
HRC-HE265(AB807427)
HE-JA13-0675(LC086313.1)
Chiba-WB-CKP4
Chiba-WB-P27001
Chiba-WB-CF-P28004
Ferret HEV(JN998607)
Rat HEV(GU345042)
Rat HEV(AB847308)
BatHEV(NC018382)
Avian HEV(KC454286)
0.05
Genotype 4
Genotype 2
Genotype 1
Genotype 3

Table 4-1. Seroprevalence of HEV infection in wild boars in Japan.

Species	Place (Year)	Anti-HEV antibody No. positive / No. tested (positive rate(%))	HEV RNA No. positive / No. tested (positive rate(%))
Wild boar	Yamaguchi (Shimonoseki) (2010-2018)	139 / 523 (26.6)	13 / 502 (2.6)
	Yamaguchi (Iwakuni) (2016-2018)	0 / 66 (0)	-
	Kagoshima (2016)	1 / 5 (20)	-
	Kumamoto (2016-2018)	3 / 90 (3)	-
	Oita (2011-2012)	9 / 46 (20)	0 / 22 (0)
	Ehime (2016-2017)	12 / 55 (22)	0 / 55 (0)
	Kagawa (2016-2017)	8 / 36 (22)	0 / 36 (0)
	Hyogo (2011-2015)	23 / 111 (20.7)	2 / 77 (2.6)
	Wakayama (2007-2013)	0 / 88 (0)	-
	Gifu (2014-2017)	5 / 116 (4.3)	0 / 112 (0)
	Toyama (2014-2017)	7 / 111 (6.3)	0 / 103 (0)
	Chiba (2015-2017)	34 / 67 (51)	4 / 55 (7)
	Tochigi (2010-2012)	12 / 220 (5.5)	0 / 88 (0)
	Gunma (2015-2017)	10 / 22 (45)	1 / 22 (5)
	Total	258 / 1556 (16.6)	20 / 1072 (1.9)

Table 4-2. Seroprevalence of HEV infection in deer in Japan.

Species	Place (Year)	Anti-HEV antibody No. positive / No. tested (positive rate(%))	HEV RNA No. positive / No. tested (positive rate(%))
Deer	Yamaguchi (2010-2018)	2 / 750 (0.3)	1 / 684 (0.1)
	Kagoshima (2015-2017)	0 / 29 (0)	-
	Yakushima (2018)	0 / 4 (0)	-
	Oita (2011-2012)	0 / 12 (0)	-
	Ehime (2016-2017)	0 / 25 (0)	0 / 25 (0)
	Kagawa (2016-2017)	1 / 25 (4)	0 / 25 (0)
	Gifu (2014-2017)	0 / 122 (0)	0 / 123 (0)
	Nagano (2015-2016)	0 / 47 (0)	-
	Yamanashi (2014-2015)	0 / 66 (0)	0 / 25 (0)
	Chiba (2014-2018)	0 / 66 (0)	0 / 61 (0)
	Tochigi (2014)	0 / 24 (0)	-
	Gunma (2015-2017)	0 / 33 (0)	0 / 33 (0)
	Hokkaido (2011-2017)	0 / 79 (0)	-
	Total	3 / 1282 (0.23)	1 / 976 (0.1)

5. GENERAL CONCLUSION

In this thesis, novel methods to examine the prevalence of HEV infection in various animal species were established, in order to reveal the prevalence of HEV in animals, ecology of HEV in nature and the risk of infection from animals to humans.

In CHAPTER 2, ELISA to detect anti-HEV antibody in mammalian species was established. Extract of cells expressing HEV capsid protein and protein A/G were used as an antigen and a reagent for detection of antibody, respectively. The newly established ELISA had better specificity and sensitivity than the previous ELISA. Seroprevalence of HEV infection among wild boars in Yamaguchi Prefecture was examined using this ELISA, confirming that 111 of 364 wild boars (30.5%) were positive for anti-HEV antibody. This ELISA was applied to humans, dogs, cats, ferrets, raccoons and masked palm civets in Japan, and anti-HEV antibodies were detected in humans, ferrets, dogs and cats. The ELISA established in this chapter is useful for serological surveys of HEV infection among various mammals, including humans.

In CHAPTER 3, ELISA using meat juices extracted from the hearts and diaphragms of wild boars, instead of serum samples, was performed, because collection of serum samples from wild animals in the field is sometimes difficult and requires special equipment and techniques. The results of ELISA using meat juice samples were significantly correlated with those using serum samples and meat juice contained one-fifth of the antibodies of serum samples. As meat juice is easily collected from wild animals in the field without special equipment and technique, ELISA using meat juice is a simple and superior method for serological survey of zoonosis among wild animals.

In CHAPTER 4, a nationwide surveillance of HEV infection in wild boars and deer in Japan was performed using our established ELISA. High prevalence of HEV infection among wild boars was observed, indicating that wild boar plays an important role as reservoir of HEV in the field. HEV RNA was detected from one deer. It is unlikely that Japanese deer might play a role as reservoir of HEV. However, deer might be a source of infection to human.

In conclusion, sensitive and specific ELISA and simple sampling method were established for surveillance of HEV among many mammalian species. For further understanding of etiology of HEV in the field, combination of these methods will be useful.

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