Studies on coronaviruses causing enteric infections in domestic animals in Japan

(日本における家畜の下痢原因コロナウイルスに関する研究)

Md. Taimur Islam

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Md. Taimur Islam

Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

Doctoral Supervisor: Kimimasa Takahashi

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Introduction

Coronaviruses (CoVs) are classified under the family *Coronaviridae* of the order *Nidovirales* (Plant et al., 2013). The order *Nidovirales* is derived from the Latin word *nidus*, meaning nest, as all the viruses in this order produce a 3' co-terminal nested set of subgenomic mRNAs during replication (de Vries et al., 1997). The name "CoV" is derived from the Latin word "*corona*", meaning crown or halo, and refers to the characteristic spikes on virions, that are shown by electron microscopy to be a royal crown or of the solar corona. Generally each CoV infects specific hosts in a species-specific manner, and infections can be either acute or persistent. Infections are transmitted commonly via respiratory and fecal-oral routes (Masters, 2006). However, two human CoVs recently emerged worldwide, severe acute respiratory syndrome CoV (SARS-CoV) and Middle East respiratory syndrome CoV (MERS-CoV), come from wild animals, revealing the host exchange during the infection (Cong and Ren, 2014).

CoVs were first identified in the 1960s from the nasal cavities of patients suffering from common cold (Geller et al., 2012). These viruses were electron microscopically shown to have characteristic crown-like spikes, after which the virus was named CoV. Thereafter, viruses with similar spikes were found in domestic animals, chickens and laboratory animals and those viruses caused serious diseases in host animals (Almeida and Tyrrell, 1967). Since there was no highly pathogenic human CoV until the discovery of highly pathogenic human CoV: SARS-CoV (Drosten et al., 2009; Ksiazek et al., 2003; Peiris et al., 2003) and MERS-CoV (Al-Hazmi, 2016), animal CoVs were major target of the research. However, the emergency of those viruses has huge impact in human society and medicine and they are currently the most studied CoVs.

The *Coronaviridae* family consists of two subfamily- *coronavirinae* and *torovirinae*. The *coronavirinae* subfamily is divided into four genera: *Alpha*, *Beta*, *Gamma* and *Delta* (Table-I). On the other hand, the *torovirinae* subfamily contains two genera, Bafinivirus and Torovirus (ToV) (Table-I) (Adams et al., 2016; Cong and Ren, 2014; Irigoyen et al., 2016; McBride et al., 2014).

Coronaviridae are a family of nonsegmented, enveloped, positive sense RNA viruses (McBride et al., 2014). The one of important features of this viral family is genome size. CoVs have the largest genomes among all RNA viruses, including those viruses with segmented genomes (Masters, 2006). The viral genome size is 26–32 kilo bases (kb) in length (Lehmann et al., 2015). Virions have average diameters of 80–120 nanometer (nm), but extreme sizes as small as 50 nm and as large as 200 nm (McIntosh, 1974). The virions are roughly spherical and have pleomorphic morphology (Masters, 2006).

Two-thirds of the CoVs genome found within the 5'-genomic region contains open-reading frame 1a (ORF) and ORF 1b. These ORF1a and ORF1b encode RNA-dependent RNA polymerase, helicase, and other nonstructural protein (nsp1-16) that are necessary for either efficient replication or pathogenesis (Fehr and Perlman, 2015; Masters, 2006). Genes for the structural proteins are encoded in the remaining one third of the genome in a fixed order: S-E-M-N (McBride et al., 2014; Tanaka et al., 2013). Proteins that contribute to the overall structure of all CoVs are the spike (S), envelope (E), integral membrane (M) and nucleocapsid (N) proteins (Snijder et al., 2003). The former three proteins exist on the viral envelope (Fig. I) (Brian and Baric, 2005; Fehr and Perlman, 2015; Masters, 2006). The S glycoprotein that constitutes the spike on the virion (Masters, 2006) facilitates receptor attachment and viral-entry into cells (Collins et al., 1982). The M glycoprotein is

the most abundant part in coronavirus virions (Sturman, 1977; Sturman et al., 1980) and determines the site of virus assembly. The E protein is a small polypeptide, which is only a minor part of virions. Because of its tiny size and limited quantity, E was identified as a virion component much later than were the other structural proteins. The N protein is the protein component of the helical nucleocapsid and binds the genomic RNA in a beads-on-a-string fashion (Masters, 2006). Some CoVs encode a second set of projections, 5–10 nm long (Guy et al., 2000; Patel et al., 1982). These shorter structures are known to be composed of the hemagglutinin-esterase (HE) protein, which is the fourth constituent of the membrane envelope in some CoVs (Masters, 2006). HE protein forms a second set of small spikes that appear among the tall S protein spikes. The HE protein also has an acetylesterase activity (Cornelissen et al., 1997).

Replication of CoVs starts with binding to its specific receptor on host cell surface and then entry to the host cells, which take place from plasma membrane or endosomal membrane. After entering into the cell, the virion is uncoated and the nucleocapsid is released into the cytoplasm of host cells, where the viral genome becomes available for translation. Since CoVs are positive stranded RNA viruses, the genome also serves as the first mRNA of infection, and is translated into the enormous replicase polyprotein. Then the replicase utilizes the genome as the template for the synthesis of new copies of negative-stranded RNA complementary to the genomic RNA and from the negative-stranded RNA subgenomic mRNAs are transcribed (Plant et al., 2013). During replication CoVs synthesize a nested set of six to ten subgenomic mRNAs in infected cells (Hussain et al., 2005) that have a common 3' end. These subgenomic mRNAs also contain a common leader sequence derived from the 5' end of the genome. Synthesis of each subgenomic

mRNA involves a discontinuous step by which the 3' body sequence is fused to the genomic 5' leader sequence (Sawicki et al., 2001). These subgenomic mRNAs are translated to viral structural proteins or nonstructural accessory proteins (Cowley et al., 2010; Kopecky-Bromberg et al., 2007). The membrane-bound structural proteins which form the viral envelope: M, S and E protein are inserted into the ER membrane, from where they transit to the endoplasmic reticulum-Golgi intermediate compartment (ERGIC). Nucleocapsids are developed from the encapsidation of progeny genomes by N protein. These coalesce of nucleocapsid with the membrane-bound proteins, develop virions by budding into the ERGIC. Finally, progeny viruses are released from infected cells via exosomal pathway.

CoVs are distributed broadly among mammals and birds, causing mainly respiratory or enteric diseases but also including hepatic, renal and neuronal infection (Fielding, 2011; Graham et al., 2013; Masters, 2006). Six different CoVs infect humans: *alpha* (229E and NL63) and *beta* (OC43, SARS-CoV, MERS-CoV and HKUI1). Among them SARS-CoV and MERS-CoV are the most publicized, although these two are of animal origin (Cong and Ren, 2014). SARS-CoV was first identified in Asia in 2003. Within the next few months, the infections spread to ca. 30 countries in North America, South America, Europe and Asia. In the first outbreak 774 patients has died (Azhar et al., 2014; Drosten et al., 2009; Ksiazek et al., 2003; Peiris et al., 2003). MERS-CoV is the newly identified CoV that has emerged in the Middle East in 2012. In January 2016, WHO has reported 1638 MERS-CoV positive human cases, including 587 deaths (36%) (Al-Hazmi, 2016).

CoVs are also responsible for a wide range of diseases in farm animals and pet animals, some of which can be serious and are a threat to the farming industry (Fehr and

Perlman, 2015). Economically important CoVs of farm animals include porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), bovine torovirus (BToV) and bovine coronavirus (BCoV), which result in enteric infection causing diarrhea in young animals. Feline coronavirus (FCoV) has two forms; feline enteric coronavirus (FECV) is responsible for minor clinical signs, but spontaneous mutation of this virus may result in feline infectious peritonitis virus (FIPV) (Rottier et al., 2005), which causes high mortality. Similarly, there are two types of CoV that infect ferrets (Murray et al., 2010). There are two types of canine coronavirus (CCoV), one causes mild gastrointestinal disease and another one to cause respiratory disease (Cong and Ren, 2014). Mouse hepatitis virus (MHV) is responsible for an epidemic murine illness with high mortality of suckling mice and immunocompromised mice (Weiss and Navas-martin, 2005). In chickens, the infectious bronchitis virus (IBV) infects not only the respiratory tract but also the uro-genital tract. This virus can spread to different organs of the chicken (Bande et al., 2015)

A huge PEDV outbreak occurred in early 2013 in the United States (US) for the first time that caused high morbidity and mortality of piglets, affecting US pig production, and spread further to Canada and Mexico (Lee, 2015). In October 2013, devastating outbreak of porcine epidemic diarrhea (PED) occurred in Japan (Masuda et al., 2015) after a period of 7 years absence of outbreak. PEDV new isolates from this outbreak are genetically related to the PEDV isolates from China and USA in 2013 (Huang et al., 2013). PEDV has the ability to infect pigs of any age, from neonates to sows or boars; however, the severity of PED in pigs differs according to age of pigs (Shibata et al., 2000). Most importantly, PEDV infection in neonatal pigs generally causes death from watery diarrhea

and dehydration. Additionally, PEDV may cause various other clinical signs, including vomiting, anorexia, dehydration, and weight loss (Pensaert and de Bouck, 1978; Song et al., 2015).

Another important virus of *Coronaviridae* family that causes enteric diseases in domestic animal is torovirus (ToV). ToV is classified in the subfamily *Torovirinae* in the family *Coronaviridae*. This virus primarily infects vertebrates (Shimabukuro et al., 2013; Snijder and Horzinek, 1993; Woode et al., 1982). There are four different species of ToV: equine, bovine, porcine, and human (Ali and Reynolds, 2000; Beards et al., 1986; Duckmanton et al., 1998, 1997; Kroneman et al., 1998)

BToV are responsible for mild to moderate diarrhea in calves (Koopmans and Horzinek, 1994). In cattle, the disease causes diarrhea and systemic signs such as pyrexia, lethargy and anorexia. In calves, it may cause neurological signs and lead to death. It infects villous and crypt enterocytes of the mid-jejunum, ileum, cecum and colon, inducing villous atrophy and necrosis of the crypts in calves (Koopmans and Horzinek, 1994; Woode et al., 1982). Respiratory infections caused by BToVs have also been reported (Hoet et al., 2003).

In the present study, new strains of PEDV were isolated from small intestine of piglets sacrificed in several prefectures in Japan during new outbreak of PED in 2014. Phylogenetic analysis of those viruses was done on the basis of S protein by using the distance-based neighbor-joining method. The author also compared the antigenicity of new isolates with that of Japanese vaccine strain by neutralization test. On the other hand, HE positive (HE+) and HE negative (HE-) strains of BToV were cloned from an isolate from a

diarrheic cattle in Niigata prefecture. The author explored the biological function of the HE protein and found that HE acts as an interferon (IFN)-α antagonist in innate immunity.

The present thesis consists of three chapters. First chapter describes the isolation of new PEDVs from the outbreak of 2014 in Japan as well as phylogenetic and antigenic characterization of those PEDV. Second chapter shows the isolation of HE+ and HE- BToVs as well as the comparison of those two viruses in terms of their biological features. Third chapter focused on the anti-IFN activity of the HE protein of BToV.

Table I: Classification of Coronaviruses (CoVs)	
Order: *Nidovirales*	
Family: *Coronaviridae*	
Subfamily: *Coronavirinae*	Subfamily: *Torovirinae*
Genus: • *Alphacoronavirus* Species: Human CoV 229E, human CoV NL63, PEDV, miniopterus bat CoV 1, miniopterus bat CoV HKU8	Genus: • Torovirus Species: BToV, equine ToV, porcine ToV, human ToV
Genus: • *Betacoronavirus* Species: SARS-CoV, MERS-CoV, human CoV HKU1, murine CoV, pipistrellus bat CoV HKU5, rousettus bat CoV HKU9, tylonycteris bat CoV HKU4, human CoV OC43	Genus: • Bafinivirus Species: White bream virus
Genus: • *Gammacoronavirus* Species: Infectious bronchitis virus, avian CoV, duck CoV, beluga whale CoV SW1	
Genus: • *Deltacoronavirus* Species: Bulbul CoV HKU11, thrush CoV HKU12, munia CoV HKU13	

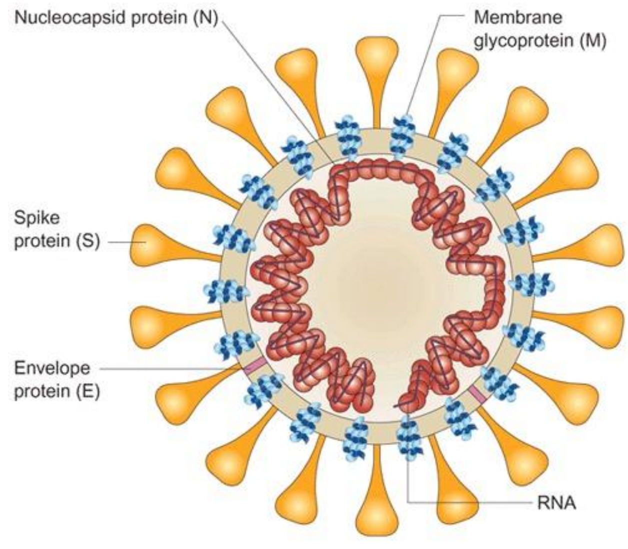

Figure I. Morphology of the SARS-CoV (Peiris et al., 2004).

Chapter 1

Phylogenetic and antigenic characterization of newly isolated PEDV in Japan

1-1. Introduction

PEDV is classified into the genus alpha-CoV in the family *Coronaviridae* of the order *Nidovirales*. It is an enveloped virus with a single-stranded, positive-sense genomic RNA of ca. 28 kb (Chen et al., 2014; Lee, 2015; Masters, 2006; Oka et al., 2014; Sung et al., 2015). PEDV infects the epithelial cells lining the small intestine of pigs and causes severe diarrhea, resulting in fatal dehydration in piglets (Lee, 2015; Liu et al., 2015; Song and Park, 2012).

PEDV virion consists of at least seven ORFs in the following order: ORF1a, ORF1b, S, ORF3, E, M, and N. The S gene is the most diverse gene (Chen et al., 2013; Oka et al., 2014). S protein is the largest surface glycoprotein of the virion and involved in viral entry into the host cells (Bosch et al., 2003). The full-length S gene is approximately 4.1 kb (Chen et al., 2013). In addition, neutralization epitopes have been found in the PEDV S protein (30–32). Therefore, the S glycoprotein is very important viral component to study the genetic relationships of different PEDV strains and the epidemiological analysis of PEDV in the field (Chen et al., 2013; Tian et al., 2013). It has been reported that S gene is considered to be an ideal region to determine the antigenic relatedness among different PEDV isolates (Chen et al., 2013; Lee et al., 2010).

PED was initially reported in Europe, and the causative virus, the PEDV CV777 strain, was first isolated in 1971 in Belgium (Chasey and Cartwright, 1978). Thereafter, PED spread to Asian countries, in which viruses were isolated from diseased pigs (Takahashi et al., 1983). In the US, PEDV was first detected in May 2013 (Stevenson et al., 2013), and a huge outbreak of PED occurred in the US thereafter. By the end of April 2014, the outbreak had spread to 30 US states, causing the death of ca. 8 million pigs, most of

which were piglets. The infection subsequently spread throughout North America, including Canada and Mexico (Vlasova et al., 2014). In October 2013, an outbreak of PED occurred in Japan after a 7-year absence. PED has affected more than 1,000 farms throughout Japan, causing the deaths of ~440,000 piglets (Masuda et al., 2015), despite vaccination of pig herds nationwide.

Vaccination of sows is the principal strategy to control and eradicate epidemic or endemic PED outbreaks. Even though PED first emerged in Europe, PED outbreaks have been become more serious and drastic in Asian countries, and therefore different kinds of PEDV vaccines have been developed in Asia. In China, CV777-attenuated or -inactivated vaccines have been regularly used against PED. Inactivated, bivalent TGEV and PEDV vaccine (Ma et al., 1995) and attenuated, bivalent TGEV and PEDV vaccine (Tong et al., 1999) are also used in Chinese pig population. Two South Korean virulent PEDV strains were also attenuated by the cell-culture adaptation and used as live or killed vaccine by either intramuscular (IM) or intraoral inoculation (Kweon et al., 1999; Lee, 2015). In Japan two different vaccines are available, both of which belong to group I virus. These vaccines were made by attenuation of the virulence by serial passages through Vero cells (Lee, 2015; Sato et al., 2011).

The present study was performed to determine why the large outbreak occurred in Japan, where PED vaccination has been performed. We first isolated two PEDV strains from infected piglet intestines, which were designated Tochigi (LC144542) and Nagasaki (LC144543). These viruses were found to be phylogenetically similar to newly isolated US strains (group II) and different from CV777 and other strains classified in group I. A neutralization test (NT) showed no significant difference in antigenicity between our new

isolates and a group I strain using a number of sera samples collected from pigs vaccinated with group I virus. Our results suggest that the PED outbreak in Japan was not caused by inefficient vaccination, but by the high virulence of newly circulating viruses.

1-2. Materials and Methods

Collection of fecal and intestinal samples from pig farm

We obtained specimens (feces and small intestines) from piglets infected by PEDV in 2014 from several prefectures in Japan and used 14 specimens PCR-positive for the PEDV N and S genes (data not shown). Seven samples were diarrheic feces, and the other seven were small intestines from piglets.

Preparation of inoculum

The transparent parts of the small intestine, indicating infection with PEDV, 10-15 cm in length, were filled with 5-10 ml phosphate-buffered saline (PBS), pH 7.2, and massaged gently to detach infected epithelial cells. PBS was collected, homogenized using a glass homogenizer and centrifuged at 5,000 rpm for 10 min at 4°C to remove cell debris. The supernatants were filtered through a 450 nm pore-size filter (Minisart®, Sartorius Stedim Biotech GmbH, Goettingen, Germany) to remove contaminating bacteria. Diarrheic feces were diluted 10-fold with PBS and treated in the same manner as the intestines, described above.

Virus isolation and propagation

To isolate the virus, we used Vero-KY5 cells (Vero) (Suzuki et al., 2015), which were kindly provided by the National Institute of Animal Health (NIAH), Japan. Vero cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS, Sigma-Aldrich, St. Louis, MO, USA), penicillin (100 IU/ml), and streptomycin (100 μg/ml) and 0.3% tryptose phosphate broth (TPB, Difco, Detroit, MI, USA). Purified suspensions were inoculated onto Vero cells prepared in 24-well plates by spinoculation, as described previously (Watanabe et al., 2006). Briefly, Vero cells seeded at a concentration of 2×10^5 per well in a 24-well culture plate (Falcon, Franklin Lakes, NJ, USA) were inoculated with 50 μl filtered samples and 400 μl DMEM per well. The plates were centrifuged at 3,000 rpm for 1 h at 4°C and then at 3,000 rpm for 1 h at 24°C. Then, cells were incubated with 0.5 ml DMEM supplemented with 5% TPB and trypsin (10 μg/ml), and cytopathic effects (CPEs) were monitored during incubation at 37°C for 2–4 days. After incubation, when cytopathic effect (CPE) was appeared (~ 4 days), cells and supernatant was collected. The cell suspension was sonicated in an ice-cold bath for 5 minutes. This suspension was used for further propagation or stored at -80°C. We also tried to isolate PEDV by conventional method, i.e., we inoculated specimens onto Vero cells and let the virus adsorb cells for 1 hour at 37°C and cultured the inoculated cells for 4-5 days in the presence of trypsin.

Immunofluorescence assay (IFA) for the detection of PEDV antigens in Vero cells

PEDV-infected Vero cells in 24-well plates were fixed with acetone/methanol (50:50, v/v) at room temperature for 5 min, and then the fixed cells were washed with PBS. Fixed

cells were inoculated with 100 times diluted anti-PEDV serum and 100 times diluted fluorescence isothiocyanate (FITC)-conjugated goat anti-swine IgG-FITC (Santa Cruz Biotechnology, Inc., CA, USA) at room temperature for 50 minutes each as first and second antibodies, respectively. After inoculation, cells were washed with PBS and then the plates were examined using a fluorescence microscope.

Extraction of RNA and RT-PCR

The initial homogenate of the specimens, from which the virus was isolated successfully, were used to isolate viral RNA using an RNA isolation kit (Direct-Zol™ RNA Miniprep, Zymo Research, CA, USA). The cDNA was prepared from the eluted RNAs and PCR was carried out with PrimeScript II 1st strand cDNA Synthesis kit and Ex Taq (Takara Bio Inc., Shiga, Japan). From the cDNA, three fragments covering the entire S gene were amplified using primers F1 and R1, F2 and R2 as well as F3 and R3 (Table 1-1). The reaction was prepared according to the manufacturer's instructions. The conditions of PCR were as follows: 98°C for 1 min, 35 cycles of denaturation at 98° C for 10 sec, annealing at 54°C for 30 sec and extension at 72° C for 1 min, followed by a final cycle of extension at 72° C for 7 min. For each reaction, ddH_2O was used as a negative control. PCR products were detected by electrophoresis on a 1.0% agarose gel with 1-kb plus DNA ladder (Invitrogen, CA, USA) as a marker.

Sequence analysis

The PCR products were purified using a DNA purification kit (NucleoSpin® Gel and PCR Clean-up kit MACHEREY-NAGEL, Düren, Germany) and then sequenced using

the primers shown in Table 1-1. The purified DNA was quantified by the use of a Qubit 2.0 spectrophotometer (Life Technologies, Carlsbad, CA) to calculate the concentrations. Purified DNA corresponding to the PEDV "S" genes were directly sequenced by Eurofins Genomics (Tokyo, Japan) with the BigDye® Terminator version 3.1 cycle sequencing kit and an Applied Biosystems 3730×l DNA analyzer (Applied Biosystems Inc., CA, USA). Nucleotide and amino acid (aa) sequences were analyzed with GENETYX® Mac software, version 9 (Genetyx Corporation, Tokyo, Japan). The aa sequences corresponding to the S protein of PEDV were aligned by using Clustal W parameters of MEGA6.06 (www.megasoftware.net) software programs. For the analysis of sequence relationships, PEDV isolates, which have been detected in pig from other countries, were used as reference.

Phylogenetic analysis

Phylogenetic analysis was performed using the aa sequences of PEDV isolates from this study as well as globally endemic PEDV strains available in GenBank based on the full length S protein. The trees were constructed using the distance-based neighbor-joining method of MEGA6.06 software. Bootstrap analysis was carried out on 1,000 replicate data sets.

Virus neutralization (VN) test

Two types of PEDV antisera were used. One type comprised 18 sera collected from herds of pigs that were vaccinated but not recently infected by PEDV, and the other comprised 17 sera from herds of pigs that were vaccinated and infected with a new PEDV

strain. Two hundred µl of serum were serially diluted in twofold steps with DMEM and then mixed with an equal volume of viral suspension containing 100 $TCID_{50}$/100 µl and incubated at 37°C for 60 min. Then, 50 µl of each dilution were inoculated into each well (4 wells per dilution) of a 96-well plate containing confluent Vero cells and incubated at 37°C for 1 h. Then, the mixture was removed, and cells were washed twice with DMEM and, finally, incubated at 37°C with 50 µl DMEM containing 5% tryptose phosphate broth (TPB) and trypsin (10µg/ml). Neutralization antibody titers were expressed as the reciprocal of the highest serum dilution that inhibited syncytium formation in at least two of four wells.

1-3. Results

Virus isolation and characterization

Virus isolation was performed on 7 intestinal homogenates and 7 fecal samples on Vero cells. Among those samples, two PEDVs were isolated by spinoculation but not by conventional method, one from Tochigi and the other from Nagasaki. Both viruses produced syncytia in Vero cells, although the syncytia were smaller than those generated by the vaccine type MK strain, which is adapted to Vero cells (Kusanagi et al., 1992) (Fig. 1-1a-c). Typical CPE was characterized by cell fusion, syncytium formation, and eventual cell detachment (Fig. 1-1). Syncytial cells contained viral-specific antigen, as revealed by immunofluorescence (Fig. 1-1e-g). Attempts at virus isolation from the remaining samples were unsuccessful. Cytotoxicity was observed in cells inoculated with several intestine samples and many of the fecal samples.

Sequence comparisons with other PEDV strains

An aa alignment of the S protein of the two novel isolates, MK and three US PEDV strains (MN-KF468752, IA1-KF468753, and IA2-KF468754), is shown in Fig. 1-2. All of the US strains and Nagasaki and Tochigi were closely related genetically (99.42–99.97% aa identity) and differed from each other by 1 to 8 aa throughout the entire S protein. However, the new isolates and the US strains were genetically different from the vaccine type MK strain, ranging from 92.84–93.15% similarity (95-99 aa differences between MK versus Tochigi, Nagasaki and the other US strain). Alignment of the aa sequence of those strains identified two regions of aa deletions in the MK strain (aa 59 to 62 and 140) compared with the other strains. There was also one region of aa insertion in MK strain (aa 160 and 161) compared with the other strains (Fig. 1-2).

Phylogenetic analysis of PEDV

A total of 45 PEDV viruses (2 isolated in this study, 3 US strains, the MK strain, 5 S-INDEL PEDV strains and 34 other globally endemic PEDV strains) were used for phylogenetic analysis based on the S protein (Fig. 1-3), which demonstrated that the new Japanese isolates were closely related to the new US isolates. Forty five PEDV strains clustered into group I or II. The above-mentioned five strains, but not the MK strain and seven Taiwan PEDV isolates as well as 5 S-INDEL PEDV strains, were clustered within group II together with 13 strains detected in China from 2011 to 2012. On the other hand, the European CV777-based vaccine strain, MK strain, Korean DR13 strain, and JS2008 were clustered into group I (Fig. 1-3). These results indicated that the PEDV currently

circulating in Japan is classified into group II, as are US isolates, but in contrast to viruses previously isolated in Japan, which are classified in group I (Fig. 1-3). These data are in agreement with previous reports (Masuda et al., 2015; Suzuki et al., 2015) that the currently prevalent PEDV in Japan is similar to US isolates (group II), but different from Japanese vaccine type viruses (group I).

Neutralization titer (NT) of different PEDV strains

The antigenic properties of the new isolates with those of the vaccine-type MK strain by NT were examined. The MK strain induces syncytium formation in the presence of trypsin (Kusanagi et al., 1992; Shirato et al., 2010), as did the new isolates Tochigi and Nagasaki, while the vaccine strain commercially available in Japan induces cell rounding and detachment without trypsin treatment. We therefore used the MK strain rather than the vaccine strain to perform NT, because the MK, Tochigi and Nagasaki strains can be examined under identical conditions. Moreover, there was no antigenic difference between Tochigi and Nagasaki in NT (data not shown).

As shown in Fig. 1-4a, all sera from pigs vaccinated but not infected by the new PEDV strain, namely pig farms vaccinated without recent PED incidence, showed almost identical neutralization titers against the MK strain and the new isolate. In some cases, the titer was twofold higher for group I virus compared with group II virus. These data indicate no significant difference in the antigenicity of viruses in terms of neutralization activity. A twofold difference in titer between group I and II viruses has also been reported by others (Wang et al., 2016). Similar results were obtained using sera from pigs vaccinated and infected with the new PEDV strain (Fig. 1-4b). No pig serum showed a higher titer to the

new isolates than to MK, suggesting that those pigs were not infected even if the farm was affected by the new PEDV strain. Alternatively, it could indicate that antiserum against group I vaccine also protected against infection by group II viruses, and thus no antibodies were elicited by group II viruses. Collectively, these data indicated that neutralizing antigenicity does not differ between MK (group I) and the new isolates (group II). This NT showed for the first time that group I and II viruses have similar neutralizing antigenicity using a number of sera from individual pigs vaccinated with group I virus.

1-4. Discussion

In the present study, we isolated two new PEDVs from piglets sacrificed during the PED outbreak in Japan. Although we firstly have tried to isolate new PEDV from more than 10 samples by conventional method, we failed isolation. Then we applied spinoculation and successfully isolated 2 viruses from 7 intestinal specimens but not from 7 feces, though isolation was not successful by conventional method. Feces were cytotoxic in spinoculation and conventional isolation. These results show that spinoculation is more efficient for isolation of PEDV from specimens than conventional isolation method and feces were not appropriate for isolation. Successful isolation of PEDV by spinoculation could be attributed to 10-100 fold enhanced attachment of coronaviruses to cells by spinoculation as previously reported (Watanabe et al., 2006).

As anticipated, the viruses that caused the 2013 outbreak in Japan, which started 6 months after the initial detection of PED in the US, were highly similar to the US isolates and to the viruses circulating in China since 2010 (Chen et al., 2014; Huang et al., 2013). New cases of PED caused by group II viruses had not been reported prior to the large PED

outbreak in September 2013 in Japan (Suzuki et al., 2015). The new PEDV currently prevalent in Japan could have originated from countries in which similar viruses are prevalent, but its origin and mechanism of spread nationwide are unknown.

We showed in this study that new Japanese isolates classified in group II do not differ antigenically from the group I vaccine type MK strain, as reported for US isolates (Lin et al., 2015). This result is not in disagreement with previous reports that CV777 (group I) and a new strain isolated in China (group II) differed in antigenicity by twofold (Wang et al., 2016). These reports are in accordance with the findings that the neutralizing epitopes of the S protein of new isolates are highly conserved and similar to those of the CV777 vaccine strain (Chen et al., 2013; Hao et al., 2014; Sun et al., 2016, 2008). It was also reported that S-INDEL PEDV strains may confer protection against more virulent PEDV isolates. The positive antisera against the S-INDEL strain neutralized non-S-INDEL types of virus (Chen et al., 2016). Moreover, sows inoculated with S-INDEL PEDV infection could reduce morbidity, mortality rate, and fecal shedding in newborn piglets challenged with a US-virulent PEDV strain (Goede et al., 2015). This is in agreement with our present findings.

In the US, PED spread rapidly nationwide from the initial case in Ohio, as well as to Canada and Mexico. The huge outbreak in the US may have been caused by the fact that no pigs were vaccinated. However, the spread of PED was similar in Japan, where pregnant sows are usually vaccinated with a virus that elicits production of antibodies that can also neutralize PEDVs of other groups. The vaccine currently used in Japan is effective against group I viruses, as evidenced by the lack of PED for 7 years in Japan prior to invasion by group II viruses. It was reported that the vaccine against group I viruses increased the

survival rate of piglets challenged with virulent wild-type PEDV, although it failed to reduce morbidity (Lee, 2015). In agreement with this report, our findings showed that group II viruses are neutralized by the antibodies elicited by a vaccine comprising a group I virus. Collectively, these findings suggest that outbreaks in countries where vaccines are available are not due to ineffective vaccination but rather are attributed to another, as-yet-unknown, cause.

Newly appearing PEDVs are reported to be more virulent than classic group I strains (Liu et al., 2015); infection and disease in piglets resulted from less than 1 infectious unit (Liu et al., 2015). This suggests that large outbreaks are due to the high pathogenicity of the viruses; escape of even a small number of viruses from antibody neutralization causes devastating diseases in piglets. As commercially available vaccines can neutralize new viruses and protect piglets via antibodies contained in colostrum and ordinary milk, factors—such as sanitation, hygiene and health conditions of lactating sows—must also be important for preventing outbreaks of highly pathogenic viruses (Lee, 2015). Moreover, PEDV-infected sows failed to endow a full amount of milk with neutralizing antibodies to their piglets, which could also have led to the high mortality rate in the present PED outbreak (Lee, 2015).

The large outbreak that occurred recently in the US was due mainly to poor biosecurity (Lee, 2015). Namely, the virus was transmitted by movement of contaminated vehicles from the farm where the PEDV outbreak occurred to PEDV-free farms (Lowe et al., 2014). Also, transmission by contaminated food was suspected (Dee et al., 2014). To prevent large outbreaks, a high level of biosecurity and highly effective vaccines are required.

1-5. Conclusion

Two different strains of PEDV were isolated from diseased piglets and they were classified into group II virus, but antigenically related to group I by neutralization test. To prevent infection by highly pathogenic viruses like those shown in this chapter, establishment of rigid biosecurity, in which PEDV infection is contained at an early phase, is critical. Also, vaccination with group II virus strains could assist to protect pigs from large outbreaks of PEDV.

Table 1-1: List of primers used for sequencing of S gene of PEDV			
Fragments of S gene	Primer	Primer sequence (5′– 3′)	Position in PEDV genome
S1	F1	gaatggtaagttgctagtgcg	20562-20582
	R1	cctgagaacacttgagttggc	22039-22059
	F-4	gggccccactgctaataatg	21035-21054
	R-4	ctcatatgagctgggatggc	21090-21109
	F-5	ggagctgctgtgcagcgtgc	21558-21577
	R-5	ccttcagcaagaatgacagagg	21610-21631
S2	F2	cagggaaattgtcatcacc	21818-21836
	R2	ccaccgatgagagacgcac	23494-23512
	F-6	ggtgccaaccttattgcatc	22209-22228
	R-6	gggcaattactgtcctgtg	22333-22351
	F-7	ggtgagggtatcattaccc	22608-22626
	R-7	ggctaacaactgtccagaatc	22668-22688
	F-8	gcacccacggttactggg	22956-22973
	R-8	gcacaatcaacactaacaggcg	23038-23059
S3	F3	cgctctgtggcagatctagtc	23403-23423
	R3	cattgagctccaactcttgg	24877-24896
	F-9	ccgtacagctgcaacacaac	23791-23810
	R-9	gctgataatctgccggtgatg	23891-23911
	F-10	gttgtgtggtcacctatgtc	24307-24326
	R-10	cgatgtaatctgggattac	24354-24372

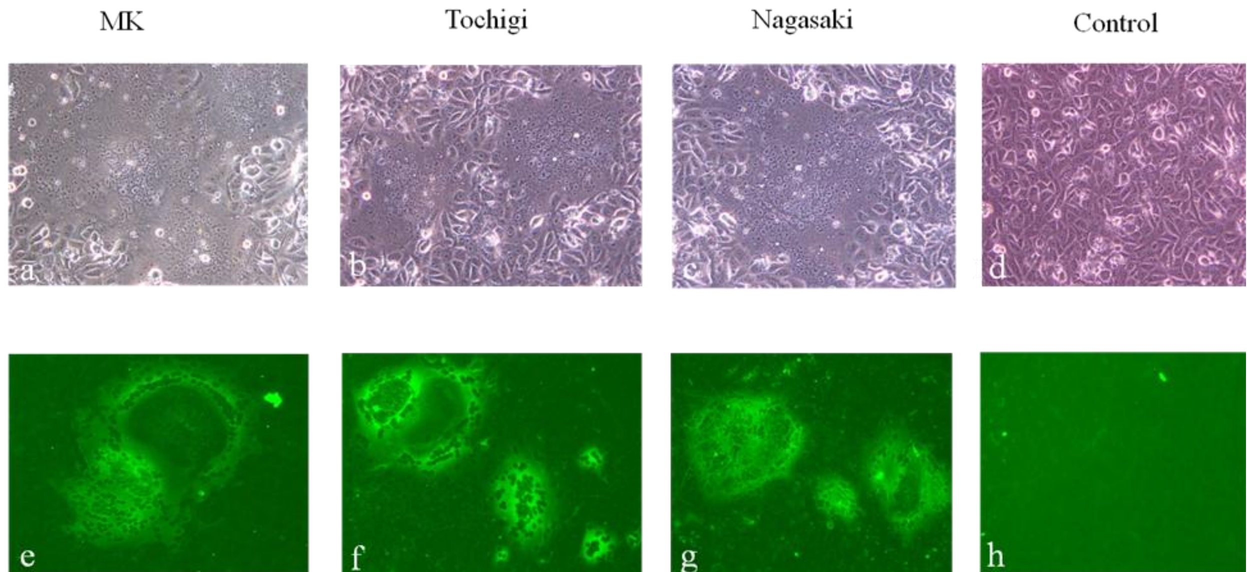

Figure 1-1: Cytopathic effects and detection of viral antigen by immunofluorescence in Vero cells infected with the MK, Tochigi and Nagasaki strains. At 48 h postinfection, cytopathic effects were observed by phase-contrast microscopy (a, b, c and d). At the same time, PEDV-infected Vero cells were fixed with acetone-methanol, and the presence of virus-specific antigen was determined by immunofluorescence assay using anti-PEDV pig serum collected from pig vaccinated by group I vaccine but not infected by newly appearing PEDV and fluorescence isothiocyanate-conjugated goat anti-swine IgG (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) (e, f, g and h).

```
Tochigi    1:MKSLTYFWLFLPVLSTLSLPQDVSRCSANTNFRRFFSKFNVQAPAVVVLGGYLPIGENQG 60
Nagasaki   1:.......................T.................................... 60
MN         1:.......................T.................................... 60
IA1        1:.......................T.................................... 60
IA2        1:.......................T.................................... 60
MK         1:....I.............I....T..QTT..................I......SMNS-- 58
             ****.*************.****.**...******************.******......
                     SP                                             Del

Tochigi   61:VNSTWYCAGQHPTASGVHGIFVSHIRGGHGFEIGISQEPFDPSGYQLYLHKATNGNTNAT 120
Nagasaki  61:............................................................ 120
MN        61:............................................................ 120
IA1       61:............................................................ 120
IA2       61:............................................................ 120
MK        59:--.S.H.GTGIE.D.......L.YVDS.Q..............................I 116
             ..*.*.*.....*.*******.*....*.******************************.
             Del

Tochigi  121:ARLRICQFPNIKTLGPTANNDVTTGRNCLFNKAIPAHMS--EHSVVGITWDNDRVTVFSD 178
Nagasaki 121:.........S.............................--.........Y......... 178
MN       121:.........S.............................--................... 178
IA1      121:.........S.............................--................... 178
IA2      121:.........S.............................--................... 178
MK       117:.........DN........-..................QDNR.I..............A. 175
             *********..********.******************.  .*.******.*******.*
                                Del                 Ins

Tochigi  179:KIYYFYFKNDWSRVATKCYNSGGCAMQYVYEPTYYMLNVTSAGEDGISYQPCTANCIGYA 238
Nagasaki 179:............................................................ 238
MN       179:...............................................F............ 238
IA1      179:............................................................ 238
IA2      179:............................................................ 238
MK       176:...H..I.........R...KRS.......T................Y.E...VD.S... 235
             ***.**.*********.***...*******.****************.*.***..*.***

Tochigi  239:ANVFATEPNGHIPEGFSFNNWFLLSNDSTLVHGKVVSNQPLLVNCLLAIPKIYGLGQFFS 298
Nagasaki 239:............................................................ 298
MN       239:............................................................ 298
IA1      239:............................................................ 298
IA2      239:............................................................ 298
MK       236:.......S......................L...............W............. 295
             *******.**********************.***************.*************

Tochigi  299:FNQTIDGVCNGAAVQRAPEALRFNINDTSVILAEGSIVLHTALGTNFSFVCSNSSNPHLA 358
Nagasaki 299:............................................................ 358
MN       299:............................................................ 358
IA1      299:............................................................ 358
IA2      299:........................................................L... 358
MK       296:....M.......SA................................L...........K. 355
             ****.*******..********************************.*********.*.*

Tochigi  719:SLSSSTFNSTRELPGFFYHSNDGSNCTEPVLVYSNIGVCKSGSIGYVPSQSGQVKIAPTV 778
Nagasaki 719:............................................................ 778
MN       719:............................................................ 778
IA1      719:............................................................ 778
IA2      719:............................................................ 778
MK       716:...N....N.......................................I.D......... 775
             ***.****.***************************************.*.*********
                                             NE-2          NE-3

Tochigi  779:TGNISIPTNFSMSIRTEYLQLYNTPVSVDCATYVCNGNSRCKQLLTQYTAACKTIESALQ 838
Nagasaki 779:............................................................ 838
MN       779:............................................................ 838
IA1      779:............................................................ 838
IA2      779:............................................................ 838
MK       776:............................................................ 835
             ************************************************************

Tochigi  839:LSARLESVEVNSMLTISEEALQLATISSFNGDGYNFTNVLGVSVYDPASGRVVQKRSFIE 898
Nagasaki 839:............................................................ 898
MN       839:............................................................ 898
IA1      839:............................................................ 898
IA2      839:............................................................ 898
MK       836:.....................................................H...... 895
             *****************************************************.******

Tochigi  899:DLLFNKVVTNGLGTVDEDYKRCSNGRSVADLVCAQYYSGVMVLPGVVDAEKLHMYSASLI 958
Nagasaki 899:............................................................ 958
MN       899:............................................................ 958
IA1      899:............................................................ 958
IA2      899:............................................................ 958
MK       896:............................................................ 955
             ************************************************************

Tochigi  959:GGMVLGGFTSAAALPFSYAVQARLNYLALQTDVLQRNQQLLAESFNSAIGNITSAFESVK 1018
Nagasaki 959:............................................................ 1018
MN       959:............................................................ 1018
IA1      959:............................................................ 1018
IA2      959:............................................................ 1018
MK       956:...A.....A.V.........................................L...... 1015
             ***.*****.*.*.***************************************.******
                                          HR-1

Tochigi 1019:EAISQTSKGLNTVAHALTKVQEVVNSQGAALTQLTVQLQHNFQAISSSIDDIYSRLDILS 1078
Nagasaki 1019:............................................................ 1078
MN      1019:............................................................ 1078
IA1     1019:............................................................ 1078
IA2     1019:............................................................ 1078
MK      1016:.................N..........S......I........................ 1075
             *****************.**********.******.************************
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Tochigi   359:TFAIPLGATQVPYYCFLKVDTYNSTVYKFLAVLPPTVREIVITKYGDVYVNGFGYLHGL 418
Nagasaki  359:............................................................ 418
MN        359:............................................................ 418
IA1       359:............................................................ 418
IA2       359:............................................................ 418
MK        356:I.T....V.E...........................K...................... 415
              .*.****.*.***************************.**********************

Tochigi   419:LDAVTINFTGHGTDDDVSGFWTIASTNFVDALIEVQGTAIQRILYCDDPVSQLKCSQVAF 478
Nagasaki  419:............................................................ 478
MN        419:............................................................ 478
IA1       419:............................................................ 478
IA2       419:............................................................ 478
MK        416:......................V............K......................S. 475
              **********************.************.**********************.*

Tochigi   479:DLDDGFYPISSRNLLSHEQPISFVTLPSFNDHSFVNITVSASFGGRSGANLIASDTTING 538
Nagasaki  479:.............................................H.............. 538
MN        479:.......H.....................................H.............. 538
IA1       479:.............................................H.............. 538
IA2       479:.............................................H.............. 538
MK        476:.............................T...........A...H.............. 535
              *******.*********************.***********.***.**************

Tochigi   539:FSSFCVDTRQFTISLFYNVTNSYGYVSKSQDSNCPFTLQSVNDYLSFSKFCVSTSLLASA 598
Nagasaki  539:....................................................A....... 598
MN        539:............................................................ 598
IA1       539:............................................................ 598
IA2       539:............................................................ 598
MK        536:.............T............................................G. 595
              *************.**************************************.*****.*

Tochigi   599:CTIDLFGYPEFGSGVKFTSLYFQFTKGELITGTPKPLEGVTDVSFMTLDVCTTYTIYGFK 658
Nagasaki  599:.........................E..........................K....... 658
MN        599:....................................................K....... 658
IA1       599:....................................................K....... 658
IA2       599:....................................................K....... 658
MK        596:................L...................................K....... 655
              ****************.********.**************************.*******
                          NE-1

Tochigi   659:GEGIITLTNSSFLAGVYYTSDSGQLLAFKNVTSGAVYSVTPCSFSEQAAYVDDDIVGVIS 718
Nagasaki  659:............................................................ 718
MN        659:............................................................ 718
IA1       659:............................................................ 718
IA2       659:............................................................ 718
MK        656:............................................................ 715
              ************************************************************

Tochigi   1079:ADVQVDRLITGRLSALNAFVAQTLTKYTEVQASRKLAQQKVNECVKSQSQRYGFCGGDGE 1138
Nagasaki  1079:............................................................ 1138
MN        1079:............................................................ 1138
IA1       1079:............................................................ 1138
IA2       1079:............................................................ 1138
MK        1076:............................................................ 1135
               ************************************************************

Tochigi   1139:HIFSLVQAAPQGLLFLHTVLVPSDFVDVIAIAGLCVNDEIALTLREPGLVLFTHELQNHT 1198
Nagasaki  1139:............................................................ 1198
MN        1139:............................................................ 1198
IA1       1139:............................................................ 1198
IA2       1139:............................................................ 1198
MK        1136:......................G...N..........G...................TY. 1195
               **********************.***.**********.*******************..*

Tochigi   1199:ATEYFVSSRRMFEPRKPTVSDFVQIESCVVTYVNLTRDQLPDVIPDYIDVNKTLDEILAS 1258
Nagasaki  1199:............................................S............... 1258
MN        1199:............................................................ 1258
IA1       1199:............................................................ 1258
IA2       1199:............................................................ 1258
MK        1196:....................................S....................... 1255
               ************************************.*******.***************
                                  HR-2
Tochigi   1259:LPNRTGPSLPLDVFNATYLNLTGEIADLEQRSESLRNTTEELQSLIYNINNTLVDLEWLN 1318
Nagasaki  1259:............................................................ 1318
MN        1259:............................................................ 1318
IA1       1259:............................................................ 1318
IA2       1259:............................................................ 1318
MK        1256:.........S...................H...Y........R................. 1315
               *********.*******************.***.********.*****************

Tochigi   1319:RVETYIKWPWWVWLIIFIVLIFVVSLLVFCCISTGCCGCCGCCCACFSGCCRGPRLQPYE 1378
Nagasaki  1319:............................................................ 1378
MN        1319:............................................................ 1378
IA1       1319:............................................................ 1378
IA2       1319:............................................................ 1378
MK        1316:...........................................G................ 1375
               *******************************************.****************
                          TM                                   NE-4
Tochigi   1379:VFEKVHVQ                                                     1386
Nagasaki  1379:........                                                     1386
MN        1379:........                                                     1386
IA1       1379:........                                                     1386
IA2       1379:........                                                     1386
MK        1376:A.......                                                     1383
               .*******
```

Figure 1-2: Alignment of the S-protein amino acid sequences of six PEDV strains (Tochigi, Nagasaki and MK are described in the text, and MN, IA1 and IA2 are US isolates). Signal peptide (SP), deletion (Del), insertion (Ins), neutralizing epitope 1 to 4 (NE-1 to NE-4), heptad repeat 1 and 2 (HR-1 and HR-2) and transmembrane domain (TM) are indicated by boxes.

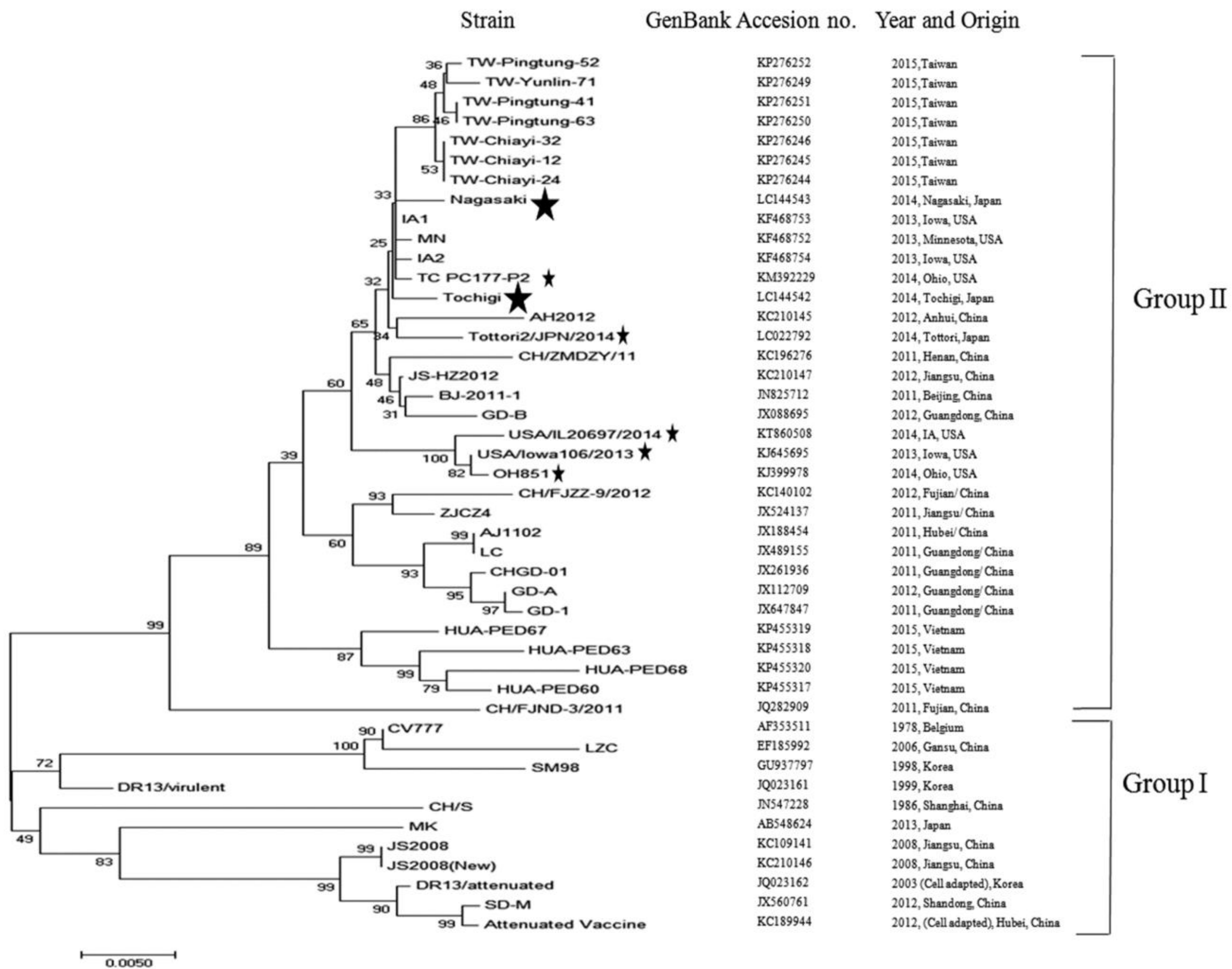

Figure 1-3: Phylogenetic analysis of the entire amino acid sequence of the S proteins of two new PEDV isolates (Tochigi and Nagasaki) and those of 43 previously published PEDVs. The trees were constructed using the distance-based neighbor-joining method of the MEGA 6.06 software. Bootstrap analysis was carried out on 1,000 replicate datasets, and values are indicated adjacent to the branching points. The viruses identified in this study are indicated by large asterisks. S-INDEL PEDV strains are indicated by small asterisks. The scale represents the number of amino acid substitutions per site.

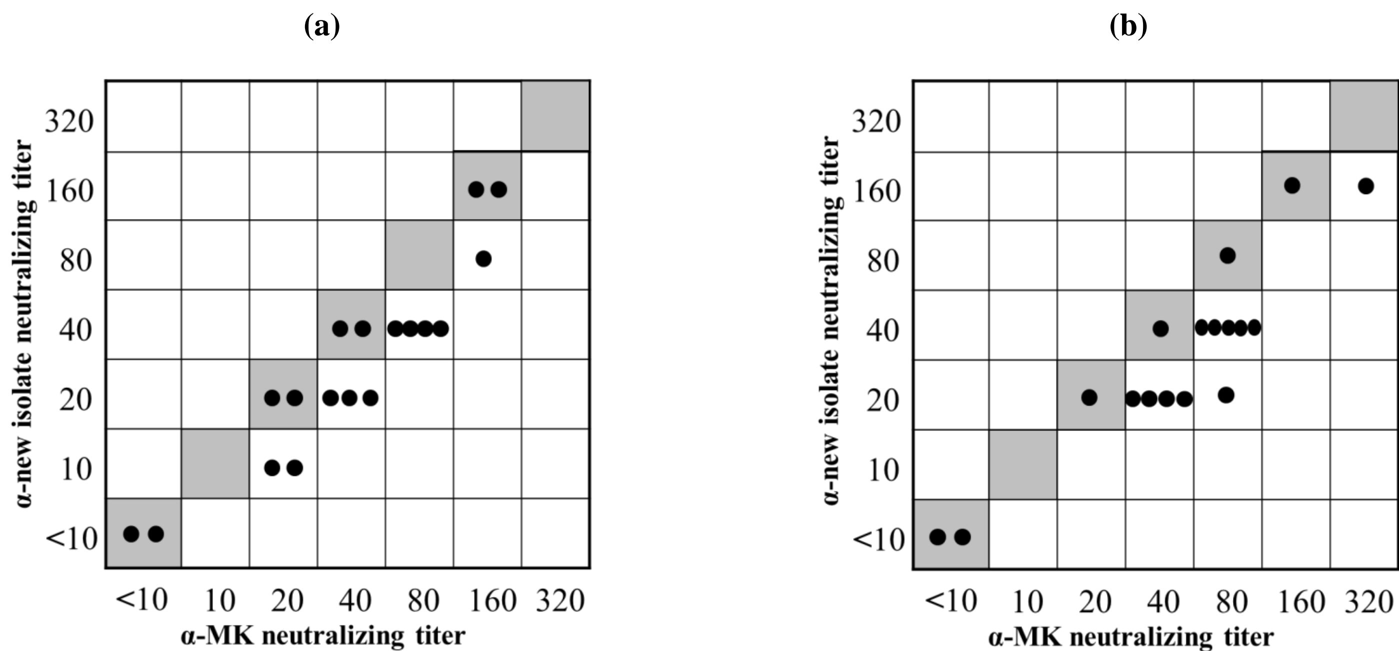

Figure 1-4: Neutralizing titers of various pig sera against the new isolates (Tochigi and Nagasaki) and the MK vaccine strain. Two types of antisera against PEDV were used. One group comprised 18 sera collected from herds of pigs that were vaccinated but not recently affected by PEDV (a), and the other group comprised 17 sera from herds of pigs that were vaccinated and affected by the new PEDV (b). Sera were collected from different pig farms. • individual pig serum.

Chapter-2

Biological characterization of the HE protein of BToV

2-1. Introduction

ToV is an enveloped virus with a single stranded, positive sense genomic RNA approximately 28 kb long and its virion size ranges from 120–140 nm in diameter (Cornelissen et al., 1997; Horzinek, 1999; Snijder and Horzinek, 1993). The virion consists of the S, M, and HE proteins on the envelope, and the genome is associated with the N protein, which is wrapped by the envelope (Draker et al., 2006). The S protein is the largest surface glycoprotein of the virion and is thought to be involved in viral entry and pathogenesis (Kirisawa et al., 2007; Snijder et al., 1990). Electron microscopy has shown that HE consists of projections of approximately 6 nm on the virion surface, which are interspersed among spikes of approximately 20 nm composed of the S protein (Bridger et al., 1978; Cornelissen et al., 1997; Sugiyama and Amano, 1981). The HE protein of ToV is similar to the HE protein of the influenza C virus and was thought be acquired by ToV as a result of recombination with the influenza C virus (de Groot, 2006; Luytjes et al., 1988; Snijder et al., 1991).

The ToV Berne strain was first isolated in Switzerland in 1972 from a horse with diarrhea (Weiss et al., 1983). The Breda strain of bovine ToV (BToV) was isolated from a calf with diarrhea in Iowa in 1979 (Weiss et al., 1983; Woode et al., 1982) and is endemic in cattle herds, with asymptomatic cows possibly acting as reservoirs. Newborn calves generally develop clinical symptoms of diarrhea, typically lasting 2–13 days (Koopmans et al., 1991, 1990). Only the equine and BToVs have been isolated from cultured cells (Kuwabara et al., 2007; Weiss et al., 1983). The other ToVs have only been detected in specimens by reverse transcriptase (RT)-polymerase chain reaction (PCR) (Ito et al., 2007; Snijder and Horzinek, 1993). However, growth of the Breda strain in cultured cells is poor,

and thus, studies of BToV have been limited. Recently, a new cell line, human rectal tumor-18 (HRT-18) cells, has been shown to be susceptible to BToV, and several different BToVs were successfully isolated and maintained with this cell line (Aita et al., 2012; Ito et al., 2010; Kuwabara et al., 2007; Shimabukuro et al., 2013).

BToV with the full length HE gene was initially isolated from diarrheal feces; however, the viruses lost the HE protein as a result of mutation of the HE gene following several passages in HRT-18 cells (Aita et al., 2012; Ito et al., 2010). These findings suggest that the HE protein is important for replication and pathogenesis in animals, but is not essential for replication in cell culture. Furthermore, HE may hinder virus replication in cultured cells.

In the present study, we explored the biological activity of the HE protein, using a virus with (HE+) and without (HE-) the HE protein, and found that HE suppresses BToV infection in cultured cells.

2-2. Materials and Methods

Cells and viruses

HRT-18, Human embryonic kidney 293T (HEK 293T), and Vero cells used in the present study were maintained in DMEM supplemented with 10% heat-inactivated FCS, penicillin (100 IU/ml), and streptomycin (100 μg/ml). HRT-18 cells, which are susceptible to BToV (Kuwabara et al., 2007), were used for BToV propagation and infection. The Aichi strain (Aita et al., 2012; Ito et al., 2010; Shimabukuro et al., 2013), as well as the Niigata-2 (Nig-2), and Niigata-3 (Nig-3) strains, isolated from diarrheal specimens from two different cattle in the Niigata prefecture (Aita et al., 2012), were used. Vero cells were

used to prepare the Sindbis virus, kindly provided by Dr. Hiroshi Shirasawa (Chiba University), which was used for the IFN experiments. The supernatant of Sindbis virus-infected cells was isolated 36 h post infection (pi), centrifuged at 5,000 rpm for 10 min, and stored at -80°C. HEK 293T cells were used for transfection of the HE gene.

Virus isolation by spinoculation

The Nig-2 and Nig-3 strains of BToV were isolated from diarrheic cattle from two different farms of the Niigata prefecture by spinoculation, as previously described (Watanabe et al., 2006). Briefly, the diarrheic feces collected from the diseased cow were diluted with nine volumes of PBS, pH 7.2 to make a 10% suspension of the specimen and the mixture was homogenized with a glass homogenizer. The homogenates were centrifuged at 5,000 rpm at 4°C for 30 min, and the supernatant was filtered through a 450 nm filter. HRT-18 cells, prepared in 24-well plates at a concentration of 2×10^5 cells in 0.5 mL per well, were incubated for 24 h and inoculated with 50 µL of the 10% fecal suspension and 400 µL of DMEM per well. The plates were centrifuged at 3,000 rpm for 2 h at 4°C. Cells were washed twice with warm DMEM and incubated for 4 d with DMEM at 37°C; the CPEs were observed. Newly prepared HRT-18 cells in 24-well plates were inoculated with the collected supernatant after evaluation of the CPEs. Viruses were passaged 15 times. We cloned Nig-3-3 (HE+) after two passages, and Nig-3-8 (HE-) after seven passages, in HRT-18 cells by two and three times limited dilution, respectively. We also isolated Nig-2-8 after seven passages of Nig-2 in HRT-18 cells.

Extraction of RNA and RT-PCR

Viral RNAs were extracted from the fecal samples and the supernatants of infected cells following each passage using Direct-Zol RNA Miniprep according to the manufacturer's instructions. The cDNA was prepared from the eluted RNAs and PCR was carried out with PrimeScript II 1st strand cDNA Synthesis kit and Ex Taq. The primers used for the amplification of the BToV HE genes by RT-PCR were described previously (Shimabukuro et al., 2013). The primers targeting the HE gene of BToV were as follows: forward primer, 5' GATGCTGAGTTTAATATTC 3' and reverse primer, 5' CCTAATAACTACTTAAAC 3'. The reaction was prepared according to the manufacturer's instructions. The PCR conditions were as follows: 35 cycles of denaturation at 98°C for 10 sec, annealing at 45°C for 30 sec, extension at 72°C for 90 sec, and a final extension at 72°C for 7 min. For each reaction, ddH_2O was used as a negative control. PCR products were detected by electrophoresis on a 1.0% agarose gel with 1-kb plus DNA ladder.

Sequence analysis

RT-PCR products were purified using a DNA purification kit (NucleoSpin Gel and PCR Clean-up kit; Macherey-Nagel, Düren, Germany). The size of the DNA fragments was estimated with a marker. The purified PCR products corresponding to the BToV HE genes were sequenced by Eurofins Genomics with the BigDye Terminator version 3.1 cycle sequencing kit and an Applied Biosystems 3730×1 DNA analyzer. For sequence similarity analysis, the BToV Aichi strain (Aita et al., 2012) was used as a reference. The accession

numbers of the HE gene of Nig-2, Nig-3 (HE+), and Nig-3 (HE-) are AB661457, AB661458, and AB661459, respectively (National Center for Biotechnology Information).

Expression of the HE protein

We expressed the HE protein of the Aichi strain in HEK 293T cells. The Aichi strain propagated in cell culture possessed a truncated (Trc-HE) HE gene (Aichi-TC), while the Aichi strain with the full-length (FL-HE) HE gene (Aichi-LIC) produced by site-directed mutagenesis was reported previously (Shimabukuro et al., 2013). We also expressed the HE gene with a C terminal HA tag. These genes were cloned into the pCAGGS expression vector and the vectors were transfected with TransIT® 293 (Mirus, Tokyo, Japan), as previously described (Shimabukuro et al., 2013).

Detection of HE protein by indirect immunofluorescence (IIF)

HRT-18 cells cultured in 24-well plates were infected with the Nig-3-3 (HE+) and Nig-3-8 (HE-) strains at an MOI of 0.05. To stain the intracellular proteins, infected HRT-18 cells were fixed 18 h pi with 4% paraformaldehyde for 30 min at room temperature and permeabilized with 0.1% Triton X-100 for 20 min at room temperature. Cells were incubated with mouse anti-HE, anti-S, and anti-M antiserum for 2 h at room temperature. After two washes with PBS, cells were incubated with fluorescence isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Life Technologies, Eugene, OR, USA) for 2 h at room temperature (Ujike et al., 2016).

Growth kinetics of Nig-3-3 (HE+) and Nig-3-8 (HE-) in HRT-18 cells

HRT-18 cells in 24-well plates at 80–90% confluence were washed once with DMEM and infected with Nig-3-3 and Nig-3-8 at a multiplicity of infection (MOI) of 0.05. After 1 h adsorption at 37°C, viral inoculums were removed, cells were washed with DMEM, and 0.5 mL DMEM was added. Viruses in the supernatant were collected at specified intervals and the supernatant was clarified by centrifugation at 5,000 rpm for 5 min at 4°C. Virus titers in the culture media were determined in a 96-well plate by $TCID_{50}$, as previously described (Reed and Muench, 1938).

Effect of HE protein on the growth of Sindbis virus

HEK 293T cells cultured in 24-well plates were transfected with the pCAGGS expression vector harboring the FL-HE (LIC) and Trc-HE (TC) genes of the Aichi strain at 1 μg/well by TransIT® 293. After 48 h of transfection, HEK 293T cells were infected with the Sindbis virus at an MOI of 0.05. The virus titers were determined 36 h pi in 96-well plates by $TCID_{50}$ using Vero cells.

Statistical analysis

Statistical analysis was performed using SPSS software (ver. 11.5; SPSS Inc., Chicago, IL, USA). The replication of Nig-3-3 (HE+) and Nig-3-8 (HE-) in HRT-18 cells was analyzed with Student's *t* test. Differences were considered to be statistically significant at $p < 0.05$.

2-3. Results

Virus isolation and comparison of HE amino acid sequences

Virus inoculums were prepared from the diarrheal specimens Nig-2 and Nig-3 and spinoculated into HRT-18 cells. The CPEs, characterized by the aggregation and enlargement of cells, were observed 3–4 days later, and the cells eventually detached from the plastic surface. There was no difference in the CPEs between Nig-2 and Nig-3 (Fig. 2-1). RT-PCR analysis of the S and HE genes indicated that both the cells and the supernatant were positive for BToV (data not shown). A mixture of the supernatant and the cells was inoculated in newly prepared HRT-18 cells. Virus passages were repeated 15 times and the viruses collected at each passage were examined for the presence of HE by amplification of the HE gene, as described in the materials and methods.

The nucleotide sequences of the RT-PCR products from the isolates of the different passages revealed that the Nig-3 strain contained the FL-HE gene even after seven passages. At the eighth passage, a mutation in nucleotide 327 arose in the HE gene of Nig-3, creating a stop codon in the 109th amino acid (Fig. 2-2a), upstream of the transmembrane domain of the HE gene. Thus, the resulting HE protein of Nig-3-8 is not incorporated into virions but becomes truncated and soluble (Fig. 2-2b). On the other hand, the Nig-2 strain contained the FL-HE gene even after 15 passages in HRT-18 cells. The Aichi (TC) strain was passaged more than eight times through HRT-18 cells and acquired a stop codon at amino acid 161 (Shimabukuro et al., 2013). The HE gene of Aichi (LIC) was produced by site-directed mutagenesis of the Aichi (TC) HE gene and consists of the FL-HE sequence (Shimabukuro et al., 2013), as depicted in Fig. 2-2.

Detection of BToV proteins in infected cells

We were unsuccessful in our attempts to produce antibodies for Western blotting analysis against the S, M, or HE proteins; however, we were able to visualize the proteins with IIF. Thus, we examined whether the S, M, or HE proteins were expressed with IIF. HRT-18 cells infected with the Nig-3-3 (HE+) and Nig-3-8 (HE-) strains were probed with anti-HE, anti-S, and anti-M serum to detect expression of the HE, S, and M proteins, respectively, at 18 h pi. Both the S and M proteins of Nig-3-3 and Nig-3-8 were detected within the cells (Fig. 2-3b and c). The HE protein of Nig-3-3 was detected in the cells (Fig. 2-3a), while there was no HE staining in Nig-3-8-infected HRT-18 cells (Fig. 2-3a). These results suggest that the Trc-HE protein was not expressed, or if it was expressed, the antibody failed to detect the protein. It is currently unknown why the Trc-HE protein could not be detected in infected cells.

Comparison of the growth of Nig-3-3 (HE+) with Nig-3-8 (HE-) in HRT-18 cells

We cloned Nig-3-3 (HE+) (third passage) and Nig-3-8 (HE-) (eighth passage) with the limiting dilution method and used these viruses for further experiments. To compare their growth, HRT-18 cells cultured in 24-well plates were infected with the viruses at an MOI of 0.05 and the growth kinetics were examined. As shown in Fig. 2-4, Nig-3-8 (HE-) grew more efficiently than Nig-3-3 (HE+): the infectious titer of Nig-3-8 (HE-) was approximately 10- to 20-fold higher than that of Nig-3-3 (HE+). This result clearly demonstrated that Nig-3-8 (HE-) grew more efficiently than Nig-3-3 (HE+), suggesting that the presence of the HE protein inhibits BToV growth in cultured cells.

Effect of HE protein on the growth of Sindbis virus

We examined whether HE expressed in HEK 293T cells influences infection of the Sindbis virus. We expressed the FL-HE (LIC) and Trc-HE (TC) protein of Aichi strain by transfection. As shown in Fig. 2-5, Sindbis virus infection was suppressed by the expressed FL-HE protein, consistent with the result that BToV with the full length HE gene grows less efficiently in HRT-18 cells than BToV with the Trc-HE gene (Fig. 2-4). The HE protein may negatively impact virus infection in general, since this result was evident with infection of BToV and Sindbis viruses unrelated to BToV.

2-4. Discussion

Although BToV infection is not frequently encountered in the clinical field, it is considered an important etiological agent because it causes diarrhea and decreases milk production in adult cattle. Moreover, seroconversion to BToV has been observed in adult cows showing winter diarrhea (Hoet et al., 2003; Ito et al., 2007; Koopmans et al., 1991, 1989).

HE proteins are retained not only in ToV, but also in some of the beta-CoVs (de Groot, 2006). The equine ToV BEV strain was the first to be isolated and its ability to grow in tissue culture has been reported. This virus does not have an HE protein, but contains a sequence similar to the HE gene. The loss of the HE protein in BEV is thought to be the result of mutation during the passage of cultured cells (Cornelissen et al., 1997; Snijder et al., 1991), similar to the events that occurred in BToVs (Aita et al., 2012; Ito et al., 2010). BToV was recently isolated in cultured HRT-18 cells, most of which had lost the HE protein after multiple passages, however, a few viruses contained the HE protein even after

several passages (Aita et al., 2012; Ito et al., 2010). In the present study, we compared BToVs with and without the HE protein to clarify its biological function.

With the use of HRT-18 cells, BToV can be isolated and maintained (Aita et al., 2012; Ito et al., 2010; Kuwabara et al., 2007; Shimabukuro et al., 2013). Among the isolates, Nig-2 retained the HE protein even after 15 passages in HRT-18 cells, while Nig-3 and Aichi lost HE after 6–8 passages (Aita et al., 2012). These short and soluble HE proteins were not detected by IIF, possibly because of the lack of antibodies against HE. Since we were able to obtain the virus Nig-3 with HE after three passages (Nig-3-3) and that without HE after eight passages (Nig-3-8), we compared these viruses in terms of their ability to replicate in cultured cells and their resistance to IFN. We assumed that HE plays an important role in viral replication in animals, since all of the BToVs isolated from clinical diarrheic specimens contained the FL-HE gene.

Our present study showed that the Nig-3-8 virus without the HE protein multiplied more efficiently in cultured cells than Nig-3-3 with HE. This may indicate that HE negatively impacts BToV infection in HRT-18 cells. This effect of HE was also observed with infection of the unrelated Sindbis virus: HEK 293T cells expressing the HE protein decreased infection of the Sindbis virus (Fig. 2-5). It has also been reported that the HE-deficient mouse hepatitis virus (MHV) grew more efficiently than the HE-positive virus (Lissenberg et al., 2005). These results suggest that HE has an inhibitory effect on the replication of various viruses in cultured cells.

2-5. Conclusion

The function of HE protein of BToV has not yet been understood. In this chapter, comparison of HE+ and HE- strains was carried out in terms of replication in cultured cells. HE- virus grew more efficiently than HE+ one, which suggested that HE has some adverse effect on the virus growth. This is in good agreement with the finding that HE negative viruses are selected after repeated passages in cultured cells.

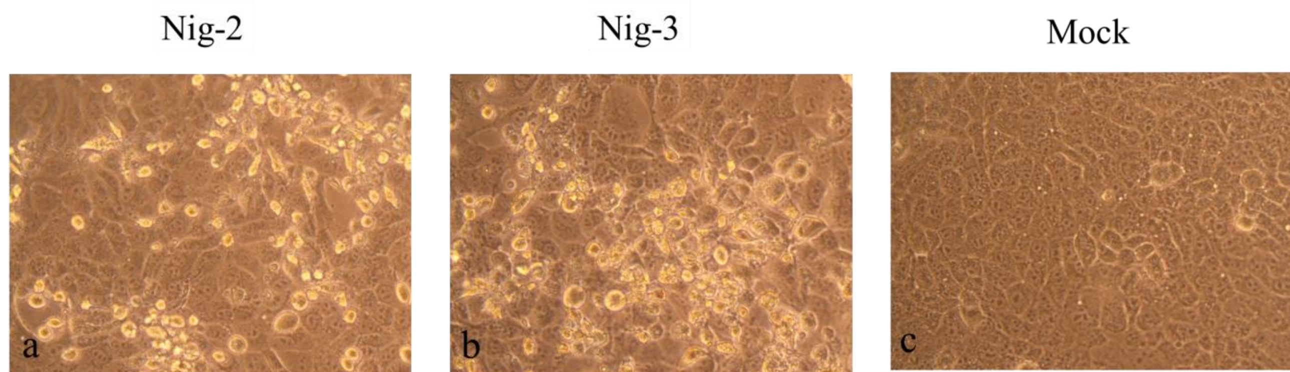

Figure 2-1: The cytopathic effects (CPEs) of newly isolated bovine toroviruses (BToVs) in human rectal tumor-18 (HRT-18) cells. (a) Niigata-2 (Nig-2) 72 h after inoculation, (b) Niigata-3 (Nig-3) 96 h post-infection (pi), and (c) mock-infected HRT-18 cells.

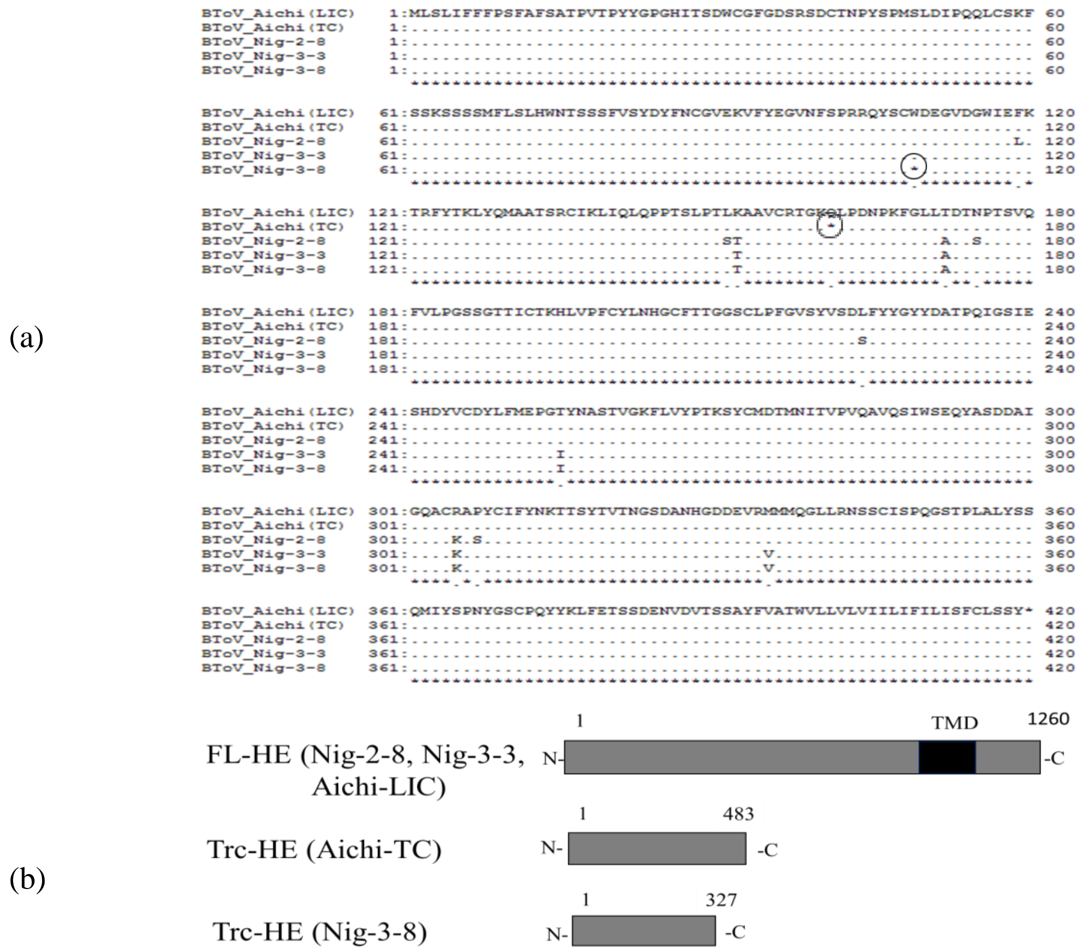

Figure 2-2: Amino acid comparison of various BToV hemagglutinin-esterase (HE) proteins. (a) Comparison of amino acid sequences of HE proteins of the Aichi strain with the full-length (FL-HE) HE gene (Aichi-LIC) and truncated (Trc-HE) HE gene (Aichi-TC), as well as the HE sequences from the Nig-2-8, Nig-3-3, and Nig-3-8 virus strains. Nig-3-8 and Aichi (TC) have a truncated HE protein due to the stop codon indicated by the circles. (b) Schematic diagram of each HE protein. Nig-2-8 and Nig-3-3 contains the FL-HE protein, while the others have the Trc-HE protein due to the stop codon that arose during

passage in HRT-18 cells. Aichi (LIC) indicates the FL-HE protein, which was produced by site-directed mutagenesis from Aichi (TC).

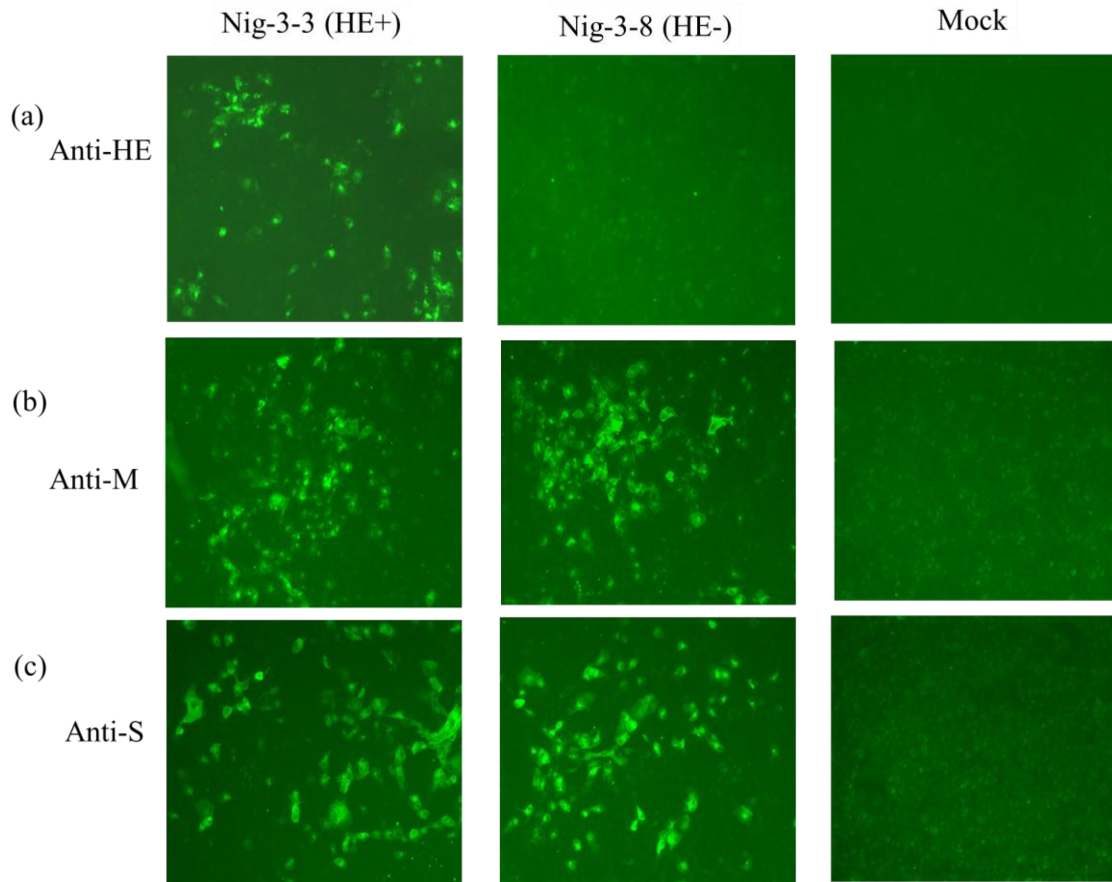

Figure 2-3: Detection of the HE, membrane (M), and spike (S) proteins of the Nig-3-3 and Nig-3-8 strains by indirect immunofluorescence (IIF). HRT-18 cells prepared in 24-well plates were infected with Nig-3-3 (HE+) and Nig-3-8 (HE-) strains at an MOI of 0.05 and examined for the presence of each protein 18 h pi. Infected cells were stained with mouse antibodies raised against each protein and fluorescein isothiocyanate (FITC)-conjugated anti-mouse goat serum. (a) anti-HE, (b) anti-M, and (c) anti-S antibodies.

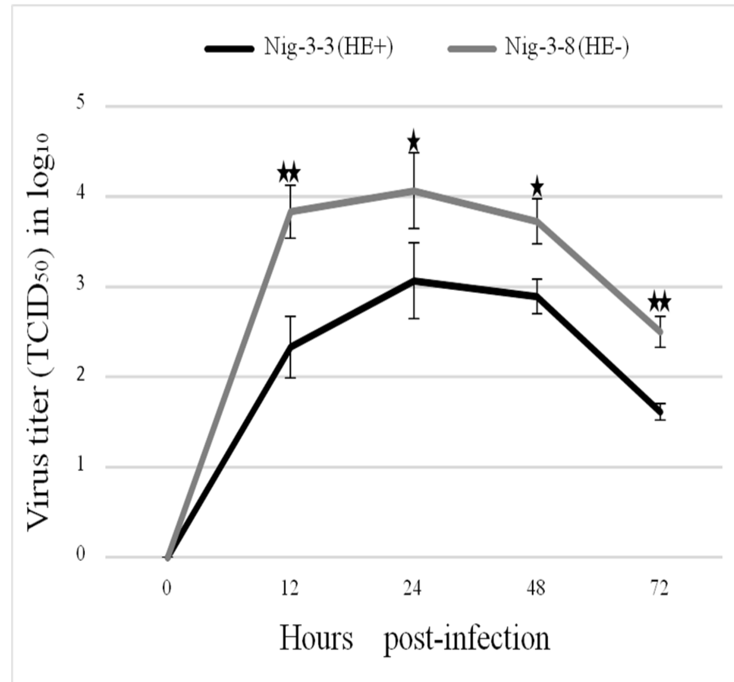

Figure 2-4: Comparison of the growth of the Nig-3-3 strain with the HE protein (HE+) with the Nig-3-8 strain without HE (HE-) in HRT-18 cells. HRT-18 cells in 24-well plates were infected with the viruses at an MOI of 0.05. At the indicated intervals, the culture supernatants of the infected cells were isolated and virus titers were determined by $TCID_{50}$. *p* values were determined with Student's *t* test. Significant differences are indicated with one ($p < 0.05$), two ($p < 0.01$), or three asterisks ($p < 0.001$).

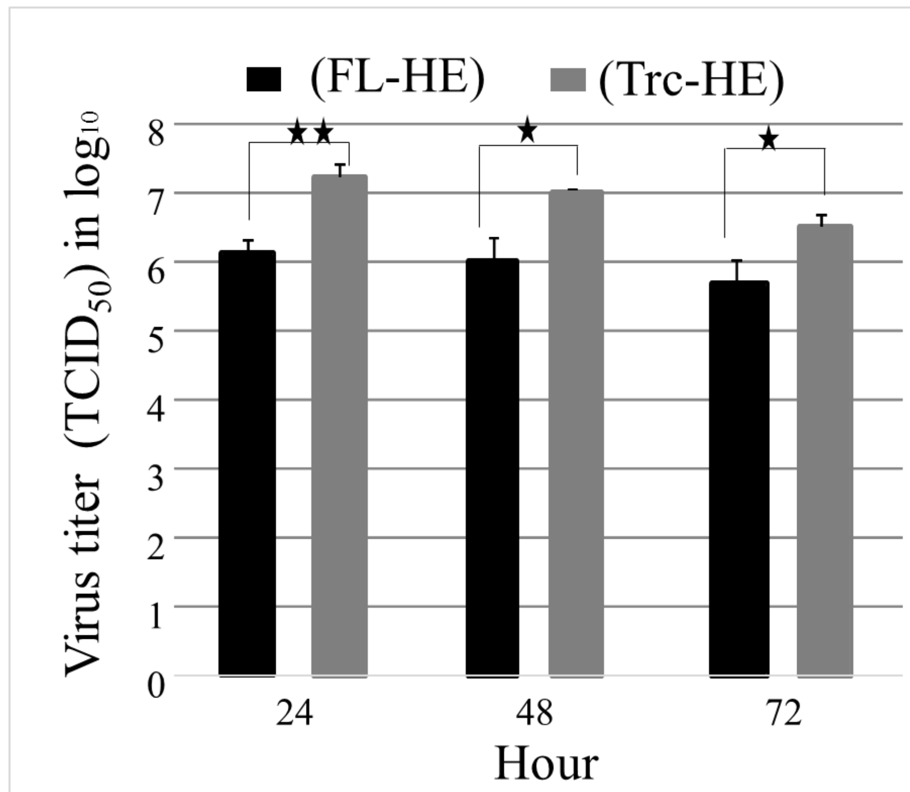

Figure 2-5: Comparison of growth of the Sindbis virus in HEK 293T cells expressing the FL-HE or Trc-HE protein. HEK 293T cells transfected with FL-HE and Trc-HE genes, were infected with the Sindbis virus and infection was monitored for 24–72 h.

Chapter-3

Anti-IFN activity of HE protein of BToV

3-1. Introduction

Innate immunity is the first step of animal defense mechanism after viral infection. As a first step following viral infection, the host defense system secretes type I IFNs, IFN-α, and IFN-β as anti-viral factors (Goodbourn et al., 2000; Gotoh et al., 2002, 2001; Huang et al., 2003). The response to viral infection consists of an innate, or nonspecific component, and an adaptive, or specific defense. The innate response is considered the first line of immune defense. Generally, CoVs contain a number of structural and nonstructural proteins that act as IFN antagonists to overcome the host defense mechanism. Protein 7a of feline infectious peritonitis virus, protein 5a of MHV, nsp1 of severe acute respiratory syndrome, N protein of MHV, nsp2 of infectious bronchitis virus, and protein 7 of transmissible gastroenteritis virus have been reported to act as IFN antagonists (Cruz et al., 2011; Dedeurwaerder et al., 2014; Kamitani et al., 2006; Koetzner et al., 2010; Wang et al., 2009; Ye et al., 2007). However, there is no information is available for the IFN antagonism of any protein of BToV.

Previous findings suggested that, HE protein is an obstacle for virus replication in cultured cells; however, HE must have some importance for viral pathogenesis in host animals. In the present study, we have explored that HE protein works as an IFN-α antagonist, which may explain why the original isolates of BToV contained the full length HE protein.

3-2. Materials and Methods

In situ acetylesterase assay

Subconfluent monolayers of HRT-18 cells grown in 24-well plates were infected with the Nig-3-3, Nig-3-8, and Aichi strains (TC) of BToV. At 24 h pi, the cells were fixed with citrate-acetone-formaldehyde, stained for acetylesterase activity with a α-naphthyl acetate esterase detection kit (Sigma-Aldrich, MO, USA) according to the manufacturer's instructions, and examined by phase-contrast microscopy. HEK 293T cells transfected with the FL-HE gene of Nig-2-8, the Aichi strain (LIC), and the Trc-HE gene of the Aichi strain (TC) of BToV using TransIT® 293 were examined to detect acetylesterase activity. After 48 h of transfection, HEK 293T cells were stained to detect acetylesterase activity of the HE protein with the same kit.

Detection of HE protein by Western blotting

Subconfluent HEK 293T cells cultured in 24-well plates were transfected with the pCAGGS vector harboring the FL-HE gene of the Aichi strain (LIC), the Trc-HE gene of the Aichi strain (TC), the FL-HE gene of the Aichi (LIC) strain tagged with HA (FL-HE+HA), and the Trc-HE gene of Aichi (TC) tagged with HA (Trc-HE+HA). Mock transfection was done with solution containing no vector. After 48 h of transfection, cell lysates were analyzed with an anti-HA antibody (mouse monoclonal antibody [MAb] clone 12CA5; Boehringer, Mannheim, Germany) (Taguchi and Matsuyama, 2002) and anti-mouse IgG conjugated with horseradish peroxidase (Rockland, Gilbertsville, PA, USA), as described previously (Ujike et al., 2016). Protein bands were visualized with enhanced

chemiluminescence prime Western blotting detection reagent (GE Healthcare, Tokyo, Japan) with an LAS-4000 imager (Fujifilm, Tokyo, Japan).

IFN sensitivity assay

The relative sensitivity of the various BToVs to exogenously added human IFN-α (hIFN-α) and hIFN-β was measured in HRT-18 cells. Briefly, cells cultured in 24-well plates to 80–90% confluence were incubated with hIFN-α and hIFN-β at various concentrations. After 24 h of incubation at 37°C, HRT-18 cells were infected with various BToVs at an MOI of 0.05 and incubated in DMEM containing IFN for another 36 h at 37°C. Virus titers in the supernatant were determined by $TCID_{50}$. The Sindbis virus was used as an IFN-sensitive control virus.

Effect of IFN on Sindbis virus infection in HE protein expressing HEK 293T cells

HEK 293T cells cultured in 24-well plates were transfected with the pCAGGS expression vector harboring the FL-HE (LIC) and Trc-HE (TC), FL-HE+HA, and Trc-HE+HA genes of Aichi strain at 1μg/well by TransIT® 293. Mock transfection was done with solution containing no vector. After 48 h of transfection, HEK 293T cells were treated with IFNs for 24 h, or not treated, and infected with the Sindbis virus at an MOI of 0.05. The virus titers were determined 36 h pi in 96-well plates by $TCID_{50}$ using Vero cells to determine the IFN effect.

To quantify the amount of mRNA for IFN, we performed real-time PCR. HEK 293T cells cultured in 24-well plates were transfected with pCAGGS expression vector harboring the FL-HE (LIC) and Trc-HE (TC) genes of the Aichi strain using TransIT® 293. Mock

transfection was performed with solution containing no vector. Then, 48 h after transfection, the cells were directly lysed with TRI Reagent (Zymo Research, CA, USA) at 300 μl/well and tRNA (Roche Diagnostics GmbH, Mannheim, Germany) was added at 30 μl (5 μg/ml) per well. Then the samples were incubated for 5 min at room temperature. Chloroform was added (60 μl), and the samples were mixed vigorously, left for 5 min at room temperature, and spun at 12000 g for 10 min at 4°C. The aqueous phase was transferred to a fresh tube. Then, 150 μl isopropanol was added for 5 min and samples were spun at 12000 g for 8 min at 4°C. The white RNA pellet was washed with 300 μl 75% ethanol and treated with DNase I (Takara Bio Inc., Shiga, Japan) to remove any contaminating DNA according to the manufacturer's instructions. The absence of contaminating DNA was confirmed in control experiments in which the reverse transcriptase enzyme was omitted.

The levels of hIFN-α mRNA expression in transfected cells were quantified using a StepOnePlus™ Real Time PCR system (Applied Biosystems, Foster City, CA, USA) and analyzed with StepOne™ Software v2.3. A Thunderbird® SYBR® qPCR mix kit (Toyobo, Osaka, Japan) was used for real-time monitoring of amplification in accordance with the manufacturer's instructions. Each reaction was performed in triplicate. The relative mRNA levels were normalized relative to GAPDH. The primer sequences were as follows: IFN-α forward (5' GACTCCATCTTGGCTGTGA 3'), IFN-α reverse (5' TGATTTCTGCTCTGACAACCT 3'), GAPDH forward (5' GAGTCAACGGATTTGGTCGT 3') GAPDH reverse (5' GACAAGCTTCCCGTTCTCAG 3') (Colantonio et al., 2011). PCR conditions were as follows: denaturation at 95°C for 1 min followed by 40 cycles of 95°C for 15 s and 60°C

for 45 s. A standard curve for RT products was generated with twofold serial dilutions of cDNA prepared from plasmid-transfected HEK 293T cells.

Statistical analysis

Statistical analysis was performed using SPSS software (ver. 11.5; SPSS Inc., Chicago, IL, USA). The IFN sensitivity of Nig-3-3, Nig-3-8, Nig-2-8, Aichi and Sindbis virus were calculated by one-way analysis of variance (ANOVA). Differences were considered to be statistically significant at $p < 0.05$.

3-3. Results

Esterase activity of the HE proteins

To examine whether HE retains esterase activity, the Nig-3-3 strain with FL-HE, as well as the Nig-3-8 and Aichi strains with the Trc-HE protein, were tested for acetylesterase activity with the substrate α-naphthyl acetate 24 h pi. Esterase activity, as demonstrated by widespread distribution of garnet-colored cells, was observed in Nig-3-3-infected HRT-18 cells but not in Nig-3-8- and Aichi (TC)-infected cells, as well as mock-infected cells (Fig. 3-1a). We further examined whether the esterase activity of the virus was a result of the HE protein. HEK 293T cells transfected with FL-HE from Nig-2-8 or Aichi-LIC, or Trc-HE from Aichi-TC were tested for esterase activity. The presence of garnet-colored cells indicated esterase activity in FL-HE expressing HEK 293T cells but not in cells transfected with the Trc-HE gene or mock-transfected HEK 293T cells (Fig. 3-1b). These results strongly suggest that the FL-HE protein from Nig-3-3 retained esterase activity, but that the esterase activity of the Trc-HE protein was lost.

Effect of IFN on Nig-3-3 (HE+) and Nig-3-8 (HE-) replication

Although the HE protein may hinder viral growth in cultured cells, the HE protein likely has an important biological function for viral growth in animals and possibly for viral pathogenesis in the host, since specimens isolated from diseased cattle contain the virus with the HE protein. One possibility is that the HE protein provides anti-innate immunity, which has been proven in a number of virus-encoded proteins (Cruz et al., 2011; Dedeurwaerder et al., 2014; Kamitani et al., 2006; Koetzner et al., 2010; Wang et al., 2009; Ye et al., 2007). We examined whether HE affects viral resistance to IFN, which was evaluated by investigating the effect of hIFN-α on Nig-3-3 and Nig-3-8 growth in HRT-18 cells. The Sindbis virus was used as an IFN-sensitive control virus. HRT-18 cells were infected with the three viruses at an MOI of 0.05 and virus growth was compared in the presence or absence of IFN-α. Although no significant virus reduction was detected with Nig-3-3 infection, there was a significant reduction of Nig-3-8 infection 36 h pi. Following treatment with 10 and 100 IU/ml IFN-α, we observed a statistically significant decrease in infection compared with the infection in the absence of IFN ($p < 0.05$ and $p < 0.01$ with 10 and 100 IU/ml, respectively) (Fig. 3-2a). Furthermore, we examined the effect of 100 IU/ml of IFN on virus infection at 12, 24, and 36 h pi. As shown in Fig. 3-2b, the titer of Nig-3-8 was significantly reduced ($p < 0.05$), though no significant difference was observed in Nig-3-3-infected cells. These results indicated again that Nig-3-8 is sensitive to IFN-α, while Nig-3-3 is not. In both experiments, infection with the Sindbis virus was drastically suppressed by IFN treatment, consistent with previous reports (Overall et al., 1980; Petrillo-Peixoto et al., 1980; Ryman et al., 2000). These findings suggest that HE functions

as an IFN antagonist. To confirm this, we examined the anti-IFN-α activity of the Aichi (HE-) and Nig-2 (HE+) BToV strains. Anti-IFN activity of these viruses was examined using the same procedure as for Nig-3-3 and Nig-3-8. As shown in Fig. 3-2c, the infection of Nig-2-8 (HE+) was not reduced by IFN-α treatment, but Aichi (HE-) infection was significantly suppressed at 10 and 100 IU/ml IFN-α ($p < 0.05$ and $p < 0.01$, respectively). These findings strengthen the notion that HE is involved in anti-IFN-α activity.

To examine the effect of IFN-β on Nig-3-3 and Nig-3-8 infection, HRT-18 cells were treated with various concentrations of IFN-β and infected with Nig-3-3 (HE+) or Nig-3-8 (HE-), similar to the IFN-α experiment. IFN-β treatment had no significant anti-viral effect on Nig-3-3 or Nig-3-8 infection, whereas there was a significant ($p < 0.05$) reduction in the replication of the Sindbis virus following IFN-β treatment (Fig. 3-2d). These results suggest that HE works as an IFN-α antagonist, and IFN-β failed to suppress the growth of both HE+ and HE- viruses.

Effect of HE expression on the anti-viral effect of IFN

We examined whether HE functions as an IFN-α antagonist using the Sindbis virus. We expressed the FL-HE and Trc-HE protein of Aichi with or without the HA tag by transfection and HE expression was confirmed by Western blotting (Fig. 3-3a). We detected the expected protein bands of FL-HE+HA and Trc-HE+HA with the anti-HA antibody. The molecular weights of FL-HE+HA and Trc-HE+HA were approximately 55 kDa and 20 kDa, respectively. We failed to detect the FL-HE and Trc-HE proteins without the HA tag because HE-specific antibodies are not currently available. Then we examined the effect of IFN-α on the growth of the Sindbis virus in HEK 293T cells expressing FL-HE,

Trc-HE protein, and also in mock transfected cells by comparing virus titers in the culture media 36 h pi. We found that infection with the Sindbis virus was not reduced by IFN-α treatment when FL-HE was expressed, while it was reduced when Trc-HE was expressed and also in mock transfected cells (Fig. 3-3b). These results clearly indicate that HE functions as an IFN-α antagonist.

As shown in Fig. 3-3b, IFN-α suppressed Sindbis virus infection in HEK 293T cells expressing Trc-HE but not in FL-HE-expressing cells, suggesting that HE is an IFN-α antagonist. However, it was also possible that HEK 293T cells transfected with a vector harboring FL-HE (LIC) produced higher levels of IFN-α than those transfected with a vector containing Trc-HE (TC) and the cells expressing higher levels of IFN might be less responsive to extrinsic IFN treatment. Therefore, we measured the amount of IFN-α in the supernatants of FL-HE, Trc-HE, and mock-transfected HEK 293T cells using an IFN-α bioassay with Sindbis virus. However, we did not detect any IFN activity in the culture fluids of transfected cells (data not shown). Next, we compared the IFN-α mRNA levels among the cells described above. We transfected HEK 293T cells with pCAGGS expression vector harboring the FL-HE (LIC) or Trc-HE (TC) gene of the Aichi strain and mock transfectants. Forty-eight hours later, we examined the levels of IFN-α mRNA by real-time PCR. There were no significant differences among cells transfected with vector containing FL-HE or Trc-HE (Fig. 3-3c). However, the level of IFN-α mRNA was six fold higher in transfected cells than in mock-transfected control cells (Fig. 3-3c). These results suggest that the anti-IFN activity triggered by transfection with a vector containing the FL-HE gene was not due to the higher level of IFN produced as a result of transfection but rather was more likely due to the anti-IFN activity of HE protein expressed by transfection.

The slightly higher replication of Sindbis virus in mock-transfected cells compared to cells transfected with vectors containing the HE gene with or without IFN-α treatment (Fig. 3-3b) was likely due to the lower level of IFN-α production in mock-transfected cells as shown in Fig. 3-3c.

3-4. Discussion

The FL-HE protein derived from Nig-3-3 and the Aichi (LIC) manipulated gene exhibited esterase activity in infected cells; however, esterase activity was not detected in cells infected with BToV with Trc-HE (Nig-3-8 and Aichi strains-TC). Since the active site for esterase is located in amino acids 38–142 and 285–350 of the HE protein (Langereis et al., 2009), the HE proteins produced from the Trc-HE genes are soluble and do not contain all of the active regions and may lack esterase activity. These short and soluble HE proteins were not detected by IIF, possibly because of the lack of antibodies against HE with the C-terminal deletion. Expression of FL-HE and Trc-HE with the HA tag were successfully recognized by Western blot with the anti-HA antibody.

Innate immunity is the first reaction of the animal defense mechanism following viral infection. CoVs encode a number of structural and nonstructural proteins within their genome that act as IFN antagonists (Cruz et al., 2011; Dedeurwaerder et al., 2014; Kamitani et al., 2006; Koetzner et al., 2010; Wang et al., 2009; Ye et al., 2007). In the present study, we examined the sensitivity of the Nig-3-3 and Nig-3-8 virus strains to IFN and found that Nig-3-3 was more resistant to IFN-α than Nig-3-8, but IFN-β did not reduce the infection of either viruses, suggesting that HE functions as an IFN-α antagonist. Anti-IFN activity of HE is also supported by the observation that infection of Nig-2 with HE is

not affected by IFN-α, but infection with the Aichi virus without HE is reduced by IFN-α treatment.

The IFN resistance of each virus may be affected by proteins other than HE, since the viruses used in the IFN experiments may have different backgrounds outside the HE gene. Thus, we examined HE expression and found that HEK 293T cells expressing HE exhibited similar Sindbis virus infection in the presence and absence of IFN-α, while virus growth was suppressed in HE-negative HEK 293T cells. These results strongly suggest that the HE protein is responsible for the anti-IFN activity of BToVs.

Most of the viral proteins that exhibit anti-IFN activity are not membrane proteins (Cruz et al., 2011; Dedeurwaerder et al., 2014; Kamitani et al., 2006; Koetzner et al., 2010; Wang et al., 2009; Ye et al., 2007). However, some membrane proteins, such as the M protein of Middle East respiratory syndrome coronavirus (Yang et al., 2013) and NS4B protein of flaviviruses, predicted to be a membrane protein, also act as IFN antagonists (Muñoz-jordán et al., 2005). This suggests that membrane proteins such as HE may function as anti-IFN factors through mechanisms other than non-membrane proteins. Ongoing studies are evaluating how HE functions as an anti-IFN factor, which could elucidate a new pathway for IFN-mediated resistance.

Our results show that HE functions as an IFN antagonist, and this is in agreement with the observation that the HE protein exists in viruses in clinical specimens, but is lost after passage in cell culture. IFN is an important mediator of innate immunity following virus infection in animals and acute infection normally produces high levels of IFN. However, the amount of IFN in HRT-18 cells infected with BToV is below the detection level (data not shown).

3-5. Conclusion

It was demonstrated in the chapter 2 that the HE protein has a negative effect on BToV growth in cell culture. In this chapter, studies were performed whether HE has something to do with the innate immunity, since BToVs isolated from clinical specimens always retain HE, suggesting that HE is essential for growth of the virus in animals. It was shown in this chapter that, HE protein acts as an IFN-α antagonist in innate immunity, strongly suggesting that HE plays an important role in the pathogenesis of BToV infection.

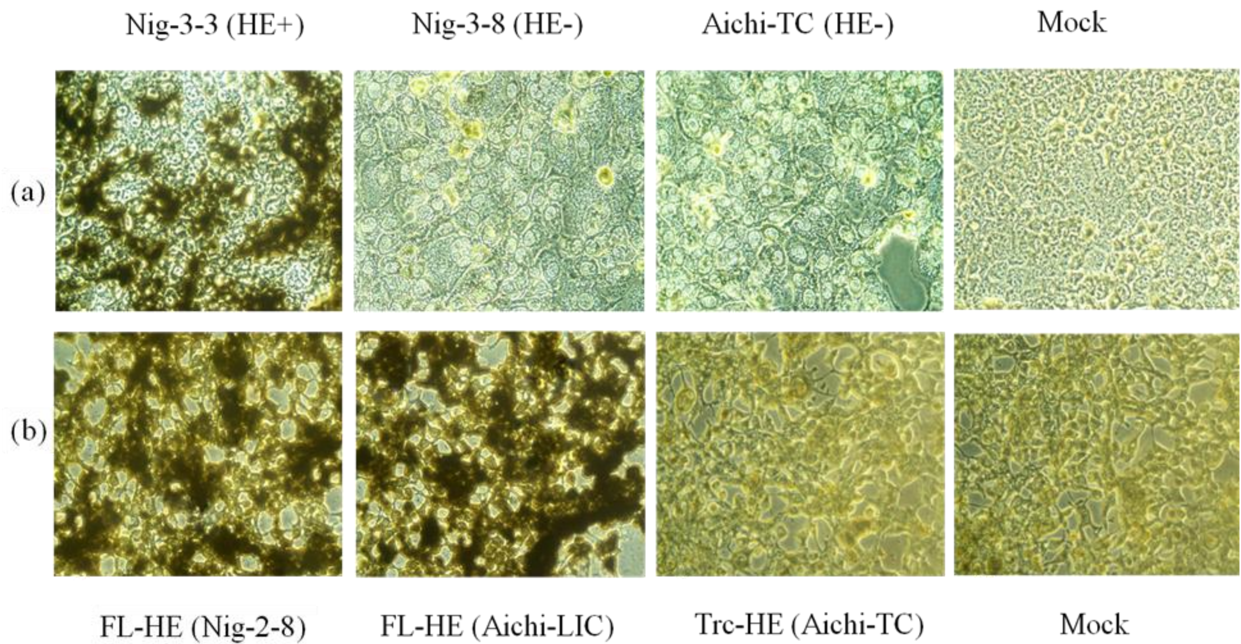

Figure 3-1: Esterase activity of BoTV HE proteins. (a) HRT-18 cells infected with Nig-3-3, Nig-3-8, or the Aichi (TC) strain were tested for acetylesterase activity with the substrate α-naphthyl acetate. Mock-infected cells were used as a negative control. (b) Human embryonic kidney 293T (HEK 293T) cells transfected with Nig-2-8, Aichi (LIC), or Aichi (TC) HE gene were tested for acetylesterase activity, as described above. Mock-transfected cells were used as a negative control.

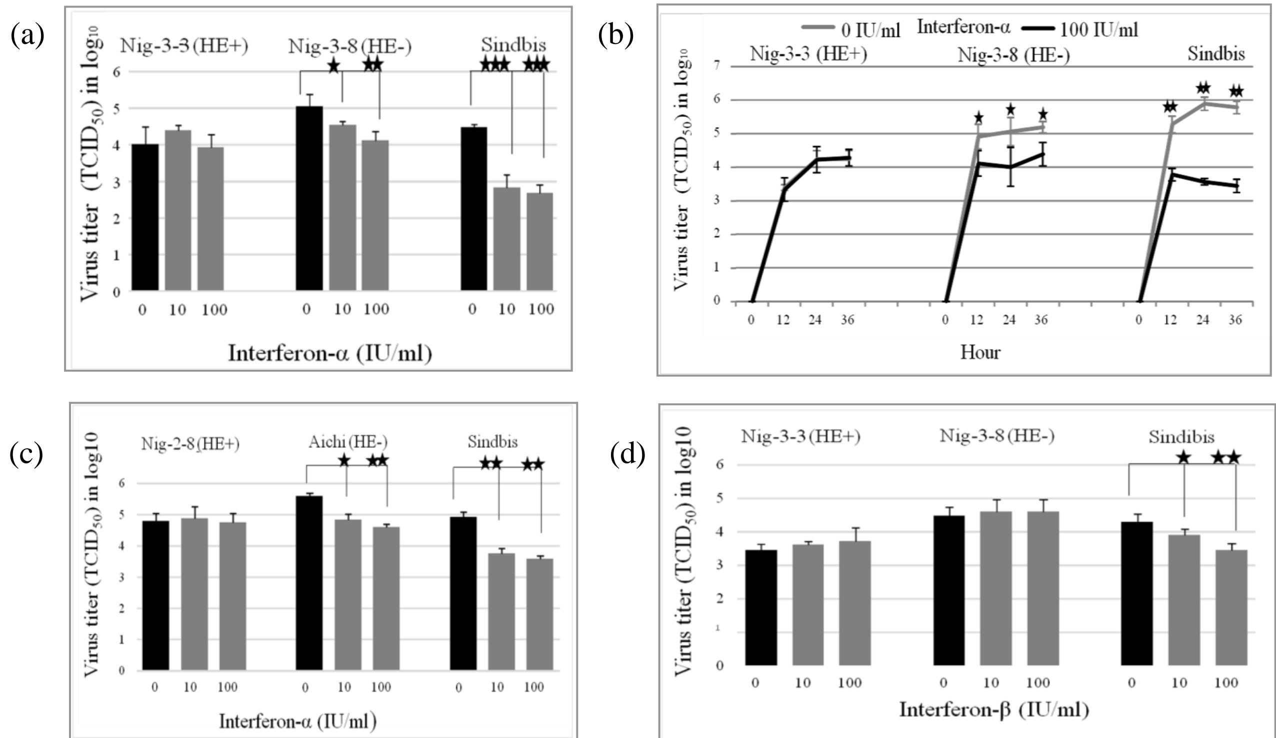

Figure 3-2: Effect of interferon (IFN)-α on the infection of Nig-3-3 (HE+) and Nig-3-8 (HE-). (a) HRT-18 cells in 24-well plates were incubated with human IFN-α (hIFN-α) at 0 IU, 10 IU and 100 IU/ml and incubated at 37°C for 24 h. The cells were infected with Nig-3-3 (HE+), Nig-3-8 (HE-), or the Sindbis virus at an MOI of 0.05 and virus titers in the culture media were examined 36 h pi. (b) HRT-18 cells were treated with 100 IU/ml hIFN-α for 24 h and infected with the Nig-3-3, Nig-3-8, or Sindbis viruses. Virus titers in the culture media were examined at 12, 24, and 36 h pi. (c) HRT-18 cells were treated with 0, 10, and 100 IU/ml hIFN-α for 24 h and infected with the Nig-2-8 (HE+), Aichi (HE-), or Sindbis viruses. Virus titers in the culture media were examined 36 h pi. (d) HRT-18 cells in 24-well plates were incubated with hIFN-β of various concentrations and infected with BToVs, as well as the Sindbis virus. Virus titers were examined 24 h pi. Titers are

expressed as the mean ± standard deviation (SD) of four replicates. p values were calculated by one-way ANOVA. Significant differences are indicated with one ($p < 0.05$), two ($p < 0.01$), or three asterisks ($p < 0.001$).

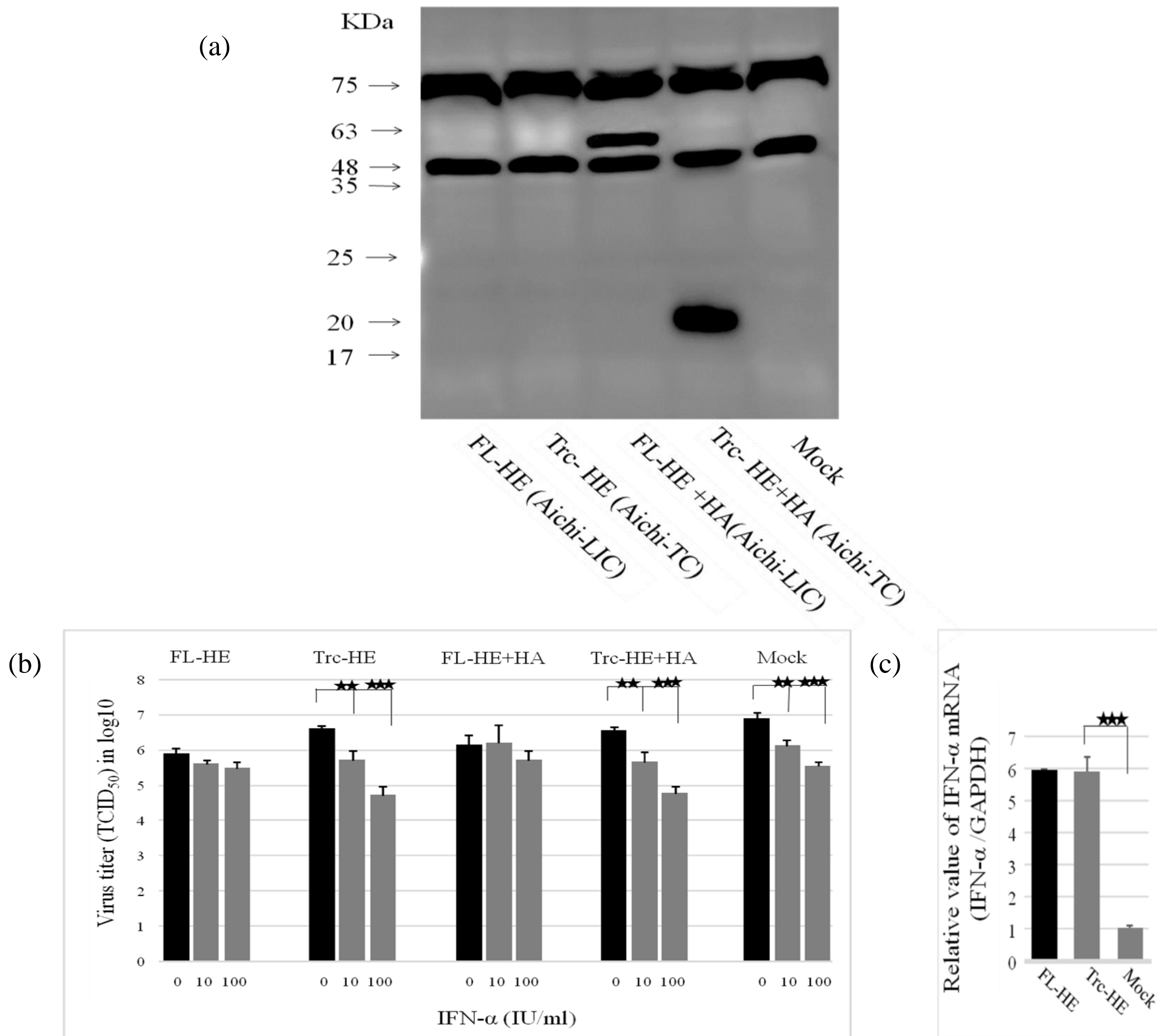

Figure 3-3: Effect of HE expression in HEK 293T cells on hIFN-α activity. (a) Western blot analysis of HE in HEK 293T cells transfected with FL-HE, Trc-HE, FL-HE+HA, and Trc-HE+HA genes, as well as mock transfection with solution containing no vector. Proteins were detected with anti-HE and anti-HA antibodies. (b) Effect of different concentrations of hIFN-α on Sindbis virus infection. HEK 293T cells transfected with FL-HE, Trc-HE, FL-HE+HA, Trc-HE+HA, and mock were treated with hIFN-α at 0, 10, and

100 IU/ml. After 24 h, the cells were infected with the Sindbis virus and virus titers in the culture media 36 h pi were determined using Vero cells. Mock transfection was done with solution containing no vector. (c) Quantification of mRNA expression levels of hIFN-α in transfected cells. HEK 293T cells were transfected with expression vector harboring FL-HE and Trc-HE genes. Mock transfection was done with solution containing no vector. Titers are expressed as the mean ± SD of three replicates. Significant differences are indicated with one ($p < 0.05$), two ($p < 0.01$), or three asterisks ($p < 0.001$).

Summary

Coronaviruses (CoVs) cause a wide range of diseases in farm and domestic animals, some of which are a threat to the farming industry and has serious effect on the economy. Economically important CoVs of farm animals include porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), bovine torovirus (BToV) and bovine coronavirus (BCoV), which result in enteric infection causing diarrhea in young animals. In 2013, a huge porcine epidemic diarrhea (PED) outbreak has occurred in Japan after a period of 7 years without any outbreak, which causes high morbidity and mortality in young piglets. On the other hand, BToV causes mild to moderate diarrhea in claves. BToV with the full length hemagglutinin-esterase (HE) gene was always isolated from diarrheal feces; however, the viruses lost the HE protein as a result of mutation of the HE gene following several passages in cultured cells. These findings suggest that the HE protein is important for replication and pathogenesis in animals, but is not essential for replication in cell culture. In the present study, phylogenetic and antigenic characterization of newly isolated PEDV in Japan is described. On the other hand, biological characterization of the HE protein of BToV focusing on anti-interferon (IFN) activity is examined.

1. Phylogenetic and antigenic characterization of newly isolated PEDV in Japan

PEDV causes severe diarrhea and dehydration, which usually lead to high morbidity and mortality, especially in piglets. Though vaccination program has been performed in pig farms throughout the whole Japan, PED has re-emerged. In the first chapter, we describe the isolation of new PEDV strains from recent outbreaks in Japan and the phylogenetic relatedness of the newly isolated strains with PEDVs circulating worldwide. Furthermore,

we attempted to reveal the antigenic relationship among the new strains and vaccine strain. To evaluate the mechanism by which a large outbreak of PED occurred in Japan, where the majority of sows are vaccinated, we isolated two new strains of PEDV from the intestines of piglets and found that they are more similar to US isolates (group II PEDV) than to the Japanese vaccine strain (group I PEDV). We compared the antigenicity of the vaccine type strain and newly isolated strains by means of a neutralization test using sera from a number of pigs from various farms; the results revealed that they are antigenically similar. This is the first report of the similarity of group I and II viruses using sera from individual pigs vaccinated with group I virus. These data suggest that the large outbreak of PED in Japan may not be attributed to inefficient vaccination but may be due to the extremely high virulence of the newly appearing viruses. It was also suggested in the literature that the poor biosecurity is one of the major causes of huge PED outbreak worldwide.

2. Biological characterization of the HE protein of BToV

BToV, which causes diarrhea in calves, contains the HE protein on the viral envelope when isolated from the host, although HE is often lost from the virion after multiple passages in cultured cells. This suggests that HE protein may be important for replication or pathogenesis in infected animals, but is not indispensable for the replication in cultured cells. In the second chapter, we explored the biological functions of the HE protein. We isolated the BToV Niigata-3 (Nig-3) from diarrheal specimen of the cattle. Nig-3-3 with HE (HE+) from the viruses with 3rd passage level and Nig-3-8 without HE (HE-) at 8th passage level were obtained by cloning using human rectal tumor (HRT-18) cells. The mutation has arisen in the HE gene of Nig-3, which makes a stop codon at the

amino acid number 109 of HE gene. We cloned Nig-3-3 and Nig-3-8 using HRT-18 cells and compared their growth in cultured cells. Nig-3-8 (HE-) grew more efficiently than Nig-3-3 (HE+), suggesting the possibility that HE inhibits BToV growth in cultured cells. Human embryonic kidney 293T (HEK 293T) cells expressing the HE protein also suppressed the infection of the Sindbis virus. These results suggest that HE protein is an obstacle for the growth of various viruses in cultured cells.

3. Anti-IFN activity of HE protein of BToV

In the previous chapter, it was revealed that HE protein is not essential for the replication but is an obstacle for virus replication in cultured cells. Although the HE protein may hinder viral growth in cultured cells, the HE protein likely has an important biological function for viral growth in animals and possibly for viral pathogenesis in the host, since specimens isolated from diseased cattle contain the virus with the HE protein. In this chapter, we explored the anti-IFN activity of the HE protein of BToV using IFN-α and IFN-β on the growth of HE+ and HE- viruses. IFN-α depressed the growth of HE- viruses, but not that of HE+ ones, whereas IFN-β has no influence on their growth. HE protein expressed in HEK 293T cells were examined whether it acts as IFN-α antagonist by using Sindbis virus. The infection of Sindbis virus in HEK 293T cells expressing HE protein was not affected by the IFN-α treatment, though the infection was depressed in cells expressing the truncated HE protein after treatment with IFN-α. This indicated that HE protein acts as an IFN-α antagonist. These results collectively suggest that HE plays an important role in the pathogenesis in BToV infections as an IFN antagonist to innate immunity.

As studied above, vaccination with group II virus strains as well as good biosecurity could assist to protect pigs from large outbreaks of PED. On the other hand, we found that HE protein suppresses BToV infection in cultured cells. More importantly, we showed that HE functions as an IFN antagonist, which indicates that HE is an important pathogenic factor for the infection in animals. The studies on two different CoVs causing enteric diseases shown in this thesis will contribute to the development of anti-viral strategies as well as delineation of the pathogenic mechanisms of the CoV infection.

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Abbreviations

BCoV: bovine coronavirus

BToV: Bovine torovirus

cDNA: Complimentary Deoxyribonucleic Acid

CoV: Coronavirus

CoVs: Coronaviruses

CPE: Cytopathic effect

DMEM: Dulbecco's modified Eagle's medium

E: Envelope

FCoV: Feline coronavirus

FCS: Fetal calf serum

FECV: Feline enteric coronavirus

FIPV: Feline Infectious Peritonitis virus

FITC: Fluorescence isothiocyanate

FL: Full length

HE: Hemagglutinin-Esterase

HEK: Human embryonic kidney

HRT: Human Rectal Tumor

IBV: infectious bronchitis virus

IFN: Interferon

IU: International Unit

kb: Kilo base

M: Membrane

MHV: Mouse hepatitis virus

MHV: Mouse hepatitis virus

MOI: Multiplicity of Infection

N: Nucleocapsid

Nig: Niigata

nm: Nanometer

PBS: Phosphate-buffered saline

PBS: phosphate-buffered saline

PED: Porcine epidemic diarrhea

PEDV: Porcine epidemic diarrhea virus

Pi: Post inoculation

RT-PCR: reverse transcriptase polymerase chain reaction

S: Spike

TCID: Tissue culture infective dose

TGEV: Transmissible gastroenteritis virus

TPB: Tryptose phosphate broth

Trc: Truncated

US: United States

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