Studies on mechanisms of evolution of coronaviruses

コロナウイルスの進化メカニズムの解析

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September 2014

Index

1. General introduction

1.1. History of feline and ferret coronaviruses

1.1.1 Feline coronavirus (FCoV)

Feline infectious peritonitis (FIP) was first described in 1963 as an important disease of cat (Holzworth, 1963). In 1968, virus particles were observed in the lesional tissue of experimentally infected cats (Zook *et al*., 1968) by electro-microscopic study. In 1978, Pedersen *et al*. (1978) reported the close genetic relationship of FIP virus (FIPV) with coronavirus (CoV) of dog and pig. In 1984, it was reported that FIPV was divided into two serotypes, feline CoV (FCoV)-like virus and canine CoV (CCoV)-like virus (Pedersen *et al*., 1984a). Now, FCoV-like virus is designated as type I FCoV and CCoV-like virus is designated as type II FCoV. Some reports indicated that type II FCoV emerged by double recombination between type I FCoV and CCoV (Motokawa *et al.*, 1996, Herrewegh *et al.*, 1998)

FIP is one of the most important diseases in cat, but there was no report on FIP until the late 1950's, indicating that FIP appeared suddenly in the late 1950's. However, it is still unknown the reason for sudden emergence of FIP.

1.1.2. Ferret coronavirus (FRCoV)

In 2000, it was reported that novel coronavirus infected domestic ferret (*Mustela putorius*) in the United States and the virus was a causative agent of epizootic

catarrhal enteritis (ECE) (Williams *et al*., 2000). This coronavirus was designated as ferret coronavirus (FRCoV). In 2006, FIP-like disease in domestic ferret was first reported and the causative agent was also FRCoV (Martinez *et al*., 2006). Now, FRCoV inducing ECE was designed as ferret enteric coronavirus (FRECV) and FRCoV inducing FIP-like disease was designated as ferret systemic coronavirus (FRSCV). In 2010, FRCoV could be genetically divided into two types, I and II, by the difference of 3'-terminal of spike gene and FRSCV and FRECV belonged to type I and type II FRCoV, respectively (Wise *et al*., 2010). However, type I FRCoV was found from many rectal swabs of healthy ferrets in the Netherland, indicating that almost type I FRCoV did not cause severe disease, but only some variants, FRSCV, caused systemic disease like FIP (Provacia *et al*., 2011).

1.2. Virus properties

CoVs are enveloped and have a large single-stranded, positive-sense RNA (26-32kb). The morphology of CoV is called "crown-like", because spike (S) protein is protuberant from virion. The size of viral particle is 120-160nm.

Both FCoV and FRCoV belong to order *Nidovirales*, family *Coronaviridae*, subfamily *Coronavirinae*, genus *Alphacoronavirus 1* (International Committee on

Taxonomy of Viruses (ICTV)). Especially, FCoV belongs to species *Alphacoronavirus 1* including CCoV, transmissible gastroenteritis coronavirus (TGEV) and porcine respiratory coronavirus (PRCoV). FRCoV also seems to belong to species alphacoronavirus-1, but has not been assigned, yet.

The 5' two-thirds of the CoV genome consists of two open reading frames (ORFs 1a and 1b) that encode a non-structural polyprotein, including RNA-dependent RNA polymerase (RdRp). The 3' one-third of the genome consists of ORFs encoding structural proteins, S, membrane (M), envelope (E) and nucleocapsid (N), and some non-structural proteins (nsp), 3a, 3b, 3c, 7a and 7b.

1.2.1. S protein

S protein is a class I viral fusion protein with a molecular mass of 180-205 kilodalton (kDa) (Bosch *et al.*, 2003, Olsen, 1993). S protein forms a trimer, projects from the surface of virions and plays important roles in entry step, interaction with viral receptor that existed on the surface of target cells and acceleration of fusion process (Olsen, 1993, de Groot *et al*., 1989, Weiss and Navas-Martin, 2005). Type II FCoV, CCoV and TGEV uses amino peptitase N (APN) as a receptor (Tresnan *et al*., 1996, Delmas *et al*., 1992, Hohdatsu *et al*., 1998), but receptor(s) of type I FCoV and FRCoV

have not been identified. S protein was divided into two domains, S1 and S2. S1 domain composes globular and possesses receptor binding domain (RBD). S2 domain composes membrane bound stalk and possesses fusion peptide (Olsen 1993, de Groot *et al*., 1989, Weiss and Navas-Martin, 2005, Pedersen, 2014). S protein of CoV showed high immunogenicity and induced virus-neutralizing (VN) antibody (Corapi *et al*., 1992, de Groot *et al*., 1989, Klepfer *et al*., 1995, Li *et al*., 2013). Especially, S1 domain tended to show high immunogenicity (Kida *et al*., 1999, Takano *et al*., 2011, Li *et al*., 2013). Because of the high immunogenicity, S protein was one of the candidates for vaccine development (Klepfer *et al*., 1995, Li *et al*., 2013).

1.2.2. Other proteins

N protein is the most abundant viral proteins and plays an important role in encapsidation and packaging of viral RNA. Since antibody against N protein was induced efficiently, N proteins of CoV were used as antigens for serological test.

E and M proteins were located on the surface of virion. There were some reports that M protein could induce the protective immune response (Fleming *et al.*, 1989, Vennema *et al*., 1991)

The 5' two-thirds of genome consists of two ORFs, 1a and 1b, that encode

non-structural polyprotein including RdRp, papain-like protease, helicase and 3' to 5' exoribonuclease (ExoN) (Perlman and Netland, 2009).

FCoV possesses other nsp, 3a, 3b, 3c, 7a and 7b, as accessory proteins. The functions of these proteins were still unclear. However, intact 3c protein was thought to be essential for replication in intestinal tract of cats (Pedersen *et al.*, 2009). Furthermore, there are some reports on relationship between pathogenicity of FCoV and nsp 3c and/or 7b (Vennema *et al*., 1992, 1998, Chang *et al*., 2010, Pedersen *et al*., 2009).

1.3. Epidemiology

1.3.1. FCoV

FCoV infection is worldwide and ubiquitous in domestic cats and wild felids, such as African lion, cheater, jaguar and so on (Colby and Low, 1970, Colly, 1973, Poelma *et al*., 1974, Fowler, 1978, Theobald *et al*., 1978, Juan-Salles *et al*., 1998., Pedersen, 1983, Watt *et al*., 1993). Some reports indicated that type I FCoV is predominant in the field (Hohdatsu *et al*., 1992, Vennema, 1999, Kummrow *et al*., 2005). Shiba *et al*. (2007) carried out serosurvey in Japan, showing that 63.3% of cats were sero-positive to FCoV and 98% of sero-positive cats possessed antibody to type I FCoV and only 2% did to type II FCoV. Cats infected with FCoV shed virus in the feces and

FCoV spread by fecal-oral routes in cat population (Pedersen *et al*., 2008).

Although FCoV infection is ubiquitous, the occurrence of FIP is rare and sporadic. FIP was the most serious cause of death of kittens, because FIP was diagnosed in 8.4% of dead kittens (17 of 203 cats) in the United Kingdom (Cave *et al*. 2002). Some risk factors for FIP were identified. One of the factors was multi-cat household (Addie *et al.,* 2009). Age is also an important risk factor and 70% of FIP cases occurred in cats less than 1 year old (Rohrer *et al*., 1993, Hartman, 2005). It was reported that some breeds, such as Abyssinians, Beggals, Himalayans, Rexes, Ragdolls and Birmians, had a high risk to become FIP (Pesteanu-Somogy *et al.*, 2006).

1.3.2. FRCoV

In 2000, the FRCoV was detected from ferrets with ECE in USA (Williams *et al*., 2000) and since then, the existence of FRCoV was reported from animals with two types of diseases, ECE and FIP-like disease in USA and Europe (Wise *et al*., 2006, Garner *et al*., 2008, Martinez *et al*., 2006, 2008, Graham *et al*., 2012). On the other hand, FRCoV was detected from 63% of rectal swabs of asymptomatic ferrets in the Netherland (Provacia *et al*., 2011).

In Japan, there was only one report that the domestic ferret became FIP-like

disease and pathologically diagnosed (Michimae *et al*., 2010). However, there was no epidemiological information on FRCoV in Japan.

1.4. Diseases and pathogenicities

1.4.1. FCoV

FCoVs are divided into two biotypes based on the pathogenicity in cats. One is feline enteric coronavirus (FECV) and another is FIPV. FECV infection is not severe, most of cats infected with FECV are asymptomatic and some kittens occasionally show enteritis. On the other hand, FIPV infection causes severe and lethal disease, FIP, in cat.

Clinical features of FIP were divided into two forms. One is wet (effusive) form and another is dry (non-effusive) form (Montali and Strandberg, 1972). The most common form of FIP is wet form (Pedersen, 2009). The cat with wet form of FIP shows the exudation in the abdomen and/or pleural cavity and inflammatory condition on visceral serosa. The exudation is yellow-tinged and mucinous fluid. Abdominal distension is the most common physical finding in wet form of FIP. Pleural effusion induces dyspnea to cats. Dry form of FIP was characterized by pyogenic granuloma at parenchymatous organs and there is no inflammatory exudation. Kidney and mesenteric lymph nodes frequently show the lesion and liver and hepatic lymph nodes do less

frequently. Involvement at central nervous system (CNS) and eyes are also frequent in cats with dry form of FIP and FIP is the most frequent cause of uveitis/chorioretintis in cats (Goodhead, 1996, Peiffer *et al*., 1991).

The target cell of FECV is enterocyte in intestine and that of FIPV is macrophage and monocyte. Therefore, the acquisition of macrophage tropism was an essential step to become FIPV from FECV. However, FECVs may be detected in blood (Meli *et al*., 2004) and this phenomenon make veterinarian difficult to diagnose dry form of FIP.

The genetic marker of FIPV, genetic basis for the difference in macrophage tropism between FECV and FIPV, is still unknown, but some candidates, the mutations in S protein, 3c and 7b, were suggested. Licitra *et al*. (2013) examined a furin cleavage site in S protein of 30 samples of FECV and 22 samples of FIPV, showing that all FECV had a conserved furin cleavage motif but most FIPV showed one or more substitution and that these substitutions modulated cleavage by furin. Chang *et al*. (2012) determined the full genome sequence of eleven strains of FIPV and eleven strains of FECV and found two significant amino acid differences in S2 protein between FIPV and FECV. Furthermore, it was reported that difference between FECV and FIPV might be due to functional mutation in 3c gene, because all FECV possessed intact 3c

gene but many FIPV did truncated 3c gene by mutation or deletion. Now, it was thought that intact 3c is essential for replication in gut (Chang *et al*., 2010, Pedersen *et al*., 2009). The function of 7b protein is still unknown. Some reports indicated that most of FIPV maintained intact 7b gene and FECV possessed truncated or mutated 7b gene (Vennema *et al*., 1992, 1998). However, Lin *et al.* (2009) reported that three strains of FIPV showed deletion in 7b gene. Hence, the relationship between virulence of FCoV and intact 7b gene is still unclear.

It was thought that cellular immunity was crucial for onset of FIP. The cellular immunity also plays an important role in determination of either dry or wet form of FIP. If the cellular immunity fails to be developed and the humoral immunity occurs, the cat will show wet form of FIP. If the cellular immunity is developed but weak, the cat will show dry form of FIP. If the cat develops sufficient level of cellular immunity, the cat might not become FIP (Pedersen, 1987, 2009, 2014, Vermeulen *et al*., 2013).

1.4.2. FRCoV

Ferrets with ECE show general clinical signs of lethargy, anorexia, and vomiting in addition to an ECE-specific clinical sign, foul-smelling green diarrhea with high levels of mucus. Although the morbidity is very high, the mortality rate is low

(<5%) (Murray *et al*., 2010).

FRSCV infection induces ferrets to be the disease like dry form of FIP. The ferrets with the FIP-like disease show the palpating mass in abdominal cavity by pyogenic granuloma at parenchymatous organs and then die. FRSCV was found in cytoplasma of macrophage in the lesion (Martinez *et al*., 2006).

1.**5. Diagnosis and treatment**

It is difficult for many veterinarians to diagnose FIP, especially dry form, because there was no test that can clearly diagnose FIP. Veterinarian should diagnose comprehensively using lots of information of cat, historical patient information, physical finding and laboratory abnormalities. The cat with FIP showed several abnormalities in hematological profiles. A mild to moderate non-responsive anemia that is typical finding of chronic disease is recognized. In white blood cells of FIP cats, absolute lymphopenia and neutrophilia were commonly observed (Pedersen, 1976, Paltrinieri *et al*., 1998, Sparkes *et al.*, 1991). A common finding of laboratory test is elevation of total serum protein that was induced by a rise in gamma globulin (Platrieri *et al*., 2001, Sparkes *et al*., 1994). Low albumin/globulin ratio (<0.8) is considered to be diagnostic for FIP (Addie *et al*., 2009). Increase of liver enzyme, urea and creatinine

depend on the degree and site of organ damage.

The effusion in abdominal and/or pleural cavity in FIP cats is valuable for diagnosis. When the fluid was pulled away using needle tip, a string will often be formed because the fluid is mucious. The effusion is yellow-tinged and mucinous fluid. The protein contents ranges from 3.9 to 9.8 mg/μl and cell counts rages from 1,600 to 25,000/μl (Pedersen, 2009). Gene detection from effusion by RT-PCR is useful for diagnosis of FIP and quantification by real-time RT-PCR shows high level of viral RNA.

There are many tests for detection of antibody to FCoV, such as indirect immunofluorescence assay (IFA), VN test and enzyme-linked immunosorbent assay (ELISA) (Barlough *et al*., 1982, Pedersen, 1976, Pratelli, 2008). Unfortunately, existence of antibody to FCoV does not prove to be FIP (Hartmann *et al*., 2003). However, there is no doubt that the cats with very high titer (>1,600) in IFA test are likely to be FIP and negative titer means non-FIP (Pedersen, 1976).

Because there is no effective treatment for FIP, most cases of FIP are fatal. Recently, cyclosporine A has been examined as one of candidates of treatment for FIP (Tanaka *et al*., 2012, 2013). However, the effect in natural case is still unknown. Feline interferon omega inhibits FIPV infection *in vitro* (Mochizuki *et al*., 1994), but this

treatment was ineffective in natural case of FIP (Ritz *et al*., 2007).

1.6. Prevent and control

There is only one vaccine available for FIP. The vaccine was made from temperature-sensitive type II FCoV and was inoculated intranasaly (Christianson *et al*., 1989, Gerber *et al*., 1990). However, the efficacy of the vaccine is in doubt (Fehr *et al*., 1997). Therefore, there was no efficient vaccine to prevent FIP.

Antibody-dependent enhancement of infection (ADE) of FIPV prevents the development of efficient vaccine. Briefly, antibody to FIPV induces efficient infection to macrophage via Fc receptor (Olsen *et al*., 1992). Vennema *et al*. (1990) constructed recombinant vaccinia virus expressing S protein and tested vaccine efficacy. After challenge of virulent FIPV, vaccinated cats were dead earlier than non-vaccinated cats.

Most important way to prevent FIP is decrease the risk factors, such as multi-cat household. It was reported that proper management could decrease the incidence of FIP in catteries (Pedersen *et al*., 1995).

1.7. Evolution and emergence of coronavirus

Recently, emerging CoV infections, such as severe acute respiratory syndrome

(SARS)-CoV and Middle-East respiratory syndrome (MERS)-CoV, have been big concern of public health. It has been believed that these viruses originated from bats and camels zoonosis. During transmission among natural host, viruses evolved by mutation and/or recombination. These mutations and recombination changed viral properties, such as host range and pathogenicity (Kocherhans *et al*., 2001, Vijgen *et al*., 2005, Sheahan *et al*., 2008).

There are three reasons for the frequent mutation and recombination of CCoV (Bolles *et al*., 2011). First, RdRp has low fidelity. The mutation rate approaches 2.0×10^{-6} mutations per site per round of replication (Eckerle *et al*., 2010). Second, there is a unique RNA replication mechanism using the transcription regulatory sequence (TRS) motif and that is known as the "copy choice" mechanism. It was thought that this unique mechanism induces homologous RNA recombination in CoVs (Pasternak *et al*., 2006, Lai *et al*., 1985). Third, CoV possesses the largest genome (26-32kb) among RNA viruses.

Since SARS-CoV occurred in 2003, research on CoV accelerated. However, experimental animals such as mouse and ferret were used in most studies on CoV evolution, but it seems to be not sufficient for analysis of the evolution of CoV because they are not natural hosts. Analysis of CoVs evolution should be carried out using their

natural hosts. In this thesis, we analyzed evolution of CoVs using natural hosts.

In Chapter 1, pathogenesis of type I FIPV with a large deletion in the 5'-terminal region of spike gene was analyzed. In Chapter 2, mechanism of emergence of pathogenic coronaviruses in cats was analyzed. In Chapter 3, two types of FRCoV in Japan were genetically characterized.

2. CHAPTER 1

Feline infectious peritonitis virus with a large deletion in the 5'- terminal region of spike gene retains its virulence for cats

2.1 Abstract

In this study, Japanese strain of type I FIPV, C3663, was found to have a large deletion of 735 bp within the gene encoding the S protein, with a deduced loss of 245 amino acids of the *N*-terminal region of the S protein. This deletion is similar to that observed in PRCoV when compared to TGEV, which correlates with reduced virulence. By analogy to PRCoV, we expected that the pathogenicity of C3663 may be attenuated in cats. However, two of four cats inoculated with C3663 died of FIP, and a third C3663-inoculated cat showed FIP lesions at 91 days after challenge. These results indicate that the 5'-terminal region of the S gene is not essential for the development of FIP.

2.2. Introduction

FIP is a progressive, systemic fatal disease in domestic and wild felids. The causative agent of FIP is FCoV belonging to the order *Nidovirales*, family *Coronaviridae*, subfamily *Coronavirinae*, genus *Alphacoronavirus,* species *Alphacoronavirus 1*. Other members of this species include CCoV, TGEV and PRCoV.

FCoVs are classified into two biotypes based on their pathogenicity in cats. One is FECV; the second is FIPV. FECV infection is asymptomatic in cats, occasionally causing enteritis in kittens, but FIPV infection causes severe and lethal disease in cats. However, there is no clear marker to distinguish between FIPVs and FECVs (Pedersen *et al.*, 1981; Pedersen, 1987, Kennedy *et al*., 2001).

FIPVs and FECVs are antigenically divided into two types (I and II) based on their reactivity with monoclonal antibodies (MAbs) raised against the S protein (Fiscus and Teramoto, 1987, Hohdatsu *et al.*, 1991b, 1992). This distinction relates to differences in the nucleotide sequence of the gene encoding the S protein (Motokawa *et al*., 1996, Herrewegh *et al*., 1998). There are also some differences in the biological characteristics between types I and II. Type II can grow well *in vitro*, while type I exhibits poor growth in cell culture (Pedersen *et al.*, 1984a). As a result, it has been difficult to isolate type I FIPVs from cats exhibiting FIP. In Japan, only three strains of

type I FIPV (C3663, KU-2, and Yayoi) have been isolated (Hayashi *et al.*, 1981, Hohdatsu *et al.*, 1991b, Mochizuki *et al.*, 1997). In this study, we describe the genetics and pathogenesis of a recent Japanese type I FIPV isolate (C3663).

2.3. Materials and methods

2.3.1. Cell

Felis catus whole fetus (fcwf)-4 cells (Jacobse-Geels and Horzinek, 1983) were maintained in 5% CO_2 at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, CA, USA) containing 10% (v/v) fetal calf serum (FCS; Hyclone Laboratories, UT, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, CA, USA).

2.3.2. Viruses

Type I FCoV strains C3663 and Yayoi were propagated in fcwf-4 cells. C3663 was isolated from a cat with FIP in Kagoshima in 1994 (Mochizuki *et al.*, 1997). Yayoi, which has been used as the Japanese prototype strain of type I FCoV, was isolated from a cat with FIP in Tokyo, originally by serial passage in the brain of suckling mice with subsequent adaptation to fcwf-4 cells (Hayashi *et al.*, 1981). C3663 and Yayoi were classified as type I FCoV by using FCoV type-specific MAbs, which were kindly provided by Dr. Hohdatsu (Hohdatsu *et al.*, 1991a, b).

2.3.3. Extraction of RNA

FCoVs were inoculated to fcwf-4 cells in 35-mm dishes (Sumitomo Bakelite,

Tokyo, Japan) and then incubated at 37 °C until a cytopathic effect (CPE) was observed. RNA was isolated from the infected cells using the RNeasy® Mini kit (Qiagen, Hilden, Germany).

2.3.4. Reverse transcription (RT)-PCR

cDNAs of C3663 were reverse-transcribed using oligo-dT M4 primer using TaKaRa RNA LA PCR™ kit (AMV) Ver 1.1 (Takara, Shiga, Japan). The reactions were carried out at 30 °C for 10 min, 42 °C for 30 min, and 70 °C for 15 min, using a Little Gene (Toyobo, Osaka, Japan) cycler. PCR amplifications of subgenomic RNA were performed using forward primer 52F (5'-ACT AGC CTT GTG CTA GAT TT-3') with one of the following reverse primers: 24991R (5'-TCA CCA AAA CCT ATA CAC AC-3'), 26218R(5'-CTT CAT TTT GTT TAG TTC AAA C-3'), M-R(5'- TAA GCC CAT CCT GTA GCA GT-3'), NR(5'-TAA TAA ATA CAG CGT GGA GGA AAA C-3') or M13 primer M4 (5'-GTT TTC CCA GTC ACG AC-3'). Each amplification was performed using an initial denaturation at 94 °C for 2 min, followed by 40 cycles at 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 2 min, and a final extension at 72 °C for 10 min. To detect subgenomic RNA encoding the S protein among the PCR products generated using primer pair 52F and 26218R, semi-nested PCR was carried out with primer pair 52F and S-R (5'- TGT TGR CAC TTR ATT CTA TT-3'; where R (purine) is a mixture of A and G) using KOD-plus-ver.2 (Toyobo), with an initial denaturation at 94 °C for 2 min, followed by 40 cycles at 98 °C for 10 sec, 55 °C for 30 sec, 68 °C for 2 min.

cDNAs of the Yayoi genome were generated by reverse transcription of RNA from infected cells with random 9-mer primers using TaKaRa RNA LA PCR™ kit

(AMV) Ver 1.1 (Takara). PCR amplification of sequences from the cDNA was performed using forward primer FIP 20390F (5'-TAA TGG CAA GCT ACT AAA CT-3') and reverse primer 24991R. The reaction was performed by an initial denaturation at 94 °C for 2 min, followed by 40 cycles at 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 5 min, and a final extension at 72 °C for 15 min.

To confirm the large deletion in the 5'-terminal region of S gene of C3663, we designed primers that specifically spanned the region in question and consisted of forward primer Yayoi S 46F (5'-GAT GCT CCT CAT GGT GTT AC-3') and reverse primer Yayoi S 1058R (5'-CTC AAA ACA TCT GCC GTG AC-3'). PCR from cDNAs was performed by an initial denaturation at 94 °C for 2 min, followed by 40 cycles at 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 2 min, and a final extension at 72 °C for 15 min.

For all PCR reactions, products were electrophoresed on agarose gels, stained with ethidium bromide, and visualized under ultraviolet light.

2.3.5. Cloning and sequencing

PCR products were cloned using the TOPO-TA cloning kit (Invitrogen) or purified using QIAquick® PCR Purification Kit (Qiagen) according to the instructions of the manufacturers. Plasmid DNAs containing genes of the C3663 strain were purified using the QIAprep® Spin Miniprep kit (Qiagen) for sequencing. Nucleotide sequences of the Yayoi S gene were determined directly from the PCR product. Sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, VA, USA), and the results were analyzed using ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems). The phylogenetic tree was constructed using the deduced amino

acid sequences of partial S proteins (278 -1467 amino acids) and MEGA5 software (Tamura *et al.*, 2011). For this phylogenetic analysis, we incorporated the S protein sequences from the following strains, as obtained from the databases: type I FCoV strains C1Je (GenBank accession number DQ848678) and NTU2/R/2003 (DQ160294); type II FCoV strains 79-1146 (DQ010921) and 79-1683 (X80799); type I CCoV strain 23/03 (AY307021); type II CCoV strain INSAVC-1 (D13096); TGEV strains virulent Purdue (DQ811789) and Miller M6 (DQ811785); and PRCoV strain RM4 (Z24675).

2.3.6. Animal experiment

To investigate the pathogenicity of C3663, four specific-pathogen free (SPF) cats (male, 6 months old; Liberty Research, NY, USA) were inoculated intra-orally with 10 ml of a viral solution containing 3.9×10^6 plaque forming unit (PFU) of C3663 per cat (No. 1-4). Clinical signs, body weights, and temperatures were recorded daily. Blood was collected every week under anesthesia with ketamine (Daiichi Sankyo, Tokyo, Japan). Serum amyloid A (SAA) was measured by Mitsubishi Chemical Medience (Tokyo, Japan). Cat sera were stocked at -80 °C until use for VN test and quantification of viral RNA using real-time RT-PCR method by canine-lab corporation (Tokyo, Japan). All animal experiments were approved by the ethics committee for animal experiments, Faculty of Agriculture, Yamaguchi University.

2.3.7. VN test

Complement in sera was inactivated by heating at 56 °C for 30 min before VN test. VN test was performed by 75% plaque-reduction neutralization test ($PRNT_{75}$). The inactivated sera were diluted with DMEM containing 2% FCS. C3663 was diluted to

approximately 1.0×10^3 PFU/ml with DMEM containing 2% FCS, mixed with equal volume of diluted sera or DMEM containing 2% FCS as control and incubated at 37 °C for 1 hr. Then 50 µl of the mixtures were inoculated onto fcwf-4 cell monolayers in 24-well plates (Sumitomo Bakelite) and adsorbed at 37 °C for 1 hr. After adsorption, the mixtures were removed and the infected cells were overlaid with 0.8% (w/v) agarose (Seaplaque® GTG agarose; Lonza, Basel, Switzerland) in DMEM containing 10% FCS. The infected cells were incubated at 37 °C until CPE was observed, at which point the cells were fixed with phosphate-buffered formalin. The fixed cells were stained with crystal violet and the number of plaques was counted. VN titers were expressed as the highest serum dilution showing 75% or more plaque reduction compared with the number of plaques in control wells (Shiba *et al.*, 2007).

2.4. Results

2.4.1. Nucleotide sequences of FCoV C3663

Sequence analysis and alignments for a total of 8,245 bp of C3663 were used to identify the following genes: S (3,669 bp; encoding a 1,222-residue protein), ORF 3a (213 bp; encoding a 70-residue protein), ORF3b (222 bp; encoding a 73-residue protein), ORF3c (714 bp; encoding a 237-residue protein), E (249 bp; encoding a 82-residue protein), M (786 bp; encoding a 261-residue protein), N (1,131 bp; encoding a 376-residue protein), ORF7a (306 bp; encoding a 101-residue protein), and ORF7b (621 bp; encoding a 206-residue protein). The nucleotide sequence of C3663 was deposited to the DNA Data Bank of Japan (DDBJ) as Accession No: AB535528.

Interestingly, the alignment of the S genes indicated that C3663 has a large deletion of 735 bp (capable of encoding 245 amino acids) at the 5'-terminus of the S

gene (Figure 2-1a). To confirm this deletion, RT-PCR was performed on RNA from C3663- or Yayoi-infected cells using forward primer Yayoi S 46F and reverse primer Yayoi S 1058R which span the corresponding domain of the S gene. PCR products of 278 bp and 1013 bp were obtained from C3663- and Yayoi-infected cells, respectively (Figure 2-1b).

A phylogenetic tree was constructed for the S proteins from the deduced amino acid sequences (residues 278 -1467) using MEGA5 software (Tamura *et al.*, 2011). The result showed that C3663 is a type I FCoV (Figure 2-1c). The S protein of C3663 exhibited 89-93% amino acid identity to those of other type I FCoVs and a much lower identity (<50%) to those of type II FCoVs or type II CCoVs.

2.4.2. Pathogenicity of C3663 in cats

The pathogenicity of C3663 with the large deletion was investigated by infecting SPF cats. Four SPF cats were inoculated intra-orally with C3663 (Nos. 1-4)

One cat (No. 2) was found dead on post-inoculation day (PID) 21 and a second cat (No. 3) was euthanized because of severe clinical signs on PID 37. The two remaining cats (Nos. 1 and 4) survived until PID 91 (the end of the observation period), at which time both were euthanized. At necropsy, one of the surviving cats (No. 1) exhibited FIP lesions but cat No.4 did not show any lesions. All cats showed clinical signs after inoculation. Anorexia was observed in three cats (Nos. 2, 3 and 4) and vomiting in two cats (Nos. 3 and 4). Lethargy and weight loss were observed in two cats (Nos. 2 and 3) (Figure 2-2a). Dyspnea was observed in one cat (No. 2) on PID 20 and 21. In cat No. 3, jaundice was observed on PID 33-37 and melena on PID 35 and 37. Furthermore, the concentration of SAA increased in all cats (Figure 2-2b). Cat Nos. 2, 3

and 4 showed a rapid increase during the acute phase. Cat No.1 intermittently showed increased levels of SAA and, despite a lack of clinical signs, exhibited a high concentration of 49.6 μg/ml at the end of our observation period. In all cats, viral RNA was detected in sera and VN activity to C3663 was observed (data not shown).

2.4.3. Postmortem examination

Following postmortem examination no lesions were found in cat No. 4. However, cat Nos. 1 and 2 showed pleural effusion and pyogranulomatous lesions in the pleural cavities. Pleural effusion and ascites were observed in cat No. 3. Lesions were seen in the kidneys, liver, stomach, intestine, pancreas, diaphragm and lung by macropathology, and confirmed as being pyogranulomatous by histopathological examination.

2.5. Discussion

In this study, it was found that C3663 has a large (735 bp) deletion in the 5' -terminus of the S gene. While C3663 has been adapted to propagation in tissue culture and the deletion may therefore have occurred *in vitro*, there is in fact evidence for naturally occurring FCoVs with similar deletions. The type I FCoV field variants UU16 (Accession No. FJ938058) and UU21 (HQ012369) have deletions of 705 and 792 bp, respectively, in the 5'-terminal region of their S genes, whereas field variant UU3 (FJ938061) has a small 126-bp deletion. It therefore appears that C3663-like FCoVs are present and maintained under field conditions.

To examine the pathogenicity of C3663, four SPF cats were intra-orally inoculated with C3663. Three of four SPF cats exhibited typical FIP during the

observation period and two died within one month of inoculation. Adaptation of FIPV to propagation in tissue culture often results in a loss of pathogenicity (Pedersen and Black, 1983, Pedersen and Floyd, 1985, Christianson *et al*., 1989, Kiss *et al*., 2004). Conceivably, the deletion in the S gene of C3663 might well have resulted in virus attenuation. In fact, a naturally occurring mutant of TGEV, PRCoV suffered a similar deletion in the S gene (Wesley *et al*., 1991) which caused a loss of virulence and a change in tissue tropism. PRCoV exhibits reduced sialic acid binding and hemagglutination activity (Schultze *et al*., 1996). It replicates efficiently in the respiratory tract, but, different from TGEV, does not replicate in the small intestine (Cox *et al*., 1990). In contrast, we demonstrate that FIPV C3663 is highly virulent. Our findings unequivocally show that large deletions in the 5'-terminal region of the FCoV S gene are tolerated without loss of pathogenicity.

In conclusion, we succeeded in efficiently inducing FIP in cats by inoculation with tissue culture adapted type I FIPV, C3663, using a natural route of infection (oral inoculation). Furthermore, we confirmed that the 5'-terminus of the S gene is not essential for the development of FIP.

2.6 Figure legends

Figure 2-1. Confirmation of the S gene deletion in Japanese strain of type I FIPV C3663. (a) Schema of the S genes of Yayoi and C3663. The box framed by a dotted line shows the deletion (735 bp) in the S gene of C3663 compared to Yayoi (AB695067). Arrowheads indicate the position and orientation of the primers, Yayoi S 46F and Yayoi S 1058R, used to confirm the deletion. (b) Confirmation of the deletion by RT-PCR using Yayoi S 46F and Yayoi S 1058R. (c) Phylogenetic tree using amino acid sequences of S proteins excluding the distinct *N*-terminal domains. The tree was constructed using MEGA5. Accession numbers of the sequences used are AB695067 (Yayoi), DQ848678 (C1Je), DQ160294 (NTU2/R/2003), DQ010921 (79-1146), X80799 (79-1683), AY307021 (23/03), D13096 (INSAVC-1), DQ811789 (TGEV-Purdue), DQ811785 (TGEV-Miller M6), and Z24675 (PRCoV-RM4).

Figure 2-2. (a) Normalized body weight among cats following inoculation with FIPV-C3663. Body weights were normalized using the weight on post-inoculation day (PID) 0 as 100%. (b) The concentration of SAA in cat sera. The limit of detection of SAA is < 2.5 μg/ml.

2.7 Figures

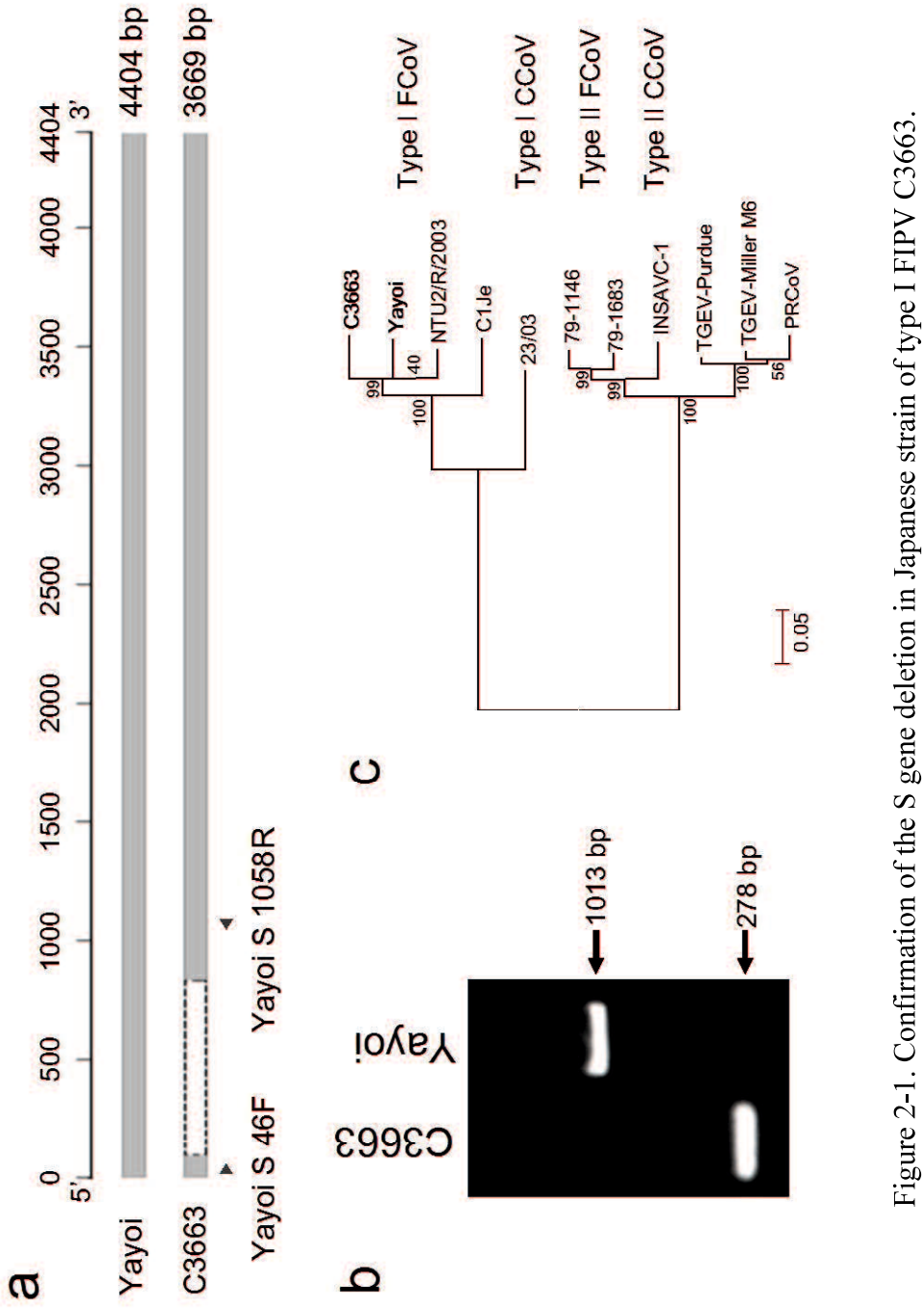

Figure 2-1. Confirmation of the S gene deletion in Japanese strain of type I FIPV C3663.

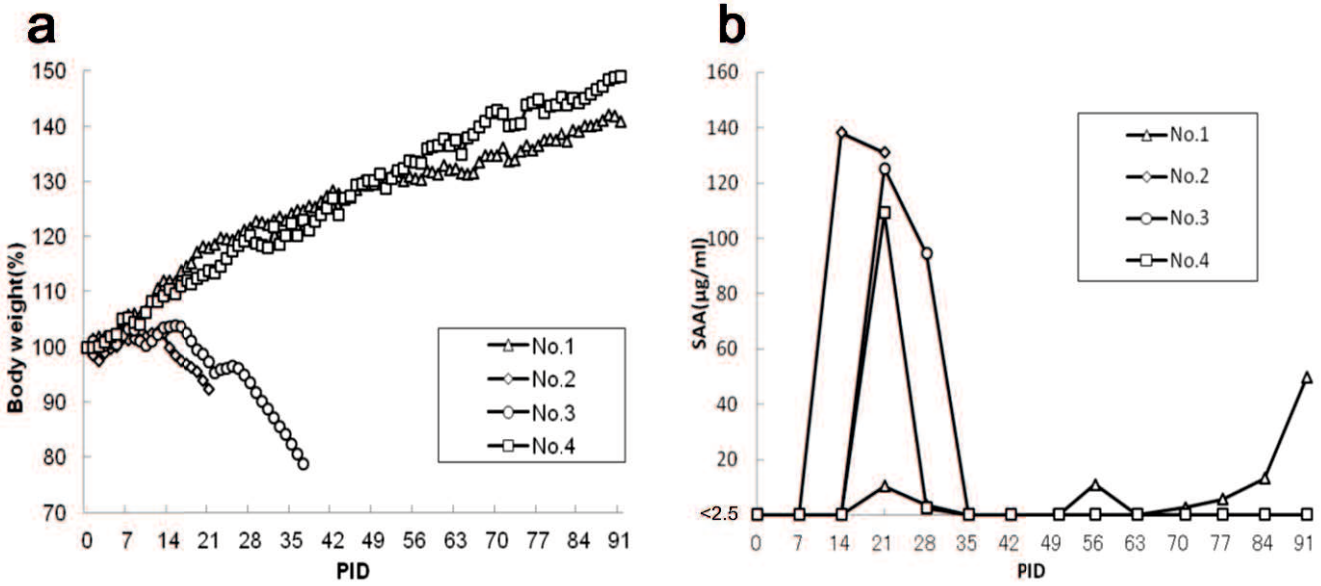

Figure 2-2. Body weight and SAA after inoculation.

3. CHAPTER 2

Emergence of pathogenic coronaviruses in cats by homologous recombination between feline and canine coronaviruses

3.1. Abstract

Type II FCoV emerged via double recombination between type I FCoV and type II CCoV. In this study, two type I FCoVs, three type II FCoVs and ten type II CCoVs were genetically compared. The results showed that three Japanese type II FCoVs, M91-267, KUK-H/L and Tokyo/cat/130627, also emerged by homologous recombination between type I FCoV and type II CCoV and their parent viruses were genetically different from one another. In addition, the 3'-terminal recombination sites of M91-267, KUK-H/L and Tokyo/cat/130627 were different from one another within the genes encoding membrane and spike proteins, and the 5'-terminal recombination sites were also located at different regions of ORF1. These results indicate that at least three Japanese type II FCoVs emerged independently. Sera from a cat experimentally infected with type I FCoV was unable to neutralize type II CCoV infection, indicating that cats persistently infected with type I FCoV may be superinfected with type II CCoV. Our previous study reported that few Japanese cats have antibody against type II FCoV. All of these observations suggest that type II FCoV emerged inside the cat body and is unable to readily spread among cats, indicating that these recombination events for emergence of pathogenic coronaviruses occur frequently.

3.2. Introduction

CoVs (order *Nidovirales,* family *Coronaviridae*, subfamily *Coronavirinae*) are enveloped and have a large single-stranded, positive-sense RNA. Most CoVs cause enteric and/or respiratory diseases in mammals and birds. The 5' two-thirds of the CoV genome consists of two ORFs, 1a and 1b, that encode a non-structural polyprotein, including RdRp. The other third of the genome consists of ORFs encoding structural proteins, S, M, E and N, and some nsp, 3a, 3b, 3c, 7a and 7b (Woo *et al*., 2010). TRS are located at 5'-distal position in each mRNA and play an important role in the RNA replication of CoV (Makino *et al*., 1991, Pasternak *et al*., 2006).

CoVs frequently undergo mutation and recombination, and there are three reasons for this (Bolles *et al*., 2011). First, CoV RdRp has low fidelity. Although CoV encodes nsp14, which possesses 3'→5' exonuclease activity for proofreading, the mutation rate approaches 2.0×10^{-6} mutations per site per round of replication (Eckerle *et al*., 2010). Second, there is a unique RNA replication mechanism using the TRS motif that is known as the "copy choice" mechanism, which induces homologous RNA recombination in CoVs (Lai *et al*., 1985, Pasternak *et al*., 2006). Third, CoV possesses the largest genome (26-32kb) among RNA viruses. Furthermore, heterologous recombination that *Betacoronavirus* subgroup A has the hemagglutinin esterase gene

originated from influenza C virus (Luytjes *et al*., 1988, Zeng *et al*., 2008). These mutation and/or recombination events change viral properties, host range and pathogenicity.

FCoV is classified into genus *Alphacoronavirus*, species *Alphacoronavirus 1*, and includes CCoV, TGEV and PRCoV. FCoV is distributed worldwide in cats and mainly induces mild intestinal inflammation in kittens (Pedersen *et al*., 1984b). FCoV inducing enteric disease is known as FECV. On the other hand, cats infected with FCoV rarely develop the more severe disease, FIP, which is caused by a mutant virus that is referred to as FIPV. In addition, FCoVs can be divided into two serotypes, types I and II, based on antigenicity (Fiscus and Teramoto. 1987, Hohdatsu *et al*., 1991b, Shiba *et al*., 2007). These serotypes differ primarily in growth characteristics in cell culture and in receptor usage. Type II FCoV is able to use fAPN as its receptor, but type I FCoV cannot (Pedersen *et al*., 1984a, Hohdatsu *et al*., 1998). Recently, it was revealed that the S protein was solely responsible for the differences in types I and II FCoV with regard to growth characteristics in cell culture and fAPN usage (Tekes *et al*., 2010).

CCoV was first isolated in 1971 from dogs with moderate to severe enteritis in Germany (Binn *et al*., 1974). CCoV is widespread in the dog population and is one of the most important canine enteropathogens (Carmichael, 1978, Rimmelzwaan *et al*.,

1991, Tennant *et al*., 1993, Bandai *et al*., 1999, Naylar *et al*., 2001, Yeşilbağ *et al*., 2004, Schulz *et al*., 2008). CCoVs were also divided into two genotypes; I and II. Before 2000, it was thought that CCoV had only one genotype, but strain Elmo/02 with a type I FCoV-like S gene was detected in Italy (Pratelli *et al*., 2003). The Elmo/02 strain possessed a novel ORF3 gene that was absent from other *Alphacoronavirus 1* between the S and ORF3a genes (Lorusso *et al*., 2008). Finally, this type I FCoV like-CCoV was designated type I CCoV and the reference CCoV was designated type II CCoV. Surprisingly, 36.9%-76.8% of dogs with diarrhea were co-infected with both types I and II CCoV (Pratelli *et al*., 2004, Decaro *et al*., 2010a, Soma *et al*., 2011). Furthermore, type II CCoV was divided into two subtypes, IIa and IIb (Decaro *et al*., 2009). In type IIb CCoV, the 5'-terminal region of the S gene was similar to that of TGEV and it was thought that type IIb CCoV emerged via recombination between type IIa CCoV and TGEV (Decaro *et al*., 2009). Recently, a type IIa CCoV strain CB/05 with high virulence was reported in Europe (Buonavoglia *et al*., 2006). CB/05-infected pups showed clinical signs such as lethargy, vomiting, diarrhea and acute lymphopenia, and the viral genome was observed in extraintestinal tissues including brain (Buonavoglia *et al*., 2006, Decaro and Buonavoglia, 2008). Furthermore, immune response induced by enteric CCoV did not protect dogs from infection with CB/05 (Decaro *et al*., 2010b).

However, there is little genetic information on CCoV in Japan.

In this study, to clarify the mechanisms of emergence of type II FCoV, three type II FCoVs isolated in Japan were genetically and antigenetically compared with ten Japanese type II CCoVs and two Japanese type I FCoVs.

3.3. Materials and methods

3.3.1. Cells

fcwf-4 cells (Jacobse-Geels and Holzinek, 1983) were grown in the same condition described in CHPTER 1.

3.3.2. Viruses

Type I FCoV strains C3663 and Yayoi, type II FCoV strains M91-267, KUK-H/L and Tokyo/cat/130627 and type II CCoV strains fc1, fc4, fc7, fc9, fc76, fc100, fc94-039, fc97-022, fc00-089 and fc00-016 were analyzed in this study (Table 1). Type I and II FCoVs, excluding Tokyo/cat/130627, were characterized by IFA using MAbs that were kindly provided by Dr. Hohdatsu (Hohdatsu *et al*., 1991a, b). Yayoi strain was isolated from a cat with a non-effusive form of FIP in Tokyo by serial passage in suckling mouse brain, and was then adapted to fcwf-4 cells (Hayashi *et al*., 1981). C3663 strain was

isolated from a cat with an effusive form of FIP in Kagoshima in 1994 (Mochizuki *et al*., 1997). The pathogenicity of C3663 and Yayoi in cats was characterized in CHAPTER 1 (Terada *et al*., 2012). M91-267 strain was isolated from a cat with an effusive form of FIP in Miyazaki in 1991 (Mochizuki *et al*., 1997). Three SPF cats were experimentally infected with M91-267, and all of these died from FIP (unpublished data). KUK-H strain was isolated from a cat with an effusive form of FIP in Kagoshima in 1987, and KUK-H/L that formed large plaques was plaque-purified from the KUK-H strain (Mochizuki *et al*., 1997). KUK-H/L caused lethal FIP in cats (Mochizuki *et al*., 1997). RNA sequences of Tokyo/cat/130627 were obtained from FIP ascites in a cat in Tokyo in 2013. The FIPV spread quickly in a cattery, and more than twenty cats developed FIP. Type II CCoV strains, fc1, fc4, fc7, fc9, fc76, fc100, fc94-039 and fc97-022, were isolated between 1990 and 1997 in Japan (Bandai *et al*., 1999), and fc00-016 and fc00-087 were isolated in 2000 in Japan (Mochizuki *et al*., 2001).

3.3.3. RT- PCR

Each virus, excluding Tokyo/cat/130627, was inoculated onto a fcwf-4 cell monolayer and was incubated until CPEs were observed. RNA was then extracted from fcwf-4 cells using an RNeasy® Mini kit (Qiagen) and RT reaction was carried out at

30°C for 10 min, 42°C for 30 min, 70°C for 15 min and 5°C for 5 min with random 9-mer oligonucleotide primers or 42°C for 30 min, 70°C for 15 min and 5°C for 5 min with oligo dT-adaptor primer using a TaKaRa RNA LA PCR^{TM} kit (AMV) Ver.1.1 (TaKaRa).

For amplification of partial S genes of type II CCoVs and type II FCoVs, primers CCVSF (5'-AGC ACT TTT CCT ATT GAT TG-3') and CCVSR (5'-GTT AGT TTG TCT AAT AAT ACC AAC ACC-3') were used (Naylar *et al*., 2002). For amplification of the N gene, primers NF (5'-CTA AAG CTG GTG ATT ACT CAA CAG-3') and NR (5'-TAA TAA ATA CAG CGT GGA GGA AAA C-3') were used (Wang and Lu, 2009). PCR was carried out at 94°C for 2 min, followed by 40 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 2 min and final extension at 72°C for 10 min using a TaKaRa RNA LA PCR^{TM} kit (AMV) Ver.1.1 (TaKaRa). PCR products were analyzed electrophoretically and the amplified products were purified using a QIAquick PCR Purification kit (Qiagen) for sequence analysis.

In order to amplify the subgenomic mRNA of CCoV fc1, PCR was performed using 52F (5'-ACT AGC CTT GTG CTA GAT TT-3') as a forward primer and CCVScenR (5'-CCA GTT TTT ATA ACA GCT G-3'), N-RR2 (5'-GCG CAA TAA CGT TCA CCA-3') and M13 primer M4 as reverse primers. Primer 52F recognized the

TRS conserved among *Alphacoronavirus*es (Terada *et al*., 2012). The reaction was carried out under the same conditions as mentioned above.

For sequence analysis of ORFs M, N, 7a and 7b of M91-267 and KUK-H/L, we carried out TA cloning. RNA was extracted from fcwf-4 cells infected with M91-267 or KUK-H/L using an RNeasy® Mini kit (Qiagen). Extracted RNA was reverse-transcribed with oligo dT-Adaptor primer using a TaKaRa RNA LA PCR™ kit (AMV) Ver.1.1 (TaKaRa) as mentioned above. To amplify the region including ORFs M, N, 7a and 7b, primers 52F, M13 primer M4 were used for PCR with a TaKaRa RNA LA PCR™ kit (AMV) Ver.1.1 (TaKaRa). PCR products were directly cloned into pGEM-T Easy (Promega, Madison, WI) according to the manufacturer's instructions. Plasmid DNAs were extracted from *E. coli* strain JM109 using a QIAprep Spin Miniprep Kit (Qiagen). Purified plasmid DNAs were applied for sequencing analysis.

Viral RNA of Tokyo/cat/130627 was extracted from FIP ascites in a cat using a QIAamp Viral RNA Mini Kit (Qiagen). For sequence analysis, five fragments of the Tokyo/cat/130627 gene between the 3'- terminus of ORF 1b and poly A were amplified using the following primer pairs: 1bF (5'-TTG ATT CAA AGA TTT GAG TAT TGG-3')-CCVSR; CCVSF-S2cenFR3 (5'-GTG TCA ATT CAG GTA CAG-3'); S2cenFF2 (5'-GAG TGC TGA TGC ACA AGT-3')-N-RR3 (5'-GCC ACC ATA CAA

TGT GAC-3'); N-RF4 (5'- AGT TCA GCA TTG CTG TGC TC-3')-N4 (5'-CAT CTC AAC CTG TGT GTC AT-3'); and N1 (5'-MMA AYA AAC ACA CCT GGA AG-3')-oligo dT-Adaptor primer. RT-PCR was carried out using a QIAGEN OneStep RT-PCR Kit (Qiagen) according to the manufacturer's instructions. Reactions were carried out at 45°C for 45 min and 95°C for 15 min, followed by 40 cycles at 94°C for 10 sec, 55°C for 30 sec, 68°C for 3 min, and a final extension at 68°C for 15 min. For amplification of partial RdRp genes, primer IN-6 (5'- GGT TGG GAC TAT CCT AAG TGT GA -3') and IN-7 (5'- CCA TCA TCA GAT AGA ATC ATC AT -3') were used. This primer pair can amplify nucleic acids from many coronaviruses in the subfamily *Coronavirinae* (Poon *et al*., 2005). Reactions were carried out at 50°C for 30 min and 95°C for 15 min, followed by 40 cycles at 94°C for 1 min, 48°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products were analyzed electrophoretically and amplified products were purified using a MinElute PCR Purification Kit (Qiagen) for sequence analysis.

3.3.4. Nucleotide sequences

Sequencing was performed using same methods described in CHAPTER 1. For sequence analysis, primers shown in Table S3-1 were used and nucleotide sequences

were deposited to DDBJ under the accession numbers listed in Table 1.

3.3.5. Homology search and phylogenetic analysis

Homologies among strains were analyzed using GENETYX® Ver.8 (GENETYX Corporation, Tokyo, Japan) and phylogenetic trees were constructed by the neighbor-joining method (Saitou and Nei, 1987) using MEGA5.0 software (Tamura *et al*., 2011) based on nucleotide pairwise distance. For construction of the phylogenetic tree, we referred to the following sequences; type II FCoV 79-1146 (Accession no. DQ010921), 79-1683 (JN634064), DF-2 (JQ408981) and NTU156/P/2007 (GQ152141), type I FCoV C3663 (AB535528), Yayoi (AB695067 for S), UCD1 (AB088222 for S, AB086902 for N), Black (EU186072), NTU2/R/2003 (DQ160294), RM (FJ938051), UCD11a (FJ917519), UCD5 (FJ917522), UCD12 (FJ917521), UCD13 (FJ917523), UCD14 (FJ917524), UU2 (FJ938060), UU16 (FJ938058), UU18 (HQ012368), UU20 (HQ392471), UU21 (HQ012369), UU23 (GU553362), type II CCoV 1-71 (JQ404409), v1 (AY390342 for S, AY390345 for N), K378 (KC175340), NTU336/F/2008 (GQ477367), 5821 (AB017789 for S), TGEV Purdue (DQ811789), and PRCoV ISU-1 (DQ811787). Analysis of similarity in the 3' region of the genome, excluding poly A, was carried out using Simplot version 3.5.1 (Lole *et al*., 1999).

3.3.6. Sera from cats

Sera collected from two SPF cats experimentally inoculated with FIPVs were used. One cat was inoculated intra-orally with type I FCoV C3663 (3.9×10^6 PFU/cat) and showed an effusion form of FIP in CHAPTER 1 (Terada *et al*., 2012). Another cat was inoculated intraperitoneally with type II FCoV M91-267 (1.0×10^6 PFU/cat) and also showed an effusive form of FIP (unpublished data). When clinical symptoms were severe, cats were euthanized under anesthesia. These sera were obtained in our previous experiments carried out under approval by the ethics committee for animal experiments, Faculty of Agriculture, Yamaguchi University.

3.3.7. VN test

VN test was performed the same as described in CHAPTER 1.

3.4. Results

3.4.1. Comparison of 3'-region among type II CCoVs, and type I and II FCoVs

Nucleotide sequences of the 3'-region of the genomes, excluding the poly A, of type II CCoV fc1 (8,959b) and type II FCoVs, M91-267 (8,889b), KUK-H/L (8,930b)

and Tokyo/cat/130627 (8,831b), were determined (DDBJ Accession No. AB781790 for fc1, AB781788 for M91-267, AB781789 for KUK-H/L and AB907624 for Tokyo/cat/130627) (Table 3-1). Because of a mutation in the start codon (ATG→ACG), Tokyo/cat/130627 lacked ORF3b. In addition, type II FCoVs, M91-267 and Tokyo/cat/130627 possessed a truncated ORF 3c (Figure 3-1). When compared with KUK-H/L, M91-267 had a 35-nucleotide deletion in the ORF 3c gene, resulting in a truncated ORF 3c. In comparison with C3663, Tokyo/cat/130627 showed a 25-nucleotide deletion in the ORF 3c gene, resulting in truncated ORF 3c gene. Deduced amino acid sequences for ORFs S, 3a, 3b, 3c, E, M, N, 7a and 7b in type II FCoVs were compared with those of type I FCoV C3663 and type II CCoV fc1 (Table S3-2, S3-3). Both M91-267 and KUK-H/L showed low identities with type I FCoV C3663 in ORFs S, 3a, 3b, 3c and E and high identities in ORFs N, 7a and 7b (Table S3-2). In contrast, the two strains showed high identities with type II CCoV fc1 in ORFs S, 3a, 3b, 3c and E and low identities in ORFs N, 7a and 7b (Table S3-3). In ORF M, the identities among type I FCoV, type II FCoV and CCoV were neither high nor low (Table S3-2, S3-3). Interestingly, comparison between Tokyo/cat/130627 and type I FCoV showed low identities in ORF S and high identities in ORFs 3a, 3c, E, M, N, 7a and 7b, while comparison with type II CCoV fc1 showed high identity only in ORF S

and low identities in ORF 3a, 3c, E, M, N, 7a and 7b (Table S3-2, S3-3).

3.4.2.Comparison of partial RdRp genes among type II CCoVs and type I and II FCoVs

Nucleotide sequences of partial RdRp gene in ORF1b (394b) of 15 Japanese CoVs were determined and deduced amino acid sequences were compared (Tables S3-2, S3-3, S3-4 and Figure 3-2A). In comparison with type I FCoVs, C3663, KUK-H/L and Tokyo/cat/130627 showed higher identity in RdRp than M91-267 (Table S3-2). On the other hand, the sequence of RdRp of M91-267 was more similar to that of type II CCoV fc1 than type I FCoV C3663 (Table S3-3). All CCoV strains possessed high homology with fc1 strain and M91-267, but showed low homology with KUK-H/L and Tokyo/cat/130627 (Table S3-4).

Phylogenetic analysis using partial RdRp genes showed that Japanese type II strains could be divided into two different groups; feline CoV and canine CoV (Figure 3-2A). KUK-H/L and Tokyo/cat/130627 belonged to feline CoV group and M91-267 belonged to canine CoV group. The other foreign type II FCoVs belonged to the type II CCoV group.

3.4.3. Comparison of partial S genes among type II CCoVs and type I and II FCoVs

Nucleotide sequences of partial S genes (692b) of 15 Japanese CoVs were determined and deduced amino acid sequences were compared (Table S3-5 and Figure 3-2B). In comparison with type I FCoV C3663, all type II FCoVs showed low identity. All CCoV strains possessed high homology with fc1 strain and type II FCoVs, but showed low homology with type I FCoV C3663 (Table S3-5).

Phylogenetic analysis using partial S genes showed that all type II FCoVs were more similar to type II CCoV than type I FCoV (Figure 3-2B). Furthermore, Japanese type II FCoVs were more similar to Japanese type II CCoV than type II FCoVs and type II CCoVs from other countries. In addition, Japanese FCoVs belonged to different subgroups; KUK-H/L belongs to a cluster with fc1. M91-267 belongs to the other cluster with fc76 and fc94-039. Tokyo/cat/130627 belongs to the cluster with Taiwanese strain NTU156/P/2007 (Figure 3-2B).

3.4.4. Comparison of N genes among type II CCoVs and type I and II FCoVs

Nucleotide sequences of N genes (1149b) of 15 Japanese CoVs were determined and deduced amino acid sequences were compared (Tables S3-2, S3-3, S3-6

and Figure 3-2C). In comparison with type I FCoV C3663, all type II FCoVs showed higher identity than type II CCoV fc1 (Table S3-2). All CCoV strains possessed high homology with fc1 strain, but showed low homology with types I and II FCoV (Table S3-6).

Phylogenetic analysis using N genes showed that FCoV strains and type II CCoV strains were genetically divided into different groups. In the feline CoV group, Japanese type II FCoVs M91-267, KUK-H/L and Tokyo/cat/130627 belonged to different clusters (Figure 3-2C). KUK-H/L was similar to Yayoi, M91-267 was similar to C3663, and Tokyo/cat/130627 was similar to Taiwanese strain NTU156/P/2007 (Figure 3-2C). Japanese CCoVs formed one cluster with Taiwanese strain NTU336/F/2008.

3.4.5. Recombination sites of type II FCoVs

Simplot analysis showed that the similarity of Tokyo/cat/130627 to CCoV fc1 changed at the 3'-terminal region of the S gene, and those of M91-267 and KUK-H/L changed within the M gene (Figure 3-3).

The M genes were compared among types I and II FCoV and type II CCoV (Figure 3-4A). The alignment showed that the 5'-terminal region of the M genes of

M91-267 and KUK-H/L was similar to that of CCoV fc1, but the 3'-terminal region was similar to type I FCoV C3663 (Figure 3-4A). The M gene of Tokyo/cat/130627 was similar to type I FCoV C3663. Furthermore, the nucleotide sequences indicated that the recombination sites of these two viruses, M91-267 and KUK-H/L, were different. Among these CoVs, two conserved regions were located at 133-177 and 325-366 in the M gene. KUK-H/L was similar to type II CCoV upstream of the first conserved region (region 133-177), but was similar to type I FCoV downstream of the region. On the other hand, M91-267 was similar to type II CCoV upstream of the second conserved region (region 325-366), and was similar to type I FCoV downstream of the region.

The alignment data using type I FCoV C3663, type II FCoV M91-267, KUK-H/L and Tokyo/cat/130627, and type II CCoV fc1 showed that the recombination site of Tokyo/cat/130627 was in the 3'-terminal of the S gene. Among these FCoVs and CCoVs, region 4183-4202 of the S gene was completely conserved (Figure 3-4B). Upstream of the conserved region, Tokyo/cat/130627 was more similar to type II CCoV fc1 than type I FCoV C3663, and downstream of the conserved region, Tokyo/cat/130627 was more similar to type I FCoV C3663 (Figure 3-4B).

3.4.6. Cross-neutralization activity to CCoV by sera collected from cats infected with FCoV

In order to examine whether cats with VN antibody against type I FCoV can be infected with type II CCoV, cross-neutralizing activity of sera from cats experimentally infected with FCoVs was examined (Table 3-1). Cat serum against type I FCoV C3663 was able to neutralize infection of type I FCoV strains C3663 and Yayoi (1:6400 and 1:2000, respectively), but not those of type II CCoV and type II FCoV (less than 1:10) (Table 3-1). On the other hand, cat serum against type II FCoV M91-267 was able to neutralize infection of type II FCoV (1:6400-1:25600), CCoV (1:200-1:9051) and type I FCoV (1:80-1:160) (Table 3-1).

3.5. Discussion

Type II FCoV emerged as a result of recombination events between type I FCoV and type II CCoV (Motokawa *et al*., 1996, Herrewegh *et al.*, 1998). Recently, one additional full genome sequence of type II FCoV NTU156/P/2007 was determined, and this facilitated understanding of the mechanisms responsible for emergence of type II FCoV (Lin *et al*., 2013). The prevalence of type II FCoV in the cat population is lower than that of type I, but the reasons for this remain uncertain (Shiba *et al*., 2007,

Vennema, 1999, Addie *et al*., 2003, Benetka *et al*., 2004, Kummrow *et al.*, 2005, Hohdatsu *et al.*, 1992). In this study, numerous FCoV and CCoV isolates from Japan were genetically characterized, and the emergence of type II was discussed.

Our phylogenetic and sequence analysis clearly indicated that type II FCoVs emerged by different recombination events between type I FCoV and type II CCoV. In addition, other type II FCoVs isolated from the USA (79-1683 and 79-1146) and Chinese Taipei (NTU156/P/2007) also showed different origins (Herrewegh *et al*., 1998, Lin *et al*., 2013). These results indicated that type II FCoV independently emerged in different cats and did not spread very easily. Our previous study also showed that many cats possess VN antibody to type I FCoV, but only a few cats in Japan possess VN antibody to type II FCoV (Shiba *et al*., 2007), supporting the notion that type II FCoV does not readily spread among the cat population. The reasons why type II FCoV is unable to spread among the cat population are unclear.

Two of three stains of Japanese type II FCoV, M91-267 and Tokyo/cat/130627, possessed the truncated ORF 3c gene (Figure 3-1). An intact 3c gene is apparently essential for efficient replication of FCoV in the intestinal tract, resulting in the secretion of FCoV from feces and transmission of FCoV among cat population (Pedersen, 2009, Pedersen *et al*., 2012). On the other hand, many FIPV possessed

truncated 3c gene and cats with FIP did not excrete virus in feces (Chang *et al*., 2010, Vennema *et al*., 1998 Pedersen *et al*., 2009). Furthermore, one outbreak of type II FIPV with intact ORF 3c gene occurred in Taiwan. In early stage of the outbreak, the type II FIPV possessed intact 3c gene, but lost it in the late stage (Wang *et al*., 2013). These results indicated that type II FCoVs might not be excreted in feces and spread in cat population, because most of them lost intact 3c gene.

It is interesting that all FCoVs, both types I and II, possessed the 5'- and 3'-termini of the FCoV genome, but not CCoV. These regions may be essential for growth of FCoV in cats and double recombination may be required to maintain both the 5'- and 3'-termini of FCoV. Type II FCoVs possessed two types of RdRp elements derived from type I FCoV or type II CCoV (Figure 3-2A), suggesting both types of RdRp were able to function during replication and transcription in cat body. Furthermore, it was thought that the region upstream of RdRp might be essential for FCoV infection to cats. In addition, it has been reported that N protein is important for viral particle production (Masters, 2006), and the N gene is conserved among FCoVs. Therefore, the N protein of FCoV, but not CCoV, may be essential for replication of FCoV in cats. Interestingly, simplot analysis showed four other candidate recombination sites, one in the 3c gene, two in the N gene and one in the 7a gene, which showed high identity between CCoV

and type I FCoV (Figure 3-3). If the M or N genes of type I FCoV are not necessary for growth of FCoV in cats, other recombinant type II FCoVs using these possible recombination sites must occur. Further analysis of type II FCoV is necessary to clarify the recombination events of CoV in cats.

Four full genome sequences of type II FCoVs (79-1146, 79-1683, DF-2 and NTU156/P/2007) are deposited in GenBank. We also reported one-third of the full genome of three type II FCoV strains (M91-267, KUK-H/L and Tokyo/cat/130627) and one type II CCoV fc1. Six of seven type II FCoV strains emerged by recombination events at the E or M gene. However, the recombination event of Tokyo/cat/130627 occurred at the 3'-terminal of the S gene. The nucleotide sequences indicated that M91-267, KUK-H/L and Tokyo/cat/130627 originated from type I FCoV strains similar to C3663, Yayoi and NTU2/R/2003, respectively, and that the central region, including the S gene, was acquired from type II CCoV strains similar to fc94-039, fc1 and fc00-089, respectively. In addition, the recombination sites were clearly different (Figure 3-3 and 3-4A, B). These results indicated that the recombination events between type I FCoV and type II CCoV occurred independently. In addition, original viruses of foreign type II FCoVs, 79-1146, 79-1683 and NTU156/P/2007 differed from those of these three Japanese type II FCoVs, indicating that the recombination events occurred

among cat populations all over the world.

Sera from cats experimentally inoculated with type I FCoV C3663 could not neutralize type II CCoV infection (Table 3-1), suggesting that the cat infected with type I FCoV could not prevent type II FCoV infection. On the other hand, the cat infected with type II FCoV could neutralize type I FCoV infection (Table 3-1). In addition, many sera from type II FCoV-infected cats in the outbreak could cross-neutralize type I FCoV infection, and those from type I FCoV-infected cats in the field could not cross-neutralize type II FCoV infection (our unpublished data). These results suggested that the cross-reactivity to type I FCoV in type II FCoV-infected cats might be induced by other viral protein except for S protein. Further analysis will be required to clarify the cross VN activity in type II FCoV-infected cats.

Type II CCoV was able to use “feline” aminopeptidase N as a receptor to infect cats (Tresnan *et al*., 1996, McArdle *et al*., 1990) and type I FCoV-infected cats did not possess VN antibody against type II CCoV infection (Table 3-1), indicating that cats infected with type I FCoV could be superinfected with type II CCoV from dogs. Our hypothesis on the mechanism of emergence of type II FCoV is shown in Figure 3-5. Cats infected with type I FCoV were unable to produce VN antibody against type II CCoV. Hence, cats had the possibility of superinfection with type II CCoV. The

recombination event between type I FCoV and type II CCoV occurred inside the cat body, leading to emergence of type II FCoV.

CoVs, such as SARS-CoV, tend to change their host range by mutation and/or recombination (Graham and Baric, 2010). Homologous recombination is a significant factor for change of host range. Therefore, investigations into homologous recombination of CoVs may help to clarify the mechanisms responsible for changes in host range.

3.6 Figure legends

Figure 3-1. Schema of feline and canine coronaviruses.

(A) Schema of type II CCoV. Each ORF is indicated by squares. Arrowheads indicate location of primers for amplification of partial RdRp, partial S and full N genes. **(B)** Schema of type II CCoV fc1, type II FCoV M91-267, KUK-H/L and Tokyo/cat/130627, and type I FCoV C3663 and Yayoi. Blue boxes indicate ORFs originating from type II CCoV. Red boxes indicate ORFs originating from type I FCoV.

Figure 3-2. The phylogenetic trees using partial RdRp(A), partial S (B) and N (C) genes.

Type I FCoVs, type II FCoVs and type II CCoVs are shown in red, green and blue, respectively. Swine CoV (TGEV and PRCoV), FRCoV and human CoV (HCoV) are shown in black. GenBank accession numbers are shown in parentheses.

Figure 3-3. Simplot analysis of canine and feline coronaviruses.

Similarity between nucleotide sequences of 3' region of genome of type II CCoV fc1, type I FCoV Black, and type II FCoVs KUK-H/L, M91-267 and Tokyo/cat/130627. Horizontal axis refers to nucleotide position of fc1. Upper region of the plot map shows

ORF structure in type II CCoV fc1 and corresponds to nucleotide positions in the plot map. A similarity of 1.0 indicates 100% identity with the nucleotide sequence. Parameters for calculation were as follows: window size, 200 bp; and step size, 40 bp.

Figure 3-4. Alignment of M and 3'-terminal of S genes in canine and feline coronaviruses.

(A) Alignment of M genes of CCoV and FCoV strains. Two regions in squares are conserved regions among type II CCoV fc1, type II FCoVs M91-267, KUK-H/L and Tokyo/cat/130627 and type I FCoV C3663. (**B)** Alignment of 3'-terminal of S genes of CCoV and FCoV strains. Square indicates conserved region. Nucleotide sequences originating from type II CCoV and type I FCoV are shown in blue and red, respectively. Dots indicate the same sequences with type II CCoV fc1.

Figure 3- 5. Hypothesis of emergence of type II FCoV.

Some cats persistently infected with type I FCoV are superinfected with type II CCoV which is excreted from dogs. Inside the cat body, type II FCoV emerges by homologous recombination and induces severe clinical disease, FIP. Diseased cats do not spread type II FCoV.

3.7 Figures and table

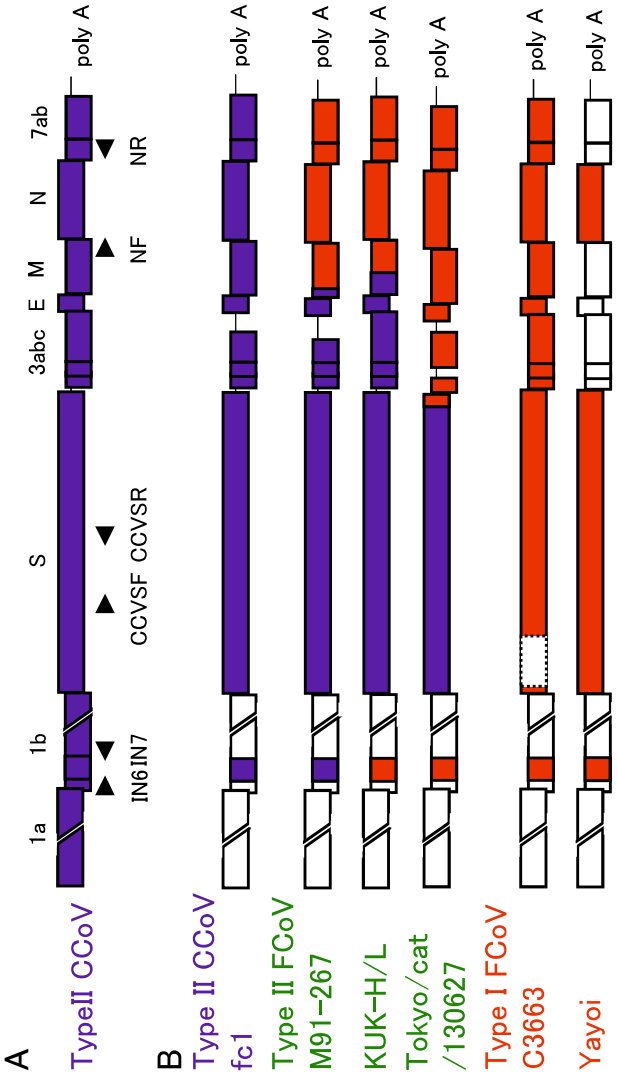

Figure 3-1. Schema of feline and canine coronaviruses

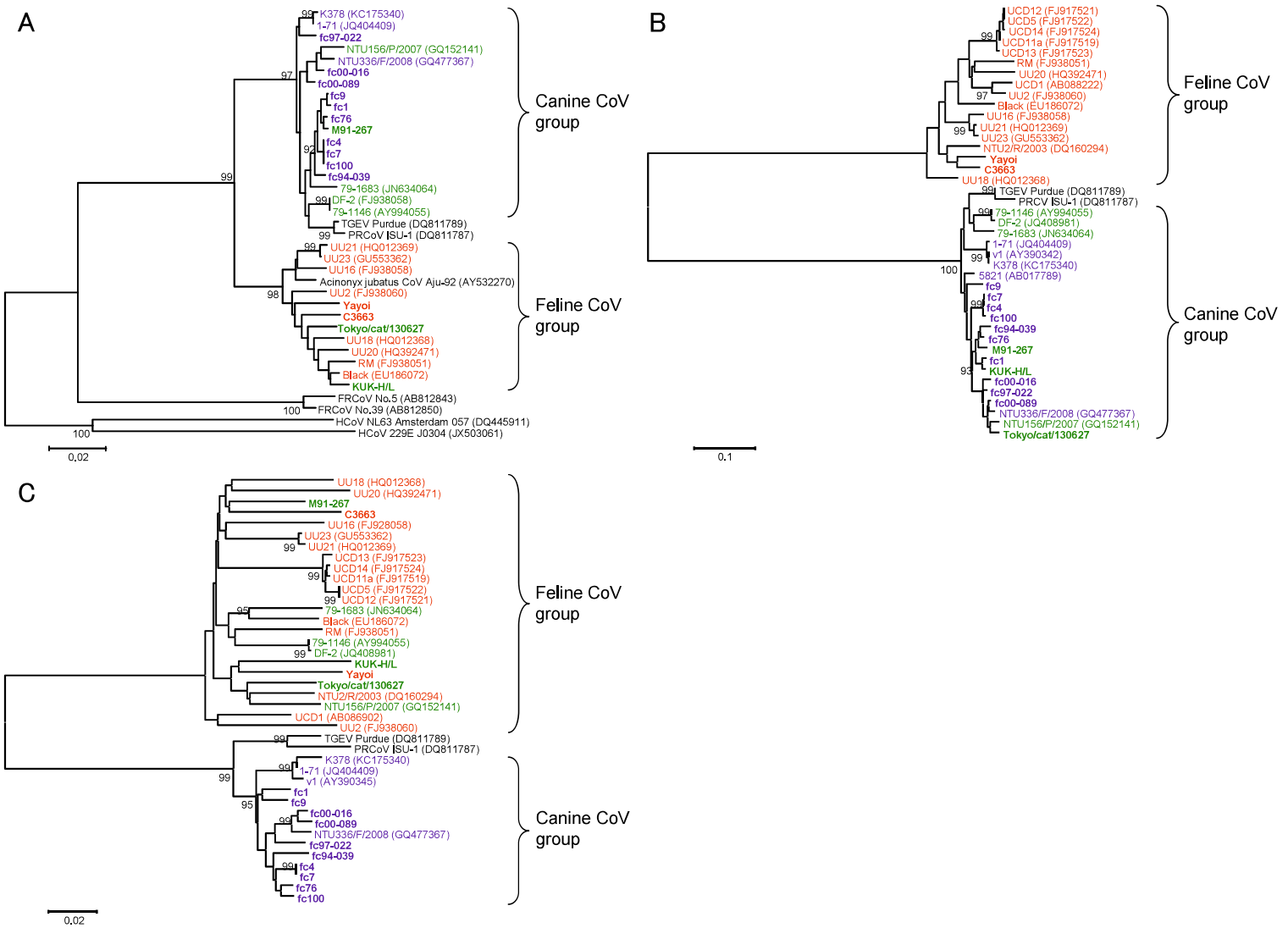

Figure 3-2. The phylogenetic trees using partial RdRp(A), partial S (B) and N (C) genes.

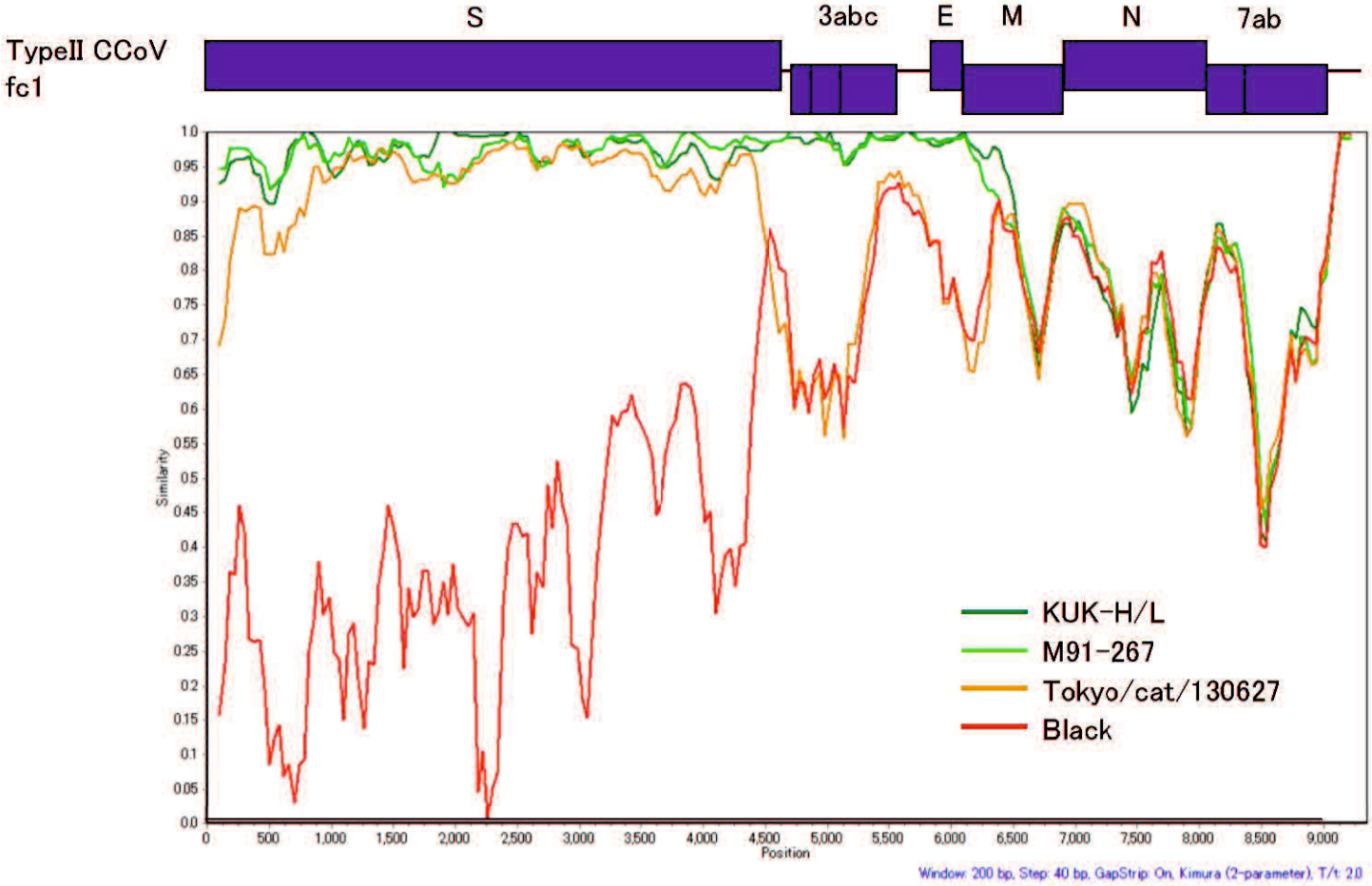

Figure 3-3. Simplot analysis of canine and feline coronaviruses.

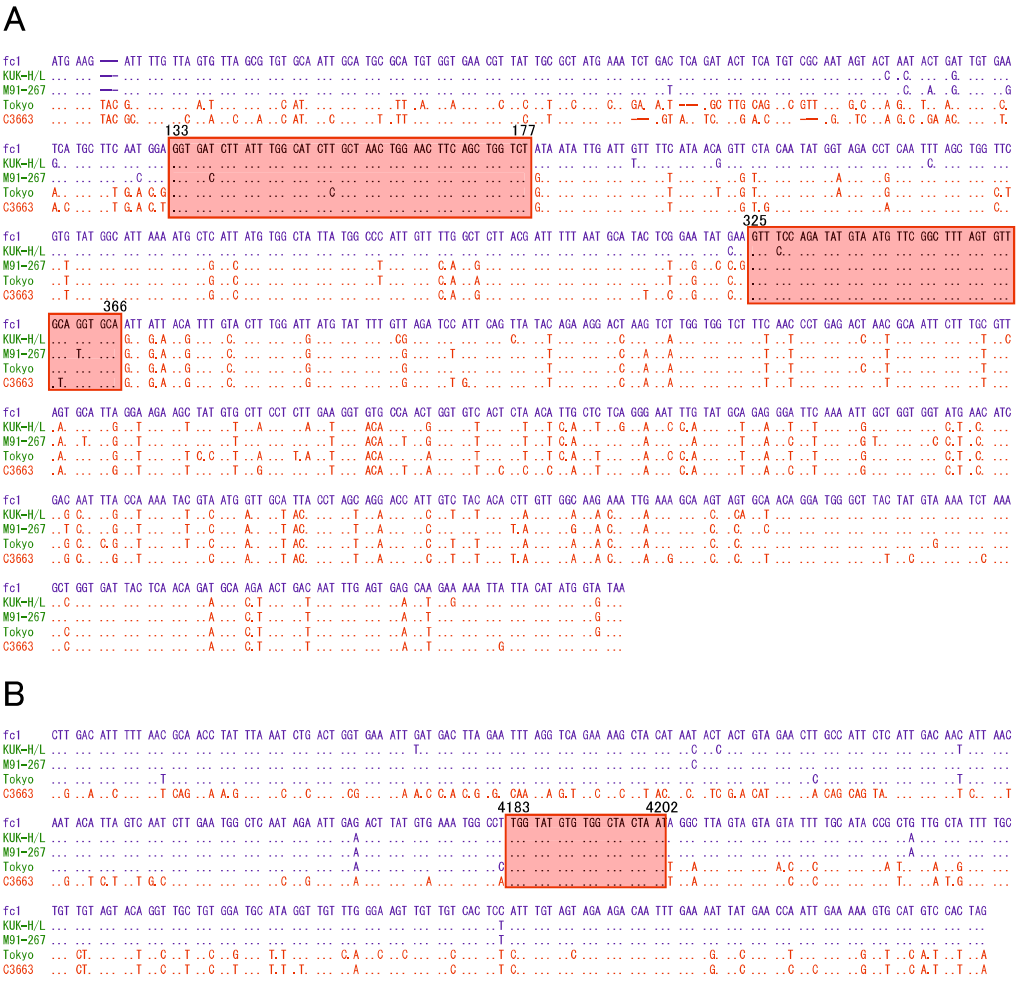

Figure 3-4. Alignment of M and 3'-terminal of S genes in canine and feline coronaviruses.

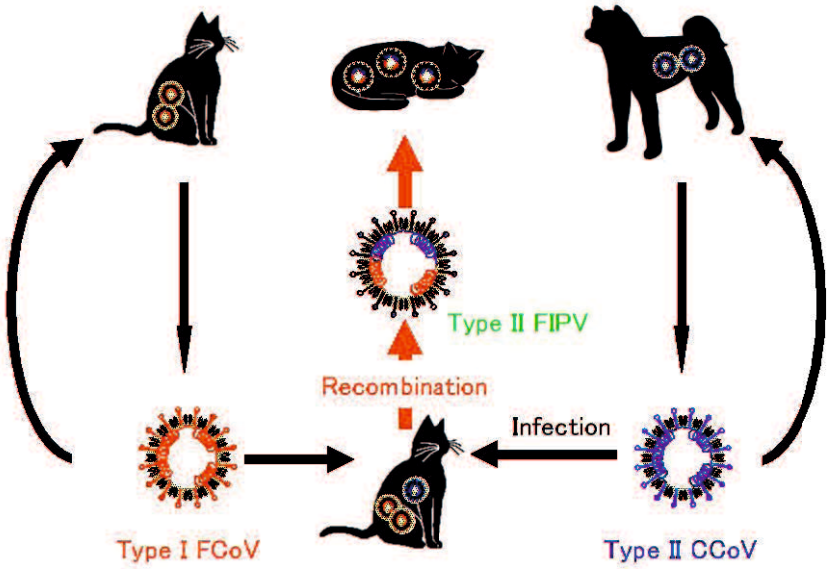

Figure 3- 5. Hypothesis of emergence of type II FCoV.

Table 3-1. Canine and feline coronaviruses analyzed in this study: nucleotide sequence acquisition numbers and serum cross-neutralizing activity

Virus	Strain	Accession No.				VN titers	
		RdRp	S - poly A	Partial S	N	Type I[a]	Type II[b]
Type II CCoV	fc1	AB781791	AB781790	AB781790	AB781790	<1:10	1:400
	fc4	AB907625		AB781807	AB781797	<1:10	1:4525
	fc7	AB907626		AB781808	AB781798	<1:10	1:1600
	fc9	AB907627		AB781809	AB781799	<1:10	1:1600
	fc76	AB907628		AB781810	AB781800	<1:10	1:9051
	fc100	AB907629		AB781811	AB781801	<1:10	1:1131
	fc97-022	AB907631		AB781812	AB781802	<1:10	1:2263
	fc94-039	AB907630		AB781813	AB781803	<1:10	1:2263
	fc00-016	AB907632		AB781814	AB781804	<1:10	1:1600
	fc00-089	AB907633		AB781815	AB781805	<1:10	1:200
Type II FCoV	M91-267	AB781792	AB781788	AB781788	AB781788	<1:10	1:25600
	KUK-H/L	AB781793	AB781789	AB781789	AB781789	<1:10	1:6400
	Tokyo/cat/130627	AB907634	AB907624	AB907624	AB907624	N.D.	N.D.
Type I FCoV	C3663	AB781794	AB535528[c]	AB535528[c]	AB535528[c]	1:6400	1:80
	Yayoi	AB781795		AB695067[c]	AB781806	1:2000	1:160

N.D.: Not done

[a] Serum was collected from the cat that was inoculated intraorally with type I FCoV C3663 (Terada *et al*., 2012).

[b] Serum was collected from the cat that was inoculated intraperitoneally with type II FCoV M91-267 (unpublished data).

[c] (Terada *et al*., 2012)

3. 8. Supplementary tables

Table S3-1. Primers used in this study

Primer	Gene	Sequence (5' to 3')	Position[a]
1bF	ORF1b	TTGATTCAAAGATTTGAGTATTGG	1-24
1bR	S	AGGCACAATGTAAGCACAATCATGG	379-375
1bFF1	ORF1b	AACGTGCCATGATTGTGC	349-366
1bF2F	S	GTGGTTATTACCCTACAGAG	526-545
1bF2R	S	CTCTGTAGGGTAATAACCAC	526-545
1bFF2	S	GCTCAAGTACTGCCACAT	1006-1023
1bFR2	S	CATATGCAGCACTGTGTG	1044-1027
CCVSF	S	AGCACTTTTCCTATTGATTG	1686-1705
CCVSR	S	GTTAGTTTGTCTAATAATACCAACACC	2483-2457
CCVScenF	S	TAAGTAACATCACACTACC	2026-2044
CCVScenR	S	CCAGTTTTTATAACAGCTG	2194-2176
CCVScenRR2	S	AACAGTAACGCGGTCCAT	1572-1555
CCVScenRF2	S	TACAGTGAGCGAGTCAAG	1499-1516
CCVScenRF3	S	GCATACATTAGTGGCCGT	930-947
CCVScenRR3	S	CAGTACTTGAGCGAGAGT	1017-1000
CCVScenRF4	S	GCTCATACCACATTGCTTCG	391-410
CCVScenRR4	S	CGACGTACTCGAAGCAAT	419-402
CCVScenRF5	S	AACATGGCACAAGAGTGCTG	1019-1038
CCVScenRR5	S	CAGGTTGTAAGACTGACCAC	959-940
SF2	S	TCTTGGTATGAAGCGTAGTGG	1979-1999
SR2	S	TACCAATAGCTTGATTGAAAGC	3591-3570
SR3	S	GCAGTTAGGTGGCTTAAAGC	3727-3708
S2cenF	S	CTATTCTGTGACACCATGTG	2564-2583
S2cenR	S	GCGCTTGCTCAATAGTTTGA	3039-3020
S2cenFF1	S	CATCTGTTGAGGCGTTCA	3115-3132
S2cenFR1	S	CTAGCCAAGAACCACCTA	3195-3178
S2cenFF2	S	GAGTGCTGATGCACAAGT	3797-3814
S2cenFR2	S	TCAGCCTGTCAACTTGTG	3825-3808
S2cenFF3	S	CTGGACTGTACCTGAATTG	4343-4361
S2cenFR3	S	GTGTCAATTCAGGTACAG	4365-4348
S2cenFF4	3a	GACACACTTCTTGAGGCT	4919-4936
S2cenFR4	3a	TGGAGAGACCAAGCTTAG	4970-4953
S2cenFR5	S	AGTCTACAACACGTCTTCTAC	4689-4669
NF	M	CTAAAGCTGGTGATTACTCAACAG	6847-6870
NR	7a	TAATAAATACAGCGTGGAGGAAAAC	8119-8095
NcenF	N	AGAGGAAGGCAACAATCCAA	7464-7483
NcenR	N	CCTGCAGTTCTCTTCCAGGT	7660-7641
N-R	N	CACCATCCTTTGCAACCCAG	7293-7274
N-R-2	N	CAGATCTAGGCTGAGAACCA	7440-7421
N1	N	MMAAYAAACACACCTGGAAG	7630-7649
N4	N	CATCTCAACCTGTGTGTCAT	8063-8044
N-RF1	M	TGGCCTTACCATCGATCA	6719-6736
N-RR1	M	CGATGGTTCTACTAGGTG	6780-6763
N-RR2	M	GCGCAATAACGTTCACCA	6196-6179
N-RR2-2	E	GCTTCGTCGGGATTATATGC	6111-6092
N-RF2	M	AGCGTGTGCAATTGCATG	6155-6172
N-RF2-2	E	CTTCTTCTGGCTCCTGTTGA	5932-5951
N-RR3	Between 3c and E	GCCACCATACAATGTGAC	5637-5620
N-RF3	Between 3c and E	GAGAAGTTCTCACAGCTC	5595-5612
N-RF4	3c	AGTTCAGCATTGCTGTGCTC	5319-5338
N-FF	7a	CTGACAGTAGTCTGCGTGTA	8222-8241
N-F2F	7b	GCATCTAGAGTGTGCTCACA	8881-8900
NR-R	7b	ACTCTCACACTCAACACGAG	8605-8586
3-R	3' UTR	GTGTATCACTATCAAAAGGAATA	9315-9293
52F	TRS	ACTAGCCTTGTGCTAGATTT	

a: Position is shown based on the fc1 sequence.

Table S.3-2 Comparison of ORF identities between C3663 and other coronaviruses

	Identity with type I FCoV C3663 (%) (amino acids)									
	RdRp	S	3a	3b	3c	E	M	N	7a	7b
fc1	**95.4**	48.4	69.0	59.7	71.5	76.8	83.4	74.9	81.2	65.9
M91-267	**94.7**	48.7	69.0	56.7	63.6	76.8	**90.0**	**92.8**	**95.1**	**91.3**
KUK-H/L	**98.5**	48.6	69.0	59.7	79.1	78.0	**89.6**	**89.9**	**96.0**	**92.2**
Tokyo/cat/130627	**98.5**	49.2	**90.0**		**91.1**	**93.9**	**92.4**	**91.8**	**95.1**	**87.9**

Bold numbers indicate that the identity is over 85%

Table S3-3. Comparison of ORF identities between fc1 and other coronaviruses

	Identity with type II CCoV fc1 (%) (amino acids)									
	RdRp	S	3a	3b	3c	E	M	N	7a	7b
C3663	**95.4**	48.4	69.0	59.7	71.5	76.8	83.4	74.9	81.2	65.9
M91-267	**99.2**	**97.5**	**100**	**98.6**	**97.7**	**100**	**90.1**	76.4	81.2	58.8
KUK-H/L	**94.7**	**96.5**	**95.8**	**98.6**	**96.2**	**97.6**	**89.7**	74.6	82.2	59.7
Tokyo/cat/130627	**94.7**	**94.6**	68.6		74.6	78.0	83.5	76.4	82.2	59.3

Bold numbers indicate that the identity is over 85%

Table S3-4. Amino acid sequence identities of partial RdRp among type II CCoV and types I and II FCoV

	fc1	C3663	M91-267	KUK-H/L	Tokyo/cat/130627
fc4	**100.0%**	**95.4%**	**99.2%**	94.7%	94.7%
fc7	**100.0%**	**95.4%**	**99.2%**	94.7%	94.7%
fc9	**100.0%**	**95.4%**	**99.2%**	94.7%	94.7%
fc76	**100.0%**	**95.4%**	**99.2%**	94.7%	94.7%
fc100	**100.0%**	**95.4%**	**99.2%**	94.7%	94.7%
fc97-022	**99.2%**	**96.2%**	**98.5%**	**95.4%**	**95.4%**
fc94-039	**100.0%**	**95.4%**	**99.2%**	94.7%	94.7%
fc00-016	**100.0%**	**95.4%**	**99.2%**	94.7%	94.7%
fc00-089	**100.0%**	**95.4%**	**99.2%**	94.7%	94.7%

Bold numbers indicate that the identity is over 95%.

Table S3-5. Amino acid sequence identities of partial S protein among type II CCoV and types I and II FCoV

	fc1	C3663	M91-267	KUK-H/L	Tokyo/cat/130627
fc4	**96.1%**	29.3%	**97.0%**	**96.5%**	**94.8%**
fc7	**95.7%**	29.3%	**96.5%**	**96.1%**	**94.3%**
fc9	**93.9%**	30.1%	**95.2%**	**94.3%**	**93.0%**
fc76	**98.3%**	27.8%	**96.1%**	**98.7%**	**95.7%**
fc100	**95.2%**	29.3%	**96.1%**	**95.7%**	**93.9%**
fc97-022	**97.4%**	29.3%	**95.2%**	**97.8%**	**97.0%**
fc94-039	**98.3%**	27.8%	**97.0%**	**98.7%**	**95.7%**
fc00-016	**97.4%**	28.8%	**95.2%**	**97.8%**	**96.5%**
fc00-089	**97.8%**	28.9%	**95.7%**	**98.2%**	**97.4%**

Bold numbers indicate that the identity is over 90%.

Table S3-6. Amino acid sequence identities of N protein among type II CCoV and types I and II FCoV

	fc1	C3663	M91-267	KUK-H/L	Tokyo/cat/130627
fc4	**98.2%**	75.9%	77.5%	75.4%	77.2%
fc7	**98.2%**	75.9%	77.5%	75.4%	77.2%
fc9	**97.9%**	75.4%	77.0%	75.4%	77.0%
fc76	**97.9%**	75.9%	77.5%	75.7%	77.5%
fc100	**97.6%**	75.7%	77.2%	75.4%	77.2%
fc97-022	**96.9%**	75.4%	76.7%	74.6%	76.4%
fc94-039	**97.6%**	74.6%	75.9%	74.6%	76.2%
fc00-016	**96.9%**	75.1%	76.4%	74.6%	76.4%
fc00-089	**96.6%**	74.6%	75.9%	74.1%	75.9%

Bold numbers indicate that the identity is over 90%.

4. CHAPTER 3

Genetic Characterization of Coronaviruses from Domestic Ferrets, Japan

4.1. Abstract

We detected FRCoVs in 44 (55.7%) of 79 pet ferrets tested in Japan and classified the viruses into two genotypes on the basis of genotype-specific PCR. Our results show that two FRCoVs that cause FIP–like disease and ECE are enzootic among ferrets in Japan.

4.2. Introduction

An ECE was first recognized in domestic ferrets (*Mustelo putorius furo*) in the United States in 2000 (Williams *et al*., 2000). The causative agent of ECE was demonstrated to be a novel FRCoV belonging to the genus *Alphacoronavirus* (Williams *et al*., 2000, Wise *et al*., 2006). Ferrets with ECE showed general clinical signs of lethargy, anorexia, and vomiting and had foul-smelling, green mucous–laden diarrhea. A systemic infection of ferrets closely resembling FIP was subsequently reported among ferrets in the United States and Europe. The causative agent was also shown to be an *Alphacoronavirus*, which was named FRSCV (Garner *et al*., 2008, Martínez *et al*., 2006). This virus was found to be genetically distinct from those associated with ECE and from two viruses assigned to different genotypes, I and II: genotype I, which included the agent of FIP-like disease, and genotype II, which included the causative

agent of ECE (Wise *et al*., 2010). Other cases of ECE and ferret infectious peritonitis have since been described in the United States and in Europe (Wise *et al*., 2006, Garner *et al*., 2008, Martínez *et al.*, 2006, 2008, Graham *et al.*, 2012). One case of pathology-confirmed FIP-like disease has been described among domestic ferrets in Japan (Michimae *et al.*, 2010). The goal of this study was to determine the prevalence of CoV among domestic ferrets seen by veterinarians in various parts of Japan.

4.3. Materials and methods

4.3.1. Samples

Fecal samples were collected during August 2012–July 2013 from 79 ferrets from 10 animal hospitals scattered across 5 prefectures in Japan. Oral swab specimens from 14 of 79 ferrets were also collected. Most of the ferrets were brought to veterinarians for clinical signs such as diarrhea, abdominal masses, and hypergammaglobulinemia; some had signs unrelated to CoV infection or were asymptomatic (Table 4-1).

4.3.2. RT- PCR

RNA was extracted from fecal and oral swab samples by using the QIAamp Viral RNA Mini Kit (QIAGEN), and RT-PCR was performed by using the QIAGEN

OneStep RT-PCR Kit (QIAGEN) according to the manufacturer's instruction. For amplification of RdRp gene in the ORF 1b region, CoV consensus primers IN-6 and IN-7 were used as described in CHAPTER 2.

On the basis of additional sequence data from RT-PCR products using IN-6 and IN-7, a new primer pair was designed: forward FRCoV RdRp-F1 (5′-GTT GGT TGC TGC ACA CAT AG-3′) and reverse FRCoV RdRp-R1 (5′-GGA GAA GTG CTT ACG CAA ATA-3′). RT-PCR was carried out by using the QIAGEN OneStep RT-PCR Kit (QIAGEN) and reactions were carried out at 50°C for 30 min and 95°C for 15 min, followed by 40 cycles at 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, and a final extension at 72°C for 7 min.

To differentiate between two genotypes in the positive samples from our testing, RT-PCR that amplified the partial S gene was carried out by using two pairs of genotype-specific primers: forward primer 5′-CTG GTG TTT GTG CAA CAT CTA C-3′ and reverse primer 5′-TCT ATT TGC ACA AAA TCA GAC A-3′ for genotype I, and forward primer 5′-GGC ATT TGT TTT GAT AAC GTT G-3′ and reverse primer 5′-CTA TTA ATT CGC ACG AAA TCT GC-3′ for genotype II (Wise *et al.*, 2010). Reactions were carried out at 50°C for 30 min and 95°C for 15 min, followed by 40 cycles at 94°C for 30 sec, 53°C for 30 sec, 72°C for 30 sec, and a final extension at

72°C for 7 min.

RT-PCR products were analyzed electrophoretically and amplified products were purified using a MinElute PCR Purification Kit (Qiagen) for sequence analysis.

4.3.3. Nucleotide sequences

Sequencing was performed using same methods described in CHAPTER 1.

4.3.4. Homology search and phylogenetic analysis

Homologies among strains were analyzed using GENETYX® Ver.8 (GENETYX Corporation) and phylogenetic trees were constructed by the neighbor-joining method (Saitou and Nei, 1987) using MEGA5.0 software (Tamura *et al*., 2011) based on nucleotide pairwise distance. For construction of the phylogenetic tree, we referred to the following sequences;, FRSCV MSU-S (GU459055), FRSCV MSU-1 (GU338456), FRSCV WADL (GU459056), FRECV MSU1 (DQ340561), FRECV 1202 (GU459057), FRECV MSU-2 (GU338457) FRCoV 4E98 (JF260914), FRCoV 511c (JF260913), mink coronavirus WD1127 (accession no. HM245925), WD1133 (HM245926), type I FCoV Black (EU186072), type II FCoV 79-1683 (JN634064), 79-1146 (DQ010921), type II CCoV NTU336/F/2008 (GQ477367), 1-71 (KC175339), TGEV virulent Purdue (DQ811789) , PRCoV ISU-1 (DQ811787), HCoV 229E VR-740 (NC_002645), NL63 Amsterdam1 (AY567487).

4.4. Results

Of 79 samples, 33 (41.8%) were positive for CoVs by RT-PCR using a primer pair, IN-6 and IN-7 (Table 2). Nucleotide sequences were determined for the amplified fragments and used to construct a phylogenetic tree (Figure 4-1). The CoVs detected in this study belonged to the genus *Alphacoronavirus* but formed a separate species from those of other species. The identities with FCoV, TGEV, PRCoV and mink coronavirus were 73.5%–75.9%, 73.5%–76.1%, 73.8%–76.1%, and 80.2%–84.0%, respectively.

Next, we attempt to construct the sensitive RT-PCR method for detection of FRCoV and designed a new primer pairs, FRCoV RdRp-F1 and FRCoV RdRp-R1. Results for RT-PCR using this new primer set showed that 44 (55.7%) of 79 samples were positive for FRCoV, which was a higher number than that obtained by using the published CoV consensus primers (55.7% vs. 41.8%) (Table 4-2). Two samples that had positive results by consensus primers had negative results by the new primers: sample 22 had many mutations in the primer binding site (Figure 4-1), whereas sample 40 had few mutations.

To differentiate between two genotypes in the positive samples from our testing, partial sequences of S gene were amplified. Among these ferrets, 30 (38.0%) were

infected with genotype I and 17 (21.5%) with genotype II; 8 (10.1%) ferrets were infected with both genotypes of FRCoVs (Figure 4-2). Samples 27 and 28 were from ferrets that lived in the same house and harbored the same FRCoV but that were born on different farms, indicating that horizontal transmission had occurred. The nucleotide sequences of the amplified genes confirmed that these CoVs also fell into genotypes I and II (Figure 4-2). There was no relationship between the genotypes of FRCoV and the type of disease (Table 4-1).

To further investigate virus transmission routes, oral swab specimens were collected from 14 of the 79 ferrets and examined by RT-PCR using primers FRCoV RdRp-F1 and FRCoV RdRp-R1. Results of RT-PCR showed that 5 (35.7%) of 14 specimens were positive.

4.5. Discussion

We established a sensitive RT-PCR method using a new primer pair to detect CoV sequences (Table 4-2). Furthermore, we determined the partial nucleotide sequences of the S gene of 23 strains and found they were clearly divided into two genotypes, I and II (Figure 4-2). These results indicated that both genotypes of FRCoV have been spreading within the ferret population in Japan for some time. The reported

FRCoVs associated with FIP-like disease, designated as genotype I by Wise *et al*. (2010), all fell within genotype I phylogenetically, whereas all published ECE-causing strains fell within genotype II. This finding leads to a possible conclusion that FIP-like disease–causing strains (i.e., FRSCVs) are variants of what has been designated genotype I FRCoVs. Because we found no relationship between the two genotypes of FRCoV and the type of disease (Table 4-1), we cannot determine whether FIP-like and ECE-like FRCoVs circulate independently as distinct entities or evolve, like FCoVs, from more ubiquitous and less pathogenic enzootic strains. Nonetheless, the addition of these 23 new isolates to the phylogenetic tree of FRCoVs tends to support the latter conclusion. Without extensive animal passage studies, virus isolation, and CoV-free ferrets, this theory may be difficult to confirm. However, additional evidence tends to link virulent pathotypes of FRCoVs to specific mutational events. Nucleotide sequences of the 3c-like protein genes of FRSCV, MSU-1 (DDBJ/EMBL-Bank/GenBank accession no. GU338456), MSU-S (GU459059), and WADL (GU459058), showed that 2, MSU-1 and WADL, possessed a truncated 3c-like protein gene (Wise *et al*., 2010), similar to that described for FIP viruses of cats (Pedersen *et al*., 2009, Chang *et al*., 2010, Pedersen, 2009). FIP-causing viruses of cats also contain a second mutation in the S gene (Chang *et al*., 2012), which was not investigated in our study. The existence of

two major genotypes of Japanese FRCoV is also reminiscent of the serotype I and II FCoV. Without FRCoVs that can be grown in cell culture, however, such serologic differentiation will be difficult.

We also showed that 35.7% (5 of 14) of ferrets secreted FRCoVs in oral cavities, providing a route leading to infection of susceptible animals. CoVs are known to cause both respiratory and intestinal diseases in various animal species; therefore, FRCoVs should be investigated in respiratory disease.

4.6. Figure legends

Figure 4-1. Phylogenetic tree constructed on the basis of the nucleotide sequences of the partial RdRp–encoding regions of FRCoVs isolated in Japan (shown in boldface; sample numbers are indicated) compared with other CoVs. The tree was constructed by the neighbor-joining method in MEGA5.0 software (Tamura *et al*., 2011); bootstrap values of >90 are shown. DDBJ accession numbers for the nucleotide sequences are shown in parentheses. HCoVs 229E and NL63, which belong to the *Alphacoronavirus* genus, were used as the outgroup.

Figure 4-2. Phylogenetic tree based on the nucleotide sequences of partial S genes of FRCoVs isolated in Japan (shown in boldface; sample numbers are indicated) compared with other CoVs. The tree was constructed by the neighbor-joining method in MEGA5.0 software (Tamura *et al*., 2011); bootstrap values of >90 are shown. Asterisks indicate samples from ferrets infected with FRCoVs of both genotypes I and II. DDBJ accession numbers for the nucleotide sequences are shown in parentheses.

4.7. Figures and tables

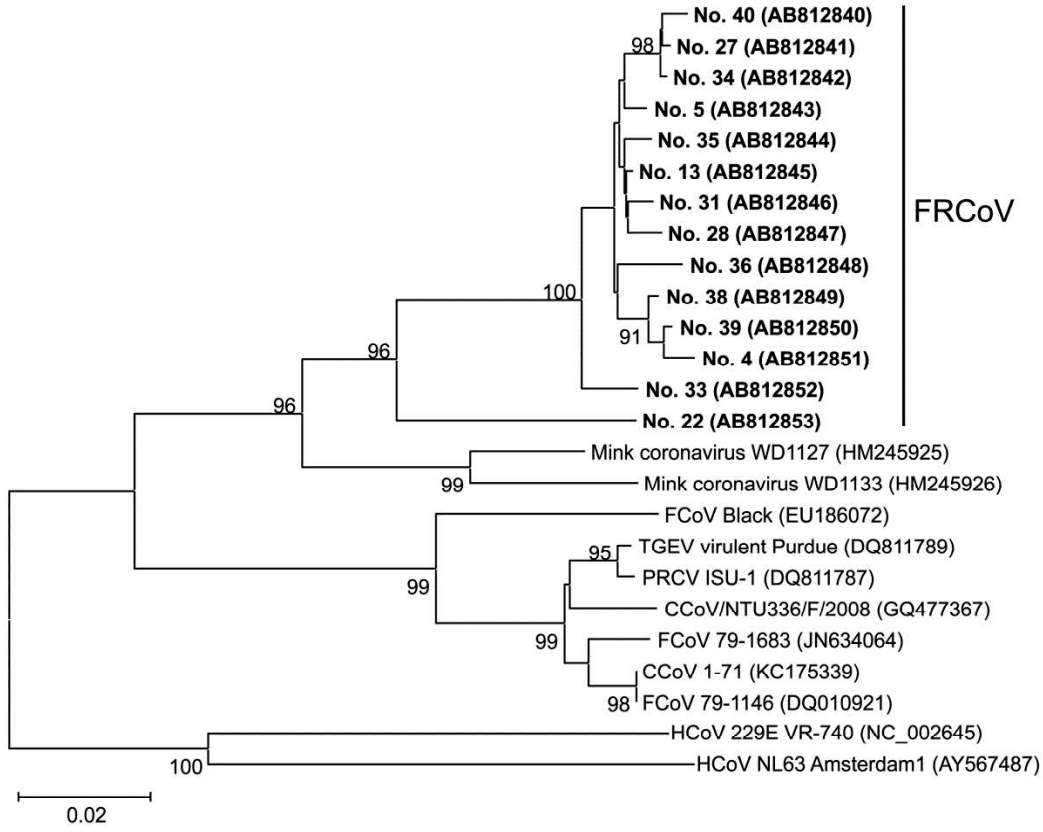

Figure 4-1 Phylogenetic tree constructed on the basis of the nucleotide sequences of the partial RdRp

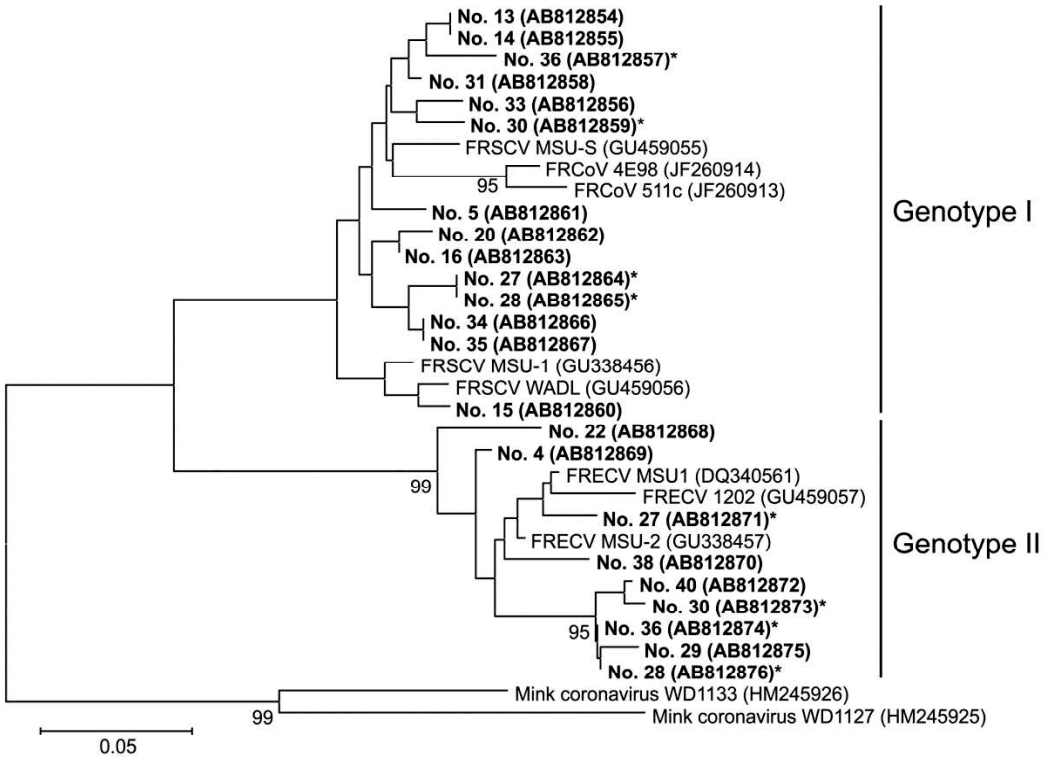

Figure 4-2 Phylogenetic tree based on the nucleotide sequences of partial S genes

Table 4-1. Detection of FRCoV from ferrets with clinical signs, Japan

Sample type	No. (%) samples			
	Diarrhea, n = 34	Hypergamma-globulinemia, n = 6	Abdominal mass, n = 14	Nonrelated signs/ asymptomatic, n = 33
All FRCoV-positive samples†	25 (73.5)	5 (83.3)	7 (50.0)	17 (51.5)
Genoype I samples‡	17 (50.0)	2 (33.3)	4 (28.6)	10 (30.3)
Genoype II samples§	7 (20.6)	1 (16.7)	4 (28.6)	7 (21.2)

*FRCov, ferret coronavirus; RT-PCR, reverse transcription PCR.

†RT-PCR was carried out by using FRCoV-specific primers.

‡RT-PCR was carried out by using type I FRCoV-specific primers (Wise *et al*., 2010).

§RT-PCR was carried out by using type II FRCoV-specific primers (Wise *et al*., 2010).

Table 4-2. Detection of FRCoV in ferret fecal samples by RT-PCR using coronavirus consensus and FRCoV-specific primers, Japan

Coronavirus consensus primers	FRCoV-specific primers		Total no. (%)
	No. positive samples	No. negative samples	
No. positive samples	31	2	33 (41.8)
No. negative samples	13	33	46 (58.2)
Total no. (%)	44 (55.7)	35 (44.3)	79

*FRCov, ferret coronavirus; RT-PCR, reverse transcription PCR.

5. General conclusion

The purpose of this thesis was to clarify the mechanism of CoV evolution. Author determined the nucleotide sequences of many strains of type I and II FCoV, type II CCoV and FRCoV, compared them and discussed on pathogenesis of natural mutant with a large deletion in S gene, the mechanism of emergence of type II FIPV and pathogenesis of two types of FRCoV.

In CHAPTER 1, the nucleotide sequences of type I FCoV C3663 and Yayoi were determined. In comparison with Yayoi, C3663 possessed 735nt deletion in 5'-terminus of S gene. Similar deletion was found not only in FCoV but also in PRCoV, suggesting that the frequency of the occurrence of the deletion in CoV must be high. Animal experiment using SPF cats showed that C3663 possessed high virulence to cats, indicating that this large deletion in S gene is not necessary for the pathogenesis of FIPV.

In CHAPTER 2, three strains of Japanese type II FCoV were genetically compared with two strains of Japanese type I FCoV and ten strains of Japanese type II CCoV. The results showed that all three type II FCoV emerged by homologous recombination and possessed different recombination sites in one another. Furthermore, it was clarified that parent viruses, type I FCoV and type II CCoV, were also different

among these type II FCoVs. These results clearly indicated that type II FCoV emerged independently. In addition, the results of VN tests showed the cats persistently infected with type I FCoV could not neutralized type II CCoV infection. These results suggested that type II FCoV emerged inside the cat body by homologous recombination independently and is unable to readily spread among cats.

In CHAPTER 3, a sensitive RT-PCR method for detection of FRCoV was constructed. The results of gene detection showed that more than 50% of ferrets in Japan were infected with FRCoV. Furthermore, it was also showed that both genotypes of FRCoV have been spreading within the ferret population in Japan. In addition, there is no significant relation between FRCoV infection and clinical disease.

6. Acknowledgments

The present studies were carried out at the Laboratory of Veterinary Microbiology, Joint Faculty of Veterinary Medicine, Yamaguchi University, Japan.

First of all, the author would like to show his greatest appreciation to his supervisor, **Dr. Ken Maeda** (Laboratory of Veterinary Microbiology, Yamaguchi University) for providing him this precious opportunity to study as a Ph. D. student with the invaluable support and advice.

The author is very grateful to all of his co-supervisors, **Dr. Masayuki Shimojima** (National Institute of Infectious Diseases) and **Dr. Rie Watanabe** (Laboratory of Veterinary Hygiene, Yamaguchi University) for giving him encourages and advices for his experiment, and **Dr. Yasuyuki Momoi** (Laboratory of Veterinary Diagnostic Imaging, Kagoshima University) for giving him insightful comments, suggestions and useful discussion, **Dr. Masami Mochizuki** (Laboratory of Emerging Infectious Diseases, Kagoshima University) and **Dr. Yumi Une** (Laboratory of Veterinary Pathology, Azabu University) for giving him many samples and viruses, useful suggestions and discussion on his experiments.

The author wants to thank to his laboratory members for their many supports in his experiments.

Finally, the author would like to thank his family for sincere supports.

7. References

Addie D, Belák S, Boucraut-Baralon C, Egberink H, Frymus T, Gruffydd-Jones T, Hartmann K, Hosie MJ, Lloret A, Lutz H, Marsilio F, Pennisi MG, Radford AD, Thiry E, Truyen U, Horzinek MC. (2009) Feline infectious peritonitis. ABCD guidelines on prevention and management. *J Feline Med Surg.* 11:594-604.

Addie DD, Schaap IA, Nicolson L, Jarrett O. (2003) Persistence and transmission of natural type I feline coronavirus infection. *J Gen Virol.* 84:2735-2744.

Bandai C, Ishiguro S, Masuya N, Hohdatsu T, Mochizuki M. (1999) Canine coronavirus infections in Japan: virological and epidemiological aspects. *J Vet Med Sci.* 61:731-736.

Barlough JE, Adsit JC, Scott FW. (1982) The worldwide occurrence of feline infectious peritonitis. *Feline Pract.* 12:26-30.

Benetka V, Kübber-Heiss A, Kolodziejek J, Nowotny N, Hofmann-Parisot M, Möstl K. (2004) Prevalence of feline coronavirus types I and II in cats with histopathologically verified feline infectious peritonitis. *Vet Microbiol*. 99:31-42.

Binn LN, Lazar EC, Keenan KP, Huxsoll DL, Marchwicki RH, Strano AJ. (1974) Recovery and characterization of a coronavirus from military dogs with diarrhea. *Proc Annu Meet U.S. Anim Health Assoc.* 78:359–366.

Bolles M, Donaldson E, Baric R. (2011) SARS-CoV and Emergent Coronaviruses: Viral Determinants of Interspecies Transmission. *Curr Opin Virol*. 1:624–634.

Bosch BJ, van der Zee R, de Haan CA, Rottier PJ. (2003) The coronavirus spike protein is a class I virus fusion protein: structural and functional characterization of the fusion core complex. *J Virol.* 77:8801-8811.

Buonavoglia C, Decaro N, Martella V, Elia G, Campolo M, Desario C, Castagnaro M, Tempesta M. (2006) Canine coronavirus highly pathogenic for dogs. *Emerg Infect Dis***.** 12:492-494.

Carmichael LE. (1978) Infectious canine enteritis caused by corona-like virus: current status and request for information. *Baker Institute Laboratory Report*. series 2, no. 9.

Cave TA, Thompson H, ReidS W, Hodgson DR, Addie DD. (2002) Kitten mortality in the United Kingdom: a retrospective analysis of 274 histopathological examinations (1986 to 2000). *Vet Rec.* 151: 497-501.

Chang HW, de Groot RJ, Egberink HF, Rottier PJ. (2010) Feline infectious peritonitis: insights into feline coronavirus pathobiogenesis and epidemiology based on genetic analysis of the viral 3c gene. *J Gen Virol.* 291:415-420.

Chang HW, Egberink HF, Halpin R, Spiro DJ, Rottier PJ. (2012) Spike protein fusion peptide and feline coronavirus virulence. *Emerg Infect Dis*. 18:1089-1095.

Christianson KK, Ingersoll JD, Landon RM, Pfeiffer NE, Gerber JD. (1989) Characterization of a temperature-sensitive feline infectious peritonitis coronavirus. *Arch Virol.* 108: 185-196.

Colby ED, Low RJ. (1970) Feline infectious peritonitis. *Vet Med Small Anim Clin.* 65: 783-786.

Colly LP. (1973) Feline infectious peritonitis. *Vet Clin North Am.* 3: 34.

Corapi WV, Olsen CW, Scott FW. (1992) Monoclonal antibody analysis of neutralization and antibody-dependent enhancement of feline infectious peritonitis virus. *J Virol.* 66: 6695–6705.

Cox E, Pensaert MB, Callebaut P, van Deun K. (1990) Intestinal replication of a porcine respiratory coronavirus closely related antigenically to the enteric transmissible gastroenteritis. *Vet Microbiol.* 23: 237-243.

Decaro N, Buonavoglia C. (2008) An update on canine coronaviruses: viral evolution and pathobiology. *Vet Microbiol*. 132:221-234.

Decaro N, Mari V, Campolo M, Lorusso A, Camero M, Elia G, Martella V, Cordioli P, Enjuanes L, Buonavoglia C. (2009) Recombinant canine coronaviruses related to transmissible gastroenteritis virus of Swine are circulating in dogs. *J Virol*. 83:1532-1537.

Decaro N, Mari V, Elia G, Addie DD, Camero M, Lucente MS, Martella V, Buonavoglia C. (2010a) Recombinant canine coronaviruses in dogs, Europe. *Emerg Infect Dis*. 16:41-47.

Decaro N, Elia G, Martella V, Campolo M, Mari V, Desario C, Lucente MS, Lorusso E, Kanellos T, Gibbons RH, Buonavoglia C. (2010b) Immunity after natural exposure to enteric canine coronavirus does not provide complete protection against infection with the new pantropic CB/05 strain. *Vaccine.* 28:724-729.

De Groot RJ, Van Leen RW, Dalderup MJ, Vennema H, Horzinek MC, Spaan WJ. (1989) Stably expressed FIPV peplomer protein induces cell fusion and elicits neutralizing antibodies in mice. *Virology.* 171:493-502.

Delmas B, Gelfi J, L'Haridon R, Vogel LK, Sjöström H, Norén O, Laude H. (1992) Aminopeptidase N is a major receptor for the entero-pathogenic coronavirus TGEV. *Nature.* 357:417-420.

Eckerle LD, Becker MM, Halpin RA, Li K, Venter E, Lu X, Scherbakova S, Graham RL, Baric RS, Stockwell TB, Spiro DJ, Denison MR. (2010) Infidelity of SARS-CoV Nsp14-exonuclease mutant virus replication is revealed by complete genome sequencing. *PLoS Pathog.* 6:e1000896.

Fehr D, Holznagel E, Bolla S, Hauser B, Herrewegh AA, Horzinek MC, Lutz H. (1997) Placebo-controlled evaluation of a modified life virus vaccine against feline infectious peritonitis: safety and efficacy under field conditions. *Vaccine.* 15: 1101-1109.

Fiscus SA, Teramoto YA. (1987) Antigenic comparison of feline coronavirus isolates: evidence for markedly different peplomer glycoproteins. *J Virol.* 61: 2607–2613.

Fleming JO, Shubin RA, Sussman MA, Casteel N, Stohlman SA. (1989) Monoclonal antibodies to the matrix (E1) glycoprotein of mouse hepatitis virus protect mice from encephalitis. *Virology.* 168:162-167

Fowler ME. (1978) Zoo and wild animal medicine. *Philadelphia: WB Saunders:* 660

Garner MM, Ramsell K, Morera N, Juan-Sallés C, Jiménez J, Ardiaca M, Montesinos A, Teifke JP, Löhr CV, Evermann JF, Baszler TV, Nordhausen RW, Wise AG, Maes RK, Kiupel M. (2008) Clinicopathologic features of a systemic coronavirus-associated disease resembling feline infectious peritonitis in the domestic ferret (*Mustela putorius*). *Vet Pathol.* 45:236–246.

Gerber JD, Ingersoll JD, Gast AM, Christianson KK, Selzer NL, Landon RM, Pfeiffer NE, Sharpee RL, Beckenhauer WH. (1990) Protection against feline infectious peritonitis by intranasal inocu- lation of a temperature-sensitive FIPV vaccine. *Vaccine.* 8: 536-542.

Goodhead AD. (1996) Uveitis in dogs and cats: guidelines for the practitioner. *J S Afr Vet Assoc.* 67:12-19.

Graham RL, Baric RS. (2010) Recombination, reservoirs, and the modular spike: mechanisms of coronavirus cross-species transmission. *J Virol.* 84:3134-3146.

Graham E, Lamm C, Denk D, Stidworthy MF, Carrasco DC, Kubiak M. (2012) Systemic coronavirus-associated disease resembling feline infectious peritonitis in ferrets in the UK. *Vet Rec.* 171:200–201.

Hartmann K, Binder C, Hirschberger J, Cole D, Reinacher M, Schroo S, Frost J, Egberink H, Lutz H, Hermanns W. (2003) Comparison of different tests to diagnose feline infectious peritonitis. *J Vet Intern Med.* 17: 781-789.

Hartmann K. (2005) Feline infectious peritonitis. *Vet Clin North Am Small Anim Pract.* 35: 39–79.

Hayashi T, Yanai T, Tsurudome M, Nakayama H, Watabe Y, Fujiwara K. (1981) Serodiagnosis for feline infectious peritonitis by immunofluorescence using infected suckling mouse brain sections. *Jpn J Vet Sci.* 43: 669–676.

Herrewegh AA, Smeenk I, Horzinek MC, Rottier PJ, de Groot RJ. (1998) Feline coronavirus type II strains 79-1683 and 79-1146 originate from a double recombination between feline coronavirus type I and canine coronavirus. *J Virol.*

72:4508-4514.

Hohdatsu T, Okada S, Koyama H. (1991a) Characterization of monoclonal antibodies against feline infectious peritonitis virus type II and antigenic relationship between feline, porcine, and canine coronaviruses. *Arch Virol.* 117: 85–95.

Hohdatsu T, Sasamoto T, Okada S, Koyama H. (1991b) Antigenic analysis of feline coronaviruses with monoclonal antibodies (MAbs): preparation of MAbs which discriminate between FIPV strain 79-1146 and FECV strain 79-1683. *Vet Microbiol.* 28: 13–24.

Hohdatsu T, Okada S, Ishizuka Y, Yamada H, Koyama H. (1992) The prevalence of types I and II feline coronavirus infections in cats. *J Vet Med Sci.* 54: 557–562.

Hohdatsu T, Izumiya Y, Yokoyama Y, Kida K, Koyama H. (1998) Differences in virus receptor for type I and type II feline infectious peritonitis virus. *Arch Virol*. 143:839-850.

Holzworth JE. (1963) Some important disorders of cats. *Cornell Vet.* 53: 157-160.

Jacobse-Geels HE, Horzinek MC. (1983) Expression of feline infectious peritonitis coronavirus antigens on the surface of feline macrophage-like cells. *J Gen Virol.* 64: 1859–1866.

Juan-Sallés C, Domingo M, Herráez P, Fernández A, Segalés J, Fernández J. (1998) Feline infectious peritonitis in servals (Felis serval). *Vet Rec.* 143: 535-536.

Kennedy M, Boedeker N, Gibbs P, Kania S. (2001) Deletions in the 7a ORF of feline coronavirus associated with an epidemic of feline infectious peritonitis. *Vet Microbiol.* 81: 227-234.

Kida K, Hohdatsu T, Fujii K, Koyama H. (1999) Selection of antigenic variants of the S glycoprotein of feline infectious peritonitis virus and analysis of antigenic sites involved in neutralization. *J Vet Med Sci.* 61:935-938.

Kiss I, Poland AM, Pedersen NC. (2004) Disease outcome and cytokine responses in cats immunized with an avirulent feline infectious peritonitis virus (FIPV)-UCD1 and challenge-exposed with virulent FIPV-UCD8. *J Feline Med Surg.* 6: 89-97.

Klepfer S, Reed AP, Martinez M, Bhogal B, Jones E, Miller TJ. (1995) Cloning and expression of FECV spike gene in vaccinia virus. Immunization with FECVS causes early death after FIPV challenge. *Adv Exp Med Biol.* 380: 235-241.

Kocherhans R, Bridgen A, Ackermann M, Tobler K. (2001) Completion of the porcine epidemic diarrhoea coronavirus (PEDV) genome sequence. *Virus Genes.* 23:137-144.

Kummrow M, Meli ML, Haessig M, Goenczi E, Poland A, Pedersen NC, Hofmann-Lehmann R, Lutz H. (2005) Feline coronavirus serotypes 1 and 2:

seroprevalence and association with disease in Switzerland. *Clin Diagn Lab Immunol*. 12:1209-1215.

Lai MM, Baric RS, Makino S, Keck JG, Egbert J, Leibowitz JL, Stohlman SA. (1985) Recombination between nonsegmented RNA genomes of murine coronaviruses. *J Virol.* 56:449-456.

Li J, Ulitzky L, Silberstein E, Taylor DR, Viscidi R. (2013) Immunogenicity and protection efficacy of monomeric and trimeric recombinant SARS coronavirus spike protein subunit vaccine candidates. *Viral Immunol*. 26:126-132.

Licitra BN, Millet JK, Regan AD, Hamilton BS, Rinaldi VD, Duhamel GE, Whittaker GR. (2013) Mutation in spike protein cleavage site and pathogenesis of feline coronavirus. *Emerg Infect Dis*. 19:1066-1073.

Lin CN, Su BL, Huang HP, Lee JJ, Hsieh MW, Chueh LL. (2009) Field strain feline coronaviruses with small deletions in ORF7b associated with both enteric infection and feline infectious peritonitis. *J Feline Med Surg*. 11:413-419.

Lin CN, Chang RY, Su BL, Chueh LL. (2013) Full genome analysis of a novel type II feline coronavirus NTU156. *Virus Genes.* 46:316-322.

Lole KS, Bollinger RC, Paranjape RS, Gadkari D, Kulkarni SS, Novak NG, Ingersoll R, Sheppard HW, Ray SC. (1999) Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. *J Virol.* 73:152-160.

Lorusso A, Decaro N, Schellen P, Rottier PJ, Buonavoglia C, Haijema BJ, de Groot RJ. (2008) Gain, preservation, and loss of a group 1a coronavirus accessory glycoprotein. *J Virol.* 82:10312-10317.

Luytjes W, Bredenbeek PJ, Noten AF, Horzinek MC, Spaan WJ. (1988) Sequence of mouse hepatitis virus A59 mRNA 2: indications for RNA recombination between coronaviruses and influenza C virus. *Virology*. 166:415-422.

McArdle F, Bennett M, Gaskell RM, Tennant B, Kelly DF, Gaskell CJ. (1990) Canine coronavirus infection in cats; a possible role in feline infectious peritonitis. *Adv Exp Med Biol.* 276:475-479.

Makino S, Joo M, Makino JK. (1991) A system for study of coronavirus mRNA synthesis: a regulated, expressed subgenomic defective interfering RNA results from intergenic site insertion. *J Virol.* 65:6031-6041.

Martínez J, Ramis AJ, Reinacher M, Perpiñán D. (2006) Detection of feline infectious peritonitis virus–like antigen in ferrets. *Vet Rec.*158:523.

Martínez J, Reinacher M, Perpiñán D, Ramis A. (2008) Identification of group 1 coronavirus antigen in multisystemic granulomatous lesions in ferrets (*Mustela putorius furo*). *J Comp Pathol*. 138:54–58.

Masters PS. (2006) The molecular biology of coronaviruses. *Adv Virus Res.* 66: 193-292.

Meli M, Kipar A, Müller C, Jenal K, Gönczi E, Borel N, Gunn-Moore D, Chalmers S, Lin F, Reinacher M, Lutz H. (2004) High viral loads despite absence of clinical and pathological findings in cats experimentally infected with feline coronavirus (FCoV) type I and in naturally FCoV-infected cats. *J Feline Med Surg.* 6:69-81.

Michimae Y, Mikami S, Okimoto K, Toyosawa K, Matsumoto I, Kouchi M, Koujitani T, Inoue T, Seki T. (2010) The first case of feline infectious peritonitis–like pyogranuloma in a ferret infected by coronavirus in Japan. *J Toxicol Pathol.* 23:99–101.

Mochizuki M, Nakatani H, Yoshida M. (1994) Inhibitory effects of recombinant feline interferon on the replication of feline enteropathogenic viruses in vitro. *Vet Microbiol.* 139(1-2):145-152.

Mochizuki M, Mitsutake Y, Miyanohara Y, Higashihara T, Shimizu T, Hohdatsu T. (1997) Antigenic and plaque variations of serotype II feline infectious peritonitis coronaviruses. *J Vet Med Sci.* 59: 253–258.

Mochizuki M, Hashimoto M, Ishida T. (2001) Recent epidemiological status of canine viral enteric infections and Giardia infection in Japan. *J Vet Med Sci*. 63:573-575.

Montali RJ, Strandberg JD. (1972) Extraperitoneal lesions in feline infectious peritonitis. *Vet Pathol.* 9: 109-121.

Motokawa K, Hohdatsu T, Hashimoto H, Koyama H. (1996) Comparison of the amino acid sequence and phylogenetic analysis of the peplomer, integral membrane and nucleocapsid proteins of feline, canine and porcine coronaviruses. *Microbiol Immunol.* 40: 425-433.

Murray J, Kiupel M, Maes RK. (2010) Ferret coronavirus-associated diseases. *Vet Clin North Am Exot Anim Pract.* 13:543-560.

Naylor MJ, Monckton RP, Lehrbach PR, Deane EM. (2001) Canine coronavirus in Australian dogs. *Aust Vet J*. 79:116-119.

Naylor MJ, Walia CS, McOrist S, Lehrbach PR, Deane EM, Harrison GA. (2002) Molecular characterization confirms the presence of a divergent strain of canine coronavirus (UWSMN-1) in Australia. *J Clin Microbiol.* 40:3518-3522.

Olsen CW, Corapi WV, Ngichabe CK, Baines JD, Scott FW. (1992) Monoclonal antibodies to the spike protein of feline infectious peritonitis virus mediate antibody-dependent enhancement of infection of feline macrophages. *J Virol.* 66: 956–965.

Olsen CW. (1993) A review of feline infectious peritonitis virus: molecular biology, immunopathogenesis, clinical aspects, and vaccination. *Vet Microbiol*. 36:1-37.

Paltrinieri S, Cammarata MP, Cammarata G, Comazzi S. (1998) Some aspects of humoral and cellular immunity in naturally occuring feline infectious peritonitis. *Vet Immunol Immunopathol.* 65:205-220.

Paltrinieri S, Grieco V, Comazzi S, Cammarata Parodi M. (2001) Laboratory profiles in cats with different pathological and immunohistochemical findings due to feline infectious peritonitis (FIP). *J Feline Med Surg*. 3:149-159.

Pasternak AO, Spaan WJ, Snijder EJ. (2006) Nidovirus transcription: how to make sense...? *J Gen Virol*. 87:1403-1421.

Pedersen NC. (1976) Serologic studies of naturally occurring feline infectious peritonitis. *Am J Vet Res.* 37:1449-1453.

Pedersen NC, Ward J, Mengeling WL. (1978) Antigenic relationship of the feline infections peritonitis virus to coronaviruses of other species. *Arch Virol.* 58:45-53.

Pedersen NC, Boyle JF, Floyd K, Fudge A, Barker J. (1981) An enteric coronavirus infection of cats and its relationship to feline infectious peritonitis. *Am J Vet Res.* 42: 368–377.

Pedersen NC. (1983) Feline infectious peritonitis and feline enteric coronavirus infections. Part I: feline enteric coronavirus. *Feline Pract.* 13:13-19.

Pedersen NC, Black JW. (1983) Attempted immunization of cats against feline infectious peritonitis, using avirulent live virus or sublethal amounts of virulent virus. *Am J Vet Res.* 44: 229-234.

Pedersen NC, Black JW, Boyle JF, Evermann JF, McKeirnan AJ, Ott RL. (1984a) Pathogenic differences between various feline coronavirus isolates. Coronaviruses; molecular biology and pathogenesis. *Adv Exp Med Biol.* 173: 365-380.

Pedersen NC, Evermann JF, McKeirnan AJ, Ott RL. (1984b) Pathogenicity studies of feline coronavirus isolates 79-1146 and 79-1683. *Am J Vet Res*. 45:2580-2585.

Pedersen NC, Floyd K. (1985) Experimental studies with three new strains of feline infectious peritonitis virus FIPV-UCD2, FIPV-UCD3, and FIPV-UCD4. *Compend Cont Educ Pract Vet.* 7:1001-1011.

Pedersen NC. (1987) Virologic and immunologic aspects of feline infectious peritonitis virus infection. *Adv Exp Med Biol.* 218: 529–550.

Pedersen NC, Addie D, Wolf A. (1995) Recommendations from working group of the international coronavirus and feline infectious peritonitis workshop. *Feline Pract.* 23:108-111.

Pedersen NC, Allen CE, Lyons LA. (2008) Pathogenesis of feline enteric coronavirus infection. *J Feline Med Surg.* 10: 529-541.

Pedersen NC, Liu H, Dodd KA, Pesavento PA. (2009) Significance of coronavirus mutants in feces and diseased tissues of cats suffering from feline infectious peritonitis. *Viruses.* 1:166-184.

Pedersen NC. (2009) A review of feline infectious peritonitis virus infection: 1963-2008. *J Feline Med Surg*. 11:225-258.

Pedersen NC, Liu H, Scarlett J, Leutenegger CM, Golovko L, Kennedy H, Kamal FM. (2012) Feline infectious peritonitis: role of the feline coronavirus 3c gene in intestinal tropism and pathogenicity based upon isolates from resident and adopted shelter cats. *Virus Res.*165:17-28.

Pedersen NC. (2014) An update on feline infectious peritonitis: virology and immunopathogenesis. *Vet J.* [Epub ahead of print].

Peiffer Jr RL,Wilcock BP. (1991) Histopathologic study of uveitis in cats: 139 cases (1978-1988). *J Am Vet Med Assoc.* 198:135-138.

Perlman S, Netland J. (2009) Coronaviruses post-SARS: update on replication and pathogenesis. *Nat Rev Microbiol*. 7:439-450.

Pesteanu-Somogyi LD, Radzai C, Pressler BM. (2006) Prevalene of feline infectious peritonitis in specific cat breeds. *J Feline Med Surg.* 8:1-5.

Poelma FG, Peters JC, Mieog WHW, Zwart P. (1974) Infectise Peritonitiss bei Karakal (Felis caracal) and Nordluchs (Felis lynx lynx). *Proceedings Erkrankungen der Zootiere, 13th International Symposium of Zoo Veterinarians; Helsinki, Finland* 249-253.

Poon LL, Chu DK, Chan KH, Wong OK, Ellis TM, Leung YH, Lau SK, Woo PC, Suen KY, Yuen KY, Guan Y, Peiris JS. (2005) Identification of a novel coronavirus in bats. *J Virol.* 79(4):2001-2009.

Pratelli A, Martella V, Decaro N, Tinelli A, Camero M, Cirone F, Elia G, Cavalli A, Corrente M, Greco G, Buonavoglia D, Gentile M, Tempesta M, Buonavoglia C. (2003) Genetic diversity of a canine coronavirus detected in pups with diarrhoea in Italy. *J Virol Methods*. 110:9-17.

Pratelli A, Decaro N, Tinelli A, Martella V, Elia G, Tempesta M, Cirone F, Buonavoglia C. (2004) Two genotypes of canine coronavirus simultaneously detected in the fecal samples of dogs with diarrhea. *J Clin Microbiol.* 42:1797-1799.

Pratelli A. (2008) Comparison of serologic techniques for the detection of antibodies against feline coronaviruses. *J Vet Diagn Invest.* 20: 45-50.

Provacia LB, Smits SL, Martina BE, Raj VS, Doel PV, Amerongen GV, Moorman-Roest H, Osterhaus AD, Haagmans BL. (2011) Enteric coronavirus in ferrets, the Netherlands. *Emerg Infect Dis.* 17:1570–1571.

Rimmelzwaan GF, Groen J, Egberink H, Borst GH, UytdeHaag FG, Osterhaus AD. (1991) The use of enzyme-linked immunosorbent assay systems for serology and antigen detec- tion in parvovirus, coronavirus and rotavirus infections in dogs in The Netherlands. *Vet Microbiol.* 26:25-40.

Ritz S, Egberink H, Hartmann K. (2007) Effect of feline interferon-omega on the survival time and quality of life of cats with feline infectious peritonitis. *J Vet Intern Med.* 21:1193-1197.

Rohrer C, Suter PF, Lutz H. (1993) The diagnosis of feline infectious peritonitis (FIP): a retrospective and prospective study. *Kleintierpraxis.* 38: 379-383.

Saitou N, Nei M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol.* 4:406-425.

Schultze B, Krempl C, Ballesteros ML, Shaw L, Schauer R, Enjuanes L, Herrler G. (1996) Transmissible gastroenteritis coronavirus, but not the related porcine respiratory coronavirus, has a sialic acid (N-glycolylneuraminic acid) binding activity. *J Virol.* 70:5634-5637.

Schulz BS, Strauch C, Mueller RS, Eichhorn W, Hartmann K. (2008) Comparison of the prevalence of enteric viruses in healthy dogs and those with acute haemorrhagic diarrhoea by electron microscopy. *J Small Anim Pract.* 49:84-88.

Sheahan T, Rockx B, Donaldson E, Corti D, Baric R. (2008) Pathways of cross-species transmission of synthetically reconstructed zoonotic severe acute respiratory syndrome coronavirus. *J Virol.* 82:8721-8732.

Shiba N, Maeda K, Kato H, Mochizuki M, Iwata H. (2007) Differentiation of feline coronavirus type I and II infections by virus neutralization test. *Vet Microbiol.* 124: 348-352.

Soma T, Ohinata T, Ishii H, Takahashi T, Taharaguchi S, Hara M. (2011) Detection and genotyping of canine coronavirus RNA in diarrheic dogs in Japan. *Res Vet Sci*. 90: 205–207.

Sparkes AH, Gruffydd-Jones TJ, Harbour DA. (1991) Feline infectious peritonitis: a review of clinicopathological changes in 65 cases, and a critical assessment of their diagnostic value. *Vet Rec.* 129:209-212.

Sparkes AH, Gruffydd-Jones TJ, Harbour DA. (1994) An appraisal of the value of laboratory tests in the diagnosis of feline infectious peritonitis. *J Am Anim Hosp Assoc.* 30:345-350.

Takano T, Tomiyama Y, Katoh Y, Nakamura M, Satoh R, Hohdatsu T. (2011) Mutation of neutralizing/antibody-dependent enhancing epitope on spike protein and 7b gene of feline infectious peritonitis virus: influences of viral replication in monocytes/macrophages and virulence in cats. *Virus Res.* 156:72-80.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. (2011) MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol Biol Evol* 28: 2731-2739.

Tanaka Y, Sato Y, Osawa S, Inoue M, Tanaka S, Sasaki T. (2012) Suppression of feline coronavirus replication in vitro by cyclosporin A. *Vet Res*. 43:41.

Tanaka Y, Sato Y, Sasaki T. (2013) Suppression of coronavirus replication by cyclophilin inhibitors. *Viruses.* 5:1250-1260.

Tekes G, Hofmann-Lehmann R, Bank-Wolf B, Maier R, Thiel HJ, Thiel V. (2010) Chimeric feline coronaviruses that encode type II spike protein on type I genetic background display accelerated viral growth and altered receptor usage. *J Virol.* 84:1326-1333.

Tennant BJ, Gaskell RM, Jones RC, Gaskell CJ. (1993) Studies on the epizootiology of canine coronavirus. *Vet Rec*. 132:7-11.

Terada Y, Shiozaki Y, Shimoda H, Mahmoud HY, Noguchi K, Nagao Y, Shimojima M, Iwata H, Mizuno T, Okuda M, Morimoto M, Hayashi T, Tanaka Y, Mochizuki M, Maeda K. (2012) Feline infectious peritonitis virus with a large deletion in the 5' terminal region of spike gene retains its virulence for cats. *J Gen Virol.* 93:1930-1934.

Theobald J. In: Fowler ME, ed. (1978) Zoo and wild animal medicine. *Philadelphia: WB Saunders*: 650-667

Tresnan DB, Levis R, Holmes KV. (1996) Feline aminopeptidase N serves as a receptor for feline, canine, porcine, and human coronaviruses in serogroup I. *J Virol.* 70:8669-8674.

Vennema H, de Groot RJ, Harbour DA, Horzinek MC, Spaan WJ. (1990) Early death after feline infectious peritonitis virus challenge due to recombinant vaccinia virus immunization. *J Virol.* 64:1407-1409.

Vennema H, de Groot RJ, Harbour DA, Horzinek MC, Spaan WJ. (1991) Primary structure of the membrane and nucleocapsid protein genes of feline infectious peritonitis virus and immunogenicity of recombinant vaccinia viruses in kittens. *Virology*. 181:327-335.

Vennema H, Rossen JW, Wesseling J, Horzinek MC, Rottier PJ. (1992) Genomic organization and expression of the 3' end of the canine and feline enteric coronaviruses. *Virology.* 191:134-140.

Vennema H, Poland A, Foley J, Pedersen NC. (1998) Feline infectious peritonitis viruses arise by mutation from endemic feline enteric coronaviruses. *Virology.* 243:150-157.

Vennema H. (1999) Genetic drift and genetic shift during feline coronavirus evolution. *Vet Microbiol*. 69:139-141.

Vermeulen BL, Devriendt B, Olyslaegers DA, Dedeurwaerder A, Desmarets LM, Favoreel HW, Dewerchin HL, Nauwynck HJ. (2013) Suppression of NK cells and regulatory T lymphocytes in cats naturally infected with feline infectious peritonitis virus. *Vet Microbiol*. 164:46-59.

Vijgen L, Keyaerts E, Moës E, Thoelen I, Wollants E, Lemey P, Vandamme AM, Van Ranst M. (2005) Complete genomic sequence of human coronavirus OC43: molecular clock analysis suggests a relatively recent zoonotic coronavirus transmission event. *J Virol.* 79:1595-1604.

Wang YY, Lu CP. (2009) Analysis of putative recombination hot sites in the S gene of canine coronaviruses. *Acta Virol*. 53:111-112.

Wang YT, Su BL, Hsieh LE, Chueh LL. (2013) An outbreak of feline infectious peritonitis in a Taiwanese shelter: epidemiologic and molecular evidence for horizontal transmission of a novel type II feline coronavirus. *Vet Res*. 44:57.

Watt NJ, MacIntyre NJ, McOrist S. (1993) An extended outbreak of infectious peritonitis in a closed colony of European wildcats (Felis sylvestris). *J Comp Pathol.* 108:73-79.

Weiss SR, Navas-Martin S. (2005) Coronavirus pathogenesis and the emerging pathogen severe acute respiratory syndrome coronavirus. *Microbiol Mol Biol Rev*. 69:635-664.

Wesley RD, Woods RD, Cheung AK. (1991) Genetic analysis of porcine respiratory coronavirus, an attenuated variant of transmissible gastroenteritis virus. *J Virol.* 65: 3369–3373.

Williams BH, Kiupel M, West KH, Raymond JT, Grant CK, Glickman LT. (2000) Coronavirus-associated epizootic catarrhal enteritis in ferrets. *J Am Vet Med Assoc*. 217: 526–530.

Wise AG, Kiupel M, Maes RK. (2006) Molecular characterization of a novel coronavirus associated with epizootic catarrhal enteritis (ECE) in ferrets. *Virology*. 349:164–174.

Wise AG, Kiupel M, Garner MM, Clark AK, Maes RK. (2010) Comparative sequence analysis of the distal one-third of the genomes of a systemic and an enteric ferret coronavirus. *Virus Res.* 149:42–50.

Woo PC, Huang Y, Lau SK, Yuen KY. (2010) Coronavirus genomics and bioinformatics analysis. *Viruses*. 2:1804-1820.

Yeşilbağ K, Yilmaz Z, Torun S, Pratelli A. (2004) Canine coronavirus infection in Turkish dog population. *J Vet Med B Infect Dis Vet Public Health.* 51:353-355.

Zeng Q, Langereis MA, van Vliet AL, Huizinga EG, de Groot RJ. (2008) Structure of coronavirus hemagglutinin-esterase offers insight into corona and influenza virus evolution. *Proc Natl Acad Sci USA.* 105:9065-9069.

Zook BC, KingNW, Robinson RL, McCombs HL. (1968) Ultrastructural evidence for the viral etiology of feline infectious peritonitis. *Pathol Vet.* 5:91-95.

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9. Publication list

9.1 Original papers

Terada Y, Matsui N, Noguchi K, Kuwata R, Shimoda H, Soma T, Mochizuki M, Maeda K*. Emergence of Pathogenic Coronaviruses in Cats by Homologous Recombination between Feline and Canine Coronaviruses. *PLoS One* 2014 9(9):e106534.

Shimoda H, Saito A, Noguchi K, **Terada Y**, Kuwata R, Akari H, Takasaki T, Maeda K*. Seroprevalence of Japanese encephalitis virus infection in Japanese macaques (*Macaca fuscata*). *Primates* 2014 55(3):441-445

Hara Y, **Terada Y**, Yonemitsu K, Shimoda H, Noguchi K, Suzuki K, Maeda K*. High Prevalence of Hepatitis E Virus in Wild Boar in Yamaguchi Prefecture, Japan. *Journal of Wildlife Diseases* 2014 50(2):378–383.

Terada Y, Minami S, Noguchi K, Mahmoud H.Y.A.H., Shimoda H, Mochizuki M, Une Y, Maeda K*. Genetic characterization of coronaviruses from domestic ferrets in Japan. *Emerging Infectious Diseases* 2014 20(2):284-287.

Mahmoud HY, Andoh K, Hattori S, **Terada Y**, Noguchi K, Shimoda H, Maeda K*. Characterization of Glycoproteins in Equine Herpesvirus-1. *Journal of Veterinary Medical Science* 2013 75(10):1317-21.

Hara Y, Suzuki J, Noguchi K, **Terada Y**, Shimoda H, Mizuno T, Maeda K*. Function of feline signaling lymphocyte activation molecule as a receptor of canine distemper virus. *Journal of Veterinary Medical Science* 2013 75(8):1085-1089.

Shimoda H, Mahmoud HYAH, Noguchi K, **Terada Y**, Takasaki T, Shimojima M, Maeda K*. Production and characterization of monoclonal antibodies to Japanese encephalitis virus. *Journal of Veterinary Medical Science* 2013 75(8):1077-1080.

Andoh K, Takasugi M, Mahmoud HYAH, Hattori S, **Terada Y,** Noguchi K, Shimoda H, Bannai H, Tsujimura K, Matsumura T, Kondo T, Maeda K*. Identification of a major immunogenic region of equine herpesvirus-1 glycoprotein E and its application to enzyme-linked immunosorbent assay. *Veterinary Microbiology* 2013 164(1-2):18-26.

Noguchi K, Shimoda H, **Terada Y**, Shimojima M, Kohyama K, Inoshima Y, Maeda K*. Isolation of a novel herpesvirus from a Pacific white-sided dolphin. *Archives of Virology* 2013 158(3): 695-699.

Shimoda H, Inthong N, Noguchi K, **Terada Y**, Nagao Y, Shimojima M, Takasaki T, Rerkamnuaychoke W, Maeda K*. Development and application of an indirect enzyme-linked immunosorbent assay for serological survey of Japanese encephalitis virus infection in dogs. *Journal of Virological Methods* 2013 187(1):85-89.

Terada Y, Shiozaki Y, Shimoda H, Mahmoud HYA, Noguchi K, Nagao Y, Shimojima M, Iwata H, Mizuno T, Okuda M, Morimoto M, Hayashi T, Tanaka Y, Mochizuki M, Maeda K*. Feline infectious peritonitis virus with a large deletion in the 5' terminal region of spike gene retains its virulence for cats. *Journal of General Virology* 2012 93: 1930-1934.

9.2 Review

寺田　豊、前田　健 :「フェレットコロナウイルス感染症」*The Japanese Society of Exotic Pet Medicine* (JSEPM) (寄稿) 2013: 15:1-10.

鈴木絢子、秋山今日子、西尾陽平、田丸精治、亀尾由紀、中野仁志、野口慧多、**寺田　豊**、下田　宙、鈴木和男、渡部　孝、吉澤未来、後藤　慈、佐藤　梓、池辺祐介、佐藤　宏、前田　健: Recent endemic of canine distemper virus（イヌジステンパーウイルスの最近の流行）*The Yamaguchi Journal of Veterinary Medicine* (山口獣医学会誌) (総説) 2012 39: 1-12

学位論文要旨
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Studies on mechanisms of evolution of coronaviruses
コロナウイルスの進化メカニズムの解析

コロナウイルス(CoV)はニドウイルス目コロナウイルス科に属するウイルスである。CoV は人を含む多くの哺乳類や鳥類に感染し、呼吸器症状や消化器症状を引き起こす。近年では重症急性呼吸器症候群(SARS)CoV や中東呼吸器症候群(MERS)CoV といった動物由来の新興 CoV 感染症が大きな問題となっている。

CoV は RNA 依存性 RNA ポリメラーゼ(RdRp)の不正確さや独特な転写機構など、様々な性状から遺伝子変異や組換えが非常に起きやすいウイルスである。そしてこれらの現象は CoV の連続的あるいは非連続的進化を促進し、その結果として宿主域や病原性の劇的な変化を引き起こす。

これまでの CoV 研究の多くは、自然宿主と異なる動物種を用いているが、CoV の進化と宿主域・病原性との関連を厳密に調べるためには、自然宿主において解析する必要がある。そこで本研究では、猫及びフェレットに致死的疾患を引き起こす猫コロナウイルス(FCoV)及びフェレットコロナウイルス(FRCoV)をその自然宿主である猫及びフェレットを用いて解析することにより、CoV の進化と宿主域・病原性との関連を調査した。更に、その進化のメカニズムを解明し、新興コロナウイルスの出現機序を解明に向けた基礎データの蓄積を目的とした。本研究は以下の 3 章より構成される。

第 1 章　I 型 FCoV C3663 株が有する S 遺伝子の巨大な欠損とその病原性の解析

FCoV は猫に対する病原性の違いから 2 種類に分類される。ひとつは感染してもその多くが無症状であり、たまに軽度の腸炎を引き起こす猫腸コロナウイルス(FECV)で、もうひとつは猫に致死性疾患である猫伝染性腹膜炎 (FIP) を引き起こす FIP ウイルス(FIPV)である。さらに FCoV はスパイク(S)遺伝子の特徴により I 型と II 型に分類されている。野外の FCoV の多くが I 型 FCoV であるにも関わらず、*in vitro* での増殖が良く、感染実験で FIP を再現することが容易な II 型 FCoV を用いた研究が数多く実施されている。本章では、I 型 FCoV を用い

たFIP研究の動物モデルの構築を目的として、I型FCoV C3663株の遺伝子解析とともに猫に対する病原性を検討した。

まず、I型FCoV C3663株のS遺伝子及びその下流8,170塩基と同じI型FCoVであるYayoi株のS遺伝子4,404塩基の塩基配列を決定した。C3663株とYayoi株のS遺伝子の塩基配列を比較した結果、C3663株S遺伝子内の5'末端領域に245 アミノ酸をコードする 735 塩基の巨大な遺伝子欠損が認められた。次にC3663株の猫に対する病原性を検討するため、SPF猫(6ヶ月齢、♂)4頭にC3663株 3.9×10^{6}PFU/10ml を経口投与した。その結果、C3663株接種猫の4頭中2頭が感染後21日と37日にFIPで死亡し、他の2頭の内1頭が感染後91日目の剖検時にFIP特有の病変が認められた（発症率75%)。即ち、欠損を有するC3663株は非常に強い病原性を有することが判明した。

C3663株のS遺伝子の欠損と同様の欠損は豚伝染性胃腸炎ウイルス(TGEV)と豚呼吸器CoV(PRCoV)の間にも認められており、このような欠損はCoVでは比較的起きやすい変異と考えられた。本研究により、C3663株を用いたI型FCoVによるFIP感染実験系の確立に成功し、さらにS遺伝子のダイナミックな変異と病原性の関連性が示された。

第2章　II型猫コロナウイルスの出現機序の解明

FCoVは主にS遺伝子の違いからウイルス学的にI型及びII型FCoVに分けられる。そして、II型FCoVは、I型FCoVとII型犬コロナウイルス（CCoV）との組換えによって出現すると考えられているが、そのメカニズムなどの詳細はいまだ十分には調べられていない。本章ではII型FCoVの出現メカニズムを解明することを目的に、国内分離株であるII型FCoV 3株とII型CCoV 1株のRdRp遺伝子の一部とS遺伝子及びその下流の塩基配列を決定した。さらに、II型CCoV 9株のRdRp遺伝子の一部、S遺伝子の一部及びヌクレオカプシド(N)遺伝子全長を決定し、第1章で塩基配列を決定したI型FCoV C3663株及びYayoi株とともに比較解析を実施した。

RdRp遺伝子を基に作成した系統樹により、II型FCoVのRdRp領域はウイルス株によってI型FCoVまたはII型CCoVに由来した。すなわち、各II型FCoVの組換え領域はこのRdRp遺伝子前後の異なる部位に存在することが判明した。S遺伝子を基にした系統樹により、II型FCoVはCCoV群の間にランダムに入っていることが示され、N遺伝子を基にした系統樹により、II型FCoVはI型FCoV群の間にランダムに入っていることが示された。このことから各II型FCoVはそれぞれ異なるI型FCoV及びII型CCoVに由来することが判明した。

3株のII型FCoVをI型FCoV C3663株及びII型CCoV fc1株とともに比較した結果、2株(M91-267, KUK-H/L 株)はメンブレン遺伝子のそれぞれ異なる部位

で、1 株(Tokyo/cat/130627)は S 遺伝子の 3’末端で組換えを起こしていた。この組換え部位の塩基配列は CoV 間で高度に保存されており、高度保存領域が相同組換えによる II 型 FCoV の出現に関与していると考えられた。また、I 型及び II 型 FCoV 感染猫血清を用いて I 型及び II 型 FCoV、II 型 CCoV に対する中和試験を実施した結果、I 型 FCoV 感染猫血清には II 型 CCoV に対する中和活性がないことが示された。以上の結果から、II 型 FCoV は I 型 FCoV が持続感染している猫に II 型 CCoV が重感染し、猫体内で相同組換えにより出現するというメカニズムが強く示唆された。

第 3 章　　フェレットコロナウイルスの国内における蔓延状況

フェレットコロナウイルス(FRCoV)は 2000 年にその存在が初めて報告された新興コロナウイルスである。FRCoV はフェレットにカタル性腸炎を引き起こす弱毒型(FRECV)と、猫伝染性腹膜炎(FIP)に類似した全身感染を引き起こす強毒型(FRSCV)が存在する。また、欧米で蔓延している FRCoV は S 遺伝子の配列の違いから I 型及び II 型に分類される。本章では、これまで不明であった国内で飼育されているフェレットの FRCoV 感染状況を調査するために FRCoV の遺伝子検出系を確立し、実際に野外に応用した。

我々は 5 都県、10 動物病院から送付された糞便 79 検体及び口腔スワブ 14 検体を用いて遺伝子検出を実施した。第 2 章で用いた RdRp 遺伝子の一部を増幅する CoV コンセンサスプライマーを用いた結果、79 頭中 33 頭(41.8%)の糞便から CoV 遺伝子が検出された。得られた塩基配列を基に新規 FRCoV 検出プライマーを設計し、糞便からの遺伝子検出を実施した結果、79 頭中 44 頭(55.7%)が陽性となり、CoV コンセンサスプライマーを用いた場合に比べ、より高い陽性率が示された。

次に、S 遺伝子を増幅する I 型及び II 型特異的プライマーを用いて、国内のフェレットに感染している FRCoV の型別を実施した。その結果、国内のフェレットからも I 型(30/79:38.0%)及び II 型 FRCoV(17/79:21.5%)が検出された。8 頭(10.1%)のフェレットには両型の FRCoV が同時感染していた。フェレットに感染している FRCoV の型と臨床症状との明確な関連性は認められなかった。口腔スワブからは 14 頭中 5 頭(35.7%)で FRCoV 遺伝子が検出され、口腔を介した水平伝播の可能性も示唆された。

以上のことから、新規に確立した遺伝子検出法によって国内のフェレットから FRCoV 遺伝子が 50%以上と高率に検出された。更に、国内においても 2 種類の FRCoV がすでに広く蔓延していることが判明した。本研究はフェレットのサンプルを提供して頂いた多くの動物病院の先生の御協力により実施された。

本研究では、S 遺伝子の巨大な欠損を有する高病原性の I 型 FCoV の存在が確認された。同様の欠損は豚の CoV である TGEV から PRCoV が出現した際にも認められており、組織特異性や病原性の違いに関与しているといわれている。このような変異は CoV では比較的起きやすい変異であると考えられることから、今後同様の変異を起こすコロナウイルスの出現も危惧される。II 型 FCoV と多くの CCoV との塩基配列を比較した研究により、II 型 FCoV が相同組換えによって猫の体内でそれぞれ独立に出現するというメカニズムが強く示された。更には、この出現の頻度が予想以上に高いことが示された。国内において、すでに 2 種類の FRCoV が広く蔓延していることが判明した。このことは、国内の動物に様々な CoV が数種類感染しており、相同性組換えや欠損などにより新興 CoV が出現する可能性が危惧された。本研究の成果は新興 CoV の出現予測に大いに役立つと考えている。