ROLE OF THE VIRAL SPIKE PROTEIN IN FELINE AND CANINE CORONAVIRUS PATHOGENESIS

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The coronavirus spike protein is a critical determinant of cell tropism and pathogenicity. It is expressed on the viral surface and is responsible for binding the host cell receptor and initiating fusion of the viral and host cell membranes. The work presented herein is an investigation into the genetic and biochemical properties of spike that influence the *in vivo* pathogenesis of feline and canine coronaviruses. A targeted sequencing approach was undertaken to identify mutations in a key viral activation site that correlate with the development of systemic disease after feline coronavirus infection. A similar strategy was used to characterize the spike proteins of canine coronaviruses associated with fatal enteritis in dogs and a thorough histopathologic characterization was performed in an attempt to independently confirm reports pantropic canine coronavirus variants.

BIOGRAPHICAL SKETCH

Beth Licitra is from the Hudson Valley region of New York State. She became interested in research as a teenager while volunteering at her local health department. Her experience trapping mosquitoes for West Nile Virus surveillance initiated her life-long interest in infectious diseases and public health. She received her BS in Biology from Cornell University in 2006. Her previous work experiences include a fellowship in Emerging Infectious Diseases at the Centers for Disease Control and Prevention in Atlanta, Georgia. She is an alumna of the Cornell Veterinary Leadership Program, Class of 2009. Her dissertation research was conducted under the mentorship of Dr. Gary Whittaker. She is a combined DVM/PhD student and will complete the DVM portion of her degree in 2016. Beth has two young sons, Dean and Mark Licitra.

This work is dedicated to my mother, Cathleen, who taught me the value of an education and without whom none of this would have been possible.

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CHAPTER ONE

Introduction to Feline and Canine Coronaviruses

Licitra BN, Duhamel GE, Whittaker GR. 2014. Canine enteric coronaviruses: emerging pathogens with distinct recombinant spike proteins. Viruses **6**:3363-76.

1.1 Why Study Coronaviruses?

Severe Acute Respiratory Syndrome (SARS), which emerged in 2002, and Middle East Respiratory Syndrome (MERS), which emerged in 2012 (1, 2) are two dramatic examples of coronaviruses' remarkable ability to jump between host species. Both viruses have ancestral reservoirs in bats and have infected other mammals on their paths to emergence in humans (3, 4). Coronavirus emergence and evolution is influenced by mutation and recombination events that occur against the background of their huge RNA genomes. How coronaviruses evolve to infect new host species is an area of active research.

The aim of this work is to identify changes in the coronavirus genome that are associated with the ability to enter new cell types. It focuses on feline and canine coronaviruses, two closely related pathogens of cats and dogs that primarily cause intestinal disease. Feline coronavirus (FCoV) is well known for its ability to escape the intestinal tract and infect monocytes and macrophages. The ability of the virus to infect these cells is associated with fatal, immune-mediated disease. Canine enteric coronavirus (CCoV) is less well studied; however, systemic fatal infections have been reported. The work presented here provides insights into a mechanism of tropism switching that all coronaviruses – not just FCoV and CCoV – have the potential to employ.

1.2 Coronavirus Structure and Genotyping

Coronaviruses are enveloped, single-stranded, (+)-sense RNA viruses that infect

humans and a wide variety of animal species (5, 6). They are primarily pathogens of the gastrointestinal tract. Coronavirus particles are composed of four major structural proteins; spike (S), envelope (E), membrane (M), and nucleocapsid (N) (7). The association of the N protein with the genomic RNA forms the helical nucleocapsid that is surrounded by an icosahedral structure composed of the viral M protein. The virus enters cells upon receptor binding and membrane fusion mediated by the S protein (8). Like many other (+)-sense RNA viruses, it replicates within the cytoplasm in association with a complex membranous network known as a viral factory (9). Coronaviruses employ a unique mechanism of replication. The (+) strand RNA viral genome is transcribed into a full-length (–) strand RNA genomic template and (–) strand subgenomic templates for mRNA synthesis. RNA recombination is believed to occur during this process of (–) strand RNA synthesis. Subsequently, the (–) strand templates are transcribed to form the (+) strand RNA genomes and (+) strand nested subgenomic mRNAs (10). While 3'-exonuclease activity allows some degree of proofreading (11), the coronavirus genome is highly prone to mutation during replication. During virus assembly, the virion buds into the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) where it enters the secretory pathway of the cell, allowing maturation and processing of the heavily glycosylated viral spike protein. The virus exits the cell via exocytosis.

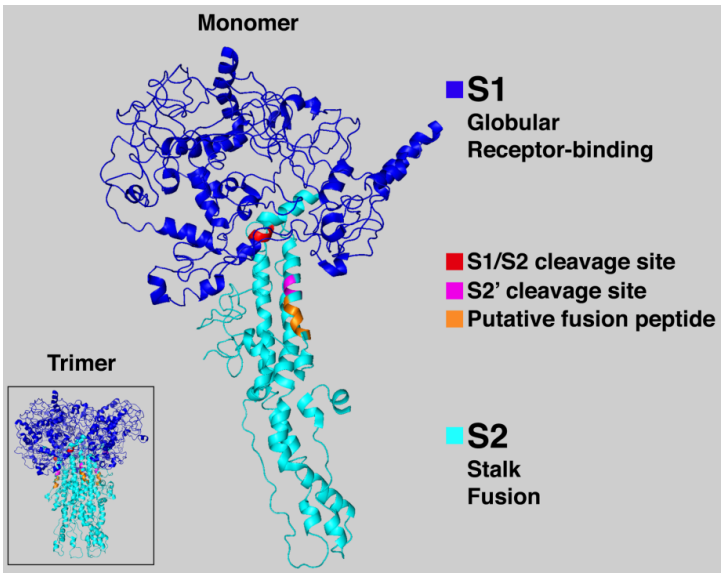
Coronaviruses are phylogenetically divided into several genera, termed alpha, beta, and gamma (12). The existence of a fourth genus – deltacoronavirus – has also been suggested (13). Classification of coronaviruses into different genera is based in part

on the presence or absence of small open-reading frames situated downstream of the genes encoding the main structural proteins.

1.3 Spike (S) Structure and Function

Spike is the most variable protein in the coronavirus genome. It varies in length from 1100 amino acids in infectious bronchitis virus (IBV) to 1400 amino acids in FCoV. It is expressed as a homotrimer on the surface of viral particles (Figure 1.1). Spike consists of two protein subunits, designated S1 and S2. The S1 subunit interacts with host-cell receptors and attachment factors. It makes up the highly exposed globular head of the S protein and is under intense selective pressure from host antibodies. The S2 domain is responsible for fusion of the viral and host-cell membranes (14, 15). It is highly conserved among coronaviruses and makes up the stalk region of the protein. In some coronaviruses, the boundary of the S1 and S2 subunits contains a pattern of amino acids, or motif, that is recognized by host proteases and is referred to as the S1/S2 cleavage site (16, 17). A second cleavage motif is located slightly downstream and is termed S2' (18). The S2' cleavage motif is present in all coronaviruses and cleavage at this site likely required to expose the viral fusion peptide and initiate the infection process.

Figure 1.1[1] This representation shows the trimeric (inset) and monomeric forms of the coronavirus spike protein and is based on the three-dimensional predicted model found in the PDB database (PDB entry 1T7G, (19)). The S1 and S2 domains as well as the cleavage sites and putative fusion peptide are highlighted.



[1] Adapted from S. Belouzard, Millet, J.K., **Licitra, B.N**. and Whittaker, G.R. Mechanisms of Coronavirus Cell Entry Mediated by the Viral Spike Protein. *Viruses* **2012**, 4, 1011-1033; doi:10.3390/v4061011

Receptor Binding

Infection of the host cell is initiated when S binds to the extracellular domain of specific membrane-bound receptors on the host cell surface. Coronaviruses take advantage of host aminopeptidase-N (APN), angiotensin converting enzyme-2 (ACE-2), dipeptidyl peptidase-4 (DPP4), carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), heparin sulfate, sialic acid, and calcium dependent (C-type) lectins to gain entry into the host cell (Table 1.1) Coronaviruses' ability to interact with a variety of host proteins and sugars helps them to infect a diverse range of mammalian and avian species.

Table 1.1 Coronavirus genera, species, and host receptor usage

Genus	Species	Receptor, co-receptor
Alphacoronavirus	- Alphacoronavirus-1 comprising: FCoV-1 CCoV-1 FCoV-2 CCoV-2 TGEV Porcine Respiratory Coronavirus (PRCoV) - Human coronavirus 229E - Human coronavirus NL63 - Porcine Epidemic Diarrhea Virus (PEDV)	unknown (20), DC-SIGN (21) unknown APN (22), DC-SIGN (21, 23) APN (24) APN (22), sialic acid (25) APN (26) APN (22) ACE2 (27) APN? (28) (29)
Betacoronavirus	- Betacoronavirus-1 comprising: Bovine coronavirus (BCoV) Human coronavirus OC43 (HCoV-OC43) Equine coronavirus (ECoV) Canine respiratory coronavirus (CRCoV) - Mouse hepatitis virus (MHV) - Human SARS-CoV - Human MERS-CoV	sialic acid (30) sialic acid (30) presumed to be sialic acid presumed to be sialic acid CEACAM1(31) ACE2 (32), DC/L-SIGN (33) DPP4 (34)
Gammacoronavirus	- Avian coronavirus comprising: Infectious Bronchitis Virus (IBV) Pheasant coronavirus Turkey coronavirus	sialic acid (35), DC/L-SIGN (36) presumed to be sialic acid presumed to be sialic acid
Deltacoronavirus	- Bulbul coronavirus HKU11 - Thrush coronavirus HKU12	unknown

APN = aminopeptidase N, ACE2 = angiotensin converting enzyme 2, CEACAM1 = carcinoembryonic antigen-related cell adhesion molecule 1, DPP4 = dipeptidyl peptidase-4, DC-SIGN = dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin, L-SIGN = liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin

Membrane Fusion

S is a class 1 fusion protein that behaves similarly to influenza virus hemagglutinin (HA), retrovirus envelope (Env) and paramyxovirus fusion (F) (37, 38). Fusion must be tightly regulated in order to target the virus to permissive cell types and reduce the number of abortive infections. Class 1 fusion proteins are primed for fusion by proteolytic cleavage, and activated by receptor binding, low pH, or a combination of the two (39). This sequence of events is required to destabilize the S protein, triggering conformational changes that result in exposure of a string of hydrophobic amino acids known as the fusion peptide. The fusion peptide inserts into the closely opposed host cell membrane. At this point, heptad repeats within the S2 domain form coiled-coil structures that draw the viral and host membranes into direct opposition and allow the creation of a fusion pore through which viral genome enters the host cytoplasm (8, 40, 41).

Fusion occurs either at the cell surface, or after endocytosis of the viral particle. For many coronaviruses, it is still unclear which point of entry is used. Recent evidence suggests that coronaviruses have the ability to utilize either pathway dependent on their proteolytic priming and pH activation. For example, SARS-CoV enters the host cell via endocytosis and cathepsin L-mediated spike protein cleavage (42); however if this pathway is blocked, the virus can enter via trypsin or elastase-mediated cleavage at the cell surface (43).

Modification of fusion requirements is one way in which viruses acquire the ability to infect new cell types. For example, Mouse Hepatitis Virus (MHV) strain JHM does

not need to bind its receptor in order to initiate fusion. This infection strategy is termed receptor-independent spread. S proteins on virons budding from infected cells trigger fusion of adjacent host cell membranes, leading to propagation of the virus via syncytia formation. MHV-JHM's enhanced fusogenicity is the result of an unstable S protein, an adaptation that carries the fitness cost of indiscriminate fusion and abortive infection, but may confer an advantage during the process of adaptation to a new host (44).

Cleavage Activation

The coronavirus spike protein is activated by proteolytic cleavage at one or two cleavage sites (S1/S2 and S2') (10). In other virus systems, notably Influenza A and Newcastle Disease, amino acid substitutions at similar sites can result in changes in viral virulence and pathogenicity (39). Depending on the individual coronavirus, cleavage at S1/S2 may or may not occur (8). S2' cleavage is likely a more universal requirement, and is linked directly to exposure of the viral fusion peptide (14, 18, 29). Cleavage at the two sites can occur via the action of a wide range of proteases, e.g., trypsin or trypsin-like proteases, cathepsins, elastase, or furin (45).

Cleavage may occur at one or more points in the coronavirus life cycle. The first opportunity is in the progenitor cell during viral egress through the trans-Golgi network where furin may cleave multi-basic amino acid motifs located at the boundary of the S1 and S2 subunits. *In vivo*, the cleaved S1 and S2 subunits remain tenuously associated by hydrogen bonds. Cleavage at the S1/S2 site is dispensable, but when present, is thought to result in destabilization of spike and increased fusogenicity.

Viruses that lack a S1/S2 furin motif are released from the host cell in an uncleaved state. The S2' cleavage site lies about 100 amino acids downstream of S1/S2, immediately proceeding the hydrophobic viral fusion peptide. Regardless of whether or not cleavage occurs at S1/S2, cleavage at S2' is likely required for infection. Cleavage at this position frees the buried fusion peptide so it can insert into the host cell membrane. Since this event is linked directly to fusion, it is not generally initiated until the virus binds its receptor. Target-cell proteases such as cathepsins B and L are known to cleave the S2' site (18, 46). Modification of viral proteolytic cleavage motifs - either by amino acid substitutions or post-transcriptional modifications such as glycosylation - has the potential to modulate S fusogenicity and infectivity.

1.4 Introduction to Feline Coronavirus (FCoV)

FCoV is a pathogen of both wild and domestic cats. It is endemic in multi-cat environments such as catteries and shelters (47). All cats that are exposed to FCoV develop mild or sub-clinical enteritis; however a small subset of infected animals will progress to a systemic, invariably fatal immune-mediated disease. This dichotomy in clinical signs (mild enteritis versus fatal immune mediated organ failure) led to the classification of FCoVs into two biotypes: feline enteric coronavirus (FECV) and feline infectious peritonitis virus (FIPV). The term biotype refers to the virus associated with one of two clinical outcomes: FECVs cause mild self-limiting enteritis while FIPVs cause systemic disease and death. The majority of the scientific evidence suggests that FIPVs arise from FECVs by mutation(s) that occur spontaneously within each affected cat. The immune response of the cat plays an

important role in determining whether FIPVs will arise and if so, whether they spread systemically via activated monocytes and macrophages.

1.5 FCoV Pathogenesis and Clinical Signs

Feline enteric coronavirus (FECV)

FECV replicates within the intestinal epithelium. Most cats are asymptomatic, but some present with fever, anorexia and mild diarrhea (48, 49). This form of FCoV is ubiquitous among cats in multi-cat environments such as catteries and shelters. Monocyte-associated viremia is common (50-52), but productive infection of these cells is abortive and is not sustained longer than 24 hours (53). Virus is shed in the feces and is transmitted by the fecal-oral route. Primary infection lasts several months and virus shedding is highest at this time, particularly among kittens. A subset of cats clear the virus, but most continue to shed at low levels for up to two years (48, 54). Colonic columnar epithelial are thought to serve as a reservoir for persistent infection and shedding (49, 52). Immunity is not long lasting and re-infection is common (55). The virus can survive up to seven weeks in cold, dry conditions and can be transmitted by fomites such as clothing and brushes (56).

Feline infectious peritonitis virus (FIPV)

In 1-5% of FECV infections (48), the virus gains the ability to *replicate efficiently* within blood monocytes and tissue macrophages. The key difference between FECV and FIPV is that FIPV initiates productive infection of monocytes, leading to their activation. Feline infectious peritonitis (FIP) is a monocyte-triggered disease that involves generalized activation of monocytes, macrophages, and endothelial cells.

The characteristic lesion of FIP is granulomatous phlebitis (57). FIPV infection of susceptible feline monocytes triggers up-regulation of mitogen-activated protein kinase (MAPK) leading to the production of the pro-inflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1β (58). Enhanced expression of the adhesion molecules CD18 and CD11a facilitates emigration of FIPV-infected monocytes into tissues, resulting in the formation of granulomatous lesions (57, 59). Monocyte and macrophage derived vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs) are suspected to play roles in the increased vascular permeability that characterizes FIP (57, 60).

The individual immune responses play an important role in determining which cats develop FIP. The monocytes of some cats are refractory to infection with both biotypes of FCoV (53). Resistance is associated with the development of an IFN-γ driven cellular immune response (61, 62). In susceptible cats, productive infection of monocytes and macrophages triggers a TNF- α response that quickly becomes dysregulated (58, 61, 63). The outcome is immune mediated tissue destruction, organ failure, and death (64).

Clinical signs are initially non-specific and include fever, lethargy, anorexia, and weight loss. Laboratory tests often reveal a non-regenerative anemia, neutrophilia with a left shift, plus a profound lymphopenia due to cytokine mediated depletion of CD8+ and CD4+ T-cells (65-67). Hyperbilirubinemia and elevated serum aspartate aminotransferase (AST) are also common findings (68). In cats that mount a predominantly Th2-mediated humoral immune response, the disease is characterized by the development of a protein rich effusion within the body cavities (69). This form

of the disease is known as effusive, or wet FIP, and it progresses rapidly. The time from diagnosis to death can be as short as a few days. This is due in part to a phenomenon known as antibody-dependent enhancement in which antibodies against FIP serve to opsonize the virus and allow it to enter monocytes and macrophages by a specialized receptor that recognizes antibodies attached to invading pathogens. (70, 71). This receptor is called Fc after the region of the antibody it binds – the so called fragment crystalizible. Uptake via this pathway actually leads to enhancement of infection and the development of more fulminant disease (72). Less commonly, cats will present with the non-effusive, or dry form of FIP. This form of the disease is associated with a mixed Th1/Th2 response that prolongs the clinical course of infection, but is ineffective in clearing the virus (73). Antibody, antigen complexes may appear as white keratic precipitates on the surface of the cornea. Uveitis, which is defined as inflammation of the pigmented structures of the eye, may manifest as a darkening of the iris and abnormal pupil shape. Virus mediated inflammation centered around the ventricles in the brain results in neurological signs including ataxia, tremors, and/or seizures (74). The course of dry FIP is more protracted and cats may live for months before eventually succumbing to the disease.

FIP has a predilection for kittens and young cats (75). It often strikes more than one animal in a household and has been known to devastate shelters and catteries. Age, overcrowding, feline leukemia virus (FeLV) co-infection, and genetic predisposition are known risk factors for the development of FIP (64).

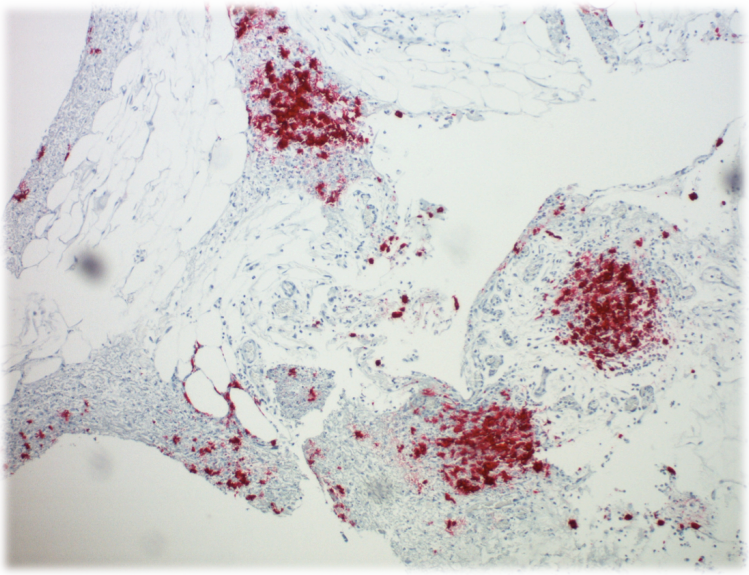
There are two proposed theories regarding the origin of FIP. The first, and most widely supported, is the internal mutation hypothesis (76, 77). In this paradigm, every case of FIP arises uniquely from mutation of FECV within the affected cat. An important supposition of the internal mutation hypothesis is FIP is not readily transmissible, making infection of monocytes an evolutionary dead-end for the virus. The second theory on the origin of FIP is the circulating strain hypothesis (78, 79). It states that both FECV and FIPV are actively circulating among cats. Whether or not an animal develops FIP depends upon which circulating variant it is exposed to. This theory has the advantage of explaining how outbreaks of FIP - which are commonly encountered by breeders and shelter veterinarians - might occur. The internal mutation and circulating strain hypotheses need not be mutually exclusive. It is entirely plausible that some strains of FECV are more likely to transition to FIPV than others. Increasing lines of evidence suggest that multiple mutations are associated with the transition from FECV to FIPV. For example, changes in the viral accessory genes 3c and the structural protein spike both correlate with the development of FIP (80, 81). If a given FECV strain already possesses one or more of the predisposing mutations, then the switch to FIPV is increasingly likely. This hypothesis, while attractive, remains untested.

1.6 FCoV Diagnosis

Primary differential diagnoses for effusive FIP include heart disease, neoplasia, and cholangiohepatitis. Non-effusive cases of FIP are more challenging to diagnose, especially when ocular and neurological signs are absent. Several informative tests can be run on serum or effusion including total protein, albumin/globulin ratio, γ-

globulin, coronavirus antibody titer, and coronavirus specific RT-PCR (82). The gold standard for FIP diagnosis remains tissue biopsy followed by immunohistochemistry revealing granulomatous lesions that contain coronavirus antigen (Figure 1.2).

Figure 1.2 Photomicrograph of omentum from a case of FIP. Coronavirus antigen is present within pyogranulomas and stains red in this immunohistochemistry assay. Image provided by ANTECH diagnostics and used with permission.



1.7 FCoV Serotypes

Two serotypes of FCoV are circulating in cats. They can be differentiated by the antigenic and genetic properties of their spike protein (83). Feline coronavirus type 1 (FCoV-1) is more prevalent clinically but grows poorly in cell culture and is therefore understudied when compared to feline coronavirus type 2 (FCoV-2), which is easily propagated *in vitro* but accounts for less than 10% of clinical cases (84). FCoV-2 is the result of homologous recombination and displays a spike protein that is most

similar to CCoV-2 (85). Both FCoV-1 and FCoV-2 infection can result in clinical signs of FECV or FIPV. The terms serotype and biotype are distinct in meaning: serotypes are defined by the genetic and antigenic properties of the virus (FCoV-1 vs FCoV-2), while biotypes are defined by clinical outcome (FECV vs FIPV).

FCoV-1

The prototype FCoV-1 viruses include FIPV TN406 (more commonly known as FIPV-Black) and FECV Rogers-Morris (FECV-RM) (86, 87). FECV-RM does not replicate in cell culture and is propagated through fecal-oral transmission in laboratory cats. Like FECV-RM, most FCoV-1 viruses cannot be isolated in tissue culture. It has been hypothesized that this is due to the requirement of a receptor or co-receptor that is not expressed on cultured cells (20). The FCoV-1 host cell receptor is unknown. FIPV-Black was isolated in the early 1980's and grows well in feline AKD (lung) and CrFK (kidney) cell lines. FIPV-Black is pathogenic to cats in its low-passage form, but has reduced virulence after tissue culture adaptation (88). Circulating FCoV-1 viruses can be sequenced from the feces of cats living in multi-cat environments as well as from the tissues of cats diagnosed with FIP (89).

FCoV-2

The prototype FCoV-2 viruses include FIPV WSU-79-1146 and FECV WSU-79-1683, both of which were isolated in 1979 (90). Type 2 FCoVs use the receptor aminopeptidase N (fAPN) (22). They display a CCoV spike protein (85) and have the ability to infect canine as well as feline cell lines. Efficient replication in feline

macrophages is limited to FIPV-1146 (91). *In vivo,* 1146 is the most virulent of all FIPV isolates, while FECV-1683 causes inapparent enteritis (48).

Receptor Binding

Aminopeptidase N (APN) has been identified as the receptor for many *Alphacoronaviruses*, including FCoV-2, CCoV-2, TGEV, and human coronavirus (HCoV)-229E (22). FCoV-1 has been suggested to use a different, yet unidentified receptor (20). The dendritic cell specific ICAM-3-grabbing non-integrin (DC-SIGN) expressed on dendritic cells, macrophages, and monocytes has been shown function as a co-receptor for both type 1 and type 2 FCoVs (21, 23). Sialic acid binding is essential for the enterotropism of closely related TGEV (25, 92), but its role in FCoV enterotropism remains unstudied.

Membrane Fusion

Fusion occurs either at the cell surface, or after endocytosis of the viral particle. For FCoVs, it is still unclear which point of entry is used. The dependence of FECV-1683 on endosomal cathepsins and low pH suggests that it enters through the endosomal pathway while the pH and cathepsin L independence of FIPV-1146 suggests that it may fuse directly at the cell surface (46).

Priming by Proteolytic Cleavage

FCoV-1 spike has an unusual feature for *Alphacoronaviruses*, the presence of a furin-like cleavage motif at the S1/S2 boundary (16) (Figure 1.3). Furin is a serine protease that belongs to a family of enzymes called proprotein convertases (PCs) (93). Along

with furin, six other PCs proteases, PC1, PC2, PC4, PC5, PACE4 and PC7, share the same multi-basic amino acid recognition motif: [R/K]-[X]-[R/K]-[R], where X is any amino acid and R and K are the basic amino acids arginine and lysine, respectively. The role of furin-like enzymes in the proteolytic priming of FCoV-1 is yet to be determined.

Like most *Alphacoronaviruses*, FCoV-2 viruses lack a furin cleavage site. They rely on cleavage by endosomal proteases such as cathepsin L and B (46). Cathepsins are a group of cysteine proteases within the papain family of proteases (94). FIPV-1146 can be activated by cathepsin B in order to initiate infection. In contrast, FECV-1683 requires cathepsin B, cathepsin L, and low pH (46). Proteolytic activation motifs are known pathogenicity determinants for Newcastle Diseae and Influenza A viruses. Amino acid mutations in proteolytic activation motifs within the coronavirus S protein could help explain the tropism switch from that defines the transition from FECV to FIPV.

Figure 1.3 Multiple sequence alignment of the spike proteins of FCoV-1 viruses FECV-RM (ACT10854.1) and FIPV-Black (BAC05493.1) and FCoV-2 viruses FIPV-1146 (AGZ84516.1) and FECV-1683 in the region of the two activation sites (S1/S2 and S2') and the viral fusion peptide (underlined) (14, 18). The alignment was created using ClustalX. Basic residues likely involved in cleavage activation are colored red, / indicates the putative location of cleavage.

	S1/S2 furin motif	S2'cathepsin motif
FECV-RM	785 HTQPRRSRR/STPN	973 PTIGKR/SAVEDLLF
FIPV-Black	783 HTQAKRSRR/PTSH	969 PKIGAR/SAVEDLLF
FECV-1683	768 WTTT--------	954 SKRKYR/SAIEDLLF
FIPV-1146	765 WTTT--------	956 SKRK/YGSAIEDLLF
		fusion peptide

1.8 FCoV Prevention and Treatment

Controlling FCoV is challenging and largely impractical due to its endemic circulation among communally housed cats (74). In shelters and catteries, regular disinfection of litter-boxes and food bowls can help to reduce the number of infectious viral particles in the environment, but will not eliminate FCoV (64). Although a vaccine is commercially available, the American Association of Feline Practitioners Advisory Board does not recommend it due to its limited efficacy. It is a temperature sensitive mutant administered as an intranasal formulation (95) that provides protection against FCoV-2 but may not be cross-protective against the more prevalent FCoV-1 strains (74). Additionally, it cannot be administered until after 16 weeks, at which point most kittens are already seropositive (96). Antibody-dependent enhancement of FIP has made development of more effective vaccines challenging (97, 98). Catteries seeking to minimize FIP are encouraged to eliminate overcrowding, actively manage litter boxes and litter dust, breed a minimum number of litters, and restrict breeding to adults that have not previously produced kittens that have died of FIP (74). Sadly, there are no effective treatments for FIP. Palliative treatment followed by euthanasia is the standard of care for this disease.

1.9 FCoV Summary

A combination of host, viral, and environmental factors contribute to the development of FIP. Overcrowded conditions cause stress and increase the likelihood of co-infection, both of which may result in immunosuppression and failure to control

coronavirus replication. In addition, overcrowding increases the viral load in the environment. Host factors such as age and genetic background also affect an animal's chances of developing FIP. Lastly, viral factors that enhance tropism for monocytes and macrophages are suspected to play a critical role. Mutations in the viral proteins 3c and S are associated with FIP, but the exact combination of viral factors required for the transition from FECV to FIPV remains unknown.

1.10 Introduction to Canine Enteric Coronaviruses (CCoV)

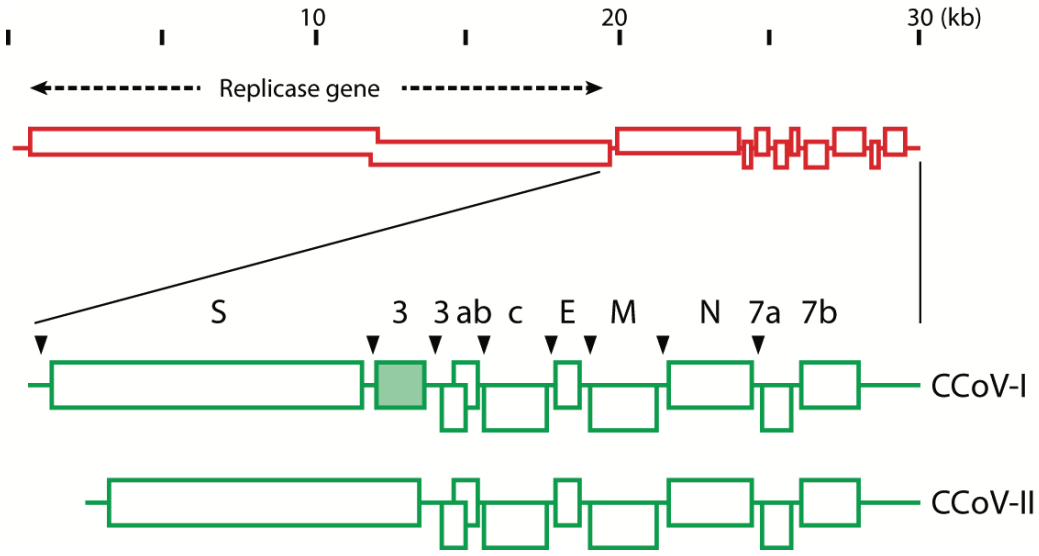
CCoV is a common infection of dogs, particularly those housed in large groups such as kennels, shelters, and breeding facilities. CCoV was first recognized as a pathogen of dogs following virus isolation of the prototype 1-71 virus in 1971 during an outbreak of gastroenteritis in military dogs (99). Classically, CCoV was considered to cause only self-limiting enteritis with mild diarrheal disease (100). Recently, CCoV has emerged as a significant pathogen in veterinary medicine, and is increasingly found to be an important cause of disease.

1.11 CCoV Structure and Genotyping

There are two known types of canine coronaviruses: CCoV is a member of the alphacoronavirus genus (12), and the more recently identified canine respiratory coronavirus (CRCoV) is a member of the betacoronavirus genus (101). CCoV is closely related to transmissible gastroenteritis virus (TGEV) of pigs, ferret coronavirus and FCoV of cats (12); whereas CRCoV is more closely related to bovine coronavirus (102). All enteric CCoVs (along with the related viruses of cats, pigs, and ferrets) are

given the same strain designation (*Alphacoronavirus-1*) from a taxonomic perspective. There are two distinct serotypes of CCoV: type 1 and type 2 (103, 104). CCoV-1 and CCoV-2, though closely related to each other, have markedly different spike proteins (105) - a situation analogous to that in cats, where feline coronaviruses (FCoV) also exist as two equivalent serotypes (type 1 and type 2, based on antigenically distinct spike proteins (106). It has been proposed that type 1 CCoVs and FCoVs evolved from a common ancestral virus, and that the canine and feline type 2 lineages arose from multiple recombination events with an unidentified genetic source (107). A summary of the genome organization of CCoV-1 and CCoV-2 is shown in Figure 1.4.

Figure 1.4 Image representation of the genomes of CCoV-1 and CCoV-2. Adapted from Lorusso *et al. (107)*.



CCoV-1 viruses were initially based on analysis of CCoVs with a variant M gene (108)—with Elmo/02 as the prototype strain (108, 109). Like other CCoV's, these

variant viruses can be sequenced from the feces of young dogs with diarrheal disease. It is now known that the major differences between viruses belonging to CCoV-1 and CCoV-2 are primarily found within the spike protein, and account for the distinct serological properties of these two viruses.

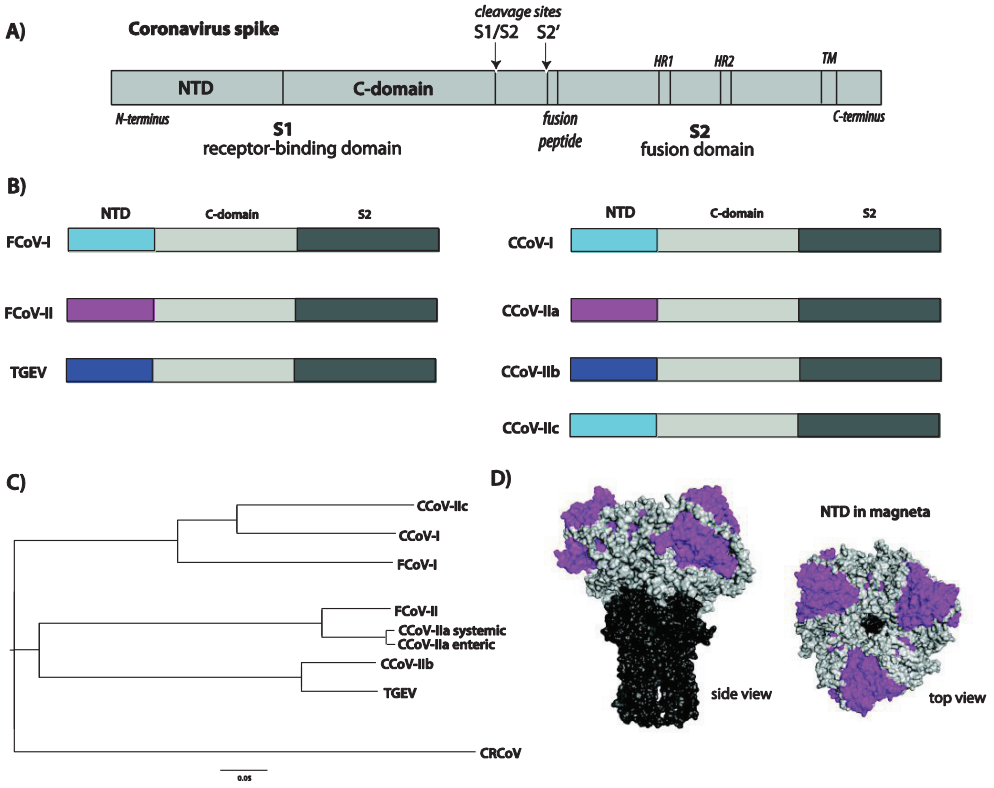
CCoV-1 isolates are not culturable in cell culture systems. *In vivo*, CCoV-1 viruses are thought to co-circulate extensively with CCoV-2 viruses, often occurring as co-infections (110-115). This extensive co-circulation, combined with the capacity for recombination inherent in coronavirus replication, suggests that natural selection of novel recombinant viruses is not uncommon. Such a situation has been proposed for the divergent CCoV-A76 as well as other similar viruses that have CCoV-1/CCoV-2 recombinant spike proteins (24, 116). This indicates that CCoV-1-like viruses have been in circulation in dogs for a considerable time prior to the identification of Elmo/02.

The first identified CCoV strain 1-71, is now categorized as a type 2 virus (CCoV-2). Further classification of CCoV-2s into distinct subtypes (CCoV-2a, CCoV-2b) has been proposed, based on the sequence of the first 300 amino acids of the spike protein, a region known as the *N*-terminal domain (NTD). The NTD is an important determinant of intestinal tropism in closely related TGEV (25, 117). The CCoV IIa and IIb classifications are not officially accepted within CCoV taxonomy, but are widely cited in the literature. CCoV-2a viruses have a NTD consistent with the prototype CCoV. These viruses exist in two biotypes that differ in pathogenicity and tissue tropism. The “classical” CCoV-2a biotype is restricted to the small intestine,

where it causes enteritis. In contrast, the emergent "pantropic" CCoV-2a biotype can spread systemically, causing leukopenia (118-120). The viruses CB/05 and 450/07 have been studied extensively as prototype pantropic CCoVs. (111, 119-122). The second variant, CCoV-2b, is genetically distinct from CCoV-2a, with the CCoV-2b spike gene having a TGEV-like NTD (123, 124). Like TGEV, CCoV-2b causes enteritis in neonatal animals. CCoV-2b RNA has been detected by PCR assays in various organs outside of the intestinal tract, primarily in dogs that are co-infected with canine parvovirus (112, 123). Isolate 341/05 has been proposed as a prototype CCoV-2b (123).

Based on the finding of a novel NTD in A76-like viruses, we propose the creation of a new subgroup, CCoV-2c, which consists of CCoV type 2 viruses with a CCoV-1- or FCoV-1-like *N*-terminal domain. These viruses have been reported in both the United States and Sweden (24, 116). Other viruses with similar biological properties may have also been identified in the past, including an atypical CCoV from a breeding facility in 1997 (125). As CCoV-A76 has been isolated and characterized (24), this serves as a convenient prototype CCoV-2c. An image of the various spike protein domains for CCoVs, and a summary of NTD phylogeny, is shown in Figure 1.5.

Figure 1.5 (**A**) Image representation of a coronavirus spike protein. Key features are identified, including the *N*-terminal domain (NTD) and C-domain within the S1 receptor-binding domain, and the fusion peptide, two heptad repeats (HR1 and HR2) and transmembrane domain (TM) in the S2 fusion domain. The two cleavage sites for protease activation (S1/S2 and S2') are shown; (**B**) Image representation of the different domains present within the spike proteins of alpha-coronaviruses of cats (FCoV), dogs (CCoV) and pigs (TGEV). The different NTDs are color-coded to indicate homology and proposed recombination events across the species, with the remainder of S1 depicted in light gray and S2 in dark gray; (**C**) Phylogenetic tree of the NTDs present within the spike proteins of representative coronaviruses of cats (FCoV), dogs (CCoV) and pigs (TGEV) (The following virus sequences were used for alignment. NTD = aa 1-279 (based on CCoV-A76). FCoV-1 = Black (BAC05493.1); FCoV-2 = WSU-79-1146 (AGZ84516.1); CCoV-2a = CCoV CB/05 (AAZ91437.1); CCoV-2b = CCoV 341/05 (ACJ63231.1); CCoV-1 = CCoV Elmo/02 (AAP72149.1); CCoV-2c = CCoV-A76 (AEQ61968.1); CRCoV = CRCoV4182 (ABG78748.1); TGEV (CAB91145.1)). The tree was created using ClustalX [45] and FigTree software [46]; (**D**) Structural model of a coronavirus spike protein based on PDB file 1T7G [47], in surface rendering. The NTD is colored magenta, the remainder of S1 is light gray and the S2 domain is colored dark gray. Two views of the trimeric spike are shown, a side view and a top view.



CCoV-2 viruses (such as 1-71) typically grow readily in cell culture, with A-72 cells (canine tumor fibroblast cells derived from unknown tissue type) widely used for virus propagation. 1-71 also grows well in a variety of feline cell lines (e.g., CRFK), but, interestingly, not in many other canine cell lines (24).

1.12 Functional Aspects of the CCoV S Protein

Receptor Binding and Host Tropism

The coronavirus spike protein is a major antigenic determinant and is also responsible for host cell receptor binding and viral entry (126). Aminopeptidase *N* (APN) has been shown to act as a common receptor for many alphacoronaviruses, including FCoV and TGEV (127). Although each virus would be assumed to utilize a species-specific homolog in its respective host during infection, the feline homologue (fAPN) has been shown to act as a common receptor for type 2 FCoV, type 2 CCoV and TGEV (22). This property is unlike most other coronaviruses, which have highly species-specific receptors. Such broad receptor-binding ability has likely played a role in the zoonotic transfer and genetic recombination events that have defined the evolution of animal alphacoronaviruses. Feline and human APN are also receptors for the human alphacoronavirus HCoV-229E, but not for another human alphacoronavirus, NL63 [43,44]. Presumably, the canine APN acts as the *in vivo* receptor for all CCoV-2s, as shown for 1-71 (24). In the case of the CCoV-2c A76, the presence of a divergent APN-binding domain within the spike glycoprotein, the "*C*-domain", has resulted in the ability to use cAPN but not fAPN as a receptor. The relative lack of glycosylation on fAPN compared to cAPN may account for these differences.

The receptor determinants for the serotype 1 FCoV/CCoV group are much less certain. While there is some evidence for fAPN as an FCoV type 1 receptor (22), other studies have concluded that there is a distinct receptor for FCoV type 1 (20, 128). Overall, receptor determinants for CCoV type 1 viruses remain essentially unknown.

In addition to a specific proteinaceous receptor, there are indications that lectin-based interactions via sugar moieties, on either the virus or the host, may play a role in the receptor-binding complex for FCoV types 1 and 2 and TGEV (21, 23, 25, 117, 129) and the same situation may also apply to CCoVs. Many coronaviruses also bind sialic acid, which can be an important determinant of tissue tropism and pathogenesis, but this has not been investigated for FCoVs or CCoVs.

Activation by Proteolytic Cleavage

The FCoV/CCoV type 1 and 2 lineages present an interesting and distinct difference in spike protein cleavage-activation. The type 1 lineage shows the presence of two distinct protease cleavage motifs (R-R-S/A-R-R-S/A at S1/S2 and G/K-R-S at S2'), whereas the type 2 lineage is missing an obvious motif at S1/S2 and has a single distinctive R-K-R-Y/F-R-S cleavage motif at S2'. This implies that the viruses in these two lineages have quite distinct means of cleavage activation. A multiple sequence alignment summarizing these sites is shown in Figure 1.6. While mutations in the FCoV cleavage site have been linked to a change of cleavability by the protease furin (which cleaves readily at R-R-S/A-R-R-S/A motifs) and correlate with gain of macrophage tropism and the development of FIP in cats (130), there is currently no evidence for cleavage site mutations in highly pathogenic CCoV strains.

Figure 1.6 Multiple sequence alignment of the spike proteins of representative coronaviruses of cats (FCoV) dogs (CCoV and CRCoV) and pigs (TGEV) (The following virus sequences were used for alignment. FCoV-1 = RM (ACT10854); FCoV-2 = WSU-79-1683 (AFH58021.1); CCoV-2a = CCoV CB/05 (AAZ91437.1); CCoV-2b = CCoV 341/05 (ACJ63231.1); CCoV-1 = CCoV Elmo/02 (AAP72149.1); CCoV-2c = CCoV-A76 (AEQ61968.1); CRCoV = CRCoV4182 (ABG78748.1); TGEV (CAB91145.1).), in the region of the two activation sites (S1/S2 and S2'). The alignment was created using ClustalX [45]. The expected cleavage motif is in bold.

	S1/S2	S2'	
CCoV-I	HTHTV**RRARRA**VQTGTTITA	AQPG**GRS**AIEDLLF	alphacoronavirus
FCoV-I	NHTQP**RRSRRS**TPNSVTTYT	PKIG**KRS**AVEDLLF	alphacoronavirus
CCoV-IIa	WTTT--------PNFYYYSI	S**KRKYRS**AIEDLLF	alphacoronavirus
CCoV-IIb	WTTT--------PNFYYYSI	S**KRKYRS**AIEDLLF	alphacoronavirus
CCoV-IIc	YIVT--------PSFYYYSI	S**KRKFRS**TIEDLLF	alphacoronavirus
FCoV-II	WTTT--------PNFYYYSI	S**KRKYRS**AIEDLLF	alphacoronavirus
TGEV	WTTT--------PNFYYYSI	S**KRKYRS**AIEDLLF	alphacoronavirus
CRCoV	GYSTQ**RRSRRS**ITTGYRFTN	NKVS**SRS**AIEDLLF	betacoronavirus, group A

1.13 CCoV Pathogenesis and Clinical Presentation

CCoV is transmitted by the fecal-oral route; however, it is unclear whether transmission by other routes including aerosols can occur. After ingestion, CCoV typically infects and replicates within the cytoplasm of mature epithelial cells along the sides and the tip of intestinal villi, while sparing the epithelium that lines the intestinal crypts (Figure 1.7). Infected villous enterocytes undergo degeneration characterized by shortening, distortion and loss of microvilli of the brush border, leading to sloughing of necrotic cells into the lumen. The loss of mature villous enterocytes causes atrophy of intestinal villi, which become attenuated in an attempt to maintain the integrity of the intestinal barrier. In response to the loss of villous enterocytes, there is an increase in mitotic activity of the crypt epithelium and a net expansion of the pool of immature enterocytes. Together, these changes in the small intestinal morphology translate into a loss of normal digestive and absorptive functions and the clinical signs of diarrhea and dehydration in affected dogs (131).

CCoV is generally thought of as a mild, but highly contagious, enteritis of young dogs, most often under 12 weeks of age (100, 132, 133) In some cases, CCoV infection can be fatal, particularly in puppies co-infected with other pathogens such as canine parvovirus (134, 135). Therefore, the range of clinical signs from loose stools to severe watery diarrhea with high morbidity and variable mortality is mainly determined by the age at the onset of infection, the level and type of pathogen exposure, and the degree of maternal transfer of immunity.

In recent years, an increasing number of reports of infections by highly virulent

CCoVs have also been documented in puppies without apparent coinfections (110, 112, 116, 121, 136-139). In the case of the "pantropic" CCoV-2a viruses, infection results in a fatal multisystemic illness, with various clinical signs similar to canine parvovirus infection, and including high fever, hemorrhagic gastroenteritis, neurological signs, and lymphopenia. The most consistent clinical sign of pantropic CCoV-2a is leukopenia (118-120). Most studies of pantropic CCoV-2a report the presence of viral RNA in various tissues, including lungs, lymph nodes, liver, spleen, kidneys, urinary bladder and brain. Evidence for replication of these pantropic viruses outside of the gastrointestinal tract is based on demonstration of CCoV antigen by immunohistochemical staining of lung tissue taken from a single dog (111, 122), and reports of virus isolation from various visceral organs (111, 118, 119). Viral isolation is often unsuccessful, even from tissues with the highest viral RNA levels (112, 119). The genetic markers for pantropic CCoV-2a's are currently unknown. TGEV-like CCoV-2b has also been associated with systemic spread, but only in cases of co-infection with canine parvovirus. Some features of systemic infection that are similar to severe acute respiratory syndrome (SARS) in humans and feline infectious peritonitis (FIP) in cats, have been documented for systemic fatal CCoV-2 infections (122), including pulmonary alveolar damage, fibrinous exudation and macrophage involvement. In spite of similarities between these viruses and frequent sub-clinical infections, it is clear that systemic and lethal FIP is a much more common outcome for FCoV infection of cats, compared to CCoV infection of dogs.

1.14 Emergence of CCoV Variants

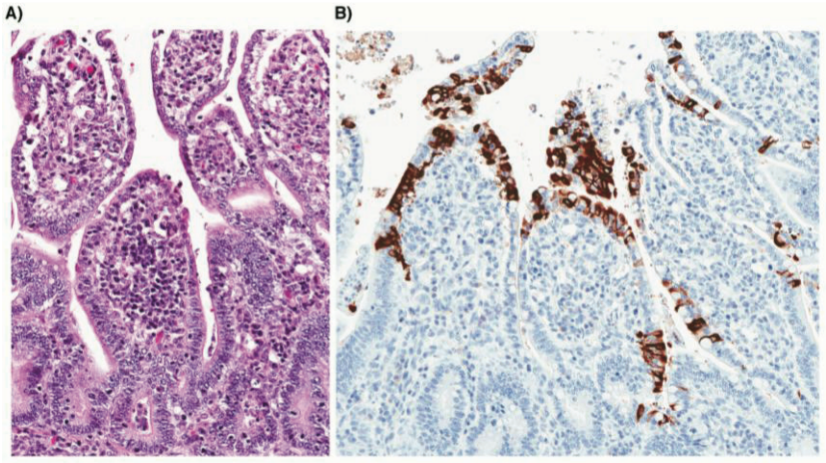
It is clear that coronaviruses have high potential for emergence of novel variants with altered tropism and pathogenesis. In large part this can be explained by several factors: a combination of recombination and mutation within the viral genome and that many coronaviruses exist as quasi-species, the availability of natural reservoirs for the virus and the "modular" nature of the viral spike protein (140). Recent studies have highlighted the extraordinary complexity of canine coronavirus genomics. The increased likelihood of high-density housing for dogs increases the viral load of coronavirus in the population, and increases the likelihood of novel viruses emerging within dogs. It is now well-established that the feline coronavirus FIPV WSU-79-1146 arose following recombination with a canine coronavirus (85), and other recombinant canine-feline viruses have been identified (141). Increased co-housing of dogs with other species, particularly cats, also increases the chance of novel recombinant coronaviruses emerging across species.

1.15 Laboratory Diagnosis

Definitive diagnosis of CCoV-1 induced disease is currently difficult; thus, it is not commonly done or available. The virus can be identified by visualization of viral particles in stool specimens following negative staining and examination by transmission electron microscopy; however, this is not a routinely available diagnostic tool. Virus isolation can be achieved for CCoV-2, but not for CCoV-1 viruses, and again, this is not commonly available. The definitive test is post-mortem identification of viral antigen by immunofluorescence or immunohistochemical staining of tissue

sections (Figure 1.7). The most useful ante-mortem tests are RT-PCR-based, which are highly sensitive assays (110, 142, 143) A common PCR test can reliably detect alphacoronaviruses (51), and more specific PCR tests can be employed for further characterization into specific serotypes or genotypes; however, because of the highly variable nature of CCoV genomes, novel variants may be missed with this approach. Serological tests are of limited use since they can only confirm exposure to CCoV and cannot currently discriminate between infecting CCoV serotypes or genotypes.

Figure 1.7 Photomicrographs of small intestine taken from a two-week-old puppy with typical lesions of CCoV infection. (**A**) Severe atrophy of intestinal villi with attenuated low cuboidal to squamous enterocytes (hematoxylin and eosin stain; 20× original magnification). (**B**) Coronavirus antigen is present within the cytoplasm of infected villous enterocytes (immunohistochemistry; 20× original magnification).



1.16 Vaccination and Treatment

A variety of inactivated and modified-live virus vaccines are commercially available and designed to prevent infection with CCoV (131). Current vaccines are safe, but provide only incomplete protection—in that they reduce, but do not eliminate, CCoV replication in the intestinal tract (125, 144). As these vaccines are likely based on the

classical CCoV-2a viruses, protection against CCoV-1 strains with these vaccines is unlikely, and protection against the variant type 2 strains is uncertain. Treatment of CCoV-1nduced gastroenteritis is mainly by supportive care, including good maintenance of fluid and electrolytes. There are no available anti-viral drugs for treatment of CCoV infections.

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CHAPTER TWO

Mutation in Spike Protein Cleavage Site and Pathogenesis of Feline Coronavirus

Licitra BN, J.K. M, Regan AD, Hamilton BS, Rinaldi VD, Duhamel GE, Whittaker GR. 2013. Mutation in spike protein cleavage site and pathogenesis of feline coronavirus. Emerging Infectious Diseases **19:**1066-1073.

2.1 Introduction

Feline infectious peritonitis (FIP) is a fatal infection that affects domestic and wild members of the family Felidae and is caused by a feline coronavirus (FCoV) of the family *Coronaviridae*, subfamily *Coronavirinae*, genus *Alphacoronavirus*, species *Alphacoronavirus*-1 (*1*). The FCoV genome is approximately 29 kB and has 11 open reading frames encoding replicative, structural, and accessory proteins (*2*). Two serotypes have been identified. Serotype 1 FCoVs are highly prevalent clinically (*3–5*) but grow poorly in cell culture and are therefore under evaluated when compared with serotype 2 FCoVs, which are easily propagated *in-vitro* but less prevalent.

Within each serotype, there are 2 biotypes, each causing distinct disease outcomes. Feline enteric coronavirus (FECV) of serotypes 1 and 2 infects enterocytes, causing mild and generally self-limiting infections. FECV spreads efficiently through the oral–fecal route, and chronically infected cats can shed infectious virus in feces for a year or longer (*6,7*). The second biotype found in both serotypes, FIP virus (FIPV) is found less frequently but causes FIP.

The current understanding is that FIPV arises during in vivo infection from a genetic mutation of FECV (*8–11*). A long-standing hypothesis is that FIP viruses arise from internal mutation of endemic FECVs (*12*), which is believed to occur in approximately 1%–5% of enteric infections, resulting in the ability of the virus to infect blood monocytes and tissue macrophages. The resulting productive infection of these cells, a hallmark of FIP, enables systemic spread and results in macrophage activation, with concomitant immune-mediated events leading to death. To date, the precise mutation

or mutations that cause a shift in FCoV biotype have not been identified.

As with other RNA viruses, coronavirus replication is error-prone; the estimated mutation rate is approximately 4 × 10 nucleotide substitutions/site/year (*13*,*14*). It has been suggested that mutations in the 3c and 7b genes may be involved in the transition to FIPV (*1*,*12*,*15*). Because FCoV spike protein plays critical roles in receptor binding (S1) and fusion (S2), we focused on structural changes in this protein and potential role in altered cellular tropism. In particular, acquisition of macrophage tropism for a serotype 2 FCoV has previously been mapped to the spike gene (*16*), further suggesting that key mutations within spike protein may be important for the biotype switch.

The coronavirus spike protein is a class I fusion protein, which typically requires activation by cellular proteases. Mutation of the proteolytic cleavage site often has profound implications for disease progression (*17*,*18*). Until recently, FCoVs were thought to have uncleaved spike proteins. However, a functional furin cleavage site has been identified in 2 serotype 1 FECVs, located at the shared boundary of the S1 and S2 subunits (*19*). Furin is a ubiquitous proprotein convertase enriched in the trans-Golgi network and is well-conserved among mammals (*20*). Furin cleaves a wide range of protein precursors into biologically active products at a consensus motif R-X-K/R-R, where R is the basic arginine residue, X is any residue, and K is the basic lysine residue (*21*).

In this article, we establish a novel approach to studying FIP that complements previous work. Instead of performing a mutation study based mainly on comparative

genetic analysis (*15*,*22–24*), we focus on S1/S2, a functionally relevant site, and study variations between the biotypes and their functional effects. This rationale could provide a better means to uncover functionally important mutations that account for FIP.

We considered that mutations at the S1/S2 site could alter proteolytic cleavage and modify S fusogenic properties, leading to tropism expansion, systemic spread and, ultimately, FIP. We investigated genetic variations at the S1/ S2 site of serotype 1 FECVs and compared these sequences to those present in viral RNA recovered from tissues of cats with FIP. Fluorogenic peptide cleavage assays were conducted to assess the effects of substitutions found in the S1/S2 site. We document a junction mutation at S1/S2 that arises during development of FIP. Our study has uncovered a molecular basis for FIP that has potential to lead to developments in diagnostics, prevention, and therapies.

2.2 Materials and Methods

FCoV Sequence Analysis

Clinical and demographic data are reported in Technical Appendix Table 1. Fecal samples from asymptomatic infected domestic cats were solicited from shelters and veterinarians throughout the United States. RNA was extracted by using QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA). FCoV primers that detect most circulating strains were used to screen all fecal samples (*25*). RNA extracted from FIPV-TN406 (Black) laboratory-adapted strain was used as a positive control.

We analyzed 22 FIPV-positive tissue samples (Veterinary Pathology Archives, Cornell University, Ithacan, NY, USA) from 11 cats with FIP. Diagnosis of FIP was based on the standard method of immunohistochemical evaluation by board-certified pathologists. Each sample was retrieved from formalin-fixed, paraffin-embedded tissue blocks from which sections were stained by using FIPV 3–70 antibody (Custom Monoclonals, Sacramento, CA, USA). Positively stained regions were thinly sectioned and RNA was extracted by using RecoverAll (Ambion, Foster City, CA, USA).

Fecal samples collected from FCoV-positive housemates, cats 234 and 304, were processed as previously described in this section. After the referring veterinarian made a diagnosis of FIP in cat 234, the owner elected to euthanize the animal. Fresh tissue was harvested and RNA extracted by using MagMAX Express (Life Technologies, Grand Island, NY, USA).

For all samples, 50 μL reverse transcription PCRs (RTPCRs) were performed with One-Step RT-PCR (QIAGEN) by using gene-specific S primers, encompassing S1/S2. The PCR primers sequences are found in Technical Appendix Table 2. PCR conditions were 30 min at 50°C, 15 min at 95°C, and 39 or 35 cycles of 1 min at 94°C, 1minat 55°C, 1 or 1.5 min at 72°C, and 10 min at 72°C. PCR products were purified by using a QIAquick Gel Extraction Kit (QIAGEN). Sanger sequencing was performed at the Life Sciences Core Laboratories (Cornell University). Nucleotide archive accession numbers are shown in Technical Appendix Table 4. DNA sequences were translated into protein sequences and alignments were performed by using Geneious 5.4 (Biomatters Ltd., Auckland, New Zealand). Sequence logos were

generated by using Weblogo 3.1 (http://weblogo.threeplusone.com/). Statistical analysis was performed by using 2-tailed Fischer exact test. In the test, the numbers of FIPV-infected and FECV-infected cats were counted. For each category of FIPV or FECV infection, cats harboring viruses with or without mutations at the S1/S2 site were counted.

Furin Cleavage Assay

Fluorogenic 12-mer peptides were designed and synthesized by RS Synthesis, Louisville, KS, USA (Technical Appendix Table 3). Purified recombinant human furin was purchased from NEB (Ipswich, MA, USA). For each reaction, 1 unit of enzyme was used in 100 μL final volume by using the reaction buffer 100 nmol/L HEPES, 0.5% Triton X-100, 1 mmol/L $CaCl_2$, 1 mmol/L 2-mercaptoethanol, pH 7.5. Peptides were diluted to 50 μmmol/L. Reactions were performed in triplicate at 30°C and fluorescence was measured with a SpectraMax fluorometer (Molecular Devices, Sunnyvale, CA, USA), enabling Vmax determination. Results for each peptide are expressed as percent cleavage by furin compared with the canonical sequence.

To perform comparative analysis of the S1/S2 cleavage site between FECVs and FIPVs, we identified cases of FIP that were confirmed postmortem by using immunohistochemistry, the standard for FIP diagnosis; archival immunohistochemistry-positive formalin-fixed tissues were used as the source of FIPV RNA. To ensure good quality sequence information from archival material, the RT-PCR amplicon size was limited to 160bp (including the S1/S2 site). This same

region was then amplified from fecal material from coronavirus-positive healthy cats.

2.3 Results

FECV S1/S2

Sequencing of the S1/S2 site of 30 S sequences from FECV fecal samples revealed an extremely well-conserved motif at the amino acid level (Figure 1, panel A). In particular, arginine (R) residues are found exclusively at the most critical positions for furin recognition and cleavage (P1, P2, and P4) in all sequences analyzed (Figure 2, panel B). The P1′ position is extremely well conserved, because serine (S) is found in 100% of cases. The P5 position is also well conserved, evidenced by a clear majority of basic residues found (96.6% arginine or lysine [K]; Figure 2, panel B). At P3, limited variability is found (76.7% serine and 23.3% alanine [A]). Overall, 100% of FECV sequences analyzed contain the furin cleavage site, with a core motif of R-R-S/A-R-R-S.

FIPV S1/S2

Analysis of the S1/S2 cleavage site of FIPV sequences shows that it has much more variability, both within the narrow furin cleavage recognition motif (P4-P1) and in residues extending out of it (P8-P5 and P2′-P4′) (Figure 2A). A striking observation is that the critical positions P1 and P2 are among the most consistently mutated (Figure 3). To a lesser degree, variability extends to other positions of the cleavage motif, notably in the P1′, P3, P4, and P5 positions (Figure 2). Examination results of the entire portion of spike sequenced in this study indicate that the conserved R-R-S/A-R-

R-S motif in FECV is present within a region of the spike gene that shows a high degree of variability, in contrast to other neighboring regions that are more highly conserved (Technical Appendix Figure 1).

Correlation between FIP Status of Cats and Presence of Mutations at S1/S2

A Fisher exact test was performed to establish whether a correlation existed between the FIP status of the sampled cats and mutations at the S1/S2 site of viruses analyzed (Table 1). The test unequivocally demonstrated that there was a strong correlation ($p<0.0001$) between FIP and presence of mutations at S1/S2.

Table 2.1 Fisher Exact Test Tabulation. Status of cats sampled for feline coronavirus and mutations in spike protein cleavage site

	FECV-infected cats	FIPV-infected cats	Total
Cats harboring viruses with ≥ 1 mutated S1/S2 residue	2	10	12
Cats harboring viruses with an intact S1/S2 site	28	1	29
Total	30	11	41

FECV, feline enteric coronavirus; FIPV, feline infectious peritonitis virus; S1, receptor binding domain of spike; S2, fusion domain of spike

Fluorogenic Peptide Furin Cleavage Assay

To test whether the identified FIPV S1/S2 mutations have an effect on cleavability by furin, we performed an in vitro proteolytic assay. We used human furin for these experiments. Human and feline furin are very similar (96% identical) and are expected to cleave in an equivalent manner. However, feline furin has not been directly studied to any degree, and reagents are not readily available. Feline and human cells lines show identical rates of cleavage for a known furin target protein (PSCK-9), which contains an active furin cleavage site (Technical Appendix Figure 2). We used fluorogenic peptides containing the canonical motif (RR-S-R-R-S) or with substitutions from positions P1′ through P7 (Figure 4, panel A). The canonical peptide was efficiently cleaved by furin (Figure 4), with average Vmax of 235 Relative Fluorescence Units (RFU) per minute.

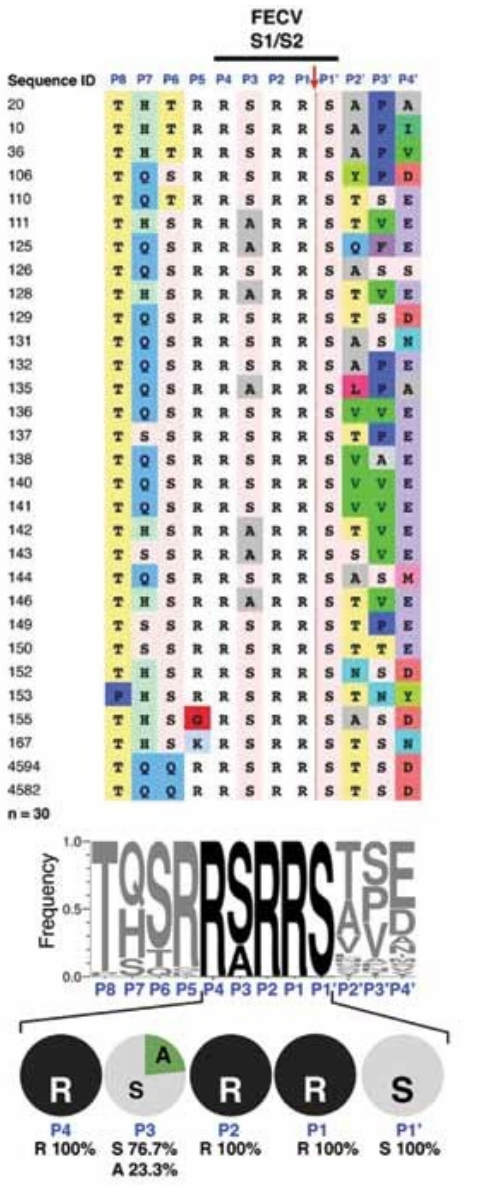

Figure 2.1 Sequence analysis of feline enteric coronavirus (FECV) spike S1/S2 site. RNA from 30 FECVs collected from 30 fecal samples obtained from subclinically infected cats was extracted, purified, and reverse-transcribed into cDNA. Sequencing of the spike gene was performed in a region surrounding the S1/S2 cleavage site. A) Sequence alignment. Sequence identification row (blue font): residue positions in the S1/S2 cleavage site from P8 to P4′. Red arrow indicates the site of furin cleavage. B) To visualize the diversity of residues at each position of the S1/S2 site, sequences were subjected to WebLogo 3.1 analysis (http://weblogo.threeplusone. com/create.cgi). Top: WebLogo for the 30 FECV S1/S2 sequences with the frequency of residue found at each position displayed. Bottom: summary of the diversity of residues for each position from P4 to P1′ and percentages of each amino acid represented.

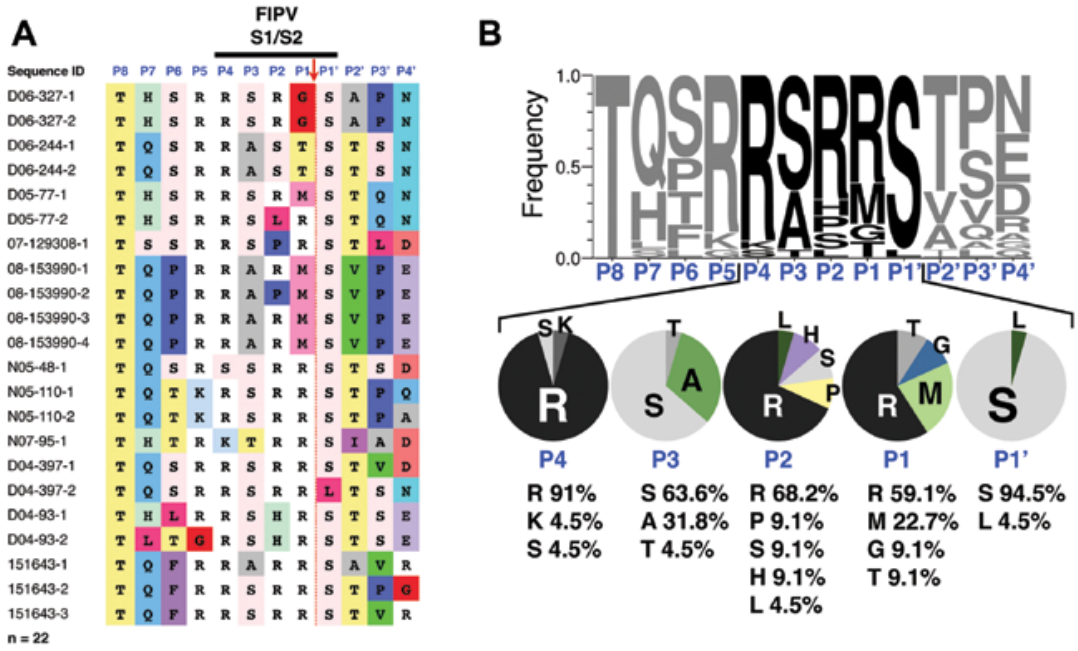

Figure 2.2 Sequence analysis of feline infectious peritonitis virus (FIPV) spike S1/S2 site. RNA from 22 FIPVs collected from 11 cats that had feline infectious peritonitis was extracted, purified, and reverse-transcribed into cDNA. Sequencing of the spike gene was performed in a region surrounding the S1/S2 cleavage site. **A)** Sequence alignment. Sequence identification row (blue font): residue positions in the S1/S2 cleavage site from P8 to P4′. Red arrow indicates the site of furin cleavage. **B)** To visualize the diversity of residues at each position of the S1/S2 site, sequences were subjected to WebLogo 3.1 analysis (http://weblogo.threeplusone.com/create.cgi). Top: WebLogo for the 22 FIPV S1/S2 sequences with the frequency of residue found at each position displayed. Bottom: summary of the diversity of residues for each position from P4 to P1′ and percentages of each amino acid represented.

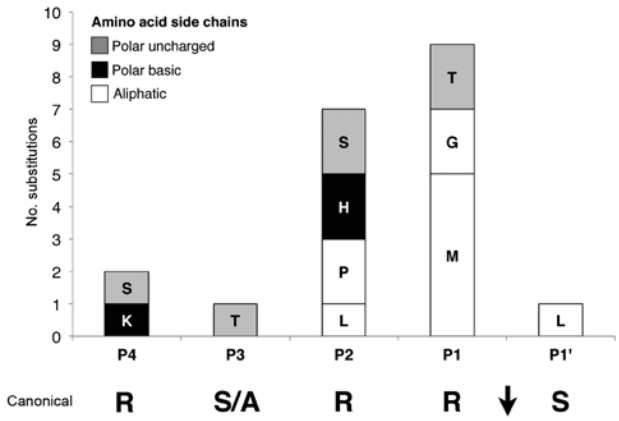

Figure 2.3 Amino acid substitution frequency at each position of the feline infectious peritonitis virus S1/S2 cleavage site. The histogram is based on feline infectious peritonitis virus S1/S2 WebLogo 3.1 analysis (http://weblogo.threeplusone.com/create.cgi), showing percentage of modification of residues at each position of the S1/ S2 site, compared with feline enteric coronavirus S1/S2 canonical sequence consensus.

Within the P4-P1′ core peptide, in the canonical background, when the P1′ serine residue is changed into a leucine (L), furin cleavage is severely diminished (8% of canonical cleavage rate), a result that shows the key role of the conserved P1′ serine. Modifications of the P1 arginine in the canonical peptide, regardless of the residue tested, for example, glycine (G), methionine (M) or threonine (T), abrogate cleavage by furin (Figure 4). Modifications at the P2 arginine residue in the canonical peptide have variable effects. When P2 arginine is changed to histidine (H), there is complete inhibition (0% of canonical cleavage). When P2 is changed to leucine or serine, cleavage efficiency is reduced by approximately 50% and 20%, respectively. When P2 is modified to proline (P), cleavage efficiency slightly increases to 129% of the canonical peptide (Figure 4). The P3 S-A substitution minimally enhances cleavage

(Figure 4). P4 arginine is another residue position that is essential for furin cleavage. In the canonical peptide, P4 R-K substitution, there is a slight decrease in cleavage efficiency (88.7% of canonical rate). In contrast, when the P4 arginine is substituted with glycine, furin cleavage is completely abrogated (Figure 4).

For positions upstream of P4, while P5 R-K and P6 T-F modifications have moderate enhancing effects on furin cleavage (149% and 162% of canonical rate, respectively), the P7 H-Q peptide shows a substantial increase in its cleavability (186% compared with canonical). The P7 H-Q P5 R-K peptide shows that the effect of each modification can be additive (232% compared with canonical peptide) (Figure 4).

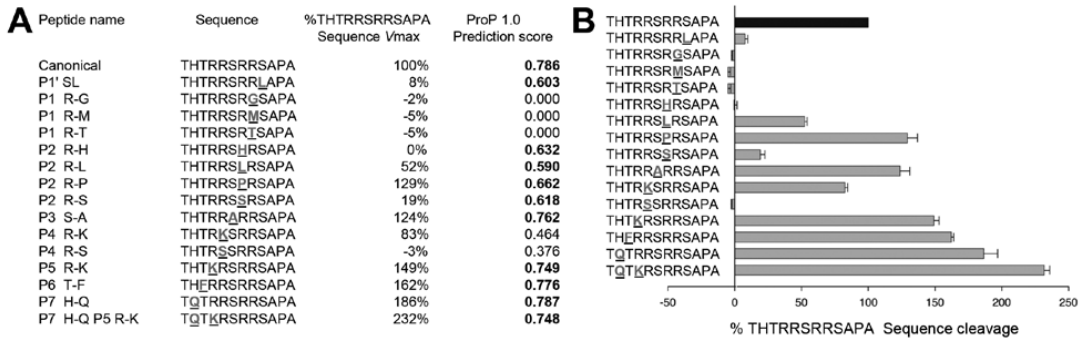

Figure 2.4 Furin cleavage assays of fluorogenic peptides. **A)** Synthetic fluorogenic peptides were generated with sequences matching consensus feline enteric coronavirus and a panel of modified sequences with substitutions (underlined) found by feline infectious peritonitis virus sequencing. Peptides (50 μmol/L) were subjected to cleavage by recombinant human furin (1 U/100 μL), at pH 7.5, 30°C, and the release of fluorescence over time was measured by a spectrofluorometer enabling calculation of the Vmax of each reaction. Peptide cleavage scores generated by the ProP 1.0 server (www.cbs.dtu.dk/services/ProP/) are also displayed. **B)** For each modified peptide (substitutions underlined), the percentage of cleavage rate compared with the canonical sequence was calculated and displayed. Cleavage assays were performed in >3 independent experiments. Error bars indicate SD for each measurement.

Functionally Relevant S1/S2 Mutation

To further confirm our findings, we analyzed the S1/ S2 sites from viral samples taken from cats 234 and 304, who lived in the same household (Table 2). At the initial sampling in 2009 (t = 1), both cats were asymptomatic for FIP and were shedding FCoV in their feces. In samples from both cats, the S1/S2 sites had a core sequence R-R-S-R-R-S consistent with the FECV consensus. Upon the second sampling in 2011/2012 (t = 2), FIP was diagnosed in cat 234. Cat 304 remained asymptomatic but continued to shed virus in feces. Notably, when the S1/ S2 sequences were analyzed at the second sampling, only the cat with FIP (234) had a change in the FECV consensus sequence (a P2 R-L mutation). While exhibiting a change in the P3 residue (S-A), the virus present in cat 304 retained the conserved S1/S2 furin cleavage motif (Table 2). These data provide direct evidence of mutations in spike linked with development of FIP in cats.

Table 2.2 Sequence of FCoV spike at S1/S2 junction in cats sampled for feline coronavirus*

Time	Cat # 234	Cat # 304
t = 1	NHTHTRRSRRˇSAPVAV	NHTHTRRSRRˇSAPVAV
t = 2	NHTHTRRSLRˇSAPVAV	NHTHTRRARRˇSAPVAV

* Underlines indicate nucleotide substitution. FCoV, feline coronavirus; S1/S2, cleavage site of spike protein; t =1 both cats disease-free; ˇ, position of cleavage; t = 2 cat #234 feline infectious peritonitis positive, cat #304 disease free

2.4 Discussion

To study FIP, we have taken an alternative approach that complements earlier studies that were based on analytical outcomes of putative FIP-causing mutations and inference of their functional consequences. We focused on the S1/S2 sequence, a specific and functionally highly relevant cleavage site within the S protein, and documented mutations between asymptomatic and highly symptomatic cats that correlated strongly with FIP. We also documented a functional S1/S2 cleavage site mutation that arose in an asymptomatic cat that subsequently developed FIP.

Our sequence data show that serotype 1 FECV from feces of asymptomatic cats contain a highly conserved furin cleavage motif at the S1/S2 site, with the following narrow range of residues: $(R{>>}K/G)^{P5}$-$(R)^{P4}$-$(S{>}A)^{P3}$-$(R)^{P2}(R)^{P1}$-$(S)^{P1'}$. In addition to the consensus R-X-K/R-R motif, additional flanking residues can also be consequential for furin-mediated cleavage (*26–28*). In particular, a serine (S) residue is critical in the P1′ position (*29*) and it is notable that all FECVs examined contained a P1′ S residue. The fact that the S1/S2 site is extremely well conserved is an indication that it is functionally essential for FECV replication in the enteric epithelium.

In contrast to the situation for asymptomatic cats infected with FECV, we found that sequences of FCoV sampled from tissue of confirmed FIP-positive cats consistently have mutations at the S1/S2 site. In the most critical position for furin cleavage, P1, we found that >40% of FIPVs have a mutation in the arginine residue, which is replaced by an aliphatic (methionine and glycine) or polar uncharged (threonine)

residue. Overall, the distinguishing feature of FIPVs is the absence of the P1 arginine, rather that the presence of any particular residue. This is corroborated by our peptide cleavage data that demonstrate that furin cleavage is fully abrogated for all P1 substitutions tested. The next most common position mutated in FIPV is P2; >30% of the FIPVs analyzed bore mutations at this position. Most mutated residues found were aliphatic (P and L). Some sequences were substituted with a polar basic (H) or a polar uncharged (S) residue. Apart from the P2 R-P substitution, peptide cleavage data indicates that all other substitutions have an inhibiting effect on furin cleavage. Of note, for murine hepatitis virus (MHV), a betacoronavirus that also harbors an S1/S2 cleavage site in its spike protein, there is a precedent for the inhibitory effect of the introduction of a histidine in the P2 position of the cleavage site. Two well-studied strains, MHV strain A59 (MHV-A59) and the neurovirulent MHV strain JHM (MHV-JHM), have a notable difference at this site. MHV-A59 has an RR-S-H-R-S sequence and is less efficiently cleaved than MHV-JHM, which has an R-R-A-S-S-R sequence (*18*). P4 is generally considered to be critical for furin cleavage, but we found limited variation in this residue position for the FIPVs tested and found mutation to the polar basic residue (K) or polar uncharged residue (S) in <5% of viruses. The peptide data indicates that, although introduction of a serine at P4 completely abrogates cleavage, the P4 R→K substitution has minimal effect. The FIPV P3 position showed small variation compared with FECV after the introduction of a polar uncharged residue (T) in 1 sample. For the P5 position, the only change was a slightly higher frequency of the lysine residue in samples from cats with FIPV. Peptide cleavage data indicated that the common S-A substitution found for FECV and FIPV P3 positions has only slightly

increasing effect on proteolysis by furin. Furthermore, the P5 R→K substitution has an enhancing effect in the peptide cleavage assay. At P1′, the conserved polar uncharged residue (S) was retained in the majority of FIPV samples, however, the introduction of an aliphatic amino acid (L) was found. It is notable that furin cleavage has been suggested to be incompatible with a hydrophobic aliphatic side chain, with a strong preference for serine in the P1′ position (*27*,*29*). In the D04-397-2 sample containing the P1′ L, the basic residues within the S1/S2 site remain identical to the ones found in FECV sequence; we suggest that disruption of furin cleavage is mediated by a mutation in P1′, rather than the more typical P1, P2 and/or P4 mutation. This hypothesis is supported by the peptide cleavage assay, where the P1′S-L peptide is unable to be cleaved by furin.

Overall, in terms of FIP-positive animals, we found that 10 of 11 cats harbored viruses with mutations in the furin motif R-R-S/A-R-R-S found in FECV of asymptomatic cats. For most FIP-positive cats, we sequenced viral RNA collected from different tissues (Technical Appendix Table 1). Our data provides strong support for the internal mutation hypothesis, as the mutations are unique to individual cats.

Of note, not all tissues from the same animal carry the same mutation. In some instances, mixed populations of viruses exist within the same animal. The majority of viruses sequenced had 1 mutation, although 5 (D06-244-1, D06244-2, 08-153990-2, N07-95-1, and D04-93-2) had 2 mutations. However, there are 2 apparent exceptions of cats harboring viruses that do not have clearly defined mutations in the furin cleavage site: samples from cat 151643 (*1–3*) and samples from cat N05-110 (*1*,*2*).

We consider that the presence of a P6 furin cleavage in samples of cat 151643 is consistent with our hypothesis of a switch in the activating protease for the virus, because this is not typical of naturally occurring furin cleavage sites. Samples 1 and 2 from cat N05-110 harbor virus with an atypical lysine residue at P5. While unusual for FECVs, a P5 lysine residue does appear to be compatible with furin cleavage, so it remains to be determined how noteworthy a P5 lysine residue versus a P5 arginine residue is in the context of a protease switch for FIPV, or whether other mutations correlated with FIPV in the case of this cat.

As part of our study, we analyzed field samples from cats harboring FCoV at different times. In cat 234, the virus underwent a transition from FECV to FIPV, and had a functionally relevant mutation in the S1/S2 motif (P2 R-L). Cat 304, living in the same house as cat 234, remained asymptomatic. Cat 304 harbored a mutated virus, but the mutation was in a functionally irrelevant position (P3 S-A). Identification of cats with FECV in which FIP subsequently develops is challenging, and while we present a single example, we consider these data to be strong evidence that mutations at the S1/S2 site are linked to a change in the pathogenic properties of the virus, and likely to be essential for the acquisition of macrophage tropism seen in FIP .

The S1/S2 cleavage site and surrounding residues of serotype 1 FIPV S sequences were found to be systematically modified by mutations. Chang et al. recently published an extensive comparative analysis of FIP mutations at the nucleotide level by performing whole-genome sequencing of FECVs and FIPVs; the authors found a site within S (nucleotide position 23531), but outside of S1/ S2, to be the most

frequently mutated in FIPV (*15*). We have undertaken an analysis of the S1/S2 sites sequenced by Chang et al. and find that our hypothesis that mutation within the S1/S2 furin motif correlates with FIP in ≈64% of their samples. There are 3 differences in methodology that may explain this lack of agreement: first, we employed immunohistochemistry to confirm the diagnosis of FIP, while Chang et al. reported using postmortem examination; second, all FIP samples in this study originate from tissue, while Chang et al. included both tissue and ascites fluid; and finally, we report multiple sequences for FIP-affected cats, while Chang et al. reported a single sequence. Sequence data from samples D04-397-1 and D04-397-2 provide evidence that both FECV and FIPV populations can be identified within an affected animal. Sequence information from a single sample may not be adequate for the detection of mutated virus.

Most mutations negatively affect furin processing, but some enhance it. Given that the majority of the FIPV S proteins still harbor basic residues at the S1/S2 boundary, it could be reasoned that the mutated site becomes more open and can be cleaved by a range of other proteases. The switch in proteolytic requirements of S that we propose may offer an explanation for the crucial tropism transition during FIP. A possible consequence of the mutations is cleavability by monocytic/macrophage-specific proteases. These could be pro-protein convertases, cathepsins, or other macrophagespecific proteases. In particular, cathepsin B, matrix metalloproteases, and furin-related PCSK1 are likely to be expressed on the surface of macrophages and recognize the hallmark residues remaining or acquired in FIPV S1/S2 cleavage site

(*30*). Matrix metalloprotease 9 is of particular interest because it was demonstrated to be upregulated in activated monocytes and macrophages during FIP (*31*). A shift in the entry pathway to enable virus entry at the cell surface instead of the endosome may simultaneously explain the ability of FIPV to infect macrophages and the macrophage resistance of FECV. It is also possible that the mutations in the S1/S2 region affect the heparin sulfate binding site in this region (*19*). However, heparin sulfate binding is a cell culture adaptation of the virus, and as so, its relevance to the clinical situation would appear to be unlikely.

A contrasting view to the internal mutation hypothesis to explain the genesis of FIP outbreaks is that there is a circulating FCOV other than FECV that is specific for FIP (*22*). For a complex disease process such as FIP, we and others consider it likely that there may be circulating FECVs that are closer to making the critical mutations necessary for FIP, possibly explaining paradoxical FIP outbreaks (*32*). Based on the data we present here, we conclude that mutation of the S1/S2 locus and modulation of a furin recognition site normally present in the S gene of FECVs is a critical contributing factor for development of FIP. Further studies could serve to analyze how S1/S2 mutations fit with the other mutations posited to account for FIP development.

2.5 Acknowledgments

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2.6 Technical Appendix

Technical Appendix Table 1. Clinical and demographic data from 44 domestic cats sampled in the continental United States from 2004–2012

Cat no.	Sample no.	FECV/FIPV	Sample Type	Year	State	Sequence
20	-	FECV	Feces	2008	PA	THTRRSRRSAPA
10	-	FECV	Feces	2008	PA	THTRRSRRSAPI
36	-	FECV	Feces	2008	PA	THTRRSRRSAPV
106	-	FECV	Feces	2010	CT	TQSRRSRRSYPD
110	-	FECV	Feces	2010	CT	TQTRRSRRSTSE
111	-	FECV	Feces	2010	CT	THSRRARRSTVE
125	-	FECV	Feces	2010	WI	TQSRRARRSQFE
126	-	FECV	Feces	2010	WI	TQSRRSRRSASS
128	-	FECV	Feces	2010	WI	THSRRARRSTVE
129	-	FECV	Feces	2010	WI	TQSRRSRRSTSD
131	-	FECV	Feces	2010	WI	TQSRRSRRSASN
132	-	FECV	Feces	2010	WI	TQSRRSRRSAPE
135	-	FECV	Feces	2010	IA	TQSRRARRSLPA
136	-	FECV	Feces	2010	IA	TQSRRSRRSVVE
137	-	FECV	Feces	2010	IA	TSSRRSRRSTPE
138	-	FECV	Feces	2010	IA	TQSRRSRRSVAE
140	-	FECV	Feces	2010	IA	TQSRRSRRSVVE
141	-	FECV	Feces	2010	IA	TQSRRSRRSVVE
142	-	FECV	Feces	2010	IA	THSRRARRSTVE
143	-	FECV	Feces	2010	IA	TSSRRARRSSVE
144	-	FECV	Feces	2010	IA	TQSRRSRRSASM
146	-	FECV	Feces	2010	CA	THSRRARRSTVE
149	-	FECV	Feces	2010	NY	TSSRRSRRSTPE
150	-	FECV	Feces	2010	NY	TSSRRSRRSTTE
152	-	FECV	Feces	2010	MI	THSRRSRRSNSD
153	-	FECV	Feces	2010	NY	PHSRRSRRSTNY
155	-	FECV	Feces	2010	NY	THSGRSRRSASD
167	-	FECV	Feces	2010	RI	THSKRSRRSTSN
4594	-	FECV	Feces	2004	MD	TQQRRSRRSTSD
4582	-	FECV	Feces	2004	MD	TQQRRSRRSTSD
D06 327	1	FIPV	Spleen	2006	NY	THSRRSRGSAPN
D06 327	2	FIPV	Mesentery	2006	NY	THSRRSRGSAPN
D06 244	1	FIPV	Mesenteric lymph node	2006	MI	TQSRRASTSTSN
D06 244	2	FIPV	Mesentery	2006	MI	TQSRRASTSTSN
D05 77	1	FIPV	Kidney	2005	NC	THSRRSRMSTQN
D05 77	2	FIPV	Cerebellum	2005	NC	THSRRSLRSTQN
07 129308	1	FIPV	Mesentery	2007	NY	TSSRRSPRSTLD
08 153990	1	FIPV	Kidney	2008	PA	TQPRRARMSVPE
08 153990	2	FIPV	Brain stem	2008	PA	TQPRRAPMSVPE
08 153990	3	FIPV	Cerebrum	2008	PA	TQPRRARMSVPE
08 153990	4	FIPV	Cerebellum	2008	PA	TQPRRARMSVPE
N05 48	1	FIPV	Cerebellum	2005	VA	TQSRSSRRSTSD
N05 110	1	FIPV	Mesenteric lymph node	2005	NY	TQTKRSRRSTPQ
N05 110	2	FIPV	Cerebellum	2005	NY	TQTKRSRRSTPA
N07 95	1	FIPV	Cerebrum	2007	NY	THTRKTRRSIAD
D04 397	1	FIPV	Spleen	2004	PA	TQSRRSRRSTVD
D04 397	2	FIPV	Mesenteric lymph node	2004	PA	TQSRRSRRLTSN
D04 93	1	FIPV	Kidney	2004	PA	THLRRSHRSTSE
D04 93	2	FIPV	Cerebrum	2004	PA	TLTGRSHRSTSE
151643	1	FIPV	Heart	2008	NY	TQFRRARRSAVR
151643	2	FIPV	Spleen	2008	NY	TQFRRSRRSTPG
151643	3	FIPV	Liver	2008	NY	TQFRRSRRSTVR
234	t1	FECV	Feces	2008	PA	THTRRSRRSAPV
234	t2	FIPV	Kidney	2011	PA	THTRRSLRSAPV
304	t1	FECV	Feces	2008	PA	THTRRSRRSAPV
304	t2	FECV	Feces	2012	PA	THTRRARRSAPV

Technical Appendix Table 2. Primers used for reverse transcription PCR amplification of the feline coronavirus spike protein S1/S2 region to detect mutation in feline coronavirus

Primer	Nucleotide sequence (5′→3′)
FFPE Fwd2	GCACAAGCAGCTGTGATTA
FFPE Rev2 homology	GTAATAGAATTGTGGCAT
442(F)	GGCAGAGATGGATCTATTTTTGTTA
Sero1rev2a(R)	ATAATCATCATCAACAGTGCC

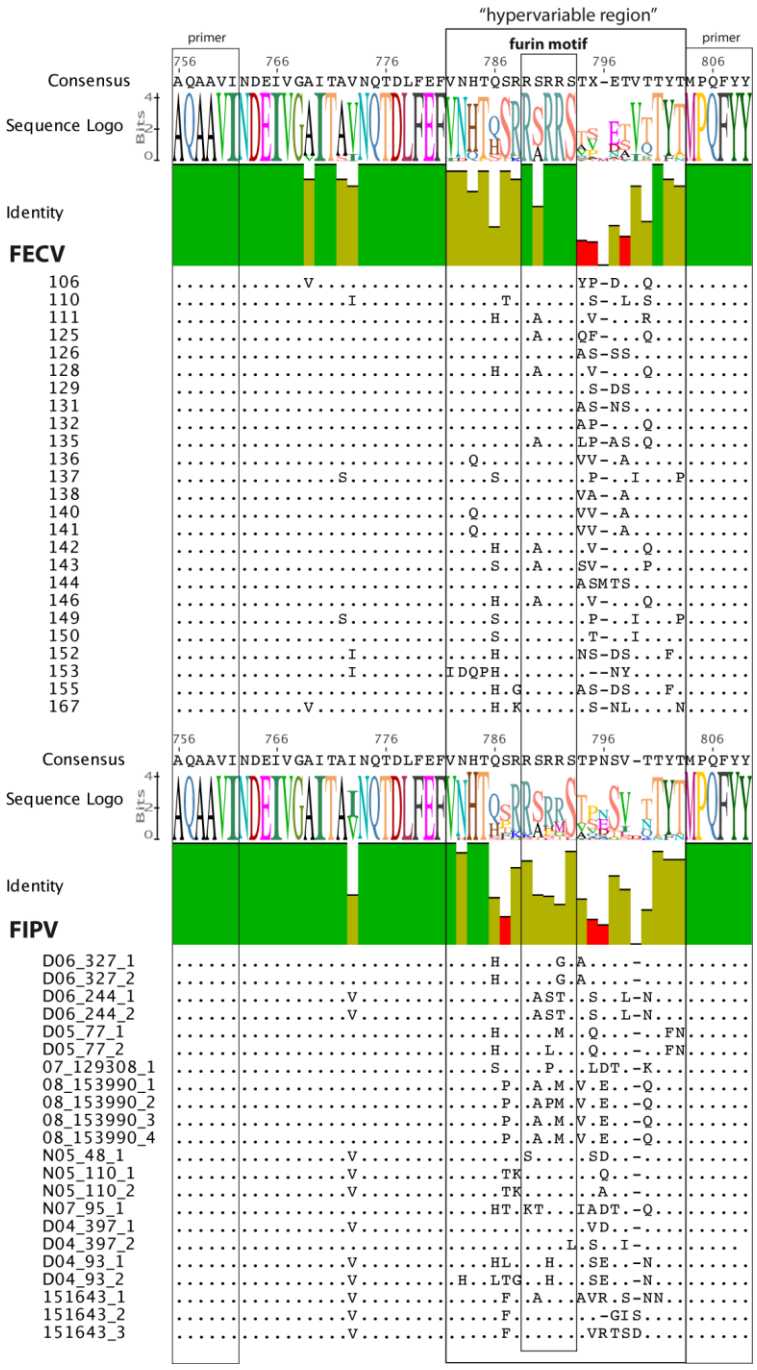
Technical Appendix Table 3. Fluorogenic peptides used in the furin cleavage assay to detect genetic mutation in feline coronavirus*

Peptide	Amino acid sequence
Canonical	THTRRSRRSAPA
P1′ S-L	THTRRSRRLAPA
P1 R-G	THTRRSRGSAPA
P1 R-M	THTRRSRMSAPA
P1 R-T	THTRRSRTSAPA
P2 R-H	THTRRSHRSAPA
P2 R-L	THTRRSLRSAPA
P2 R-P	THTRRSPRSAPA
P2 R-S	THTRRSSRSAPA
P3 S-A	THTRRARRSAPA
P4 R-K	THTRKSRRSAPA
P4 R-S	THTRSSRRSAPA
P5 R-K	THTKRSRRSAPA
P6 T-F	THFRRSRRSAPA
P7 H-Q	TQTRRSRRSAPA
P7 H-Q P5 R-K	TQTKRSRRSAPA

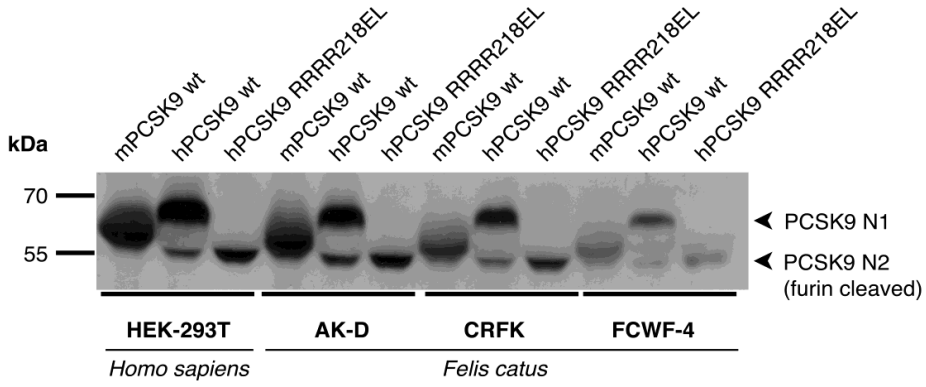
*All peptides contain a methylcoumarin acetamido/2,4-Dinitrophenyl fluorescence resonance energy transfer pair.

Technical Appendix Table 4. European Nucleotide Archive accession numbers for submitted sequences of feline coronavirus spike protein S1/S2 junction site

Cat-sample no.	Sequence	Accession no.
106	TQSRRSRRSYPD	HF954926
110	TQTRRSRRSTSE	HF954927
111	THSRRARRSTVE	HF954928
125	TQSRRARRSQFE	HF954929
126	TQSRRSRRSASS	HF954930
128	THSRRARRSTVE	HF954931
129	TQSRRSRRSTSD	HF954932
131	TQSRRSRRSASN	HF954933
132	TQSRRSRRSAPE	HF954934
135	TQSRRARRSLPA	HF954935
136	TQSRRSRRSVVE	HF954936
137	TSSRRSRRSTPE	HF954937
138	TQSRRSRRSVAE	HF954938
140	TQSRRSRRSVVE	HF954939
141	TQSRRSRRSVVE	HF954940
142	THSRRARRSTVE	HF954941
143	TSSRRARRSSVE	HF954942
144	TQSRRSRRSASM	HF954943
146	THSRRARRSTVE	HF954944
149	TSSRRSRRSTPE	HF954945
150	TSSRRSRRSTTE	HF954946
152	THSRRSRRSNSD	HF954947
153	PHSRRSRRSTNY	HF954948
155	THSGRSRRSASD	HF954949
167	THSKRSRRSTSN	HF954950
D06 327-1	THSRRSRGSAPN	HF954951
D06 327-2	THSRRSRGSAPN	HF954952
D06 244-1	TQSRRASTSTSN	HF954953
D06 244-2	TQSRRASTSTSN	HF954954
D05 77-1	THSRRSRMSTQN	HF954955
D05 77-2	THSRRSLRSTQN	HF954956
07 129308-1	TSSRRSPRSTLD	HF954957
08 153990-1	TQPRRARMSVPE	HF954958
08 153990-2	TQPRRAPMSVPE	HF954959
08 153990-3	TQPRRARMSVPE	HF954960
08 153990-4	TQPRRARMSVPE	HF954961
N05 48-1	TQSRSSRRSTSD	HF954962
N05 110-1	TQTKRSRRSTPQ	HF954963
N05 110-2	TQTKRSRRSTPA	HF954964
N07 95-1	THTRKTRRSIAD	HF954965
D04 397-1	TQSRRSRRSTVD	HF954966
D04 397-2	TQSRRSRRLTSN	HF954967
D04 93-1	THLRRSHRSTSE	HF954968
D04 93-2	TLTGRSHRSTSE	HF954969
151643-1	TQFRRARRSAVR	HF954970
151643-2	TQFRRSRRSTPG	HF954971
234-t1	THTRRSRRSAPV	HF954972
234-t2	THTRRSLRSAPV	HF954973
304-t1	THTRRSRRSAPV	HF954974
304-t2	THTRRARRSAPV	HF954975



Technical Appendix Figure 1. Multiple sequence alignment of regions of FECV and FIPV spike sequenced in this study. Complete nucleotide sequences were translated and aligned by using Geneious version RG (Biomatters Ltd). Logo y-axis bars indicate the sequence conservation at each site, expressed in bits. A consensus, sequence logo, identity histogram, and alignment are displayed for the FECV and FIPV datasets. The primer annealing regions are boxed. There is moderate to high conservation of the consensus sequence outside of a hypervariable region, which spans from position 782 to position 802. It is within this hypervariable region that the conserved FECV furin motif RS/ARRS is found.



Technical Appendix Figure 2. Feline furin can proteolytically process PCSK9, a known substrate of human furin. Human HEK-293T, and feline cell lines AK-D, CRFK, and FCWF-4 were transfected with the wild-type (wt) mouse (m), wt human (h) and a human mutant form of PCSK9 (RRRR218EL) containing a furin canonical cleavage site, enabling complete cleavage by furin. All constructs contain a C-terminal V-5 tag. After undergoing proteolytic processing by several proteases, including furin, PCSK9 is secreted in the extracellular compartment. Twenty-four hours post-transfection, the supernatant of transfected cells were harvested and subjected to Western blot analysis and detection by using mouse monoclonal anti-V5 antibodies. The ~55 kDa (N2) band of the protein corresponds to the furin-cleaved form of PCSK9.

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CHAPTER THREE

Genotypic Characterization of Canine Coronaviruses

Associated with Fatal Canine Neonatal Enteritis in the United States

Licitra BN, Whittaker GR, Dubovi EJ, Duhamel GE. 2014. Genotypic characterization of canine coronaviruses associated with fatal canine neonatal enteritis in the United States. J Clin Microbiol. **52:** 4230-8.

3.1 Abstract

Emerging canine coronavirus (CCoV) variants that are associated with systemic infections have been reported in the European Union; however, CCoV-associated disease in the United States is incompletely characterized. The purpose of this study was to correlate the clinicopathological findings and viral antigen distribution with the genotypic characteristics of CCoV in 11 puppies from nine premises in five States that were submitted for diagnostic investigation at Cornell University between 2008 and 2013. CCoV antigen was found in epithelial cells of small intestinal villi in all puppies and the colon in two of the 10 puppies where colon specimens were available. No evidence of systemic CCoV infection was found. Comparative sequence analyses of viral RNA extracted from intestinal tissues revealed CCoV-II genotype in nine out of 11 puppies. Of the nine CCoV-IIs, five were subtyped as group IIa, one as IIb, while three CCoVs could not be subtyped. One of the CCoV-IIa was isolated in cell culture. Infection with CCoV alone was found in five puppies, of which two also had small intestinal intussusception. Concurrent infections either with parvovirus (n=1), attaching-effacing *Escherichia coli* (n=4) or protozoan parasites (n=3) were found in the other six puppies. CCoV is an important differential diagnosis in outbreaks of severe enterocolitis amongst puppies between 4 days and 21 weeks of age that are housed in high population density. These findings will assist with rapid laboratory diagnosis of enteritis in puppies and highlight the need for continued surveillance for CCoV variants and intestinal viral diseases of global significance.

3.2 Introduction

Canine coronavirus (CCoV) was first recognized a pathogen of dogs in 1971 (1) and together with transmissible gastroenteritis virus (TGEV) of swine and feline coronavirus (FCoV) is a member of the family *Coronaviridae*, subfamily *Coronavirinae*, genus *Alphacoronavirus*, species *Alphacoronavirus*-1 (2). Infection with CCoV is common in young dogs, particularly those housed in large groups such as kennels, shelters and breeding facilities (3-7). Traditionally, CCoV has been reported to infect the small intestinal villous absorptive epithelial cells resulting in mild and self-limiting diarrheal disease (8, 9). Young dogs, particularly those co-infected with other enteropathogens including parvovirus, can develop severe and often fatal disease (8, 10-12). The emergence of CCoV variants that are associated with severe clinical disease, mortality and systemic infections of dogs has been reported from several countries in the European Union (EU) (13-18). Although fatal CCoV-associated disease without other pathogens was reported in two puppies in the United States in 2005, the CCoVs were not characterized (19).

CCoVs circulate as two distinct genotypes: CCoV-I and CCoV-II, and both viruses can be detected in feces and tissues obtained from infected dogs by RT-PCR (7, 20). These genotypes can be distinguished on the basis of antigenic and genetic differences in the gene encoding the surface spike protein (21, 22). The viral spike protein binds to host cell receptor and triggers fusion of the viral and cellular membranes, making it an important determinant of cellular tropism and pathogenicity (23). Genotype I CCoV cannot be propagated in cell culture, and thus, is understudied compared to genotype II

CCoV that is easily adapted to cell culture conditions. A similar situation exists with the closely related FCoV type I viruses and is suspected to be due to differential receptor requirements between genotypes (24). CCoV-II viruses use aminopeptidase N (APN) as receptor (25), while the receptor for CCoV-I viruses has not been identified. CCoV-II viruses are classified into at least two subtypes, namely CCoV-IIa and CCoV-IIb, based on the sequence of the first 300 amino acids of the spike protein, a region known as the N-terminal domain (NTD). The NTD is an important determinant of intestinal tropism in the closely related TGEV (26, 27). Although the CCoV-IIa and -IIb classification is not part of the official CCoV taxonomy, these subtypes are widely referenced in the literature. Moreover, CCoV-IIa viruses also exist as two biotypes that differ in pathogenicity and tissue tropism and have an entirely CCoV-like NTD. Productive infection and replication of the classical CCoV-IIa biotype is restricted to intestinal epithelial cells. By contrast, an emergent pantropic CCoV-IIa biotype that can spread systemically is associated with profound leukopenia (28, 29), and has been detected from the tonsils, thymus, heart, lungs, liver, pancreas, mesenteric lymph node, spleen, kidneys, urinary bladder, muscles and brain of affected dogs by RT-PCR (13-18). Isolation of virus from extra-intestinal tissues has also been reported in some instances (13, 14, 16), but also failed in multiple other instances (18). The CCoV-IIb spike gene has a TGEV-like NTD (15, 30), and like TGEV, it causes enteritis in neonatal animals. Although it is generally restricted to the small intestine, CCoV-IIb RNA has been detected in extra-intestinal tissues of dogs co-infected with canine parvovirus (15-17) or with unknown co-morbidity (18). Finally, a third CCoV-II variant with a CCoV-I NTD has been reported in both the United States and Sweden

(25, 31).

The purpose of the present study was to characterize the genotype of CCoV associated with outbreaks of fatal disease in young dogs submitted to the Animal Health Diagnostic Center (AHDC) at Cornell University between 2008 and 2013. Following localization of CCoV antigen in tissue sections by using immunostaining, the type and subtype of each virus was determined by sequencing of the NTD from purified viral RNA amplified by RT-PCR assay. Since changes in the proteolytic cleavage of the spike protein can modulate viral pathogenesis in FCoV (32), we also characterized the sequence of the spike protein cleavage motifs. Lastly, we used phylogenetic analysis to compare the sequences of the spike NTD obtained in the present study with those previously reported in the EU. The results of our study will assist with rapid laboratory diagnosis of CCoV-associated enteritis in dogs and enhance surveillance for emerging intestinal viral variants of global significance.

3.3 Methods and Materials

Diagnostic Investigation. The sample population consisted of dogs submitted to the AHDC at Cornell University between 2008 and 2013 with lesions of viral enteritis that were positive for the presence of CCoV antigen by immunohistochemical (IHC) staining. With the exception of puppies 4a and 4b in which selected tissues were collected by the referring veterinarian during a field necropsy, all cases were processed for complete necropsy including collection of multiple segments of gastrointestinal tract. In addition to gross and histopathological examinations of a standard set of tissues, bacteriological culture of intestinal specimens including

Salmonella and *Campylobacter* species and fluorescent antibody (FA) tests on fresh frozen tissue sections for CCoV, group A rotavirus and canine parvovirus were performed on all cases. At the request of the referring veterinarians, fresh tissues obtained from puppies 2, 4a, 4b and 8 were also processed for virus isolation. Dogs with respiratory signs or lesions were examined for the presence of canine distemper virus, canine parainfluenza and canine adenovirus by FA staining of frozen tissue sections.

Histopathology. Sections of brain, thymus, heart, trachea, lungs, liver, gall bladder, tongue, stomach, pancreas, small and large intestines, mesenteric lymph node, spleen, kidneys, adrenal glands, urinary bladder, skeletal muscles and bone marrow were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4-μm thickness, and stained with hematoxylin and eosin. Selected sections of intestinal tract also were stained with tissue Gram stain and a modified Steiner silver stain to further characterize bacteria when present. For IHC staining, sections of tissues were deparaffinized and processed for antigen retrieval. After blocking endogenous peroxidase activity with 3% hydrogen peroxide and treatment with normal goat or normal rabbit serum for 5 minutes (Invitrogen, Carlsbad, CA), the slides were reacted with the coronavirus- specific mouse monoclonal antibody FIPV3-70 (Custom Monoclonals International, Sacramento, CA, USA) followed by biotinylated goat anti-mouse, streptavidin-peroxidase conjugate (Invitrogen), chromogen, 3,3-diaminobenzidine-tetra hydrochloride and hematoxylin counterstain. Duplicate intestinal sections from each puppy were stained with the group A rotavirus-specific

mouse monoclonal antibody 9-10 (33) and canine parvovirus-specific rabbit polyclonal antibody CPV vp1/vp2 (Colin Parrish, Baker Institute, Cornell University). For FIPV3-70 and 9-10 antibodies, heat antigen retrieval consisted of microwave in citrate buffer at pH 6.0 for 20 minutes, whereas for CPV vp1/vp2 antibody, antigen retrieval was accomplished by digestion with pronase for 30 minutes.

Virus Isolation. Ten percent tissue pools of lung, liver, spleen and intestine from puppy 2, intestine from puppy 4a, lung and intestine from puppy 4b, and lung, liver, spleen, kidney and brain from puppy 8 were prepared in Eagle minimal essential medium (MEM-E) containing 0.5% bovine serum albumin and 10 μg/mL ciprofloxacin. After tissue disruption and low speed centrifugation, 1 mL of the filtered supernatants from puppy 2, 4a and 4b were inoculated onto monolayers of canine fibroblast-like A-72 cells (ATCC CRL-1542) and immortalized canine kidney cells (AHDC, Cornell University), while supernatant from puppy 8 was inoculated onto immortalized canine kidney cells and human colorectal adenocarcinoma HRT-18 (ATCC CCL-244) cells grown in 25-cm^2 flasks as previously described (34). Supernatants from puppy 4a and 4b also were inoculated onto canine kidney MDCK cells (ATCC CCL-34) and HRT-18 cells, respectively. The extract was allowed to remain on the monolayer for 1–2 h and then rinsed off with phosphate buffered saline (PBS). Cells were cultured at 37°C in MEM-E containing 10% gamma-irradiated fetal bovine serum. At 5-7 day intervals, monolayers were disrupted with trypsin and new monolayers established at a 1:3 split ratio. Cultures were monitored on a daily basis for the presence of cytopathic effect. CCoV isolation was confirmed by FA staining

with the mouse monoclonal antibody FIPV3-70.

RT-PCR and Genotyping. RNA was extracted from formalin-fixed and paraffin-embedded (FFPE) tissues with RecoverAll™ Total Nucleic Acid Isolation Kit according to the manufacturer's instructions (Ambion, Foster City, CA, USA). The resulting RNA was reverse transcribed into cDNA using SuperScript™ III First-Strand Synthesis System for RT-PCR (Life Technologies, Carlsbad, CA, USA). The RT reaction was primed with random hexamers. The presence of coronavirus cDNA within the sample was confirmed as previously described by PCR directed at a conserved region of the 3' UTR (35). Samples that tested positive for the presence of coronavirus RNA based on the 3' UTR were further characterized as genotype I or II using oligonucleotide primers directed against S1/S2 and S2' cleavage motifs (Fig. 1 and Table 1). Type II viruses were further characterized into subtypes on the basis of the NTD (Fig. 1 and Table 1). Previous studies on CCoV differentiate type I and type II CCoVs based on the sequence of the membrane (M) protein; however, it is unclear how changes in the M protein correlate with changes in the S protein. Therefore, we designed oligonucleotide primers against regions of the S protein that differ substantially between genotypes (Fig. 3.1 and Table 3.1). PCR was performed using Platinum Taq DNA Polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturers' instructions with an annealing temperature of 55°C. PCR products were analyzed by electrophoresis on a 0.8% agarose gel. Products of the expected size were purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA).

Figure 3.1 CCoV-I and CCoV-II spike genes with the location of N-terminal domain (NTD), S1 receptor binding domain, S1/S2 cleavage site (S1/S2), S2' cleavage site (S2'), S2 fusion domain and transmembrane domain (TM). Note that the S1/S2 furin cleavage site is only present in CCoV-I viruses.

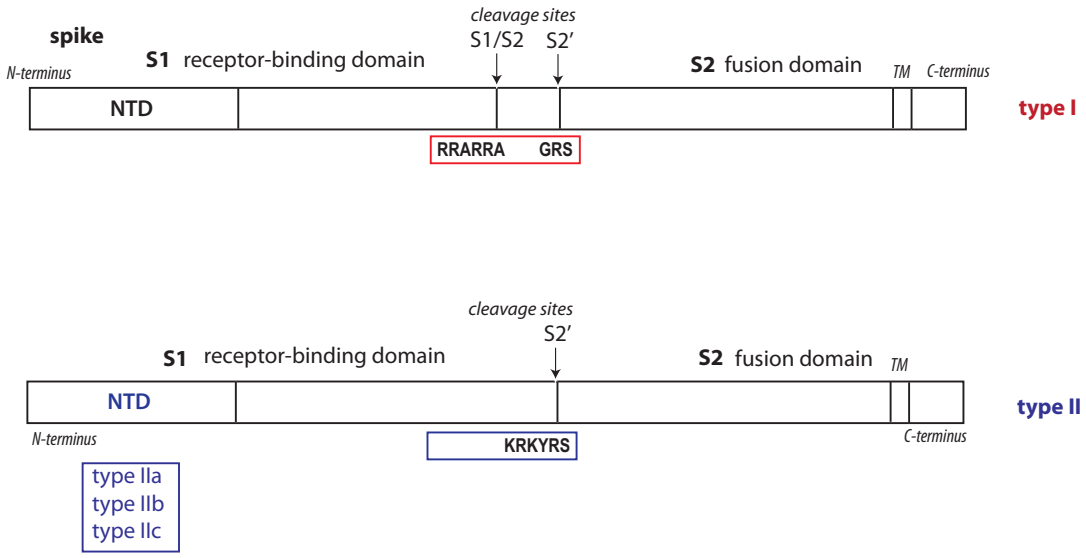


Table 3.1 Oligonucleotide primers for amplification and sequencing of the 3' UTR, spike N- terminal domain (NTD), spike S1/S2 cleavage site (S1/S2) and spike S2' cleavage site (S2')

Name	Specificity	Sense	Sequence (5' to 3')
CCV 1-1	Type 1 S1/S2 cleavage site	+	CTGCTCAAGCTGCTGTAATT
		-	TACTACTGTGTTGGTGGTGA
CCV 1-2	Type 1 S2' cleavage site	+	ATGTAATGACAGAAGTACA
		-	TACATTGCCATCTTATTATCA
CCV 2-1	Type 2 S1/S2 cleavage site	+	GCCATAGTTGGAGCTATGAC
		-	CCTATTTACAAAGAATGGCC
CCV 2-2	Type 2 S2' cleavage site	+	ATGCCATTGTAATATTGTGC
		-	CCATCAGAATGTGTGACGTTA
CCV-IIa	IIa NTD	+	ATGATTGTGATCGTAACTTG
		-	TTGTACCACACCTCTGTAGG
CCV-IIb	IIb NTD	+	GAACTATAGGCAACCATTGG
		-	TACAATGCTTTAAGATTTTC
20179[a]	Type I NTD	+	GGCTCTATCACATAACTCAGTCCTAG
CCV-IIc		-	TACATACTAGCTTCAAATC

[a]Previously described oligonucleotide (20)

Comparative Sequence Analysis. The products of the RT-PCR assays were sequenced by using Sanger di-deoxy sequencing method (Biotechnology Resource Facility, Cornell University). The CCoV RNA extracted from clinical samples was classified as CCoV-I or CCoV-II based on RT- PCR and sequencing of the spike S1/S2 and S2' cleavage sites. Sequencing was also used to distinguish CCoV-IIa and CCoV-IIb variants by using a combination of new and previously published spike-specific primers targeting the NTD (20). Oligonucleotides specific for CCoV-I NTD were also included in order to detect CCoV-I/CCoV-II recombinants (Fig. 1 and Table 1). We sequenced and aligned the CCoV-II S2' cleavage site, which is adjacent to the conserved coronavirus fusion peptide (36), to look for deviations from the CCoV-II consensus cleavage motif: K-R-K-Y-R-S, where K is the amino acid lysine, R is the amino acid arginine, Y is the amino acid tyrosine, and S is the amino acid serine. This amino acid motif is likely to be cleaved by a variety of trypsin- and cathepsin-like proteases, with cleavage occurring between the R and S residues. Comparative analysis of PCR amplified CCoV gene-specific sequences was performed on the N-terminus of the S gene using Clustal X (Conway Institute, UCD Dublin, Ireland) and viewed in Genious v6.1.7 (Biomatters Ltd., Auckland, New Zealand). Neighbor-joining trees were constructed in Clustal X using 10,000 bootstrap trials and viewed in FigTree v1.4.0 (Institute of Evolutionary Biology, Edinburgh, UK). The partial nucleotide sequences of CCoV spike genes have been deposited in the European Nucleotide Archive under the accession numbers LN624642 to LN624663.

3.4 Results

Clinical Findings. The signalments and clinical presentations of 11 dogs from 9 premises investigated in the present study are presented in Table 3.2. No sex or breed predilections were noted. Affected puppies ranged in age from 4 days to 21 weeks with a median age of 7 weeks, and multiple puppies per litter and multiple litters were affected on most premises. The puppies were mostly housed in large groups that experienced severe clinical signs of intestinal illness and mortality in Indiana (n=1), Kansas (n=4), New York (n=4), Pennsylvania (n=1), and a litter in transit between shelters located in North Carolina and Rhode Island (n=1).

Table 3.2 Clinical history of puppies with canine coronavirus enteritis in this study

Case	ID[a]	Location	Breed	Age	Description and/or clinical sign(s)
1	08-149076	NY	Golden retriever	3 weeks	Breeder with 24 adults not affected; litter of 5 puppies with weakness, dehydration, vomiting, and diarrhea (2 died, 1 recovered); in other litters, 3 puppies were affected (1 died, 2 recovered)
2	09-89334	PA	Yorkshire terrier	21 weeks	Central nervous system signs, including hypoglycemia; a littermate with diarrhea died 4 days earlier
3	09-97736	ID	Spaniel	5 weeks	Breeder with 15–20 adults not affected; puppy with 4-day history of vomiting and diarrhea died during surgery for jejunoileal intussusception; 1 littermate with intussusception and 2 others ill
4a	09-107207	KS	Maltese	8 weeks	Distributor with 800 puppies with history of respiratory signs, vomiting, and diarrhea
4b	09-110089		Basset hound	8 weeks	
5	09-123567	NY	Mixed	7 weeks	Breeding/research facility; puppies with pale mucous membranes, depression, dehydration
6a	10-51534A	KS	Bichon frise	8 weeks	Breeder with 200 adults not affected; 100 puppies with 20% mortality when 6 to 8 weeks old
6b	10-51534B		Mixed	8 weeks	1 to multiple puppies per litter died within 3–4 days of showing anorexia, vomiting, and diarrhea
7	12-120628	RI	Mixed	5 weeks	8 rescued weaned puppies in transit from NC; 4 died with weakness, lethargy, dehydration, hypothermia
8	12-159396	NY	Shepherd mixed	2 weeks	Rescue bitch from KY; 5 puppies died from a litter of 8
9	13-47387	NY	German shepherd	4 days	Breeding/boarding facility with 8 adults showing mild vomiting and diarrhea; 2 puppies died when 3 and 4 days old from a litter of 8 with abdominal pain and bloody stools

[a] ID, identifier code.

Laboratory Findings. The results of IHC staining of formalin-fixed and paraffin-embedded (FFPE) intestinal tissue sections for the presence of CCoV and other pathological, microbiological and parasitological findings in dogs investigated in this study are presented in Table 3.3. All puppies had lesions consistent with viral enteritis characterized by various degrees of atrophy of small intestinal villi (villous:crypt ratio approximately 1:2) that were lined with attenuated, low cuboidal to squamous epithelial cells (Fig. 3.2A). Immunostaining confirmed the presence of CCoV antigen within the cytoplasm of small intestinal villous epithelial cells in all of the puppies (Table 3.3 and Fig. 3.2B and 3.2C). Infection with CCoV extended from the villus:crypt junction to the tip of villi diffusely along the small intestine in puppies 5, 7, 8, and 9, multifocally in groups of epithelial cells in puppies 1, 3, 4a, 4b, and 6b, and within scattered individual epithelial cells in puppies 2 and 6a. Of the 10 puppies in which colonic sections were available, only puppies 8 and 9 showed CCoV antigen within epithelial cells along the surface and crypts of the colon. Although lymphoid depletion of Peyer's patches was present in 7 puppies, none of 10 puppies where lymphoid tissues were examined by IHC showed positive staining for the presence of CCoV antigen. Rare individual CCoV antigen positive cells, most likely antigen presenting dendritic cells, were scattered within the mesenteric lymph nodes in puppies 6a, 6b, and 9. None of the puppies were positive for the presence of group A rotavirus by FA and IHC staining or *Salmonella* and *Campylobacter* species by bacteriological culture of intestinal specimens. However, concurrent intestinal pathogens were present in six puppies. Puppy 4a had severe acute multifocal crypt epithelial cell necrosis that was associated with canine parvovirus antigen as

determined by FA and IHC staining. None of the other 10 puppies were positive for the presence of canine parvovirus antigen by FA and IHC staining. In addition to diffuse attenuation of villous epithelial cells, sections of small intestines from puppies 2 and 5 also showed multifocal epithelial cell necrosis and sloughing into the lumen that was respectively associated with large and moderate numbers of cytoplasmic coccidian parasites. Parasitological examination of fecal samples confirmed the presence of *Isospora* species and *Cystoisospora ohioensis* in puppies 2 and 5, respectively. The small intestine of puppy 2, a young Yorkshire Terrier also showed multifocal crypt ectasia, a finding associated with protein-losing enteropathy in this breed of dogs (37). The lumen of many colonic crypts in puppy 4b contained small numbers of pale eosinophilic, pear-shaped flagellated protozoan parasites consistent with mild trichomoniasis. Closely adherent Gram negative coccobacilli consistent with attaching-effacing *Escherichia coli* (AEEC) were present multifocally along the apical membrane of villous epithelial cells in sections of small intestines from four puppies; large numbers of bacteria were present in puppy 4a, while puppies 5, 6a and 6b had small numbers of adherent bacteria (38). Bacteriological culture of segments of jejunum taken from puppies 6a and 6b yielded *E. coli* isolates that were typed as O untypable:H49 and O8:H14, respectively (*E. coli* Reference Center, The Pennsylvania State University). The isolate from puppy 6b also was positive for the presence of *stxII*, encoding Shiga-like toxin type II, and isolates from puppies 6a and 6b were negative for the presence of *eae*, encoding intimin-gamma. Consistent with clinical signs of weakness and vomiting, aspiration pneumonia was present in puppies 2, 5, 6a, and 7. Other lesions including bronchopneumonia in puppies 4b and 6b and

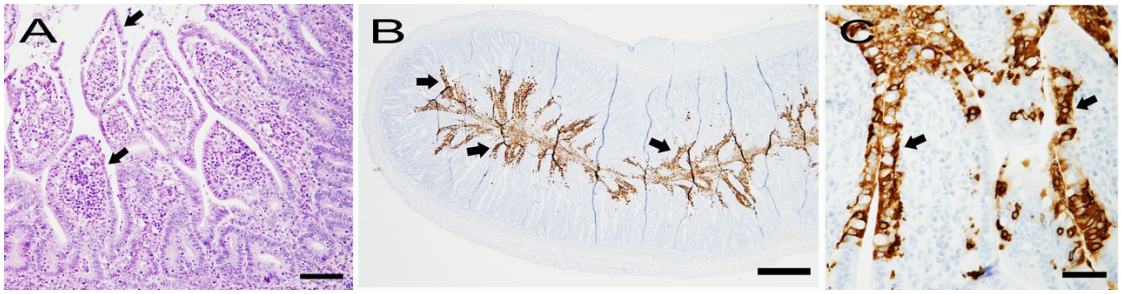
hepatocellular necrosis in puppies 8 and 9 were considered incidental findings. With the exception of puppy 4a where lung tissue was not available, none of the lung sections taken from the remaining 10 puppies were positive for the presence of CCoV antigen by IHC staining.

Table 3.3
Results of IHC staining of tissues for the presence of CCoV antigen and other findings in puppies investigated in this study[a]

Case	ID	CCoV IHC			Other finding(s)
		SI	LI	Other tissues	
1	09-149076	Pos.	Neg.	Neg.: lung, kidney, bone marrow	Peyer's patch lymphoid depletion
2	09-89334	Pos.	Neg.	Neg.: lung, urinary bladder	Protein-losing enteropathy, Isospora species, Peyer's patch lymphoid depletion, aspiration pneumonia
3	09-97736	Pos.	Neg.	Neg.: lung, liver	Jejunoileal intussusception, Peyer's patch lymphoid depletion
4a	09-107207	Pos.	NA	NA	Canine parvovirus enteritis, AEEC
4b	09-110089	Pos.	Neg.	Neg.: lung, MLN	Trichomoniasis, Peyer's patch lymphoid depletion, bronchointerstitial pneumonia
5	09-123567	Pos.	Neg.	Neg.: thymus, heart, trachea, lung, liver, pancreas, spleen	AEEC, Cystoisospora ohioensis, aspiration pneumonia
6a	10-51534A	Pos.	Neg.	Pos.: MLN (rare); Neg.: thymus, lung, stomach, pancreas	AEEC, aspiration pneumonia, bone marrow depletion, thymic and MLN lymphoid depletion
6b	10-51534B	Pos.	Neg.	Pos.: MLN (rare); Neg.: thymus, lung	AEEC, bronchopneumonia, bone marrow depletion, thymic, MLN and Peyer's patch lymphoid depletion
7	12-120628	Pos.	Neg.	Neg.: lung, tongue, stomach, MLN, liver, gallbladder, pancreas, kidney	Jejunojejunal intussusception, ulcerative gastritis, Peyer's patch lymphoid depletion, aspiration pneumonia
8	12-159396	Pos.	Pos.	Neg.: thymus, lung, liver, stomach, spleen, kidney	Mild, multifocal, acute hepatocellular necrosis
9	13-47387	Pos.	Pos.	Pos.: MLN (rare); Neg.: lung, stomach	Peyer's patch lymphoid depletion, mild, multifocal, subacute hepatocellular necrosis

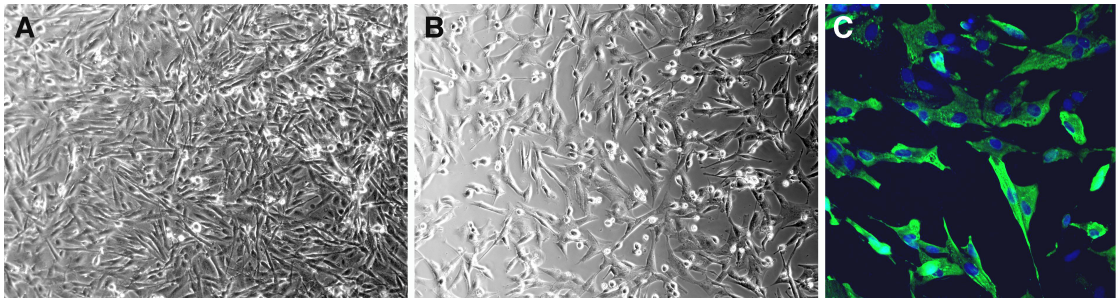
[a] SI, small intestine; LI, large intestine; Pos., positive; Neg., negative; NA, not available; MLN, mesenteric lymph node; AEEC, attaching-effacing Escherichia coli.

Figure 3.2 Photomicrographs of small intestine from puppy 8 with typical lesions of CCoV infection. (A) The villus epithelial cells are diffusely disorganized, attenuated, and low cuboidal (arrows). Note that the space between the epithelial cells and the lamina propria is an artifact of processing (hematoxylin and eosin stain; bar, 100 □m). (B) Cross-section of small intestine showing CCoV antigen in villus epithelial cells (arrows; immunohistochemical stain; bar, 500 μm). (C) Higher magnification showing CCoV antigen in the cytoplasm of villus epithelial cells (arrows; immunohistochemical stain; bar, 50 μm).



Virus Isolation. CCoV was isolated from puppy 4b and canine parvovirus was isolated from puppy 4a. CCoV cytopathic effect (CPE) consisting of cell rounding, cell death and syncytia formation was observed 24 hours post-inoculation of canine A-72 cells (Fig. 3.3A and 3.3B). The other two cell lines did not yield CCoV. Infected A-72 cells were positive for the presence of CCoV antigen by FA staining (Fig. 3.3C). Sequencing of CCoV RNA extracted from infected cell culture lysates further confirmed infection of puppy 4b with CCoV-IIa that corresponded to the RT-PCR assay and sequencing results from corresponding FFPE intestinal specimen.

Figure 3.3 Canine fibroblast-like A-72 cells, 24 hours post-infection with puppy 4b CCoV. Phase contrast microscopy of uninfected cell culture monolayer (**A**) and CCoV-infected cell culture monolayer with cytopathic effect characterized by rounding and death of individual cells (**B**) (10X original magnification). Immunofluorescence microscopy of CCoV-infected cell culture monolayer (mouse monoclonal antibody FIPV3-70 followed by Alexa Fluor 488 conjugated anti-IgG; 20X original magnification)**.**



RT-PCR, Genotyping and Comparative Sequence Analysis. Coronavirus RNA was detected by RT-PCR in nine out of 11 puppies; sufficient CCoV RNA was not recovered from puppy 1 and 6b (Table 4). Comparative sequence analysis revealed CCoV-II in all 9 cases. Consequently, the S1/S2 cleavage site was not analyzed because it is only present in CCoV-I genotype (Fig. 3.1) (39). The CCoVs from puppies 3, 4b, 5, 7 and 8 were subtyped as CCoV-IIa, while the CCoV from puppy 9 was subtyped as CCoV-IIb, and those from puppies 2, 4a, and 6a could not be subtyped. The S2' site was sequenced in eight out of the nine puppies from which CCoV RNA was successfully extracted. No variations in amino acid sequence at the cleavage site were detected (Table 3.4). Based on a neighbor-joining phylogenic tree of available CCoV NTDs (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/), CCoV-IIa viruses from puppies 3, 4b, 5, 7 and 8 clustered with other CCV-IIa viruses, including those associated with previous reports

of pantropic CCoV in the EU, while the CCoV-IIb from puppy 9 clustered with CCoV-IIb from the EU (Fig. 3.4).

Figure 3.4 Phylogenetic tree based on the spike protein N-terminal domain (NTD). Sequences are identified as CCoV-IIa or -IIb followed by the case number.

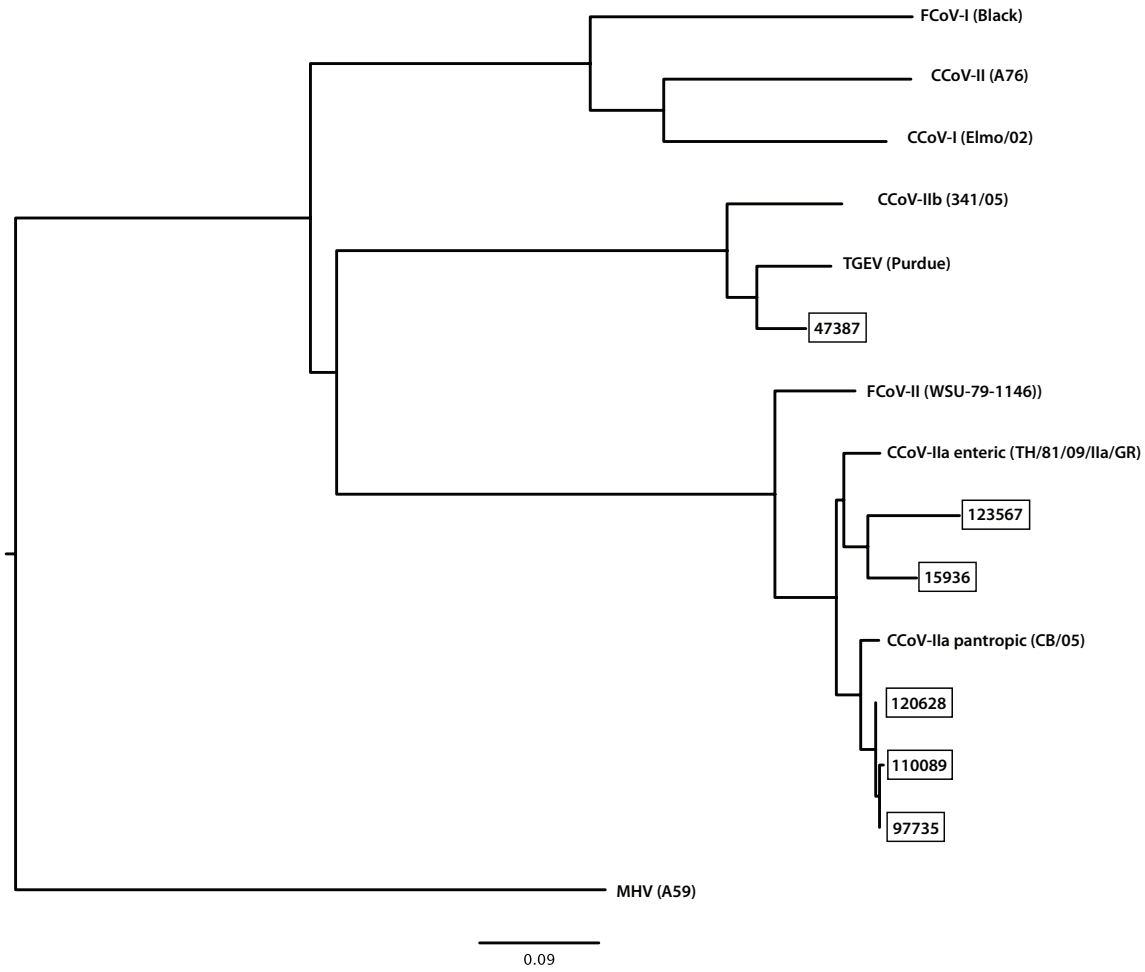


Table 3.4 Results of NTD genotyping and S2' subtyping of canine coronaviruses (CCoV) in this study. Genotyping and subtyping of reference CCoV-I and –II are included.

CCoV (case)	Genotype	Subtype	S2' Cleavage Site Sequence	S1/S2 Cleavage Site Sequence
89334-09 (2)	II	ND	**KRK**Y**R**S	A**RT**R - - - - - G
97736-09 (3)	II	a	**KRK**Y**R**S	E**RT**R - - - - - G
107207 (4a)	II	ND	**KRK**Y**R**S	D**RT**R - - - - - G
110089-09 (4b)	II	a	**KRK**Y**R**S	A**RT**R - - - - - G
123567-09 (5)	II	a	**KRK**Y**R**S	E**RT**R - - - - - G
51534-10A (6a)	II	ND	**KRK**Y**R**S	A**RT**R - - - - - G
120628-12 (7)	II	a	**KRK**Y**R**S	ND
159396-12 (8)	II	a	**KRK**Y**R**S	E**RT**R - - - - - G
47387-13 (9)	II	b	**KRK**Y**R**S	A**RT**R - - - - - G
Reference CCoV				
CB/05	II	a	**KRK**Y**R**S	A**RT**R - - - - - G
1-71	II	a	**KRK**Y**R**S	E**RT**R - - - - - G
341/05	II	b	**KRK**Y**R**S	A**RT**R - - - - - G
Elmo/02	I		QPGG**R**S	V**RR**A**RR**AVQG

The reference CCoV-IIa pantropic CB/05 (AAZ91437.1), CCoV-IIa "enteric" 1-71 (AAV65515.1), CCoV-IIb 341/05 (ACJ63231.1) and CCoV-I Elmo/02 (AAP72149) are included to highlight the conserved nature of the S2' cleavage site within genotype II viruses. Basic residues suspected to be important for cleavage activation of the spike protein are in bold. ND, not determined.

3.5 Discussion

Although closely related to pantropic CCoV-IIa, the CCoV-IIa associated with neonatal mortality in our study were restricted to the intestinal tract, and therefore, consistent with classical enteric CCoV infection (19, 40). The use of immunostaining to confirm CCoV disease in our study might explain the lack of detection of pantropic CCoV. Previous work on pantropic CCoV has relied mainly on RT-PCR to detect systemic spread. RT-PCR is very sensitive and can detect viral genome in tissues without productive viral replication or infection being present. IHC is arguably a better method for detecting clinically relevant infection because it requires high levels of viral antigen, and therefore viral replication, to yield a positive result. In addition, IHC can localize antigen to biologically relevant cellular compartments such as the cytoplasm of infected villous epithelial cells (Fig. 3.2B and 3.2C). Because detection of viral antigen by immunostaining is highly time dependent, early infection with concentrations of CCoV antigen below the detection limit of the assay cannot be ruled out completely as a reason for the lack of detection of CCoV in extra-intestinal tissues of our puppies. Confirmation of pantropic CCoV in previous reports relied primarily on RT-PCR assays; however, whether viral replication or infection is present in individual tissues cannot be conclusively confirmed by this method alone. Conversely, isolation of CCoV viruses from extra-intestinal tissues has been reported, but the clinical significance of this finding is unclear without co-localization of extra-intestinal lesions with viral antigen. Previous reports with FCoV have confirmed viral RNA by RT-PCR in tissues obtained from otherwise healthy cats (41). As with CCoV,

immunostaining is generally negative in these cases. In the same studies, the positive RT-PCR results were attributed to viremia, a finding that is common among cats experiencing asymptomatic enteric infection with FCoV (42). The possibility that viremia may be associated with low-level virus replication within tissues of healthy animals cannot be ruled out completely (41).

Viral tropism was found to extend to the large intestines in puppies 8 and 9, respectively infected with CCoV-IIa and CCoV-IIb. To our knowledge, colonic infection has only been documented in one of five experimentally-infected 10-week-old puppies, 10 days post- inoculation with the C54 reference CCoV-II (40, 43). Given that concurrent pathogens were not found, extensive intestinal infection with CCoV alone most likely accounted for the demise of puppies 8 and 9. In support of this interpretation was the presence of hepatocellular necrosis in these puppies; a common finding in animals with extensive loss of intestinal barrier integrity which results in showering of the portal circulation by toxic products. Interestingly, these were the youngest puppies (4 days and 2 weeks), and host factors such as age may have contributed to CCoV infection of colonic epithelial cells.

Lymphopenia is a common clinical finding in reports of dogs with pantropic CCoV infection. Although hemograms were not available, lymphoid depletion of the thymus, mesenteric lymph nodes or intestinal Peyer's patches was present in eight out of 10 puppies that had lymphoid tissues available. Similar lymphoid depletion was found in two puppies with fatal CCoV-associated enteritis previously described in the United States (19). Consistent with the previous report, CCoV infection of lymphoid tissues

was not found in our study. Infections with other coronaviruses including Middle Eastern Respiratory Syndrome (MERS) coronavirus, severe acute respiratory syndrome (SARS) coronavirus, equine coronavirus (ECoV), and FCoV are associated with lymphopenia (44-47). Where the cause of lymphopenia has been investigated, it is attributed to indirect mechanisms secondary to the viral infection such as cytokine-mediated apoptosis (48, 49).

All but one of the puppies in our study originated from high-density housing where outbreaks of enterocolitis were ongoing. Over half (6/11 or 54%) of our cases had concurrent intestinal infections with various combinations of pathogens. Co-infection with canine parvovirus, a known risk factor for CCoV associated mortality, was found in only puppy 4a. Severe intestinal damage can result in translocation of toxic products to extra-intestinal tissues, particularly the lungs and liver. Consistent with this observation, bronchopneumonia and hepatocellular necrosis were present in four puppies. Additionally, aspiration pneumonia, a common clinical complication seen in young debilitated puppies that are vomiting was also present in four puppies. The clinical significance of intestinal infection with AEEC in four puppies is unclear; however, the presence of small intestinal epithelial colonization by these organisms likely contributed to clinical signs of intestinal dysfunction leading to mortality in these cases. Clearly, host and environmental factors such overcrowding of puppies, co-infections with intestinal pathogens, pathogen load and degree of maternal immunity can determine the outcome of CCoV-associated enteritis. Although mutations in the S2' cleavage site were not found, it remains possible that unidentified

viral factors also could have contributed to the fatal outcomes. These factors may include variations in the NTD, a region that is known to be important for enterotropism in TGEV. Subtyping of CCoV-II was based on the amino acid sequence of the NTD (Fig. 1). Because both CCoV-IIa and -IIb viruses were associated with fatal outcomes, it appears that viruses with a CCoV-like NTD (subtype CCoV-IIa) or a TGEV-like NTD (CCoV-IIb) have the potential to cause fatal enteritis.

Intussusception was observed in the small intestine of two of the 11 puppies with fatal CCoV-associated enteritis (Table 3.3). Interestingly, puppy 3 was from a breeding facility where a littermate with small intestinal intussusception recovered following surgical resection and anastomosis. This breeder recalled having over a dozen other puppies with small intestinal intussusception over the last two years following the introduction of several breeders acquired from Sweden where CCoV outbreaks were documented around the same period (31). Interestingly, a similar association between CCoV infection and small intestinal intussusception was reported in one of the two puppies previously described in the United States (19). The pathogenesis of intestinal intussusception associated with enteric viral infection is not well understood; however, in human infants, intestinal intussusception has been associated with adenovirus and enterovirus infections as well as vaccination with a discontinued live-attenuated rhesus-human reassortant rotavirus tetravalent vaccine (50-52). The observation that CCoV- associated enteritis can sometime present with small intestinal intussusception suggests a similar pathogenesis. Our study provides detailed pathological findings in 11 puppies with fatal CCoV- associated enteritis that originated from nine premises in

five States in the United States between 2008 and 2013. Key regions of the viral spike gene were sequenced to determine if viral factors played a role in these CCoV-associated mortalities. Owing to the retrospective nature of the present study with available FFPE tissue samples collected at necropsy with variance in post- mortem intervals, sample quality and degree of RNA crosslinking by formalin fixation combined with the relatively low ratio of viral to cellular RNA within tissues and the natural variability of CCoV S gene limited our ability to use next generation sequencing methods to capture a more complete assessment of the viral populations involved in these cases. Another limitation of the present study was our inability to subtype the CCoV-II viruses from three puppies which was likely attributable to divergent NTD that could not be amplified by our PCR primers. Lastly, approximately 50% prevalence of CCoV-I and CCoV-II co-infections has been reported previously; however, we were unable to detect any CCoV-I infections in our study. Our CCoV-I primers were based on multiple alignments of previously published CCoV-I virus sequences in the region of the S1/S2 and S2' cleavage sites. It is possible that the puppies in our study were infected with divergent CCoV-I viruses that were not amplified by our S-specific primers.

This study revealed the presence of CCoV-IIb variants in the United States and highlighted the potential of CCoV-IIa and IIb to cause morbidity and mortality in puppies. Extended tissue tropism of CCoV to the large intestine was found in two puppies; however, pantropic CCoV infections were not identified. Isolation of CCoV-IIa from puppy 4b confirmed the validity of genotyping results obtained from the

corresponding FFPE tissue sections. CCoVs should be considered as a differential diagnosis and specifically sought in outbreaks of severe enteritis among puppies up to 21 weeks of age particularly when housed in high population density, but also in cases with small intestinal intussusception or enteritis associated with infections caused either by parvovirus, AEEC or protozoan parasites.

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