Review Article

Rethinking the Drivers of Coronavirus Virulence and Pathogenesis: Toward an Understanding of the Dynamic World of Mutations, Indels, and Recombination Within the Species Alphacoronavirus-1

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Alphacoronaviruses are widespread but understudied in comparison to betacoronaviruses. Recombination, mutations, and indels are hallmarks of coronavirus replication, which together facilitate viral spillover and emergence, especially within the alphacoronaviruses. The species *Alphacoronavirus-1* comprises distinct viruses of cats, dogs and pigs. In cats, high-pathogenicity feline coronavirus (FCoV) is infamous as the cause of feline infectious peritonitis (FIP), a lethal disease that can now be treated with antiviral drugs. FCoV-1 exists as two distinct genotypes (type -1 and -2) and is transmitted as a low-pathogenicity virus that causes mild or asymptomatic disease. The high-pathogenicity FCoV variants arise in cats already infected with FCoV, and while the mutations responsible for this phenotype change remain enigmatic, the main determinant of pathogenicity is the viral spike glycoprotein. FCoV-1 disease outcome is driven by a combination of both within- and between-host evolution, whereas FCoV-2 disease appears to be driven by recombination with co-circulating canine coronaviruses (CCoV). FCoV-1 virulence can be largely explained using the "furin cleavage site (FCS) disruption hypothesis," which argues that low-pathogenicity FCoV-1 contains an intact FCS while high-pathogenicity FCoV-1 has a disrupted FCS that is unable to be cleaved. FCoV-2 virulence and pathogenesis is exemplified by FCoV-23, a novel canine/feline recombinant virus that caused a widespread outbreak of severe disease in Cyprus during 2023. As such, *Alphacoronavirus-1* mayexist as adynamic "metavirome"[1] that is in a constant state of flux, presenting notable challenges for disease surveillance and management, and in risk-assessment.

 doi.org/10.32388/YYO05O

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Introduction

Alphacoronaviruses are in the family *Coronaviridae*, along with three other genera; beta, gamma, and delta coronaviruses. The alphacoronavirus genus contains of viruses that infect a wide range of hosts including humans, cats, dogs, pigs, bats, rats, and other rodents. Many of these viruses display cross-species transmission, and co-infection in a given host can lead to recombination between viral species, which drives viral evolution. Historically, alphacoronaviruses have demonstrated the ability to infect humans and spillover events from animals to humans have occurred on several occasions, underscoring the importance of monitoring these viruses[1]. For feline coronaviruses (FCoVs), a 2020 FCoV review by Jaimes et al[2] focuses on the spike protein, as it is the main driver of coronavirus cell tropism and pathogenesis[3]. In 2023, Gao et al also published a review on the two FCoV biotypes[4]. In this review, we provide an update on FCoV-1 in comparison to FCoV-2 and canine coronavirus (CCoV), and include the newly emerged feline/canine recombinant virus FCoV-23[5][6][7][8].

Feline coronavirus (FCoV) is one of the most important viruses of cats, affecting both domestic and wild felids; first recognized in 1963, it is now well established to be the cause of feline infectious peritonitis (FIP), which is typically lethal without therapeutic intervention[9][10]. FCoV is widespread, with the prevalence of infection in the US feline population estimated at 75-95% in multi-cat households[11], 25% in single-cat households, and approaching 100% in shelter/breeder situations. With an estimated 58 million owned cats in the US alone[12], FCoV represents a widespread endemic coronavirus that to date remains largely unexplored from a molecular evolution perspective, and with still many unanswered clinical questions.

FCoV infection can lead to three principal clinical outcomes[13]: 5% of cats clear the infection without viral shedding, 70-80% intermittently shed low levels of virus from their gastrointestinal tract, and 10-15% have persistent high viral load shedding. This indicates that FCoV often causes persistent infections. Furthermore, FIP may occur in 5-12% of these cases, presenting as an effusive (wet) form with fluid accumulation or a non-effusive (dry) form, often with neurological signs. FIP is prevalent in environments with high cat density, particularly affecting young cats under 2, and often triggered by stress[13].

FCoV "biotypes" (phenotypes and genotypes): FCoV has been traditionally categorized as one of two pathogenic biotypes[2]; feline enteric coronavirus (FECV) or feline infectious peritonitis virus (FIPV). Feline enteric coronavirus is the low-pathogenicity biotype, which presents with asymptomatic or mild symptoms and is proposed to infect intestinal epithelial cells based on viral shedding in the feces. Notably, the high-pathogenicity biotype, feline infectious peritonitis virus, utilizes monocytes/macrophages for systemic spread, and classically presents with peritonitis in its "wet" form. The two biotypes were originally differentiated based on the "internal mutation theory", in that a low-pathogenicity virus infects cats and then mutates[14] within an individual animal into the high-pathogenicity virus. While this basic concept has remained a mainstay of FIP pathogenesis for over a quarter century, we consider that the over-simplistic concept of an "FECV" switching to an "FIPV" is flawed, as is more recent classification of the two biotypes as "less virulent FCoV" and "FIP-associated FCoV" [15]; while pathogenesis of FIP is certainly linked to viral mutation, this is a much more complicated process than previously anticipated and measurable disease attributes for FIP are not easily quantifiable. Early studies to assign biotypes for FCoV linked the development of FIP to two small open reading frames (ORFs), 3c and 7b. However, more recent understanding has questioned the early connection to ORFs 3 and 7 [16][17], with the emphasis for the internal mutation focusing on the spike (S) gene, principally within S2 and at the S1/S2 interface [18][19]. While defined mutations such as S:M1058L are no longer thought to be high-pathogenicity FCoV-specific—and are rather associated with systemic spread of FCoV[20]—it remains likely that the predominant genetic changes controlling viral pathogenesis lie in the S gene [21]. Based on it being the principal cause of FIP, recent genomic studies linked to the biotype switch have focused on FCoV-1.

The more we uncover about the genetics of the virus, it is becoming apparent that the two "biotypes" are a simplified and outdated way to label the virus. We argue that the "biotype" framework is problematic because it explains FCoV infection through phenotypic presentation in order to make sense of the genotype (which is, in reality, the driver of pathogenesis). To understand the disease process, there is a need for more robust sequencing to be performed, so that we can use the genotype to successfully predict the phenotype. In this review, we intentionally use the terminology of "low-pathogenicity" and "high-pathogenicity" FCoV to descriptively capture the different biotypes without inference to specific markers of virulence (which remain poorly defined) and classify the two viruses as genotypes[3]. It is important to note that based on conventional taxonomic classification, all FCoVs (as well as CCoVs and certain coronaviruses of pigs) are a single virus species, *Alphacoronavirus-1* (Genus Alphacoronavirus, sub-genus

Tegacovirus, member species *Alphacoronavirus suis*.); *see* *https://ictv.global/report/chapter/coronaviridae/coronaviridae/alphacoronavirus*.

While low-pathogenicity FCoV is widely distributed, and presumably highly transmissible, “FIPV” is not generally thought to be a transmissible virus—with “outbreaks” likely resulting from multiple individual low- to high-pathogenicity conversions of FCoV within a defined location. In this context, we also need to reconsider what is meant by an outbreak for FCoV, as compared to a cluster of non-transmissible viruses. There is limited molecular epidemiology of FCoV in the literature, with the three main examples[22][23][24] describing what seem to be traditional horizontal transmission events (FCoV-2) and what might be better considered as clusters of distinct but closely-related viral variants (FCoV-1).

FCoV Serotypes: In addition to being a primary pathogenesis determinant, the FCoV spike is also a critical factor in antigenicity, and the virus has been traditionally considered to exist as two “serotypes”, I and II [4]. Historically, the term serotype was used in the early stages of discovery for FIP, prior to the availability of robust genomic information, as it was appreciated that sera from certain cats with FIP or infected with FCoV failed to cross-react/cross-protect with sera from other cats, leading to the concept that the virus exists as two distinct serotypes. This concept was reinforced by the isolation of monoclonal antibodies to what is now known as FCoV-2, whereby distinct differences in spike protein antigenicity were apparent. However, a 'serotype' may in reality only reflect minor differences in defined antigenic epitopes, and there is <50% amino acid identity between the spike proteins of the two FCoVs—as such the term serotype does not reflect their notable evolutionary differences. In 2018, we presented evidence that the ‘serotypes’ really reflect viruses representing two distinct genetic clades[5], with clade A corresponding to serotype I and clade B corresponding to serotype II; this nomenclature captures the high genetic diversity that has been recognized to date, including fundamentally distinct biological properties of the two viruses[25]. For example, FCoV-1 but not FCoV-2 contains a furin cleavage site (FCS) in the spike protein, which can prime the spike for virus entry. This proteolytic cleavage site in FCoV-1 may add a layer of control the virus can utilize for productive infection. In contrast, FCoV-2 results from recombination events between FCoV-1 and CCoV, where the recombinant genotype has the spike protein (and sometimes surrounding regions) from CCoV, with the rest of the genome from FCoV-1. Exchange of the spike protein through recombination results in antigenic shift.

FCoV-1 accounts for most coronavirus infections of cats—and cases of FIP; it is the most-studied virus in the species *Alphacoronavirus-1* from a clinical perspective. FCoV-1 cannot be readily isolated, making it

difficult to study in vitro. Therefore, robust sequencing of clinical FCoV-1 cases has been the principal means of advancing the study this virus.

The FCoV-1 Furin Cleavage Site Disruption Hypothesis. The progression of low-pathogenicity to high-pathogenicity FCoV has been linked to several "hotspot" genomic changes, including in the 7b, 3c and spike (S) genes. While multiple genomic changes likely account for ultimate conversion to FIP, mutations at the furin cleavage site (FCS) are strongly linked to high-pathogenicity FCoV-1. The FCS in FCoV-1 is a structural loop located close to the interface of the spike S1 (receptor binding domain) and S2 (fusion domain) subdomains. Furin is a ubiquitous cellular protease that minimally recognizes two critical arginine (R) residues, accompanied by a complex series of additional amino acids, often with a preference for addition basic residues, and serine (S) residues that promote docking of the cleavage loop substrate into the enzyme binding site[26][27][28]. Due to its location in spike, this region is often known as the "S1/S2 cleavage site", even though it does occur at the actual interface of the S1 and S2 subdomains[29], being found within S1 domain D (see Fig. 2 for a graphical depiction of spike domain organization). In circulating low-pathogenicity FCoV-1, the S1/S2 cleavage site contains a consensus motif for cleavage-activation by furin. While specific mechanistic information remains elusive, by analogy to the more studied betacoronaviruses (where a furin cleavage site is common), proteolytic cleavage at this site is likely to alter cell tropism and entry pathways, and possibly drive virus transmission, promote membrane fusion and affect spike stability[30].

In 2013, we showed that amino acid sequence changes in the furin cleavage site of FCoV that decreased cleavage are highly correlated with conversion to FIP[18]. Our initial molecular analysis of the S1/S2 cleavage site identified a consensus sequence in low-pathogenicity FCoV samples of (S/T/Q)-R-R-(S/A)-R-R-S in 30 fecal samples from apparently healthy cats (*i.e.*, "FECV, or low-pathogenicity FCoV") and a disruption of this motif in 22 tissue samples from cats clinically confirmed to have FIP based on immunohistochemical (IHC) analysis. In this initial pilot study, the disruption of the consensus cleavage motif was present in 100% of FIP cats—although not in all tissues. The result of FIP-positive cats having 100% mutated and apparently nonfunctional furin cleavage sites led to the hypothesis that "uncleaved" spikes are somehow functionally responsible for the "FIPV, or high-pathogenicity FCoV" biotype. Disruption of the FCS was shown to decrease proteolytic cleavage, peptide cleavage assays show this effect *in vitro*, but for FCoV-1 this has not been studied in live virus or *in vivo*. Subsequent case studies of individual cats, and follow up of a localized FIP outbreak in an animal shelter, also confirmed the 100% correlation between mutated/non-functional furin cleavage sites and high-pathogenicity FCoV[23][31][32].

Independent validation of S1/S2 mutations as drivers of FIP has been limited, in part due to technical difficulties reported by others in sequencing this region of spike—although recent epidemiology studies from China have recently provided some genomic support for this hypothesis[33]. Sequence analysis of the spike gene from a series of FIP cats in clinical trials testing antiviral drugs has also shown that the majority of S1/S2 sites were disrupted[34].

Notably, in 2023, a decade after our initial pilot study was published, an unbiased genomic analysis has provided additional support for the "FCS disruption hypothesis", which identified the FCoV-1 S1/S2 loop as a main genomic region that evolves under different selective regimes between high- and low-pathogenicity FCoVs[21]. This work also identified evidence of selection pressure acting on site "1058" (M1058L)—but not ORFs 3c or 7b. "M1058L" has long been attributed to systemic spread of FCoV (but not with FIP *per se*), and we now hypothesize that this mutation acts to stabilize the spike protein and to offset the functional traits imparted by subsequent FCS (and other) mutations. FCoV-1 spike also contains a second cleavage-activation site (S2′) that is also mutated in many FIP cases[19], but as with FCoV-2 remains poorly understood from a functional perspective.

Overall, for FCoV-1, we argue that pathogenic variants mainly derive from accumulated point mutations, with some recent evidence for insertions-deletions (indels[6] (see ref 34)); the point mutations/indels appear to be mainly present in certain 'hot-spots' within the spike, including the spike protein cleavage sites, "position 1058," and in the N-terminal domain.

FCoV Quasispecies: An understanding of FCoV-1 links to the general concept that for RNA viruses, pathogenesis is part of quasispecies diversity[35]. Over the years, this concept has been exploited to great effect in studies of human immunodeficiency virus (HIV-1)[36] and hepatitis C virus (HCV)[37], and most recently for SARS-CoV-2[38]. For HIV and HCV, it is well established that these viruses cause chronic or persistent infections in specific tissues, with the virus also present in specific "latent" compartments without productive replication. The presence of virus in such compartments plays a major role in the efficacy of antiviral drugs (which can only target the actively replicating compartment). "Sanctuary" compartments can also be established where the virus is protected from the immune response or antiviral drugs due to strong barriers between this site and other anatomical compartments, such as the central nervous system (CNS)[39]. Increasing evidence suggests that coronavirus infections can also take advantage of such persistent or sanctuary sites. Future studies of FCoV-1 represent a new way to merge population dynamics and phylogenetics to understand disease outcomes, as it has widespread tissue

distribution linked to its pathogenesis—*i.e.*, to set a novel precedent in a discipline that has been termed "phyloanatomy"[40][41][42].

FCoV Antivirals: FIP has recently lost its reputation as an invariably lethal infection due to the availability of antiviral drugs originally developed for COVID-19 and other viral diseases of humans, including hepatitis C and Ebola. There are now three basic therapeutic classes that are being used in differing ways in different countries based on the availability of approved or non-approved drugs through regulatory agencies—and with highly variable clinical management and use of molecular diagnostics; the three drug classes are nucleoside analogs (GS441524/Remdesivir®), protease inhibitors (GC376/Paxlovid®), and mutagens (molnupiravir/EIDD-2801).

The understanding of FCoV-1 infection as a quasispecies is essential for an understanding and clinical management of the antiviral drugs (such as Remdesivir®/ GS441524 and molnupiravir), which are repurposed COVID drugs rapidly coming into widespread use for treatment of FIP in cats; see refs[43][44][45][46] for examples. Without further evaluation of possible sanctuary sites and ability of viruses to be shed following treatment, treated animals may better defined as "in remission", rather than "cured". Based on its mechanism of action as a mutagen that accelerates virus evolution[44][47][48], molnupiravir may be especially problematic for FCoV-1-type infections—with viral dynamics being highly adaptive processes[49][50]. However, development of traditional anti-viral resistance (AVR) may technically be more of a problem with GS441524-like compounds, based on their widespread use and mechanism of action as a nucleoside analog[45]. Current clinical trials are also investigating the use of Paxlovid® as an FIP treatment

FCoV-2 is a recombinant of FCoV-1, in which a region of the genome—including the spike gene—is obtained from CCoV-2. Since the S genes of FCoV-1 and FCoV-2 (CCoV-2) are highly divergent (<50% amino acid identity), virus-host interactions like cell entry and tropism, antigenicity, and host range are vastly different for the two viruses. For example, in cell culture FCoV-2 grows readily, whilst FCoV-1 does not. For this reason, the mechanisms of cell entry of FCoV-2, including the molecular interaction with its host cell receptor, aminopeptidase N (APN or CD13), are well known.

FCoV-2 Pathogenesis: In contrast to FCoV-1, much less is known about the genetic diversity of FCoV-2 circulating in domestic cats, with relatively few sequences available. Comparative genetic studies have revealed different FCoV-2 variants with different recombination breaking points along the genome (Figure 1), which indicates that recombination between FCoV-1 and CCoV-2 has occurred on multiple

occasions. Genetic identification of FCoV-2 has been done mostly targeting a region in the 5'-end of the S gene[51]. Although this assay is sufficient to detect FCoV-2, differentiate it from FCoV-1, and detect co-infections, sequencing the complete genome is essential to detect different recombinant variants and to identify their origin. Likewise, sequencing a small region of S does not differentiate between FCoV-2 and CCoV-2. Differentiating whether cats are infected with FCoV-2 or CCoV-2 is also essential to understand if they act as mixing vessels for the recombination between FCoV-1 and CCoV-2. *In vitro* assays suggest that APN of the domestic cat allows entry of CCoV-2, FCoV-2, and transmissible gastroenteritis virus (TGEV[52]). However, compared to FCoV-1, FCoV-2 is much less prevalent in domestic cats[51][53][54]; typically considered to be <10% of FCoV-infected animals. FCoV-2 was reported in the feces of healthy and diseased cats, and in the pleural and abdominal fluids and tissues of diseased cats. Co-infections can occur—but seem to be rare. Although early experiments showed that different variants of FCoV-2 may be more virulent than others[55], whether FCoV-2 can be differentiated into FECV/low-pathogenicity and FIPV/high-pathogenicity is less clear. Notably, the virus, typically referred to as FECV-1683, was originally isolated from a cat that had severe clinical signs, including infection of lymphoid tissue.

Studies of FCoV-2 have disproportionally focused on cases of FIP, and assessment of natural infection by CCoV-2 in cats is limited to an early study of CCoV infection in FIP cats[56], along with a recent study of a series of captive snow leopards (*Panthera uncia*) with CCoV infection linked to severe gastroenteritis[57].

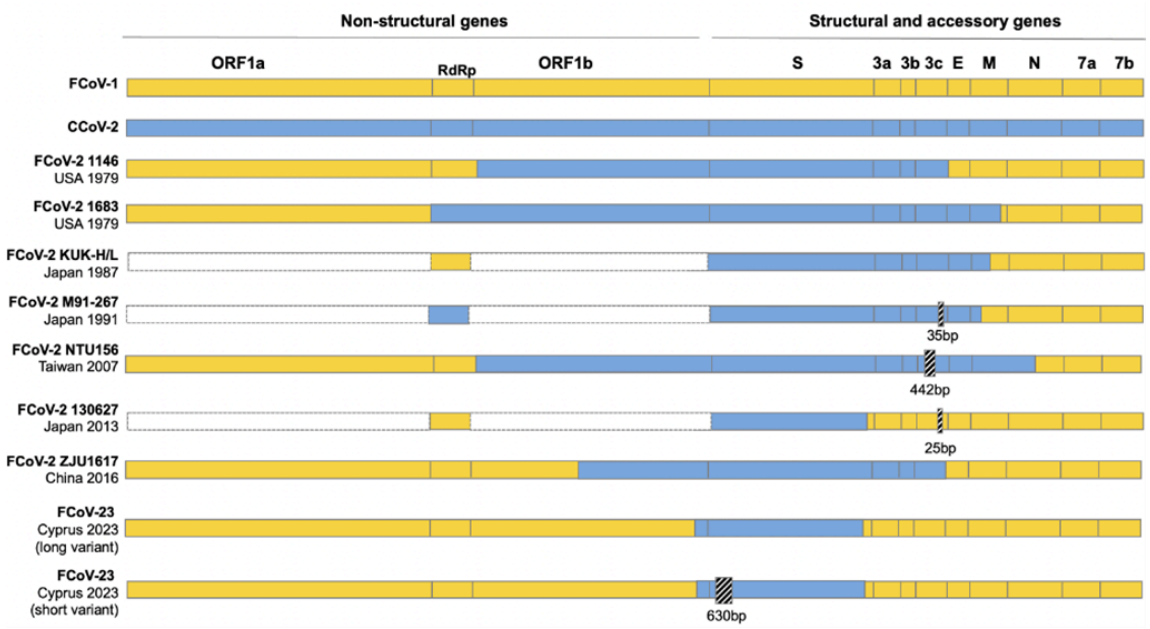

Figure 1. Graphical depiction of the genomic diversity of FCoV-2 and FCoV-23 recombinant variants reported to date. The position of each open reading frame (ORF) or gene along the genome is shown at the top. Within ORF1b, the RNA-dependent RNA polymerase (RdRp) position is also shown. The genome of FCoV-1 is in yellow, and the one of CCoV-2 is in blue. FCoV-2 is a recombinant between FCoV-1 (yellow) and CCoV-2 (blue). In white are regions of the genome from which the sequence has not yet been obtained. A black box with diagonal white lines indicates deletions. The deletion size is indicated below the box in base pairs (bp). Each variant is named to the left of its respective genome. The GenBank accession number for the FCoV-2 variants used is 1146 (AY994055), 1683 (JN634064), KUK-H/L (AB781789), M91-267 (AB781788), NTU156 (GQ152141), 130627 (AB907624).

S2' Compared to FCoV-1, and as expected for an alphacoronavirus, there is only a single readily identifiable cleavage site in the spike protein of FCoV-2 (S2'). The S2' cleavage sites of FCoV-1 and FCoV-2 have notably different consensus sequences (KR|S for FCoV-1 and RKYR|S for FCoV-2). These consensus sequences are highly conserved, except for a few notable substitutions that have been reported. An R-G substitution at the expected S2' cleavage position in a few FCoV-2 variants has been reported; this may affect cell tropism[58], but not necessarily virulence. This R-G substitution has also been observed in porcine epidemic diarrhea virus (PEDV), and may be a cell culture adaptation[59]; the same substitution also occurs with ferret CoVs, but its relationship to disease outcome in this case also remains unclear[60]. Additionally, a small minority of FCoV-1 isolates contain an R-S substitution at S2' (i.e. UU47, as well as viruses in FIP cats identified in Licitra et al., 2014).

Canine coronavirus (CCoV) is a well-established enteric pathogen of dogs—hence its alternative name canine enteric coronavirus (CECoV)[9][10], which differentiates it from canine respiratory coronavirus (CRCoV); CCoV/CECoV, like FCoV and TGEV, lie in within the *Alphacoronavirus-1* species, whereas CRCoV is distinct and is a betacoronavirus (embecovirus; species *Betacoronavirus-1*), closely related to bovine coronavirus[61][62]. As with FCoV, CCoV exists as two serotypes or types (clades), CCoV-1 and CCoV-2, with CCoV-2 being the predominant circulating form (or the one targeted for surveillance).

CCoV-2 was first isolated in 1971 and has since been found in what appears to be three distinct subtypes. Originally classified as CCoV-Ia and CCoV-IIa (here termed CCoV-2a and CCoV-2b), these subtypes have been well-documented and are differentiated by having distinct N-terminal domains (NTD) in their spike. CCoV-2b is the result of a recombination event with a TGEV-like virus; thus, it can be deduced that the CCoV-2a NTD is of canine origin, and the CCoV-2b NTD is of porcine origin. There also exists CCoVs with a third distinct NTD closely related to CCoV-1, which is in itself evolutionarily linked to FCoV-1; such viruses have been referred to as CCoV-IIc (CCoV-2c)[63][64], with other examples of recombinant viruses possibly spanning continents and long time periods; such viruses may include divergent CCoVs identified in Sweden[65], Australia[66] and China[67][68][69]. Notably, increased surveillance indicates CCoV-2c-like viruses may be the cause of ongoing winter waves of vomiting and diarrhea in dogs in the UK[70][71][72]. Also of note is the finding that CCoVs with distinct NTDs[73] have been isolated from humans, and defined as HuCCoV or CCo-HuPN-2018[1][74][75], where they are considered to have respiratory tropism.

CCoV-2a also exists as what are known as ‘pantropic’ isolates. The initial isolate (CB/05) was responsible for a severe outbreak of fatal systemic disease in a pet shop in Bari, Italy, which included broncho-pneumonia and neurological signs[76]. These viruses have since been well-reviewed in the literature[77] and have now been documented across the Mediterranean region over the past decades, as well as in other European countries[77]. CB/05-like pantropic CCoVs are typified by severe clinical signs, lymphopenia, and infection of lymphoid tissue. While sequencing was limited at the time, we note that viruses clustering with CB/05 have also been historically detected in the USA[63]. A recent evaluation of a localized outbreak from 2012 of severe enteritis in captive snow leopards used next-generation sequencing to identify a CB/05-like canine coronavirus present in the USA—further expanding the known distribution of these highly virulent viruses, as well as their capacity to infect felids[78]. Data such as these raise the question of whether the recombination event that generates FCoV-2 occurs in felids or canids.

CCoV-1 is typified by the isolate Elmö/02[79], which has high identity to FCoV-1; this virus is not well understood, and—notably—likely cocirculates extensively with the various CCoV-2 viruses[80]. This leads to challenges regarding surveillance efforts.

FCoV-23 is a recently emerged canine/feline recombinant virus which caused a large outbreak on the Mediterranean island of Cyprus during 2023, with (at the time of writing) documented spread of isolated travel-related cases in the UK[5][7][8]. This is a concerning situation, as the virus is highly virulent with most cats showing signs consistent with effusive FIP and a high degree of neurological signs along high viral loads in the colon—in cell types noted as having macrophage-like morphology. Compared to the other FCoV-2s that acquired a larger portion of their genome from CCoV-2, FCoV-23 only acquired its spike gene and a small region of Orf1b. The FCoV-23 spike gene has 97% identity to CCoV NA/09—a CB/05-like virus from Greece[81]—and is present in two forms, including one with a 630 bp deletion in the NTD that results in a 0-domain-truncated spike protein in the majority of studied cases. As noted by Attipa *et al*., the reason behind this notable outbreak may be due the 'right mutation, right time, right place' theory[6], with major roles being played by both viral factors (such as recombination and the 0-domain deletion), and environmental/community factors (the large numbers of feral cats—up to 1.5 million—on a relatively small island).

Alphacoronavirus 0-Domain Deletion Hypothesis: While unusual, the deletion of the spike NTD is not unprecedented; by far the most widely appreciated example is the tropism change of TGEV to porcine respiratory coronavirus (PRCoV)—in this case accompanied by a notable reduction in viral virulence[82]. The loss of the 0-domain through distinct and variable deletions is also associated with certain clinical isolates of PEDV[83]; notably, PEDVs with intact and deleted S proteins can co-exist in pigs, with the two viruses competing with each other (a situation quite different to that for TGEV/PRCoV)[84]. The loss of the 0-domain is also related with the emergence of HCoV-229E from its bat ancestor, where the 0-domain is actually duplicated in some 229E-like bat viruses[85]. 0-domain deletions also occur for FCoV-1, specifically with the FIPV isolates C3663[86], UU16 and UU21[87]; for FCoV-1 the pathogenic outcome of truncated vs intact spikes currently remains unknown. Alphacoronaviruses and betacoronaviruses have a fundamentally different domain organization for their spikes (square-shaped vs. V-shaped tertiary structure), and clearly the presence or absence of the alphacoronavirus spike 0-domain (which is missing in all betacoronaviruses) can have a major impact on viral tropism and pathogenesis—with its effects of particular interest in the context of FCoV-23 (Figure 2).

Figure 2. Graphical depiction of the spike proteins of selected alpha- beta- gamma- and deltacoronaviruses. The location of the S1 and S2 subdomains is shown at the top. The 0, A, B, C, and D subdomains are in yellow, green, blue, light purple, and cyan, respectively. The fusion peptide (FP) is in magenta.

doi.org/10.32388/YYO05O

The currently known receptors/attachment factors are shown below the region in spike with which they interact and are abbreviated as follows: Sia (sialic acid), O-Ace-Sia (O-acetylated sialic acid), APN (aminopeptidase N), ACE2 (angiotensin-converting enzyme 2), CEACAM (carcinoembryonic antigen-related cell adhesion molecule). The GenBank accession numbers are PEDV (AAK38656.1), TGEV (ABG89325.1), PRCoV (ABG89317.1), FCoV UU23 (ADC35472.1), FCoV UU21 (ADL71466.1), NL63 (YP_003767.1), 229E-related bat CoV with one O domain (ALK28775.1), 229E-related bat CoV with two O domains (ALK28765.1), 229E (NP_073551.1), MHV (ACO72893), BCoV (AAX38489), OC43 (AAT84362), HKU1 (AAT98580), SARS-CoV (AAP13441), MERS-CoV (009047204), HKU4 (AGP04928), HKU5 (AGP04943), IBV (ADP06471), and PDCoV (AIB07807). Figure is adapted from Hulswit et al., 2016.

Outstanding Questions and Clinical Context

Recent findings have prompted a re-analysis of the over-arching question of how we define virulence for FCoV and CCoV; does “FIP/FIPV” just mean robust macrophage tropism/spread, and a concomitant inflammatory response/cytokine storm? Notably, many FCoVs have extensive infection of lymphoid tissue which may not be picked up in traditional antibody-based immunohistochemistry approaches, for example see Fig. 4/lymph node in[88].

FCoV Methodology Differences: Aside from serological studies, it is important to note that most current diagnostic testing methodologies (either IHC or PCR) does not discriminate between FCoV-1 or FCoV-2. FCoV-1 has a lot of clinical sequencing data, but very little *in vitro* data. Alternatively, FCoV-2 has little clinical sequencing data but many *in vitro* studies. The differences between the approaches with which FCoV-1 and FCoV-2 have been studied illustrate how both surveillance of ongoing infections and basic science work need to be done in parallel. Sequencing of circulating viruses validates and reinforces results from basic science, or can provide insight into the genetic adaptations viruses gain when being propagated in cell culture. FCoV-2 is well understood from a basic science perspective because it is possible to grow and propagate in cell culture. However, because not much sequence data has been obtained from clinical FCoV-2 variants, it has not been possible to compare circulating viruses to the lab-adapted strains that have been highly studied.

FCoV Cellular Tropism: Despite many years of study, cell tropism of FCoV across the FECV/FIPV spectrum, and of CCoV, remains an open question. In part, this is because, cell culture-based studies can easily lead to misappropriation of viral tropism; coronaviruses can select tropism variants extremely easily, with a "hot-spot" of selection in the spike cleavage sites; for examples see[89][90]. While rapid cell

culture adaptation has been known for many years, the notable loss of the "furin cleavage site" of the prototype SARS-CoV-2 isolate WA-1 in VeroE6 cells readily illustrated this process to the wider scientific community; this occurred mainly through indels, but also through point mutations, see[91][92] for examples. Related to this, the passage history of FCoV-2 FECV-1683 included up to four passes in CRFK and/or fcwf-4 cells prior to the apathogenic phenotype documented upon experimental challenge of cats[55], and sequences of the original isolate are not available. FCoV-1 is almost impossible to isolate in conventional cell culture, except for the highly cell-adapted FIPV-Black virus; it has notably mutated spike cleavage sites and has likely also picked up heparin-sulfate binding activity (Whittaker lab, unpublished). While both FCoV-2 and CCoV-2 are readily isolatable, and with a well-characterized receptor (APN), a specific molecular receptor for FCoV-1 remains unidentified to date. FCoV-1 and FCoV-2 are also able to recognize Fc receptors *in vivo* [93][94], so driving antibody-dependent enhancement of infection (ADE) for FIP. Clinically, current 'gold standard', antibody-based immunohistochemistry (IHC) approaches are limited with respect to identification of specific cell types *in vivo*; RNA-based in situ hybridization (ISH) approaches are much better[88], but not commonly used.

Despite many years of study, the enteric tropism of FCoV remains unclear. While some clinical studies appear to show robust infection of epithelial cells[6][95], an experimental study of low pathogenicity FCoV-1 showed that the epithelial cells in the colon had pathology, but it was barely infected, especially compared to the lymph node macrophages[96]. For many FCoV infections, pathology may not linked to the clinical symptoms.

In the context of newly emerging viruses such as FCoV-23, we need to consider what exactly "FIP" is, clinically speaking; signs are already split into "wet" and "dry' manifestations, with dry FIP likely be a much broader category than currently recognized. For FIP, are neurological manifestations (as with FCoV-23) just the tip of the iceberg?; for example, rhinitis[32] and myocarditis[97][98] have been documented, with infection possibly also leading pancreatitis (although the latter is only well documented in ferrets, which also get FIP-like disease from a distinct but related ferret coronavirus[25]; see ref.[99]). Other clinical conditions such as liver problems, stomatitis *etc*. are also possible.

While FCoV-2 and CCoV-2 are established enteric pathogens, recent findings of FCoV-1 in the respiratory tract of FIP cats[100], as well as in respiratory[101] and conjunctival[102] samples of cats without confirmed FIP, leads to a reconsideration of an enteric route of transmission for FCoV-1, despite the preponderance of

viral RNA being shed in the feces; without the ability to readily isolate viruses it cannot be guaranteed that this viral RNA corresponds to infectious virions[103].

Perspectives

In this review, we argue that both the virus and the disease may be fundamentally different—for both FCoV-1 *vs.* FCoV-2, and for CCoV-1 *vs*. CCoV-2.

Recombination, along with mutations and generation of indels, is fundamental for coronavirus evolution —facilitating cross-species transmission and acting as a primary driver of viral spillover and emergence[104]. Within the species *Alphacoronavirus-1* there may exist a specific and dynamic "metavirome" that is in a constant state of flux and can seed the emergence of both within-host and between-host variants with highly context-dependent properties. We propose that this selection of variants having discrete pathogenic properties is driven in fundamentally different ways between FCoV-1/CCoV-1 and FCoV-2/CCoV-2—by a process of accumulated point mutations/indels and recombination events, respectively. FCoV-CCoV may not be an individual entity, and we argue that using simple, PCR methodologies for diagnosis and monitoring/surveillance may be treacherous—in that we are trying to hit a moving target; thus, there is a need for robust sequencing that embraces the inherent sequence diversity and recombination that is part of the "lifestyle" of a coronavirus. We note that available commercial FIP-specific PCR-based tests have generally not been widely adopted in the marketplace as a successful tool for clinical diagnosis.

As reported by LePoder[105], feline and canine coronaviruses have common genetic and pathobiological features, and it may be unwise to treat these viruses in an animal species-specific manner; this analogy also applies not only to the TGEV-like porcine viruses noted above, but also to coronaviruses of ferrets and mink—which also exist in different pathobiological forms, often with pyogranulomatous lesions and effusions remarkably similar to FIP in cats; these viruses are classified as either a separate subspecies *(Alphacoronavirus-2)* or sub-genus (minacovirus), and are notable pathogens of "exotics" in veterinary medicine, see[60]—but are poorly understood. Whether animals other than pigs—including wildlife species—harbor viruses that can readily recombine with FCoV/CCoV remains to be seen.

Acknowledgements

We thank Jean Millet, Javier Jaimes, Beth Licitra and all members of the Whittaker lab for helpful comments and assistance with this manuscript. Work on this topic is funded by the Cornell Feline Health Center and the Michael Zemsky Fund for Feline Disease at Cornell University College of Veterinary Medicine.

Footnotes

[1] Metavirome = sum of all viral genomes present in a sample

[2] biotype = a non-formal category, traditionally implying taxonomic connection, but applied to FCoV to imply pathogenic outcome or phenotype

[3] genotype = the genetic constitution of an individual organism

[4] serotype = a distinguishable feature of an organism based on an antibody response or test

[5] clade = a group of organisms believed to have originated from a common ancestor

[6] indel = an insertion or deletion of genetic material/sequence

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Declarations

Funding: Work on this topic is funded by the Cornell Feline Health Center and the Michael Zemsky Fund for Feline Disease at Cornell University College of Veterinary Medicine.

Potential competing interests: No potential competing interests to declare.