Rethinking the drivers of feline and canine coronavirus virulence and pathogenesis; toward an understanding of the dynamic world of coronavirus mutations, indels and recombination

a technical white paper

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Introduction

Feline coronavirus (FCoV) is one of the most important infectious diseases 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 (Ettinger, Feldman, & Cote, 2024; Sykes, 2022). It is widespread, with the prevalence of FCoV infection in the US feline population estimated at 75-95% in multi-cat households (Pedersen, 1995), possibly dropping to 25% in single-cat households and approaching 100% in shelter/breeder situations. With an estimated 58 million owned cats in the US alone (according to the American Veterinary Medical Association), FCoV represents a widespread endemic coronavirus that to date remains largely unexplored from a molecular evolution perspective, and with still many unanswered clinical questions.

According to recent guidelines from the American Association of Feline Practice (Thayer et al., 2022), FCoV infection has three principal clinical outcomes:

i) animals clear what is assumed to be an acute primary infection, with no viral shedding (about 5% of cats);
ii) animals intermittently shed low levels of virus from their gastrointestinal tract (70-80% of cats);
iii) animals develop long-term persistent shedding with high viral load for a prolonged period (10-15% of cats).

Based on these clinical outcomes, it is clear that FCoV is far from an acute infection, and it likely persistently infects cats over long periods of time.

In a subset of infected cats (about 5-12%), feline infectious peritonitis (FIP) follows the primary/persistent infection. FIP typically presents in either an effusive ("wet") or non-effusive ("dry"), form; typically associated with peritoneal/pleural effusion and neurological signs respectively. It can be prevalent in high density housing situations, such as in shelters, rescue groups and/or breeders, in young cats (<2 yrs of age), and often occurs following a stressful event (Thayer et al., 2022).

FCoV is traditionally considered to behave as one of two pathogenic "biotypes"[1]; FECV (equivalent to "low pathogenesis") or FIPV (equivalent to 'highly pathogenic")[2]—which unidirectionally progress (forwards) through an "internal mutation" (Vennema, Poland, Foley, & Pedersen, 1998); while this basic concept has remained a mainstay of FIP pathogenesis for over a quarter century, we consider that the over-simplistic (possibly even naïve) concept of an "FECV" switching to an "FIPV" is flawed; while pathogenesis of FIP is certainly linked to viral mutation, this is a much more complicated process than previously anticipated. Recent understanding has questioned the early connection to ORFs 3 and 7, and while mutations such as S:M1058L are also no longer thought to be FIPV-specific—and are rather associated with systemic spread of FCoV (Barker & Tasker, 2020)—it remains likely that the predominant genetic changes controlling viral pathogenesis lie in the spike (S) gene (Zehr et al., 2023).

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, [3]—but this terminology is also inherently flawed; 'serotype' is a widely used term and may 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

1 *biotype = a non-formal category, traditionally implying taxonomic connection, but applied to FCoV to imply pathogenic outcome*
2 *pathogenesis = a qualitative descriptor of infection based on host response; virulence = a quantitative and measurable outcome of infection*
3 *serotype = a distinguishable feature of an organism based on an antibody response or test*

notable evolutionary differences. We previously presented evidence that the 'serotypes' really reflect viruses representing two distinct genetic clades [4], with clade A corresponding to serotype I and clade B corresponding to serotype II, and with much more diversity that has been recognized to date, including fundamentally distinct biological properties (Whittaker, André, & Millet, 2018). Exchange of the spike protein through recombination results in antigenic shift, which is relevant epidemiologically, as animals that have been exposed to one virus will still be able to be infected by the other serotype/genotype (*i.e.*, there is no immunological cross-protection).

Here, we group the viruses more broadly, as "genotypes"[5] or more simply "types"—*i.e.*, FCoV-1 and FCoV-2. As with Jaimes et al, (Jaimes, Millet, Stout, André, & Whittaker, 2020), we focus on the spike protein, as it is the main driver of coronavirus cell tropism and pathogenesis (Belouzard, Millet, Licitra, & Whittaker, 2012). Such aspects of FCoV-1 have recently been covered elsewhere (Gao et al., 2023); we provide an update on FCoV-1 in comparison to FCoV-2 and CCoV, and include the newly emerged feline/canine recombinant virus FCoV-23 (Attipa et al., 2023; Warr, Attipa, Gunn-Moore, & Tait-Burkard, 2023; Atippa, Warr, Epaminondas, & O'Shea…, 2023; Hardas, Attipa, Gunn-Moore, & Epaminondas…,)

FCoV-1 accounts for the majority of coronavirus infections of cats—and cases of FIP; it is the most-studied virus in the species *Alphacoronavirus-1* from a clinical perspective. 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).

As mentioned above, the progression of FCoV (FECV) to its FIPV biotype has been linked to several genomic changes, including in the viral 7b, 3c and spike (S) genes. While multiple genomic changes likely account for ultimate conversion to FIP, a specific region of spike—the structural loop spanning the interface of the spike S1 and S2 domains—is strongly linked to the FIPV phenotype: our prior studies have shown that amino acid sequence changes in this region are highly correlated with conversion to FIP (Licitra et al., 2013). In circulating FCoV-1, this "S1/S2" domain contains a consensus motif for cleavage-activation by the cellular protease furin (*i.e.*, a furin cleavage site, or FCS). Our initial molecular analysis of S1/S2 identified a consensus sequence (S/T/Q)-R-R-(S/A)-R-R-S in 30 fecal samples from apparently healthy cats (*i.e.*, "FECV") 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. These data led to the hypothesis that "uncleaved" spikes are somehow functionally responsible for the FIPV biotype. Subsequent case studies of individual cats, and follow up of a localized FIP outbreak in an animal shelter, also confirmed this 100% correlation (André, Cossic, Davies, Miller, & Whittaker, 2019; André, Miller, & Whittaker, 2020; Healey, Andre, Miller, Whittaker, & Berliner, 2022). 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 support for this hypothesis (Ouyang et al., 2022), along with a series of FIP cats from a set of clinical trials testing antiviral drugs where the majority of S1/S2 sites were disrupted (Murphy et al., 2024). Notably, a recent unbiased genomic analysis has provided additional support for the "FCS" disruption hypothesis, which identified the FCoV-1 S1/S2 loop (along with other residues) as a region with sites evolving under different selective regimes between pathogenic and non-pathogenic FCoVs (Zehr et al., 2023). 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 (Licitra, Sams, Lee, & Whittaker, 2014), but remains poorly understood from a functional perspective.

While "FECV" is widely distributed, and presumably highly transmissible, FIPV is not generally thought to be a transmissible virus—with "outbreaks" likely resulting from multiple individual conversions events 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

4 *clade = a group of organisms believed to have originated from a common ancestor*
5 *genotype = the genetic constitution of an individual organism*

the literature, with the three main examples (Barker et al., 2013; Wang, Su, Hsieh, & Chueh, 2013; Healey et al., 2022) describing both more traditional transmission events (type-2 viruses) and what might be better considered as clusters of distinct but closely related viral variants (type-1 viruses).

For FCoV-1, we argue that pathogenic variants mainly derive from accumulated point mutations, with some recent evidence for indels[6] (Olarte-Castillo et al., 2023); the point mutations/indels appear to be mainly present in certain 'hot-spots' including the spike protein cleavage sites, "position 1058" and in the N-terminal domain.

Recent data on FCoV-1 links to the general concept that for RNA viruses, pathogenesis is part of quasispecies diversity (Vignuzzi, Stone, Arnold, Cameron, & Andino, 2006). Over the years, this concept has been exploited to great effect in studies of human immunodeficiency virus (HIV-1) (Fraser et al., 2014) and hepatitis C virus (HCV) (Raghwani et al., 2019), and most recently for SARS-CoV-2 (Lythgoe et al., 2021). 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) (Hoetelmans, 1998). Increasing evidence suggests that coronavirus infections can also take advantage of such persistent or sanctuary sites. A study of FCoV-1 may 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 discipline that has been termed "phyloanatomy" (Bons & Regoes, 2018; Lorenzo-Redondo et al., 2016; Normandin et al., 2023). We also consider that such an understanding of FCoV-1 infection in this manner is essential for an understanding and clinical management of the antiviral drugs (such as GS441524 and molnupiravir), which are rapidly coming into widespread use for treatment of FIP in cats (Zhang, 2020)—with treated animals better defined as "in remission" rather than "cured" based on the presence of sanctuary site(s). Based on its mechanism of action, molnupiravir may be especially problematic for FCoV-1-type infections (Pond & Martin, 2023; Sanderson, Hisner, Donovan-Banfield, & Hartman…, 2023)—with viral dynamics and antiviral resistance often being highly adaptive processes (Irwin, Renzette, Kowalik, & Jensen, 2016; Chomont, 2023).

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.

In contrast to FCoV-1, much less is known about the prevalence and 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, 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 (Lin et al., 2009). Although this assay is sufficient to detect FCoV-2, to differentiate it from FCoV-1, and to detect co-infections, sequencing the complete genome is essential to detect different recombination events, and to identify the origin of these recombinant variants. 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 TGEV (Tresnan, Levis, & Holmes, 1996). However, natural infection with CCoV-2 in cats has not been reported (or assessed at all). Compared to FCoV-1, FCoV-2 is much less prevalent in domestic cats (Lin et al., 2009; Shiba, Maeda, Kato, Mochizuki, & Iwata, 2007; An et al., 2011); 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 (Pedersen, Evermann, McKeirnan, & Ott, 1984), whether FCoV-2 can be differentiated into FECV and FIPV is less clear. Notably the virus typically referred to as FECV-1683 was originally isolated from a cat which had severe clinical signs, including infection of lymphoid tissue.

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

Compared to FCoV-1, there is only a single cleavage site in the spike protein (S2') with a notable R-G substitution at the expected cleavage position in certain isolates; this may affect cell tropism (Regan, Shraybman, Cohen, & Whittaker, 2008), but not necessarily virulence. This R-G substitution is also seen with porcine epidemic diarrhea virus (PEDV), and may be a cell culture adaptation (Wicht et al., 2014) or may occur in field strains; the same substitution also occurs with ferret CoVs, but its relationship to disease outcome in this case also remains unclear (Tarbert et al., 2020).

Canine coronavirus (CCoV) is a well-established enteric pathogen of dogs—hence its alternative name canine enteric coronavirus (CECoV) (Ettinger, Feldman, & Cote, 2024; Sykes, 2022), 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 (Priestnall, Mitchell, Walker, Erles, & Brownlie, 2014; Erles & Brownlie, 2008). 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, with type IIb the result of a recombination event with a TGEV-like virus; thus is 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) (Licitra, Whittaker, Dubovi, & Duhamel, 2014; Regan et al., 2012), with other examples of recombinant viruses possibly spanning continents and long time periods; such viruses may include divergent CCoVs identified in Sweden (Escutenaire et al., 2007), Australia (Naylor et al., 2002) and China (Chen et al., 2019; He et al., 2020; Wu et al., 2023). 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 (Radford et al., 2021; Stavisky et al., 2012). Also of note is the finding that CCoVs with distinct NTDs (Zehr et al., 2022) have been isolated from humans, and defined as HuCCoV or CCo-HuPN-2018 (Keusch et al., 2022; Pratelli et al., 2021; Buonavoglia, Pellegrini, Decaro, Galgano, & Pratelli, 2023), where they are considered to have respiratory tropism.

CCoV-2a also exists as what are known as 'pantropic' isolates. The initial such isolate (CB/05) was responsible for severe outbreak of fatal systemic disease in a pet shop in Bari, Italy, which included broncho-pneumonia and neurological signs (Buonavoglia et al., 2006). These viruses have since been well reviewed in the literature (Decaro & Buonavoglia, 2011) and have now documented across the Mediterranean region over the past decades, as well as in other European countries. 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 (Licitra et al., 2014). 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 (Olarte-Castillo & Whittaker, 2024).

CCoV-1 is typified by the isolate Elmö/02 (Pratelli et al., 2003), which has high identity to FCoV-1; this virus is not well understood, and—notably—likely cocirculates extensively with the various CCoV-2 viruses (Decaro et al., 2010). This leads to particular challenges with regard to 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 (Attipa et al., 2023; Warr et al., 2023; Atippa et al., 2023; Hardas et al.,). 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 large 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 (Ntafis et al., 2012)—and is present in two forms, including one with 630bp indel in the N-terminal domain 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 (Attipa et al., 2023), with a major roles being played by both viral factors (such as recombination and the 0-domain indel), and environmental/community factors

(the large numbers of feral cats—up to 1.5 million—on a relatively small island). While unusual, the deletion of the spike N-terminal domain is not unprecedented; it has been reported before, notably during the tropism change of TGEV to porcine respiratory coronavirus (PRCoV) (Wesley, Woods, & Cheung, 1991), and for FCoV-1 with the FIPV isolate C3663 (Terada et al., 2012).

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" really 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 (Sweet, Andre, & Whittaker, 2022).

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 (Le Coupanec et al., 2015; Choi, Kots, Singleton, Weinstein, & Whittaker, 2024). 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 (Sasaki et al., 2021; Lamers et al., 2021) 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 (Pedersen et al., 1984), and sequences of the original isolate are not available. FCoV-1 is almost impossible to isolate in cell culture, with the exception of 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 -2 are also able to recognize Fc receptors *in vivo* (Weiss & Scott, 1981; Takano, Kawakami, Yamada, Satoh, & Hohdatsu, 2008), 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 (Sweet et al., 2022), but not commonly used.

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 (André et al., 2020) and myocarditis (Ernandes et al., 2019; Stephenson, Swift, Moeller, Worth, & Foley, 2013) have been documented, with infection possibly also leading pancreatitis (although the latter is only well documented well in ferrets, which also get FIP-like disease from a distinct but related ferret coronavirus (Whittaker et al., 2018); see (Wills, Beaufrère, Brisson, Fraser, & Smith, 2018)). Other clinical conditions such as liver problems, stomatitis *etc*. are also possible.

While type 2 FCoV-2 and CCoV-2 are established enteric pathogens, recent findings of FCoV-1 in the respiratory tract of FIP cats (Slaviero et al., 2024), as well as in respiratory (…, Tejada, DeTar, Berliner, & Whittaker, 2021) and conjunctival (Olarte-Castillo et al., 2023) 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 (Griffin, 2022).

Perspectives

In this paper 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.

Within the species *Alphacoronavirus-1* there may exist a specific and dynamic "metavirome" that is is in a constant state of flux and can seed the emergence both within-host and between-host variants with highly context-dependent properties. In this paper, 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 virus 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 (Le Poder, 2011), 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 (Tarbert et al., 2020)—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.

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