CATS AND CORONAVIRUSES: ONE HEALTH IN THE AGE OF COVID-19

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Feline coronavirus (FCoV) circulates widely in feline populations and is associated with two common disease outcomes: mild to inapparent gastrointestinal disease or the lethal, systemic disease, feline infectious peritonitis (FIP). Anecdotally, FCoV has been associated with upper respiratory disease and a previous case report of FIP highlighted FCoV presence in the nasal cavity. Following the onset of the COVID-19 pandemic, it has become apparent that cats are susceptible to infection with SARS-CoV-2, but many questions remain regarding clinical outcome. The work presented here investigates the association of FCoV, along with other common feline pathogens, with respiratory disease in two shelter environments. Further work provides serological evidence of SARS-CoV-2 infection in cats presenting to a New York City animal hospital. Lastly, rodent and bat coronaviruses were analyzed in regards to the presence of furin cleavage sites. A defining feature of SARS-CoV-2 is the presence of a furin cleavage site, which is unique among the *Sarbecoviruses*. However, *Embecoviruses* circulate in rodent populations and nearly always contain an S1/S2 furin cleavage site, which may have important zoonotic implications. While bats are recognized as an important coronavirus reservoir, human coronaviruses OC43 (HCoV-OC43) and HKU1 (HCoV-HKU1) both have rodent origins. FCoV, likewise contains an S1/S2 furin cleavage site and loss of the furin cleavage site is associated with the severe form of the disease. Coronaviruses identified in rodents commonly

have S1/S2 furin cleavage sites. Understanding *Coronaviridae* in other animals allows for a One Health approach for future associated challenges.

BIOGRAPHICAL SKETCH

Alison Stout is originally from Northwestern New Jersey where her love for animals and infectious diseases developed as a 4-H and FFA member. She received her BS in Animal Science and Agricultural Science Education with a minor in Agricultural Business Management from Cornell University in 2011. After graduation, she spent two years working as a research technician in a laboratory focused on equine immunology and equine herpesvirus. She then attended Cornell University College of Veterinary Medicine and graduated in 2017. As a veterinary student her interest in One Health flourished, volunteering at the Cornell Wildlife Health Center and doing field work in Indonesia through Expanding Horizons. After graduating veterinary school, she spent a summer in private small animal practice, before joining the PhD program. She has completed her dissertation research in the laboratory of Dr. Gary Whittaker, in the Department of Microbiology and Immunology in the College of Veterinary Medicine.

This work is dedicated to my family for their constant support.

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ACE2	Angiotensin-converting enzyme 2
ADE	Antibody dependent enhancement
APN	Aminopeptidase N
BCoV	Bovine coronavirus
BtCoV	Bat coronavirus
CDC	Centers for Disease Control and Prevention
ChRCoV	China *Rattus* coronavirus
CsA	Cyclosporin A
CoV	Coronavirus
COVID-19	Coronavirus Disease 2019
CRCoV	Canine respiratory coronavirus
CRFK	Crandell-Rees feline kidney cells
CryoEM	Cryoelectron microscopy
DLH	Domestic long hair
DMEM	Dulbecco's Modified Eagle's Medium
DMH	Domestic medium hair
DPBS	Dulbecco's phosphate-buffered saline
DPP4	Dipeptidyl-peptidase 4
DSH	Domestic short hair
EMEM	Eagle's Minimal Essential Medium
FBS	Fetal Bovine Serum

FCoV	Feline coronavirus
FCV	Feline calicivirus
FCWF-4	*Felis catus* whole fetus-4 cells
FECV	Feline enteric coronavirus
FHV-1	Feline herpesvirus
FIP	Feline infectious peritonitis
FIPV	Feline infectious peritonitis virus
FRDC	Feline respiratory disease complex
GISAID	Global initiative on sharing avian influenza data
HCoV	Human coronavirus
HCoV-229E	Human coronavirus 229E
HCoV-HKU1	Human coronavirus HKU1
HCoV-NL63	Human coronavirus NL63
HCoV-OC43	Human coronavirus OC43
HIV	Human immunodeficiency virus
IBV	Infectious bronchitis virus
IFN	Interferon
MERS-CoV	Middle East respiratory syndrome coronavirus
MHV	Mouse Hepatitis Virus
NSSP	National Syndromic Surveillance Program
PEDV	Porcine epidemic diarrhea virus
RBD	Receptor binding domain
RSV	Respiratory Syncytial Virus

SADS-CoV	Swine acute diarrhea syndrome coronavirus
SARS-CoV	Severe acute respiratory syndrome coronavirus
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
TGEV	Transmissible gastroenteritis virus
TMPRSS2	Transmembrane protease, serine 2
URTD	Upper respiratory tract disease
USDA	U.S. Department of Agriculture
VOC	Variant of concern
WHO	World Health Organization

CHAPTER I

INTRODUCTION

1.1 Introduction to the Coronaviridae

The *Coronaviridae* is a family of large, single-stranded, positive sense, enveloped RNA viruses, belonging to the order Nidovirales (de Vries et al., 1997). The four coronavirus genera are denoted as *Alpha*-, *Beta*-, *Delta*-, and *Gammacoronaviruses* (Knipe & Howley, 2013). In general, the *Alpha*- and *Betacoronaviruses* are associated with mammalian species, while the *Delta*- and *Gammacoronaviruses* are associated with avian species (Woo et al., 2012). While seven coronaviruses have been described in humans, three have been associated with high morbidity and/or mortality: SARS-CoV-2, SARS-CoV, and MERS-CoV (Lau et al., 2005; Wendong Li et al., 2005; van Boheemen et al., 2012; Zhou et al., 2020). SARS-CoV is classified as a federal select agent, by the Federal Select Agent program, a joint program between the US Departments of Health and Human Service and Agriculture (USDA). Several other common coronaviruses circulate in people, including human coronavirus (HCoV) 229E and HCoV-NL63, which both are believed to have origins in bat species (Corman et al., 2015; Huynh et al., 2012) and HCoV-OC43 and HKU1 that are believed to have rodent origins (Cui et al., 2019).

Across the veterinary world, coronaviruses have been described amongst agricultural and companion animals, including transmissible gastroenteritis (TGEV) of swine, infectious bronchitis virus (IBV) of poultry, bovine coronavirus (BCoV) of cattle, feline coronavirus (FCoV) in cats, canine coronavirus (CCV), canine respiratory coronavirus (CRCoV), ferret coronavirus, and equine coronavirus (Saif, 2004). Swine acute diarrhea syndrome coronavirus (SADS-CoV) has recently been described in pigs (Gong et al., 2017; Pan et al., 2017; Zhou et al., 2018). Additionally, the emergence and global spread of SARS-CoV-2 has called attention to

which species, beyond humans, might be susceptible to the virus. Natural infection of several large cats at the Bronx Zoo sparked concern for anthropozoonosis, or reverse zoonosis (McAloose et al., 2020). Following this initial event, it became apparent that farm-raised mink were also highly susceptible to SARS-CoV-2 (Molenaar et al., 2020; Munnink et al., 2020; Oreshkova et al., 2020). In the laboratory setting, both ferrets and cats prove to be susceptible to the virus (Bosco-Lauth et al., 2020; Shi et al., 2020). Additionally, naturally occurring cases of SARS-CoV-2 have been observed in cats (Newman et al., 2020; Segales et al., 2020). Fortunately, as of early 2021, most documented cases of SARS-CoV-2 in cats have remained mild, though questions remain in regards to SARS-CoV-2 pathophysiology in cats. Currently, more concerning threat to cats, however, is infection with FCoV and of course, dual infections with FCoV and SARS-CoV-2 have not yet been described, but could be disastrous clinically, or allow for viral recombination, a common feature of CoVs (Graham & Baric, 2010).

FCoV is the causative agent of the invariably fatal disease, feline infectious peritonitis (FIP), which a subset of infected cats succumb to each year (Pedersen, 2014). The FCoV genome consists of 11 open reading frames and is ~30 kB (King et al., 2011). The 11 ORFs encode two replicases, the spike (S) protein, envelope (E), membrane (M), nucleocapsid (N) and five nonstructural proteins (3a, 3b, 3c, 7a, 7b) (Dye & Siddell, 2005). The spike protein in particular is a main driver of viral tropism (Belouzard et al., 2012). In the majority of cats, FCoV infection is considered an inapparent to mild gastrointestinal disease (Sykes, 2014). The Merck Veterinary Manual notes that FCoV can also be associated with respiratory disease and clinical histories of cats who succumb to lethal FCoV will occasionally note early respiratory signs,

though this could be due to other respiratory pathogens. The invariably lethal disease stemming from FCoV infection is known as feline infectious peritonitis (FIP). As has been observed in severe cases of COVID-19, including cases of multisystem inflammatory syndrome in children (MIS-C) following SARS-CoV-2 infection, the clinical course of FIP is impacted by ensuing vasculitis (August, 1984; Gupta et al., 2020; Rowley, 2020; Varga et al., 2020). FIP was first described by Holzworth in 1963, initially called chronic fibrinous peritonitis (Holzworth, 1963) and later discovered to be caused by a coronavirus (Zook et al., 1968). A disease similar to FIP has also been described in ferrets, though vasculitis is less associated with disease (Doria-Torra et al., 2016).

The COVID-19 pandemic has called for a One Health approach for solving global health challenges. The CDC defines One Health as "a collaborative, multisectoral, and transdisciplinary approach—working at the local, regional, national, and global levels—with the goal of achieving optimal health outcomes recognizing the interconnection between people, animals, plants, and their shared environment" (*One Health | CDC*, 2021). The spillover of pathogens between species is nearly inevitable and future coronavirus spillover events may come from species, beyond just bats, belonging to the order Chiroptera (Wardeh et al., 2021). FCoV is not a zoonotic pathogen. However, learning from natural models of disease, including cats with FIP and other natural diseases provides a comparative approach for solving global challenges.

1.2 FCoV Classification

Feline coronavirus is generally accepted to exist as two biotypes: Feline enteric coronavirus (FECV) and feline infectious peritonitis virus (FIPV). FECV is associated with the mild form of

disease. FIPV, however, is associated with macrophage/monocyte tropism, a hallmark of the disease FIP (Rottier et al., 2005). Additionally two serotypes exist, designated as type I and type II, based on growth in cell culture and antigenicity (Fiscus & Teramoto, 1987). While type I viruses are more often seen in disease, type II has been easier to study in vitro (Shiba et al., 2007). FCoV type II emerged as a homologous recombination between feline and canine coronavirus (Terada et al., 2014). While type I viruses are much more commonly identified via molecular methods in field studies, the ability to propagate type I viruses in cell culture is diminished in comparison to type II viruses (Tekes et al., 2010). The exception is FIPV I Black, which has previously been isolated in cell culture (Black, 1980). A number of cell lines have been used to investigate FCoV, including Crandell-Rees Feline Kidney cells (CRFK); *Felis catus* whole fetus-4 cells (FCWF-4); and feline lung epithelial cells (AK-D) (Jacobse-Geels & Horzinek, 1983; Neuman et al., 2006; Regan et al., 2010). Clinical isolates are exceedingly difficult to propagate in culture and a major area of needed improvement.

Receptor binding is an additional major difference between type I and type II FCoV. Across the coronavirus species, several common receptors have been identified, including aminopeptidase N (APN) and angiotensin converting enzyme 2 (ACE2) (Jaimes & Whittaker, 2018). FCoV type II has previously been identified to utilized feline APN (Hohdatsu et al., 1998). Both SARS-CoV and SARS-CoV-2 utilize human ACE2 (Hoffmann et al., 2020; Wenhui Li et al., 2003). Feline and human ACE2 are highly similar (85.2% sequence identity) (Stout et al., 2020). To date, no receptor has been identified for FCoV type I, however, antibody-dependent enhancement (ADE) has remained a plausible mechanism by which FCoV enters the cell, especially the macrophage/monocyte (Hohdatsu et al., 1998). In this process, non-neutralizing antibody

promotes the uptake of virus in the macrophage through Fc receptor binding (Weiss & Scott, 1981). This mechanism has been considered at play with dengue hemorrhagic fever (DHF), respiratory syncytial virus (RSV), and influenza (Arvin et al., 2020). Whether type I FCoV could utilize feline ACE is an intriguing question. ACE2 has previously been shown to be expressed on human macrophages (Keidar et al., 2007; Song et al, 2020).

An additional consideration for macrophage tropism lies with considering viral mutations, including at the S1/S2 furin cleavage site. The spike protein includes two domains, denoted S1 and S2, corresponding with receptor binding and membrane fusion. FCoV contains an S1/S2 furin cleavage site functioning in viral activation (Millet & Whittaker, 2015). Furin is a serine protease that cleaves at a minimum motif of R-X-X-R. Other proteases that are also utilized across CoVs include Transmembrane protease, serine 2 (TMPRSS2), trypsin, and cathepsins (Heald-Sargent & Gallagher, 2012). Mutations in the spike protein have unequivocally been correlated with the FIPV biotype (Licitra et al., 2013). However, this is distinct compared to SARS-CoV-2, in which loss of the furin cleavage site has been associated with viral attenuation (Johnson et al., 2021; Peacock et al., 2020).

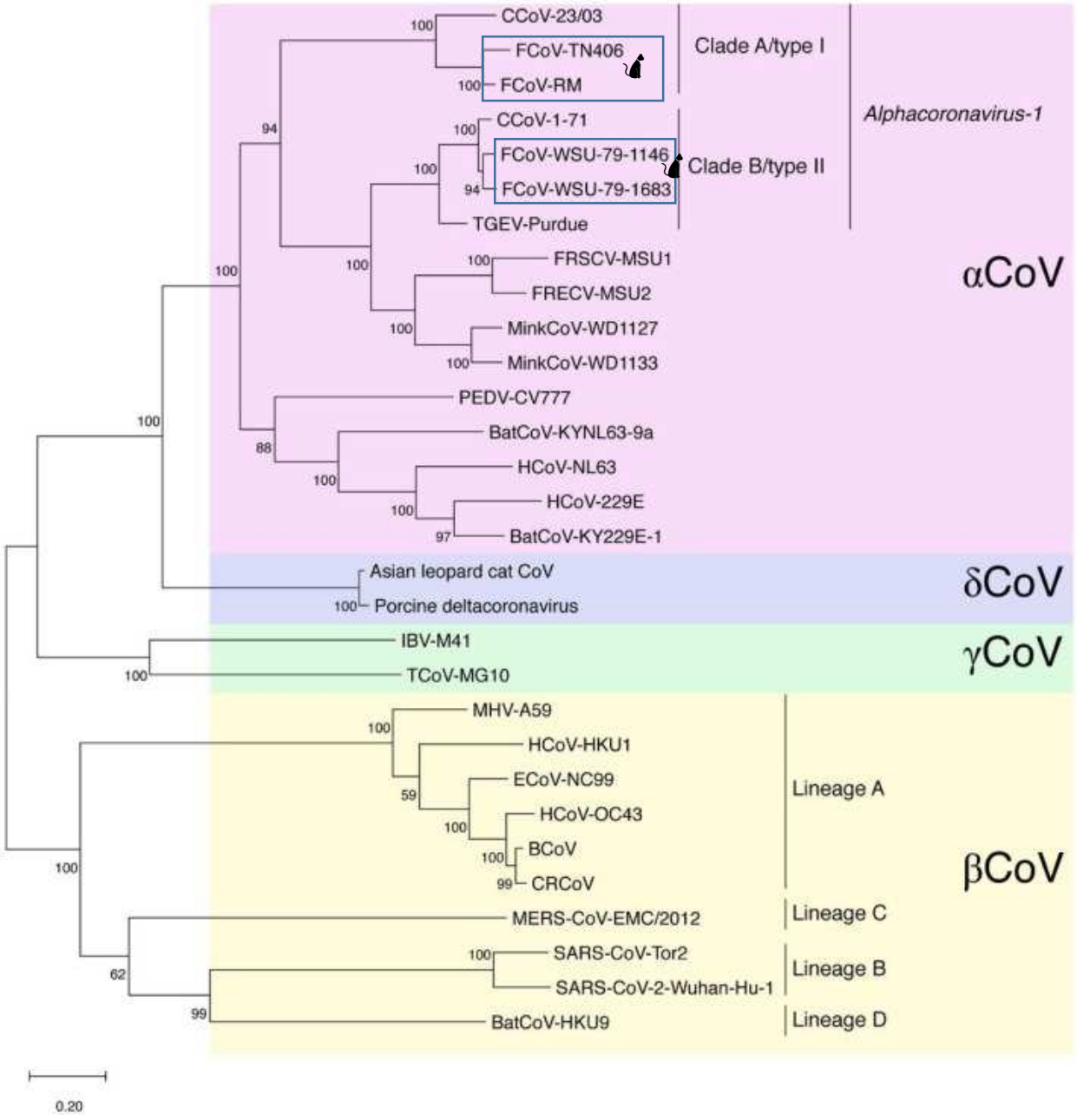

Figure 1.1: Phylogenetic overview of the *Coronaviridae* family, based on the S gene. The spike protein is a main factor driving viral tropism. Feline coronavirus is an alphacoronavirus, along with several other animal CoVs. Image adapted from Stout et al., 2020.

1.3 Clinical outcomes of FIP

FIP is frequently classified in two disease forms: effusive or non-effusive. Colloquially, these disease forms are also commonly referred to as "wet" and "dry." The effusive form is characterized by proteinaceous effusion accumulation in primarily the peritoneal or pleural spaces and non-effusive disease is characterized by granulomatous lesions across numerous organs, especially the brain (Hartmann, 2005). The mild enteric disease caused by FCoV is due to viral replication in intestinal epithelial cells (Addie & Jarrett, 1992). Case reports and other literature, however, have further highlighted possible clinical manifestations of FIP (table #1.1). The strength of evidence for disease associations is of course limited in case reports, whether it relates to FIP or COVID-19.

In the immune response to both FIP and COVID-19, the immune response including inflammatory effects, contributes to disease pathogenesis. Previous work investigating SARS-CoV has demonstrated the necessity of CD4+ T cells for viral clearance (Chen et al., 2010; Yasui et al., 2014). However, T cell depletion has been a recognized consequence of FCoV infection and has been observed to be associated with more severe cases of COVID-19 (Chen et al., 2020; de Groot-Mijnes et al., 2005; Haagmans et al., 1996). Additionally, both regulatory T cells and natural killer (NK) cells decrease in FIP disease across blood, mesenteric lymph node and spleen (Vermeulen et al., 2013). High levels of IL-6 have previously been demonstrated in FIP ascites (Goitsuka et al., 1990), and likewise, elevated IL-6 levels appear associated with disease severity and outcome in COVID-19 patients (Aziz et al., 2020). In considering the balance between cell-

mediated immunity and humoral immunity, early reports indicated an association with strong humoral immunity and a deficient T cell response resulting in FIP (Kai et al., 1992).

Table 1.1: Clinical manifestations observed in FIP cases. The strength of the evidence varies across the literature, including single case reports, nonetheless, FIP is a wide ranging disease.

Reported disease manifestations	**Reference**
Central Nervous System	
Depression	(Norris et al., 2005; Weiss et al., 1980)
Seizures	Reviewed in (Diaz & Poma, 2009; Norris et al., 2005)
Vestibular signs	Reviewed in (Diaz & Poma, 2009)
Ataxia	Reviewed in (Diaz & Poma, 2009; Norris et al., 2005)
Behavior changes	Reviewed in (Diaz & Poma, 2009)
Cranial nerve deficits	Reviewed in (Diaz & Poma, 2009)
Hyperesthesia	Reviewed in (Diaz & Poma, 2009; Norris et al., 2005)
Tetraparesis	Reviewed in (Diaz & Poma, 2009; Norris et al., 2005)
Abnormal postural reactions	Reviewed in (Diaz & Poma, 2009)
Hydrocephalus	(Krum et al., 1975)
Ocular	
Chorioretinitis	(Andrew, 2000)
Anterior uveitis	(Andrew, 2000; Norris et al., 2005)
Respiratory	
Rhinitis	(André et al., 2020)
Upper respiratory tract infection	(Montali & Strandberg, 1972)
Renal	
Renomegaly	(Norris et al., 2005)
Polyuria/polydipsia	(Montali & Strandberg, 1972; Norris et al., 2005)
↑ Blood urea nitrogen/Creatinine	(Tsai et al., 2011)
Hepatic	
Hepatic necrosis	(Oliveira et al., 2014; Weiss et al., 1980)
Hepatic lipidosis	(Oliveira et al., 2014; Trotman et al., 2007)
Coagulative necrosis	(Weiss et al., 1980)
Elevated serum alkaline phosphatase	(Zawie & Garvey, 1984)
Icterus	(Zawie & Garvey, 1984)
Hyperbilirubinemia	(Norris et al., 2005)
↑ Aspartate aminotransferase (AST)	(Norris et al., 2005)
↑ Alanine aminotransferase (ALT)	(Norris et al., 2005)
Gastrointestinal	
Diarrhea	(Weiss et al., 1980)
Vomiting	(Weiss et al., 1980)
Anorexia	(Weiss et al., 1980)
Melena	(Weiss et al., 1980)
Dermatologic	

Feline skin fragility syndrome	(Trotman et al., 2007)
Erythema, nodules, papules	(Bauer et al., 2013; Cannon et al., 2005; Declercq et al., 2008)
Cardiovascular	
DCM-like disease	(Yoshida et al., 2016)
Myocarditis	(Ernandes et al., 2019; Stephenson et al., 2013)
Pericardial effusion	(Oliveira et al., 2014)
Epicarditis	(Oliveira et al., 2014)
Pericarditis	(Hayashi et al., 1977)
Clin path	
Anemia	(Norris et al., 2005; Tsai et al., 2011)
Thrombocytopenia	(Ellis et al., 2018; Jordan et al., 1993)
↓ Albumin/Globulin Ratio	(Hartmann et al., 2003)

1.4 Epidemiology

Feline coronavirus is a ubiquitous pathogen in both domestic and wild felids. Most often, disease is considered a self-limiting to mild diarrheal disease. Nonetheless, a small proportion of cats succumb to the invariably fatal disease, feline infectious peritonitis (FIP). In one study, 1 of every 200 cats that presented to a veterinary teaching hospital was because of FIP (Rohrbach et al., 2001). Pesteanu-Somogyi et al report an FIP prevalence of 0.52% across all cat breeds (Pesteanu-Somogyi et al., 2006). Stratifying based on mixed or pure-bred, gives an FIP prevalence of 0.35% and 1.3%, respectively (Pesteanu-Somogyi et al., 2006).

Both type I and type II FCoV circulate globally. In a UK study, type II FCoV could be detected in 2% of naturally infected cats (Addie et al., 2003). Similarly, in a Swiss study looking at FCoV seropositive cats, 68% were positive for type I, 23% for both type I and II virus, and just 9% that were positive for only type II virus; of those cats that were considered to be clinically sick, antibody titers to type I virus were most common (Kummrow et al., 2005). In China, partial S

gene-based genotyping found type I FCoV in approximately 95% of FIP cases that could be sequenced; less than 5% of cats were positive for type II virus (Li et al., 2019). An additional survey in Austria also shows high prevalence of type I viruses (Benetka et al., 2004). Lastly, while a study in Japan shows high type I seroprevalence amongst all seropositive cats, paradoxically, a higher correlation between FIP and type II seroprevalence was found (Lin et al., 2009). In an outbreak scenario of FIP, type II virus was identified in the feces and other bodily tissues of cats who died of the disease (Wang et al., 2013). The prevalence of type I FCoV compared to type II may be due to the better adaptation of the virus for invading the feline host.

Animal shelters, rescues, and catteries are common sources of pet cats and important for understanding the spread of FCoV. In a case-control study of 50 cats with diarrhea and 50 cats with normal feces presenting to a shelter in Alachua County, Florida, USA, feline coronavirus was more commonly observed in cats with diarrhea (58%) compared to those deemed healthy (36%) (Sabshin et al., 2012). In those cats with diarrhea, FCoV was significantly associated with owner surrender and age. All cats that presented with diarrhea and were owner surrendered were observed to be shedding FCoV in their feces (Sabshin et al., 2012). This difference in risk may be due to increased density among owned cats compared to strays, as well as the usage of common litter boxes. Among cats with diarrhea, adults were more commonly shedding FCoV (12/15), compared to those less than 6 months old (15/35). It is possible this is due to waning maternal antibody, however, the history of these cats was unknown. In a similar study exploring enteropathogens in unowned cats in those that were experiencing diarrhea or healthy, similar levels of FCoV shedding were observed amongst cats in short term shelters (47% of diarrheic cats, 46% of healthy cats) and long term shelters (74% of diarrheic cats, 78% of normal cats)

(Andersen et al., 2018). Among trap neuter return (TNR) programs, 37% of diarrheic cats were shedding FCoV in feces, compared to 24%, though this was not statistically significant (Andersen et al., 2018). Diarrheic cats in foster care programs, however, were commonly shedding FCoV (80%) (Andersen et al., 2018). While age was not explicitly controlled for, the foster program cats tended to be under 6 months of age, compared to the TNR program, where cats tended to be over 6 months of age, or of unknown age (Andersen et al., 2018). In a sample of FCoV positive cats in an Austrian shelter, 6 cats were deemed healthy while 3 had respiratory clinical signs, 1 cat had diarrhea, and 2 cats were FIP suspect (Benetka et al., 2006). While the intent of this study was to evaluate M gene mutations, the authors also evaluated the frequency of type I and type II FCoV (Benetka et al., 2006). While no type II viruses were identified in cats, the authors designated several viruses as atypical or questionable type I viruses, which appeared related with length of stay (Benetka et al., 2006). In a study of 3000 feline adoptees, in which the shelter was noted to educate adopters in regards to FIP, 40 (1.3%) cats were identified to have developed FIP (Pedersen et al., 2012). While the intent of this study was to investigate the role of the 3c gene in intestinal tropism, of 45 cats that were deemed healthy, 31 (69%) were shedding FCoV in their feces (Pedersen et al., 2012). In an additional study, cats were screened for FECV on intake into a shelter and then followed for several weeks. Of 162 cats, approximately 33% of cats were positive for FECV via fecal sample and after a week in the shelter, 36 of 60 cats (60%) were observed to be shedding FECV (Pedersen et al., 2004). In convenience samples of 205 healthy cats at two animal care locations in Southern California, qRT-PCR amplified FCoV mRNA in a buffy coat sample of a single 8-week-old female cat, indicating replicating FCoV, while an additional 8 cats were also considered viremic based on total FCoV RNA (Fish et al., 2018). At a six month follow up, seven of the cats were reported to

have not developed FIP and the additional two cats were lost to follow-up (Fish et al., 2018). In fecal samples from fifty cats that were part of the larger study, 56% of samples were positive for FCoV (Fish et al., 2018). Only two cats were positive via both feces and buffy coat (Fish et al., 2018).

Additionally, the idea of FIP outbreaks in shelters remains as an ongoing question, especially in regards to how FIP develops. Two main hypotheses have been proposed: the internal mutation hypothesis or the circulating strain hypothesis. In the internal mutation hypothesis, cats develop FIP after infection with FECV and subsequent viral mutations (Pedersen et al., 1981). The circulating strain hypothesis, however, suggests that FIPV and FECV strains naturally circulate and thus, disease development is related with virus exposure (Brown et al., 2009). In 2013, Wang and colleagues reported on an FIP outbreak where 13 of 46 cats present in the shelter succumbed to signs consistent with FIP (Wang et al., 2013). In eight cats with FIP, all had at least one sample positive for type II FCoV (Wang et al., 2013). Of the 33 cats that remained healthy in this population, no cats were observed to be shedding type II virus; however 15 cats had untypable virus and 11 had type I virus (Wang et al., 2013). While this is only a single study, questions remain in regards to viral transmission and subsequent development of FIP. Additional factors in individual shelters must also be considered. Though shelter cats come from various backgrounds, once in a shelter environment, they share the same circulating air, are fed by the same person who may or may not adhere to strict biosecurity practices, received the same diet, and are subject to the same stressors.

1.5 Vaccines and treatment

To date, there is no effective treatment that is approved by the FDA for the treatment of FIP. Clinically, veterinarians have turned to steroids, antibiotics, and other supportive care, yet the outcomes rarely improve (Hartmann and Ritz, 2008). While Zoetis markets an intranasal vaccine to aid in preventing FIP, use of the vaccine is controversial and not widely accepted Scherk et al., 2013). In fact, the use of vaccines could enhance ADE, and thus, progression of the immune-mediated disease (Scott et al., 1992). ADE has been used to explain vascular permeability syndrome correlated with dengue vaccination and subsequent dengue exposure (Halstead, 2019). A study of a SARS-CoV vaccine demonstrated the potential of a hypersensitivity reaction on subsequent exposure to SARS-CoV post-vaccination (Tseng et al., 2012). In regards to the SARS-CoV-2 vaccines, the use of a stabilized spike protein results in a neutralizing antibody response (Jackson et al., 2020). Whether the same technology used to create the SARS-CoV-2 vaccine could be used for cats remains an open question.

In 1963 when the first clinical cases of FIP were described (prior to knowing the viral etiology), it was noted that antibiotic therapy was frequently attempted, but obviously yielded no benefit (Holzworth, 1963). Since this first report, and without an effective vaccine, numerous therapies have been attempted in cats presenting with FIP. Most recently, several reports have demonstrated the clinical benefit of the nucleoside analog GS-441524 in cats with FIP, including effusive, non-effusive, and neurologic forms of the disease (Dickinson et al., 2020; Pedersen et al., 2019). A 3C-like protease inhibitor has also shown the ability to decrease viral replication in vitro (Kim et al., 2013). While an antiviral might bring improved clinical outcomes, aside from

GS-441524, there has been little success of several antivirals tested in cats with FIP. Ribavirin, also a nucleoside analog, previously provided promising results against FCoV when studied in vitro (Weiss & Oostrom-Ram, 1989); when administered to cats as an experimental treatment, this antiviral resulted in worse outcomes in some instances (Weiss et al., 1993).

Given the inflammatory nature of FIP, therapy is frequently targeted at controlling the immune response. Though glucocorticoids are often given to cats with FIP, the clinical benefit is negligible (Hartmann & Ritz, 2008). An in vitro study evaluating cyclosporine A (CsA) against a type II FCoV virus showed a decrease in viral replication (Tanaka et al., 2012) and treatment of a 14-year-old cat with CsA, following unsuccessful interferon (IFN) treatment, resulted in clinical improvement, reduction in viral load and survival time over 260 days (Tanaka et al., 2015).

Numerous other therapies have been rethought about in regards to their potential application in FIP. Hydroxychloroquine and chloroquine are common anti-malarial drugs, with the ability to raise the pH of vacuoles and affect inflammatory processes (Fox, 1993). While in vitro chloroquine treatment results in reduction of FCoV titers, the in vivo benefits of chloroquine are less evident in cats, with no apparent improvement in survival in experimental studies (Takano et al., 2013). Itraconazole, a common antifungal, has been investigated in vitro and shown to have antiviral activity against type I FCoV, potentially through the inhibition of cholesterol (Takano et al., 2016, 2019).

Interferons have also been investigated in controlling FIP. While neither human IFN-α nor feline IFN-β solely have previously been associated with clinical improvement, high dose IFN-α in addition to *Proprionibacterium acnes* provided mild benefits (Weiss et al., 1990). IFN-ω has been investigated in FIP cats and while clinical trials did not reveal any association with improved outcome, a single cat that received this treatment survived for 200 days (Ritz et al., 2007). In a small study of cats experimentally infected with FIPV-1146, anti-tumor necrosis factor (TNF)-α demonstrated benefits for disease management (Doki et al., 2016). Additional immunotherapy through the use of convalescent plasma has not been explicitly investigated in cats, however, Feliserin has been administered in addition to other therapies (Ritz et al., 2007). Lastly, therapies have also been suggested in regards to hemostasis. Two cats with clinically diagnosed FIP showed disease improvement after administration of ozagrel hydrochloride, a thromboxane synthetase inhibitor, able to mitigate platelet aggregation (Watari et al., 1998).

1.6 Coronaviruses and One Health

There is undeniably a need for One Health in coronavirus disease research and discovery. The COVID-19 pandemic has highlighted the global impact of viral emergence. There is a need for continued surveillance, both in bats and other mammalian hosts. Bats (order Chiroptera) are frequently targeted for their role in C*oronaviridae* emergence (Lau et al., 2005), however, numerous other species likely harbor novel CoVs that may cause disease in humans, companion animals, or agricultural species (Wardeh et al., 2021). In this regard, viral discovery and open data sharing are essential. Additionally, understanding natural disease models, including CoVs in cats, can contribute to solving future global CoV challenges.

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CHAPTER II

IDENTIFICATION OF PATHOGENS IN RESPIRATORY SAMPLES FROM SHELTER CATS IN NEW YORK (NOVEMBER 2018-MARCH 2020)

Contents from this chapter were partially submitted as:

Stout, A. E., André, N. M., Tejada, M., DeTar, L., Berliner, E. A., and Whittaker, G. R. (2021). Identification of pathogens in respiratory samples of shelter cats in New York. Journal of Feline Medicine and Surgery Open Reports.

2.1 Introduction

Feline upper respiratory disease is commonly encountered in animal shelters and rescues (Dinnage et al., 2009; Gourkow et al., 2013). Typical pathogens associated with feline upper respiratory tract disease (URTD) include feline herpes virus (FHV-1), feline calicivirus (FCV), *Mycoplasma felis, Chlamydia felis,* and *Bordetella bronchiseptica* (Cohn, 2011; Gourkow et al., 2013). More recently, infections with *Streptococcus equi zooepidemicus* (Blum et al., 2010) and influenza have also been described (Lee et al., 2017). Canine pneumovirus is an emerging respiratory pathogen in dogs, but less is known in regards to respiratory infection from pneumovirus in cats, including prevalence and clinical significance (Decaro et al., 2014; Glineur et al., 2013; Maclachlan & Dubovi, 2017, pp. 327–356). Feline URTD remains a significant health and welfare concern for animals in shelters (Dinnage et al., 2009). High URTD rates can lead to increased use of antibiotics, higher cost of veterinary care, longer shelter length of stay due to time spent in isolation wards, and greater barriers to providing enrichment.

Feline coronavirus (FCoV) is also common in cat populations (Pedersen et al., 2004). Clinical presentations of FCoV infection can take two forms: a common, mild, self-limiting to inapparent diarrheal disease; or the rare, systemic, lethal disease, feline infectious peritonitis (FIP) (Brown et al., 2009; Kipar & Meli, 2014; Rottier et al., 2005). Wolfe and Griesemeir, who first suggested a possible viral etiology for FIP, noted that 2 of 16 cats with FIP presented with upper respiratory signs (Wolfe & Griesemer, 1966). In experimental infections of cats with FCoV, the virus can be detected in oropharyngeal swabs, in addition to fecal samples (Stoddart et al, 1988). Additionally, kittens with URTD signs are frequently FCoV seropositive (Addie & Jarrett, 1992). More recently, our lab has demonstrated viral antigen in the nasal cavity of a cat who

died from FIP (André et al., 2020). Though these reports support an association of FCoV with upper respiratory disease symptoms, FCoV is rarely considered a respiratory pathogen.

Investigating a role for FCoV in respiratory disease, in addition to other common respiratory pathogens, may provide insight for managing feline populations in shelters and communities. The objective of this pilot study was to investigate the role of FCoV as a component of upper respiratory disease in cats in two New York state shelters between November 2018 and March 2020.

2.2 Materials and Methods

Feline sampling & initial processing

Samples were solicited from two shelters in New York State. Cats were diagnosed with "upper respiratory disease" by the shelter veterinarian, based on nasal and/or ocular discharge, chemosis, lethargy, oral ulcerations, fever, etc. Cats who lacked clinical respiratory signs were classified as healthy. To decrease stress and the possibility of pain or discomfort, samples were obtained while anesthetized at the time of spay/neuter surgery, with the exception of any cats with URTD deemed too sick to undergo anesthesia. Sample collection occurred between November 2018 and March 2020. Because of the COVID-19 pandemic, further sampling was discontinued. For each cat, basic signalment was provided by the shelter. Age was estimated by the shelter and then categorized by life stage, in accordance with published guidelines (17). Because of the COVID-19 pandemic, further sampling was discontinued.

Samples submitted included oropharyngeal, nasal and conjunctival swabs and fecal material for each cat. To obtain conjunctival swabs, the lower eyelid was gently pulled to expose the conjunctival sac before the tip of the swab was inserted. Both eyes were swabbed and then swabs were placed in a sterile tube and wetted with a few drops of sterile saline or water to prevent drying during transport. Oropharyngeal swabs were obtained from the back of the throat, in proximity to the tonsils, utilizing 2 swabs. Nasal samples were either obtained through insertion into the nares or by swabbing discharge from the nares. Fecal material was collected from the cats' litterboxes. All samples were stored at 4° C and shipped on ice to the laboratory for further processing.

Upon delivery to the laboratory, approximately 2mL of sterile DMEM (no additives) per swab was added to the tube containing respiratory swabs from each individual site. Each tube was vortexed for 15 seconds and incubated for 30 minutes at 4°C before being aliquoted. All aliquots were stored at -20°C until submission for further testing. A total of 400mg of feces were diluted in 1.2mL of sterile phosphate buffered saline (PBS) and vortexed for 15 seconds. Fecal samples were incubated for at least 2 hours, up to overnight, at 4°C. Diluted fecal samples were diluted a second time, then centrifuged at 18,000 x g for 10 minutes and the supernatant was drawn off. Samples were stored at -20°C.

Respiratory pathogen detection

Respiratory pathogen testing was performed through the Cornell Animal Health Diagnostic Center (NYS AHDC), utilizing their diagnostic feline respiratory panel. Specifically, FHV-1,

FCV, and panleukopenia were detected through viral isolation, and *Bordetella, Chlamydia,* influenza, *Mycoplasma cynos, Mycoplasma felis,* pneumovirus and *Streptococcus zooepidemicus* by PCR. Results of the PCR tests were reported as not detected, low-, moderate-, or high-positive, but in this report were coded as "positive" or "not detected," for the purposes of data analysis.

FCoV detection

RNA extraction was performed using either MAX Express (Life Technologies, Grand Island, NY, USA) or manually E. Z. N. A.® Viral RNA Kit (Omega Bio Tek, Norcross, GA). Quantitative RT-PCR was performed using Ultraplex 1-Step Tough Mix 4X (Quantabio, Beverly, MA, USA) with primers and probe previously published by Dye and colleagues (Dye et al., 2008). Each 25 µl reaction contained 8 µl of extracted sample, 8.1 µl nuclease free water, 6.25 µl Ultraplex 1-Step Tough Mix, 1.13 µl P009 primer 10 µM, 1.13 µl P010 primer 10 µM, and 0.38 µl P9/10P probe. FIPV-TN406 was serially diluted 1:10 and used as a standard. Quantitative RT-PCR conditions were 50^{o} C for 20 minutes, 95 ^{o}C for 3 minutes, followed by 40 cycles of 95 ^{o}C for 10 seconds then 30 seconds at 60 ^{o}C, and a final hold at 4 ^{o}C. A sample was considered positive if a properly shaped amplification curve was produced and Ct value was determined.

Demographics and data analysis

A total of 39 cats were enrolled in this study. Breed of cat was determined by the shelter, and grouped together as either domestic shorthair (DSH) or other/unknown, which included domestic medium hair (DMH), domestic long hair (DLH), and a Maine-Coon Mix. Sex was designated as

male or female. Age was further categorized by life stage, in accordance with published guidelines (Vogt et al., 2010). Univariate analysis of continuous and ordinal variables (age, categorical age, and weight) were evaluated using logistic regression. For categorical demographic variables (shelter location, sex, and breed), association with the disease outcome was evaluated via Fisher's exact test using a two-sided alternative.

Ethical approval

All procedures were approved by the Cornell IACUC #2012-0116. Both animal shelters were made aware of the risks and benefits of this study, willingly participated, and gave permission to publish the results of their tested samples.

2.3 Results

Population demographics

A total of 39 cats were enrolled, of which 20 were classified as having upper respiratory disease and 19 as healthy (table 2.1). Cats with upper respiratory disease tended to be younger individuals ($p < 0.05$), with 75% kittens, compared to the healthy group. Unsurprisingly, weight was correlated with age ($\rho = 0.72$, p-value < 0.05) and cats with URTD tended to weigh less, compared to healthy cats ($p < 0.05$). Individual shelter was also associated with disease status ($p < 0.05$). Sex and breed were not associated with disease outcome.

Table 2.1: Basic characteristics of cats with and without upper respiratory disease. IQR is interquartile range. † p- value <0.05

	URTD cats (n =20)	Healthy (n = 19)
Sex		
Female –count (%)	9 (45)	10 (53)
Male – count (%)	11 (55)	9 (47)
Median age in months (IQR) †	3.12 (4.53)	24 (14.52)
Categorical age†		
Kitten (up to 6 months) – count (%)	15 (75)	1 (5)
Junior (7 months-2 years) - count (%)	4 (20)	14 (74)
Prime (3-6 years) - count (%)	1 (5)	3 (16)
Mature (7-10years) - count (%)	0 (0)	1 (5)
Shelter†		
A - count (%)	11 (55)	3 (16)
B - count (%)	9 (45)	16 (84)
Breed		
DSH - count (%)	16 (80)	15 (79)
Other/unknown (%)	4 (20)	4 (21)
Median weight in lbs (IQR)†	1.56 (0.94)	3.63 (1.43)

Pathogen detection

Of the 11 pathogens on the diagnostic panel, 5 pathogens were detected: *Bordetella,* FCV, *M. felis,* panleukopenia, and pneumovirus (table 2.2). In cats with URTD, the mean number of pathogens identified from respiratory swabs was 1.45 (SD: 1.2), with a median of 1. Fifteen cats with URTD had detectable pathogens, while nine cats were positive for >2 pathogens. The highest number of pathogens detected in a single cat with URTD was four. In healthy cats, the mean number of pathogens detected in respiratory samples was 0.58 (SD: 0.7), with a median of 0. One or two pathogens were detected in nine of the healthy cats.

FCV was significantly associated with respiratory disease status (p=0.001, odds ratio (OR): 20.27, 95% confidence interval for OR: 2.32-991.89). Pneumovirus was identified in two cats that were classified as having upper respiratory disease; however, the association was not statistically significant (p=0.49). No other pathogens detected in respiratory swabs were significantly associated with cats with upper respiratory disease. While *Bordetella* was not significantly associated with respiratory disease status (p=0.13), it was associated with shelter location (p=0.003), with 7 of 9 positive *Bordetella* samples coming from the same shelter.

Table 2.2: Specific pathogen detection in respiratory swabs. Pathogens were detected via PCR, with the exception of FHV-1, panleukopenia and FCV, which were investigated via viral isolation. *† p <0.05,* significant association with the outcome was assessed via two-sided Fisher's Exact test).

	URTD cats: n = 20 Count (%)	**Healthy cats: n = 19 Count (%)**
Bordetella	7 (35)	2 (11)
Feline calicivirus†	11 (55)	1 (5)
Chlamydia	0 (0)	0 (0)
Feline coronavirus	0 (0)	1 (5)
Feline herpesvirus type 1	0 (0)	0 (0)
Influenza virus	0 (0)	0 (0)
Mycoplasma cynos	0 (0)	0 (0)
Mycoplasma felis	8 (40)	7 (37)
Panleukopenia	1 (5)	0 (0)
Pneumovirus	2 (10)	0 (0)
Streptococcus zooepidemicus	0 (0)	0 (0)

Two male kittens with URTD had detectable pneumovirus in both nasal and oropharynx samples. Both pneumovirus-positive kittens were additionally positive for FCV and *Bordetella*. One kitten was also positive for *Mycoplasma felis* and was shedding FCoV in the feces. Available radiographs from one of the pneumovirus positive kittens were described as having a

diffuse interstitial/bronchiolar pattern. A low level of FCoV antigen was identified in a pooled respiratory sample from a single 10-week-old, male, DSH kitten in the healthy group. On intake to the shelter, the kitten was bright, alert, responsive, and euhydrated with no ocular or nasal discharge. Physical examination was unremarkable, except for the presence of fleas/flea dirt. Post-neutering, on day three of shelter intake, vomiting and diarrhea were noted

FCoV detection in feces and potentiation of respiratory pathogens

The shedding of FCoV in fecal samples was assessed across respiratory disease status to determine whether an association existed with clinical upper respiratory disease (table 2.3). FCoV was identified in 30% of fecal samples from URTD cats and 16% of healthy cats. This relationship was not significant (p=0.5).

Table 2.3: The count and percent of fecal samples that were positive for FCoV via qRT-PCR in cats with and without URTD. Significance of association was assessed via a two-side Fisher's exact test (p-value: 0.5)

	URTD: n = 20 Count (%)	**Healthy: n = 19 Count (%)**
Feline coronavirus	6 (30)	3 (16)

2.4 Discussion

In this study, five pathogens were detected in feline respiratory samples from cats with URTD. FCV was the only pathogen significantly associated with URTD in cats. Among cats with URTD, 55% of cats were positive for FCV, compared to only one (5%) of the healthy cats.

Detection of FCV was performed via viral isolation, supporting the pathogenic role. The healthy animal that was positive for FCV may have been in an early stage of infection, but was not further followed for the purposes of this study. *Bordetella* was more common in one of the shelters, despite neither shelter vaccinating cats against the pathogen. The prevalence of *Bordetella* in shelters is thought to be variable, associated with the presence of dogs, and in this study, associated with living at one shelter. Persistent shedding of *B. bronchiseptica* from cats has been experimentally observed in kittens (Coutts et al., 1996). Here, 2 of 18 healthy cats were *Bordetella* positive. The potential for asymptomatic *Bordetella* cases to introduce the bacteria into shelters remains a possibility and should be considered when designing shelter biosecurity protocols. *Mycoplasma felis* was commonly found across both disease statuses in this study and across both shelters. The role of *M. felis* in URTD has been debated, though accumulating evidence has supported an association with disease, especially conjunctivitis (Lee-Fowler, 2014). In this study, we also detected pneumovirus in two cats with clinical upper respiratory disease. Both of the affected cats, however, were also positive for *Bordetella* and FCV, which could account for the clinical signs. Since 2010, canine pneumovirus has been identified across several canine populations and associated with respiratory disease outbreaks in dogs (Mitchell et al., 2013, 2017; Piewbang & Techangamsuwan, 2019; Renshaw et al., 2010). Pneumovirus has been isolated from other cats with respiratory disease and passage of a feline virus in Balb/C mice has resulted in lung pathology and cytokine changes (Glineur et al., 2013). More research, however, is needed in regards to pneumovirus in cats and whether, like *Bordetella*, it is spread from dogs to cats.

Surprisingly, in this study, we did not detect feline herpesvirus type 1 (FHV-1). The small sample size, inclusion of only two shelters, and population constrained by those needing spay/neuter likely contributed to this observation. FHV-1 is commonly detected across feline populations and across the anatomical sites utilized in this study (Litster et al., 2015). The utilization of viral isolation, compared to PCR may have accounted for the lack of herpesvirus detection (Schulz et al., 2015); in another surveillance study in two animal shelters, viral culture for FHV-1 was also frequently negative (Foley et al., 2002). Viral isolation is highly sensitive (Burgesser et al., 1999) and at the time of investigation was the primary assay offered through the NYS AHDC as part of their feline respiratory panel. While PCR is highly sensitive, a concern is detection of DNA from recent vaccination (Maggs & Clarke, 2005). Though unlikely, sampling technique or time between sample collection to viral isolation could have both impacted the ability to detect herpesvirus via viral isolation. It is also possible that time between shelter intake and sampling was short enough that cat stress from shelter entry had not yet contributed to herpes recrudescence, or, less likely, that the cats in this young population had not yet been exposed to FHV-1. Litster and colleagues have previously demonstrated low prevalence of *Chlamydia felis* in shelters (Litster et al., 2015), though in cats with conjunctivitis, the PCR prevalence has been over 50% (Hartmann et al., 2010). Likewise, *Chlamydia* was not observed in this study. *Mycoplasma cynos* has previously been identified in one cat with conjunctivitis, though more research is required to understand its pathogenic potential in cats (Hartmann et al., 2010) and it is not surprising to not find the pathogen in this study. By comparison *Mycoplasma cynos* has been identified in samples from dogs with respiratory disease (Decaro et al., 2016; Jambhekar et al., 2019). Influenza virus remains a relatively rare occurrence in cats and has primarily been associated with outbreak situations or spillover events and was not expected to be

found in this study (Borland et al., 2020; Harder & Vahlenkamp, 2010; Hatta et al., 2018). Lastly, *Streptococcus equi* sbsp. *zooepidemicus* was first recognized as causing disease in cats in 2010, and is associated with respiratory signs in addition to pharyngeal and meningeal disease (Blum et al., 2010), and unsurprisingly, was not identified in this study.

Our lab previously reported on the presence of FCoV antigen in the respiratory passages of a cat who died of FIP (André et al., 2020). Historically, while FCoV has most been associated with enteric disease and FIP, the potential for upper respiratory disease has been recognized (Saif, 2004; Stoddart et al., 1988; Wolfe & Griesemer, 1966). Although this study did not demonstrate FCoV in any symptomatic cats, we did observe FCoV in respiratory sampling in a kitten considered healthy at the time of sampling. While this kitten developed gastrointestinal signs common of FCoV, the preclinical identification of FCoV in respiratory swabs may indicate an early entry point for the virus across the respiratory epithelium. Long-term follow up on the single kitten that was positive for FCoV in respiratory samples was not available; more specifically, whether it developed FIP. Furthermore, though not significant, 30% of URTD cats were shedding FCoV, compared to 16% of healthy cats in this small study.

The small sample size and convenience sampling are both limitations of this study and the results here may not be representative of shelter cat populations more broadly. In regards to FCoV, based on the estimates here, a sample size of approximately 140 cases and 140 controls would be needed to detect whether a statistically significant difference exists in regards to FCoV fecal shedding and upper respiratory disease status. In part because of the COVID-19 pandemic, the decision was made to discontinue sampling. In this study, younger cats were significantly more

likely to be clinical, test positive for respiratory pathogens, and test positive for FCoV. Age is frequently a contributing or confounding factor in studies of infectious disease. Older cats in this study may have acquired immunity against the investigated pathogens, either through previous infection or vaccination, but exposure history was not available for such evaluation (and rarely is). Furthermore, environments in which a young cat is at risk for acquiring respiratory disease may be the same environments in which they are at risk for acquiring GI disease. The population of cats sampled in this study was constrained by our humane protocols which encouraged sampling at the time of spay/neuter surgery. This resulted in both the healthy and sick populations being younger than the general population of cats in these shelters and as a whole. Future studies of these pathogens may increase their yield by targeting younger cats; prevalence studies should strive to sample more evenly across cat age groups.

Finally, only two shelters participated in this study, and shelter location was a risk factor for the types of respiratory disease detected. Cats sampled in this study were selected by veterinarians and staff at each shelter at their convenience, not at random, and not blindly. Both of these shelters have staff veterinarians, which means they are potentially able to care for animals that are sicker than shelters without veterinarians on staff.

2.5 Conclusions

URTD remains a challenging disease complex to manage, especially in densely housed populations of cats. In this pilot study, we identified two symptomatic cats that were shedding pneumovirus. These cats were also positive for other pathogens, so it remains inconclusive

whether pneumovirus contributed to their respiratory disease. Respiratory samples from a single cat without respiratory disease symptoms were positive for FCoV; following sampling, this cat proceeded to develop gastrointestinal signs of disease. This may have been due to FCoV infection. While we did not detect a statistically significant association between fecal shedding of FCoV and respiratory disease, the small sample size limited the power of this study.

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2.7 References

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CHAPTER III

SARS-CoV-2 SEROSURVEY OF HEALTHY, OWNED CATS PRESENTING TO A NEW YORK CITY ANIMAL HOSPITAL

3.1 Introduction

SARS-CoV-2, the causative agent of the COVID-19 pandemic is a recently-emerged *Betacoronavirus* that is associated with significant morbidity and mortality in human patients (Wu et al., 2020; Zhou et al., 2020). While SARS-CoV-2 primarily poses a threat to humans, natural infections in domestic animals have been described, including in domestic cats, non-domestic felids, dogs, and farm-raised American mink (McAloose et al., 2020; Munnink et al., 2020; Newman et al., 2020; Oreshkova et al., 2020; Sit et al., 2020; Wu et al., 2020; Zhou et al., 2020).

Natural and experimental infections have been reported in several cats, zoo-housed lions and tigers, mink, and ferrets (Halfmann et al., 2020; Munnink et al., 2020; Newman et al., 2020; Oreshkova et al., 2020; Shi et al., 2020; Wang et al., 2020). Experimental infections have shown cats to be a robust infection model for SARS-CoV-2, more so than experimentally infected dogs (Bosco-Lauth et al., 2020; Shi et al., 2020). The rapid movement of SARS-CoV-2 across the globe has prompted surveillance for the virus in numerous species to better understand transmission and associated risk. In outbreaks of SARS-CoV-2 on mink farms, both zoonotic and reverse zoonotic transmission has been reported (Munnink et al., 2020). As of March 2021, transmission from feline species to humans has not been observed and remains unlikely to drive significant transmission. However, reverse zoonotic transmission remains a concern in owned animals, zoo-housed animals, and other group-housed animals, including cats in shelter environments or those being boarded for other purposes.

The natural spread of SARS-CoV-2 infection from human to animal has been demonstrated in numerous case reports, finding either the virus/viral antigen or seroconversion (Garigliany et al.,

2020; Newman et al., 2020; Ruiz□Arrondo et al., 2020; Sailleau et al., 2020; Temmam et al., 2020; Zhang et al., 2020). In a cohort of cats residing in Wuhan, China, the initial epicenter of the COVID-19 pandemic, a serological response against SARS-CoV-2 was demonstrated in approximately 15% of cats owned by COVID-19 patients, presenting to a pet hospital, or enrolled as stray cats (Zhang et al., 2020). While Zhang and colleagues did not provide clinical histories to the cats enrolled in this study, making it challenging to extrapolate their results to the general feline population, this early data piece made indicated that cats could mount an immune response against the virus. Interestingly, only a subset of those cats with a positive titer had a documented exposure to a human patient, and serum from several cats classified as stays was able to neutralize virus (Zhang et al., 2020). By comparison, neither SARS-CoV-2 antigen nor antibody were detectable in a small sample of 9 cats and 21 dogs residing in France, despite 2 of the owners testing positive (Temmam et al., 2020). The lack of viral detection, however, may have been confounded by time of testing, social differences in cat-human-interactions, etc. However, even in larger samples of animals, including over 900 dogs and cats in Italy, PCR was unable to identify positive animal cases, despite a small percentage (~6%) of cats seroconverting (Patterson et al., 2020). Nonetheless, in a separate French study of 22 cats, 1 animal that belonged to a confirmed COVID-19 case, was positive for the virus via rectal swab and showed respiratory and gastrointestinal signs consistent with infection (Sailleau et al., 2020). In two cats in New York, specifically Nassau and Orange counties, infection with SARS-CoV-2 was associated with respiratory signs, as well as non-specific lethargy (Newman et al., 2020). A cat in Belgium residing in a household of a COVID-19 patient, also tested positive for SARS-CoV-2 and demonstrated gastrointestinal signs, respiratory signs and lethargy (Garigliany et al., 2020). This cat seroconverted and recovered without further complications (Garigliany et al., 2020). A

cat in Spain was also infected with SARS-CoV-2 and following euthanasia, necropsy revealed the cat to have hypertrophic cardiomyopathy (HCM) (Segalés et al., 2020). However, it is unclear whether SARS-CoV-2 infection was the inciting cause of the HCM or whether it was not previously detected. Most recently, there has been some evidence that a SARS-CoV-2 variant of concern (VOC), the British variant B.1.1.7 may be able to cause myocarditis in domestic cats (Ferasin et al., 2021). Despite this, other cats that have been considered to be naturally infected by owners have remained free of clinical disease (Barrs et al., 2020) and in one cat whose owner developed severe COVID-19, the cat remained asymptomatic, despite numerous underlying chronic inflammatory conditions and a positive qRT-PCR test of an oropharyngeal swab (Ruiz Arrondo et al., 2020). Natural infections in non-domestic felids have been observed and have been associated with upper respiratory signs (Wang et al., 2020).

In a small sample of laboratory housed cats, asymptomatic infection and transmission among cats has been demonstrated (Halfmann et al., 2020). Molecular analysis has shown identical viruses to transfer from humans to cats, but it remains challenging to know whether cats naturally pass virus between other feline housemates (Neira et al., 2021). In experimentally infected cats, viral RNA has been detected across both respiratory tissue and the small intestine after inoculation (Shi et al., 2020). Additionally, necropsy of experimentally infected cats has confirmed lesions in respiratory tissues (Gaudreault et al., 2020). Seroconversion is evident in cats, despite not showing clinical signs (Bosco-Lauth et al., 2020; Gaudreault et al., 2020).

In addition to susceptibility to SARS-CoV-2, cats are frequently infected with feline coronavirus (Kummrow et al., 2005; Pedersen, 2009). While FCoV is most often associated with mild to

inapparent gastrointestinal disease, a small proportion of cats succumb to the invariably fatal disease, feline infectious peritonitis (FIP) (Pedersen et al., 1981). Like COVID-19 in human patients, FIP is associated with systemic vasculitis (August, 1984; Gupta et al., 2020). Across feline populations, a majority of cats are serologically positive against FCoV (Kummrow et al., 2005; Zhao et al., 2019). In one of the first studies of FCoV seroprevalence between 9-60% of cats were seropositive depending on geographic region (Horzinek & Osterhaus, 1979). Interestingly, sera from 49 Australian feral cats were all considered negative for FCoV, utilizing the Immunocomb test, an in house assay that detects IgG against FCoV (Bell et al., 2006). Sera from cats in the Netherlands have also demonstrated reactivity against other coronaviruses, including other human and swine coronaviruses (Zhao et al., 2019). Unsurprisingly, infection and seroconversion by SARS-CoV has previously been demonstrated in cats (Martina et al., 2003). Lung pathology in cats following SARS-CoV has been evident and associated with ACE2-expression, the SARS-CoV receptor (Brand et al., 2008). Human and feline ACE2 share a high identity, potentially driving the ability for interspecies transmission of SARS-CoV and SARS-CoV-2 (Stout et al., 2020). During the SARS-CoV outbreak in Hong Kong, the Amoy Garden Complex apartments offered a unique case study of cats becoming infected, including the environmental sources of the virus (Lun & Qu, 2004).

Here, we present serological data from cats presenting to a small animal veterinary clinic in the Upper East Side of New York City (NYC), NY, USA. New York City was an early epicenter of COVID-19 in the United States (Arnold, 2020) with cases identified in Manhattan, Bronx, Queens, and Brooklyn by early March of 2020 (Gonzalez-Reiche et al., 2020). In similar time frames across human populations in New York City, mounting seroprevalence was observed

(Stadlbauer et al., 2020) and in obstetric patients in NYC tested for SARS-CoV-2, 15.4% of 215 women participating in screening were positive for the virus, with only 4 women having symptoms of infection (Sutton et al., 2020). Home to a large population of both people and pets, understanding previous SARS-CoV-2 infection in cats is essential for public health surveillance and cats may help act as sentinel species. Though the role of cats in furthering human transmission is considered small, veterinarians and pet owners must be aware of potential SARS-CoV-2 infection in cats, as an animal health risk and public health surveillance.

3.2 Materials and Methods

Participant recruitment and sampling

Feline participants from NYC presenting to a private practitioner veterinarian were enrolled between June and January of 2021. From each cat, approximately 2mL of whole blood was collected and allowed to clot. Serum was removed, stored at 4^{o}C, and sent to the Cornell Animal Health Diagnostic Center (NYS AHDC) on ice pack. Sampling from cats was approved by the Cornell IACUC 2012-0116 and all sampling was performed by licensed veterinary professionals, employed by the collaborating veterinary practice. Basic signalment was collected from each cat.

Multiplex test against SARS-CoV-2 epitopes

Serological status was evaluated using a proprietary, multiplex assay, developed through the Cornell Animal Health Diagnostic Lab, Serology Section. The assay evaluates antibody reactivity against three SARS-CoV-2 proteins or protein domains (N, the receptor-binding domain (RBD) of S, and S1 subunit). Based on validation with human serum, reactivity against

N or RBD is considered positive, while reactivity against S1 is considered cross-reactivity and not specific for previous SARS-CoV-2 exposure.

Viral neutralization

Vero E6 cells, obtained from the American Type Culture Collection (ATCC), were seeded 24 hours prior to infection, under BSL-2 conditions, at a density of $2x10^4$ cells/well of a flat-bottom 96-well plate in 100uL of Dulbecco's modified Eagle medium (DMEM) (Cellgro) supplemented with 25mM HEPES (Cellgro) and 10% HyClone FetalCloneII (GE) at 37ºC and 5% CO_2. For neutralization assays, under BSL-3 conditions, each heat inactivated sample was 4-fold serially diluted in Eagle's Minimum Essential Medium (EMEM) (Cellgro) supplemented with 4% heat inactivated fetal bovine serum (FBS) (Gibco) and mixed with an equal volume of SARS-CoV-2 isolate USA-WA1/2020 from the Biological and Emerging Infections Resources Program (BEI Resources) for a final virus concentration of 100 $TCID_{50}$. Dilutions were performed in triplicate. Following a one-hour incubation at 37ºC, media was removed from the Vero E6 cells and replaced with the serum/virus mixture. Plates were incubated for 72 hours at 37ºC and 5% CO_2. After incubation, serum/virus was removed and plates were fixed with 5% paraformaldehyde for 15 minutes and then stained with crystal violet for 10 minutes. The plates were washed with water and then allowed to dry. The virus neutralization titer (VNT) was determined as the highest dilution in which two or three of the wells were not-infected, based on visualization of the crystal violet staining. Samples were considered negative if there was no obvious viral neutralization at the 1:4 dilution.

FCoV Serology

FCoV serology was performed through the NYS Animal Health Diagnostic Center, utilizing a commercially available ELISA for antibody detection, reported as a signal to noise (S/N) ratio and then classified as positive, negative, or inconclusive.

Statistical analysis

Statistical analysis was performed in GraphPad Prism 9 (San Diego, CA). A p-value of 0.05 was considered significant.

3.3 Results

SARS-CoV-2 serology, FCoV serology, and SARS-CoV-2 viral neutralization was performed on a total of 15 cats. Of these cats, five cats had evidence of viral neutralization, against SARS-CoV-2 with VNT ranging from 256 to 4096, the reciprocal of the highest dilution for which the majority of replicates were still negative for infections. Comparing viral neutralization with the three epitopes evaluated via multiplex, the RBD epitope response was significantly different ($p=0.0013$) across samples that were considered to have a positive VNT compared to those which were negative (figure 3.1). The N epitope response was frequently higher in samples that were positive via VNT, but this relationship was not statistically significant ($p=0.0992$). VNT results were not predictive of the S1 epitope multiplex results, as was previously observed with human serum during test development.

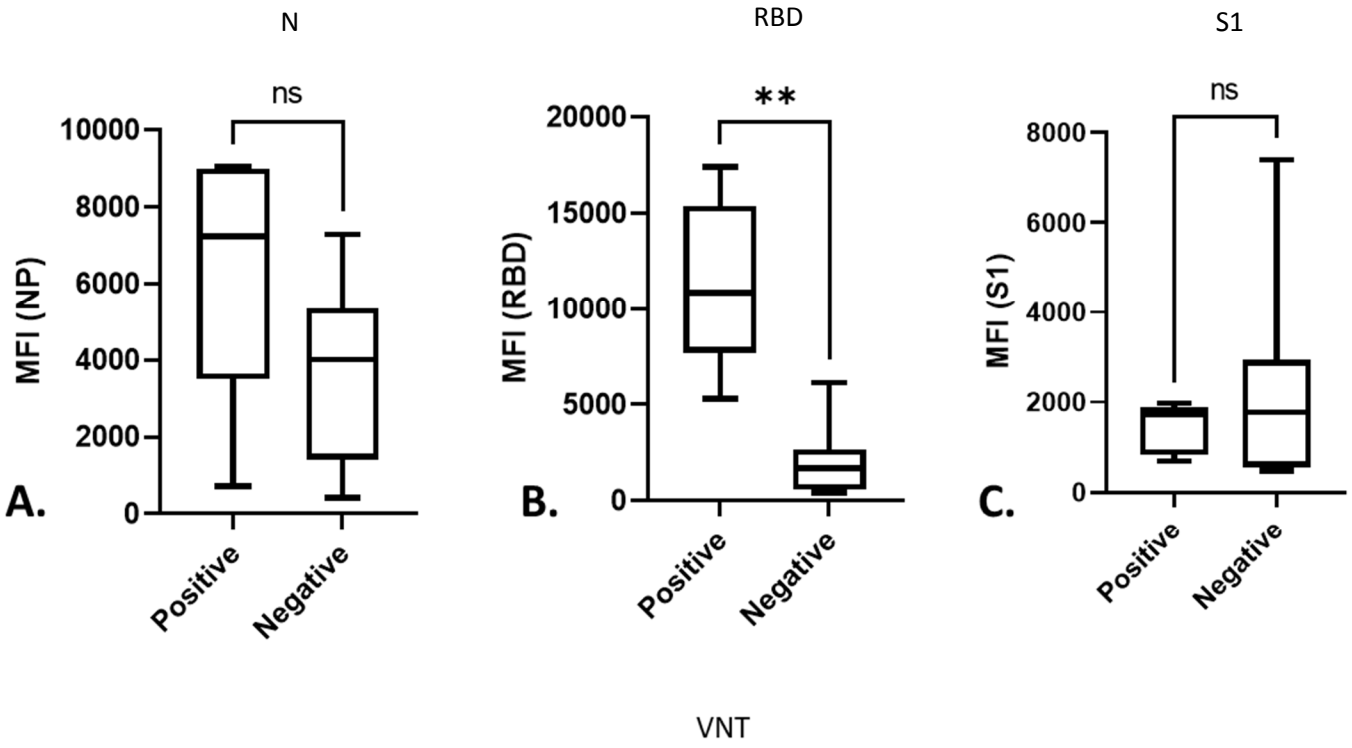

Figure 3.1: Feline SARS-CoV-2 antibody response, evaluated via multiplex against three viral epitopes compared to viral neutralization. N is antibody against nucleocapsid. RBD is antibody specifically against the receptor binding domain of the spike protein and S1 is a subunit of the spike protein. Five cats were initially confirmed to have viral neutralizing antibodies, while 10 cats had neutralizing titers under 1:4 and considered negative. A) Cat samples with viral neutralizing antibodies did not show a statistically significant difference when evaluating the NP epitope. B) Cat samples with viral neutralizing antibody responses had higher levels of antibodies against the RBD (p-value of 0.0013), based on a Mann-Whitney test. C) The S1 response tended to be low and there was no difference across cats with and without neutralizing titers.

Next, the results of viral neutralization and the multiplex assay were compared against the FCoV status of each cat. Across the three epitopes, NP antibody levels tended to be higher in cats classified as FCoV (p=0.028) (figure 3.2). Neither of the other two SARS-CoV-2 antibody levels were associated with FCoV status. Lastly, we compared viral neutralization with the FCoV S/N ratio. Cats with neutralizing antibody against SARS-CoV-2 had lower levels of S/N ratios against FCoV (figure 3.3).

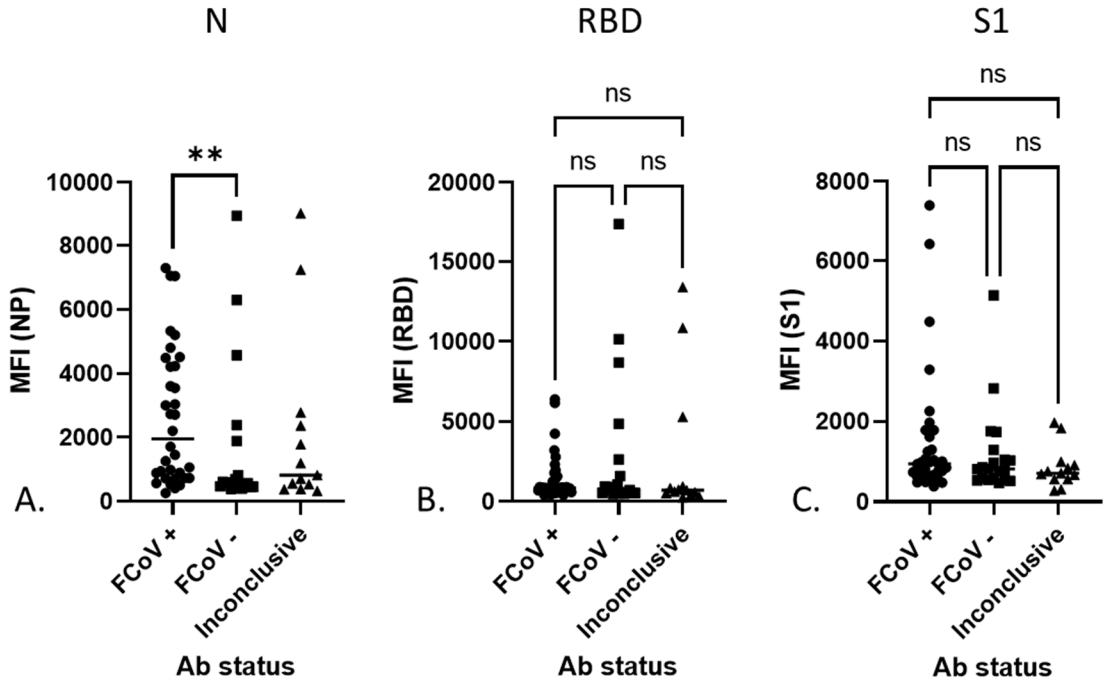

Figure 3.2: Concurrent serological testing for feline coronavirus (FCoV) and SARS-CoV-2 demonstrates that classification as FCoV positive is associated with higher levels of antibodies against the nucleocapsid (N) protein of SARS-CoV-2 compared to the receptor binding domain (RBD) or S1 subunit of the spike protein. A) Cat samples considered positive for FCoV had higher antibody levels specifically against NP (p-value of 0.0178), based on the Kruskal-Wallis test followed by Dunn's multiple comparisons test. B) FCoV serological status was not associated with differences in the response against RBD. C) FCoV serological status was not associated with differences in the response against S1.

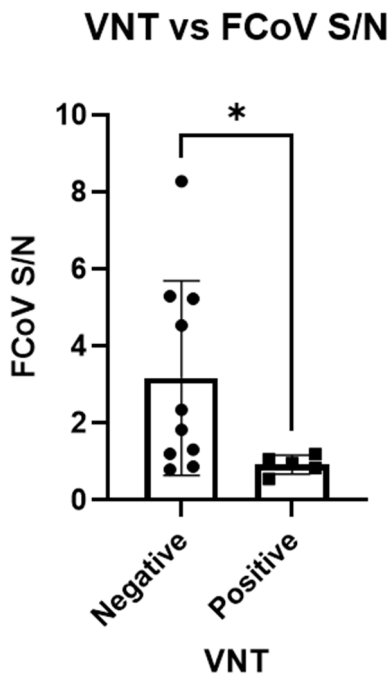

Figure 3.3: Cat samples that lacked SARS-CoV-2 viral neutralizing ability tend to have higher levels of FCoV antibody. Samples were classified as to whether SARS-CoV-2 virus neutralization was observed. Those samples which had notable titers how lower FCoV antibody levels. S/N is the signal to noise ratio.

3.4 Discussion

While our study did not specifically investigate viral shedding, it is evident that cats can be infected with SARS-CoV-2 and mount robust immune responses against the virus. In this regard, cats may be useful sentinel species for understanding SARS-CoV-2 spread in human populations. Virus neutralization was observed in five cats tested here, indicating a specific response against the virus and not because of cross-reactivity. Cat S109, for example, showed high responses against all three viral domains and was negative when tested via viral neutralization. This is not unexpected and indicates cross-reactivity and not seroconversion against SARS-CoV-2. In this regard, the elevated response against S1 is a useful control with the multiplex assay. Another cat, Cat S155, had a robust response against NP, however, no virus

neutralization was observed. This may have been due to timing of sampling, including whether this was early in the course of infection. Nonetheless, correlates of immune protection can be challenging to quantify. Although it is unknown whether these cats could naturally be reinfected and develop clinical disease, studies in non-human primates with SARS-CoV-2 have shown that reinfection did not result in clinical disease and a study with SARS-CoV-2 experimentally-infected cats did not result in disease (Bao et al., 2020; Bosco-Lauth et al., 2020). In relation to FCoV and the development of FIP, antibody-dependent enhancement (ADE) has been long considered an important player in disease pathophysiology, whereby non-neutralizing antibodies enhance virus uptake into the monocyte/macrophage and drive systemic virus dissemination (Hohdatsu et al., 1998). The long-term consequences of SARS-CoV-2 infection in cats remains unknown, including whether infection could result in FIP-like disease. To date, no cases of FIP-like disease have been reported in any of the case reports of SARS-CoV-2-infected cats. This could be due to lack of testing. Alternatively, it has been suggested in humans that prior exposure to other human coronaviruses may provide protection against SARS-CoV-2 (Ma et al., 2020), despite little evidence supporting this (Loos et al., 2020; Yang et al., 2020). All of the samples tested here were obtained after the onset of the pandemic. FCoV and SARS-CoV-2 serology was performed at the same time, so it is challenging to conclude if prior FCoV exposure may be protective in cats. However, other serological studies have not supported cross-reactivity between SARS-CoV-2 and FCoV (Zhang et al., 2020).

To date, there have not been any cases of SARS-CoV-2 observed in cats living in a shelter or other colony-type environment. While the risk of cats in these scenarios was not evaluated here, it remains prudent for shelter workers to continue practicing biosecurity practices and to

encourage visitors to do the same. While the behaviors that a pet owner engage in with their own cat may be different than that of group-housed cats (for example, sharing a bed), the movement of SARS-CoV-2 into animal populations is possible, as demonstrated both by the ongoing outbreaks of the virus in mink farms and cases in non-domestic felids at several zoos. Transmission studies have indicated that cats can spread the virus between themselves (Bosco-Lauth et al., 2020; Halfmann et al., 2020). Further, while the cats in this study were nearly all indoor housed, another concern may be the ability of cats that enjoy an indoor and outdoor lifestyle to spread the virus to wildlife species. By comparison, though rare, exposure and disease development from FCoV have been reported in wildlife species, including lions, cheetahs, and mountain lions (Foley et al., 2013; Kennedy et al., 2003; Stephenson et al., 2013). While it remains unknown how these animals were exposed, fecal spread, direct contact, and predation remain possibilities and it is feasible SARS-CoV-2 could spread from domestic to wild species, following a pattern similar to FCoV. In zoo-housed, non-domestic felids, respiratory signs have been documented following SARS-CoV-2 infection (Wang et al., 2020). It remains unknown across zoo populations if SARS-CoV-2 infection is sporadic or whether asymptomatic infection occurs. The World Organisation for Animal Health (OIE), as of March 22, 2021 lists numerous cases of SARS-CoV-2 in non-domestic felids, including tigers, lions, a snow leopard, and pumas (OIE, 2021). Further seroprevalence studies across zoo facilities would provide further clarification in addition to ensuring conservation goals are still being met at such institutions.

Cats are regularly exposed to SARS-CoV-2 and seroconvert, even in the absence of clinical signs. The role of cats in transmitting the virus to date is minor, however, SARS-CoV-2 infection

from a One Health perspective and comparative medicine paradigm may help provide for further insight into human pathogenesis, vaccine studies, and disease ecology.

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CHAPTER IV

ANALYSIS OF PREDICTED FURIN CLEAVAGE SITES IN THE SPIKE PROTEINS OF BAT AND RODENT CORONAVIRUSES: IMPLICATIONS FOR VIRUS EVOLUTION AND ZOONOTIC TRANSFER FROM RODENT SPECIES

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4.1 Introduction

The *Coronaviridae* are single-stranded, positive sense, enveloped RNA viruses (Masters & Perlman, 2013). They are classified, by genera, as *Alpha*-, *Beta*-, *Gamma*-, and *Deltacoronaviruses* (Payne, 2017). Current classification schemes for the *Coronaviridae* utilize the replicase domain of the ORF1ab gene; however, the spike protein, is a critical mediator of viral tropism and an important contributor to the virus's natural history and potential for spillover (Whittaker et al., 2018). The *Alpha*- and *Betacoronaviruses* are most often associated with mammalian species, while the *Gamma*- and *Deltacoronaviruses* are largely avian-associated (Woo et al., 2012). Across bat and rodent species, both *Alpha*- and *Betacoronaviruses* have been identified. Members of the *Coronaviridae* are further classified into subgenera. In bats specifically, these include *Setracovirus, Myotacovirus, Rhinacovirus, Colacovirus, Pedacovirus, Decacovirus, Minunacovirus, Duvinacovirus*, and *Nyctocovirus*, from the *Alphacoronavirus* genus, in addition to *Nobecovirus, Hibecovirus, Sarbecovirus*, and *Merbecovirus*, which are *Betacoronaviruses* (Wong et al., 2019) (table 4.1).

Table 4.1: Coronavirus subgenera associated with the Chiroptera and Rodentia orders.

	Order Chiroptera	**Order Rodentia**
α CoV	*Decacovirus*	*Luchacovirus*
	Duvinacovirus	
	Colacovirus	
	Setracovirus	
	Minunacovirus	
	Myotacovirus	
	Nyctocovirus	
	Pedacovirus	

	Rhinacovirus	
β CoV	*Nobecovirus*	*Embecovirus*
	Hibecovirus	
	Sarbecovirus	
	Merbecovirus	

Coronaviruses from rodents fall within two subgenera: the *Luchacoviruses* (within the *Alphacoronavirus* genus) and *Embecovirus* (within the *Betacoronavirus* genus) (Wang et al., 2015) (table 4.1). The rodent *Alphacoronaviruses* have previously been shown to form a monophyletic group (Tsoleridis et al., 2019). The *Embecovirus* subgenera, previously classified as lineage 2a, additionally includes human coronavirus HKU-1, several mammalian coronaviruses such as equine coronavirus and dromedary camel coronavirus HKU23 (Woo et al., 2014), but lacks any representation of bat-associated coronaviruses (Wong et al., 2019). With discovery of the China *Rattus* coronavirus (ChRCoV) HKU24, Lau and colleagues have provided evidence that rodent coronaviruses are the ancestors to the *Embecovirus* subgenus (Lau et al., 2015; Wong et al., 2019). While bats are frequently implicated as the source of new and emerging coronaviruses, rodents need also to be considered as a potential source of zoonotic spillovers, given previous observations. The natural ability of coronaviruses to recombine adds to the emergence and transmission of new viruses and there are many examples of recombination reported: the prototypic coronavirus mouse hepatitis virus (MHV), an *Embecovirus,* has been noted for its ability to recombine (Keck et al., 1988; Makino et al., 1986) and the dromedary camel coronavirus HKU23 appears to have had a recombinant history, based on similarity between portions of HKU23 and RodentCoV-IM2014 (So et al., 2019).

Globally, over 2000 rodent species have been described and encompass a wide range of habitats (Wilson & Reeder, 2005). Rodents have previously been implicated in transmitting numerous pathogens to humans, including hantavirus, Lassa fever, *Francisella tularensis*, and *Yersinia pestis*. In a study involving rodents destined for human consumption, coronaviruses were frequently identified (Huong et al., 2020). Among some of the rodents sampled, bat coronavirus 512/2005 and chicken infectious bronchitis virus (IBV) were identified; and although this may not be an indication of active infection, as the authors note, it does provide evidence of ongoing transmission opportunities across animal species (Huong et al., 2020). Two coronaviruses of human importance have been hypothesized to have rodent origins: HCoV-OC43 and HCoV-HKU1, with HCoV-OC43 emerging via a bovine intermediate (Cui et al., 2019). Surveillance efforts have recently identified OC-43, and sequences that cluster with OC-43, in a small number of free-living rodent species (McIver et al., 2020; Wang et al., 2020). In addition to zoonotic rodent transmission concerns, Yang and colleagues have hypothesized a potential role of rodents in the emergence of swine acute diarrhea syndrome (SADS-CoV) coronavirus (Yang et al., 2020). Other hypotheses regarding SADS-CoV suggest it may have origins with the bat coronavirus HKU2, which has previously been classified as a *Rhinacovirus*, genus Alphacoronavirus based on the replicase gene, despite the spike protein resembling that of *Betacoronaviridae* (Lau et al., 2007). A virus identified in *Apodemus chevrieri*, AcCoV-JC34 has been considered to have a unique spike protein compared with other alphacoronaviruses, being only 1126 residues, possessing predicted protease cleavage sites at S1/S2 (RRAR/AR) and S2' at residue 674 (R-S), and phylogenetically clustering with the bat coronavirus HKU-2 (Ge et al., 2017). Coronaviruses in rodents have been identified across several countries and from a

number of rodent host species, including *Bandicota savilei, Rattus exulans, Rattus tanezumi, Rattus norvegicus, Rattus argentiventer, Leopoldamys neilli, Rattus andamanensis, Rattus losea, Maxomys surifer, Mus cervicolor, Berylmys berdmorei, Mus caroli, Mus cookie, Niviventer fulvescens, Berylmys bowersi, Dremomys refigenis,* and *Menetes berdmorei* (McIver et al., 2020; Wu et al., 2021) (Table 4.2).

Table 4.2: Surveillance studies investigating coronavirus shedding across rodent species

Species	**Location**	**Time period**	**# positive/ Sample size**	**Ref**
Apodemus sp.	France	2014-2016	16/206	(Monchatre-Leroy et al., 2017)
Apodemus agrarius	China	2011-2013	10/444	(Wang et al., 2015)
	China	2014-2015	1/49	(Wang et al., 2020)
Apodemus chevrieri	China	2011	21/98	(Ge et al., 2017)
	China	2014-2015	24/193	(Wang et al., 2020)
Apodemus ilex	China	2011	1/17	(Ge et al., 2017)
Apodemus latronum	China	2014-2015	3/6	(Wang et al., 2020)
Apodemus sylvaticus	United Kingdom	Not given	0/9	(Tsoleridis et al., 2016)
Arvicola terrestris	France	2014-2016	0/35	(Monchatre-Leroy et al., 2017)
Bandicota indica	China	2014-2015	2/5	(Wang et al., 2020)
Deomys ferrugineus	Democratic Republic of Congo, Republic of Congo	2006-2018	1/1	(Kumakamba et al., 2020)
Eothenomys cachinus	China	2014-2015	1/1	(Wang et al., 2020)
Eothenmys fidelis	China	2011	1/62	(Ge et al., 2017)
Eothenomys miletus	China	2014-2015	3/131	(Wang et al., 2020)
Field rat (*Rattus* sp. and *Bandicota* sp.)	Viet Nam	2013-2014	239/702**	(Huong et al., 2020)

Hystrix sp.	Viet Nam	2013-2014	20/331***	(Huong et al., 2020)
Malacomys longipes	Democratic Republic of Congo, Republic of Congo	2006-2018	1/38	(Kumakamba et al., 2020)
Micromys minutus	China	2011-2013	0/2	(Wang et al., 2015)
Microtus sp.	France	2014-2016	0/9	(Monchatre-Leroy et al., 2017)
Microtus agrestis	United Kingdom	Not given	3/11	(Tsoleridis et al., 2016)
Microtus fortis	China	2011-2013	0/305	(Wang et al., 2015)
	China	2014-2015	0/10	(Wang et al., 2020)
Mus musculus	China	2011-2013	0/7	(Wang et al., 2015)
	United Kingdom	Not given	0/394 (Liver)	(Tsoleridis et al., 2016)
			0/58 (Gut)	
	China	2014-2015	0/1	(Wang et al., 2020)
Myodes glareolus	United Kingdom	Not given	1/1	(Tsoleridis et al., 2016)
	Poland	Not given	1/300	(Tsoleridis et al., 2016)
	France	2014-2016	5/80	(Monchatre-Leroy et al., 2017)
Niviventer confucianus	China	2011-2013	1/85	(Wang et al., 2015)
Niviventer eha	China	2014-2015	0/2	(Wang et al., 2020)
Niviventer fulvescens	China	2010-2012	0/97****	(Lau et al., 2015)
Niviventer niviventer	China	2014-2015	0/2	(Wang et al., 2020)
Rattus sp.	Viet Nam	2013-2014	1/1***	(Huong et al., 2020)
Rattus andamanensis	China	2010-2012	0/170****	(Lau et al., 2015)
	China	2014-2015	1/1	(Wang et al., 2020)
Rattus edwardsi	China	2011-2013	0/2	(Wang et al., 2015)
Rattus lossea	China	2011-2013	14/301	(Wang et al., 2015)
Rattus losea	China	2014-2015	0/15	(Wang et al., 2020)
Rattus fulvescens	China	2011-2013	0/4	(Wang et al., 2015)
Rattus nitidus	China	2014-2015	0/2	(Wang et al., 2020)
Rattus norvegicus	United Kingdom	Not given	4/95* (Liver)	(Tsoleridis et al., 2016)
	China	2010-2012	3/359****	(Lau et al., 2015)
	China	2011-2013	4/262	(Wang et al., 2015)
	China	2014-2015	2/101	(Wang et al., 2020)
Rattus tanezumi	China	2010-2012	0/9****	(Lau et al., 2015)
	China	2011-2013	1/53	(Wang et al., 2015)

	China	2014-2015	2/159	(Wang et al., 2020)
Rattus rattus	China	2010-2012	0/24****	(Lau et al., 2015)
Rattus rattus sladeni	China	2014-2015	0/18	(Wang et al., 2020)
Rhizomys sp.	Viet Nam	2013-2014	6/96***	(Huong et al., 2020)
Sciuridae sp.	Viet Nam	2013-2014	0/1	(Huong et al., 2020)

*Authors noted that some animals had both liver and gut samples positive (2).
**26 of the animals were co-infected; viruses identified were murine coronavirus and Longquan Aa coronavirus
***Virus identified were bat coronavirus 512/2005 or infectious bronchitis virus
****Specifically tested for HKU24 via RT-PCR

The spillover of coronaviruses from animals to humans has the potential for major global health implications. Following the 2002-2003 severe acute respiratory syndrome coronavirus (SARS-CoV) outbreak, surveillance studies began to recognize the role that bat species play as reservoirs for novel coronaviruses (Lau et al., 2005; Li et al., 2005). Ten years later, Middle East respiratory coronavirus (MERS-CoV) emerged in humans and was found to cluster phylogenetically with two bat viruses, *Tylonycteris* bat coronavirus HKU4 (BtCoV-HKU4) and *Pipistrellus* bat coronavirus HKU5 (BtCoV-HKU5) (van Boheemen et al., 2012) in the *Merbecovirus* clade (group 2c coronaviruses). Further investigation identified NeoCoV from South African *Neoromicia capensis* bats and suggested it as the sister group to the MERS-CoV-camel clade (Corman et al., 2014). MERS-CoV involved the dromedary camel as an intermediate host in the spillover to humans (Ferguson & Van Kerkhove, 2014; Haagmans et al., 2014; Memish et al., 2014). The most recent SARS-CoV-2 outbreak has been traced to bat origins, with a high similarity to a previously identified bat coronavirus RaTG13 (96% genome-wide sequence identity) (Zhou et al., 2020b), as well as several other bat coronaviruses, such as RpYN06 (94.5% sequence identity) (Zhou et al., 2021) and bat-SL-CoVZC45 and bat-SL-CoVZXC21 (Lu et al., 2020). The role of an intermediate host in the spread of SARS-CoV-2 to humans remains an open area of investigation. A major difference between SARS-CoV-2 and RaTG13 is the insertion of a

polybasic, furin cleavage site (P-R-R-A-R) between the S1/S2 regions of the spike protein of SARS-CoV-2 (Walls et al., 2020; Wang et al., 2020; Wrobel et al., 2020). RmYN02, identified in *R. malayanus* has 93.3% genomic nucleotide identity with SARS-CoV-2 and while the identity is only 71.9% (nucleotide sequence) in the spike protein, RmYN02 has a closely homologous sequence (P-A-A-R) at the S1/S2 cleavage site, but lacking basic amino acids needed for furin cleavage (Zhou et al., 2020). A major factor for understanding coronavirus tropism lies with the spike protein, consisting of S1 and S2 domains that carry out the receptor binding and membrane fusion functions of the virus, respectively (Masters & Perlman, 2013). SARS-CoV-2 and SARS-CoV both utilize ACE-2 as their receptor, while MERS-CoV utilizes DPP4 (Hoffmann et al., 2020; Li et al., 2003; Raj et al., 2013). Equally important to the presence of a usable receptor is the presence of a functional protease for S activation (Millet & Whittaker, 2015). Numerous proteases have been investigated across the *Coronaviridae*, including Transmembrane protease, serine 2 (TMPRSS2), which acts to activate SARS-CoV-2, as well as furin, trypsin, and cathepsins (Heald-Sargent & Gallagher, 2012). SARS-CoV-2 contains a furin cleavage site and loss of this site has been associated with virus attenuation (Johnson et al., 2021; Peacock et al., 2020). Comparatively, MERS-CoV is highly unusual in that it contains both an S1/S2 furin cleavage site and an S2' furin cleavage site (Millet & Whittaker, 2015). Among two closely related bat coronaviruses, BatCoV-HKU5 and BatCoV-HKU4, HKU5 is also cleaved by furin, though HKU4 may be more closely related to MERS-CoV and can utilize the same receptor, DPP4 (Lau et al., 2013; Yang et al., 2014).

Furin is a ubiquitously expressed serine protease that functions across numerous physiological processes (Braun & Sauter, 2019). In addition to several other proprotein convertases, furin has the ability to cleave and activate viral proteins (Seidah & Prat, 2012). Differences in furin-like

activity across animal hosts may impact viral processing or alter viral tropism. It is perhaps fitting to note that proteolytic processing of the coronavirus spike protein was first recognized in the model rodent coronavirus murine hepatitis virus, MHV-A59 (Frana et al., 1985), with later analyses demonstrating the implication of furin in the proteolytic cleavage of its spike protein (de Haan et al., 2004).

Furin cleavage sites are not unique to the *Coronaviridae*. The highly pathogenic influenza strain, H5N1, for instance also contains a polybasic furin cleavage site in the hemagglutinin H5 protein (Decha et al., 2008). Additionally, within the *Nidovirales* (the order within which the *Coronaviridae* are placed), a furin cleavage site has been predicted in an insect nidovirus isolated from *Culex* mosquitoes (Kuwata et al., 2013). Other viral proteins that contain furin cleavage sites include, for example, human immunodeficiency virus (HIV) envelope glycoprotein gp160, herpesvirus glycoprotein B, tick-borne encephalitis virus envelope protein prM, and Ebola virus GP (Braun & Sauter, 2019; Izaguirre, 2019; Stadler et al., 1997).

Based on the known ability of bats and rodents to harbor coronaviruses, comparing the spike protein and furin cleavage sites of viruses across these two hosts may help elucidate origins of novel coronaviruses and guide future surveillance efforts. Through the use of two publicly available programs used to accurately predict furin cleavage sites, PiTou and ProP (Duckert et al., 2004; Tian et al., 2012), we provide an overview of furin cleavage across spike protein sequences in rodent and bat species. PiTou utilizes a hidden Markov model, specifically targeting 20 amino acid residues surrounding furin cleavage sites that are important for binding and solvent

accessibility (Tian et al., 2012). The final score in PiTou is based on log-odds probability. ProP utilizes an artificial neural network to predict furin cleavage (Duckert et al., 2004). In ProP, every lysine and arginine residue (designated P1) and flanking residues are given a score of between 0 and 1, with a score above 0.5 being a predicted furin cleavage site.

4.2 Furin cleavage across bat and rodent species

The protease furin cleaves at a distinct multi-basic motif containing paired arginine residues; furin requires a minimal motif of R-X-X-R, with a preference for an addition basic residue; i.e., R-X-B-R (Seidah & Prat, 2012). While most studies have focused on human furin, previous work has indicated furin-like proteases in the megabat *Pteropus* (El Najjar et al., 2015). The presence of suitable protease activators to enable viral infections further adds to the mystery that enables bats to act as viral reservoirs without seemingly showing clinical signs. Many, but not all, coronaviruses contain a cleavage site at S1/S2, which primes the spike protein for fusion and may increase its affinity for the viral receptor but may also make it structurally unstable. As such S1/S2 is considered dispensable for virus infection. The presence of S1/S2 furin cleavage sites in viruses uniquely identified in bats is shown in figure 4.1A. Across the *Coronaviridae*, the S2' cleavage site is also present, with cleavage here considered a required event for viral infection (White &

Whittaker, 2016). Bat sequences which have predicted furin cleavage sites at S2' are additionally shown in figure 4.1B.

A.

Isolate	Subgenus	S1/S2 Sequence	Furin Score	
			PiTou	ProP
Bat Hp-betacoronavirus/Zhejiang2013	HibeCoV	714 - CVNYTADTRLRTAR | AADRAL - 733	8.19	0.61
(Putative) Zaria bat coronavirus	HibeCoV	696 - DTCLNITRGRVGSR | SAGHLK - 715	2.27	0.20
Bat coronavirus HKU5-1	MerbeCoV	732 - LCAIPPTTSSRVRR | ATSGAS - 751	10.26	0.82
BtPa-BetaCoV/GD2013	MerbeCoV	733 - LCAIPPTTSSRLRR | ATSGVP - 752	10.21	0.81
Pipistrellus abramus bat CoV HKU5-related	MerbeCoV	738 - LCAIPPTTSTRVRR | ATSGVS - 757	8.40	0.72
Bat coronavirus HKU5-2	MerbeCoV	737 - LCAIPPTTSTRFRR | ATSIPD - 756	7.06	0.59
Pipistrellus bat coronavirus HKU5	MerbeCoV	737 - LCAIPPTTSTRFRR | ATSGVS - 756	7.01	0.73
Coronavirus Neoromicia/PML-PHE1/RSA/2011	MerbeCoV	732 - LCAIPPNTNLRSGR | STFGLG - 751	2.17	0.56
Bat coronavirus (BtCoV/A434/2005)	Unclassif.	725 - LCAIPPTISTRLRR | ATSGVS - 744	8.11	0.61
Bat coronavirus PREDICT/PDF-2180	Unclassif.	733 - LCAIPPNTNLRVGR | STFGLG - 752	1.28	0.54

B.

Isolate	Subgenus	S2' Sequence	Furin Score	
			PiTou	ProP
Pipistrellus abramus bat CoV HKU5-related	MerbeCoV	877 - LQIPQVTTGERKYR | SAIEDL - 896	0.35	0.571
Bat coronavirus HKU5-1	MerbeCoV	871 - LQIPQVTTGERKYR | STIEDL - 890	-0.56	0.507
Coronavirus BtRt-BetaCoV/GX2018	NobeCoV	796 - MGCLGSSCNSRNYR | SALSDL - 815	-5.18	0.527
Rousettus bat coronavirus HKU 9	NobeCoV	789 - MGCLGSNCGGKSHR | SALSEL - 808	0.09	0.16
Bat coronavirus 1B	MinunaCoV	876 - FDLTLALPRQRQSR | SAIEDL - 895	5.3	0.592
229E-related bat coronavirus	DuvinaCoV	865 - IPSLPTSGSRVAGR | SAIEDI - 884	0.8	0.212
BtMf-AlphaCoV/GD2012-b	Unclassif.	876 - FDLTLALPRERQSR | SAIEDL - 895	4.98	0.525
Bat coronavirus MfulBtCoV/3709	Unclassif.	879 - FDLTLALPRQRQSR | SAIEDL - 898	5.3	0.592

Figure 4.1: Examples of predicted furin cleavage sites identified in coronaviruses associated with chiropteran species. Over 150 spike sequences from bat associated CoVs were screened for furin cleavage sites, including those in both the *Alpha*- and *Betacoronavirus* genera, using the programs PiTou and ProP. A) Unique S1/S2 furin cleavage sites predicted in bat associated coronaviruses. B) Unique S2' furin cleavage sites predicted across bat associated coronaviruses. Associated NCBI accession numbers are as follows: Bat Hp-betacoronavirus/Zhejiang2013 (YP_009072440), (Putative) Zaria bat coronavirus (ADY17911), Bat coronavirus HKU5-1 (ABN10875), BtPa-BetaCoV/GD2013 (AIA62343), Pipistrellus abramus bat coronavirus HKU5-related (QHA24687), Bat coronavirus HKU5-2 (ABN10884), Pipistrellus bat coronavirus HKU5 (AGP04938), Coronavirus Neoromicia/PML-PHE1/RSA/2011 (AGY29650), BtCoV/A434/2005 (ABG11962), Bat coronavirus PREDICT/PDF-2180 (YP_009361857), BtRt-BetaCoV/GX2018 (QJX58383), *Rousettus* bat coronavirus HKU 9 (AVP25406), Bat coronavirus 1B (ACA52157), 229E-related bat coronavirus (ALK28781), BtMf AlphaCoV/GD2012-b (AIA62241), Bat coronavirus MfulBtCoV/3709 (AMB43191), Bat coronavirus MfulBtCoV/3736-1 (AMB43194), Bat coronavirus MfulBtCoV/3759-1 (AMB43195), Bat coronavirus MsBtCoV/4001-1 (AMB43196), Bat coronavirus MsBtCoV/4068 (AMB43198), *Rhinolophus* bat coronavirus HKU32 (QCX35178), *Rousettus* bat coronavirus GCCDC1 (QKF94914), *Hipposideros* bat coronavirus HKU10 (AFU92131), Bat coronavirus HKU9-3 (ABN10927), Bat coronavirus HKU9-1 (ABN10911), Alphacoronavirus Bat-CoV/P.kuhlii/Italy/3398-19/2015 (YP_009755890), NL63-related bat coronavirus

(YP_009824967), *Chaerephon* bat coronavirus/Kenya/KY41/2006 (ADX59458), Miniopterus bat coronavirus/Kenya/KY33/2006 (ADX59488), SARS-like coronavirus BatCoV/BB9904/BGR/2008 (ALJ94036), and *Rousettus* bat coronavirus/Kenya/KY06/2006 (ADX59474).

The S1/S2 cleavage site was first identified in coronaviruses infecting laboratory rodents; for example, the mouse hepatitis virus (MHV) strain JHM contains a strong S1/S2 cleavage site in addition to a second predicted furin cleavage site just distal. The presence of S1/S2 furin cleavage sites in viruses uniquely identified in rodents is shown in figure 4.2A. Most often, a second, overlapping, S1/S2 furin cleavage site was predicted by PiTou but not by ProP, although an exception is the rodent coronavirus RtBi-CoV/FJ2015, in which both PiTou and ProP predicted adjacent furin cleavage sites. This redundancy may indicate a needed feature for rodent-associated *Coronaviridae*. The majority of the rodent coronaviruses with S1/S2 furin cleavage sites were classified as *Embecoviruses*, a subgenus well known for this particular feature (Wu & Zhao, 2021). A weakly predicted cleavage site was also detected in the *Alphacoronaviruses,* AcCoV-JC34 and Lucheng Rat Coronavirus-Lijiang-170. A total of 21 unique potential S1/S2 furin cleavage sites are shown in figure 4.2A. It is important to note, however, that isolate-specific differences exist; for example, two isolates of HKU24 are shown in figure 4.2A, but possess disparate predicted furin cleavage sites. Lastly, the virus with the strongest predicted furin cleavage site was RtR-CoV-T12006A based on PiTou. ProP also gave a strong furin cleavage prediction score (0.88), the highest score observed from ProP, but shared by several other viruses. S2' furin cleavage sites additionally occur naturally in rodent coronaviruses and are shown in figure 4.2B. It is worthwhile to note that the rodent coronaviruses with S2' furin cleavage sites also possess S1/S2 furin cleavage sites. Compared to S1/S2 furin cleavage sites in rodent coronaviruses, S2' furin cleavage sites are less common.

Given the well-conserved nature of the S2' cleavage site, it is unsurprising that the predicted scores in the rodent coronaviruses are relatively similar to the predicted scores from bat - associated coronaviruses.

A.

Isolate or strain	Classification	S1/S2 Sequence	Furin Score	
			PiTou	ProP
Murine hepatitis virus strain JHM	EmbeCoV	756 - GLCVDYSKSRRARR | SVSTGY - 775	13.70	0.88
		755 - AGLCVDYSKSRRAR | RSVSTG - 774	4.48	0.37
Longquan RI rat coronavirus Longquan-189	EmbeCoV	740 - GFCVDYSTARRKRR | DLSTGY - 759	12.69	0.84
		739 - SGFCVDYSTARRKR | RDLSTG - 758	4.02	0.45
Betacoronavirus HKU24, Longquan-723	EmbeCoV	751 - GYCVDYSATRRSKR | DLNTGY - 770	12.63	0.79
Longquan Rl rat coronavirus Ruili-66	EmbeCoV	740 - GFCVDYSTARRKRR | EISTGY - 759	11.38	0.80
Betacoronavirus HKU24, Lijiang-41	EmbeCoV	750 - GYCVDYSATRRAKR | DLNTGY - 769	10.34	0.75
Rat coronavirus Parker	EmbeCoV	743 - GLCVNYSTAHRARR | SVSTGY - 762	10.00	0.88
Rat sialodacryoadenitis coronavirus SDAV-681	EmbeCoV	743 - GLCVNYSIAHRARR | SVSTGY - 762	9.77	0.86
Longquan Rl rat coronavirus Longquan-708	EmbeCoV	747 - GLCVNYSTSHRARR | SISTGY - 766	9.25	0.86
Betacoronavirus HKU24, HKU24-R05005I	EmbeCoV	750 - GYCVDYSSTWRAKR | DLNTGY - 769	-5.47	0.74
MHV Strain A59	EmbeCoV	701 - MGAGLCVDYSKSRR | ADRSVS - 720	0.58	0.17
		704 - GLCVDYSKSRRADR | SVSTGY - 723	-5.00	0.79
Coronavirus AcCoV-JC34	LuchaCoV	495 - CNSSDVVTFSRRAR | ARTLTD - 514	0.15	0.28
RtRe-CoV/Tl2006A	Unclassif.*	737 - GLCVDYSKARRSRR | SVSTGY - 756	15.45	0.88
		736 - SGLCVDYSKARRSR | RSVSTG - 755	2.16	0.22
RtRt-CoV/Tb2018	Unclassif.*	737 - GLCVDYSKARRARR | SVSTGY - 756	13.17	0.87
		736 - SGLCVDYSKARRAR | RSVSTG - 755	3.48	0.31
Rodent coronavirus RtMm-CoV/GD2015	Unclassif.*	740 - GFCVDYSTARRKRR | ALSTGY - 759	13.07	0.80
		739 - SGFCVDYSTARRKR | RALSTG - 758	6.91	0.59
Rodent coronavirus RtAp-CoV/Tibet2014	Unclassif.*	750 - GYCVDYSSARRSKR | ALSTGY - 769	12.96	0.82
Rodent coronavirus RtMm-CoV-1/IM2014	Unclassif.*	735 - GYCVDYSTSRRNKR | SLSTGY - 754	12.81	0.88
Rodent coronavirus RtBi-CoV/FJ2015	Unclassif.*	740 - GFCVDYSSARRKRR | DLSTGY - 759	12.71	0.84
		739 - SGFCVDYSSARRKR | RDLSTG - 758	4.43	0.51
Rodent coronavirus RtAs-CoV/IM2014	Unclassif.*	756 - CVDYQSQSTRRARR | AVDAPT - 775	12.20	0.78
		755 - YCVDYQSQSTRRAR | RAVDAP - 774	3.80	0.38
Rodent coronavirus RtMruf-CoV-2/JL2014	Unclassif.*	752 - GYCVDYSKTRRAKR | ATSTGY - 771	11.14	0.77
		749 - MGSGYCVDYSKTRR | AKRATS - 768	1.34	0.16
RtRe-CoV/Tl2009	Unclassif.*	749 - GLCVNYSTAHRARR | SISTGY - 768	8.71	0.85
Rodent coronavirus RtNn-CoV/SAX2015	Unclassif.*	743 - GFCVDYSTAHRERR | EISTGY - 762	5.90	0.76

B.

Isolate	S2' Sequence	Furin Score	
		PiTou	ProP
RtRt-CoV/Tb2018	887 - SSCSEGTTVSRTGR | SAIEDL - 906	5.53	0.613
RtRe-CoV/Tl2006A	887 - SSCAEGTTVSRTGR | SAIEDL - 906	5.44	0.613

Figure 4.2: Furin cleavage sites are commonly found in rodent associated coronaviruses. A) Examples of predicted S1/S2 furin cleavage sites in rodent associated coronaviruses. Longquan RI rat coronavirus Longquan-189 (AID16649) shared the same S1/S2 furin cleavage site as Longquan Rl rat coronavirus Longquan-370 (AID16655). Murine hepatitis virus strain JHM (YP_209233) shares the same S1/S2 furin cleavage sites as several other MHV strains (ACN89763, ACN89705, ACN89696, ACN89722), as well as Murine coronavirus MHV-3 (ACN89743) in which sites occur at residues 770 and 769, versus 769 and 768. Longquan Rl rat coronavirus Longquan-708 (AID16661) shares the same S1/S2 cleavage site as Murine coronavirus MHV-1 (ACN89742), in which the predicted furin cleavage site occurs at residue 758. Betacoronavirus HKU24 Lijiang-41 (QOE77297) shares the same S1/S2 furin cleavage site as Betacoronavirus HKU24 Lijiang-53 (QOE77307) and Betacoronavirus HKU24 Ruili-874

(QOE77327), in addition to several other accession numbers (AYR18625, AYR18679, AYR18634, AYR18607, AYR18652, AYR18670, and AYR18643). Coronavirus AcCoV-JC34 (YP_009380521) shares the same S1/S2 furin cleavage site as Lucheng Rn rat coronavirus Lijiang-170 (QOE77268), which were only predicted by PiTou. RtRt-CoV/Tb2018 (QIM73854) shared the same S1/S2 predicted cleavage sites as RtRt-CoV/Tk2011 (QIM73813). Rodent coronavirus RtMm-CoV/GD2015 (ATP66756) shared the same S1/S2 predicted cleavage site as Rodent coronavirus RtRn-CoV/YN2013 (ATP66727). RtRe-CoV/Tl2009 (QIM73841) shared the same S1/S2 furin cleavage site as RtRt-CoV/Tn2018 (QIM73848). B) S2' furin cleavage sites predicted in rodent association coronaviruses, both of which were noted as unclassified, but our analysis supported as belonging to the *Embecovirus* subgenus.

4.3 Comparative protein models

The coronavirus spike (S) protein is a type I viral fusion protein and is heavily glycosylated (Bosch et al., 2003; Kirchdoerfer et al., 2016; Shajahan et al., 2020; Walls et al., 2016b). Cryo-electron microscopy is optimal for understanding the S protein structure and several structures have previously been resolved, including those of MHV and SARS-CoV (Gui et al., 2017; Walls et al., 2016a). In the absence of direct structure determination, protein modeling can help predict features of the S protein, including whether a given furin cleavage site might be accessible. Protein models of individual monomers that assemble into trimers are shown in figure 4.3, highlighting the expected protein structure for two bat sequences, with and without predicted S1/S2 furin cleavage sites (figure 4.3 A&B) and a rodent spike protein with a predicted S1/S2 furin cleavage site (figure 4.3 C). An extended loop allows for furin accessibility and is demonstrated in the two models with predicted furin cleavage sites, each from a bat and rodent associated virus (figure 4.3 A&C). Across rodent and bat species, it is likely that furin cleavage can be achieved. All of the protein models shown are from viruses that have been found naturally circulating in bat or rodent populations.

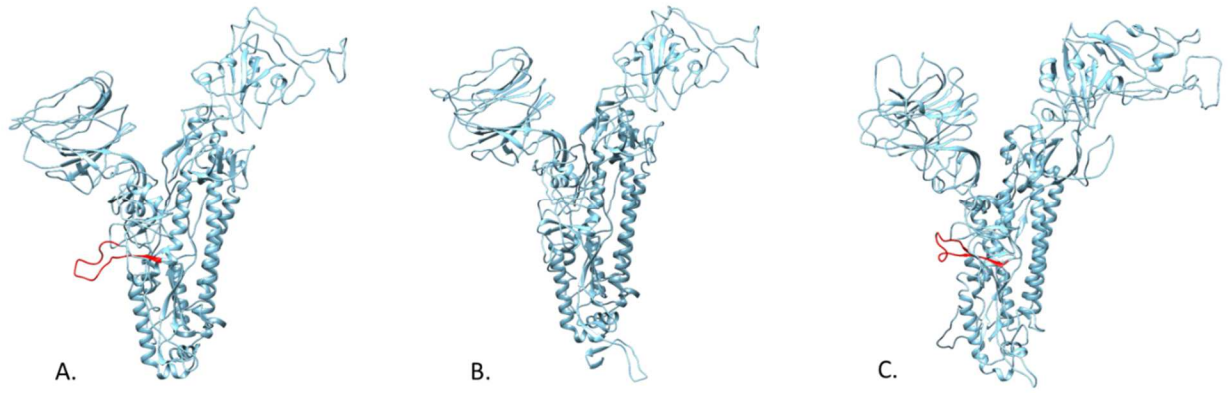

Figure 4.3: Protein models of three spike proteins across bat and rodents. The red area (A. and C.) of the spike protein corresponds with the predicted S1/S2 furin cleavage site and surrounding residues. These extended loops allow for furin accessibility. The protein models were built using the program UCSF Chimera (v.1.15rc, University of California), through the Modeller extension (Pettersen et al., 2004). A) Protein model of the spike protein of HKU5-1, a bat associated CoV, based off SARS-CoV (PDB N. 5X58). B) Spike protein model of a bat CoV lacking a predicted furin cleavage site (Bat coronavirus BM48-31/BGR/2008, accession # YP_003858584.1). Model was built using SARS-CoV (PDB N. 5X58). C) Protein model of a rodent associated coronavirus, Longquan RI rat coronavirus, isolate Longquan-189, based off MHV strain A59 (PDB #6VSJ).

4.4 Discussion

Continued surveillance in bat and rodent species, concomitant with laboratory experimentation and computational analysis, is essential for understanding the overall disease ecology of coronaviruses in these two host groups. The Chiroptera and Rodentia encompass vast lineages and inhabit unique ecological niches from bats roosting in caves to mice living in apartment buildings. The spillover of coronaviruses between these species and into humans, livestock, and other species remains a significant threat to One Health and global health. Across species, it is also important to consider host adaptations that may facilitate their ability to act as viral reservoirs. In bats, the ability to harbor and shed coronaviruses, with limited or inapparent clinical signs has remained an ongoing question (Irving et al., 2021; Letko et al., 2020). In comparison, wild rodent

species harboring coronaviruses have been investigated to a lesser extent, despite the fact that rodents can show disease associated with coronavirus infection in laboratory settings. An excellent example are Golden Syrian Hamsters (*Mesocricetus auratus*) that have proven to be a robust and effective animal model for SARS-CoV-2 infection (Chan et al., 2020; Sia et al., 2020).

SARS-CoV-2 has remained a global challenge and like most diseases, rodent models are helpful in guiding our understanding. In laboratory mice, transgenic humanized ACE2 mice better recapitulate SARS-CoV-2 infection and pathology (Sun et al., 2020; Winkler et al., 2020). However, new SARS-CoV-2 variants of concern (VOCs) have expanded the host range of SARS-CoV-2, allowing infection of laboratory mice (Montagutelli et al., 2021). Challenges of deer mice (*Peromyscys maniculatus*) with SARS-CoV-2 have demonstrated infection and transmission (Fagre et al., 2020; Griffin et al., 2020). Understanding coronavirus adaptation and transmission among rodent species is an important area of investigation both from the One Health perspective, as well as understanding spillover between rodents. Additional important areas of study include how/why some rodents harbor unique viruses, as well as what are the underlying host differences that previously allowed one rodent species (laboratory mice) to be largely unaffected by SARS-CoV-2 while another rodent species (hamsters) readily develop disease. In mice infected with MHV, disease has been associated with encephalomyelitis, wasting, and mortality in naïve animals (Cheever et al., 1949; Houtman & Fleming, 1996). Furthermore, a case report has described a wasting syndrome in guinea pigs with a presumed coronavirus infection (Jaax et al., 1990).

The activation of the S protein is a complex process and furin is a commonly used protease to activate fusion machinery. Among the alphacoronaviruses, the presence of an S1/S2 furin cleavage site is infrequently observed, with FCoV as a notable example. FCoV normally possesses a furin cleavage site at the S1/S2 boundary, but loss of basic residues in that site is associated with the systemic, highly fatal disease feline infectious peritonitis (FIP) (Licitra et al., 2013). A second alphacoronavirus, canine coronavirus 23/03, also possesses a furin cleavage site, but the role in disease is less well defined (Decaro et al., 2015). In bat spike sequences from alphacoronaviruses, no obvious S1/S2 furin cleavage site has been identified based on ProP and PiTou predictions. In contrast, two proposed alphacoronaviruses from rodents, AcCoV-JC34 and Lucheng Rn rat coronavirus Lijiang-170, did have a shared, weakly predicted furin cleavage site (S-R-R-A-R), based on the PiTou program. A caveat to using bioinformatics programs lies in the correlation between a software prediction and biological plausibility. A predicted site, for example, may not be accessible to furin and thus, non-functional. Furthermore, we have focused solely on furin in this present study, although the actions of other proteases also need to be considered.

Based on our results reported in this study, it is possible that viruses with S1/S2 furin cleavage sites are more commonly found in *Coronaviridae* circulating naturally in rodents. Over three times as many S protein sequences from bats (179) were publicly available and screened when compared to available rodent sequences (55). While MHV was included in our screening, 41 rodent viruses were from previous surveillance work and can be considered naturally occurring. Of these 41 rodent sequences, 32 (78%) had potential S1/S2 furin cleavage sites. In the S protein sequences from bats, only 11 of 179 (6%) sequences had predicted S1/S2 furin cleavage sites. For some CoVs, such as HKU5, numerous accessions are publicly available through sources such as NCBI,

so it is challenging to strictly compare the frequency of furin cleavage sites across the species that sequences come from, but it does seem apparent that rodents regularly harbor viruses with strong S1/S2 furin cleavage sites. With regard to the S2' furin cleavage site, the proportion of rodent versus bat sequences possessing this site is relatively similar, being found in 3 of 41 (7.3%) naturally occurring rodent spike sequences and in 13 of 179 (7.3%) bat sequences. Host differences may additionally contribute to these observed differences. Both rodent and bat associated coronaviruses have been associated with human disease and both must continue to be investigated, including in regular surveillance studies.

The *Coronaviridae* lend themselves well to zoonotic spillover. The unique spike protein determines host tropism and is largely a balance between receptor binding ability and the presence of an acceptable host cell protease for spike protein activation. Bats and rodents have both been implicated as reservoirs for ancestral coronavirus species. The common presence of predicted furin cleavage sites across rodent coronaviruses, suggests that rodents warrant further consideration as a putative source of new viral emergences.

4.5 References

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CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

5.1 Feline coronavirus

Feline coronavirus (FCoV) remains a challenge across populations of cats. Though there is data to support the use of a nucleoside analog for systemic disease associated with FCoV (Pedersen et al., 2019), the drug currently lacks US Food and Drug Administration approval. A major improvement for preventing feline infectious peritonitis (FIP) may come in the form of a vaccine. While older vaccine technologies were associated with worse outcomes following FCoV infection (Scott et al., 1992), new technologies, including mRNA vaccines may be an option for preventing future FIP cases. The development of mRNA vaccines against SARS-CoV-2 utilizes a stabilized, pre-fusion spike immunogen (Jackson et al., 2020), thereby minimizing the chances of antibody dependent enhancement (ADE), a feature thought to be associated with early FCoV vaccine "failure." Utilizing this same technology for feline coronavirus vaccines, especially higher risk populations such as those in shelters may help protect against FIP. For many cats, shelter entry often includes vaccination for common feline pathogens, given the potential for infectious disease spread and the public health consequences, for example rabies vaccination. The core feline vaccine, noted as FVRCP, covers three pathogens: feline herpesvirus, calicivirus, and panleukopenia. While vaccinating on entry to the shelter is a step towards improved feline health and welfare, delayed immune responses, stress, and previous exposure create challenges for "vaccinating away" common feline pathogens. In our survey of respiratory cases, 55% of cats with upper respiratory disease were positive for calicivirus. Likewise, shedding of FCoV in the feces was relatively common across cats. Thus, the benefits of a vaccine for FCoV may be best targeted at queens who can pass on maternal immunity or by ensuring vaccination occurs prior to exposure. Additionally, although the SARS-CoV-2 mRNA vaccines have thus far proven safe in human populations (Polack et al.,

2020), the same should not automatically be assumed for cats. Already thirty years ago, it was noted that certain vaccines for cats were associated with the development of fibrosarcoma in these animals, specifically termed injection site sarcoma (Saba, 2017). Lastly, the administration of vaccines, even those of veterinary importance, also involves a sociological perspective. Hendra, for instance, is lethal to horses, yet numerous barriers for using a vaccine for Hendra have been described by owners, including worries about vaccine safety and cost (Manyweathers et al., 2017). Additional qualitative study of Hendra vaccine use by horse owners identified motivators for vaccine use including wanting to protect horses and people and feeling a peace of mind (Wiethoelter et al., 2017). With the longstanding concern that FCoV vaccines can worsen the disease and even lead to FIP, potentially because of ADE, attention and subsequent commitment will be required to address vaccine hesitancy. Roger's Diffusion of Innovation theory indicates that adoption of new technology occurs gradually and involves social and behavioral aspects; adoption of human vaccines often occurs in a similar manner (Agyeman et al., 2009; Rosen & Goodson, 2014) and one would expect the same for veterinary vaccine uptake.

Decades of FCoV work has guided a better understanding of the virus, however, an open question remains with regards to the entry receptor used by type I FCoV. Type II FCoV utilize Aminopeptidase N (fAPN) (Hohdatsu et al., 1998), while the receptor for type I viruses remain unknown. This question creates challenges both clinically and from the basic science perspective, including the limited ability to isolate type I FCoV in culture, with exception of the lab adapted strain FCoV Black, which is culturable in macrophage-like, *Felis catus* whole fetus 4 (FCWF-4) cells. Angiotensin-converting enzyme-2 (ACE2) is utilized by several CoVs,

including SARS-CoV, SARS-CoV-2 and HCoV-NL63 (Hoffmann et al., 2020; Hofmann et al., 2005; Li et al., 2003). Given the similar vasculitides between COVID-19 in humans and FIP in cats, it is possible that feline ACE2 serves as a receptor for FCoV. Previous work has demonstrated ACE-2 expression in the feline gastrointestinal tract, the lower respiratory tract, and numerous other tissues such as the kidneys (Brand et al., 2008; Chiocchetti et al., 2020; Sun et al., 2021). ACE2 expression and activity on human macrophages/monocytes appears variable, including based on health status (Keidar et al., 2007; Song et al., 2020). Further investigation of feline ACE2, including expression on macrophage/monocyte populations, as well as the interaction of FCoV with fACE2 may help further elucidate associated pathogenesis. Dual immunostaining, for fACE2 and FCoV, of tissues from cats with and without FIP would support the role of this fACE2 as a receptor in addition to in vitro work, including co-immunoprecipitation, surface plasmon resonance, and CRISPR-Cas9. While FCoV is not considered zoonotic, a single report identified FCoV-like strains in three samples submitted for influenza testing (Silva et al., 2014). If type I FCoV is determined to utilize fACE2, the potential for zoonotic transmission may need to be reconsidered, given the similarity between human and feline ACE2 (Stout et al., 2020), though this is likely a rare event. FCoV infection and replication in non-permissible cells transfected with hACE2, in addition to primary human cell lines, would provide further evidence whether type I FCoV zoonosis is possible. Lastly, investigation of FCoV spillover, through molecular and serological detection, in high risk populations such as veterinarians and shelter workers would provide further knowledge for the potential of zoonotic spread. By comparison, canine coronavirus has also been detected in human samples (Xiu et al., 2020). The frequency of these events is presumed to be rare and humans would be assumed to be dead-end hosts. Nonetheless, understanding the surrounding

disease ecology is helpful for preventing the emergence of novel pathogens, especially given the recombinogenic potential of coronaviruses.

The natural pathogenesis of FCoV provides a comparative model for SARS-CoV-2 and COVID-19. Understanding the combination of host, virus, and environmental factors pertinent to both FCoV and other coronaviruses can help promote both animal and human health, including similarities and differences between clinical pathology trends, immune responses, viral activation, shedding patterns, vasculitis development, neurological consequences, long term disease, and risk factors for worse outcomes.

5.2 SARS-CoV-2 and felid species

It is apparent that domestic and non-domestic cats are susceptible to SARS-CoV-2 infection. A primary question for feline health is whether there are long term consequences of SARS-CoV-2 infection or whether systemic disease can develop, especially in light of new variants. A recent preprint noted the potential for cats to develop congestive heart failure and other cardiac abnormalities in cats infected with the B.1.1.7 variant of SARS-CoV-2 (Ferasin et al., 2021). In addition to domestic cats, serostudies in zoo-housed non-domestic felids would help with understanding how frequently SARS-CoV-2 is being transmitted into these species and whether asymptomatic infection is possible. Additionally, for individuals destined to be released into the wild for species conservation, understanding SARS-CoV-2 shedding would be helpful for species survival and potentially preventing spillback opportunities. With FCoV, previous work has indicated that testing five consecutive fecal samples had the highest probability of detecting the virus, compared to other testing strategies (Gaffney et al., 2012). This strategy may be also

appropriate for SARS-CoV-2 testing in non-domestic felids. Nonetheless, repeat sampling and molecular detection of SARS-CoV-2 in cats, both with clinical signs of SARS-CoV-2 and asymptomatic, would provide guidance for quarantine procedures and viral dynamics in non-domestic felids.

5.3 Coronavirus ecology, surveillance, and spillover potential

From a disease ecology standpoint, the origin of FCoV remains unclear. Bats and rodents have been primarily associated with coronavirus spillovers in humans (Cui et al., 2019). It is feasible that one of these orders was also responsible for a spillover event into cats. Most recently, two papers have considered the ecological interactions of cats and bats and the potential for infectious disease spread between these two species, including, rabies, for instance (Oedin et al., 2021; Salinas-Ramos et al., 2021). As most cat owners know, cats are also efficient rodent hunters. Rodents occupy numerous ecological niches, from living in urban environments, hitchhiking on shipping containers, and occupying rural environments. The first cases of FIP were described at Angell Memorial Animal Hospital in Boston, MA, USA (Holzworth, 1963). A number of pathways may have thus provided a pathway for a naturally circulating rodent coronavirus spillover into cats, whether that might have been a shelter with a rodent problem, cats providing rodent control in Boston Harbor, or owned cats occupying a city building and catching mice or rats. Indeed, one study investigating circulating coronaviruses in rodents, several rodents were even noted to have been obtained as a result of cat attacks (Tsoleridis et al., 2019). Coronavirus surveillance in rodents in the Northeast, including in Boston, has not previously been performed and while it may not further our understanding of where type I FCoV originated from, such investigation would still provide a catalogue of circulating viruses. Further, it is possible that CoV spillover from bats into cats may have led to FCoV as we know

it. *Alphacoronaviruses* circulate in bat species. Like rodents, little is known about CoVs circulating in bats in the Northeast. Nine bat species can be found in New York State specifically: Northern Bat (*Myotis septentrionalis*), Little Brown Bat (*Myotis lucifugus*), Indiana Bat (*Myotis sodalist*), Eastern Pipistrelle (*Perimyotis subflavus*; formerly *Pipistrellus subflavus*), Big Brown Bat (*Eptesicus fuscus*), Small footed bat (*Myotis leibii*), Red Bat (*Lasiurus borealis*), Hoary bat (*Lasiurus cinereus*), and Silver-haired bat (*Lasyionicterius noctivagans*) (Stegemann & Hicks). In the Little Brown Bat, *Alphacoronavirus* isolates have been identified in bats in Manitoba, Canada and Colorado (Misra et al., 2009; Osborne et al., 2011; Subudhi et al., 2017). Additionally, *Alphacoronaviruses* circulate in Big Brown Bats in Colorado (Osborne et al., 2011). Surveillance in bat species in the Northeast would be helpful to confirm *Alphacoronavirus* circulation.

Both rodents and bats are important reservoirs for CoVs. In a unique rodent coronavirus AcCoV-JC34, a weakly predicted S1/S2 furin cleavage site exists (F-S-R-R-A-R | A-R), which is a somewhat rare observation for *Alphacoronaviruses*, though it should be noted that phylogenetically, the spike protein of AcCoV-JC34 creates a unique cluster compared to other *Alphacoronaviruses* (Ge et al., 2017) (figure 5.1). Notably, FCoV also contains an S1/S2 furin cleavage site. This sequence is in further need of biochemical analysis, including a proteolytic cleavage assay of a peptide derived from the S1/S2 site. The development of an AcCoV-JC34 spike pseudoparticle and survey of viral entry on numerous cell lines, including those of feline origin, may help understand spillover potential for this specific CoV. Phylogenetic analysis of ORF1ab indicates a close relationship between several additional rodent CoVs and FCoV (Tsoleridis et al., 2019).

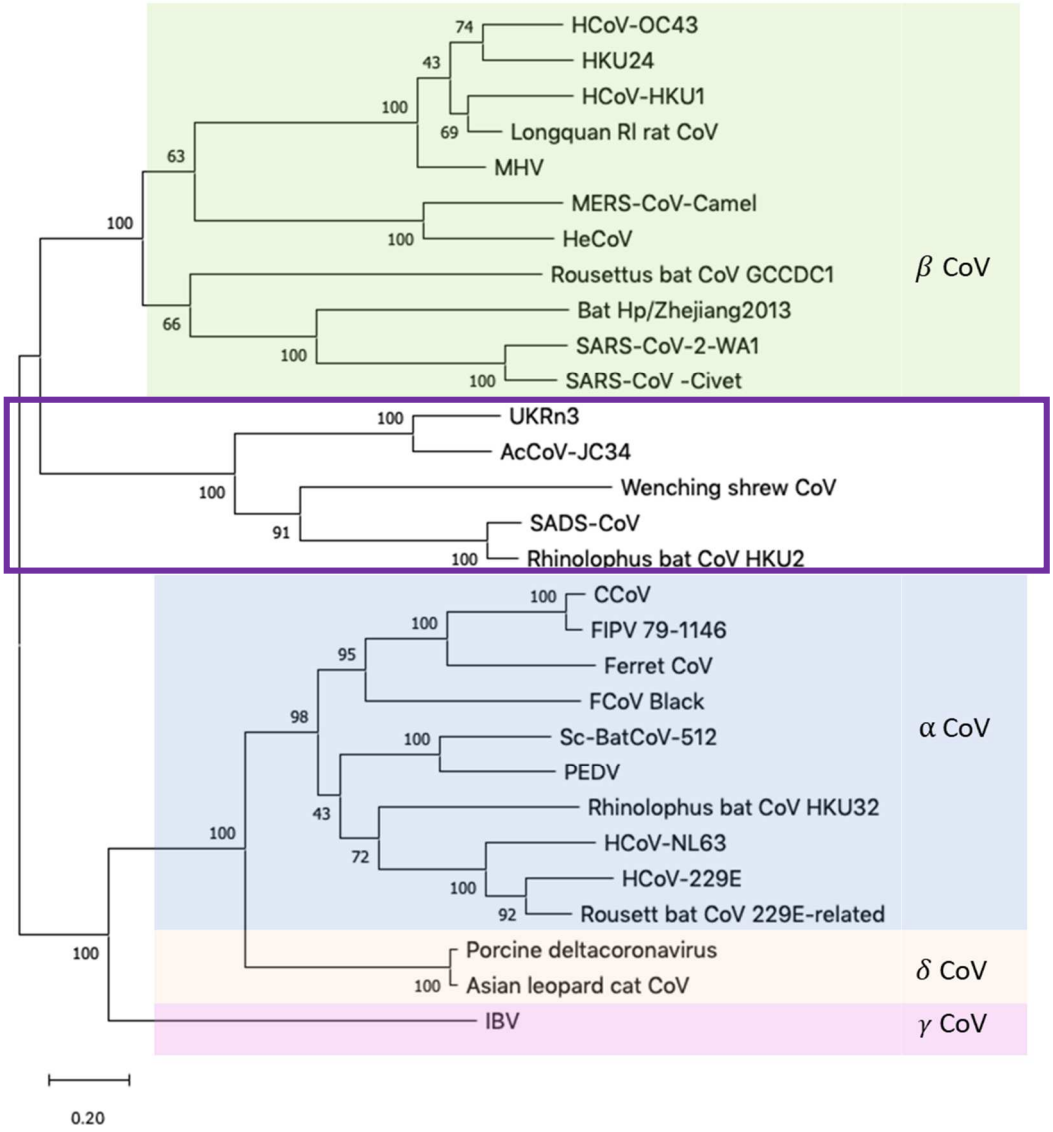

Figure 5.1: Phylogenetic analysis of spike proteins show a unique cluster compared to described genera and includes the rodent coronavirus AcCoV-JC34. Sequences were aligned using MUSCLE alignment and then the phylogenetic tree was built using MEGAX (Kumar et al., 2018). Bootstrap values are based on 100 iterations. Image created by A. Choi.

Preventing spillover events of novel viruses is essential, however, this does not come without significant challenges. Recently, a publicly available resource has been developed, known as *Spillover: Viral Risk Ranking*, to quantify the risk of virus spillovers from wildlife species in

collaboration with the Global Virome Project (Grange et al., 2021). With novel pathogens and infectious disease outbreaks, collaboration and open data sharing is essential (World Health Organization, 2016). The previously developed platform known as the global initiative on sharing avian influenza data (GISAID) provides one tool for open sharing and with the SARS-CoV-2 pandemic, has played a key role for sequence sharing (Elbe & Buckland-Merrett, 2017). The continued adoption of new technologies will also be invaluable in preventing future viral spillovers. For instance, the US National Syndromic Surveillance Program (NSSP) was first developed because of bioterrorism concerns (Gould et al., 2017). Integrating such programs with next-generation sequencing has the capacity to identify novel pathogens rapidly. Additionally, the use of artificial intelligence integrated into disease surveillance programs has the ability to raise alert systems before a human may even recognize disease. Thermal sensors, for instance have experimentally shown the potential to identify cases of foot and mouth disease, prior to clinical signs (Rainwater-Lovett et al., 2008). Likewise, in dairy cattle, the use of pedometers is an early warning for disease onset. The integration of similar technologies into additional species has the potential for early detection of pathogens and protection of the public health. Lastly, in addition to early detection and characterization of novel pathogens, traceability must be considered, potentially through the use of use of Block-Chain (Zhu et al., 2021).

5.4 Conclusions

One Health is a useful and necessary approach when thinking about infectious diseases. Cats are beloved family members, yet also host to numerous infectious diseases, some of which have zoonotic potential. FCoV infection, while not zoonotic, provides a comparative model in regards to severe COVID-19. While human infection is of utmost concern, it has become apparent that cats are also infected with SARS-CoV-2. Preventing feline infection with SARS-CoV-2,

however, is still recommended, given the many remaining questions regarding infection or further virus spread. Solving infectious disease challenges requires global collaboration and as technologies continue to become cheaper, there are many opportunities for solving those challenges faced by humans, beloved pets, or wildlife species.

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