SEROLOGICAL SCREENING OF COMPANION-ANIMAL CATS FOR SARS-COV-2 SPIKE-SPECIFIC ANTIBODIES

by

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(Under the Direction of Stephen Mark Tompkins)

ABSTRACT

Near the end of December 2019, cases of pneumonia with unknown cause were being reported to the World Health Organization (WHO) in Wuhan, China. Months later, the world went into lockdown due to a coronavirus outbreak more paralyzing than the 2002 Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) and 2012 Middle East Respiratory Syndrome Coronavirus (MERS-CoV) outbreaks. The cause of this recent and ongoing pandemic is a virus named SARS-CoV-2 and it has raised the concern for species-jump due to cases of reverse zoonotic infection and arising variants. Many counties have performed serosurveillance studies to uncover which animals are susceptible to SARS-CoV-2 infections and the possibility of animal-to-human spread. In this study, we assess the seropositivity rate of client owned cats in Athens, Georgia that have been infected with SARS-CoV-2 through testing samples collected by the Veterinary Teaching Hospital of the University of Georgia between May 2020 and August 2021. We utilize recombinant Spike and Receptor Binding Domain (RBD) as capture antigens for ELISAs, and analyze our data using Normalized Absorbance Ratio (NAR). Our results show that out of the 335 samples received and tested, 4 samples (1.2%) were believed to be true SARS-CoV-2 reactive.

INDEX WORDS: Enzyme-Linked Immunosorbent Assay, SARS-CoV-2, Receptor Binding Domain, Spike, Normalized Absorbance Ratio, feline

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CHAPTER 1

INTRODUCTION

The word Coronavirus (CoV) is derived from the Latin words “virus” meaning “venom” and “corona” meaning “crown” or “wreath”. These words describe the name of a tiny biological agent, with an outer membrane composed of spikes resembling a crown, that can cause mild to severe illness in a variety of animals. Coronaviruses (CoVs) are enveloped, single-stranded, positive sense RNA viruses. CoVs have many natural hosts, ranging from wild birds to mammals, now including domesticated birds and humans [1]. Signs of infection vary by host, but CoVs commonly cause respiratory, gastrointestinal, and systemic diseases [2]. In human adults, the mildest form of respiratory disease comes from coronaviruses 229E, NL63, OC43, and HKU1, which contribute up to 30% of common cold cases [3]. In canines, Canine enteric coronavirus (CCoV) is commonly found in wild and domestic dogs worldwide and causes mild gastrointestinal signs, such as diarrhea [2]. However, viruses, such as CoV, demonstrate genetic plasticity and “jump” from species to species and/or mutate to cause disease in a new host species. An example of this mutation is a genetic recombination event in which a new, more virulent coronavirus is created in an existing host, as the case with FIP *(*feline infectious peritonitis) or Feline enteric coronavirus (FECV) type 2 emerged from Canine coronavirus (CCoV) and FECV [2, 4]. Alternatively, a CoV may enter a new host species and adapt to its new environment resulting in a new CoV and new endemic species. Coronavirus adaptation into new species have been responsible for many disease outbreaks in the 21st century, three occurring within the first two decades. Severe acute respiratory syndrome coronavirus (SARS-

CoV) emerged in 2002 causing the disease severe acute respiratory syndrome (SARS). The Middle East respiratory syndrome coronavirus (MERS-CoV) outbreak that began in 2012 causes Middle East respiratory syndrome (MERS). Most recently the 2019-2022 pandemic coronavirus disease 2019 (COVID-19) caused by SARS-CoV-2, a virus related to SARS-CoV, continues to cause disease as a global pandemic [1].

Ironically, SARS-CoV-2 has shown a more complex and frightening host "jump". Rather than the typical animal-to-human transmission, this pandemic has shown that human-to-animal, or reverse zoonotic, cases are possible and occurring. There have been reported cases of animals being infected with the virus by their owners and caretakers. One of the most reported and studied by researchers are the susceptibility of domestic animal such as cats, and dogs. The reported susceptibility of cats to infection with SARS-CoV-2 and other serosurveillance surveys raised the possibility that companion animal cats could be infected with SARS-CoV-2, serve as a reservoir, and/or support zoonotic infection of the cat owners. It has been shown that cats have a low frequency of SARS-CoV-2 infection, except for a few cats that were susceptible to severe infections due to having comorbidities and underlying conditions [5]. An example of human-to-cat transmission is the case where three cats owned by a confirmed Covid-19 patient were diagnosed with SARS-CoV-2 infection after two consecutive positive anal swab specimens during the fourth outbreak in Heilongjiang Province [6, 7]. However, there was a case where a cat-to-human transmission occurred in Thailand, when a healthy 32-year-old veterinarian was sneezed on by an infected cat owned by a confirmed infected person [8]. Not much is understood of this case yet, but it opens up the possibility that another zoonotic event is there.

Thus, the aim of this study was to assess whether client-owned cats in Georgia had been infected with SARS-CoV-2 by completing a serosurveillance study utilizing convenience

samples of cat serum from the University of Georgia Veterinary Teaching Hospital and a validated SARS-CoV-2 enzyme-linked immunosorbent assay (ELISA). I wanted to test for the presence of IgG antibodies and define the frequency of seropositivity in our samples. To do this, I utilized purified recombinant Spike and RBD proteins from SARS-CoV-2 (Wuhan-1) as capture antigens in the ELISA, and validated the assay using cat serum pooled from a SARS-CoV-2 experimentally-infected cat in our lab as our known seropositive sample and serum from naïve, specific pathogen-free cats as our known seronegative sample. I modified an ELISA procedure from a previous study done where researchers developed a protocol for human serosurveillance [9]. De-identified serum samples from hospital clients were collected between May 2020 and August 2021, tested first in a SARS-CoV-2 RBD ELISA, and then positive samples confirmed using a second SARS-CoV-2 Spike ELISA. I hypothesized that similar to other studies performed, I would see low or similar seropositivity.

CHAPTER 2

LITERATURE REVIEW

Pandemic CoVs

SARS-CoV emerged in the Guangdong Province, Foshan, China in November 2002 and continued to infect humans until July 2003 [10]. The etiological agent was described as causing an atypical pneumonia that did not respond to existing antimicrobial treatment [11]. This outbreak spread swiftly to several parts of the world, causing over 8000 infection cases and approximately 800 deaths; a mortality rate of 10% [1, 12]. Ten years later, in 2012, MERS-CoV was identified in Jeddah, Saudi Arabia with a case of acute pneumonia and renal failure leading to death [10]. Despite interventions, the virus targeted adults and spread to more than 27 countries, including the Middle East, Europe, North America, and Asia [13]. MERS continues to have outbreak events in small communities in the Middle East, bringing the total to more than 2,200 cases and having a 35% mortality rate [14, 15]. The most recent CoV to emerge, SARS-CoV-2, has infected over 534 million people and caused over 6.3 million deaths globally as of June 2022 [16]. The causative agent made its appearance on 31 December 2019, when the WHO China Country Office was informed of cases of pneumonia with unknown cause in Wuhan, China [17]. In early January 2020, the cause was identified as Novel Coronavirus (2019n-CoV) but was later renamed as SARS-CoV-2 due to similarities in genetic, transmissions and pathogenicity with SARS-CoV [18, 19]. As the pandemic continues, several variants have emerged with increased transmission rates compared to the ancestral virus as well as variants that can efficiently evade vaccine-elicited immunity against infection.

There are four genera for the subfamily Coronavirinae; alpha- (α), beta- (β), gamma- (ɣ), and delta- (δ) coronaviruses, with the β-coronaviruses further classified into viral lineages A-D [18]. Based on protein sequences, birds are thought to be the natural reservoir for ɣ- and δ-coronaviruses, while the reservoirs for α- and β-CoVs are thought to be bats and other mammalian species [20, 21]. The emerging HCoVs, i.e. SARS, MERS, and SARS-CoV-2, all reside in the β genera.

Natural reservoirs/hosts are the long-term ecological habitat for viral and parasitic populations; this holds to any animal species and is not restricted to those in a geographic range [21]. These hosts must meet three characteristics: 1.) they can contain a higher genetic virus diversity, 2.) they can harbor the virus continuously, and finally, 3.) they can be naturally infected beyond geographic range [21]. There is often another host that serves as the bridge between species, with this intermediate host allowing for mutation/adaptation and assisting in zoonotic transmission.

This intermediate host adaptation was proposed for SARS-CoV, as the virus would need to have evolved human-compatible receptors within its carnivorous zoonotic sources, but it was later found that an endangered Horseshoe bat genus served as the primordial natural host and harbored similar human-infecting viruses [21]. Although these human-infecting viruses are members of the same species, they do not share the same viral clade that made the jump from carnivores to humans and initiated the SARS pandemic [21]. The viral ancestor of SARS continues to exist in its natural reservoir (i.e. a bat species), which harbors different viral populations and contributes new variants [21]. Furthermore, all HCoVs can be traced back to bat origin, jumping to mice and other domesticated animals, based on evolutionary evidence [20].

The emergence of three pathogenic human coronaviruses, SARS-CoV-2, SARS-CoV, and MERS-CoV brings the total number of human coronavirus species up to seven. These emerging CoVs are generally very virulent possibly due to the lack of immunity in humans, the emergence as spill-over events, or with these viruses having distinct virulence determinants. This is in contrast to the four, endemic, community-acquired human coronaviruses HCoV; OC43, HKU1, NL63, and 229E, which circulate in human populations year-round without an animal host intermediate. However, SARS-CoV-2 has features similar to all the other CoVs, being highly transmissible as with the four community-acquired HCoVs, and having the potential to cause severe disease like SARS/MERS-CoV [20].

CoV Genome and Structure

Coronaviruses have the largest genome of all RNA viruses; approximately 26-32 kilobase [14]. Typically, positive-sense, single-stranded, RNA viruses display a mutation rate of up to 10^{-4} substitutions per nucleotide per cell infection [22]. Subgenomic RNAs are generated, and template switching increases homologous recombination among genes from different CoV lineages and viruses co-infecting the host [2, 14]. Unlike DNA polymerase, which proofreads and repairs errors before continuing transcription, RNA-dependent RNA polymerase (RdRp) does not proofread and continues copying the RNA genome. This lack of correction results in RNA viruses having high frequencies of genetic recombination and mutation compared to other viruses [1, 23]. However, due to the CoV's large genome, a rapid mutation rate is a dangerous characteristic as deleterious mutations in larger genomes can quickly compromise viral fitness by encoding a lethal mutation [22]. To solve for this, CoVs encode for a non-structural protein, nsp14 or ExoN, a proofreading 3′-to-5′ exoribonuclease that corrects errors made by RdRp [22].

With a mutation rate of 4×10^{-6} substitutions per nucleotide per cell infection, CoV mutation rates are still higher than double-stranded DNA viruses, and allows for 1.) extra plasticity in the genome, 2.) increased spike protein evolution, 3.) the potential for intraspecies variablility, 4.) 'host jumping', and 5.) the emergence of novel coronaviruses [14, 22]. It is found that the Spike protein contains the majority of mutations [24]. These characteristics are also responsible for emergence of new variants of SARS-CoV-2, such as the delta, lambda, and omicron variants.

The similarities that CoVs share in their genomes make these viruses easy to identify; including the 16 nonstructural proteins following the structural Spike (S), Envelope (E), Membrane (M), and Nucleocapsid (N) proteins [14]. The nucleocapsid (N) protein is responsible for binding the viral RNA genome and forming the RNA capsid. The transmembrane (M) protein focuses on using the host cellular membranes to join the virus and host factors together, making new virus particles. The envelope (E) protein, despite being abundant in an infected cell, makes up only a small portion of the protein content of virions. However, it has three functions: participating in viral assembly, release of virions, and contributing to pathogenesis. These proteins can be found localized in the endoplasmic reticulum, Golgi apparatus, and ER-Golgi intermediate compartment of the host cell, respectfully [17]. While the N, M, and E proteins assist this family of viruses in their structural success within the host; the spike (S) protein is what contributes the most.

The Spike protein plays an important role in host receptor recognition, viral attachment, and entry of host cells. For SARS-CoV-2 and several other human coronaviruses, the angiotensin converting enzyme 2 (ACE2) is the primary receptor. The Spike protein forms trimers protruding from the surface of the viral envelope. These trimers, represented in Figure 1, are composed of a signaling peptide on an N-terminus, an S1 subunit, and an S2 subunit adding up to 1273 amino

acids in length [25]. The S1 subunit is further divided into 2 sections; an N-terminal and a Receptor Binding Domain (RBD), while the S2 subunit is composed of 5 parts. The functional component of the S1 subunit is the RBD, represented in red in Figure 1, which is responsible for recognizing and binding to the ACE2. Binding of RBD to ACE2 then signals a conformational change in which the heptapeptide repeat domain of the S2 subunit will fuse the viral envelope and host cell membrane, resulting in delivery of the contents of the virion [25]. Although the Spike protein is able to bind with ACE2 through the RBD region, it has been shown that the C-terminal domain (CTD) of the S1 subunit has a larger surface area, more directly interacting residues, and a higher affinity for ACE2. These residues most essential for binding to the Spike protein are less conserved compared to the receptor-binding motif, which makes it less ideal for drug targeting and the RBD a critical target for neutralizing antibodies (nAbs) [25]. Due to the importance of the Spike protein, the virus has developed a tactic for evading surveillance from the host immune system by coating the Spike protein with polysaccharide molecules to camouflage it during entry [25]. This is just one of the many immune evasion methods used by SARS-CoV-2.

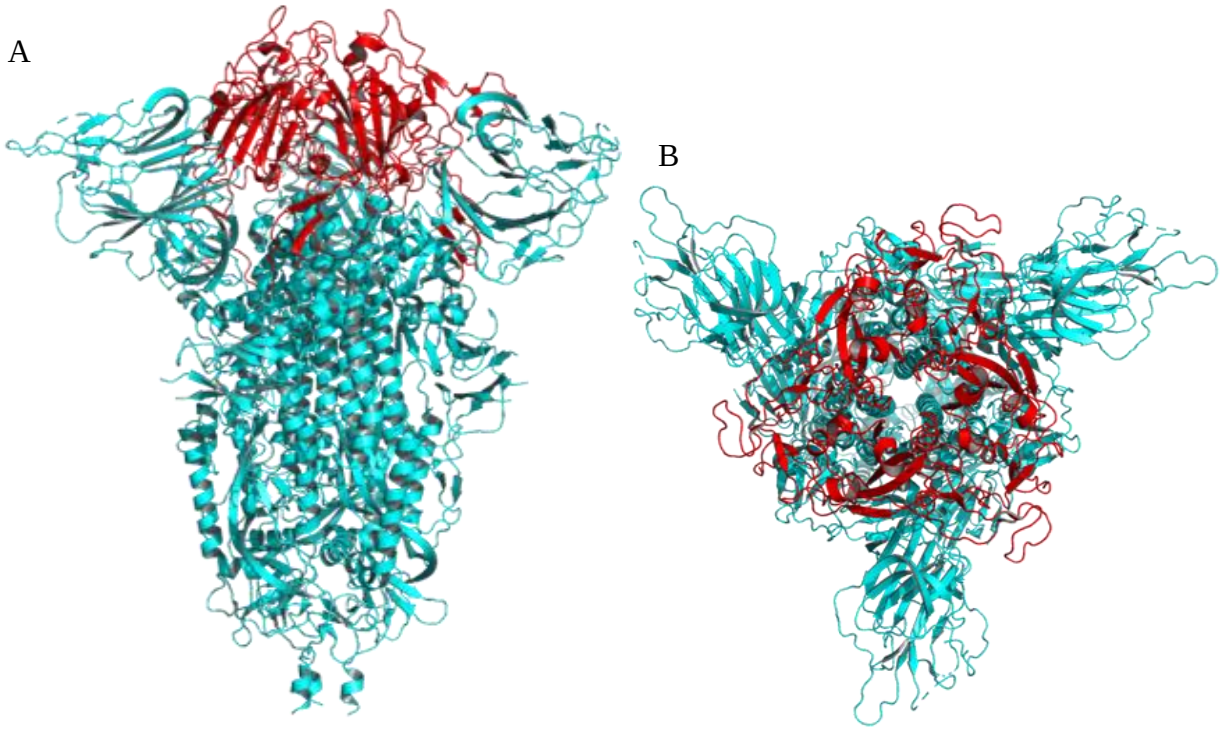


FIGURE 1: 3D Ribbon Structure of SARS-CoV-2 RBD and Spike Protein
A. Side view and B. top view of SARS-CoV-2 Spike trimers in closed conformation with RBD (red). Protein Data Bank: 7DF3

Host Immune Response

Upon SARS-CoV-2 infection, cells release cytokines to signal an innate immune response; this limits the spread of the virus and initiates the adaptive immune response, which will later clear the infection. When double-stranded RNA and 5'-triphosphate-bearing RNA molecules are detected in the cytosol [26], intracellular Pattern Recognition Receptors (PRR), such as Rig-like receptors (RLRs), and Toll-Like Receptors (TLR), will activate a signaling cascade that results in the synthesis and secretion of type I interferons alpha (IFN-α) and beta (IFN-β) [27]. This helps slow virus replication and contributes to the initiation of adaptive immunity, i.e. humoral immunity and T-cell mediated immunity. Humoral immunity is mediated by secreted antibodies which neutralize the virus, preventing entry into the host cell, and direct

the innate and adaptive immune cells (i.e. NK cells and macrophages) to the location of pathogens for elimination [28, 29]. Antibodies also improve the immune response by activating the complement system, which enhances B cell proliferation and differentiation into plasma cells, producing and secreting specific antibodies to control viral replication [29]. Even after the infection is cleared, virus-specific antibodies produced *de novo* by memory B cells and plasma cells provide long-term immunity and protection from future encounters with the same pathogen [28, 29]. One of the primary antibody isotypes elicited after a SARS-CoV-2 infection is Immunoglobulin G (IgG). IgG is first detected at about 12 days after initial infection, peaks at around 25 days, plateaus, and then gradually decreases after 60 days [30, 31]. At 6 months post-infection, IgG titer decreases by 68.9% but remains stable until 12 months post-infection, when titer decreases by an additional 86% from the level at 6 months [30, 31].

Coronaviruses, like other viruses, have developed robust suppression/evasion mechanisms to avoid the innate immune response. One example is that both alpha- and betacoronaviruses encode numerous gene products to block interferon (IFN) signaling in infected cells [26]. Several less-conserved viral gene products, such as the 'accessory proteins', also have innate immune suppressor features to help regulate the immune response. These accessory genes are believed to be acquired from different linages [26], with this acquisition possibly due to the nature of CoV RNA and genome replication, i.e. recombination by the RdRp. Due to these characteristics and the emergence of new variant strains of SARS-CoV-2, there have been difficulties with vaccine efficacy.

Vaccines and Treatments

With the rapid spread and growing numbers of hospitalizations and deaths due to SARS-CoV-2, rapid development of a vaccine was imperative. Coronavirus vaccines have been under development since the SARS and MERS outbreaks, with research showing that neutralizing Abs, such as monoclonal Abs, antigen binding fragments, single-chain variable region fragments, and single-domain antibodies are the best avenue due to targeting the RBD, S1-NTD, or S2 regions [25]. In late 2020- early 2021, four companies released candidate vaccines varying in effectiveness, number of doses required, and potential side effects. Pfizer and Moderna were the first to receive the FDA Emergency Use Authorization (EUA) and distribution, followed by Johnson & Johnson (J&J) in the United States [32]. These vaccines were produced and manufactured with the EUA status prior to receiving FDA approval. The AstraZeneca vaccine was the first to receive EUA by the European Medicines Agency in the United Kingdom [32]. Both Pfizer and Moderna use mRNA, while the latter two use adenovirus vectors. These mRNA vaccines were first authorized as a 2-dose vaccination regimen, and as of 2021, there is an additional booster for each. The J&J viral vector vaccine is a single-dose vaccine but has lower protection levels while also having an association with an increased risk of blood clots. AstraZeneca is also a 2-dose vaccine and has shown reduced effectiveness compared to the J&J vaccine but with fewer side effects. All of these vaccines have the common goal of eliciting antibody responses against the spike protein to protect against CoV infection. While cellular immune responses are elicited and important for induction of protective immunity, virus neutralizing antibody titers appear to be critical for prevention of infection and severe disease [32].

Even with several effective vaccines, new challenges continue to arise as SARS-CoV-2 mutates, resulting in variant viruses having increased transmission rates and evading infection or vaccine-elicited immunity. The most recent variant, omicron, which emerged in late 2020, is transmitted very efficiently, reducing the effectiveness of non-pharmaceutical interventions (e.g. non-surgical masks, social distancing, or isolation) and is only partially hindered by vaccination. Recent studies have shown that our current vaccines do not protect from infection by the omicron variant; however, full vaccination or prior infection combined with vaccination can lower disease severity [33]. The vaccine-evading variants have forced calls for vaccine updates, additional vaccine boosters, and new vaccines to control the spread of these viruses. This is critical as other seasonal viruses return (e.g. influenza and RSV).

At the start of the pandemic, the common symptoms of SARS-CoV-2 infection were loss of taste and smell, fever, dry cough, shortness of breath, muscle aches, and chest pains. However, when patients were being admitted to the hospital due to low CO_2 levels, they were ultimately placed on ventilatory support. Patients would rapidly decline within 2 weeks as their hyperactived immune systems produces “cytokine storm”, damaging the surrounding organs and resulting in death [34]. Over the course of the pandemic, we have discovered other ways that SAR-CoV-2 damages the body, sometimes affecting the heart or the central nervous system, even in asymptomatic cases [35]. We continue to learn of the long-term effects that SARS-CoV-2 patients experience months after recovery from infection. While vaccines are available, there is still a need to find a treatment for COVID-19 to address the surge of infected persons admitted to and remaining in hospitals. Antibodies are not only elicited by vaccines but can also serve as therapeutic infusions. Early in the pandemic, several monoclonal antibodies (mAb, S230, m396, and CR3014) were surveyed and assessed for cross-reactivity to SARS-CoV-2 due to their

efficient binding to the RBD protein of SARS-CoV [36]. Unfortunately, using ELISA and other assays to examine binding ability it was revealed that most of these antibodies failed to bind SARS-CoV-2, except for CR3022. The human mAb CR3022 showed potential as a candidate for therapeutic purposes alone or integrated with other new SARS-CoV-2-specific mAbs [36]. In 2020, an antibody treatment was given the EUA. Casirivimab and imdevimab are monoclonal antibodies that specifically target the spike protein and prevent viral attachment and entry [37]. As noted by the FDA, this treatment is only for mild to moderate SARS-CoV-2 cases, as it showed high success rate when administer intravenously together but was of no benefit to those hospitalized.

Feline Coronavirus

Feline coronaviruses (FCoVs) are part of the Alphacoronavirus genus. They are further separated into two distinct biotypes: feline enteric coronavirus (FECV) and feline infectious peritonitis virus (FIPV) [2]. FECV is highly contagious and common in domestic cat populations, especially multi-cat homes. It primarily infects the epithelial cells of the intestinal tract, resulting in mild gastrointestinal (GI) upset or lack of clinical (subclinical) signs. FECV is spread via fecal-oral transmission with sub clinically infected cats playing an important role in transmission, shedding virus for between 2-14 days following infection [2].

In contrast, FIPV causes the clinical syndrome feline infectious peritonitis (FIP), a highly inflammatory and systemic disease that is nearly 100% fatal once clinical signs appear. Feline infectious peritonitis virus is not transmitted via the fecal-oral route but arises by mutations in the less virulent FECV from within the hosts [38]. The mutations that give rise to FIPV take place in open reading frame 3abc and substitutions in the furin cleavage site [2]. Researchers

have shown their point mutations in the spike protein and/or 3c gene are sufficient to alter the tropism of the virus and allow binding to a different receptor on monocytes and macrophages [2, 17, 39]. Aside from mutations, males cats under 2 years old, older cats, immunosuppressed cats, and specific purebreds, like Abyssinian, Bengal, Birmans, Ragdoll, and Rex breeds, have higher disease prevalence [2, 38]. Although only a small percentage of cats develop FIP, the emotional effects take a toll, as there is no definitive diagnosis *ante mortem* [38]. Most diagnostic tests cannot differentiate between FECV and FIPV, especially if the cats do not present with body cavity effusions. Although effusion can occur in other etiologies, analysis of the fluid coupled with presentation of abnormalities and other test results can rule out other diagnoses and increase the probability of presence of FIP [38].

Clinical signs of FIP are presented in one of two forms: “wet” (effusive) and “dry” (granulomatous), or rarely, a combination of the two [2]. Effusive FIP is more common, as most cats present for fluid build-up in body cavities and difficulty breathing as a result. In contrast, granulomatous FIP lacks effusion and instead presents with multifocal granuloma formation in a variety of organs [2]. Overall clinical signs, regardless of form, include anorexia, lethargy, weight loss, pyrexia, ocular disease, severe inflammation in the organs, and neurological abnormalities, such as ataxia, seizures, nystagmus, hyperesthesia, and/or cranial nerve deficits [2]. With the lack of diagnostic tools, multiple invasive tests are required and evaluated in combination, such as: sampling of cerebral spinal fluid, macroscopic and cytologic examination of effusions (with which most FIP cats present), and aqueocentesis (collecting a sample of aqueous humor from the eyes) [38].

Feline enteric coronaviruses are further classified into two serotypes based on reactivity to neutralizing antibodies: Type I and Type II [39]. FECV Type I is the more common of the two

serotypes, (80-90% of clinical cases); however, serotype II was reported at a 30% prevalence in Japan [39]. Both serotypes of FECV can cause FIP. FECV serotype II emerged from a double recombination event following cross-species transmission of a cat infected with both FECV type I and Canine Coronavirus (CCoV) serotype II [2, 4, 39, 40]. In 1996 and 1998, two strains of FECV type II were found to have template switching in the S and M genes, giving rise to viruses that encoded like CCoV on the S-protein but that had M, N, 7a and 7b FCoV type I proteins [4]. This is an example of how through a spillover event and recombination, a new form of an already existing virus emerged.

Ongoing studies of the evolution of SARS-CoV-2, and specific mutations appearing in the SARS-CoV-2 lineages, reinforce the need to better understand the emergence and changes of this novel coronavirus, as well as the potential role(s) of animal species. There are continuing concerns of the possibility of companion or other animals contributing to transmission, maintenance, and/or evolution of the virus. There have been several, confirmed, reverse zoonotic events in zoos, farms, and clinics where animals were infected with SARS-CoV-2 through human caretakers and pet owners. The US Wildlife Conservation Society reported cases in which 4 tigers and 3 lions were infected [41]. According to the CDC and the USDA, other cases of human to animal infection were found in minks, otters, non-human primates, hyenas, coatimundi, binturong, and ferrets [42, 43]. All of these animals have displayed clinical signs including respiratory and gastrointestinal signs, with the worst outcome being death in more exotic species [8, 43, 44].

With concerns of companion animal infections, serosurveys were conducted in Croatia and Minnesota during the early months of the pandemic looking for evidence of prior SARS-CoV-2 infection in feline and canine companion animals using serum ELISAs [45, 46]. The

study in Croatia looked at the occupational risk for vet staff working with cats and dogs that may come from COVID-19 positive households. They tested serum from more than 500 dogs and more than 100 cats using microneutralization (MNT) assays, and used dog serum only for ELISA testing. Using the MNT assay, they determined that there were SARS-CoV-2 neutralizing serum antibodies in 0.31% dogs and 0.76% cats tested, and recorded 7.56% ELISA reactivity from dog sera [46]. The study in Minnesota measured the presence of IgG antibodies in 239 cat and 510 dog serum samples against SARS-CoV-2 N and RBD proteins using ELISAs. The researchers tested against N protein first and detected N-specific antibodies in 1% of dog serum samples and 7.9% (19/239) of the cat serum samples. They further tested the 19 positive cat serum samples, for RBD protein-specific antibodies and determined that 7 of these samples were positive, for a 1.2% (9/510) RBD seropositivity rate [45]. Both studies concluded that cats were more susceptible to SARS-CoV-2 compared to dogs.

Scientists from Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, and the National High Containment Laboratory for Animal Diseases Control and Prevention performed experiments using different laboratory, companion and domestic animals to evaluate concerns regarding which animal species could serve as a reservoir for SARS-CoV-2, how it behaves in other animals, and which model could be used to accurately test the efficiency of antiviral treatments and vaccines for humans [47]. Dogs, chickens, pigs, and ducks were intranasally inoculated and nasal washes and rectal swabs were collected on days 2, 4, 6, 8, 10, 12, and 14 post-infection for viral RNA detection and virus titration. On day 14, sera was collected from all dogs for antibody detection by an ELISA, and of the 6 dogs tested, 4 were seronegative for SARS-CoV-2 [47]. The process of sera collection was repeated for the 3 other animal species. With ferrets, researchers intranasally inoculated and collected nasal washes and

rectal swabs for days 2, 4, 6, 8, and 10 post-infection and euthanized 2 subjects on day 13 to observe for viral RNA detection in the organs. For cats, a similar experiment was performed in which they were inoculated; however, 2 were scheduled to be euthanized on days 3 and 6 post infection to evaluate viral replication in the organs. From this study, the researchers were able to conclude that ferrets and cats are highly susceptible to SARS-CoV-2; dogs have low susceptibility; and pigs, chickens, and ducks are not susceptible to the virus [47]. Several other studies confirmed that cats are among the animals susceptible to SARS-CoV-2 infection [48-50]. Thus, it is important to determine the frequency of SARS-CoV-2 infection in companion animal cats. Furthermore, understanding potential cross-reactivity between SARS-CoV-2-elicited and FECV-elicited antibodies in FCoV-positive cats is important to accurately define SARS-CoV-2 infection in cats through serosurveillance [51].

CHAPTER 3

METHODOLOGY

Samples and Sample Processing

We analyzed convenience samples collected in an unbiased fashion through blood draws from the UGA Veterinary Teaching Hospital in Athens, GA. Blood was collected from feline patients presenting for routine wellness visits or walk-ins. All samples were processed on site at the hospital; serum was transferred into screw cap blood collection tubes and then hand delivered to the lab. Samples were labeled by the hospital with patient ID and characteristics of the patients, such as breed, gender, name, blood test, and/or status of blood draw. A total of 469 samples were collected between Clarke and Walton Counties during the middle of the COVID-19 pandemic (approximately September 2020 to May 2021). All samples were stored at -20°C.

Upon receipt in the lab, the serum samples were thawed, aliquoted into 2 mL screw cap micro centrifuge tubes, and stored at 4°C. Two aliquots of each sample were made; one 2 mL centrifuge tube contained about 100 µL of serum from stock tubes, and the other 2 mL tube containing the reminder for long-term storage at -80°C. Both tubes were labeled with lab identification number, and aliquot dates and volumes were recorded. In some cases, several serum samples were collected from the same patient on multiple visits. In these cases, only the earliest sample was selected for processing and testing. Extra samples were stored at -20°C. After sorting, aliquoting, and annotating, we had 335 unique serum samples to assay for SARS-CoV-2-specific antibodies.

Experiment 1

Samples were tested using an indirect ELISA method. Greiner 96-well, flat-bottom ELISA plates were used for all assays. Plates were coated with 100 µL of purified RDB protein (stock concentration 2.56 µg/µL) per well at a concentration of 0.2 µg/µL (20 µg per plate) diluted in 1x Phosphate Buffer Solution (PBS Corning, 21-040-CV). Plates were covered and incubated overnight at 4°C and either used the next day or stored for up to 4 days before use in an assay. After incubation, plates were washed 3x with 300 µl of 0.05% PBS-T (Phosphate Buffer Solution with Tween 20) using an automated plate washer (BioTek 405 TS washer). Plates were patted dry to remove residual wash buffer, blocked with 200 µl of blocking buffer made from 3% nonfat dry milk in 0.05% PBS-T, incubated for 2 hours at room temperature, and then washed as before. Serum samples were heat-inactivated in a 56°C dry bath for 40 mins before use and diluted in blocking buffer for addition to ELISA plates. Dilutions for all samples, including negative control sera, were repeated in duplicate and started at 1:100 with 10-fold dilution to 10^{-4}. Initial assays using positive and negative control sera, defined the 1:100 dilution as appropriate for a low background with negative serum, while the positive control serum had to be further diluted to avoid saturation of the assay, enable titration of the positive response, and to conserve the unique reagent. Therefore, negative and experimental sera were diluted at 1:100, 1:1,000, and 1:10,000 while the positive control serum was diluted at 1:500 and then 50-fold to 1:25,000, and 1:1,250,000 (Figure 2).

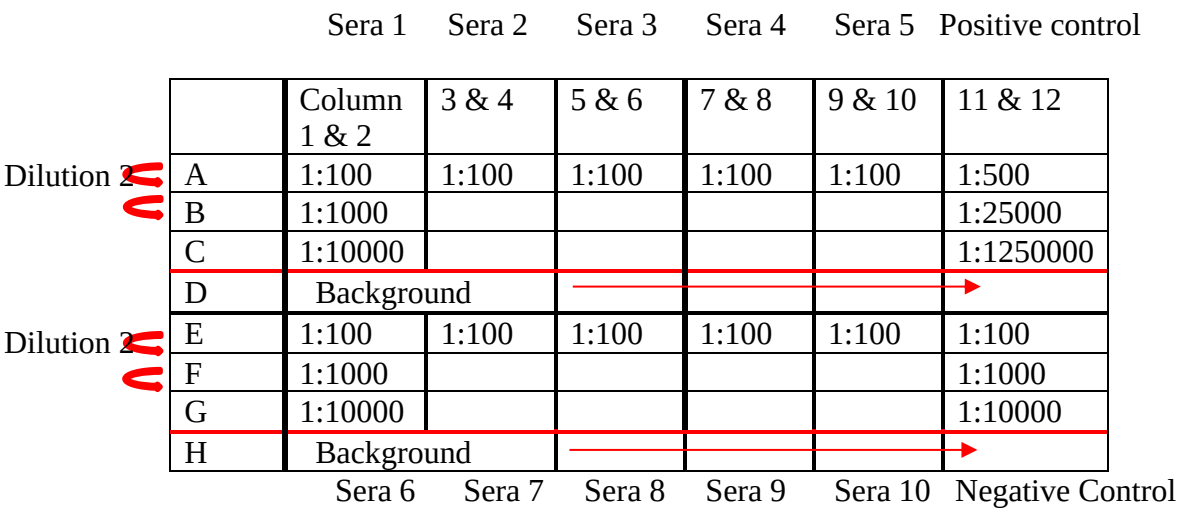


FIGURE 2: Plate layout for 96-well RBD ELISA

Sera were diluted in separate tubes, and then 100 µl was transferred to each well of the coated and blocked ELISA plate in duplicate. The plates were covered, incubated for 2 hours at room temperature, and washed by 0.05% PBS-T. Goat anti-feline IgG HRP-conjugated antibody (Bethyl Laboratories, A20-120AP) was diluted at 1:10,000 in blocking buffer, 100 µL was added per well, and plates were incubated for 1 hour at room temperature. Plates were washed as above with a final rinse with 300 µL of distilled H_2O. Vector Laboratories TMB substrate kit (SK-4400) was used for the detection of horseradish peroxidase activity (HRP), prepared in distilled H_2O following manufacturer's instructions at 50 µl per well, developed for 5 minutes, and then 50 µl of 1 N H_2SO_4 was added to stop the reaction. Plates were read at an absorbance of 450 NM using a BioTek, Cytation7 spectrophotometer.

Experiment 2

Similar to the SARS-CoV-2 RBD ELISA process, Greiner 96-well plates were coated with 100 µL of purified Spike protein (stock concentration 2 µg/µL) per well at a concentration

of 0.2 µg/µL (20 µg per plates) diluted in 1x Phosphate Buffer Solution (Corning, 21-040-CV). When used, all serum samples, including positive and negative controls, were repeated in duplicate, diluted in blocking buffer, and had a starting dilution of 1:100 with 10-fold up to 10^{-7} (Figure 3). Preliminary results for the Spike protein ELISA showed that the positive control did not need to be at a lower concentration than its negative counterpart. Remaining steps of the ELISA for Spike protein were performed exactly as with the RBD ELISA, including the use and dilution of secondary antibody (Goat anti-ferret IgG HRP-conjugated antibody), the final wash process, and TMB preparation and development period. After the 5 minutes, 50 µl of 1 N H_2SO_4 was added to stop the reaction, and plates were read at absorbance of 450 nm by the BioTek, Cytation7 spectrophotometer.

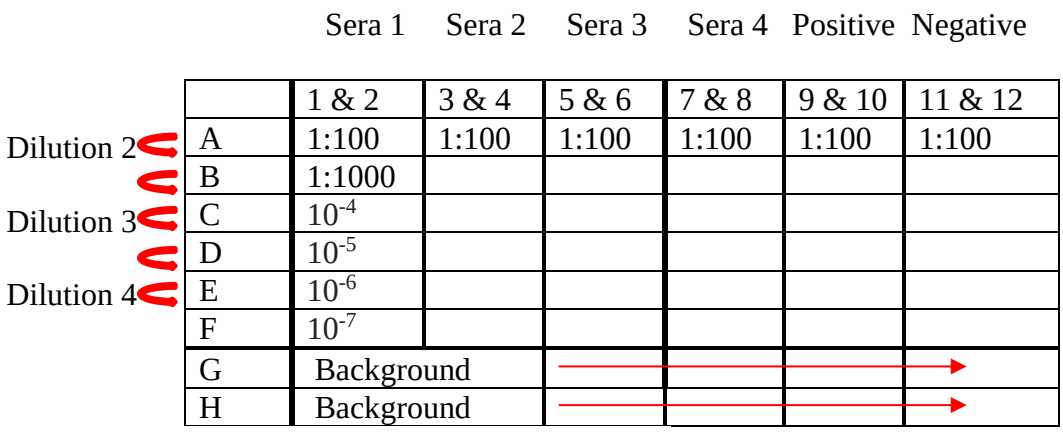


FIGURE 3: Plate layout for 96-well Spike ELISA

CHAPTER 4

RESULTS

The purpose for this study was to define the seropositivity rate of companion animal cats for SARS-CoV-2. We utilized convenience samples from the UGA Veterinary teaching hospital to test 335 unique serum samples collected over the span of 15 months for positive antibodies against SARS-CoV-2 RBD and Spike proteins. We utilized an ELISA, with serum from an experimentally infected cat as the positive control. Our positive and negative (serum from an SPF cat) control sera were included on every plate to enable comparison across plates and assays. As seen in Supplemental Figures, the raw optical density (OD) readings from each sample, although varying between samples, remained remarkably consistent within samples overall throughout the study, even though experiments were completed over several days and weeks. Rather than analyze all dilutions of serum for each sample we decided to compare the OD value at the 1:1,000 dilution as a middle ground where there would be less possibility for antibody non-specific binding resulting in false positives, but sufficient serum antibody levels to provide positive signal suggesting a true positive test result.

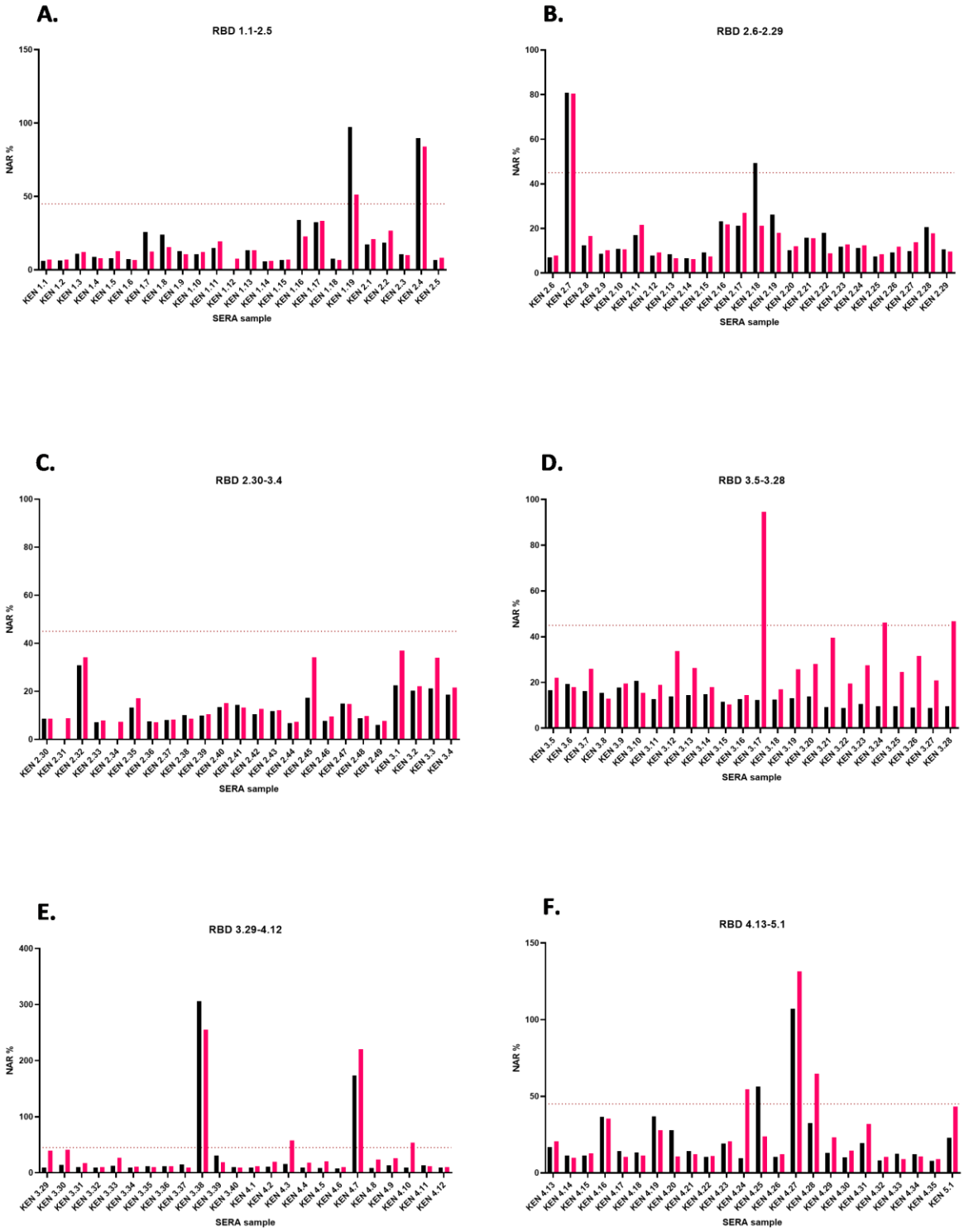
Our first approach to analyze data across plates was to calculate the Stimulation Index (SI) as a way to normalize our data. The net means and standard deviations of the OD values of our background wells were calculated. We applied the calculations from our background into the formula (avg mean+3*SD), and found the difference in values for the samples after subtracting each OD value by the sum of the formula. Using the difference in our 1:1000 dilution rows, we divided by the average difference of the 1:1000 negative control and found the SI for each

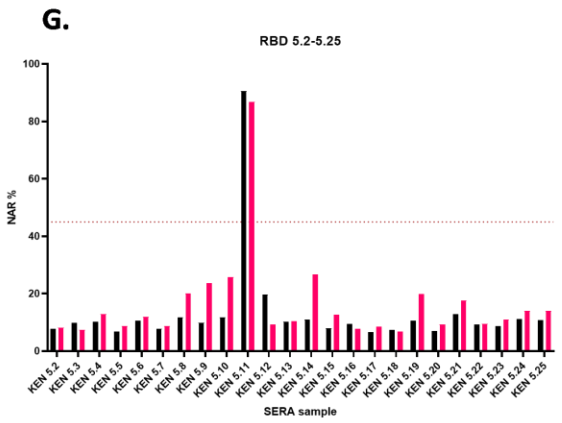
sample. After reviewing all the SI values as a whole, this was determined to not be the best analysis approach. Several samples had a much higher SI value than the positive control, while other SI values were negative even if they appeared positive on the plates. This was because small changes in the negative control (background) could result in large changes in the SI from plate to plate. When the background control had an atypically high value in one of the wells, the SI could be negative, thus, skewing the results and making it difficult to compare inter- and intra-experimental results.

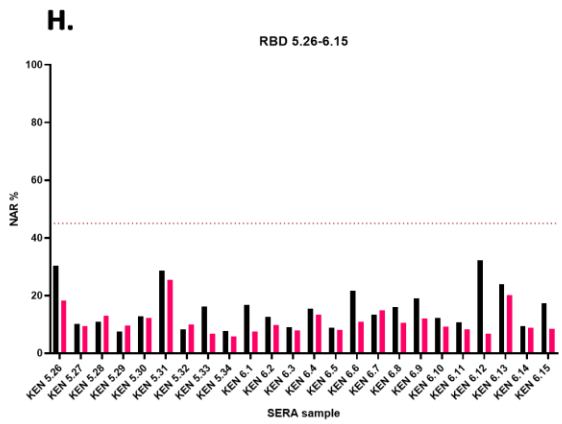
We then utilized the Normalized Absorbance Ratio (NAR) as an alternative approach to normalize all of our ELISA data. Similar to the Simulation Index, NAR normalizes the samples but uses the positive control value as the constant across all the plates. Ramanakumar *et al.* compared the use of NAR and SI for 2,000 ELISAs for the diagnosis of Human Papilloma Virus [52]. They screened cervical specimens for HPV and, afterwards, tested for antibodies against HPV-16 proteins. With NAR, the authors were able to control variations in intra- and interassay performance and minimize measurement errors [52]. There was a better expression of seroreactivity even when comparing the control serum in the same and other ELISA plates, in replicates, and with different dilutions.

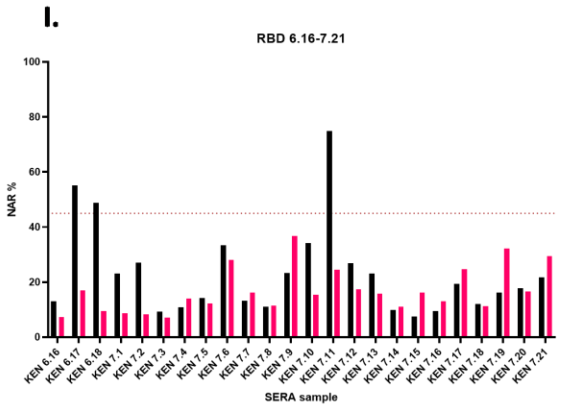
To calculate NAR for our RBD portion of the serostudy, we divided the net mean OD reading of each sample at 1:1,000 dilution by the mean OD reading of the positive control at 1:25,000. To further ensure that our results were consistent, sera were tested a second time at a later date and compared against previous runs. Figures 3 and 4 present the NAR values for each serum sample run twice (red vs black bars/symbols), in duplicate. The majority of samples had NAR values <25% against RBD antigen (Figure 4). This signal was comparable to the negative control serum at the same dilution (Supplemental Figures). Based upon variability of our

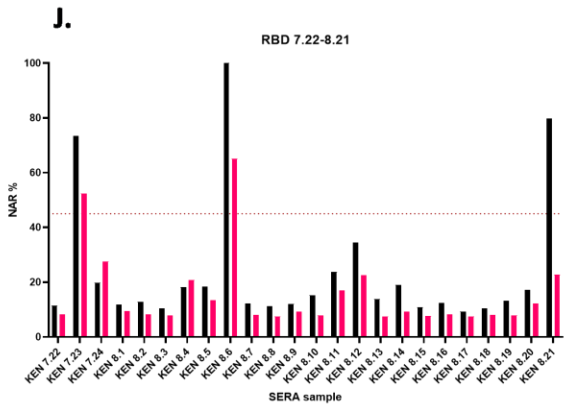
"negative" responses we set our threshold for a positive result at a NAR of 45%. We selected serum samples with ≥45% NAR in at least one assay. Of the 335 serum samples tested against SARS-CoV-2 RBD protein, 25 samples were positive for a 7.5% RBD seropositivity rate. In the study by Dileepan *et al*. [45] the authors observed a 7.9% positivity rate in the N protein screened ELISA of cat serum. Based upon this similar seropositivity rate for our primary screen, we moved forward with secondary testing of the 25 samples with >45% NAR.

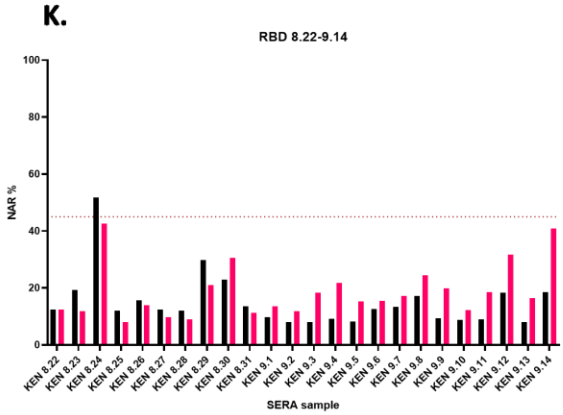

A.
RBD 1.1-2.5
NAR %
SERA sample
B.
RBD 2.6-2.29
NAR %
SERA sample
C.
RBD 2.30-3.4
NAR %
SERA sample
D.
RBD 3.5-3.28
NAR %
SERA sample
E.
RBD 3.29-4.12
NAR %
SERA sample
F.
RBD 4.13-5.1
NAR %
SERA sample

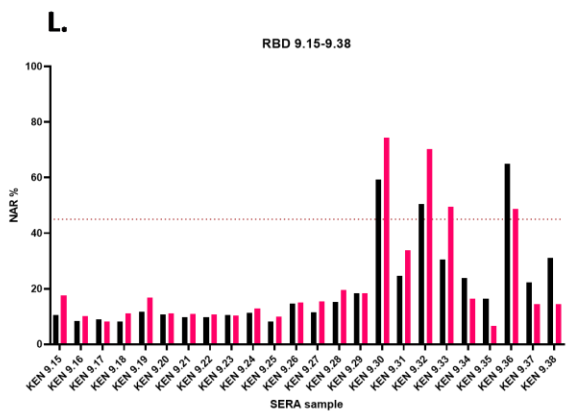

G.
RBD 5.2-5.25
NAR %
SERA sample


H.
RBD 5.26-6.15
NAR %
SERA sample


I.
RBD 6.16-7.21
NAR %
SERA sample


J.
RBD 7.22-8.21
NAR %
SERA sample


K.
RBD 8.22-9.14
NAR %
SERA sample


L.
RBD 9.15-9.38
NAR %
SERA sample

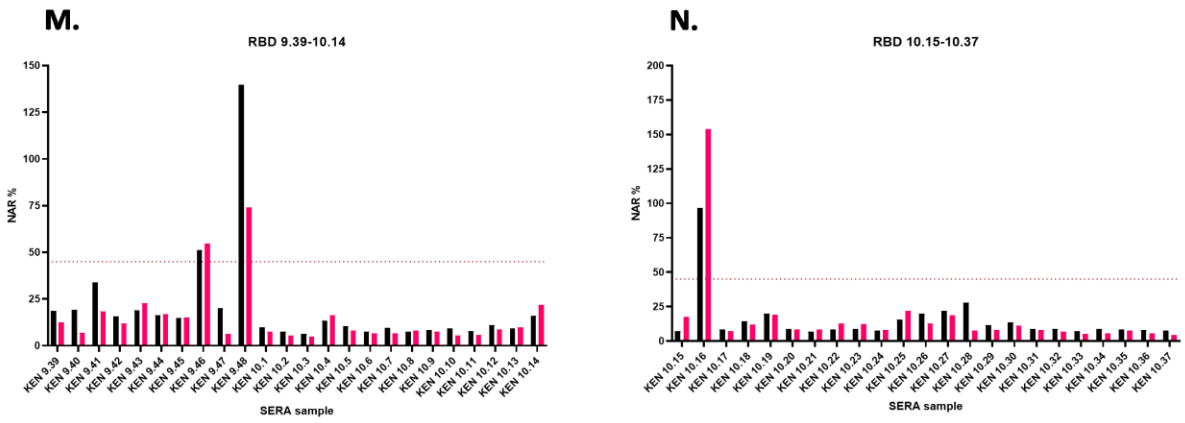


FIGURE 4: Normalized Absorbance Ratio for samples 1.1-10.37
NAR values at 1:1000 dilution were plotted to compare the consistency of samples during runs at different time points. First and second runs are distinguished in red and black. The average between each replicate was calculated and most samples remained relatively similar in value no matter the run. The red dotted line indicates our cutoff value.

The RBD positive serum samples were further tested by ELISA for antibodies against Spike protein to verify the potentially positive result and confirm that these were truly positive against SARS-CoV-2. The serum samples were diluted from 1:100 to 1:10^{-7}, and binding to RBD was compared to binding to full-length, soluble, Spike protein. Figure 5 shows the OD values for the full dilution curve for each serum sample against RBD (A and C) and Spike (B and D). We again calculated NAR values for each sample, normalizing the 1:1,000 experimental sample dilutions to the 1:1,000 positive control serum sample. Figure 5E shows the NAR values for RBD (black) and Spike (red) antigens for each serum at a 1:1,000 dilution. Where both RBD and Spike signals are high, we interpret this to indicate SARS-CoV-2 specific serum antibodies and a true seropositive result. Where RBD is positive, but Spike is negative, we interpret this to be a false positive result, likely due to cross-reactive antibodies binding to the highly-conserved RBD protein. Based upon these assumptions, of the 25 selected samples we ran against Spike and RBD, we defined 4 samples (1.2% of cat sera) to be true SARS-CoV-2-reactive samples.

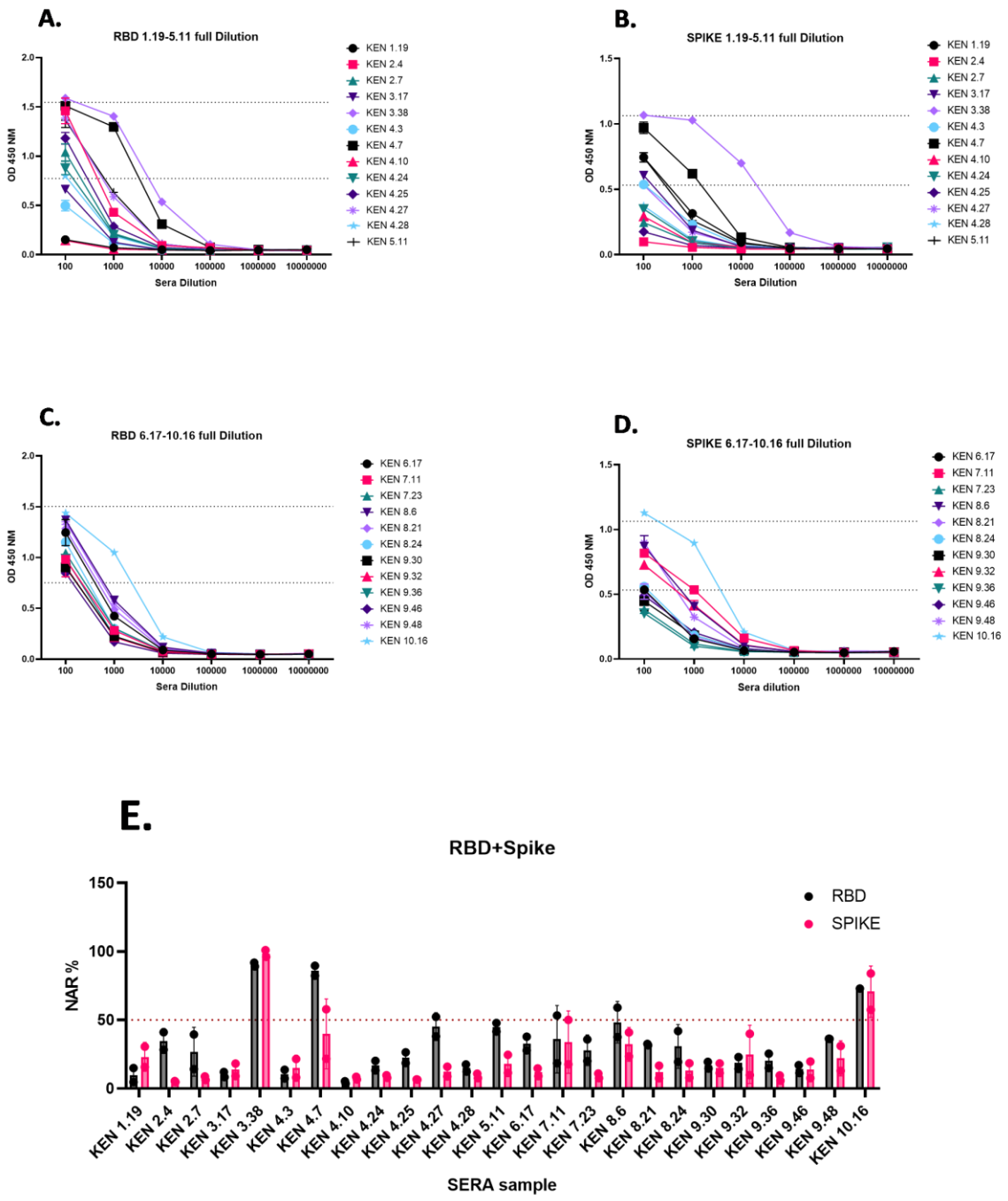


FIGURE 5: Normalized Absorbance Ratio of Full Dilution Spike vs. Receptor Binding Domain ELISA

A-D show optical density in RBD (A, C) and Spike (B, D) ELISAs with serum samples diluted as indicated. E shows the average NAR at 1:1,000 dilution for each sample with the red dotted line indicating our cutoff value. Red indicates Spike protein, while black indicates RBD protein.

KEN 3.38, 4.7, 7.11, and 10.16 were positive for RBD and Spike binding, with NAR RBD and Spike values at 89% and 96%, 82% and 58%, 18% and 50%, and 73% and 84%, respectively. We received information from the hospital regarding age, gender, collection date, and presenting concerns at the time of blood collection for the 25 RBD positive samples as seen in Table 1. Sample KEN 3.38 is a 2-year-old, neutered male who presented for positive toxoplasmosis diagnosis, gastrointestinal issues, and chronic coughing, with a confirmed parasitic infection. There wasn't any available information for KEN 4.7. KEN 7.11 is a 10-year-old, neutered male who presented for ureteral obstruction, with no underlying conditions. KEN 10.16 is a 1-year-old, healthy, spayed female who presented no complaints and needed bloodwork for an upcoming procedure. In review of the companion animal data, there are no notable consistencies between the SARS-CoV-2 Spike seropositive cats. However, the number of positive samples/cats is too low to derive even potential associations.

CHAPTER 5

DISCUSSION

According to *National Geographic* and the USDA, there have been animals in farms, zoos, and even aquariums testing positive for COVID-19 for the first time, with some animals developing severe clinical signs, succumbing to infection, or requiring euthanasia due to complications [43, 44]. Several groups have conducted serosurveillance studies to test the frequency of SARS-CoV-2 infection in agricultural, zoo, sport, and companion animals with the goals of assessing risk to these animals and the potential for zoonotic and reverse-zoonotic infections. As mentioned previously, there were two studies conducted in Croatia and Minnesota to determine the presence of antibodies in cats and dogs, where in researchers looked at either the N- and Spike proteins, or the N-protein and RBD [45, 46].

Similar to my research, both studies used hospital-acquired samples to assess seroreactivity to SARS-CoV-2 antigens using ELISAs. However, the Croatian study tested cat serum using a virus microneutralization test, while the study in Minnesota tested against N protein and then RBD protein to define seropositive cats. While the nucleocapsid (N) protein is immunogenic and has been widely used in serological assays for SARS-CoV diagnostics, it is more relatively highly conserved across coronaviruses [53, 54] and may detect cross-reactive antibodies. However, the authors of the Minnesota study suggested that their N-based ELISA was more specific and sensitive than the RBD ELISA to detect SARS-CoV-2 antibodies in pet cats [45]. Nonetheless, our seropositivity rate of 1.2% was comparable to the feline seropositivity rate by MNT assay from the Croatian study (0.76%) and the N and RBD seropositivity rate of

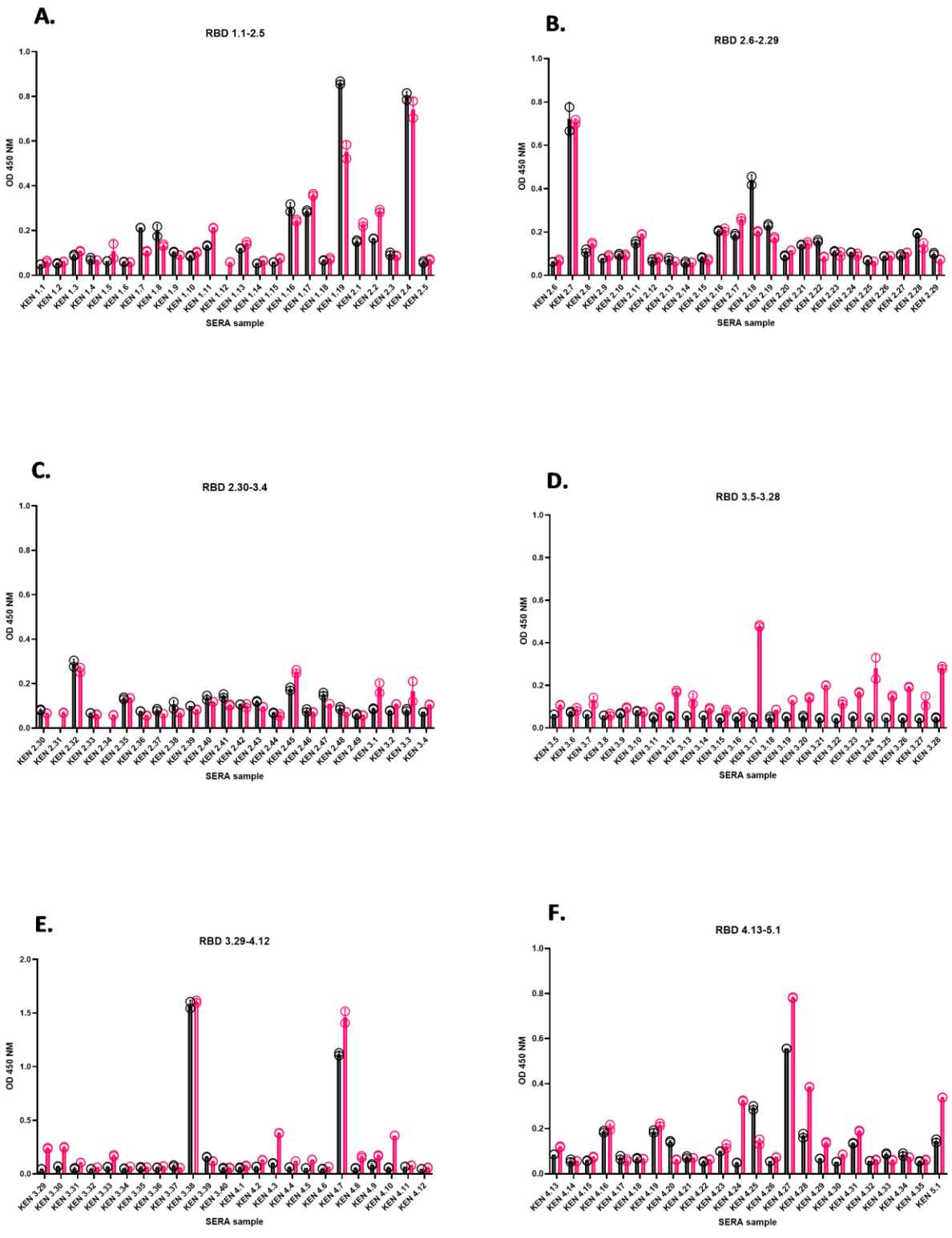
1.25% in the Minnesota study. Thus, we believe our results to be representative of the SARS-CoV-2 seropositivity rate for companion animal cats.

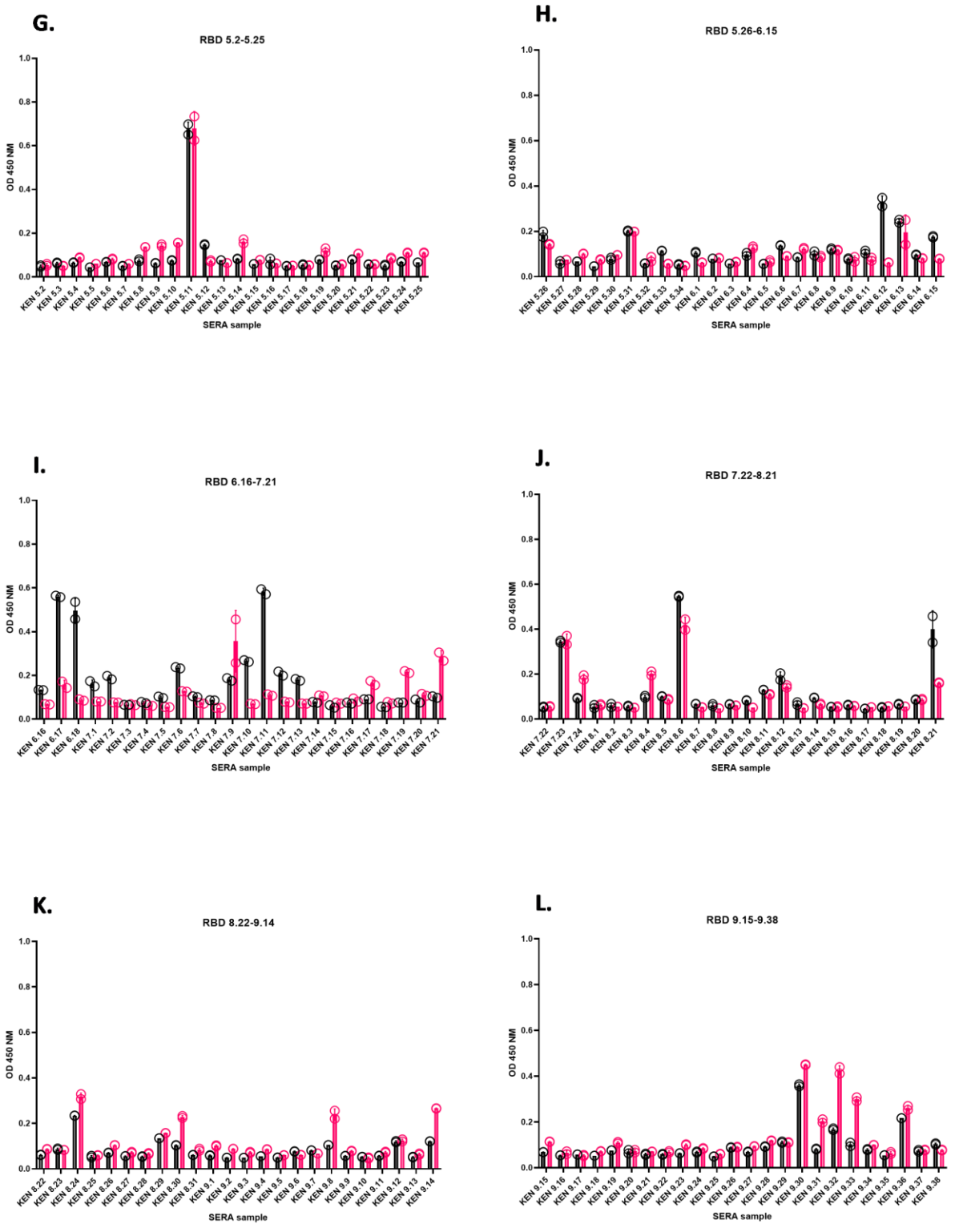
Nonetheless our study did have some limitations. First, it is possible that the positive results we achieved were due to FECV infection and a cross-reactive antibody response. We planned to address this possibility by testing our 25 RBD positive samples against a FCoV-I RBD ELISA, but expression of the recombinant proteins was unsuccessful. A second limitation was the use of convenience samples; in this case, the sera were discarded diagnostic samples. Using serum samples from cats being treated for respiratory infection or another infectious disease could increase the likelihood of identifying as false positive samples. Lastly, the samples assessed here were collected early during the pandemic, prior to the emergence of SARS-CoV-2 variants having altered spike sequences and increased transmissibility. It is possible that these new variants have increased the potential to transmit to and infect cats, and so our study underestimates the risk associated with SARS-CoV-2 variant infection of companion animal cats or subsequent reverse-zoonotic infection of humans.

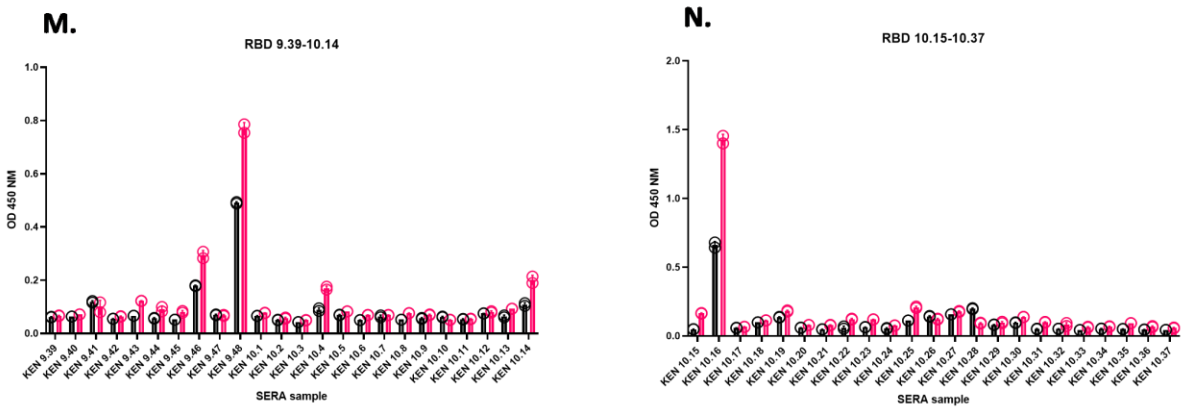
A study published in 2022 by Ghent University in Belgium assessed the cross-reactivity of SARS-CoV-2 antibodies against FECV, OC43, and 229E [54]. The two aims of this study were as follows: 1.) to evaluate the frequency at which coronavirus cross-reactive antibodies and cross-neutralization are formed between a panel of pre-pandemic human plasma samples and SARS-CoV-2 and a panel of Covid-19 sera towards HCoVs, and 2.) to profile the antibody response against the most conserved part of S2 in both panels [54]. They achieved this by using pepscan analysis and located a specific 12-mer peptide that encompasses the SARS-CoV-2 S2′ cleavage site and N-terminal of the Fusion Peptide, which showed high cross-reactivity. Researchers then isolated COVID-19 antibodies specific to the spike region, and evaluated the

binding and neutralizing abilities against HCoV-OC43, HCoV-229E and a FCoV. They found from the 12-mer peptide that two peptides, PEP71 and PEP72, showed higher reactivity, considering that they are homologous to SARS-CoV-2 PEP3. Seeing as how PEP3 is well-conversed and highly immunogenic, they decided to further test against a non-human coronavirus, FECV. They found that all seropositive FECV samples showed reactivity to PEP3 (in SARS-CoV-2), PEP71 (in OC43), and PEP72 (in 229E), and concluded that FECV elicits antibodies that cross-react to these three peptides [54]. Thus, it is possible that the antibody responses we detected were due to cross-reactivity to FECV spike peptides.

To conclude, there was low presence of anti-SARS-CoV-2 antibodies in the hospital samples collected between May 2020 and August 2021 of the pandemic, suggesting a low risk of spillover from humans to feline companion animals. Continued studies assessing risk associated with SARS-CoV-2 infection of cats and other companion animals are needed. For future consideration, the next studies should confirm the specificity of these results using FeCoV proteins against our 25 positive SARS-CoV-2 RBD samples. Another consideration would be to include factors used in the previously mentioned studies, such as analysis of our 335 serum samples for antibodies against SARS-CoV-2 N protein, and consider using Spike proteins from other human coronaviruses. Any further testing may provide insight into cross-reactivity, evolution of spike protein, and the jump from host-to-host, especially as the pandemic continues to evolve.


A.
RBD 1.1-2.5
OD 450 NM
SERA sample
B.
RBD 2.6-2.29
OD 450 NM
SERA sample
C.
RBD 2.30-3.4
OD 450 NM
SERA sample
D.
RBD 3.5-3.28
OD 450 NM
SERA sample
E.
RBD 3.29-4.12
OD 450 NM
SERA sample
F.
RBD 4.13-5.1
OD 450 NM
SERA sample


G.
RBD 5.2-5.25
H.
RBD 5.26-6.15
I.
RBD 6.16-7.21
J.
RBD 7.22-8.21
K.
RBD 8.22-9.14
L.
RBD 9.15-9.38
OD 450 NM
SERA sample



SUPPLEMENTAL FIGURES: Optimal Density readings of samples 1.1-10.37
Red bars represent first ELISA runs with samples, while the second, more recent, runs are colored black. Samples were run in duplicates and data points of each sample at 1:1000 dilution are represented in open circles over their respected bars. Runs were plotted side by side to compare consistency.

Table 1: Characteristics of 25 selected feline samples and presenting complaint

Lab ID	Gender	Breed	Age (yrs)	Collection date (MM/YYYY)	Presenting Complaints
KEN 1.19	M/N			08/2020	CKD/pancreatitis obstipation/IBD
KEN 2.4				06/2020	research cat
KEN 2.7	M/N		13	08/2020	hepatopathy
KEN 3.17	M/N		13	02/2021	Hypertrophic cardiomyopathy
KEN 3.38	M/N		2	04/2021	toxo +/Gi signs/chronic cough
KEN 4.3	M/N	DSH	18	01/2021	Small T cell (CD3+) lymphoma
KEN 4.7				12/2020	
KEN 4.10	M/N		6	12/2020	hemolytic anemia
KEN 4.24	F/S	DSH	4.5	01/2021	facial mass
KEN 4.25	F/S	Mcoc	8	12/2020	renal lymphoma/CKD
KEN 4.27	F/S	Ocicat	7	12/2020	chronic bronchitis or feline asthma
KEN 4.28	M/N	Siam	10	01/2021	CKD/bilat subs
KEN 5.11	M/N	DMH	14	10/2020	hyperthyroid
KEN 6.17	M/N	DSH	16	12/2020	central vestibular dz
KEN 7.11	M/N	DSH	10	08/2020	ureteral obstruction
KEN 7.23	M/N	DSH	7	08/2020	LSA/Lymphadenopathy
KEN 8.6				01/2021	
KEN 8.21	M/N	DLH	15	01/2021	CKD
KEN 8.24	M/N	DSH	9	01/2021	neuro/cardio/syncope
KEN 9.30	M/N	DSH	13	06/2021	prior kidney xplant/herpes flare/neuro dz
KEN 9.32	M/N	DSH	6	06/2021	swollen eye/biliary obstruction
KEN 9.36	F/S	DLH	5	05/2021	lily exposure
KEN 9.46	F/S	DMH	18	05/2021	hemoabdomen
KEN 9.48	F/S	DSH	4	07/2021	chronic gingivitis/stomatits
KEN 10.16	F/S	DSH	1	08/2021	Preop bw

Aside from listing gender, breed and age for the 25 samples selected for further ELISA testing, the dates the samples were collected and the presenting complaint for that hospital visit on those dates are listed.

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