Molecular Biology and Epidemiology of Human and Feline Coronaviruses

by

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Abstract

Coronaviruses (CoVs) are RNA viruses belonging to the *Coronaviridae* family and known to infect various hosts such as pigs (porcine epidemic diarrhea virus), cats (feline infectious peritonitis virus), birds (avian coronavirus), and even humans (human coronavirus, HCoV) including severe acute respiratory syndrome coronavirus SARS-CoV-2 with different disease severity. Emerged in 2019, the human coronavirus, SARS-CoV-2, has caused millions of deaths worldwide which radically changed our lives and influenced our lifestyles and habits. Feline coronavirus (FCoV) causes feline infectious peritonitis (FIP), a disease of felids that has been considered irremediably deadly.

A functional surveillance system remains fundamental to understanding the currently circulating virus, the evolution of the virus, risk factors for severe disease, and for taking public health measures. Monitoring mammalian domestic and wildlife populations for coronavirus will not only provide information about the health of threatened or endangered species but also be needed to understand how coronavirus is spread between humans and animals. Designed PCR in combination with fluorescence resonance energy transfer (FRET) could enable efficient diagnosis and active surveillance for mutations and variants. This surveillance effort is essential to provide sustainable platforms for pandemic control, targeted drug discovery, and the development of safe and effective antivirals to combat coronaviruses.

Knowing SARS-CoV-2 variants is vital for formulating effective control policies during the pandemic, so we developed a rapid diagnostic technique, a reverse transcription FRET-PCR technique. This technique targeted the two most common mutations in the SARS-CoV-2: A23403G in the genome coding for spike protein and C14408T in the genome coding for RNA-

dependent RNA polymerase. The RT FRET-PCRs identified the mutants from the classic variant in ATCC control viruses and feline and human clinical samples based on a high-resolution melting curve analysis.

During the COVID-19 pandemic, active surveillance is essential to determine the prevalence of circulating variants of SARS-CoV-2. The Delta RT-FRET-PCR can diagnose COVID-19 patients and simultaneously identify if they are infected with the Delta variant. After confirming the performance of FRET RT-PCR, we also investigated using this technique to see if this target-specific design of RT-FRET-PCR could detect all SARS-CoV-2 strains and simultaneously identify the Delta variant. Delta RT-FRET-PCR established in this study could detect as few as ten copies of the DNA target and 100 copies of the RNA target per reaction performed on reference SARS-CoV-2 strains and human nasal swab samples positive for the Delta and non-Delta strains. The melting temperature of the PCR products obtained for SARS-CoV-2 Delta variants (around 56.1 °C) was consistently higher than the same for non-Delta variants (about 52.5°C).

To provide more complete data on SARS-CoV-2 infections in dogs and cats in the United States, we conducted a serosurvey on convenience serum samples from dogs (n=1,336) and cats (n=956) collected from 48 states of the USA in 2020. An ELISA targeting the antibody against nucleocapsid identified eleven positive and two doubtful samples in cats and five positive and five doubtful samples in dogs. A surrogate neutralization assay detecting antibodies blocking the attachment of the spike protein to ACE2 was positive, with three of the ELISA positive and doubtful samples and one of 463 randomly selected ELISA negative samples. These four positive samples were confirmed by SARS-CoV-2 virus neutralization testing. The four positives were from cats in New York (n=1), Florida (n=1), and New Jersey (n=2). The serosurvey results,

one of the largest yet completed on dogs and cats globally, support the World Organization for Animal Health (OIE) and the Centers for Disease Control (CDC) positions that currently, there is no evidence that pets play a substantial role in the spread of SARS-CoV-2 in humans.

While serological and molecular evidence of SARS-CoV-2 infection has been reported in white-tailed deer (*Odocoileus virginianus*) from the USA, deer sera from the U.K. (n=1,748) were found to be negative by a serosurvey. To further understand the geographical distribution of SARS-CoV-2 infected deer, a serosurvey was performed on archived deer serum samples collected from the Auburn University Captive Facility in Camp Hill, Alabama, between Oct 2019 and Jan 2022. A surrogate SARS-CoV-2 virus neutralization test identified one positive sample, which was later determined to be negative by the virus neutralization testing performed at USDA National Veterinary Services Laboratories. In addition, rectal and nasopharyngeal swabs from deer collected in January and February 2022 were negative by SARS-CoV-2 PCR. Of 72 people who had close contact with the deer over the study period, 29 completed a voluntary questionnaire that showed three had been infected with the SARS-CoV-2 during the study period. Our finding was that the deer we studied appeared not to have been exposed to SARS-CoV-2 despite human infections in the facility.

While antiviral drugs such as GS441524 have been used to treat FIP in some countries, such as Australia and the UK, no Food and Drug Administration (FDA)-approved drugs are available to veterinary clinicians for FIP treatment in the USA. In addition, limited data is available for the antiviral efficacy and toxicity of these antiviral drugs against FIPV. We studied six types of antiviral drugs for their cytotoxicity, effect on the cell, and antiviral efficacies in Crandell Reese Feline Kidney (CRFK) cells. The GS441524 molecule showed inhibition of FIPV replication irrespective of initial inocula (2.5×10^3, 2.5×10^2, 2.5×10^1 $TCID_{50}$) and

incubation period (48 and 72 hours). No significant difference was observed in the FIPV inhibition for 24, 48, and 72 hours with 98-99% inhibition by GS441524 (25 μM) as long as the drug was applied at the time of or immediate after the FIPV inoculation. Cytotoxicity assay and viability assays showed that six drugs were safe to be used with essentially no cytotoxicity with the concentration as high as 250 μM for Ruxolitinib, 125 μM for GS441524, 63 μM for Teriflunomide, Molnupiravir, and Nirmatrelvir, and 16 μM for Ritonavir. In the dose-response analysis conducted in CRFK cells, GS441524, Nirmatrelvir, and Molnupiravir were identified as the top three drugs with selectivity for FIPV with SI values of 165.54, 113.67, and 29.27, respectively.

In conclusion, we developed and validated specially designed RT-FRET-PCR to detect and differentiate SAR-CoV-2 mutants from classical strains and the Delta variant of SARS-CoV-2. Highly sensitive and specific diagnostic assays established in this work can be used for further active surveillance and longitudinal studies and better understand the ecology of SARS-CoV-2 in domestic and wild animals. In the surveillance conducted on domestic animals, no evidence was found that cats and dogs play a substantial role in the spread of SARS-CoV-2 to humans. In the serological and molecular surveillance conducted on deer, no evidence has been found that deer play a role in spreading SARS-CoV-2 in humans. The FIPV antiviral efficacy study showed that three of six tested drugs (GS441524, Nirmatrelvir, and Molnupiravir) are safe antivirals strongly effective in inhibiting FIPV replication. This data suggest that Nirmatrelvir and Ritonavir may bring new hopes for FIPV treatment besides GS441524 and could be an alternative to treat infection with GS441524-resistant FIPV strains in cats. The in vitro antiviral efficacies of six drugs from this work warrants future studies to explore further their treatment efficacies in vivo and side effects in FIP therapy.

The information gained so far on FCoV could be of interest and might give ideas on different pathogenic aspects of SARS-CoV-2 that are still unclear and vice versa. This sharing of knowledge may serve as a basis for the rapid development of therapeutics for COVID-19, such as GS441524, as well as for studies on the possible interaction between FCoV and SARS-CoV-2 which may occur due to the close relationship between people and cats as companion animals.

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List of Abbreviations

CC_{50}=50% cytotoxic concentration

EC_{50}=50% effective concentration

CCoVs=Canine Coronaviruses

CDC=Centers for Disease Control and Prevention

COVID-19=Coronavirus Disease 2019

CoVs=Coronaviruses

CPE=Cytopathic effect

CRFK=Crandell Reese Feline Kidney (*Felis catus* kidney cells)

CTD=C terminal domain

E=Envelope

ER=Endoplasmic reticulum

ERGIC=Endoplasmic reticulum-golgi intermediate compartment

fAPN=Feline aminopeptidase N

FCoVs=Feline Coronaviruses

FDA=Food and Drug Administration

FECV=Feline Enteric Coronavirus

FIPV=Feline Infectious Peritonitis Virus

FIP=Feline Infectious Peritonitis

FRET=Fluorescence Resonance Energy Transfer

hpi=Hours post-inoculation

HCoV-229E=Human Coronavirus 229

HCoV-NL63=Human Coronavirus NL63

ICTV=International Committee on Taxonomy of Viruses

IFN=Interferons

Kb=Kilobases

kDa=Kilodaltons

M=Membrane

N=Nucleocapsid

NSPs=Non-structural proteins

NTD=N terminal domain

OIE=The World Organization for Animal Health

ORFs=Open reading frames

PCR=Polymerase chain reaction

PEDV=Porcine Epidemic Diarrhea Virus

PFU=Plaque forming unit

pp=Polyproteins

PPV=Positive predictive value

RdRp=RNA-dependent RNA polymerase

RT=Reverse transcription

S=Spike

SARS-CoV=Severe Acute Respiratory Syndrome CoV

SI= Selectivity indices

SPs=Structural proteins

ssRNA+=Single-strand positive-sense RNA

$TCID_{50}$=50% tissue culture infective dose

TGEV=Transmissible Gastroenteritis Virus

TRS=Transcription regulatory sequence

UTR=Untranslated region

WHO=World Health Organization

Chapter 1 Literature review

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1.1 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)

1.1.1 Overview

1.1.1.1 Introduction and history

The 'Severe Acute Respiratory Syndrome coronavirus type 2' (SARS-CoV-2), the causative agent of coronavirus disease 2019 (COVID-19), was first identified in China in late December 2019. This virus belongs to the *Betacoronavirus* genus of the *Coronaviridae* family. Coronaviruses have the largest genomes (26–32 kb) among RNA viruses (Mousavizadeh and Ghasemi 2021). The distinguishing feature of coronaviruses is the club-shaped spike-like projections emanating from the surface of the virion. These projections appear like a crown under the electron microscope, prompting the name coronaviruses as "corona" means "crown" in Latin (Fehr and Perlman 2015). The interspecies transmission of these animal viruses to human beings is considered an emerging threat to human health. Wild bats are considered a reservoir of coronaviruses among other animals due to the sequence similarity of some of their coronaviruses to human coronaviruses (Poon, Chu et al. 2005).

Since the first confirmed case of COVID-19 until September 2022, this disease has devastated the world's population, with a global pandemic resulting in more than 607 million confirmed cases and almost 6.4 million deaths worldwide (https://covid19.who.int). Since the influenza pandemic of 1918, this situation rapidly leads to managing the clinical syndrome elicited by SARS-CoV-2, the primary stressor for all healthcare systems worldwide. Many countries continue to endure several waves of outbreaks of COVID-19.

SARS- CoV- 2 transmission, primarily through cough or sneeze droplets from an infected person, is the primary driver of the worldwide spread of this disease in the current pandemic, with a latency period of about 2–14 days. The patient's clinical presentation after

infection varies from asymptomatic to severe, with most SARS-CoV-2 conditions not being severe (Wu and McGoogan 2020). The leading causes of death associated with COVID- 19 are respiratory failure, septic shock, renal failure, hemorrhage, and cardiac failure (Rai, Kumar et al. 2021). The most common symptoms include fever, dry cough, and dyspnea (Wang, Hu et al. 2020). The less common gastrointestinal symptoms include diarrhea, nausea, vomiting, and abdominal discomfort, with a varied prevalence among different studied populations. However, gastrointestinal symptoms are frequent in hospitalized patients, and these become more pronounced as the severity of the disease increases (Gu, Han et al. 2020). Vaccines have been developed to control the global spread of COVID-19 and have been rapidly implemented worldwide. Non-pharmaceutical interventions such as physical distancing, hand washing, test-trace-isolate, travel restrictions, closures of businesses and schools, and stay-at-home orders also have proved their effectiveness in reducing the spread of the virus before the advent of vaccines (Flaxman, Mishra et al. 2020, Fuller, Hakim et al. 2021, Piovani, Christodoulou et al. 2021).

1.1.1.2 Animal host and spillover

Scientists believe that SARS-CoV-2 emerged due to the viral spillover from animals to humans. The full-length genome sequence of bat coronavirus named 'RaTG13', detected in *Rhinolophus affinis* from Yunnan province, China, is found to be 96.2% identical to that of SARS- CoV-2 (Zhou, Yang et al. 2020). This high genetic similarity between these two viruses supports the hypothesis that bats are possible reservoirs of SARS- CoV-2. Beyond bats, pangolins could be another potential wildlife host linked to SARS-CoV-2, with sequence similarity up to 92.4%. Interestingly, the receptor-binding motif of the RBD of pangolin coronaviruses has only one amino acid variation from SARS- CoV-2 (Xiao, Zhai et al. 2020).

However, the presence of clinical signs and histopathological changes in infected pangolins suggested that pangolins are unlikely to be the reservoir of these coronaviruses.

Studies showed that domestic (dogs, cats) and wild animals (ferrets, lions, mink, pumas, rodents, snow leopards, and tigers) are susceptible to SARS-CoV-2 infection (Bashor, Gagne et al. 2021). However, minks have been shown not only to be susceptible to natural infection of SARS-CoV-2 but also to develop severe illnesses. Since the first report in the Netherlands in April 2020, the outbreak of SARS-CoV-2 infection has been reported in mink firms from other countries, including the USA, Denmark, Italy, Sweden, Canada, Poland etc. characterized by respiratory signs and increased mortality (Oreshkova, Molenaar et al. 2020, Badiola, Otero et al. 2021, Boklund, Hammer et al. 2021, Hammer, Quaade et al. 2021, Oude Munnink, Sikkema et al. 2021). Furthermore, the rapid virus dissemination among minks and the associated mutations has resulted in the emergence of a new mink-associated virus variant, and transmission to humans and further community spread has been described (Devaux, Pinault et al. 2021, Larsen, Fonager et al. 2021). Therefore, minks could represent a potential animal reservoir for SARS-CoV-2 (Sharun, Tiwari et al. 2021).

Recent studies showed white-tailed deer were susceptible to SARS-CoV-2 (Bashor, Gagne et al. 2021). Serological and molecular evidence indicated the exposure of white-tailed deer to SARS-CoV-2 in the USA (Palermo, Orbegozo et al. 2022, Vandegrift, Yon et al. 2022). Thus, new animal reservoirs of SARS-CoV-2 could emerge with a unique potential to maintain, disseminate, and drive the novel evolution of the virus. However, there is a significant gap in our knowledge of susceptible animal host species and potential new reservoirs.

1.1.1.3 Morphology

This virus is an enveloped, positive-sense, single-stranded RNA virus with a genome size of 29,903 nucleotides (Lu, Zhao et al. 2020). The diameter of each virion is 50-200 nm. The virion can exist in different forms- mainly in a spherical shape but also in pleomorphic and oval shapes (Wang, Zhang et al. 2020).

1.1.1.4 Structural proteins

Like other coronaviruses, SARS-CoV-2 has four structural proteins known as the spike (S, 1273 amino acids), envelope (E, 75 amino acids), membrane (M, 222 amino acids), and nucleocapsid (N, 419 amino acids) proteins. These proteins are required to produce a structurally complete viral particle (Lu, Zhao et al. 2020).

The trimeric S protein is a Class I transmembrane protein. This protein mediates binding to host cell-surface receptor angiotensin-converting enzyme-2 (ACE2) and fusion of virus and host cell membranes through its two subunits: S1 and S2, respectively (Ou, Liu et al. 2020). The S1 subunit contains two distinct domains, the N-terminal domain (NTD) and the C-terminal domain (CTD), as the host receptor-binding domain (RBD) (Lan, Ge et al. 2020). It has been found that modifications in S protein may lead to different mechanisms and differential intensity of entry into the host cells.

The structural E protein, the most conserved protein across the studied CoVs, is a small polypeptide with a range of 8.4-12 kDa. This protein consists of the hydrophobic transmembrane domain and the charged cytoplasmic tail. The E protein has been reported as a single- spanning membrane protein with its N- terminus translocated across the membrane, and the C- terminus exposed to the cytoplasmic side. (Alam, Kamau et al. 2020). This protein participates in the

formation of new virions, mostly built from the host cell material. This protein is characterized by a large amount of valine with a lower GC percentage than the analogous structural protein SARS-CoV-1 (Bianchi, Benvenuto et al. 2020).

M-glycoprotein is the most abundant protein in the outer membrane. This multispanning M protein is characterized by three transmembrane domains with its C-terminal inside and N-terminal outside. The amphipathic region at the end of the third transmembrane domain is highly conserved across *Coronaviridae* members. Apart from this region, other regions of M protein show variability in protein sequences (Jiang, Hillyer et al. 2020). Both M and E proteins play a role in viral packaging.

The N-nucleocapsid protein, a structural component of the nucleocapsid, is a phosphoprotein ranging from 43 to 50 kDa. This protein has three conserved domains: N arm, central linker (CL), and C tail. The NTD and CTD are the protein's critical structural and functional domains. The N protein plays a vital role in RNA binding and packaging (Mariano, Farthing et al. 2020).

1.1.2 Variants of SARS-CoV-2

Adaptive mutations play an essential role in altering the virus's pathogenic potential and ability to evade the immune system and complicate vaccine development against the virus (Giovanetti, Benedetti et al. 2021). Like other RNA viruses, SARS-CoV-2 is prone to genetic evolution to facilitate the adaptation to the new hosts and accumulation of mutations over time. This event leads to emerging variants with possibly different characteristics than their ancestors. During the early phase of the pandemic, genetic evolution was minimal; the initial global dominant variant was called D614G and had higher transmissibility (Korber, Fischer et al. 2020).

Since then, multiple variants of SARS-CoV-2 have been identified. Based on the impact on public health, those variants were mainly categorized as variants of concern (VOCs) and variants of interest (VOIs). The Centers for Disease Control and Prevention (CDC) and World Health Organization (WHO) have independently established a classification system for distinguishing SARS-CoV-2 variants into VOCs and VOIs. The WHO uses the Greek alphabet letters to name the variants of SARS-CoV-2.

1.1.2.1 SARS-CoV-2 Variants of Concern (VOCs)

As of September 2022, the WHO updated the nomenclature of five SARS-CoV-2 VOCs since the beginning of the pandemic such as Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2) and Omicron (B.1.1.529) (Table 1.1). All of these reported VOCs, except the Delta variant, have a common amino acid substitution of N501Y in the S protein, which results in increased affinity of the S protein to the ACE2 receptor, enhancing the viral attachment to its host cells. Some reports show that this single mutation alone can increase the affinity between RBD and ACE2 ten-fold compared to the ancestral strain (RBD-N501) (Liu, Wei et al. 2021, Liu, Zhang et al. 2021).

The Alpha variant, with its reported 43%-82%, increased transmissibility, contains 17 mutations in the viral genome, of which eight result in amino acid substitutions in the S protein (Δ69-70 deletion, Δ144 deletion, N501Y, A570D, P681H, T716I, S982A, D1118H) (Davies, Abbott et al. 2021). This variant was reported in the USA at the end of December 2020 with increased severity and mortality of the disease (Davies, Jarvis et al. 2021).

The Beta variant, with an increased risk of transmission and reduced neutralization by monoclonal antibody therapy, harbors nine amino acid substitutions (L18F, D80A, D215G,

R246I, K417N, E484K, N501Y, D614G, and A701V) in its spike protein (Wang, Casner et al. 2021). Among those, three substitutions (K417N, E484K, and N501Y) are located in the RBD with increased binding affinity for the receptor ACE (Mwenda, Saasa et al. 2021). This beta variant was reported in the USA at the end of January 2021.

The third VOC, Gamma variant, contains ten amino acid substitutions in its spike protein (L18F, T20N, P26S, D138Y, R190S, H655Y, T1027I, V1176, K417T, E484K, and N501Y) of which three (L18F, K417N, E484K) are located in the RBD similar to the Beta variant with reduced neutralization by monoclonal antibody therapies (Wang, Casner et al. 2021).

The fourth and deadly VOC, the Delta variant, contains ten amino acid substitutions (T19R, G142D, 156del, 157del, R158G, L452R, T478K, D614G, P681R, D950N) in its spike protein. This variant was first identified in the USA in March 2021 and was the most dominant variant until recently replaced by the SARS-CoV-2 Omicron variant. After its initial detection in India in December 2020, this variant rapidly spread worldwide (Mishra, Mindermann et al. 2021).

The fifth variant, the Omicron, was quickly added to the VOC variant list due to more than 30 changes in its spike protein along with the sharp rise in the number of cases observed in South Africa in late November 2021 (Vaughan 2021). The daily case numbers in the USA skyrocketed to over a million by December due to the spreading of the Omicron variants, with one of its subvariants (BA.5) making up more than 88% of cases. Its increased transmissibility, even more than Delta, is due to more than 30 changes to the spike protein of the virus (Callaway 2021).

1.1.3 Diagnosis of SARS-CoV-2

Identifying individuals infected with SARS-CoV-2, either symptomatically or asymptomatically, with rapid and accurate testing is crucial in containing and mitigating the COVID-19 pandemic and developing appropriate control measures. Following observation of clinical signs consistent with COVID-19, various tests based on detecting SARS-CoV-2 or antibodies to it can be used to diagnose COVID-19 definitively. In addition, screening for variants and their spread is essential in understanding the dynamics of the COVID-19 pandemic.

1.1.3.1 Nucleic acid-based detection tests

The diagnostics gold standard is the nucleic acid amplification test by RT-qPCR for the molecular detection of SARS-CoV-2. Using polymerase chain reaction (PCR) in real-time (RT-qPCR) is the convenient, sensitive, accurate, rapid, and globally accepted testing method for the detection of SARS-CoV-2 infection (Udugama, Kadhiresan et al. 2020, van Kasteren, van der Veer et al. 2020). Many detection kits targeting ORF1b (including RdRp), N, E, M, or S genes are commercially available, with detection times ranging from several minutes to hours, depending on the technology.

Molecular detection is affected by the sample source and disease onset. The viral load is higher in upper respiratory samples, such as oropharyngeal and nasopharyngeal swabs. However, samples can be collected from various respiratory sources, including throat swabs, posterior oropharyngeal saliva, sputum, and bronchial fluid (Corman, Landt et al. 2020, Lu, Wu et al. 2020). Viral nucleic acid can also be detected in blood, urine, and fecal material, even when respiratory samples are negative (Zhang, Du et al. 2020).

Recently, nucleic acid amplification test has included other techniques such as reverse transcription loop-mediated isothermal amplification, isothermal amplification platforms with nicking endonuclease amplification reaction, and transcription-mediated amplification (Kuo, Realegeno et al. 2021).

1.1.3.2 Antigen detection tests

Antigen detection tests are immunoassays that detect the presence of a specific viral protein using nasopharyngeal, pharyngeal, nasal, or throat swab specimens for laboratory-based, point-of-care tests and self-tests (Mina, Parker et al. 2020, Peto and Team 2021). Compared to nucleic acid-based detection techniques such as RT-PCR, antigen detection test is less expensive, mostly giving results in approximately 15–30 min; however, they are generally less sensitive.

The fluorescent immunochromatographic assay is another point-of-care test that is small, and easy to use. It provides results promptly (within 5–20 min) with high sensitivity and specificity in respiratory samples (Porte, Legarraga et al. 2020).

The chemiluminescence enzyme immunoassay is also a rapid (about 30 min) and sensitive point-of-care test to detect antigens of SARS-CoV-2. When the antigen in the sample reacts with the antibody (chemiluminescent substrate), the enzyme converts the substrate to the reaction product, emitting light photons instead of color development. The total emission is read by an automated chemiluminescence analyzer (Rai, Kumar et al. 2021).

1.1.3.3 Antibody detection tests

SARS- CoV-2 serological tests can detect antibodies to N or S protein, which is particularly useful in late phases after disease onset or for retrospective studies. However, available serological tests differ in their sensitivity and specificity.

Enzyme-linked immunosorbent assay (ELISA) is considered the gold standard for laboratory testing for SARS-CoV-2 to assess IgM and/or IgG to one of two viral proteins: S or N. The positive detection rates for the S protein-based ELISA and the N protein-based ELISA are 82.2% and 80.4%, respectively (Liu, Liu et al. 2020). Recently, an ELISA kit was developed targeting the RBD region from S protein with a specificity of 99.3% that could detect many antibodies (Peterhoff, Gluck et al. 2021).

The lateral flow assay-based colloidal gold immunolateral flow chromatography kit, which is robust and simple, is available for serum, plasma, and whole blood to co-detect virus-specific IgG and IgM. This kit has rapid detection in 15 min and requires only 10–20 μl of serum (Li, Yi et al. 2020, Sheridan 2020). There is also a lateral flow immunoassay based on duel-mode quantum dot nanobeads for the simultaneous detection of SARS-CoV-2 specific IgM and IgG (Wang, Yang et al. 2020).

1.1.4 Vaccination

Vaccines provide moderate, but short-lived protection from SARS-CoV-2 infection but are highly effective in controlling hospitalization and deaths associated with COVID-19 (Polack, Thomas et al. 2020, Baden, El Sahly et al. 2021, Voysey, Clemens et al. 2021, Chemaitelly, Ayoub et al. 2022, Taylor, Whitaker et al. 2022). Over 280 vaccine candidates undergo clinical

and pre-clinical trials, categorized as mRNA, inactivated pathogen, protein subunit, viral vector-based, etc. However, as of January 2022, nine vaccines are already being assessed in Phase IV clinical trials, validated for use by WHO, and have obtained Emergency Use Listing (Table 1.2). Among those vaccines, the FDA has currently authorized COVID-19 vaccines from Pfizer-BioNTech, Moderna, Novavax, and Johnson & Johnson's Janssen in the USA.

Although most vaccine regimens are of two doses, a third dose (booster dose) has been included in the vaccination schedule. Studies showed some waning of immunity after two doses, and protection increases after a third dose (Saiag, Goldshmidt et al. 2021).

As of September 2022, more than 12.6 billion COVID-19 vaccines have been deployed worldwide (https://covid19.who.int) to decrease the rate of SARS-CoV-2 infections. However, rising numbers of breakthrough infections may have caused a significant negative impact on global public health security and increased speculation on the possibility of vaccine failure, triggering panic in society.

1.1.5 Treatment

At the onset of the SARS-CoV-2 pandemic, it was urgent to alleviate this new viral illness with experimental therapies and drug repurposing. Significant progress has been made due to the intense clinical research efforts that have resulted in various novel therapeutic options at an unparalleled speed. Available therapeutics under EUA for the management of COVID-19 are i) antiviral medications such as remdesivir, molnupiravir, and ritonavir in combination with nirmatrelvir; ii) anti-SARS-CoV-2 monoclonal antibodies such as sotrovimab; iii) anti-inflammatory drugs such as dexamethasone; iv) immunomodulators agents such as baricitinib, tocilizumab, etc. (Coopersmith, Antonelli et al. 2021). The utility of these treatments is mainly

based on the severity of the illness and certain risk factors. Antiviral medications and antibody-based therapies are likely more effective during the early phase of infection when SARS-CoV-2 replication is greatest before or soon after the onset of symptoms. Anti-inflammatory therapies such as immunomodulating therapies, corticosteroids, or a combination of these therapeutics may help battle the later phase of the illness of hyperinflammatory state, induced by the release of cytokines and the coagulation system's activation (Gandhi, Lynch et al. 2020).

1.1.6 Conclusion

The COVID-19 pandemic had devastating economic and social effects worldwide, with evolving variants of SARS-CoV-2 that will likely remain a part of our lives for many years. Worldwide routine surveillance of SARS-CoV-2 variants and their impact on virulence and currently used therapeutics will require scientists to determine if vaccines and other therapies need to be updated periodically. New emerging variants may penetrate herd immunity, infect unvaccinated individuals, and facilitate vaccine escape. However, most studies have suggested that currently available vaccines are still effective against the currently circulating variants and may provide protection against severe disease outcomes.

1.2 Feline coronavirus (FCoV)

1.2.1 Overview

1.2.1.1 Introduction and historical aspects

Feline coronavirus (FCoV) infection, a common disease in wild and domestic cats, was first called "some important disorders of cats" in 1963 at the Angell Memorial Animal Hospital in Boston, United States (Holzworth 1963). Subsequent studies by electron microscopy

identified a coronavirus in this disease (Ward, Munn et al. 1968). The relationship of FCoV to other previously reported coronaviruses (CoVs) such as human coronavirus 229E and mouse hepatitis virus) was first described by Ward in 1970 after observing viral particles in FCoV-infected animal tissues (Ward 1970). Furthermore, the close relationship of FCoV to other animal coronaviruses in dogs and swine was reported (Pedersen, Ward et al. 1978, Herrewegh, Smeenk et al. 1998). Using autochthonous peritoneal cells, FCoV was first isolated in 1976 (Pedersen 1976). The isolated strain was named "TN-406" (later known as feline infectious peritonitis virus serotype I strain Black or FIPV I Black) (Black 1980). It was first propagated in cell culture using *Felis catus* kidney cells (CRFK) (Pedersen, Boyle et al. 1981). Due to the isolation and propagation difficulties with the virus, few cell culture-adapted strains of FCoV are available. Feline enteric coronavirus (FECV) serotype II strain 79-1683 or FECV II 79-1683 (former WSU 79-1146) and FIPV serotype II strain 79-1146 or FIPV II 79-1146 (former WSU 79-1683) have been used in models to study FCoV since their isolation in 1987 (McKeirnan, Evermann et al. 1987).

FCoV infection usually presents either asymptomatically or is the cause of mild and transient gastrointestinal signs, such as diarrhea. However, up to 10% of cases result in the fatal disease of feline infectious peritonitis (FIP) (Tasker (2018). Due to the virulence of FIPV in infected cats, FCoV has been a focus of study for several decades. Some promising results have been found in several new drug treatments. However, a definitive diagnosis of this infection antemortem is still very challenging (Felten and Hartmann 2019).

1.2.1.2 Taxonomy

According to the International Committee on Taxonomy of Viruses (ICTV), the members of the family Coronaviridae (subfamilies *Orthocoronavirinae* and *Letovirinae*), a monophyletic cluster, belong to the order *Nidovirales* together with the *Arteriviridae*, *Mesoniviridae*, and *Roniviridae* (Lauzi, Stranieri et al. 2020). The diversity of viruses in the main subfamily *Orthocoronavirinae* is encompassed by four viral genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*. The *Alphacoronavirus* and *Betacoronavirus* genera include viruses derived from the bat gene pool and principally infect mammals. Viruses in the *Gammacoronavirus* and *Deltacoronavirus* genera are derived from avian and swine gene pools and infect birds and mammals (Woo, Lau et al. 2012).

Feline coronaviruses (FCoVs) can be grouped with canine coronaviruses (CCoVs) and porcine transmissible gastroenteritis virus (TGEV), as they all belong to the genus *Alphacoronavirus*, subgenus *Tegacovirus*, and species *Alphacoronavirus 1*. According to their serological and genetic properties, FCoVs are classified into types I and II. Recently, a non-taxonomical classification (clades A and B) in *Alphacoronavirus* 1 has been proposed based on the viral spike protein of FCoVs, a significant driver of viral pathogenesis and tropism (Jaimes, Millet et al. 2020). Other distantly related species in the genus *Alphacoronavirus* include human coronavirus 229 (HCoV-229E), human coronavirus NL63 (HCoV-NL63), and porcine epidemic diarrhea virus (PEDV) (Lauzi, Stranieri et al. 2020), ICTV, 2019).

1.2.1.3 Morphology

FCoVs are large, enveloped, single-strand positive-sense RNA ($ssRNA^{+}$) viruses. FCoV structure comprises a helically symmetrical nucleocapsid protecting the viral genome and an

outer envelope (Pedersen 1976). FCoV virions have a size range between 80 and 120 nm, a spherical shape, and a moderate level of pleomorphism typified by club-like spikes (S) of glycoprotein on the virion's surface (about 12-24 nm). The glycoprotein projections give the virus the crown-like appearance for which coronaviruses are named (Barcena, Oostergetel et al. 2009, Fehr and Perlman 2015). The RNA genome of FCoV is approximately 29 kilobases (kb) in length and is consistent with the typical genome organization of coronaviruses (Olsen 1993).

1.2.1.4 Structural proteins, non-structural proteins and accessory proteins

The FCoV genome has 11 open reading frames (ORFs) encoding four structural proteins (namely spike -S, nucleocapsid -N, envelope -E, matrix -M) and seven non-structural proteins (the accessory proteins: 3a, 3b, 3c, 7a, and 7b; the replicases 1a and 1b). The 5′ untranslated regions (UTR) (310 nucleotides) are comprised of the leader sequence and the transcription regulatory sequence (TRS). The core-TRS motif (5′-CUAAAC-3′ core-TRS motif) is conserved in all FCoVs (Dye and Siddell 2005, Tekes, Hofmann-Lehmann et al. 2008). The 3′ UTR (around 275 nucleotides in length, is followed by a poly(A) tail.

Structural proteins play essential roles in viral genome protection and facilitate the interactions of virions with susceptible cells. The S protein (~150 kDa) is a class I viral fusion protein characterized by predominant α-helical secondary structures and a trimeric organization of its pre-fusion and post-fusion state (White, Delos et al. 2008). S protein has two domains- the S1 domain responsible for receptor binding and the S2 domain required to fuse the viral and cellular membranes during viral entry (Bosch, van der Zee et al. 2003). Regarding FCoV pathogenesis, the S protein is considered the most crucial antigenic element among the viral structural proteins due to the role it plays in cell receptor binding and inducing the fusion

between the viral and the cellular membranes (Bosch, van der Zee et al. 2003, Belouzard, Millet et al. 2012, Jaimes and Whittaker 2018). Serotype classification of FCoV S has been established based on antigenic differences of S proteins (Hohdatsu, Okada et al. 1991).

The viral helical nucleocapsid consists of multiple copies of the RNA binding protein N, (50 kDa), which protects genomic RNA by binding the RNA with its two domains: the N-terminal domain (NTD) and C-terminal domain (CTD). The viral envelope protein, E, is small (~ 8 to 12 kDa) in size and is a type III membrane protein. This protein is less abundant than proteins M and S. Both the C-terminal endodomain and N-terminal ectodomain of the E protein have ion channel activity (Fehr and Perlman 2015). The function of the viral E protein has been associated with the assembly at the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) (Kipar and Meli 2014).

M protein is a medium-sized (about ~ 25 to 30 kDa) N-linked glycosylated protein randomly distributed along with the viral envelope and anchored through three transmembrane domains (Masters and Perlman, 2013) (Armstrong, Niemann et al. 1984). However, this protein is less antigenic because of its extensive C-terminal endodomain and small ectodomain (about 10% of the N-terminal portion). During viral maturation and assembly, M protein plays a vital role in viral membrane remodeling at the ERGIC (Neuman, Kiss et al. 2011, Kipar and Meli 2014).

Non-structural proteins (NSPs) are expressed from the replicase gene, which comprises two large open reading frames (ORF) 1a and 1b. The translation of the FCoV replicase gene leads to the production of polyproteins (pp), pp1a, and pp1ab through a ribosome frameshifting mechanism. These polyprotein products are then processed by virus-encoded proteinases into 16 NSPs (Hagemeijer, Rottier et al. 2012, Kipar and Meli 2014). Together, the 16 NSPs of the

replicase complex are involved in viral genome replication and the generation of transcription templates for the structural and accessory proteins (Fehr and Perlman 2015).

Like other alphacoronaviruses, FCoVs possess five accessory genes, *3a*, *3b*, *3c*, *7a*, and *7b*, with typical locations at two different genome positions (Dye and Siddell 2005, Tekes, Hofmann-Lehmann et al. 2008). The first location is in between the S and E genes. While FCoVs and CCoVs possess three accessory ORFs (3a, 3b, and 3c) at this location, TGEV contains only two (3a and 3b) in the exact location. The second location for additional accessory genes is downstream of the N gene preceding the 3' UTR. FCoVs and CCovs contain two accessory ORFs (7a and 7b) at this second location, while TGEV has only one (7a). ORF 3c is well conserved among the alphacoronavirus genus, and an intact 3c gene is necessary for replicating enteric FCoV (Chang, de Groot et al. 2010). The FCoV 3a and 3b proteins comprise 71-72 amino acids and are well conserved within pathotypes. They lack predicted hydrophobic segments, so they are probably located and exert their function in the cytoplasm. However, the role of these accessory proteins remains unknown (Meszaros, Olasz et al. 2018, Acar, Stroobants et al. 2019). ORF 7a encodes a small hydrophobic membrane protein of 101 aa (~10 kDa) with an N-terminal cleavable signal sequence and a C-terminal transmembrane domain and is more or less well-conserved among FCoVs. It has been shown that the 7a protein is a type I interferon (IFN) antagonist and protects the virus from the antiviral activity by interfering with the IFN response (Dedeurwaerder, Desmarets et al. 2013, Dedeurwaerder, Olyslaegers et al. 2014). ORF 7b encodes a soluble glycoprotein of ~24 kDa (207 AA) and is reported to function as a virokine, which acts as an immune-modulator of host immune responses (Kennedy, Abd-Eldaim et al. 2008).

1.2.1.5 Viral entry and replication

The exact mechanism of viral attachment by FCoV to host cells remains unknown. With poor sequence similarity (~30%) of the S1 domains between CoVs serotype I and II, different receptors for cell entry are strongly suggested between those serotypes. Type II FIPVs attach to the cell surface via feline aminopeptidase N (fAPN), a 150-kDa glycoprotein cellular receptor with metalloprotease activity. However, fAPN is not a functional receptor for serotype I (Dye, Temperton et al. 2007), and the primary receptor for type I is still unknown. Feline C-type lectin dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (fDC-SIGN, CD209) has a role as a co-receptor for both FCoV serotypes (Regan and Whittaker 2008, Van Hamme, Desmarets et al. 2011). However, it is unclear if these receptors are crucial solely for FECV binding to target enterocytes or whether they also play a role in the infection of macrophages. FIPVs could enter their target macrophages through the Fc-receptors used for complement binding, promoting FIPV II entry without a fAPN receptor in vitro (Jaimes and Whittaker 2018). The attachment of the viral S protein to the host cell membrane receptor is the key determinant of viral tropism and host range (Cham, Chang et al. 2017).

After binding the viral S protein to the receptor in the host cell membrane, the receptor-bound virus is endocytosed (Burkard, Verheije et al. 2014). The fusion of the viral envelope and host cell membranes is crucial for the virus to establish infection mediated by a fusion peptide in the second domain (S2) of the S protein. Two additional biochemical events are thought necessary to induce the membrane fusion at the endocytic vesicle: protease activation or cleavage of FCoV S protein and a decrease in the pH of the endosome (Fehr and Perlman 2015, Millet and Whittaker 2015). Proteases, such as furin-like proteases, have been shown to mediate

coronavirus activation. Depending on the serotype of the virus, one or more site-specific proteolytic activations may be required.

Type I viruses require two specific activation sites at the S1/S2 boundary and within S2. In contrast, FCoV II viruses are only reported to have the S2 site (Licitra, Millet et al. 2013, Millet and Whittaker 2015). Proteolytic activation is not necessarily limited to the endosome. The cleavage of the S protein can occur at different stages of the virus life cycle depending on the coronaviruses and host cells. The timing of the cleavage event, which can occur during S biosynthesis and virus entry into target cells, is a critical factor in modulating the pathogenicity, cell and tissue tropism, and host range (Millet and Whittaker 2015). Furin-like proteases are suggested to activate FCoV S at the S1/S2 site, while cathepsins activate the S2' for the endosomal route of entry (Licitra et al., 2013; Regan et al., 2008). The second event required to induce the FCoV-cell membrane fusion is a drop in the endosomal pH, which may play a role in protease activity and facilitate the unfolding of S after activation (Regan and Whittaker 2008, White and Whittaker 2016). Still, in vitro experimentation has shown that FIPV II 79-1146 fusion is less dependent on a low pH when compared to FECV II 79-1683 (Regan, Shraybman et al. 2008). Additional ionic factors (i.e., Ca2+ ions) may also drive S-mediated fusion, as furin proteolytic enzymes are calcium-dependent serine proteases.

After the fusion of the membrane, the FCoV nucleocapsid releases the viral genome into the cytoplasm for viral genome replication and protein synthesis. Replicase proteins must be synthesized before genome replication (Masters and Perlman, 2013). After viral uncoating, the replicase genes, encoded by the ORFs rep1a and rep1b, are immediately translated through ribosome frameshifting. The two polyproteins produced, pp1a and pp1ab, cleaved to 11 and 16 nonstructural proteins, respectively. After processing by virus-encoded proteinases, the replicase

proteins assemble to form the membrane-bound replication–transcription complex in the cytoplasm of the infected cell (Dye and Siddell 2005, Fehr and Perlman 2015). Replicase proteins mediate replication of the genomic ssRNA+ into multiple sub-genomic mRNAs (sgRNAs) and subsequent transcription of the remaining structural and accessory genes (Dye and Siddell 2005, Enjuanes, Almazan et al. 2006). The maturation of FCoV structural proteins (S, M, and E) occurs at the endoplasmic reticulum (ER) in the cytoplasm, where the proteins are inserted and processed.

Glycosylation of S, M, and E proteins occurs during the transport of the viral proteins through the secretory pathway into the ERGIC (Krijnse-Locker et al., 1994). In a parallel process at the same compartment, N proteins bind recently synthesized viral genomic ssRNA+ molecules to be enclosed into the mature virions. The M protein directs most protein-protein interactions required for the assembly of coronaviruses through the interaction with N protein at the ERGIC. These interactions promote the completion of the virion assembly. The new viruses are transported to the cell surface through secretory vesicles and released in an endosome-cell membrane fusion process, not regulated by the virus (Fehr and Perlman 2015, Jaimes and Whittaker 2018). FCoVs are released from the basolateral side facing the inner environment of the epithelial cell membrane (Rossen, Kouame et al. 2001).

1.2.1.6 Type I and type II feline coronaviruses

To better understand the distinctive features of the two serotypes of FCoV (serotypes I and II), it is crucial to consider the evolution of FCoV in the context of the other viruses in Alphacoronavirus 1 species, including CCoV. Both FCoV and CCoV are believed to originate from a common ancestor. During their evolutionary process, several independent recombination

events led to the emergence of FCoV and CCoV, as well as the appearance of novel, chimeric FCoVs with S proteins derived from CCoV (Herrewegh, Smeenk et al. 1998, Decaro, Martella et al. 2007, Whittaker, Andre et al. 2018). Double homologous recombination events make FCoV type I and II differ genetically and antigenically, whereas the S protein antigenic differences derive from the genetic origin of each serotype. The consequence of recombination causes the replacement of approximately one–third (~10 kb) of the FCoV serotype I genome, including the S gene and the neighboring regions with CCoV genome equivalents (Herrewegh, Smeenk et al. 1998, Terada, Matsui et al. 2014). The FCoV S1 domain of serotypes I and II have poor sequence identity (~30%). Therefore, these serotypes may use different receptors for cell entry (Hohdatsu, Izumiya et al. 1998, Dye, Temperton et al. 2007, Tekes, Hofmann-Lehmann et al. 2010).

Serotype I is the original serotype of FCoV and predominates worldwide (Kipar and Meli 2014, Haake, Cook et al. 2020). Serotype I has great epidemiological importance, making up 80-95% of natural infections in Europe and America. Serotype II is less common in the field and is mainly observed in Asia, with a reported 25% of natural infections (Benetka, Kubber-Heiss et al. 2004, Pedersen, Liu et al. 2009, An, Jeoung et al. 2011). Serotype II FCoVs continuously arise through independent recombination events during co-infection with serotype I and CCoV (Wang, Su et al. 2013).

Serotype II FCoVs replicate well in feline tissue culture cells. In contrast, serotype I strain isolation and cell culture adaptation are considered difficult (Lewis et al., 2015). Studies of recombinant FCoV type I viruses carrying an FCoV type II S protein demonstrated faster replication in cell culture and an expansion of receptor usage. These results indicate that the adaptability of FCoV in cell culture is related to the S protein (Tekes, Hofmann-Lehmann et al.

2010). Most of the available cell culture-adapted FCoV strains correspond to serotype II. As a result, most studies of FCoV have been based on serotype II strains. Studies on serotype I, the clinically significant serotype of FCoV, have been neglected due to continued isolation and culture difficulties (Dye and Siddell 2005, Tekes, Spies et al. 2012).

1.2.2 Pathogenesis

1.2.2.1 Feline enteric coronavirus

According to the pathogenicity in animals, FCoVs of serotypes I and II are separated into two biotypes: FECV, defined as the "ubiquitous enteric biotype/avirulent biotype", and feline infectious peritonitis virus (FIPV), defined as the "virulent biotype" that causes FIP in individual cats (Kipar, Kremendahl et al. 1998, Pedersen 2014). Since distinguishable markers between FECV and FIPV have remained unclear, these strains cannot be distinguished serologically or morphologically. In old cats, oral FECV infection often leads to mild, nonspecific clinical signs, such as transient anorexia. Infection can induce mild to severe enteritis in young kittens following the wane of maternal antibodies.

Although FECV RNA can be detected in the entire gastrointestinal tract, blood, and various tissues of persistently infected cats, experimental infections have indicated that the lower gastrointestinal tract is the primary site of viral replication and persistence. FECVs are tropic to the apical epithelium of the intestinal villi from the lower part of the small intestines to the caecum in acute infections (Kipar, Meli et al. 2010, Vogel, Van der Lubben et al. 2010). However, they can also infect monocytes, albeit inefficiently, and spread throughout the body (Porter, Tasker et al. 2014).

FCoV infection ranges from 36% to 75% (Paris, Wills et al. 2014, McKay, Meachem et al. 2020). Kittens can be exposed at a young age, as the persistently infected queen can shed the virus in feces for extended periods. Carrier cats play an essential role in the persistence of FECVs in cat populations. However, serological or PCR-based test results for FCoV should be interpreted cautiously. Most FECV-infected cats exhibit mild clinical signs unless co-infected with other enteropathogens. Treatment for coronaviral enteritis in cats is symptomatic and supportive (Pedersen 2009, Pedersen 2014).

1.2.2.2 Origin of feline infectious peritonitis virus

Understanding of FIP pathogenesis remains at a basic level. Several hypotheses were developed to explore the mechanism by which it occurs. The "internal mutation theory", postulates that FIP develops from a mutation or recombination event, allowing infection of monocytes and macrophages (Vennema, Poland et al. 1998). However, no specific mutation causing a shift in the FCoV biotype has been identified. Differing only in pathogenicity, FECV and FIPV are considered two distinctly different pathotypes (Vennema 1999, Pedersen, Liu et al. 2009). The second hypothesis of FIP development suggests that any FCoV can cause FIP. Host factors (e.g. immune response) and viral factors (e.g., formation of quasispecies) are proposed determinants for the development of FIP (Battilani, Coradin et al. 2003, Kipar, Meli et al. 2006). Thus, the pathogenesis mechanism of FIP remains elusive.

1.2.2.2.1 Internal mutation theory

Controversy remains on the origin of FIP. While FECVs and FIPVs were considered different species in early investigations, they were later proposed to be closely related viruses

with distinct virulence properties due to their high sequence similarity. This observation guided the internal mutation theory that FIPV evolves from FECV by specific viral mutations in infected cats. The presence of "circulating virulent–avirulent FCoV" is an alternative hypothesis proposed in one study, demonstrating FIP development only upon infection with the virulent FCoV type. This result suggests the independent coexistence of virulent and avirulent FCoVs in cat populations (Brown, Troyer et al. 2009) but has not carried much support from other studies.

Further studies have broadly strengthened the "internal mutation" theory of FIPV development (Chang, Egberink et al. 2012, Pedersen, Liu et al. 2012, Barker, Tasker et al. 2013, Porter, Tasker et al. 2014, Barker, Stranieri et al. 2017). FECV mutants that attain the FIP biotype have gained tropism for macrophages and monocytes, allowing the virus to stray from its typical infection of the mature intestinal epithelium and become a systemic pathogen (Figure 1.1). The clinical fate of FIP varies depending on the type and strength of the humoral immune response to macrophage infection (Pedersen, Liu et al. 2009, Pedersen, Liu et al. 2012).

Studies in the past decade have sought to identify mutations in the accessory and S genes associated with FIP development. The ORF *3c* accessory gene was a significant focus in studies of FECV to FIPV conversion. Mutations that increase macrophage tropism were initially thought to reside solely in the *3c* gene (Vennema, Poland et al. 1998). These findings were corroborated by subsequent studies, which demonstrated *3c* mutations in two-thirds of FIPVs, and altered host cell tropism (Hsieh, Huang et al. 2013). Comprehensive sequence analyses of FECVs (gut localized) and FIPVs (more systemic) suggested that an intact *3c* gene is not required for systemic replication of FIPVs. Instead, the *3c* gene is deemed essential only for viral replication in the gut (Chang, Egberink et al. 2012, Pedersen, Liu et al. 2012, Bank-Wolf, Stallkamp et al. 2014). While these findings do not support mutations in the *3c* gene as FIP virulence markers,

the contribution of this gene to the increased viral fitness in monocytes/macrophages may still be considered necessary in the development of FIP.

Although one study in Persian cats implicates NSP 7a and 7b mutations in FIP (Kennedy, Boedeker et al. 2001), this study has not been further supported. Currently, mutations in the *7a* gene are not considered crucial in the biotype switch. Furthermore, since deletions in the *7b* gene can occur naturally in FECVs, *7b* mutations are not considered significant in FIP development (Lin, Su et al. 2009).

Lately, FIP pathogenesis research has focused on the relationship between S gene mutation and systemic FIPV. Due to the S protein's importance in receptor binding and viral entry, mutations in the S gene alone or in combination with other genes could be responsible for altered target cell tropism during FECV–FIPV transition. Two-point mutations, M1058L and S1060A, were identified by analyzing 11 FECV and 11 FIPV full-length genome sequences (Chang, Egberink et al. 2012). These mutations caused minor changes in single amino acids (i.e. methionine to leucine, serine to alanine) at positions 1058 (M1058L) and 1060 (S1060A) within the FIPV S protein. These mutations were observed in most FIPVs studied (approximately 96%), but not in any FECVs. Diagnostic assays have since been developed from observed sequence differences between the S genes of FECVs and FICVs. Mutational analysis of short fragment sequences of the S gene derived from fecal and tissue samples of both FECV and FIPV demonstrated the presence of methionine at position 1058 in most fecal samples and leucine at the same position in the majority of tissue samples of both biotypes. From these findings, the M1058L substitution was postulated to have more involvement in macrophage infectivity than subsequent host-virus immune interactions (Porter, Tasker et al. 2014, Decaro, Mari et al. 2021).

In addition to M1058L and S1060A, isoleucine to threonine substitution was reported at position 1108 (I1108T) in the heptad repeat 1 (HR1) region of FIPVs but not of FECVs. The I1108T mutation alters fusogenic activity in the S protein, which may affect the cellular tropism of FIPV, a link to FIP development that has not yet been confirmed. (Bank-Wolf, Stallkamp et al. 2014, Lewis, Porter et al. 2015). Another study investigating FECV-FIPV discriminatory mutations in the S protein reported amino acid substitutions at the furin cleavage site or proximity to the furin cleavage site between the S1 and S2 domains (Licitra, Millet et al. 2013). While all FECVs contained intact and functional furin cleavage motifs, point mutations in the S gene were identified in 10 of the 11 FIPVs with these amino acid substitutions. Amino acid substitutions at the protease cleavage site could disrupt the efficiency of furin-mediated S protein cleavage. This disruption may indirectly affect viral spread, disease progression, and FIP development (Bosch, Rossen et al. 2008).

FECV–FIPV transition requires positive selection for mutant viruses able to replicate in macrophages and unfit for replication in enterocytes. The ultimate targets are a distinct population of precursor monocytes/macrophages with a specific affinity for endothelium of venules in the serosa, omentum, pleura, meninges, and uveal tracts. The identification of these viral mutations is based only on comparative sequence analyses. Interestingly, no particular tissue lesions have been identified as sites for viral mutation and FECV-FIPV transition. The anatomical location of FIPV development may be apparent at some point between the intestine and FIP-associated lesions. However, experimentally, no assumed functional changes concerning cell tropism and the emergence of highly virulent FIPV from FECV have been proven.

1.2.2.2.2 Quasispecies theory

Viral quasispecies refers to a heterogeneous population structure of closely related viruses. Its fidelity mechanism relies on RNA-dependent RNA polymerase (RdRp) and is associated with a high mutation rate. Genetic diversity and viral quasispecies have been well documented in coronaviruses (Denison, Graham et al. 2011, Alluwaimi, Alshubaith et al. 2020). In FCoV infections, viral quasispecies have been demonstrated in individual cats and between cats living in the same household. More extensive viral quasispecies formation has been reported in FIP infections compared to healthy animals (Battilani, Coradin et al. 2003). Other studies have demonstrated that viral subpopulations can differ by organs in a single cat. The heterogeneity of the FCoV genome is related to disease severity, clinical form of FIP, cellular tropism, and pathogenicity in the affected animals (Kiss, Kecskemeti et al. 2000, Battilani, Coradin et al. 2003). However, it is still unclear what association between genetic diversity and pathogenesis can be attributed to quasispecies dynamics (Moya, Holmes et al. 2004).

1.2.2.2.3 The immune response related to viral-host interaction

Host factors are likely an essential prerequisite for FIP development and include host immune response and the ability of monocytes to sustain FCoV replication, breed, and genetics. The pathogenesis of FIP is complex, with many unresolved issues relating to the immune system's role. As mentioned earlier, FECV–FIPV switch for FIP pathogenesis is postulated to be accompanied by the infection of monocytes and macrophages, which facilitates systematic dissemination (Stoddart and Scott 1989, Rottier, Nakamura et al. 2005). Therefore, intrinsic resistance of macrophages to FCoV infection is an essential immunopathological feature against FIP pathogenesis.

Macrophages are the predominant inflammatory cells in FIP lesions called pyogranulomas. Viral antigens can be detected in macrophages isolated from pyogranulomatous lesions and monocytes (progenitors of tissue macrophages) isolated from effusions. Studies with FIPV-infected monocytes showed that viral antigens are expressed on the plasma membrane of 50% of the infected cells. These viral antigens are internalized after the addition of antibodies. As a result, the plasma membrane of the infected cells clears all visually detectable viral antigens (Dewerchin, Cornelissen et al. 2006). However, it is unknown if FIPV affects MHC I expression on the surface of FIPV-infected cells in FIP cats. Although the FCoV is not directly infecting $CD4^+$ or $CD8^+$ T-cells, the infected macrophages release tumor necrosis factor alpha (TNF-α), causing apoptosis and depletion of CD4 T cells, especially CD8 T cells. This event causes the inhibition of cell-mediated lysis during a chronic FIPV infection (Haagmans, Egberink et al. 1996, de Groot-Mijnes, van Dun et al. 2005).

Humoral immunity against FIPV is not protective against the progression of viral replication. Consequently, this makes the outcome of FIPV infection much more complicated and highly involved in cell-mediated immunity. The antibody titer is not effective for the elimination of the virus. Inversely, it enhances FIP development in vitro (Petersen and Boyle 1980) and in cats previously immunized against FCoV in vivo (Takano, Kawakami et al. 2008). This phenomenon is explained as antibody-dependent enhancement (ADE). In FIPV infection, ADE activity is induced by antibodies to the FIPV S protein; this might help the spread of the virus in an infected cat by facilitating the virus uptake through the formation of virus-antibody complexes. Complexes are then taken up by uninfected monocytes/macrophages via the Fc receptor (Hohdatsu, Nakamura et al. 1991, Takano, Kawakami et al. 2008, Takano, Morioka et al. 2014).

Several studies have revealed significant differences in the composition and functional state of hemolymphatic tissues collected from FCoV-infected cats with and without clinical signs of FIP, which suggests a role for these tissues in FIP pathogenesis. FCoV-infected cats without FIP clinical signs have been shown to exhibit different B and T cell hyperplasia with a high rate of lymphocyte proliferation (Paltrinieri, Ponti et al. 2003). On the other hand, experimentally infected animals with FIP have shown the depletion of B and T cell zones in the spleen, mesenteric lymph nodes, and thymus. In particular, there is generally marked thymus atrophy in young cats due to enhanced lymphocyte apoptosis and TNF-alpha expression by lymphocytes. TNF-alpha expression is exceptionally high in lymphatic tissues with FIP lesions (Kipar, Kohler et al. 2001, Dean, Olivry et al. 2003). These findings are supported by a hemogram of lymphopenia, which is observed in the terminal stage of FIP. Lymphopenia coincides with a persistent drop in circulating CD4+ and CD8+ T-cell numbers and an increased apoptosis rate in peripheral blood mononuclear cells (PBMCs) throughout the disease (de Groot-Mijnes, van Dun et al. 2005, Takano, Hohdatsu et al. 2007). Takano et al. demonstrated that cats infected with FIP displayed higher expression of IL-6 in PBMCs than the same cells from specific pathogen-free cats. These findings suggest that IL-6 is involved in the immune-complex mediated vasculitis development and, therefore, in FIP pathogenesis (Takano, Azuma et al. 2009). The action of IL-6 to recruit and activate T cells and macrophages, expand cytotoxic T lymphocytes, downregulate acute phase proteins, modulate the differentiation of plasma cells and promote an increase of vascular permeability. IL-6 contributes to immune-mediated destruction in cats with neurological clinical signs of FIP (Foley, Rand et al. 2003). FCoV-infected asymptomatic cats showed higher levels of IL-10 transcription in the spleen than cats with FIP. IL-10 negatively regulates the expression of β-integrins in monocytes, reducing their ability to adhere to endothelial cells, and

causing vasculitis (Kipar, May et al. 2005). Significantly lower IL-12 p40 mRNA levels have been found in the lymphatic tissues of cats infected with FIP. This suggests a lack of IL-12 may inhibit an effective immune response and allows monocyte/macrophage activation. This could be a consequence of impaired T-cell-mediated macrophage activation (Kipar, Meli et al. 2006).

Berg et al. reported high levels of IFN-γ mRNA in tissues with inflammatory lesions of FIP (Berg, Ekman et al. 2005). This finding indicated that the inflammatory response does not solely control FIP infection. However, the cytokine profile in these tissues could reflect the local cytokine response more adequately than in PBMC (Berg, Ekman et al. 2005). Another study showed the serum concentration of IFN-γ in cats with FIP was not significantly different from clinically normal FCoV-infected animals living in catteries with low disease prevalence, supporting previous findings. Moreover, IFN-γ concentrations in effusions of cats with FIP were 40-fold higher than in the serum of the same animal due to IFN-γ production within FIP lesions. (Giordano and Paltrinieri 2009). These findings support the hypothesis that cats, although resistant to FCoV infection, have a strong and 'systemic' cell-mediated immune response, as measured by serum IFN-γ production. CMI is likely involved in the pathogenesis of FIP, albeit at a tissue level, as evidenced by the high IFN-γ concentration of the FIP effusions.

Systemic inflammatory reaction is another factor involved in the FIP pathogenesis where the concentrations of acute-phase proteins (APPs) are expected to increase. APPs are plasma proteins produced by hepatocytes during systemic inflammation (Ceron, Eckersall et al. 2005). The central feline APP is an α1-acid glycoprotein (AGP), and AGP concentration often increases in cats with FIP (Ceciliani, Grossi et al. 2004, Paltrinieri, Metzger et al. 2007). This AGP is hyposialylated, i.e., a decrease in the degree of sialylation, a posttranslational modification (Ceciliani, Grossi et al. 2004). Paltrinieri et al. investigated the relationship between the amount

of FCoVs shed in feces with the sialylation pattern of serum AGP in FCoV-infected non-symptomatic cats. They demonstrated that FCoV-infected cats do not develop FIP despite large amounts of viral RNA shed in the environment. Hypersialylation of AGP may be a possible reason for this and may also protect the host from the development of FIP (Paltrinieri, Gelain et al. 2008).

Another strong argument can be made for the role of genetic factors in FIP pathogenesis. In vitro studies suggested that monocytes from different cats do not have equivalent susceptibility to FCoV infection with the same strain of FCoV. Cellular factors, influenced by genetic background and/or differentiation/activation status, are significant in determining the development of FIP (Dewerchin, Cornelissen et al. 2005, Tekes, Hofmann-Lehmann et al. 2010). In natural FCoV infections, a higher incidence of infection has been reported in very young or geriatric cats, purebreds, and immune-compromised animals, such as those previously infected with the feline leukemia virus or feline immunodeficiency virus (Norris, Bosward et al. 2005, Pesteanu-Somogyi, Radzai et al. 2006, Drechsler, Alcaraz et al. 2011).

1.2.3 Clinical and pathological features of FIP

1.2.3.1 Prevalence

FIP, a severe and usually fatal disease, remains a major killer of young cats. Although seropositivity for FCoVs in domestic cats is approximately 20-60% and reaches 90% in animal shelters or multi-cat households, FIP morbidity is low and rarely surpasses 5% of FCoV-infected animals (Pedersen 2009). The proportion of chronic shedders and the overall frequency of viral shedding are considered risk factors in catteries. Young (6 months to 2 years), purebred, intact, and male cats are significantly more likely to be diagnosed with FIP. Breed susceptibility has

been postulated (Pesteanu-Somogyi, Radzai et al. 2006). However, FIP occurrence may connect more to specific familial lineages than to breed (Kennedy 2020). In addition, FIP may be more common in cats that live in multi-cat households, shelters, or catteries. Other factors that are thought to be associated with an increased disease prevalence include stress and genetic factors (Worthing, Wigney et al. 2012).

1.2.3.2 Transmission, shedding, and persistence

FCoV transmission occurs by a fecal-oral route, and the virus primarily infects enterocytes. FECV is associated with asymptomatic persistent enteric infections with the virus continuously or intermittently shed in the feces. In contrast, FIPV causes feline infectious peritonitis (FIP), which is usually a fatal systemic disease. Persistently-infected FECV carriers play an essential role in the epidemiology of FIP. In an experimental setting, consistent shedding of FECV isolates has been reported as early as two days and for up to 2 weeks post-infection. A steep decline in fecal viral loads and intermittent shedding could occur for varying times or even lifelong (Meli, Kipar et al. 2004, Vogel, Van der Lubben et al. 2010, Felten, Klein-Richers et al. 2020). The amount of virus shed by cats with FIP is lower than that shed by diarrheic or healthy carriers.

Furthermore, replication is significantly lower in the intestines than in organs in FIP (Hornyak, Balint et al. 2012). While FECV transmits horizontally between cats, this transmission of FIPV should be considered rare. However, as it is not impossible, there is a potential risk of transmission to other cats living in the same environment (Wang, Su et al. 2013, Takano, Yamada et al. 2019).

The virus can persist in both intestinal and extra-intestinal sites. The colon is the primary site of FCoV persistence, where viral antigen has been found in differentiated enterocytes. However, the virus was shown to persist in several other tissues, with macrophages allowing additional sources for recurrent systemic spread. These findings suggest that following initial viremia, FIP can develop in infected animals at any stage. This could even include after the virus is cleared from the intestine (Kipar, Meli et al. 2010). The occurrence of a FIP outbreak is associated with different factors, such as the environment (stress, crowding, concurrent infections), the virus (virulence, mutation rate, and replication rate), and the host (individual differences in the immune response to FCoV, genetics).

1.2.3.3 Clinical features

FIP is clinically recognized as either a ‘wet’ or ‘dry’ form of the disease. The most classic presentation is the wet form and describes a non-parenchymatous infection with inflammatory exudation into body cavities (Pedersen 2009, Haake, Cook et al. 2020). The dry form of FIP lacks effusion and is most difficult to recognize; it is characterized by granulomatous lesions developing in various organs. (Haake, Cook et al. 2020). There is an occasional occurrence of focal non-effusive FIP, typically presenting as a palpable mass in the abdominal cavity (Tasker 2018). While infected cats rarely manifest both forms of FIP simultaneously, progression from one form to another is possible, and warrants repeated clinical evaluations (Pedersen 2009, Tasker 2018). In necropsy, evidence of both effusion and granulomatous lesions is common, making the distinction between the wet and dry forms of FIP less clear (Tasker 2018) (Figure 1.2).

Clinical signs of FIP can vary widely depending on the host's immune response to infection and the disease's form (Kennedy 2020). The initial signs and symptoms of illness may be vague and typically include chronic fluctuating pyrexia, worsening malaise, anorexia, weight loss, jaundice, and lymphadenomegaly. Notedly, pyrexia is non-responsive to antibiotics and non-steroidal anti-inflammatory medication (Pedersen 2009, Tasker 2018, Haake, Cook et al. 2020). It takes several days to weeks to develop other symptoms. While the wet form of FIP typically presents clinical signs sooner than the dry form, the incubation period is highly variable and still considered unknown in natural infections (Pedersen 2009, Haake, Cook et al. 2020) (Barker 2020). Once clinical signs develop, the disease is nearly 100% fatal (Haake, Cook et al. 2020).

While routine laboratory tests cannot definitively diagnose FIP, they may support clinical suspicions. Changes in hematology and serum biochemistry are often nonspecific. The most common changes indicated on hemograms are nonregenerative anemia, neutrophilia with left shift or toxic change, lymphopenia, eosinopenia, monocytosis, thrombocytopenia, and microcytosis without anemia. On serum biochemistry panels, hyperbilirubinemia, hyperglobulinemia, polyclonal gammopathy, hypoalbuminemia, and low albumin: globulin (A:G) ratio are all common signs (Barker 2020). An A:G ratio greater than 0.8 is unsupportive of FIP; however, lower ratios may not be sufficient to rule in FIP as a diagnosis (Tasker 2018). Alpha1-acid glycoprotein (AGP) levels, an acute-phase protein, are often markedly raised in FIP cases. Levels above 1.5 to 2 mg/mL of AGP support the diagnosis of FIP (Barker 2020).

In the wet form of the disease, the most common physical exam finding is abdominal distention due to effusion. However, effusion can be found in other places, such as the thoracic cavity, scrotum, and pericardium (Pedersen 2009, Felten and Hartmann 2019). Ultrasonography

is considered more sensitive than radiography in identifying effusion, and when available, effusion samples should always be collected for diagnostic purposes in FIP suspect cases. When sampled or observed at necropsy, FIP effusions will have a clear-yellowish color and viscous consistency. They are exudates due to their high protein concentration but can also be described as modified transudates due to poor cellularity (Tasker 2018).

Rivalta test can be utilized routinely to quickly and inexpensively identify whether an effusion is an exudate. While the Rivalta test is considered a crude test, it has an excellent negative predictive value with 91-100% sensitivity for excluding FIP as a diagnosis (Felten and Hartmann 2019). Performing the Rivalta test requires placing a drop of effusive fluid on the surface of a mixture of 8 mL distilled water and a drop of 98% acetic acid. If the drop of effusion fluid maintains an attachment to the surface of the mixture, it is considered a positive result and indicates that the effusion is an exudate. The disappearance of the drop suggests a negative result. The specificity of the Rivalta test only ranges between 66-81% in ruling in FIP and cannot differentiate exudation produced by other causes. Therefore, routine cultures and cytology are also recommended to rule out other possible causes of exudation, such as lymphoma and bacterial infection (Tasker 2018). Like blood serum, effusions in FIP cases are often characterized by low A:G ratios and high AGP concentrations. However, when effusion samples are tested, AGP concentrations show the highest sensitivity and specificity (93%) for FIP (Tasker 2018).

A subset of FIP cases, particularly cats with the dry form of the disease, present with ocular and/or neurologic involvement. Ocular and neurologic signs are only seen in less than 9% of cats with the wet form, whereas, in the dry form, ocular and/or CNS involvement is associated with 60% of affected cats. Changes in iris color can be a frequent early sign of FIP disease and

may distort the pupil. Keratin precipitates on the cornea can also be found. However, the two predominant ocular signs in dry FIP infections are uveitis and chorioretinitis; FIP is the most common cause of these signs in cats (Pedersen 2009). FIP is the leading cause of disease in the central nervous system (Crawford, Stoll et al. 2017) and is considered the most common cause of spinal disease in cats under two years of age (Pedersen 2009). When the CNS is affected, lesions are usually surface-oriented in the brain and spinal cord, affecting the leptomeninges, ependyma, ventricles, choroid plexus, and neuroparenchyma (Rissi 2018). Neurologic signs are variable depending on the localization of lesions in the CNS. Signs may include pathological nystagmus, abnormal mentation, ataxia, and paresis. Magnetic resonance imaging (MRI) may help localize neuropathologic changes (Crawford, Stoll et al. 2017).

1.2.4 Diagnosis of FCoVs

The primary considerations for diagnosing FIP are age, origin, clinical signs, and physical examination of a cat infected with FCoV. Cats between the ages of 4 and 36 months from high-density environments that manifest a persistent and undulating antibiotic unresponsive fever are prime suspects for FIP. There are very few infectious diseases other than FIP that have this signalment. Choosing what tests to run is difficult because none can give a definitive antemortem diagnosis of FIP (Table 1.3). The postmortem diagnosis of FIP relies on a combination of histological examination and detection of viral antigens in lesions and is, therefore, much more uncomplicated. A veterinary clinician should have a high index of suspicion for FIP for a patient with specific signalment, clinical signs, medical history, positive results on indirect and/or direct virus detection tests, and other host blood parameters.

1.2.4.1 Detection of antibodies

Measurement of serum antibodies is helpful in the detection of FCoV infection. However, the antibody is best for screening and managing FCoV infection in catteries and quarantine since antibody titers can be correlated to shedding intensity and frequency (Drechsler, Alcaraz et al. 2011). There are a variety of serum antibody tests for FCoV, including enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence antibody test (IFAT), virus neutralization, and rapid immunochromatographic test (RIM) (Addie, le Poder et al. 2015). A Positive FCoV antibody test indicates that the cat has been infected with FCoV and has seroconverted; seroconversion usually occurs 2–3 weeks after initial infection.

Antibody tests such as FCoV/FIP Immuno-comb and speed F-Corona are susceptible to detecting low titers. Those tests are considered point-of-care tests and give qualitative or semiquantitative results. In the IFAT test for FCoV, virus-infected cells are fixed on slides, the test sample and a secondary fluorophore-labeled antibody are added, and bound antibodies indicate infection. ELISA and the immunochromatographic test are comprised of viral antigens bound to the membrane, and the test sample is added. Bound antibodies are detected using a secondary labeled antibody (Addie, le Poder et al. 2015). IFATs observed the cross-reactivity of FCoV with other closely related coronavirus species based on TGEV and FCoV (serotypes 1 and 2) (Zhao, Li et al. 2019).

The likelihood of seropositivity in clinically healthy cats from multi-cat households is high, but only a small percentage will develop FIP. This makes antibody tests of sera of limited value in diagnosing FIP (Bell, Toribio et al. 2006). Approximately 10% of cats with FIP are seronegative. This false negative issue of FIV has been addressed in a study that showed a correlation of lower signal in FCoV antibody samples containing high virus load using reverse

transcription-polymerase chain reaction (RT-PCR). This seronegativity could be due to the presence of the virus in the sample binding antibodies and, therefore, unavailable to the serological test (Meli, Burr et al. 2013). As a result, clinicians do not always perform serology in suspected cases. High antibody titers (>1:1600) in cats from endemic environments (such as those in multi-cat households) make a diagnosis of FIP increasingly likely (Hartmann, Binder et al. 2003). It is also noted that different serology results can be obtained from the same sample depending on the target antigen, the method used, and variations between laboratories (Hartmann 2005).

The anti-FCoV antibody detection in effusion has a sensitivity of 86% and a specificity of 85% in cats with FIP confirmed by histopathology (Hartmann, Binder et al. 2003). Though these numbers sound promising, the diagnostic value of anti-FCoV antibody measurement is low in serum and effusion and has similar limitations to serum antibody detection. Detecting anti-FCoV antibodies in the cerebrospinal fluid (CSF) of cats with neurologic FIP is unsuitable for definitive antemortem diagnosis of FIP. Previous studies postulated that anti-coronavirus antibodies were derived from antibody-containing blood crossing the impaired blood-brain barrier, which did not necessarily indicate intrathecal antibody production or the presence of FCoV in the CNS (Boettcher, Steinberg et al. 2007, Soma, Saito et al. 2018). As a result, anti-FCoV antibody detection has only limited diagnostic value, and a positive FCoV antibody test is not confirmatory of FIP.

1.2.4.2 Analysis of effusion samples

Analysis of any effusion samples in a suspected case of FIP can provide strong support for diagnosis, so collecting these samples should always be a priority. FIP-associated effusions

are usually clear due to low cellularity (<5000 nucleated cells/mL), straw-yellow (reflecting hyperbilirubinemia present), and viscous/sticky due to protein-rich content. Total protein concentration is often greater than 35 g/l (>50% globulins), but it can be lower than 30 g/L following repeated drainage. With sufficient cells, viral antigen demonstration in macrophages confirms the diagnosis with a very high positive predictive value (PPV) (Hartmann, Binder et al. 2003, Hartmann 2005).

Using a cut-off of 1.55 mg/mL of AGP concentration to measure acute phase protein in effusions has a 93% sensitivity and specificity, making it very useful in differentiating FIP and non-FIP cases (Hazuchova, Held et al. 2017). Immunostaining and molecular diagnostics of FIP-associated effusions are discussed later.

1.2.4.3 Molecular detection of viral RNA

RT-PCR, especially real-time RT-PCR, is a sensitive and frequently used method to detect FCoV RNA in different materials (feces, blood, tissues, effusions CSF or other cytology samples) from FCoV-infected animals as well as suspected cases of FIP. However, this test cannot differentiate between the pathotypes of FCoV (Kiss, Kecskemeti et al. 2000). In RT-PCR assay, both cell-associated subgenomic mRNA and virion-associated genomic RNA have been amplified by primers to determine the relative abundance of each (Barker, Stranieri et al. 2017).

Since FCoV RNA can be amplified outside of the gastrointestinal tract in cats without FIP, RT-PCR positive for FCoV in blood, effusion, or any other body fluid or tissue is not a definitive identification of FIP. However, it is crucial to remember that cats with FIP have much higher viral loads than asymptomatic FECV-infected cats (Meli, Kipar et al. 2004, Porter, Tasker et al. 2014). For this reason, quantitative assays (i.e. RT-qPCR) directed at the 5' end (viral

replicase complex gene) of the genome should be used to avoid viral overestimation. Because transcription starts at the 3' end of the genome, more subgenomic mRNA contains the viral 3' sequence. In addition, due to error-prone FCoV replication, any viral mutation at the site of primer and/or probe binding can result in the loss of efficiency and sensitivity of PCR. As a result, an ideal RT-PCR assay should be able to quantify the viral load to facilitate diagnosis.

The primary purpose of detecting FCoV RNA in the feces is to determine the kinetics of the viral shedding and to manage better catteries (Addie and Jarrett 2001, Drechsler, Alcaraz et al. 2011). Due to the systematic spread of FCoVs, diagnostic tests to identify viremia are only supportive of other tests for the diagnosis of FIP despite the absence or presence of FIP (Meli, Kipar et al. 2004, Drechsler, Alcaraz et al. 2011). RT-PCR for FCoV on effusions, collected both antemortem and postmortem, was found to have a sensitivity between 72% and 100% and a specificity from 83% to 100% on a small number of experimental samples tested (Barker, Stranieri et al. 2017, Felten, Weider et al. 2017, Aalaei, Khatibjoo et al. 2018). These findings indicate that preventing the virus in effusions has a high PPV. On the contrary, a negative result does not rule out FIP. RT-PCR for FCoV on other cytology samples, such as CSF, has a much lower sensitivity (42% to 63%) with similar specificity of 100% (Doenges, Weber et al. 2016, Emmler, Felten et al. 2020). Cats with neurologic or ocular manifestations of FIP were found to have significantly higher positive results than cats without these manifestations (Doenges, Weber et al. 2016). Tissue samples for RT-PCR of FCoV have usually collected postmortem. The sensitivity of tissue RT-PCR varied from 88% to 90% in individual cats (Hornyak, Balint et al. 2012, Barker, Stranieri et al. 2017). However, samples collected from cats with the more advanced clinical disease increased FCoV diagnostic sensitivity, while these patients were found to have higher viral copy numbers (Barker, Stranieri et al. 2017).

Loop-mediated isothermal amplification (LAMP) has recently been described for detecting FCoV RNA in-house in which targeted complementary DNA (cDNA) is amplified. Unlike RT-PCR, a thermal cycler is not required; instead, constant temperature is used, which makes LAMP a cheaper and more robust option for FCoV testing. This system detects DNA amplification as an increase of turbidity with reported overall 100% specificity but poor sensitivity. Further optimization of this technique needs to be explored to validate LAMP as a reliable diagnostic tool for FIP (Stranieri, Lauzi et al. 2017). Another recent technique, *7b* gene RT-PCR, has been proposed as an alternative to immunohistochemistry (IHC) in tissues with histopathological changes consistent with FIP; it uses primers to detect all FCoV *7b* genes. This study had a higher number of positive results for FCoV than IHC. Thus, it can be helpful for samples obtained by minimally invasive techniques if tissue biopsies are impossible (Emmler, Felten et al. 2020).

Although molecular diagnostics, primarily RT-qPCR, are not a reference standard for diagnosing FIP, these tests provide increased and quicker support for diagnosis with minimally invasive sample sources. Overall, a positive RT-PCR test result from effusion samples and tissue can provide strong evidence for the diagnosis of FIP. On the contrary, a negative result does not rule out FIP. With increasing knowledge of virus-host pathophysiological interaction in FCoV infection and constant improvement of molecular techniques, the diagnosis of FIP can be definitive in the near future.

1.2.4.4 Mutation analysis

One mutation or a combination of mutations can lead to the development of the FIP pathotype and trigger cell tropism. FCoV mutation analysis helps differentiate FCoV pathotypes

(i.e., FECV from FIPV) by identifying those mutations following the detection of FCoV RNA by RT-PCR (Chang, Egberink et al. 2012). Several molecular techniques, such as pyrosequencing, Sanger sequencing, or PCR with sequence-specific hydrolysis probes, can characterize the targeted sections of FCoV genomic sequences of RT-PCR positive samples.

In 2012, Chang et al. described two alternative amino acid mutations (M1058L and S1060A) in the putative fusion peptide encoded by the S gene in FCoV and FIPV; substitutions in this gene (23531A>T/C and 23537T>G respectively) distinguish FIPV from FECV in 95% of cases (Chang, Egberink et al. 2012). On the contrary, other researchers believe that fusion peptide mutations can only be implicated in monocyte/macrophage tropism (Porter, Tasker et al. 2014). They do not substantially improve the ability to diagnose FIP compared to the detection of FCoV alone (Barker, Stranieri et al. 2017, Felten, Weider et al. 2017). The latter group has calculated that using S gene mutation analysis as a confirmatory step to detect FCoV by RT-PCR can only slightly increase specificity for the FIP diagnosis in tissue samples (from 92.6 to 94.6%) with a moderate reduction of sensitivity from 89.8% to 80.9%.

Other amino acid differences in the furin cleavage motif, encoded by the S gene, can be of diagnostic value. It is found that FECVs have a conserved furin cleavage motif, whereas FIPVs have ≥one substitution in the same motif (Licitra, Millet et al. 2013). In several studies, the *3c* gene was present in the truncated form in most strains obtained from animals with FIP (Pedersen, Liu et al. 2012, Bank-Wolf, Stallkamp et al. 2014). As a result, the *3c* gene is also a candidate marker for distinguishing between FECV and FIPV when the mutant spectrum is considered (Hora, Tonietti et al. 2016).

All these amino acid differences could lead to FIP and thus can be used as markers of FCoVs associated with FIP. However, the presence of low levels of FCoV or FCoV sequence

with variability (due to the quasispecies nature of FECV) makes the technique unsuccessful as targeted sequencing techniques cannot generate results. In addition, the presence and detection of FIP-specific mutations can only make this technique helpful in diagnosing FIP. Furthermore, the sample source is also essential because these S gene markers have less chance of being present in feces but are more likely in the tissues of cats with clinical FIP (Barker, Stranieri et al. 2017).

1.2.4.5 Histological examination

The traditional gold standard for the definitive diagnosis of FIP is histopathology of tissue lesions with immunohistochemistry to detect FCoV antigens. Samples of affected tissues, such as kidney, liver, or mesenteric lymph nodes, are often collected at postmortem examination or via antemortem ultrasound-guided biopsy, laparoscopy, or laparotomy with a high index of suspicion of FIP. Unfortunately, many cats clinically ill with FIP may be poor anesthetic candidates. Any cat with signs suspicious of FIP that dies or is euthanized should have an autopsy with the collection of tissue samples for histopathological analysis. After the tissue sample is stained with hematoxylin and eosin (H & E), the characteristic histopathological changes of FIP with localized inflammation containing macrophages, neutrophils, lymphocytes, and plasma cells is generally regarded as being reliable for FIP diagnosis. Proliferated inflammatory cells may surround these vascular lesions, characteristic of "wet FIP." In dry FIP, focal accumulations of inflammatory cells and necrotic-proliferative lesions are typical of granulomatous lesions (Benetka, Kubber-Heiss et al. 2004, Pedersen 2014). Positive histopathological tests along with immunostaining for FCoV antigen can confirm the FIP diagnosis. However, if the histopathological lesions are absent, the interpretation is difficult; this

is often the result of sampling non-affected organs or tissues in the case with a high index of suspicion of FIP (Giordano, Paltrinieri et al. 2005, Tasker 2018).

1.2.4.6 Immunostaining

Immunostaining has been used for two decades to look for the presence of FCoV antigen within histological specimens of the lesions consistent with FIP and has a high PPV (Tammer, Evensen et al. 1995). Therefore, many pathologists consider it the "reference standard" and an essential component of FIP diagnosis, particularly in histologically inconclusive cases (Pedersen 2009, Giori, Giordano et al. 2011, Felten and Hartmann 2019). These assays include immunohistochemistry (IHC) of formalin-fixed cell pellets and tissue (Felten and Hartmann 2019), immunocytochemistry (ICC) on cytological samples (Felten, Matiasek et al. 2017), and immunofluorescence (IF) of cytologic preparations (Litster, Pogranichniy et al. 2013). In these tests, a monoclonal or polyclonal antibody against FCoV antigens is used as a reagent to provide visual details about antigen abundance. Surgical biopsies of granulomatous lesions from affected tissues are used for definitive antemortem diagnosis; Random Tru-Cut needle biopsies or fine-needle aspirates are often not helpful (Giordano, Paltrinieri et al. 2005). Immunostaining macrophages from effusions for FCoV have sensitivity from 57% to 100% and specificity from 71% to 100% (Felten, Matiasek et al. 2017, Tasker 2018). False-positive results were also reported for cats with neoplasia and cardiac disease (Litster, Pogranichniy et al. 2013, Felten, Matiasek et al. 2017).

The gold standard test for FCoV detection remains histopathology using IHC on affected tissue, often only done postmortem (Ziolkowska, Pazdzior-Czapula et al. 2017, Rissi 2018). IHC was reported to have excellent sensitivity of 97–100% in cats with histopathologically confirmed

FIP. There is up to a 100% chance of excluding FIP in cats with other diseases when they are diagnosed by histopathology of affected tissues (Tammer, Evensen et al. 1995, Rissi 2018). However, this test has high cost and time implications. In addition, IHC requires an invasive sampling procedure, so this test is often not possible in sick and debilitated cats. The IF test has higher sensitivity with effusive fluid samples in the "wet form" of FIP (Litster, Pogranichniy et al. 2013, Felten, Matiasek et al. 2017). However, IF tests in tissue samples in "dry" FIP cases are far inferior compared to IHC, especially in neurologic FIP (Rissi 2018), as a result, IHC is considered more sensitive and reliable than IF for the diagnosis of FIP.

In ICC staining, successful detection of FCoV antigen has been reported in the CSF collected post-mortem in cases of neurologic FIP (Ives, Vanhaesebrouck et al. 2013). The ICC test can also be done with aqueous humor samples with a sensitivity of 64% and specificity of 81.8%, thus making it especially valuable in non-effusive cases (Felten, Matiasek et al. 2018). However, further evaluation of the usefulness of this technique is required.

A positive test result is highly predictive of FIP, whereas a negative result does not exclude FIP. The false negativity is due to poor cellularity of the effusions, masked FCoV antigen by FCoV antibody and/or the relatively low sensitivity of the method utilized. More reliable results can be obtained by concentrating cell preparations in pellets, parallel cytological staining, and using formalin-fixed, paraffin-embedded cell pellets prepared from the effusions (minimum 1 ml) (Hartmann, Binder et al. 2003).

1.2.5 Treatment

Until recently, FIP treatment only focused on providing supportive care (appetite stimulants, antioxidants, fluid therapy, etc.) and alleviating symptoms. Removing fluid from a

cat with fluid buildup in the chest and/or abdomen can ease breathing. However, with the advent of novel antiviral medication, two different approaches have been considered to treat FIP: 1. direct inhibition of FCoV replication and 2. Modifying the immune response of affected cats using non-specific immunostimulants or immunosuppressive agents. These approaches can be used individually or in combination to treat FIP (Pedersen 2014).

Corticosteroids, drugs to treat chronic inflammatory diseases, have long been used to suppress negative aspects of the immune responses, including the humoral responses, and to provide palliative relief. This medication is most useful when the lesions are focal and restricted to a single tissue, such as anterior uveitis (Legendre, Kuritz et al. 2017). Feline cytokines such as interferon-omega have often been used for immune response modification. However, there is no convincing evidence of effect in a placebo-controlled trial (Ritz, Egberink et al. 2007). Hydroxychloroquine is a broad-range anti-inflammatory and antimalarial drug. This drug has been shown to inhibit FIPV replication in vitro when used with recombinant feline IFN-ω (Takano, Satoh et al. 2020). At low drug concentrations (2.5 mM), the antifungal itraconazole has demonstrated in vitro anti-FIPV activity (Takano, Akiyama et al. 2019).

Antiviral therapies targeting viral proteins responsible for RNA synthesis have become the mainstay in treating acute and chronic RNA virus infections in humans. However, interest in developing antiviral drugs for animal infections is slower to develop, especially for cats. FIPV causes systemic disease (FIP) with high mortality. Effective drug development is imperative for animal health as no safe vaccine is available and FIP kills 0.3-1.4% of cats worldwide. No drugs are currently approved in the USA for treating FIP in cats.

GS441524 is an adenosine nucleotide analog core of remdesivir, originally developed to treat Ebola virus infection (Warren, Jordan et al. 2016). This antiviral becomes phosphorylated to

form the active nucleoside triphosphate through intracellular phosphorylation (Cho, Saunders et al. 2012, Warren, Jordan et al. 2016). Being incorporated into the genome of virions, this molecule interferes with the RNA-dependent RNA polymerase (RdRp) mediated transcription by competing against the endogenous ATP, which leads to the premature termination of viral replication (Sheahan, Sims et al. 2017). Studies showed that GS-441524 was found to be more bioavailable and well-tolerated in cats than remdesivir and was found to be a promising oral antiviral drug against coronavirus for treating FIPV (Murphy, Perron et al. 2018, Pedersen, Perron et al. 2019).

Another synthetic adenosine nucleotide analog, Mutain X, is available as both oral and injectable formulations. This medication is not related to the treatment of FIP directly. However, in limited research, this drug has been reported to stop fecal shedding of the virus when administered orally for four days (Addie, Curran et al. 2020). An experimental process using small interfering RNA has shown efficacy in limiting virus replication in vitro for FCoV and induces posttranscriptional gene silencing (McDonagh, Sheehy et al. 2011, Anis, Wilkes et al. 2014, McDonagh, Sheehy et al. 2015).

1.2.6 Conclusions

FCoV is one of cats' most important infectious diseases due to its high prevalence and high mortality rate in the FIP form. Both pathotypes, FECV and FIPV, exhibit differences at the genomic and functional levels. Systemic infection is the critical event in the pathogenesis of FIP, followed by the effective and sustainable viral replication in monocytes and activation of infected monocytes. However, an incomplete understanding of infection biology and the pathogenesis of FIP makes the diagnosis challenging. Diagnosis remains a combination of

signalment, clinical signs, suspicious laboratory findings, and virus detection. The lack of a simple method to definitively diagnose FIP remains frustrating. Treatment also remains frustrating, as readily available options are ineffective, and most affected cats succumb to the disease.

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Table 1.1 List of SARS-CoV-2 Variants of Concern (VOC)

WHO label	PANGO lineage	Country first reported	Earliest sample	Characteristic amino acid substitutions in S	Transmissibility
Alpha	B.1.1.7	United Kingdom (UK)	December 2020	69–70del, N501Y, P681H	Increased (Campbell, Archer et al. 2021)
Beta	B.1.351	Africa	December 2020	K417N, E484K, N501Y	Increased (Campbell, Archer et al. 2021)
Gamma	P.1	Brazil	January 2021	K417T, E484K, N501Y	Increased (Campbell, Archer et al. 2021)
Delta	B.1.617.2	India	December 2020	L452R, T478K, P681R	Increased (Campbell, Archer et al. 2021)
Omicron	B.1.1.529	South Africa	November 2021	P681H, N440K, N501Y, S477N, and others	Increased (Meo, Meo et al. 2021)

Table 1.2 COVID-19 vaccines having obtained emergency use listing (EUL) by WHO and distributed worldwide as of January 2022

Name of the vaccine	Developer company	Platform	Doses	Efficacy	Obtained Emergency Use Listing (EUL)	References
Comirnaty	Pfizer/BioNTech and Fosun Pharma	Modified m RNA	Two doses	95%	31 December 2020	(Garcia-Montero, Fraile-Martinez et al. 2021)
Spikevax	Moderna and National Institute of Allergy and Infectious diseases	mRNA encapsulated in lipid nanoparticle	Two doses	94.1%	30 April 2021	(Garcia-Montero, Fraile-Martinez et al. 2021)
Vaxzevria	AstraZeneca and University of Oxford	Recombinant ChAdOx1 adenoviral vector encoding spike protein of SARS-CoV-2	Two doses	72%	16 February 2021	(Garcia-Montero, Fraile-Martinez et al. 2021)
CoronaVac	Sinovac Research and Development Co.	Inactivated virus	Two doses	97.4%	1 June 2021	(Garcia-Montero, Fraile-Martinez et al. 2021)
Ad5-nCoV	CanSino Biological Inc./Beijing Institute of Biotechnology	Non-replicating adenovirus type 5 vector	One dose	97%	19 May 2022	(Zhu, Guan et al. 2020)
Janssen/ Ad26.COV2.S	Johnson and Johnson	Recombinant, replication-incompetent adenovirus type 26 vector encoding the spike protein	One dose	92%	12 March 2021	(Garcia-Montero, Fraile-Martinez et al. 2021)
BBIBP-CorV	Sinopharm, China National Biotec Group Co, and the Beijing Institute of Biological Products	Inactivated virus	Two doses	79.34%	7 May 2021	(Xia, Zhang et al. 2021)

BBV152 COVAXIN	Bharat Biotech	Inactivated virus	Two doses	78%	3 November 2021	(Loo, Letchumanan et al. 2021)
NVX-CoV2373 Nuvavax and Covovax	Novavax and Serum Institute of India	Protein subunit Matrix M adjuvant	Two doses	90%	20 December 2021/ 17 December 2021	(Garcia-Montero, Fraile-Martinez et al. 2021)

(https://www.who.int/news-room/questions-and-answers/item/coronavirus-disease-(covid-19)-vaccines)

Table 1.3 Comparison of different diagnostic tests for FCoV infection

Diagnostic Test	Sample Source	Recommendation	Comments	References
Detection of Anti-FCoV Antibodies: IFAT, ELISA	Blood serum, Effusion, CSF	Low diagnostic value	Antibody detection is not specific to FIPV; High titers (>1:1600) may be supportive	(Barker 2020)
Rivalta's test	Effusion	Exclusion Test	Nonspecific If positive, must rule out other causes of exudate	(Fischer, Sauter-Louis et al. 2012)
Delta total nucleated cell count (ΔTNC >3.4 × 109 /L)	Effusion	Confirmatory Test	Nonspecific	(Stranieri, Giordano et al. 2018)
AGP concentrations >1.55 mg/mL	Effusion	Exclusion Test	Nonspecific	(Hazuchova, Held et al. 2017)
SPE	Blood	Confirmatory Test	Nonspecific	(Stranieri, Giordano et al. 2018)
Cytology	Effusion	Exclusion Test	Nonspecific If positive, rule out other inflammatory conditions	(Stranieri, Giordano et al. 2018)
RT-qPCR	Tissue Effusion CSF Aqueous humor	Confirmatory Test	RT-qPCR cannot distinguish between FCoV pathotypes; poor sensitivity in blood	(Felten and Hartmann 2019)
RT-LAMP	Blood	Not recommended	Low sensitivity	(Stranieri, Lauzi et al. 2017)
7b gene RT-PCR	Effusion Tissue	Confirmatory Test	Can be applied to samples deemed positive by RT-qPCR and as an alternative to IHC	(Emmler, Felten et al. 2020)
Histopathology	Tissue	Confirmatory Test	Histopathology recommended in combination with IHC; invasive and often only utilized in post-mortem diagnosis	(Felten and Hartmann 2019)
IHC for FCoV antigen	Tissue	Confirmatory Test		
ICC for FCoV antigen	Effusion CSF Aqueous humor	Confirmatory Test	ICC specificity is generally more variable than IHC	(Felten, Matiasek et al. 2017, Gruendl, Matiasek et al. 2017)

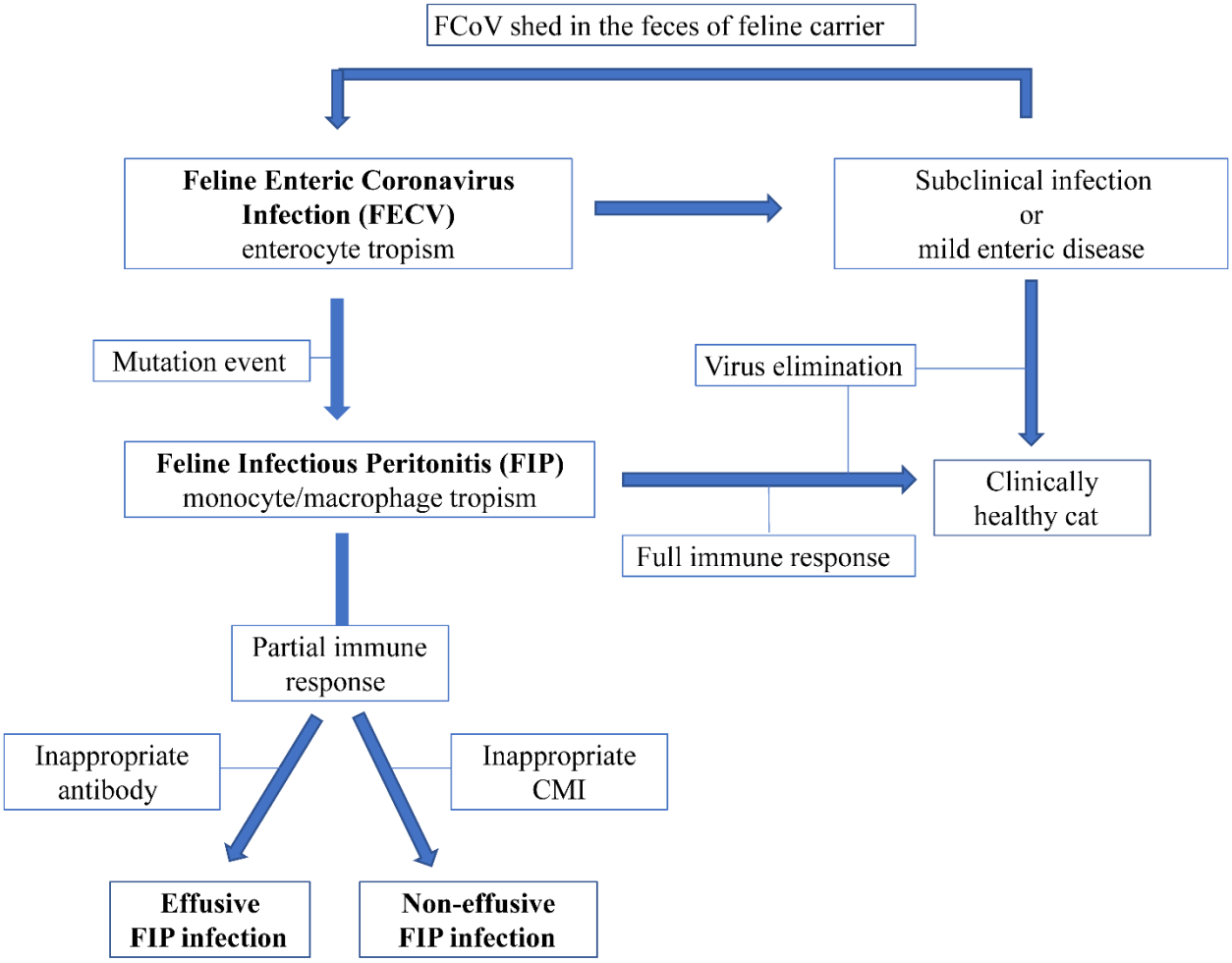

Figure 1.1 Pathogenesis algorithm of FIP. The hierarchy diagram shows the development of feline infectious infection (FIP). A mutation event allows FECV to gain tropism for monocytes/macrophages, allowing the virus to become systematic and develop FIP.

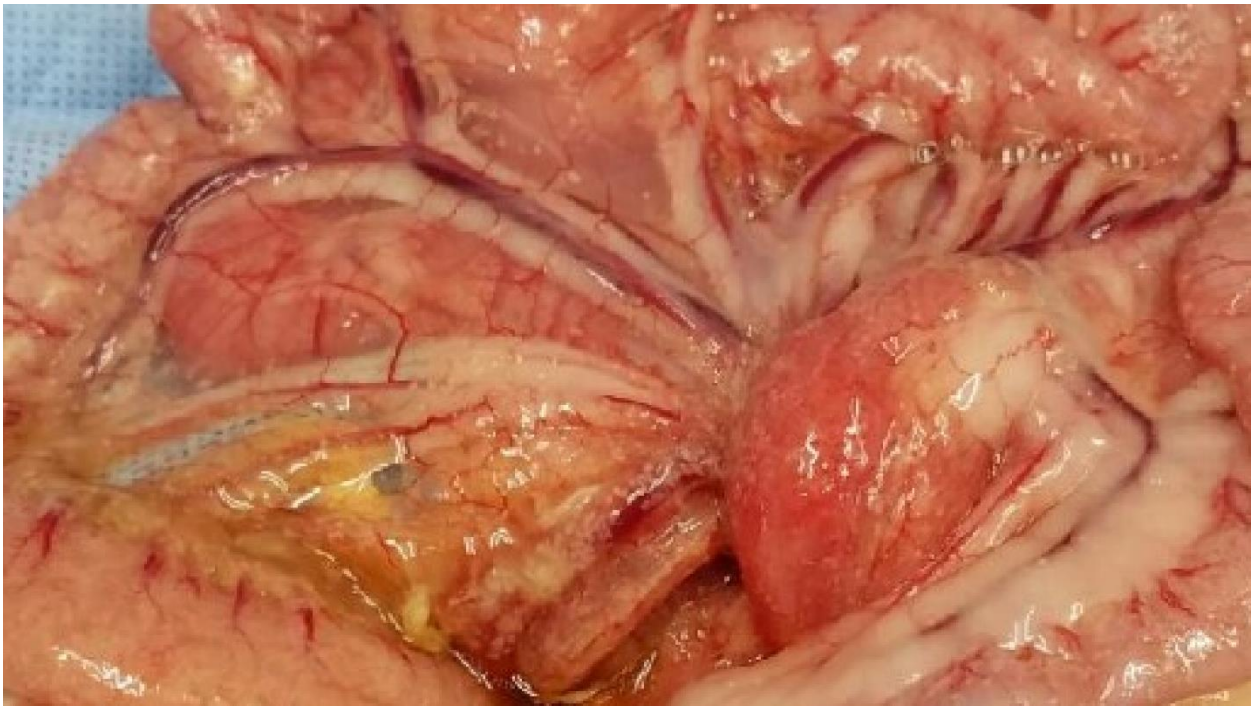

Figure 1.2 Evidence of lymphadenopathy. The presence of generalized fibrinous plaques and some yellowish fluid provide evidence for the diagnosis of FIP.

Chapter 2 High-resolution melting curve FRET-PCR rapidly identifies SARS-CoV-2 mutations

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2.1 Abstract

Reverse transcription FRET-PCRs were designed against the two most common mutations in the SARS-CoV-2 (A23403G in the spike protein; C14408T in the RNA-dependent RNA polymerase). The RT FRET-PCRs identified the mutations in ATCC control viruses and feline and human clinical samples based on a high-resolution melting curve analysis. All major makes of PCR machines can perform melting curve analysis. Thus, further specifically designed FRET-PCRs could enable active surveillance for mutations and variants in countries where genome sequencing is not readily available.

2.2 Introduction

Genetic mutations giving rise to variants of the SARS-CoV-2 continue to emerge and circulate worldwide during the COVID-19 pandemic. These mutations enable SARS-CoV-2 variants to be categorized into eight clades (https://clades.nextstrain.org/) and six major lineages (Rambaut, Holmes et al. 2020). Some emerging SARS-CoV-2 variants may have increased potential for transmissibility and virulence and lowered protection from vaccines (Tegally, Wilkinson et al. 2020, Tegally 2020, Cele, Gazy et al. 2021, Faria 2021, Larsen, Fonager et al. 2021, Tang, Tambyah et al. 2021, Zhang, Davis et al. 2021). Surveillance for variants and their spread is important in understanding the dynamics of the COVID-19 pandemic and in developing effective control policies. Screening for variants, however, is generally infrequent as it requires genome sequencing, which is expensive, time-consuming, and not readily available in most countries (Figure 2.1) (Cyranoski 2021). Comparison of sequences in GISAID and reported cases from the six countries most affected by COVID-19 reveals genotyping was only performed on under 1% of cases in the USA, Brazil, India, France, Russia, Italy, and South Africa, and 8.3% in the UK (Table 2.1).

As it is practically impossible for even the most advanced countries to sequence all positive samples, it would be beneficial if tests were available which could be readily used by laboratories around the world to identify mutations and thereby greatly facilitate the detection and tracking of SARS-CoV-2 variants. To test this concept, we developed reverse transcription (RT) FRET-PCRs against two of the most common mutations worldwide and used them to test clinical samples. Our results show that RT FRET-PCRs can be developed against mutations in the SARS-CoV-2. Developing similar RT FRET-PCRs against other mutations of interest will

enable general diagnostic laboratories worldwide to monitor variants rapidly and conveniently, thereby implementing more targeted and appropriate control programs.

2.3 Materials and methods

2.3.1 Identifying common mutations in SARS-CoV-2 variants

The A23403G and the C14408T mutations are the most common mutations from the original Wuhan strain that persists in almost all variants today (Cyranoski 2021). They are present in all Variants of Interest (VOI) and Variants of Concern (VOC) determined by the CDC (www.cdc.gov/coronavirus/2019-ncov/cases-updates/variant-surveillance/variantinfo.html) and reported to be the most common in the USA and globally (Wang, Chen et al. 2021). We confirmed this by analyzing all available high-quality SARS-CoV-2 sequences from GISAID (https://www.gisaid.org/), which revealed the A23403G and the C14408T occurred in over 99.85% (250,568 /250,945) of the five significant variants recognized today (Table 2.2). The presence of these two mutations in variants reached from 0% in 2019 to 99.59% for A23403G and 98.94% for C14408T mutation as of March 2021 (Table 2.3).

These variants were initially found in the United Kingdom (20I/501Y.V1, VOC 202012/01, or B.1.1.7), South Africa (20H/501Y.V2 or B.1.351), Brazil (P.1), Denmark (Cluster 5), and recently in the USA (CAL.20C).

2.3.2 SARS-CoV-2 reverse-transcription FRET-PCRs

Representative sequences around the mutations were aligned, and upstream and downstream primers and probes were designed to amplify and detect all SARS-CoV-2. The 6-carboxyfluorescein (6-FAM)-labeled probes were further designed to contain the unique

A23403G or C14408T mutation (Table 2.4). The 6-FAM probe was 3’ labeled as a FRET energy donor probe excited by 488 nm light. The LCRed 640 probe was 5’-labeled and 3’-phosphorylated as the acceptor probe.

Each 20 µl PCR reaction contained 2.0 U Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) and 0.0213 U ThermoScript™ reverse transcriptase (Invitrogen, Carlsbad, CA). Primers were used at one µM, the LCRed 640 probe at 0.2 µM, and the 6-FAM probe at 0.1 µM. PCR was performed on a Roche Light Cycler 480 II system (Roche Molecular Biochemicals, Indianapolis, IN). Thermal cycling was preceded by a 10-minute reverse transcription reaction at 55°C followed by a 5 min denaturation at 95°C, and 40 cycles of 10 sec @ 95°C, 10 sec @ 55°C and 10 sec @ 72°C.

Genomic RNA of two SARS-CoV-2 viruses from ATCC served as controls and as quantitative standards: 2019-nCOV/USA-WA1/2020, which does not contain the A23403G and the C14408T mutations and 201/501Y.V1, which includes both mutations). To generate quantitative standards, PCR products of the two control viruses were purified by 4 % MetaPhor agarose gel electrophoresis and quantified by PicoGreen DNA fluorescence assays (Molecular Probes, Eugene, OR).

The melting curve, which assessed the dissociation of the PCR products and labeled probes, was determined by monitoring the fluorescence from 35°C to 75°C with a temperature transition rate of 0.2°C per second. The first derivatives of F2/F1 were evaluated to determine the T_m of the probe (Figures 2.2 and 2.3). Nucleotide mismatches between the 6-FAM-probes and the SARS-CoV-2 variants result in distinct T_m values.

2.3.3 Test samples

RT FRET-PCRs were performed on ATCC controls without (2019-nCOV/USA-WA1/2020) and with the mutations (201/501Y.V1) and convenience samples of genomic RNA from the trachea of a SARS-CoV-2 positive cat (provided by Alabama Thompson Bishop Sparks State Diagnostic Laboratory), and eleven SARS-CoV-2 positive samples from human nasal swabs (provided by Kansas State Veterinary Diagnostic Laboratory, College of Veterinary Medicine, Kansas State University, USA). The Kansas lab also provided RNA from human nasal swabs found negative for SARS-CoV-2, which acted as negative controls. The PCR products of all tested samples and controls were sent to ELIM Biopharmaceuticals (Hayward, CA, USA) for DNA sequencing.

2.4 Results

The RT FRET-PCR we developed was very sensitive, detecting as few as ten copies of the gene target in a reaction (Figures 2.4 and 2.5). The control 2019-nCOV/USA-WA1/2020 without the A23403G mutation had a T_m of 63.1°C in the RT FRET-PCRs for the A23403G mutation (Figure 2.2). This was irrespective of copy number. This T_m of 63.1 °C was clearly distinguished from the T_m of 58.2 °C obtained with the control 201/501Y.V1 that had the A23403G mutation.

Similarly, there was a marked difference in the T_m of the control 2019-nCOV/USA-WA1/2020 with no C14408T mutation (54.3 °C) and that of the T_m obtained with the control 201/501Y.V1 (Figure 2.3) that had the C14408T mutation (57.7 °C). The feline and human samples all had very similar T_m (around 58 °C) in both RT FRET-PCRs, indicating that all samples carried both mutations.

Sequencing of the DNA of the PCR products further confirmed the presence of the mutations in the control sample and that the feline and human samples were variants containing both mutations.

2.5 Discussion

The RT FRET-PCRs we designed to establish if high-resolution melting curve analysis could detect mutations in the SARS-CoV-2 virus showed that the technique can rapidly and conveniently detect mutations in both control and clinical samples. RT FRET-PCRs can be performed in under two hours, and the examples we developed cannot only demonstrate not only if a sample is positive for SARS-CoV-2 but also if the mutations we targeted were present. Although we used a Roche 480 II platform, all major brands of PCR machines can perform melting curve analysis with dual-labeled probes. Thus, RT FRET-PCRs can be readily used for active surveillance and screening for mutations and variants, thereby reducing requirements for sequencing. It can also be used for large-scale retrospective molecular epidemiology studies of SARS-CoV-2 and its variants worldwide.

In conclusion, we have shown highly sensitive RT FRET-PCRs can be developed to detect SARS-CoV-2 infections and to determine if specific mutations are present. This highly specific and readily available platform should be readily and rapidly adapted to monitor the presence of other mutations and associated variants that concern countries worldwide. This technique will greatly facilitate monitoring the origins and spread of mutations in variants in the COVID-19 pandemic and more readily provide data that can be used for public health intervention programs.

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Table 2.1 Summary of sequence submission country-wise in GISAID compared to total cases

Country	Total cases*	Total WGS submission	Percentage of WGS submission
USA	31,003,585	255,570	0.82
Brazil	13,279,857	5,484	0.04
India	13,060,542	10,248	0.08
France	5,000,156	16,514	0.33
Russia	4,572,053	2,474	0.05
United Kingdom	4,385,025	363,444	8.29
Italy	3,717,602	18,176	0.49
South Africa	1,554,975	4,673	0.30
Canada	1,043,478	26,735	2.56
Bangladesh	666,132	1,070	0.16
China	101,998	1,590	1.56
Australia	29,390	17,822	60.64
New Zealand	2,561	1,231	48.07

* The high-quality SARS-CoV-2 sequences were obtained from GISAID sequences on April 28, 2021.

Table 2.2 Prevalence of A23403G and C14408T mutations in the different SARS-CoV-2 clades and variants

Clades/variants		Number of submitted sequences*	With A23403G mutation	With C14408T mutation
Clade	**L**	3,686	0, 0.00%	34, 0.92%
	S	7,902	266, 3.37%	12, 0.15%
	V	4,264	9, 0.21%	3, 0.07%
	Total	15,852	275, 1.70%	49, 0.31%
	G	85,109	85,070, 99.95%	84,455, 99.23%
	GH	166,783	166,724, 99.96%	165,623, 99.30%
	GR	145 ,342	145,320, 99.98%	145,035, 99.79%
	GRY	221,434	221,345, 99.96%	221,213, 99.90%
	GV	111,023	111,009, 99.99%	110,922, 99.91%
	Total	729,691	729468, 99.97%	727248, 99.67%
Variant	**VUI202012/01 (B.1.1.7)**	230,771	230,702; 99.97%	230,683; 99.96%
	501Y.v2 (B.1.351)	4,489	4,489; 100%	4,219; 93.99%
	501Y.V3 (P.1)	1,565	1,543; 98.59%	1,556; 99.42%
	452R.V1 (B.1.429+B.1.427)	13,774	13,771; 99.98%	13,764; 99.93%
	484K.V3 (B.1.525)	346	346; 100%	346; 100%
	Total	250,945	250851; 99.96%	250568; 99.85%

* The high-quality SARS-CoV-2 sequences were obtained from GISAID sequences on April 28, 2021.

Table 2.3 Prevalence of A23403G and C14408T mutations in SARS-CoV-2

Year	Total sequences submitted	A23403G (Spike_D614G) mutation (percentage)*	C14408T (NSP12_P323L) mutation (percentage)*
2019	22	00 (0.00%)	00 (0.00%)
2020	503,942	483,171 (95.87%)	481,451 (99.64%)
2021	730,202	727,244 (99.59%)	722,485 (98.94%)

***** data collected as of March 2021

Table 2.4 The oligonucleotides used in this study

Target of PCR	Primer/Probe	Sequences (5'-3')
A23403G	Upstream primer	TGTTCTTTTGGTGGTGTCAGT
	Downstream primer	TAGAATAAACACGCCAAGTAGGAGT
	6-FAM-probe	TTCTTTATCAGG**A**TGTTAACTGCACAGAA-6FAM
	LCRed 640 probe	LCR640-TCCCTGTTGCTATTCATGCAGATCA-phosphate
C14408T	Upstream primer	TTAAATATTGGGATCAGACATACC
	Downstream primer	GAAGTGGTATCCAGTTGAAACT
	6-FAM-probe	AAAACTTGTA**A**GTGGGAACACTGT -6FAM
	LCRed 640 probe	LCR640- GAGAATAAAACATTAAAGTTTGCA-phosphate

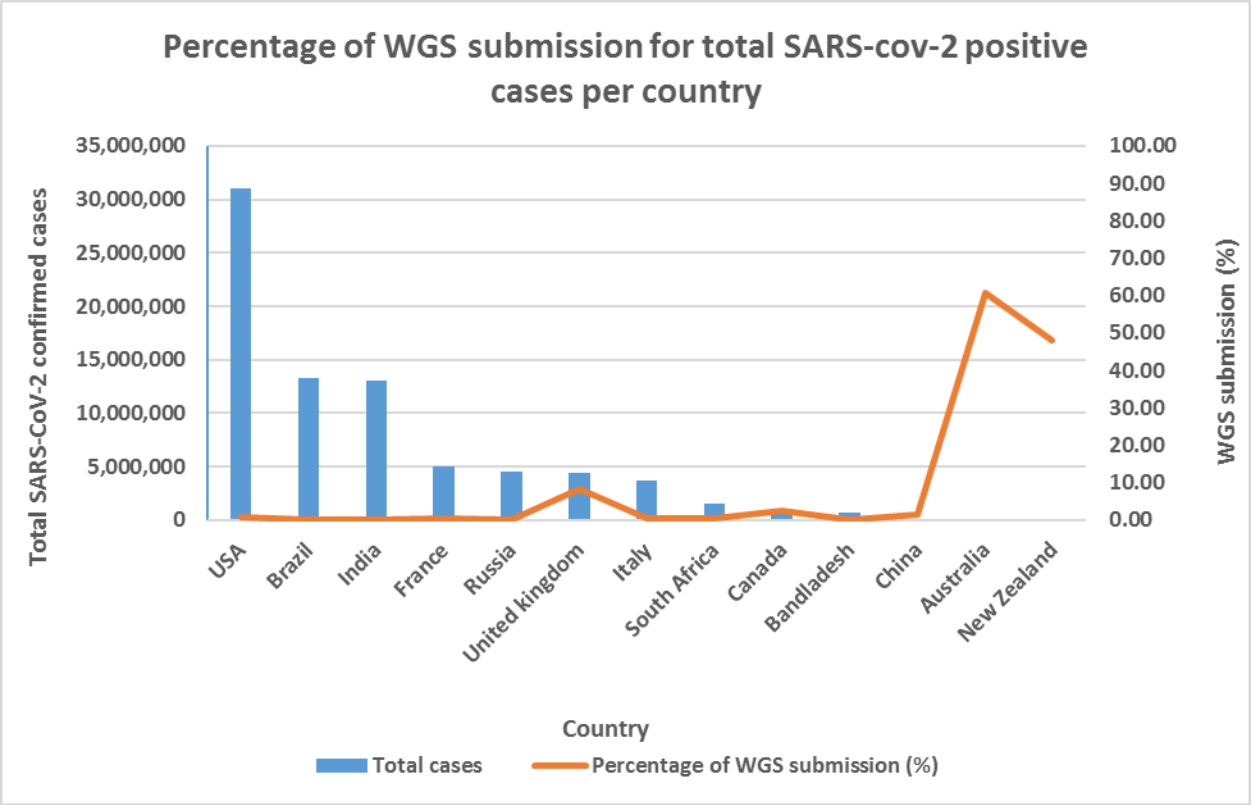

Figure 2.1 Country-wise genomic sequencing effort. Sequence submission to GISAID compared to total reported cases from the six countries most affected by the SARS-CoV-2 pandemic. This diagram reveals genotyping performed on under 1% of cases in the USA, Brazil, India, France, Russia, Italy, South Africa, and 8.3% in the UK. As screening for variants is expensive and time-consuming, this technique is not readily available and thus infrequent in most countries.

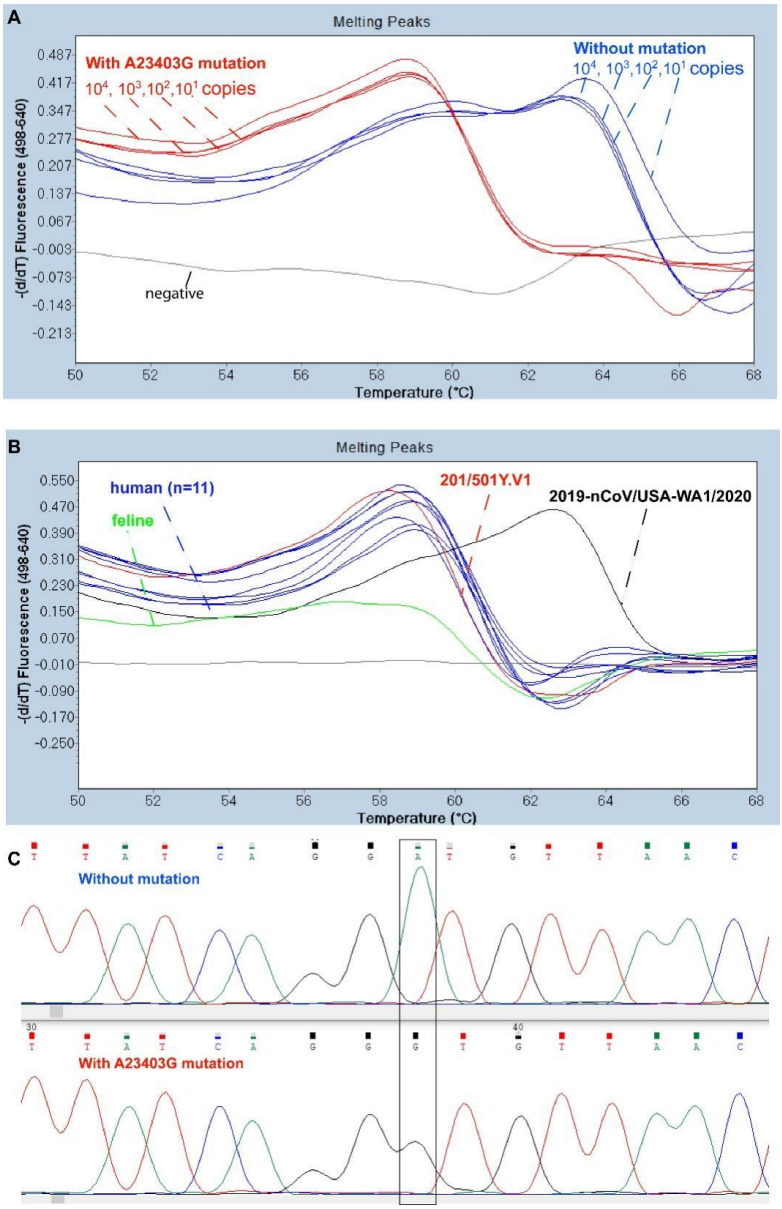

Figure 2.2 Melting temperature (T_m) analysis of SARS-CoV-2 controls and feline and human isolates with a RT-FRET-PCR for the A23403G mutation. The 6-FAM probe designed to match precisely with the SARS-CoV-2 control without the mutation (2019-nCOV/USA-WA1/2020) had a T_m of 63.1°C. This was irrespective of copy number. With the SARS-CoV-2 control that had the mutation (201/501Y.V1), an A to G mismatch with the probe (chromas graph C) resulted in a lower T_m of 58.2°C. (B) RT FRET-PCRs of the clinical samples from a cat and people all had a T_m of around 58.2°C, indicating the presence of the A23403G mutation.

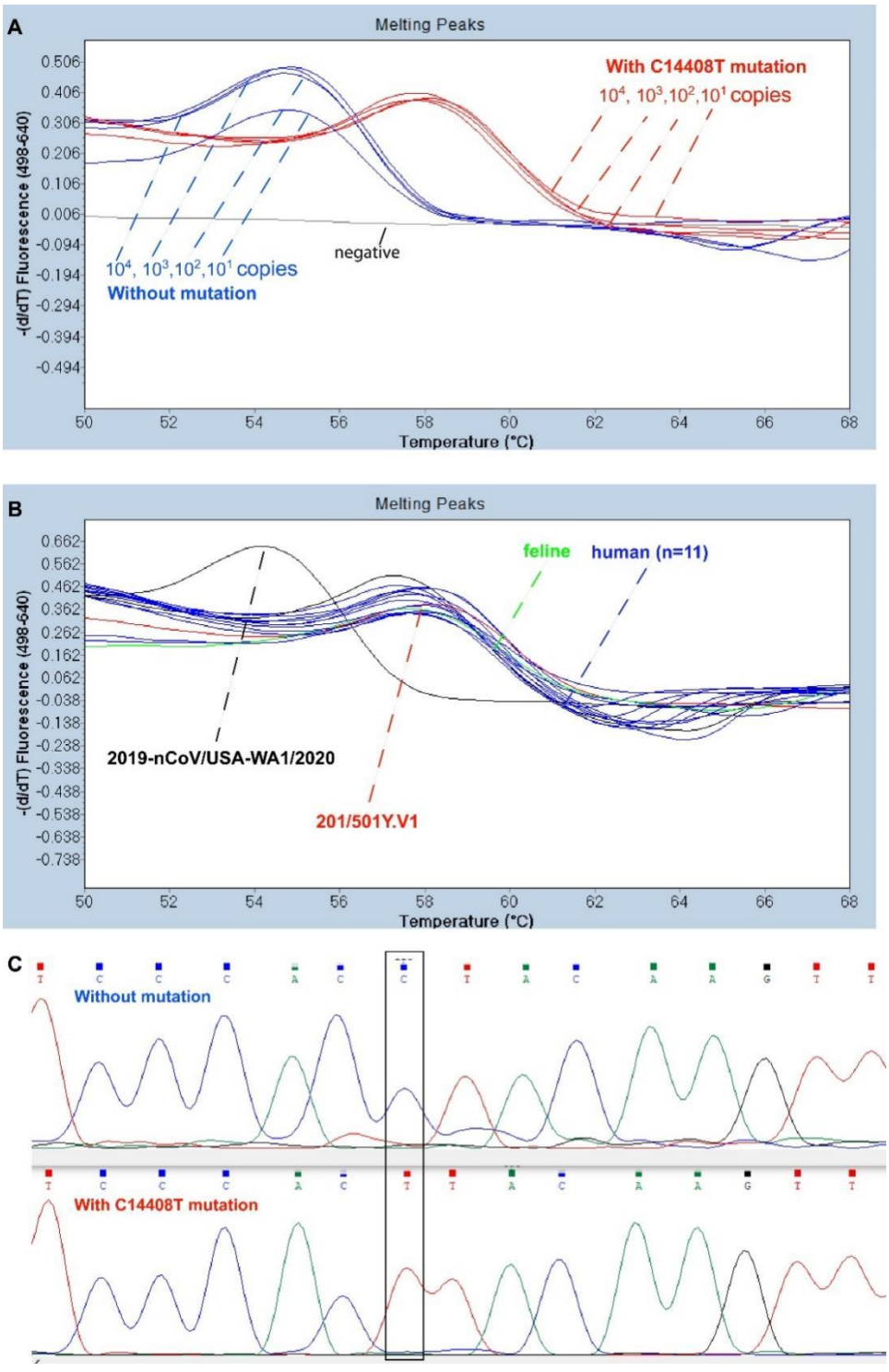

Figure 2.3 Melting temperature (T_m) analysis of SARS-CoV-2 controls and feline and human isolates with a RT-FRET-PCR for the C14408T mutation. The 6-FAM probe designed to match precisely with the SARS-CoV-2 control without the mutation (201/501Y.V1)

had a T_m of 57.7°C. This was irrespective of copy number. With the SARS-CoV-2 control that had the mutation (201/501Y.V1), an A to C mismatch with the probe (chromas graph C) resulted in a lower T_m of 54.3°C. (B) RT FRET-PCRs of the clinical samples from a cat and people all had a T_m of around 54.3°C with this RT FRET-PCR, indicating the presence of the C14408T mutation.

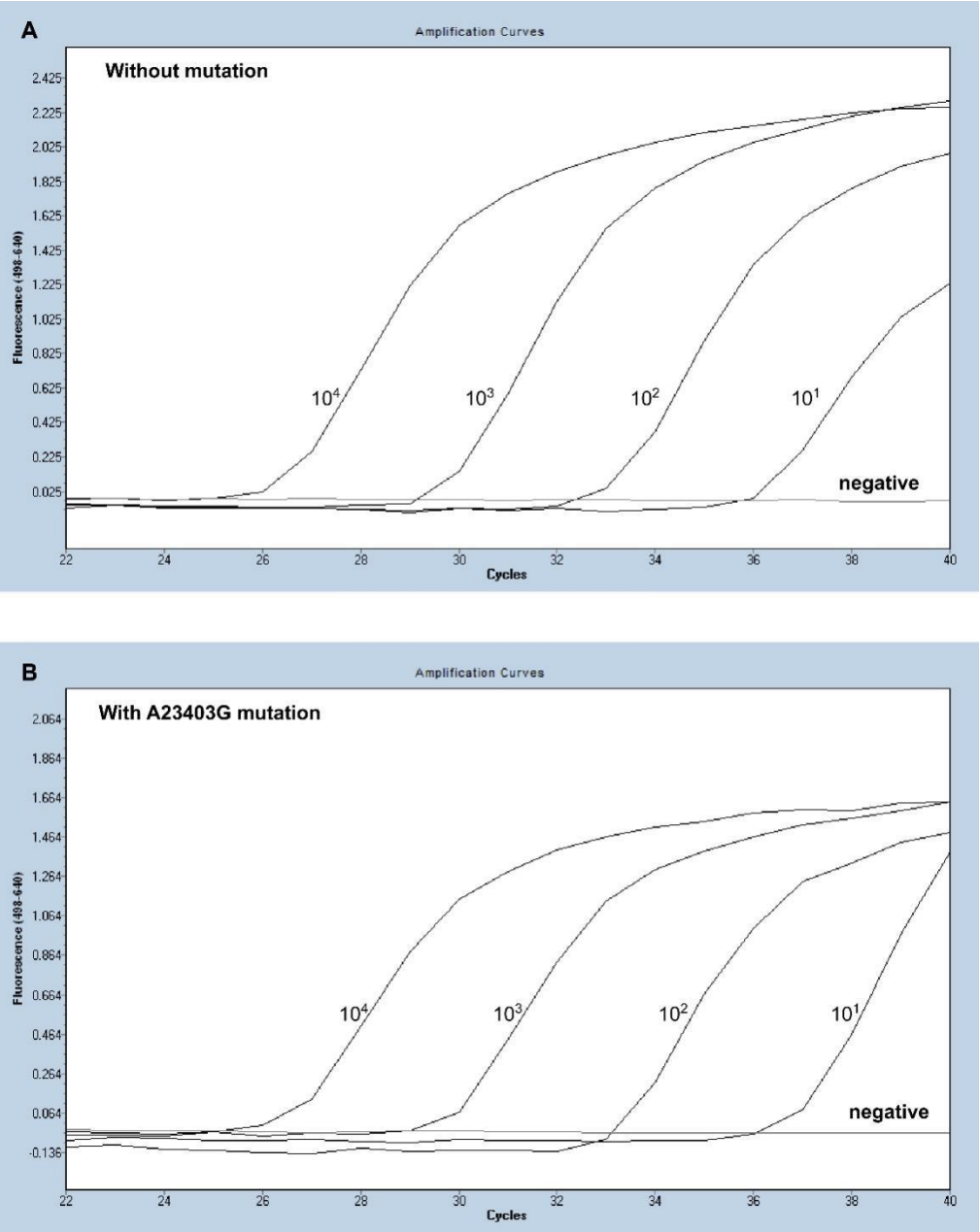

Figure 2.4 Quantitative standards of SARS-CoV-2 PCR targeting the A23403G region. The quantitative standards (10^4, 10^3, 10^2, 10^1 /10 μl) containing sequences of SARS-CoV-2 without (A) and with A23403G mutation (B) and negative control were detected by the one-step reverse transcription FRET-PCR established in this study. The detection sensitivity was ten copies per reaction system.

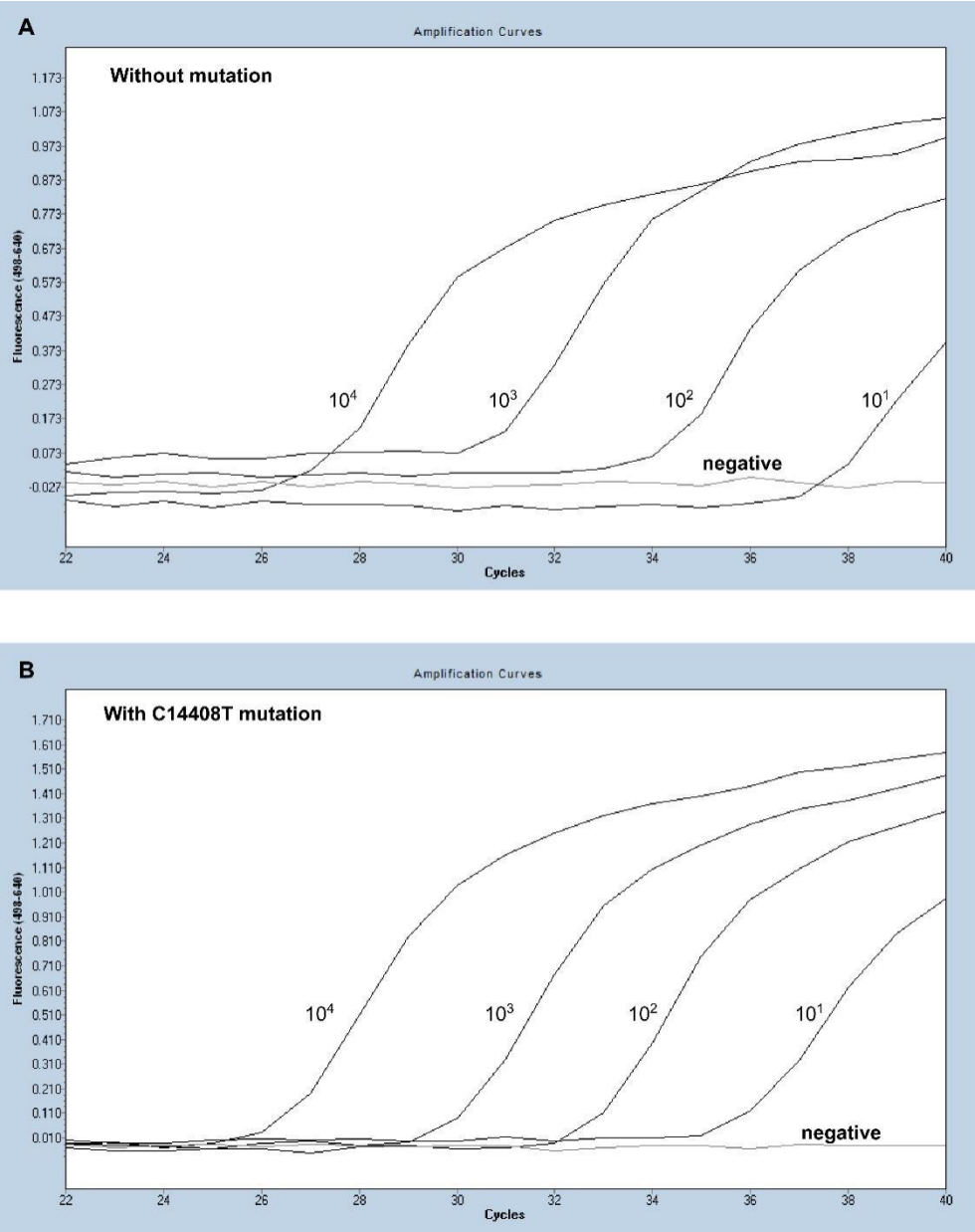

Figure 2.5 Quantitative standards of SARS-CoV-2 PCR targeting the C14408T region. The quantitative standards (10^4, 10^3, 10^2, 10^1 /10 μl) containing sequences of SARS-CoV-2 without (A) and with C14408T mutation (B) and negative control were detected by the one-step reverse transcription FRET-PCR established in this study. The detection sensitivity was ten copies per reaction system.

Chapter 3 Identification of the SARS-CoV-2 Delta variant C22995A using a high-resolution melting curve RT-FRET-PCR

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3.1 Abstract

Knowledge of SARS-CoV-2 variants is essential for formulating effective control policies. Currently, variants are only identified in relatively small percentages of cases as the required genome sequencing is expensive, time-consuming, and not always available. The Delta variant currently predominates in countries with facilities to sequence the SARS-CoV-2. Elsewhere, the prevalence of the Delta variant is unclear. To avoid sequencing, we investigated a RT-FRET-PCR that could detect all SARS-CoV-2 strains and simultaneously identify the Delta variant. The established Delta RT-FRET-PCR has been performed on reference SARS-CoV-2 strains and human nasal swab samples positive for the Delta and non-Delta strains. The Delta RT-FRET-PCR established in this study detected as few as ten copies of the DNA target and 100 copies of the RNA target per reaction. The melting points of products obtained with SARS-CoV-2 Delta variants (around 56.1 °C) were consistently higher than products obtained with non-Delta strains (about 52.5°C). The Delta RT-FRET-PCR can diagnose COVID-19 patients and simultaneously identify if they are infected with the Delta variant. The Delta RT-FRET-PCR can be performed with all major thermocycler brands meaning data on the Delta variant can now be readily generated in diagnostic laboratories worldwide.

3.2 Introduction

The SARS-CoV-2 Delta variant (B.1.617.2) has recently been classified as a variant of concern (VOC) by Public Health England (PHE), the World Health Organization (WHO), and the U.S. Centers for Disease Control (CDC) (https://www.cdc.gov/coronavirus/2019-ncov/variants/variant-info.html). Since its first detection in India in December 2020, the Delta variant has been identified in more than 130 counties (Washington, Gangavarapu et al. 2021) (https://gvn.org/covid-19/delta-b-1-617-2/). It is more than twice as transmissible as the original strain of SARS-CoV-2. It has become the major variant in many countries worldwide where genome sequencing is available to determine its presence (Raman, Patel et al. 2021). Individuals infected with this variant had relative viral loads up to 1,260 times higher than the original virus, indicating accelerated replication of this variant (Li, Deng et al. 2022). In addition, the potency of vaccine efficacy against the delta variant was three- to five-fold lower compared to the Alpha variant (B.1.1.7) (Planas, Veyer et al. 2021).

Detection of SARS-CoV-2 variants is essential for monitoring the COVID-19 pandemic and developing appropriate control policies (Janik, Niemcewicz et al. 2021). Detecting variants, however, requires genome sequencing, which is expensive and time-prohibitive for institutions outside of reference laboratories; thus only infrequently performed (Helmy, Awad et al. 2016). This is the case even in developed countries where, for example, under 1% of positive cases in the US are sequenced (Barua, Hoque et al. 2021). As it is practically impossible for even the most advanced countries to sequence all positive samples, it would be beneficial if tests were available that could be readily used by laboratories worldwide to identify delta variants conveniently. We recently reported a reverse transcription fluorescence resonance energy transfer-polymerase chain reaction (RT-FRET-PCR) that can be performed with all major brands

of PCR machines to detect mutations in the SARS-CoV-2 strains rapidly and conveniently (Barua, Hoque et al. 2021). Here, we establish a Delta RT-FRET-PCR against one mutation uniquely present in the Delta variant that can be readily used by diagnostic laboratories around the world to detect SARS-CoV-2 infections and, simultaneously, the presence of the Delta variant.

3.3 Materials and Methods

3.3.1 Unique mutation in SARS-CoV-2 Delta variant

Analysis of the available whole-genome SARS-CoV-2 sequences in GISAID (www.gisaid.org) confirmed previous reports that the C22995A (T478K) mutation is one of the most common and particular mutations present in the SARS-CoV-2 Delta variant (Cherian, Potdar et al. 2021, Kim, Liu et al. 2021, Aleem, Akbar Samad et al. 2022). The C22995A mutation is present in 99.73% (320,730 / 321,061) of the Delta variant but in only 0.006% (62 / 962,990) of classical isolates, three other variants of concern (VOC), and six variants of interest (VOI) (Table 3.1).

3.3.2 SARS-CoV-2 Delta reverse-transcription FRET-PCRs

Using the whole-genome SARS-CoV-2 sequences from GISAID, upstream and downstream primers were designed (Table 3.2) to target the spike gene with the amplicon size of 235 bp and amplify all SARS-CoV-2 strains we examined in GISAID. The acceptor fluorescent LCRed 640 probe was 5'-labeled and phosphorylated at the 3' end. The 6-carboxyfluorescein (6-FAM)-labeled probe was specifically designed to target the gene for spike protein to contain the

unique C22995A mutation (T478K), and 3' labeled as FRET energy donor probe excited by 488 nm light.

The Delta RT-FRET-PCR was performed on a Roche Light Cycler 480 II system (Roche Molecular Biochemicals, Indianapolis, IN) as described (Barua, Hoque et al. 2021). Thermal cycling was preceded by a 15-minute reverse transcription reaction at 55°C followed by 4 min incubation at 95°C. Thermal cycling consisted of 18 high-stringency step-down cycles followed by 30 relaxed-stringency fluorescence acquisition cycles. The 18 high-stringency step-down thermal cycles were 6 x 10 sec at 95°C, 10 sec at 70°C, 10 sec at 72°C; 9 x 10 sec at 95°C, 10 sec at 68°C, 10 sec at 72°C; 3 x 10 sec at 95°C, 10 sec at 66°C, 10 sec at 72°C. The relaxed-stringency fluorescence acquisition cycling consisted of 30 x 10 sec at 95°C, 10 sec at 55°C, followed by fluorescence acquisition, and 30 sec at 72°C.

The melting curve analysis that assessed the dissociation of the PCR product and the 6-FAM probe was determined by monitoring the fluorescence from 35°C to 75°C with a temperature transition rate of 0.2°C per second as described (Barua, Hoque et al. 2021). The first derivatives of F2/F1 were evaluated to determine the T_m of the probe (Figure 3.1). The PCR products of all tested samples and controls were sent to ELIM Biopharmaceuticals (Hayward, CA, USA) for DNA sequencing.

3.3.4 RT FRET-PCR quantitative standards

DNA standards for the quantitative analysis of the Delta RT-FRET-PCR were generated using the PCR products of the 2019-nCOV/USA-WA1/2020 (purchased from ATCC), and a Delta variant confirmed by whole-genome sequencing [provided by Kansas State Veterinary Diagnostic Laboratory (KSVDL), College of Veterinary Medicine, Kansas State University,

USA]. The PCR products were purified with the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and quantified with the PicoGreen DNA fluorescence assay (Molecular Probes, Eugene, OR) to generate quantitative standards. Standards for RNA quantification analysis studies consisted of 10-fold dilutions of the Quantitative Synthetic SARS-CoV-2 RNA (ATCC, USA).

3.3.5 Test samples

Samples used to evaluate the Delta RT-FRET-PCR consisted of a positive and negative control SARS-CoV-2 sample used during routine screening of samples at KSVDL. The lab also provided 30 human nasal swab samples collected in March 2021 that were positive in SARS-CoV-2 PCR tests. Seventeen of the 30 positive nasal swabs were found to be the Delta variant by genome sequencing, with the remainder not having the C22995A mutation. Finally, we also tested two non-Delta controls purchased from the ATCC (2019-nCOV/USA-WA1/2020; 201/501Y.V1).

A comparison of the melting temperatures between SARS-CoV-2 viruses with and without C22995A mutation was analyzed by the T-test (Statistica, StatSoft, Tulsa, USA). Differences at $P \leq 0.05$ were considered significant.

3.4 Results

The Delta RT-FRET-PCR established in this study was very sensitive, detecting as few as ten copies of the DNA target and 100 copies of the RNA target per reaction. This PCR for Delta was validated to rapidly and conveniently identify delta variants by testing on ATCC controls and human clinical samples.

The Delta RT-FRET-PCR was positive for the positive control sample from KSVDL, the reference SARS-CoV-2 strains from ATCC, and the 30 PCR-positive human nasal swab samples provided by KSVDL that had been confirmed positive by whole genome sequencing. When the Delta RT-FRET-PCR was performed on the seventeen nasal swab samples from KSVDL that were confirmed positive for the SARS-CoV-2 Delta variant, each had a T_m of 56.1 °C (Figure 3.1). In contrast, when the Delta RT-FRET-PCR was performed on the SARS-CoV-2 nasal swab strains that were not the Delta variant, the mismatch between the 6-FAM-probe and the strains resulted in a distinctly lower T_m value of 52.5°C. The apparent difference in the T_m between the Delta variant and non-Delta strains enabled the convenient differentiation of strains with and without the C22995A mutation. The T_m did not change with target copy numbers (Figure 3.1).

Due to the additional nucleotide mismatch between FRET probes and non-Delta SARS-CoV-2, the amplification fluorescence curves are less smooth in non-Delta SAR-CoV-2 than in the Delta variant (Figure 3.2). However, the distinctive melting curves remain sharp for SARS-CoV-2 with and without C22995A mutations (Figure 3.1).

DNA sequencing of the Delta RT-FRET-PCR products verified the presence and absence of the C22995A mutation, as indicated by melting curve analysis. The delta variant with C22995A mutation demonstrated significantly higher melting temperatures than non-Delta strains (56.13± 0.27 SD vs. 52.50 ± 0.23 SD; $p<10^{-4}$).

3.5 Discussion

The SARS-CoV-2 Delta variant has recently been classified as a variant of concern (VOC) by Public Health England (PHE), the World Health Organization (WHO), and the U.S. Centers for Disease Control (CDC) (https://www.cdc.gov/coronavirus/2019-

ncov/variants/variant-info.html). Since its first detection in India in December 2020, the Delta variant has been identified in more than 130 counties [7] (https://gvn.org/covid-19/delta-b-1-617-2/). It is more than twice as transmissible as the original strain of SARS-CoV-2. It has become the major variant in many countries worldwide where genome sequencing is available to determine its presence [8].

Data on the distribution and prevalence of the Delta variant in countries with limited access to genome sequencing would enable a more global picture of the variant to be developed. Similarly, more detailed data on the epidemiology of the Delta variant in developed countries would enable a finer-scale analysis of the dynamics and public health implications of the strain (Cyranoski 2021).

A limitation of using the RT-FRET-PCR is that the C22995A mutation it detects is also present in other variants, but these are only very rarely reported in GISAID (0.006%; 62 / 962,990), and false-positive results would be expected to be very unusual in symptomatic people. The Delta variant is just one of several SARS-CoV-2 variants that have been prevalent, so ongoing monitoring of strains from around the world will still be necessary to detect the evolution of new variants (Janik, Niemcewicz et al. 2021). However, new RT-FRET-PCRs can rapidly be designed to readily enable widespread and detailed monitoring of their spread, epidemiology, and characteristics. The sequence data we used to develop the primers for the Delta RT-FRET-PCR indicate the assay will detect all SARS-CoV-2 strains based on high-quality sequence data recorded in GISAID. Although the Delta RT-FRET-PCR detected all SARS-COV-2 strains against which it was tested in our study, further studies are indicated to more precisely define the sensitivity and specificity of the test in the diagnosis of COVID-19.

In conclusion, the Delta-RT-FRET-PCR we described above proved very sensitive in detecting all the SARS-CoV-2 strains we tested while simultaneously identifying those that were the Delta variant. Further, it can readily be performed with the thermocyclers supplied by the major vendors widely used in COVID-19 diagnostic laboratories worldwide. The Delta-RT-FRET-PCR could, then, add considerably to the available knowledge on the spread of the Delta variant around the world and facilitate the development of public health intervention programs to counter the COVID-19 pandemic.

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Table 3.1 Prevalence of the C22995A mutation in the different SARS-CoV-2 strains

SARS-CoV-2*		**Total sequences**	**With mutation**	**Without mutation**
Delta variant (B.1.617.2)		321,061	320,730 (99.73%)	871 (0.27%)
Non-Delta	Classical	4,018	0 (0.00%)	4,018 (100%)
	VOC**: Alpha (B.1.1.7)	846,338	43 (0.005%)	846,295 (99.99%)
	VOC: Beta (B.1.351)	21,269	9 (0.042%)	21,260 (99.96%)
	VOC: Gamma (P.1)	46,746	3 (0.006%)	46,743 (99.99%)
	VOI**: Zeta (P.2)	3,685	0 (0.00%)	3,685 (100%)
	VOI: Eta (B.1.525)	2,423	1 (0.04%)	2,422 (99.96%)
	VOI: Iota (B.1.526)	20,203	1 (0.005%)	20,202 (99.99%)
	VOI: Kappa (B.1.617.1)	3,804	1 (0.026%)	3,803 (99.97%)
	VOI: Lambda (C.37)	388	0 (0.00%)	388 (100%)
Total non-Delta variant		948,874	58 (0.006)	948,816 (99.99%)

***** The high-quality SARS-CoV-2 sequences were obtained from GISAID on August 10, 2021.

****** VOC: variants of concern; VOI: variants of interest

Table 3.2 The oligonucleotides used in this study

Target of PCR	Primer/Probe	Sequences (5'-3')
C22995A	Upstream primer	CAGGCTGCGTTATAGCTT
	Downstream primer	TATGGTTGGTAACCAACACC
	6-FAM-probe	CCGGTAGCAAMCCTTGTAAT-6FAM
	LCRed 640 probe	LCR640- GTGTTGAAKGWTTTAWTTGTTACTTT- phospate

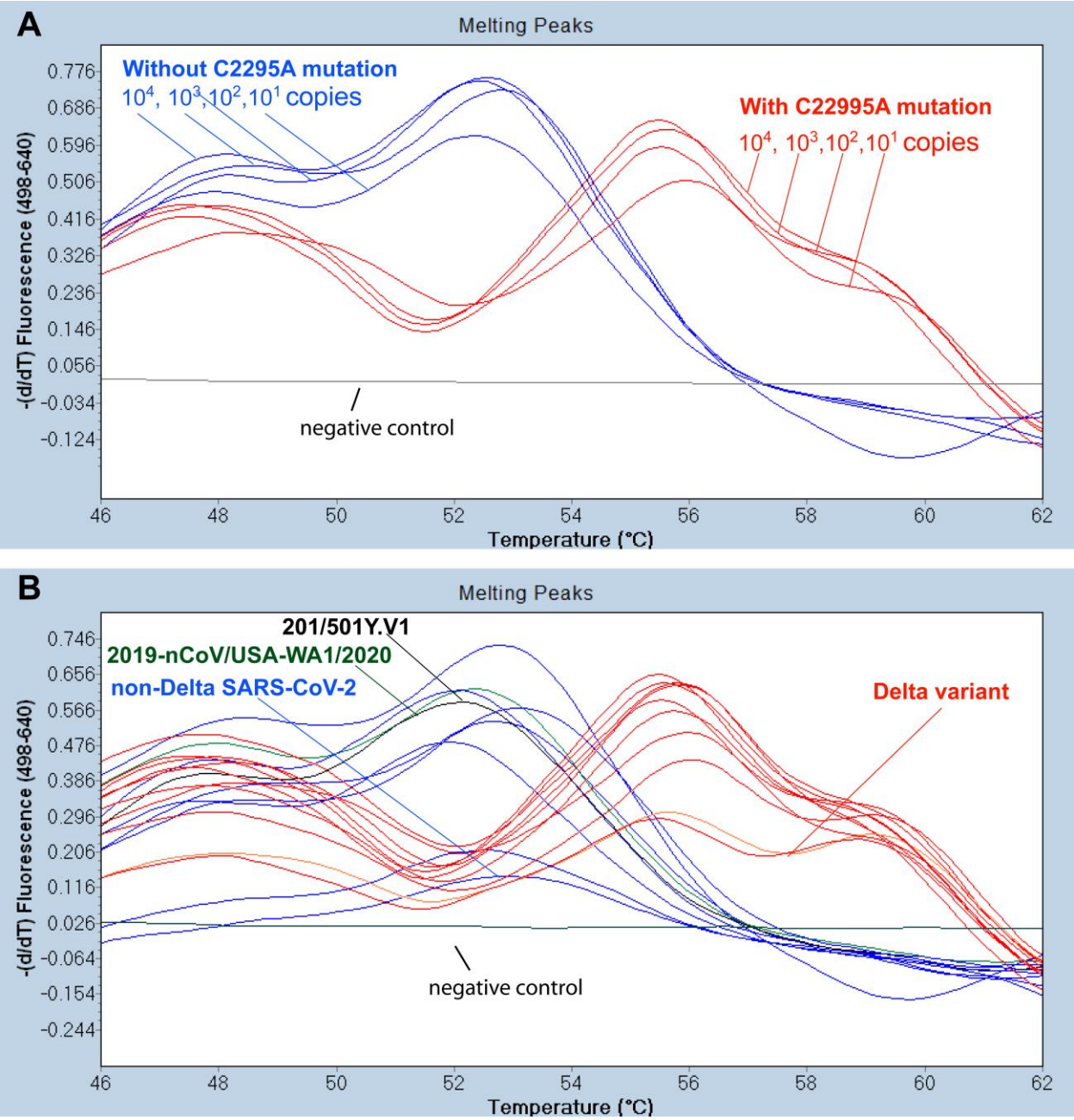

Figure 3.1 Differential melting temperatures of PCR products with non-Delta SARS-CoV-2 and the Delta variant. (A) With the 6-FAM probe designed to match the area incorporating the C22995A mutation exactly, dilutions of a control SARS-CoV-2 Delta variant (positive control from Kansas State Veterinary Diagnostic Laboratory) used in the Delta RT-FRET-PCR had a T_m of around 56.1°C. With the SARS-CoV-2 strains that did not have the mutation (2019-

nCOV/USA-WA1/2020), the A to C mismatch with the probe resulted in a lower T_m of around 52.5°C. The T_m values did not vary significantly with the copy number. The negative control was RNA from a human nasal swab negative for SARS-CoV-2 by routine diagnostic PCR. (B) T_m analysis of representative Delta RT FRET-PCR products from controls and human nasal swab samples with non-Delta variant SARS-CoV-2 (blue lines) and Delta variants (red lines), all confirmed by DNA sequencing. The T_m values for the non-Delta strains were around 52.5°C, while those of the Delta variant were clearly different at around 56.1°C.

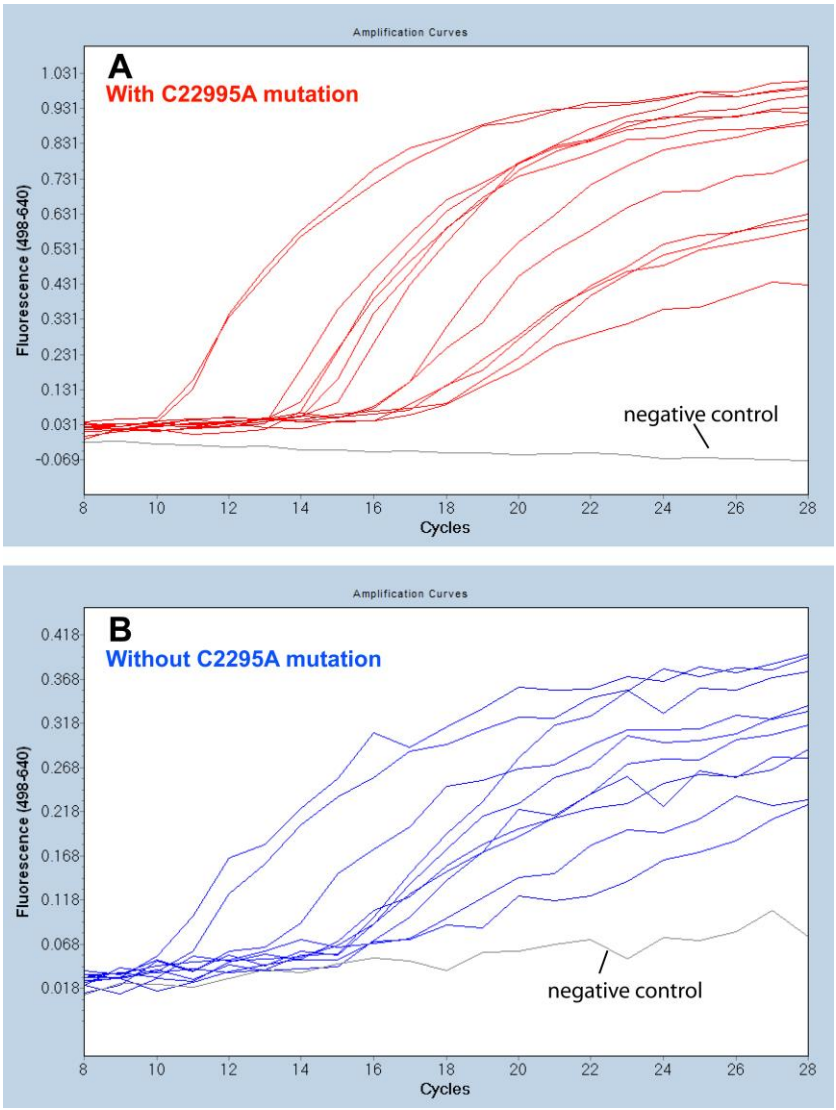

Figure 3.2 PCR amplification curves of SARS-CoV-2 with and without C22995A mutation. SARS-CoV-2 strains with C22995A mutation (A), without C22995A mutation (B), and negative control were detected by the one-step reverse transcription FRET-PCR established in this study. The additional nucleotide mismatches between FRET probes and non-Delta SARS-CoV-2 (without C22995A mutation) resulted in shaking fluorescence curves (B). However, the distinctive melting curves showed sharp fluorescence peaks for SARs-CoV-2 with and without the C22995A mutation.

Chapter 4 Antibodies to SARS-CoV-2 in dogs and cats, USA

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4.1 Abstract

To provide more complete data on SARS-CoV-2 infections in dogs and cats in the United States, we conducted a serosurvey on convenience serum samples from dogs (n=1,336) and cats (n=956) collected from 48 states of the USA in 2020. An ELISA targeting the antibody against nucleocapsid identified six positives and two doubtful samples in cats and five positive and five doubtful samples in dogs. A surrogate neutralization assay detecting antibodies blocking the attachment of the spike protein to ACE2 was positive, with three of the ELISA positive and doubtful samples and one of 463 randomly selected ELISA negative samples. These four positive samples were confirmed by SARS-CoV-2 virus neutralization testing. All were from cats in New York, Florida, and New Jersey (n=2). The serosurvey results, one of the largest yet completed on dogs and cats globally, support the OIE and CDC positions that currently, there is no evidence that pets play a role in the spread of SARS-CoV-2 in humans.

4.2 Introduction

The current Coronavirus Disease 2019 (COVID-19) pandemic is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which most likely originated from wildlife, in particular, horseshoe bats (*Rhinolophus affinis*) or Malayan pangolins (*Manis javanica*), in China in 2019 (Andersen, Rambaut et al. 2020, Sreenivasan, Thomas et al. 2021). Dogs and cats have their coronaviruses (Sharun, Sircar et al. 2020), and the question arose early in the pandemic as to whether they can also be infected with the SARS-CoV-2. As dogs and cats often live in close association with people, if they were susceptible to infection, they might become clinically ill and act as sources of infection for people.

Recent studies have shown dogs have low susceptibility to SARS-CoV-2 infections. Experimentally infected animals can seroconvert but do not show clinical signs. Viral RNA might not be detected in oropharyngeal swabs, but rectal swabs can be positive for up to 6 days post-infection (Bosco-Lauth, Hartwig et al. 2020, Shi, Wen et al. 2020). Although dogs in contact with experimentally infected dogs do not seroconvert (Shi, Wen et al. 2020), a small percentage of dogs in contact with people with COVID-19 (13%; 2/15) might become asymptomatically infected, and some can have low levels of viral RNA in nasal swabs for up to 9 days (Sit, Brackman et al. 2020).

Cats appear to be more susceptible than dogs to SARS-CoV-2 infections and become clinically ill and even die following experimental infection. The infectious virus can be recovered from the upper and lower respiratory tracts for up to 10 days, and viral RNA can be detected in nasal washes for up to 9 days (Gaudreault, Trujillo et al. 2020, Shi, Wen et al. 2020) . In-contact cats can likely become infected by respiratory droplet transmission (Gaudreault, Trujillo et al. 2020, Halfmann, Hatta et al. 2020, Shi, Wen et al. 2020). Cats appear to develop

robust immunity as they produce virus-neutralizing antibodies and are resistant to re-infection upon subsequent challenge (Bosco-Lauth, Hartwig et al. 2020). Cats (12%; 6/50) in COVID-19-positive and close contact households can develop asymptomatic infections (Barrs, Peiris et al. 2020).

The OIE and CDC have produced statements indicating that there is no evidence that pets play a role in spreading human infections with SARS-CoV-2. However, accumulating reports that cats and dogs are susceptible to SARS-CoV-2 has led to growing concerns of owners abandoning pets, fearing they might be a source of infection. Although transmission from pets to humans has not yet been demonstrated, thousands of pets have been killed and abandoned (McNamara, Richt et al. 2020). Recommendations have been made that surveillance for SARS-CoV-2 in cats should be considered an adjunct to the elimination of COVID-19 in people (Shi, Wen et al. 2020). In some areas, dogs and cats in COVID-19-positive and close contact households have been quarantined at home or in holding facilities until proven to be PCR negative (Barrs, Peiris et al. 2020). Models have been developed that indicate abandoning cats in the environment could increase the risk of infection for people (Gao, Pan et al. 2020).

Recent limited studies from Europe and China suggest that natural infections of dogs and cats are infrequent, most commonly following exposure to COVID-19 patients (de Morais, Dos Santos et al. 2020, Michelitsch, Hoffmann et al. 2020). Currently, 113 cases of SARS-CoV-2 in companion animals are reported in the U.S. (46 dogs and 67 cats), predominantly from COVID-19-positive households (www.aphis.usda.gov/aphis/ourfocus/animalhealth/sa_one_health/sars-cov-2-animals-us). The USDA reports confirmed cases in the U.S. to the OIE, and the CDC provides guidelines for protecting pets from infection and what to do if pets become infected (www.cdc.gov/coronavirus/2019-ncov/animals/pets-other-animals.html.).

Assays based on the detection of anti-SARS-CoV-2 IgG antibodies, typically detectable 7 to 21 days post-infection, can identify previous exposure, even in asymptomatic individuals. ELISAs detecting the whole virus, nucleocapsid protein, and the receptor-binding domain (RBD) of the spike receptor protein has been widely used to detect antibodies against SARS-CoV-2 in humans (Michelitsch, Hoffmann et al. 2020, Peterhoff, Gluck et al. 2021). Their usefulness in testing for infections in other species requires evaluation as there are a wide diversity of other coronaviruses in animals that might influence results. Although ELISAs can rapidly process large samples in low-level security facilities, the laborious and slow SARS-CoV-2 virus neutralization test (VNT) requires specialized biocontainment facilities (BSL3) is considered the 'gold standard'. The CDC uses it to classify a case based on presumptive laboratory evidence (www.cdc.gov/coronavirus/2019-ncov/animals/toolkit).

Recently a surrogate neutralization assay (sVNT) was developed, which detects neutralizing antibodies that block the attachment of the RBD of the spike protein to the ACE2 cell surface receptor (Tan, Chia et al. 2020). This test uses an ELISA format which is simple and rapid to perform under BSL-2 conditions. It has high sensitivity and specificity compared with the 90% plaque reduction neutralization test ($PRNT_{90}$) (Perera, Ko et al. 2021). The VNT and sVNT are thus functional assays that detect a protective immune response, while the ELISAs can detect a greater range of antibodies and may, therefore, give higher seroprevalences. For example, Hughes et al. reported that only around half of the individuals who tested positive by ELISA for antibodies to the spike protein also had high VNT titers (Hughes, Amat et al. 2021). Similarly, Zhang et al. reported that only 11 of 15 cats IgG positive in indirect ELISA targeting the RBD of the spike protein had SARS-CoV- 2 neutralizing antibodies detected by VNT (Zhang, Zhang et al. 2020).

To provide data on the exposure of dogs and cats from around the USA to SARS-CoV-2, we tested convenience samples of sera from dogs and cats across 48 states. We initially screened the sera with a commercial double antigen sandwich ELISA kit before further testing selected samples with a commercial sVNT and a classical VNT method.

4.3 Materials and Methods

4.3.1 Sera

Samples used in the study consisted of convenience samples of sera from apparently healthy dogs (n=1,215) and cats (n=831) submitted to Auburn University College of Veterinary Medicine between March and November 2020 for rabies titer testing, which is a requirement for international travel. We also tested sera from dogs (n=121) and cats (n=125) with clinical signs suggestive of hepatozoonosis and feline infectious peritonitis, respectively, that had been submitted for molecular diagnosis (Table 4.1). No data was available on the COVID-19 status of the households from which the dogs and cats originated.

4.3.2 SARS-CoV-2 Double Antigen ELISA

The ID Screen® SARS-CoV-2 Double Antigen ELISA (IDVet, rue Louis Pasteur, Grabels, France) was used to detect nucleocapsid-binding antibodies of SARS-CoV-2 in the dog and cat sera. The ELISA was performed according to the manufacturer's instructions with an S/P % (sample to positive ratio) over 60% regarded as positive. Ratios from 50-60% were considered doubtful, and those below 50% were negative (Figure 4.1).

4.3.3 SARS-CoV-2 Surrogate Virus Neutralization Test

SARS-CoV-2 Surrogate Virus Neutralization Test (sVNT) Kits were purchased from GenScript (N.J., USA) and used according to the manufacturer's instructions (Figure 4.2). The optical densities of the reactions of the test sera and the positive and negative controls supplied were read at 450 nm (OD_{450}), and percentage inhibitions were calculated as follows: percent inhibition = (1 - sample O.D. value/negative-control O.D. value) ×100. Sera with percent inhibition values of ≥20% were regarded as positive, while those with lower values were considered negative (Perera, Ko et al. 2021).

4.3.4 SARS-CoV-2 virus neutralization test

The virus neutralization test (Figure 4.3) was performed at the United States Department of Agriculture (USDA) National Veterinary Services Laboratories (NVSL), as described (Hamer, Pauvolid-Correa et al. 2020). Briefly, 25 µL of two-fold serially diluted sera (for final dilutions of 1:8 to 1:512) were pre-incubated with 25 µL of 100 TCID50/ml of SARS-CoV-2 (2019-nCoV/USA-WA1/2020) in MEM-E containing 200UI/mL penicillin, 200µg/mL streptomycin, 75µg/ml gentamicin sulfate and 6µg/mL Amphotericin B for 60 min at 37°C with 5% CO2. Each serum sample was tested in duplicate in 96-well plates. At one hour post-infection, 150µl of Vero 76 cells were added to the virus-serum mixtures. The neutralization titers were determined at three days post-infection. The titer was recorded as the reciprocal of the highest serum dilution that provided 100% neutralization of the reference virus, as determined by visualization of the cytopathic effect. Neutralizing titers of 8 and 16 were considered suspect in the absence of other positive tests; titers greater than 16 were considered seropositive. Samples positive for SARS-CoV-2 at NVSL were reported to the OIE.

4.4 Results

With the I.D. Screen ELISA, six cat sera were positive, two were doubtful, and 948 were negative (Table 4.2). With the I.D. Screen ELISA, five sera from dogs, were positive, five were doubtful, and 1,326 were negative (Table 4.3). None of the sera from the 125 cats with suspected FIP were positive in the I.D. Screen ELISA (tend to delete this sentence). Two of the 121 dogs (ID Numbers: 1098, 2013) with suspected *Hepatozoon americanum* were positive in the IDVet Screen ELISA but subsequently were negative in both the sVNT and VNT.

Twenty-six sera with the highest S/P ratios in the I.D. Screen ELISA (11 positives, seven doubtful, and six negative samples) and 434 randomly selected negative samples were further tested with the sVNT. Three of the eleven I.D. Screen ELISA positive samples and one of the 434 negative samples gave positive results in the sVNT (Table 4.2). All the I.D. Screen ELISA doubtful samples were negative in the sVNT.

When the above 26 sera with the highest S/P ratios in the I.D. Screen ELISA was further tested by VNT at the NVSL; only the samples positive in the sVNT were positive (Table 4.2). The sample the sVNT identified as positive amongst the 434 found negative by the I.D. Screen ELISA also tested positive in the VNT (Table 4.2). Three samples showed the toxic effects of VNT. One had given a doubtful result in the I.D. Screen ELISA (S/P% =49.2%) was negative in the sVNT. The remaining two were negative in both the I.D. Screen ELISA and the sVNT.

Although the VNT positive results represent only a single point in time, they suggest infection with SARS-CoV-2 and meet the USDA case definition for confirmed SARS-CoV-2 cases (CDC). While the VNT titers of the four confirmed cases ranged from 1:32 to 1:256, all four had high inhibition values with the sVNT (around 97%) (Table 4.2). Three of the four VNT-

positive cases also had high S/P ratios (>220%) in the I.D. Screen ELISA. The remaining sample (ID Number 2620), with a VNT titer of 1:32, had an S/P ratio of 48.2% in the I.D. Screen ELISA, just below the value of 50-60%, indicative of a doubtful result.

All the VNT-positive sera were from cats: one from N.Y. (ID number 2538), one from Florida (ID 2797), and two from New Jersey (ID 2620 and ID 2903).

4.5 Discussion

Overall, regarding the results of negative samples, there was a relatively good correlation between the performances of the IDVet Screen ELISA and the sVNT, with the tests agreeing on 433 of the 434 samples. However, of the 11 samples found positive for antibodies to the nucleocapsid proteins in the IDVet Screen ELISA, only 3 had neutralizing antibodies against the RBD of the spike protein, as demonstrated by a positive VNT. A variety of coronaviruses can infect dogs and cats and, in general, nucleocapsid proteins of coronaviruses are relatively conserved with significant antigenic cross-reactivity (Sun and Meng 2004, Fritz, Rosolen et al. 2021). There is thus the possibility that the IDVet Screen ELISA positives were most likely due to exposure to other animal coronaviruses.

The cat that was negative by the IDVet Screen ELISA but positive in both the GenScript sVNT and VNT most likely had been exposed to the SARS CoV-2 but not developed neutralizing antibodies against the nucleocapsid. The presence of antibodies against the spike protein in the absence of antibodies against nucleocapsid proteins is not uncommon in people (Tehrani et al., 2020; Liu et al., 2021).

Despite the USA being one of the countries most seriously affected by the COVID-19 pandemic, we found none of the convenience serum samples of dogs (0/1,336) we studied were

positive for antibodies to SARS-CoV-2. Further, a few of our convenience samples from cats (0.4%; 4/956) had serological evidence of infection. The SARS-CoV-2 positivity rate of pets in households with unknown COVID-19 status in the U.S. would thus appear to be very low (0.17%; 4/ 2,289 animals).

Our serosurvey of cats and dogs for antibodies to SARS CoV-2 adds to the growing body of information from smaller studies on infections in pets worldwide (Chen, Huang et al. 2020, Deng, Jin et al. 2020, Hamer, Pauvolid-Correa et al. 2020, Michelitsch, Hoffmann et al. 2020, Patterson, Elia et al. 2020, Temmam, Barbarino et al. 2020, Zhang, Zhang et al. 2020, Ruiz-Arrondo, Portillo et al. 2021, Stevanovic, Vilibic-Cavlek et al. 2021). Studies have shown that cats experimentally infected with ~10^5 PFU can readily transmit SARS-CoV-19 to naïve cats when placed in continuous and close confinement (Bosco-Lauth, Hartwig et al. 2020, Halfmann, Hatta et al. 2020, Shi, Wen et al. 2020). In contrast, naïve dogs closely confined with experimentally infected dogs do not become infected (Shi, Wen et al. 2020). Although animal-to-human transmission of SARS-COV-2 occurs in minks (Oude Munnink, Sikkema et al. 2021), there is currently no evidence that dogs and cats can transmit infections to people (de Morais, Dos Santos et al. 2020). Instead, reports on dogs and cats in households suffering from COVID-19 (de Morais, Dos Santos et al. 2020) indicate that people infect pets. However, such transmission appears to be low, with PCR studies showing only low positivity rates in pets in COVID-19-positive households. For example, in Spain, only 1/8 (12%) cats and 0/12 dogs tested positive in COVID-19-positive households (Ruiz-Arrondo, Portillo et al. 2021), while in Hong Kong, only 6/50 (12%) cats (Barrs, Peiris et al. 2020) and 2/15 (13%) dogs were positive (Sit, Brackman et al. 2020). Also, transmission is not inevitable. In a report of 9 cats and 12 dogs

living among 20 French veterinary students (2 with confirmed COVID-19 and 11 with clinical signs), none of the pets were seroconverted (Temmam, Barbarino et al. 2020).

The seroprevalence we found in cats in the U.S. is very similar to that of 0.69% (6/920) and 0.76% (1/131) reported in the only other large-scale serosurveys carried out to date, in Germany (Michelitsch, Hoffmann et al. 2020) and Croatia (Stevanovic, Vilibic-Cavlek et al. 2021), respectively. Both surveys were carried out on cats from households with unknown COVID-19 status in the early stages of the pandemic when the incidence of human infection was likely still relatively low. Our study was conducted until November 2020 and thus included the first COVID-19 wave of disease and the initial phases of the second wave when pet exposure would appear more likely. Other smaller surveys of cats with an unknown history of exposure have revealed seropositive animals in China (0%, 0/86; 15%,15/102)(Deng, Jin et al. 2020, Zhang, Zhang et al. 2020), France (6%, 1/16) (Sun and Meng 2004), and Italy (5.1%; 2/39)(Patterson, Elia et al. 2020) for example.

A limitation of our study is that we had no information on the COVID status of the households containing the animals we studied. We anticipate, however, that as the sera we tested were submitted for rabies titers, there was the anticipation of international travel, which would not be expected in households with active COVID-19. We suspect, then, that most of the animals we studied were from households with no history of COVID-19 patients and that have had households with active human infections might provide higher prevalence. Although seroprevalences are difficult to compare directly because of differences in serological techniques used in experiments, dogs and cats in contact with COVID-19 patients, have been reported to be eight times more likely (relative risk 8.1) to be seropositive than those in homes of unknown exposure (Fritz, Rosolen et al. 2021). Cat seroprevalences in households with COVID-19

patients have been reported from Italy (4.5%, 1/22)(Patterson, Elia et al. 2020), France (0%; 0/9; 23.5%, 8/34)(Temmam, Barbarino et al. 2020, Fritz, Rosolen et al. 2021), and the U.S. (4/ up to 34) (Hamer, Pauvolid-Correa et al. 2020), for example.

In conclusion, our data indicate that dogs and cats in the U.S. appear to be infrequently infected with SARS-CoV-2. This data supports evidence that companion animals are not a significant source of human infection. A recent study has shown animal health workers and veterinary laboratory personnel in contact with animals and their products are no more likely to become seropositive than workers with no animal contact (Stevanovic, Vilibic-Cavlek et al. 2021). Companion animals provide multifaceted health benefits to their owners, including increased emotional well-being, significant stress reduction, and increased physical activity. Such benefits are significant to senior citizens during isolation and stress induced by the COVID-19 pandemic. The media should thus be encouraged to refrain from emotive reporting on the role of pets in COVID-19. The current OIE (https://www.oie.int/scientific-expertise/specificinformation-and-recommendations/questions-and-answerson-2019novel-coronavirus/), CDC (CDC), and AVMA (https://www.avma.org/resources-tools/animal-healthand-welfare/covid-19/) recommendations relating to companion animal infections should be followed closely. Companion animal veterinarians need to be alerted to the possibility, albeit low, of SARS-CoV-2 in their patients and the most appropriate treatments and methods to prevent the spread of infection to other animals and people in the household (https://www.avma.org/resources-tools/animal-healthand-welfare/covid-19/).

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Table 4.1 Sera samples used in this study

State	Number of samples submitted			Tested positive					
				IDVet (N=2117)		VNT (N=26)		sVNT (N=460)	
	Total	Canine	Feline	Canine	Feline	Canine	Feline	Canine	Feline
Alabama	123	91	32	1	0	0	0	0	0
Alaska	0	0	0	0	0	0	0	0	0
Arizona	12	7	5	0	0	0	0	0	0
Arkansas	24	7	17	0	0	0	0	0	0
California	439	208	231	1	1	0	0	0	0
Colorado	20	17	3	0	0	0	0	0	0
Connecticut	13	2	11	0	0	0	0	0	0
Delaware	6	2	4	0	0	0	0	0	0
District of Columbia	0	0	0	0	0	0	0	0	0
Florida	177	136	41	0	1	0	1	0	1
Georgia	121	93	28	0	0	0	0	0	0
Hawaii	59	45	14	0	0	0	0	0	0
Idaho	14	7	7	0	0	0	0	0	0
Illinois	47	26	21	0	0	0	0	0	0
Indiana	18	6	12	0	1	0	0	0	0
Iowa	12	7	5	0	0	0	0	0	0
Kansas	7	3	4	0	0	0	0	0	0
Kentucky	7	6	1	0	0	0	0	0	0
Louisiana	15	12	3	0	0	0	0	0	0
Maine	2	2	0	0	0	0	0	0	0
Maryland	8	3	5	0	0	0	0	0	0
Massachusetts	81	34	47	0	0	0	0	0	0
Michigan	34	26	8	0	0	0	0	0	0
Minnesota	13	5	8	0	0	0	0	0	0
Mississippi	35	30	5	1	0	0	0	0	0

Missouri	13	10	3	0	0	0	0	0	0
Montana	3	3	0	0	0	0	0	0	0
Nebraska	4	3	1	0	0	0	0	0	0
Nevada	9	5	4	0	0	0	0	0	0
New Hampshire	9	1	8	0	0	0	0	0	0
New Jersey	12	8	4	1	0	0	2	0	2
New Mexico	5	5	0	0	0	0	0	0	0
New York	304	141	163	0	2	0	1	0	1
North Carolina	25	9	16	0	0	0	0	0	0
North Dakota	0	0	0	0	0	0	0	0	0
Ohio	52	29	23	0	0	0	0	0	0
Oklahoma	16	14	2	0	0	0	0	0	0
Oregon	5	2	3	0	1	0	0	0	0
Pennsylvania	90	66	24	0	0	0	0	0	0
Rhode Island	5	3	2	0	0	0	0	0	0
South Dakota	0	0	0	0	0	0	0	0	0
South Carolina	21	9	12	0	0	0	0	0	0
Tennessee	38	35	3	1	0	0	0	0	0
Texas	91	59	32	0	0	0	0	0	0
Utah	7	7	0	0	0	0	0	0	0
Vermont	4	2	2	0	0	0	0	0	0
Virginia	37	20	17	0	0	0	0	0	0
Washington	56	24	32	0	0	0	0	0	0
West Virginia	2	1	1	0	0	0	0	0	0
Wisconsin	11	5	6	0	0	0	0	0	0
Wyoming	3	1	2	0	0	0	0	0	0
Unknown	8	5	3	0	0	0	0	0	0
Total	**2117**	**1242**	**875**	**5**	**6**	**0**	**4**	**0**	**4**

Table 4.2 Eleven sera tested for cats by I.D. Screen ELISA, sVNT and VNT in this study

Sample ID	Species	Breed	Sex	Age	IDVet Screen S/P ratio and O.D. value	GenScript sVNT inhibition value; OD value	VNT titer
2538	Cat	Munchkin	Male	20 M	292.1%; 2.39	97.3%; 0.044	1:256
2903	Cat	DSH	Female	7 Y	240.7%; 2.34	97.4%; 0.043	1:128
2620	Cat	DSH	Female	2 Y	220.4%; 2.24	96.9%; 0.046	1:32
2410	Cat	DSH	Female	2Y2M	89.3%; 0.92	-2.1%; 1.67	negative
3213	Cat	DSH	Female	5 Y	70.4%; 0.77	-9.1%; 1.78	negative
3577	Cat	DSH	Female	2 Y	63.5%; 0.71	-10.8%; 1.81	negative
3329	Cat	Ragdoll	Female	10 M	58.8%; 0.68	-7.4%; 1.75	negative
1960	Cat	Ragdoll	Female	14 M	54.2%; 0.59	0.2%; 1.63	negative
2797	Cat	DSH	Male	6 Y	49.2%; 0.55	97.2%; 0.050	1:32
3372	Cat	Bengal	Female	5 Y	41.7%; 0.47	-2.9%; 1.68	negative
2931	Cat	DSH	Female	4 Y	41.2%; 0.44	0.3%; 1.63	negative

Table 4.3 Fifteen sera tested for dogs by I.D. Screen ELISA, sVNT and VNT in this study

Sample ID	Species	Breed	Sex	Age	IDVet Screen S/P ratio and O.D. value	GenScript sVNT inhibition value; OD value	VNT titer
1098	Dog	Pyrenees Mix	Male	8Y	129.6%; 1.21	-0.4%; 1.71	negative
107	Dog	Labrador Retriever	Female	5Y	119.6%; 1.16	7.9%; 1.51	negative
1243	Dog	Shih Tzu	Male	7Y	109.4%; 0.99	-6.8%; 1.74	negative
2013	Dog	Miniature poodle	Female	4Y	101.5%; 1.06	-1.8%; 1.66	negative
2075	Dog	Terrier Mix	Male	1Y	69.3%; 0.62	-0.5%; 1.64	negative
2166	Dog	Shiba Inu	Male	7 Y	59.5%; 0.63	-4.0%; 1.70	negative
2617	Dog	Border Collie	Female	4 Y	59.5%; 0.64	-9.5%; 1.75	negative
3180	Dog	Collie Rough Coat	Male	6 Y	54.5%; 0.61	-1.2%; 1.65	negative
1187	Dog	Karelian Bear Dog	Female	12 Y	53.3%; 0.37	1.7%; 1.60	negative
351	Dog	Mixed breed	Male	3 Y	52.0%; 0.48	-3.9%; 1.70	toxic
3496	Dog	Miniature Schnauzer	Male	10 M	47.5%; 0.49	0.3%; 1.63	negative
463	Dog	Mixed breed	Female	10 M	44.2%; 0.42	-0.1%; 1.63	toxic
462	Dog	American domestic shorthair	Female	5 M	43.0%; 0.41	-5.8: 1.73	toxic
2685	Dog	Basenji Mix	Female	21 M	38.3%; 0.43	-12.7%; 1.84	negative
3408	Dog	Border Collie	Male	1Y 4M	37.6%; 0.43	-3.8%; 1.70	negative

Figure 4.1 Double antigen sandwich ELISA detects nucleocapsid-binding antibodies of SARS-CoV-2. Unlike conventional ELISA formats, this assay uses an antigen sandwich, with the second part of the sandwich being an antigen conjugated to an enzyme to visualize specific detection. The ELISA titer >16 is considered positive, and titers 8 and 16 a suspect.

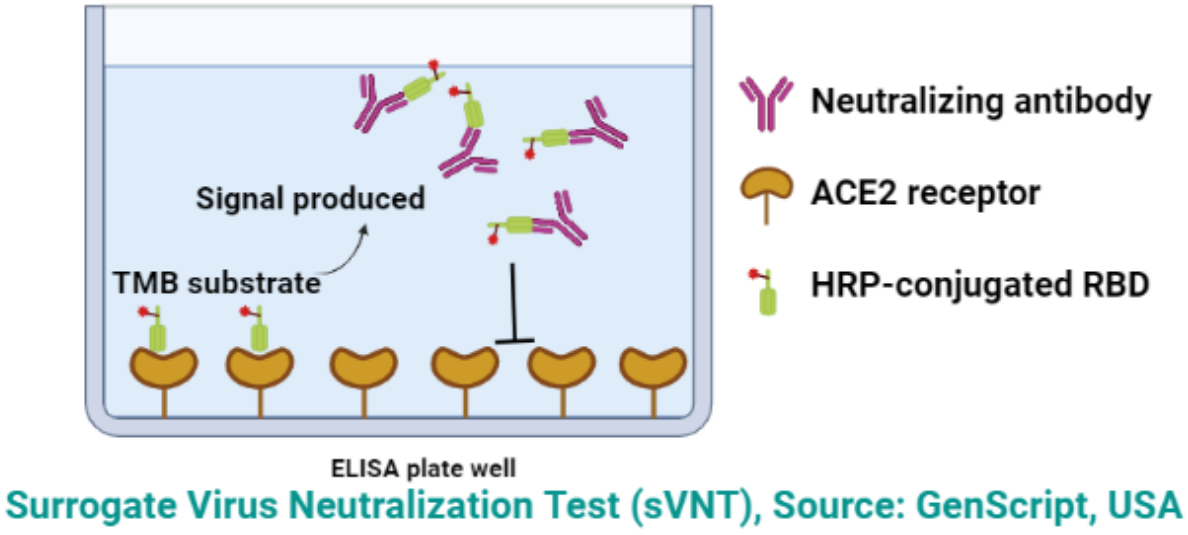

Figure 4.2 SARS-CoV-2 surrogate virus neutralization test (sVNT) where Horseradish peroxidase (HRP) conjugated to the receptor-binding domain (RBD) of the SARS-CoV2 spike protein is pre-incubated on angiotensin-converting enzyme 2 receptor-coated ELISA plate. If nAb is present, HRP-conjugated RBD is blocked from binding, resulting in an attenuated signal when a substrate is provided. The optical density value ≥20% is considered positive, whereas <20% is considered negative.

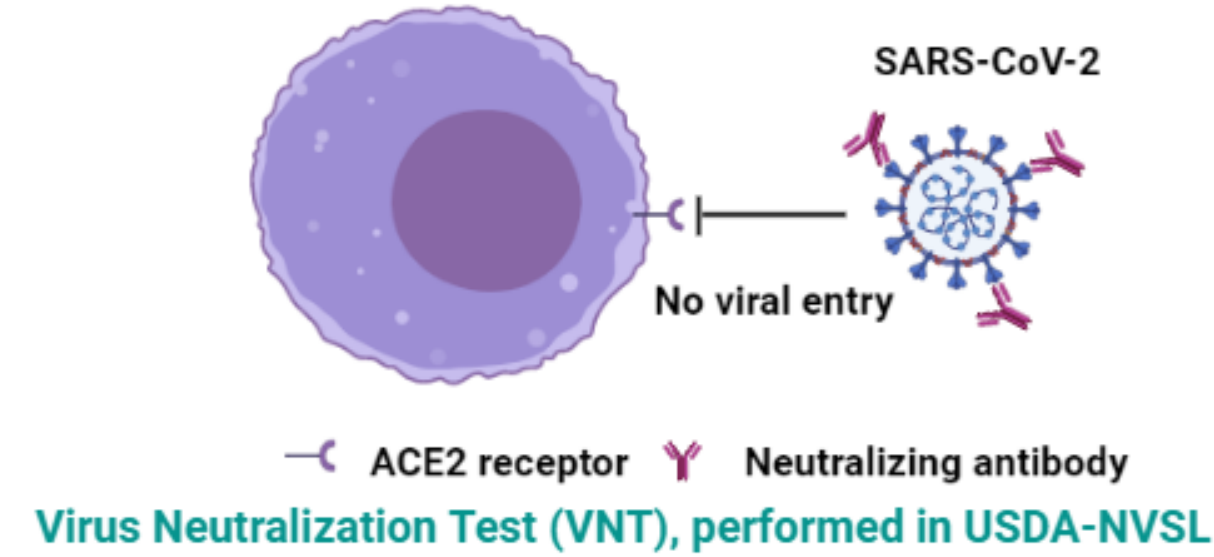

Figure 4.3 Virus neutralization tests (VNT) where anti-SARS-CoV-2 neutralizing antibodies block the SARS-CoV-2 spike protein from binding to ACE2 receptor proteins on the host cell surface, inhibiting viral entry and the formation of plaques. Sample to a positive ratio (S/P%) with ≥ 60% considered as positive whereas ≤ 50% as negative and >50% to < 60% as doubtful value.

Chapter 5 Absence of SARS-CoV-2 in a captive white-tailed deer population in Alabama, USA

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5.1 Abstract

While serological and molecular evidence of SARS-CoV-2 infection has been reported in white-tailed deer (*Odocoileus virginianus*) from the USA, deer sera from the U.K. (n=1,748) were found to be negative by a serosurvey. To further understand the geographical distribution of SARS-CoV-2 infected deer, a serosurvey was performed on archived deer serum samples collected from the Auburn University Captive Facility in Camp Hill, Alabama, between Oct 2019 and Jan 2022. A surrogate SARS-CoV-2 virus neutralization test identified one positive sample, which was later determined to be negative by the virus neutralization testing performed at USDA National Veterinary Services Laboratories. In addition, rectal and nasopharyngeal swabs from deer collected in January and February 2022 were negative by SARS-CoV-2 PCR. Of 72 people who had close contact with the deer over the study period, 29 completed a voluntary questionnaire that showed three had been infected with the SARS-CoV-2 during the study period. Our finding was that the deer we studied appeared not to have been exposed to SARS-CoV-2 despite human infections in the facility.

5.2 Introduction

SARS-CoV-2, the agent causing COVID-19 in humans, has been reported to infect domestic (dogs, cats) and wild animals (ferrets, lions, mink, pumas, rodents, snow leopards, tigers) (Mallapaty 02 August 2021). Recently, infection models demonstrated that white-tailed deer (*Odocoileus virginianus*) were susceptible to SARS-CoV-2 (Bashor, Gagne et al. 2021, Di Guardo 2021, Palmer, Martins et al. 2021, Cool, Gaudreault et al. 2022). Serological and molecular evidence indicated that white-tailed deer from the USA, such as Illinois, Iowa, Michigan, Ohio, Pennsylvania, New York, South California, and Texas (Hale, Dennis et al. 2022, Hancock, Hickman et al. 2022, Kuchipudi, Surendran-Nair et al. 2022, Palermo, Orbegozo et al. 2022, Roundy, Nunez et al. 2022, Vandegrift, Yon et al. 2022) and Canada (Kotwa, Massé et al. 2022) had been exposed to SARS-CoV-2 (Table 5.1). Vandegrift et al. reported the detection of the highly transmissible SARS-CoV-2 Omicron variant (B.1.1.529) from white-tailed deer in New York (Vandegrift, Yon et al. 2022).

Estimates suggest there may be more than 30 million white-tailed deer in rural, suburban, and urban areas in North America. They are the most sought-after game species in North America, with $35M spent yearly by the 11.4 million people who hunt the animals. In Alabama, there are an estimated 1.25 million white-tailed deer, making the human-deer population ratio approximately 4:1. There are about 225,000 licensed deer hunters in Alabama, and Alabama hunters typically harvest approximately 275,000 animals annually over a season that is 109 days in length. The interactions between humans and deer due to hunting and suburban/urban encroachment create a scenario in Alabama where deer could be exposed to SARS-CoV-2. To provide information on SARS-CoV-2 infections of white-tailed deer in a captive facility in the state of Alabama, we conducted a serological and RT-PCR survey.

5.3 Materials and Methods

5.3.1 Samples

Blood samples and nasopharyngeal and fecal swabs used in this study were collected from deer at the Auburn University Captive Facility in Camp Hill, Alabama. This is a 174-hectare high-fenced facility with a population of approximately 100 deer. Although given ad libitum supplemental feed, these deer are not domesticated and have similar behaviors to free-ranging deer outside the facility, albeit the latter have more space and a lower population density (Newbolt, Acker et al. 2017). On weekdays between October 2019 and February 2022, deer captured after being darted at feeders (Newbolt, Acker et al. 2017) had body and dental measurements taken and sera and nasopharyngeal swabs collected. This usually took 15-30 minutes and involved 2-5 people in very close contact with the deer, thus creating a high potential for human-to-deer and deer-human transmission of infectious diseases. The COVID-19 status of the deer handlers at the time of the procedures was obtained from voluntarily completed questionnaires.

All animal procedures were approved by the Auburn University Institutional Animal Care and Use Committee (PRN 2016-2964, PRN 2016-2985, PRN; 2019-3599, PRN 2019-3623). Whole blood was collected into EDTA from the jugulars of 64 deer (seven in October 2019; 25 in 2020, 30 in 2021, and two in January 2022), and serum was separated and stored at -85C. In January and February 2022, nasopharyngeal and fecal swabs were obtained from seven deer and stored in 400 µl DNA/RNA stabilization buffer (Roche Life Science) until DNA extraction and PCR as described below.

5.3.2 SARS-CoV-2 Surrogate Virus Neutralization Test

SARS-CoV-2 Surrogate Virus Neutralization Test (sVNT) Kits were purchased from GenScript (N.J., USA) and used according to the manufacturer's instructions. The optical densities of the reactions of the test sera and the positive and negative controls supplied were read at 450 nm (OD_{450}), and percentage inhibitions were calculated as follows: percent inhibition = (1 - sample O.D. value/negative-control O.D. value) ×100. Sera with percent inhibition values of ≥20% were regarded as positive, while those giving lower values were considered to be negative (25)

5.3.3 SARS-CoV-2 virus neutralization test

The virus neutralization tests were performed at the United States Department of Agriculture (USDA) National Veterinary Services Laboratories (NVSL), as described in the previous chapter (28). Briefly, 25 µL of two-fold serially diluted sera (for final dilutions of 1:8 to 1:512) were pre-incubated with 25 µL of 100 TCID50/ml of SARS-CoV-2 (2019-nCoV/USA-WA1/2020) in MEM-E containing 200UI/mL penicillin, 200µg/mL streptomycin, 75µg/ml gentamicin sulfate and 6 µg/mL Amphotericin B for 60 min at 37°C with 5% CO2. Each serum sample was tested in duplicate in 96-well plates. At one hour post-infection, 150µl of Vero 76 cells were added to the virus-serum mixtures. The neutralization titers were determined three days post-infection. The titer was recorded as the reciprocal of the highest serum dilution that provided 100% neutralization of the reference virus, as determined by visualization of the cytopathic effect. Neutralizing titers of 8 and 16 were considered suspect in the absence of other positive tests; titers greater than 16 were considered seropositive. Samples positive for SARS-CoV-2 at NVSL were reported to the OIE.

5.3.4 SARS-CoV-2 Reverse-Transcription FRET-PCRs

The High-Pure PCR Template Preparation Kit (Roche Diagnostics, Indianapolis, IN USA) was used to extract total nucleic acids from nasopharyngeal and rectal swabs according to the manufacturer's instructions and described previously (Hoque, Adekanmbi et al. 2020).

SARS-CoV-2 Reverse Transcription FRET-PCR was performed as described (Barua, Bai et al. 2022). The 6-carboxyfluorescein (6-FAM)-labeled probes were designed to contain the unique C22995A mutation (T478K), targeting the spike protein gene (Table 5.2). The 6-FAM probe was 3' labeled as a FRET energy donor probe excited by 488 nm light. The LCRed 640 probe was 5'-labeled and 3'-phosphorylated as the acceptor probe.

Each 20 µl PCR reaction contained 2.0 U Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA) and 0.0213 U ThermoScript™ reverse transcriptase (Invitrogen, Carlsbad, CA). Primers were used at 1 µM, the LCRed 640 probe at 0.2 µM, and the 6-FAM probe at 0.1 µM. PCR was performed on a Roche Light Cycler 480 II system (Roche Molecular Biochemicals, Indianapolis, IN). Thermal cycling was preceded by a 10-minute reverse transcription reaction at 55°C followed by a 5 min denaturation at 95°C and 40 cycles of 10 sec @ 95°C, 10 sec @ 55°C, and 10 sec @ 72°C.

Genomic RNA of two SARS-CoV-2 viruses from the American Type Culture Collection (ATCC) (2019- nCOV/USA- WA1/2020; 201/501Y.V1) served as controls and quantitative standards.

The melting curve, which assessed the dissociation of the PCR products and labeled probes, was determined by monitoring the fluorescence from 35°C to 75°C with a temperature

transition rate of 0.2°C per second. The distinct T_m values occurred due to the nucleotide mismatches between the 6-FAM-probes and the SARS-CoV-2 variants.

5.4 Results

In the sVNT, only one sample collected on March 10, 2021, showed an inhibition value of 36.03% and, above the 30% cut-off value, was regarded as positive. This sample and three others with the highest inhibition values (20.03%, 18.53%, 17.27%) were submitted to the USDA NVSL for VNT. All four samples were found to be negative in the virus neutralization assay. All nasopharyngeal and fecal swabs were negative for SARS-CoV-2 by quantitative SARS-CoV-2 PCR. Although we found one sample positive by sVNT, the USDA definition of a confirmed SARS-CoV-2 case in animals requires a positive VNT result, which was not the case with our sample. A variety of coronaviruses, such as bovine-like coronaviruses, have been identified in cervids in the United States, and there is the possibility that the sVNT-positive deer had been exposed to one of these viruses.

Twenty-nine of the 72 individuals who had been in close contact with the deer during handling and sampling voluntarily completed the questionnaire on their COVID-19 status at the time of contact. Three of the 29 people confirmed they were infected with SARS-CoV-2 during the study period. None reported being in contact with deer seven days before or seven days after testing positive.

5.5 Discussion

The negative test results for SARS-CoV-2 infections in the deer we studied were contrary to our expectations generated by the high prevalence reported in free-ranging deer (Chandler,

Bevins et al. 2021, Hale, Dennis et al. 2022, Kuchipudi, Surendran-Nair et al. 2022, Roundy, Nunez et al. 2022, Vandegrift, Yon et al. 2022). The research activities in the Auburn Captive Facility result in relatively high levels of human-deer contact, thereby creating favorable opportunities for transmission of SARS-CoV-2 from infected people. This is contrary to the situation for free-ranging white-tailed deer, which seldom, if ever, come into close contact with people. Further, contact between deer is higher in our facility than with free-range animals. Thus greater prevalence and rates of deer-to-deer transmission of SARS-CoV-2 would be anticipated. The stocking density in the facility is approximately five times greater than that typically seen with free-ranging deer, and our captive deer regularly come into close contact at the three permanent feeding stations in the facility. Data from a captive cervid facility reported by Roundy et al. showed very high rates (94.4%) of SARS-CoV-2 infections. This supported our hypothesis that we should have found a high prevalence if the deer in our facility had acquired SARS-CoV-2 (Roundy, Nunez et al. 2022).

At least three people who worked in our deer facility and came into close contact with the deer were positive for SARS-CoV-2 during the study period. However, they reported not having contact with deer seven days before or after testing positive for SARS-CoV-2. The remaining 43 people who came into close contact with the deer did not provide data, but it seems likely that some of them would also have been infected with the SARS-CoV-2 and would have had the opportunity to pass the infection to the deer. Wu et al. reported that many people are SARS-CoV-2 positive yet unaware because they were never tested and/or never developed symptoms (Wu, Mertens et al. 2020). They suggest that the number of people infected maybe 3 to 20 times greater than the number of cases confirmed through testing (Wu, Mertens et al. 2020). These expected high infection rates amongst the workers at the deer facility suggest that our deer were

exposed to SARS-CoV-2-positive people but did not become infected. This raises the possibility that SARS-CoV-2 spillover from humans to deer may be less common than initially suggested by other studies (Chandler, Bevins et al. 2021, Hale, Dennis et al. 2022, Kuchipudi, Surendran-Nair et al. 2022, Roundy, Nunez et al. 2022, Vandegrift, Yon et al. 2022).

Our finding of unexposed deer is not unique, with no seropositive animals in an extensive survey of 1,748 deer in the U.K. (Holding, Otter et al. 2022) and Germany and Austria (Moreira-Soto, Walzer et al. 2022). Similarly, in Texas, deer in only one of three captive facilities were found to have been infected (Roundy, Nunez et al. 2022). The patchy distribution of seropositive deer could indicate that SARS-CoV-2 spillover from infected humans to deer is low, as indicated by our study. However, while it is generally agreed that deer are directly infected with SARS-CoV-2 from humans, other potential transmission routes, such as via rodents and contaminated wastes, cannot be excluded. It appears likely, though, from the high seroprevalence seen in deer that once animals are infected, there is efficient transmission within a herd. The results of the studies indicate an urgent need for further active surveillance and longitudinal studies to more completely understand the ecology of SARS-CoV-2 in deer and other animals.

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Table 5.1 Molecular and serological prevalence of SARS-CoV-2 in deer

Country, State	Sampling period	Sample; detection method	Prevalence; SARS-CoV-2 lineages identified	References
USA: OH	Jan-Mar, 2021	Nasal swabs; RT-PCR	35.8%; 129/360; B.1.2, B.1.596, B.1.582	(Hale, Dennis et al. 2022)
USA: MI, PA, IL, NY	2011-2021	Serum; sVNT	40.0%; 152/624	(Di Guardo 2021)
USA: I.A.	Apr 2020-Jan 2021	Retropharyngeal lymph node; RT-PCR	33.2%; 94/283; B.1, B.1.1, B.1.119, B.1.2 51, B.1.234, B.1.240, B.1.264, B.1.311, B.1.362, B.1.400, B.1.459, B.1.596	(Kuchipudi, Surendran-Nair et al. 2022)
USA: NY	Dec 2021-Jan 2022	Serum; sVNT	14.5%; 19/131	(Vandegrift, Yon et al. 2022)
		nasal swabs; RT-PCR	10.3%; 7/68 B.1.1.529	
USA: TX	Jan-Feb 2021	Serum; plaque reduction neutralization assay	37.0%; 20/54	(Palermo, Orbegozo et al. 2022)
USA: TX	Sep-Nov 2021	Serum; plaque reduction neutralization assay	Facility A: 94.4%; 34/36; facility B: 0.0%; 0/16: facility C: 0.0%; 0/29	(Roundy, Nunez et al. 2022)
		Respiratory and rectal swabs; RT-PCR	0.0%; 0/80	
USA: SC	N/A	Serum; Neutralizing antibody assay	9.0%; 2/22	(Hancock, Hickman et al. 2022)
Canada: QC	Nov 2021	Nasal swabs; RPLNs; RT-PCR	1.2%; 3/251 (nasal swab); 0.0%; 0/104 (RPLNs); B.1.617.2 (lineage AY.44)	(Kotwa 2022)

		Thoracic cavity fluid; neutralizing antibody assay	5.6%; 14/251	
UK	Jan 2020-May 2021	Serum; sVNT	0.0%; 0/1748	(Holding, Otter et al. 2022)
Germany	Jan 2020-Dec 2021	Serum; sVNT	0.0%; 0/181	(Moreira-Soto, Walzer et al. 2022)
Austria	Jan 2020-Dec 2021	Serum; sVNT	0.0%; 0/51	(Moreira-Soto, Walzer et al. 2022)

Table 5.2 Oligonucleotides used in this study

Target of PCR	Primer/Probe	Sequences (5'-3')
C22995A (T478K) in spike protein	Upstream primer	CAGGCTGCGTTATAGCTT
	Downstream primer	TATGGTTGGTAACCAACACC
	6-FAM-probe	CCGGTAGCAAMCCTTGTAAT-6FAM
	LCRed 640 probe	LCR640-GTGTTGAAKGWTTTAWTTGTTACTTT-phospate

Chapter 6 *In vitro* antiviral efficacies of six drugs (GS-441524, Teriflunomide, Ruxolitinib, Molnupiravir, Ritonavir, and Nirmatrelvir) against feline infectious peritonitis virus

6.1 Abstract

Feline infectious peritonitis (FIP) caused by feline infectious peritonitis virus (FIPV) is an immune-mediated disease and is considered one of the most important infectious causes of death (estimated 0.3-1.4%) in domestic and nondomestic felids. Limited data is available for the antiviral efficacy and toxicity of these antiviral drugs against FIPV. This study tested six types of antiviral drugs for their cytotoxicity and antiviral efficacies in Crandell Reese Feline Kidney (CRFK) cells.

The cytotoxicity and cell viability of six drugs was quantified using commercially available kits. Finally, antiviral efficacies of six drugs were evaluated using quantification of FIPV by qRT-PCR in CRFK treated with six drugs. The GS441524 molecule showed inhibition of FIPV replication irrespective of initial inocula (2.5×10^3, 2.5×10^2, 2.5×10^1 $TCID_{50}$) and incubation period (48 and 72 hours). No significant difference was observed in the FIPV inhibition for 24, 48, and 72 hours with 98-99% inhibition by GS441524 (25 µM) as long as the drug was applied at the time of infection or immediately after the FIPV inoculation. Cytotoxicity assay and viability assays showed that six drugs were safe to be used with essentially no cytotoxicity with the concentration as high as 250 µM for Ruxolitinib, 125 µM for GS441524, 63 µM for Teriflunomide, Molnupiravir, and Nirmatrelvir, and 16 µM for Ritonavir. While GS441524 and Nirmatrelvir were found to have the least deleterious effects on the CRFK cells with 50% cytotoxic concentration (CC_{50}) values of 260.0 µM and 279.1 µM respectively, Ritonavir was found to be highly toxic (CC_{50} 39.9 µM).

In the dose-response analysis conducted in CRFK cells, GS441524, Nirmatrelvir, and Molnupiravir were found to be the top three drugs with selectivity for FIPV with SI values of 165.54, 113.67, and 29.27, respectively. In comparison, the selectivity of Teriflunomide,

Ruxolitinib, and Ritonavir for FIPV was very low, with SI values of 2.7, 7.8, and 2.3, respectively.

Overall, this study showed that GS441524 and Nirmatrelvir are safe antivirals and strongly effective in inhibiting FIPV replication. Compared to these drugs, Molnupiravir showed moderate efficacy against FIPV. These data suggest that Nirmatrelvir and Molnupiravir may bring new hopes for FIPV treatment besides GS441524 and could be an alternative to treat infection with GS441524-resistant FIPV strains in cats. The antiviral efficacies of six drugs from this work warrant future studies to explore further their treatment efficacies in vivo and side effects in FIP therapy.

6.2 Introduction

Feline coronavirus (FCoV), belonging to the family Coronaviridae, is a common viral pathogen of cats. Based on the pathobiology, FCoV occurs as two pathotypes, feline enteric coronavirus (FECV), causing asymptomatic enteritis infections with mild transient diarrhea, and virulent feline infectious peritonitis virus (FIPV), associated with a fatal systemic disease known as feline infectious peritonitis (FIP) (Pedersen, Boyle et al. 1981, Pedersen, Allen et al. 2008). Once the clinical symptoms such as weight loss, weakness, fever, lethargy, and ocular lesions appear, the situation signals that the cat's battle with FIPV has been lost (Pedersen 2014). Though some cats can live for weeks, months, or even years, FIP-associated mortality is exceptionally high (Pedersen 2014). The treatment for FIP is mainly symptomatic and supportive (Pedersen 2009) until recently.

GS-441524, the active triphosphate form of Remdesivir, is an adenosine nucleoside analog, inhibiting the RNA-dependent RNA polymerase and interfering with viral replication. GS-441524 has been reported to strongly inhibit FIPV replication in culture, experimentally-infected cats, and alleviate symptoms in naturally-infected cats (Murphy, Perron et al. 2018, Pedersen, Kim et al. 2018, Pedersen, Perron et al. 2019). Daily administration by subcutaneous injection caused a rapid, transient increase in serum globulin level and resolution of effusions in FIP (Murphy, Perron et al. 2018, Pedersen, Perron et al. 2019, Dickinson, Bannasch et al. 2020). Initially available in Australia and then in the UK in 2021, this novel treatment of FIP with GS-441524 and Remdesivir is available for vets to prescribe, although not yet in all countries (https://www.vettimes.co.uk/news/vet-help-sought-for-fip-treatments-study/).

Teriflunomide is an anti-inflammatory drug for rheumatoid arthritis and some other rheumatic conditions. Teriflunomide is a selective inhibitor of dihydroorotate dehydrogenase, a

key mitochondrial enzyme involved in the *de novo* synthesis of pyrimidines in rapidly proliferating cells. Recent studies showed that multiple sclerosis patients undergoing continuous teriflunomide treatment experience milder COVID-19 than those without treatment (Ciardi, Zingaropoli et al. 2020, Luetic, Menichini et al. 2021).

Ruxolitinib is a potent JAK1 and JAK2 inhibitor with a good safety profile. This drug is approved for the treatment of myelofibrosis (Harrison, Kiladjian et al. 2012) and polycythemia vera (Vannucchi 2015) characterized by over-inflammation; it also has proven to be quite promising with a short-term high dose schedule in rapidly improving COVID-19-related severe respiratory conditions (Gozzetti, Capochiani et al. 2020).

Molnupiravir (MK-4482 or EIDD-2801) is a small molecule, broad-spectrum antiviral drug and prodrug of the nucleoside analog β-D-N4-hydroxycytidine recently received FDA emergency use authorization in the USA for the treatment of symptomatic COVID-19. This drug is an inhibitor of the viral RdRp, developed initially against different RNA viruses such as influenza (Painter, Holman et al. 2021). A phase 2a clinical trial of this drug in patients with COVID-19 showed accelerated viral RNA clearance and elimination of infectious viruses (Fischer, Eron et al. 2022). This drug has recently been reported to be efficacious against FIPV (Cook, Vogel et al. 2020).

Ritonavir is an antiretroviral protease inhibitor and CYP3A inhibitor. This drug, when co-administered with other drugs, has been demonstrated to enhance the plasma concentration of those drugs (Cook, Vogel et al. 2020, Lamb 2022).

Nirmatrelvir is an antiviral drug widely used against COVID-19 (Vandyck and Deval 2021). Nirmatrelvir is a peptidomimetic that acts as a reversible, competitive inhibitor of the FCoV protease (Chia 2022). When co-administered with Ritonavir within 3 or 5 days of

COVID-19 symptom onset, Nirmatrelvir was reported to reduce hospitalizations and death by 89% (White, Schiffer et al. 2021).

Whereas no treatments for FIP have been approved in the U.S. until now, numerous studies have explored commercially available antiviral agents with the hope of antiviral drug development. In this work, we investigated the potential of six drugs (GS-441524, Teriflunomide, Ruxolitinib, Molnupiravir, Ritonavir, and Nirmatrelvir) as safe and effective FIPV antivirals (Table 6.1).

6.3 Materials and Methods

6.3.1 Antiviral drugs and reagents

Teriflunomide, GS-441524, Ritonavir, and Nirmatrelvir used in this study were purchased from MedChemExpress (Princeton, NJ, USA). Ruxolitinib was purchased from InvivoGen (San Diego, CA, USA), and Molnupiravir was from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions (5 mM) for all six drugs were prepared by dissolving or resuspending the antiviral drugs in dimethyl sulfoxide. The stock solutions of the drugs were kept at -20°C (Ruxolitinib) or -80°C (GS-441524, Molnupiravir, Nirmatrelvir, Ritonavir, Teriflunomide) according to the manufacturer for maximum solution storing time without activity loss. Drugs were further diluted for working concentration using cell culture maintenance media.

The Crandell-Reese feline kidney cells (CRFK) cells, FIPV serotype II (WSU-79-1146, GenBank DQ010921) strain, Eagle's Minimum Essential Medium (EMEM) with 4.5g/L glucose; fetal bovine serum (FBS); penicillin (100 U/mL), streptomycin (100 μg/mL), Dulbecco's phosphate-buffered saline (D-PBS), trypan blue, trypsin-EDTA, and DMSO were purchased from American Type Culture Collection (Manassas, VA, USA). Non-essential amino acids were

purchased from HyClone (Logan, UT, USA), and Corning tissue-culture flasks were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

6.3.2 Culture of CRFK cells

CRFK cells were used in this study and maintained in EMEM supplemented with 10% FBS and 1% antibiotics (penicillin and streptomycin). The cells were incubated at 37°C and 5% CO_2. When cells reached a confluent monolayer, they were trypsinized and split to propagate the cells to maintain and seed flasks/plates for experiments. Cells were enumerated using an automated Countess 3 hemacytometer (ThermoFisher Scientific, Waltham, MA, USA).

6.3.3 FIPV stock for *in vitro* experiments

CRFK cells were cultured in T75 flasks. FIPV was inoculated and propagated in 20 mL of EMEM and 10% FBS. Extensive cytopathic effect (CPE) with large areas of cell detachment was found after 72 hours of infection at 37°C. After incubation, the flasks were subjected to 3 freeze-thaw cycles to facilitate maximum virus recovery. Then, the supernatant was collected and centrifuged at 1,500g for 5 minutes to obtain cell-free viral stocks. The collected viral stock was aliquoted and stored at -80°C. This viral stock was later titered using bioassay ($TCID_{50}$) and quantified by real-time RT-PCR (qRT-PCR**)**.

6.3.4 50% tissue culture infective dose ($TCID_{50}$) of FIPV stock

Titration of FIPV was performed using a bioassay ($TCID_{50}$) method. CRFK cells were grown on a 96-well tissue culture plate (Nunc, ThermoFisher Scientific), and at 80-90% cellular confluency, 100 µL of 10-fold serially diluted FIPV stock was added to wells in six repeats.

CRFK cells free of FIPV infection and undiluted FIPV stock served as negative and positive controls, respectively. At 72 hours post-inoculation (hpi) of FIPV in CRKF cells, visualization under an inverted phase-contrast microscope showed wells with cytopathic effect (CPE) in the wells inoculated with virus dilutions as well as in positive control. In addition, the assay plate was stained with crystal violet (Sigma-Aldrich, St. Louis, MO, USA) after fixation of the cells with methanol. The FIPV titer was determined based on individual CPE (rounding of cells) observation and score record (Figure 6.1). The $TCID_{50}$ endpoint values were further calculated based on the CPE score for CPE according to the method of Reed and Muench (Lei, Yang et al. 2021).

6.3.5 Drug cytotoxicity assay

A commercially available kit (CellTox Green Cytotoxicity Assay, Promega, Madison, WI, USA) was used to determine the cytotoxicity of six antiviral drugs. In this assay, fluorescence intensity quantified cytotoxicity as the dye selectively penetrates and binds the DNA of degenerate/apoptotic/necrotic cells. CRFK cells at a density of 5×10^4 cells/well were seeded in 96-well plates and incubated at 37°C and were treated in four-well replicates with 1,000, 500, 250, 125, 63, 31, 16, 8, and 4 µM concentrations of the drug of interest at 90% confluency. After 48 hours of incubation, the DNA binding dye from the kit was applied to all wells and incubated shielded from light at 37°C for 15 minutes. Cytotoxicity of the drugs was measured using a plate reader, SpectraMax iD3 (Molecular Devices, CA, USA), with fluorescence intensity at 495/519 nm (λex/λem). The fluorescence of CRFK cells was compared to the untreated CRFK cells as the negative control and lysing reagent-treated cells (provided by the manufacturer) as the positive control. The fluorescence reading is proportional to cell death

due to the selective binding to the dye with the DNA of apoptotic/necrotic cells. The mean fluorescence value of all four replicates for each drug concentration was interpolated as percent cytotoxicity (%) ranging from 0-100%, where untreated cells were considered as 0% (baseline cytotoxicity) and cells treated with the positive control reagent as 100%.

6.3.6 Cell viability assay of the antiviral drugs

The cell viability of CRFK cells was measured using a Cell Proliferation Kit I (MTT) (Roche Applied Science, Indianapolis, IN, USA), following the manufacturer's instructions. Cells were seeded at 5×10^4 cells/well into 96-well plates and incubated at 37°C and were treated in four-well replicates with 1,000, 500, 250, 125, 63, 31, 16, 8, and 4 µM concentrations of the drug of interest at 90% confluency. After incubation for 48 hours, 10 µl of MTT solution (0.5 mg/ml in phosphate-buffered saline) was added to each well. The plate was incubated for 4 hours at 37°C to allow the formation of formazan crystals. Following incubation, 1% sodium dodecyl sulfate (100 µl) solution was added to dissolve the crystals. Then, the spectrophotometrical absorbance of the sample was measured at 575 nm wavelength using a microplate reader, SpectraMax iD3 (Molecular Devices, CA, USA) as described (Riss, Moravec et al. 2004).

6.3.7 Antiviral efficacy of the drugs

CRFK cells were seeded in 96-well plates at a density of 5×10^4 cells/well and incubated at 37°C. The culture media was discarded at approximately 90% cellular confluency, and cells were infected with FIPV with 2.5×10^4 $TCID_{50}$ or according to the experiment design. The culture plate was incubated for one hour with occasional gentle agitation every 15 minutes. After

incubating and discarding the media, fresh EMEM media (with 2% FBS) containing different dilutions of the antiviral drugs was added to the wells. CRFK cells infected with FIPV without any drug treatment served as a positive control, and CRFK cells without virus infection or drug treatment were considered a negative control. The plate was incubated in 5% CO_2 at 37°C for the desired incubation times. Then, the culture supernatant was harvested and stored for virus quantification.

6.3.8 Quantification of FIPV by qRT-PCR

Cell-free total nucleic acid was isolated from the collected supernatant/viral stock using the commercially available High-Pure PCR Template Preparation Kit (Roche Diagnostic, Indianapolis, IN, USA) following manufacturer instructions. The isolated RNA was subsequently reverse transcribed in volumes of 10 µL sample and 10 µL master mixture containing 2.0 U Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) and 0.0213 U ThermoScript™ reverse transcriptase (Invitrogen, Carlsbad, CA); and primers and probes targeted both M and N gene mRNA (Table 6.2). Roche Light Cycler 480 II system (Roche Molecular Biochemicals, Indianapolis, IN) was used for the PCR. Thermal cycling was preceded by a 10-minute reverse transcription reaction at 55°C followed by a 4 min denaturation at 95°C and 18 high-stringency step-down thermal cycles: 6 cycles of 10 sec at 95°C, 12 sec at 72°C, and 30 sec at 72°C; 9 cycles of 10 sec at 95°C, 12 sec at 70°C, and 10 sec at 72°C; 3 cycles of 10 sec at 95°C, 12 sec at 68°C, and 10 sec at 72°C. The high-stringency cycles were followed by 30 low-stringency fluorescence acquisition cycles of 10 sec at 95°C, 8 sec at 58°C with fluorescence acquisition, 30 sec at 67°C and 30 sec at 72°C and the melting curve was determined by 1 min at 95°C, 2 min at 42°C, and increasing to 74°C with continuous fluorescence reading. The positive

control and quantitative standard were commercially synthesized as target DNA cloned into an expression plasmid and used for four standards: 5,000, 500, 50, and 5 copies.

6.3.9 Statistical analysis

All statistical analyses were performed using Statistica 7.0 software package (StatSoft, Inc.). Shapiro-Wilk's W test confirmed the normal distribution of data, and Levene's test confirmed the homogeneity of variances. Data were analyzed by mean plots with 95% confidence intervals (CI) in one-way or factorial analysis of variance. Comparisons of means under the assumption of no a priori hypothesis were performed by a two-tailed Tukey honest significant difference (HSD) test. Differences at P values of 0.05 in the Tukey HSD test were considered significant.

6.4 Results

6.4.1 Determination of the $TCID_{50}$ of FIPV stocks

Using endpoint dilution assay, the titer of FIPV was determined as 2.5×10^4 $TCID_{50}$/mL and used in the subsequent studies unless otherwise mentioned.

6.4.2 Effect of initial inocula and incubation times on anti-FIPV efficacy of GS441524

Initial virus inoculum and incubation time are two of the many factors that can affect *in vitro* antiviral efficacy. Here, we used GS441524 as an example to identify the effect of those factors on the drug in the inhibition of FIPV in CRFK calls (Table 6.3).

CRFK cells with 80-90% confluency were infected with three FIPV inocula such as 2.5×10^3, 2.5×10^2, and 2.5×10^1 $TCID_{50}$ and incubated in the presence or absence of GS441524

molecules (25 µM) for different incubation times of 48 hours and 72 hours to identify the effect of initial virus inoculum on the anti-FIPV efficacy of GS441524 in different time points. The quantitative viral load was measured by qRT-PCR in FIPV-infected CRFK cells. The result demonstrated that the GS441524 molecule inhibited FIPV-associated replication with no difference in log value genome copy number in CRFK cells, irrespective of initial inocula and incubation times (Figure 6.2).

The findings of this experiment indicate that the GS441524 molecule is very effective against FIPV irrespective of the initial viral dose inoculated for at least 72 hours. Therefore, 48 hours of incubation with the highest inoculum of FIPV were used in the remaining assays.

6.4.3 Effect of addition time and removal of GS441524 on the anti-FIPV efficacy

In the use of antiviral drugs to treat viral infections, the drugs can be applied at different stages of infection, different doses, and different frequencies. To simulate the effect of drug use in real life, we designed an experiment with five different times of addition and removal of GS441524 (Figure 6.3).

Confluent monolayers of CRFK cells in 96-well plates were inoculated with FIPV (2.5×10^4 $TCID_{50}$) for 1 hour. The GS441524 (25 µM) was applied according to the study design. Plates were withdrawn at 24, 48, and 72 hours. Compared to the control wells (no drug), the inhibition of FIPV replication in the presence of GS441524 in CRFK cells did not differ up to 72 hpi with different initial drug application time-points, except for the -2 hpi group (Table 6.4). In the latter group, the FIPV RNA copies were significantly higher at 72 hours (275-fold) than at 24 (0.79-fold) and 48 hours (11.75-fold). The peak viral load of -2 hpi wells at 72 hours of

incubation is similar to the no-drug group at 48 hours, indicating that the initial antiviral activity observed results from limited initial inhibition by GS441524.

The results indicated antiviral efficacy did not differ significantly if GS441524 was applied during or after FIPV infection but dropped significantly if the drug was removed. In the following viral efficacy experiments, the drug was applied at +1 hpi time point.

6.4.4 Cytotoxicity and effect on cell viability of antiviral drugs

Determination of the cytotoxicity and effect on cell viability of drugs was a prerequisite to antiviral efficacy assay. Cytotoxicity of different drugs was quantified using a commercially available kit (CellToxTM Green assay) in CRFK cells by the intensity of the fluorescence at 485-500nm$_{Ex}$/520-530nm$_{Em}$ for four well replicates using different concentrations of the drugs (1,000, 500, 250, 125, 63, 31, 16, 8, 4 µM). All antiviral drugs demonstrated no cytotoxicity up to 63 µM except Ritonavir (Figure 6.4). Ritonavir showed toxicity to cells at a concentration as low as 31 µM. On the other hand, the cytotoxicity of Ruxolitinib was undetectable at a high concentration of 250 µM. GS441524 was non-toxic to cells at a concentration as high as 125 µM.

Cell viability assay was also performed using Cell Proliferation Kit I (MTT assay) for CRFK cells, treating the confluent monolayer of cells with the same dilutions of the antiviral drugs as used in the cytotoxicity assay (Figure 6.4). Quantifying MTT staining of CRFK cells treated with varying doses of different drugs showed a dose-dependent loss of cell viability. The findings demonstrated the six drugs as safe to be used with essentially no cytotoxicity with the concentration as high as 125 µM for GS441524; 62.5 µM for Teriflunomide; 250 µM for Ruxolitinib; 63 µM for Molnupiravir; 16 µM for Ritonavir; and 63 µM for Nirmatrelvir. Cell

viability data should inversely correspond with the cytotoxicity of the drugs. However, the drug cytotoxicity and cell viability data at high drug concentrations did not match. In addition, visual inspection of the antiviral drug-treated wells immediately before applying the fluorescent dye and plate readings revealed differences in cell morphology (CPE) between the treated and untreated CRFK cells. The inconsistency between the visual assessment of drug-treated wells and the fluorescence assay led to the possibility that an overall decrease in the cell number in the drug-treated wells resulted in degradation and loss of nucleic acid, thus being unavailable for fluorescence binding and detection in the CellTox assay. As a result, when the cell viability is near 0% at a high drug concentration, the corresponding concentration's percent cytotoxicity is much lower than 100%.

6.4.5 Antiviral efficacies of the tested drugs

Antiviral efficacies of six drugs were evaluated after the cytotoxicity and viability assays. We used serial 1:10 dilutions of six drugs (GS441524, Teriflunomide, Ruxolitinib, Molnupiravir, Ritonavir, and Nirmatrelvir) to observe the ranges of drug concentration to be effective against FIPV (Table 6.5).

A dose-response analysis was conducted in CRFK cells infected with FIPV (2.5×10^4 $TCID_{50}$) and treated with 10-fold dilutions of the drugs. The viral load of FIPV was quantified by qRT-PCR at 48 hpi. All tested drugs showed anti-FIPV efficacy at a concentration as low as 5 µM compared to the no-drug group (Figure 6.5). GS441524 was effective against FIPV at a concentration as low as 0.5 µM with 85% FIPV inhibition with no CPE in cells. The anti-FIPV efficacy of Nirmatrelvir was also promising, with 80% inhibition of IPV replication at a concentration as low as 5 µM with no CPE in cells. However, this drug did not have efficacy as

low as 0.5 µM. While Teriflunomide and Molnupiravir harbored dose-dependent antiviral activities, Ritonavir showed a sudden drop in FIPV antiviral efficacy if the concentration changed from 50 µM to 5 µM with increasing copy number of FIPV up to two-log values (Table 6.5). Interestingly, this drug showed CPE in cells at both concentrations of 50 µM and 5 µM with toxicity to cells at a concentration as low as 31 µM in the cytotoxicity assay. As a result, it can be conjectured that the low FIPV copy number at 50 µM of Ritonavir may be a result of cytotoxicity more than drug efficacy. A similar observation is also applicable to Ruxolitinib with the change of the drug concentration from 500 µM to 50 µM causing a two-log value increase in FIPV copy number where cytotoxicity was found with concentration as low as 250 µM.

Further, the drugs were serially diluted two-fold to observe the interim range of concentration of the drugs to be effective against FIPV in CRFK cells (Figure 6.6). Based on the findings in this study, GS441524 showed anti-viral efficacy at a concentration as low as 0.98 µM with a 22.5% reduction of FIPV copy number with significant inhibition of FIPV (98.25%) at a concentration of 7.8 µM where no cytopathic effect was found in cells (Table 6.6). Next to GS441524, Nirmetrelvir showed 98.33% inhibition of FIPV replication at the same concentration with no CPE of cells at a concentration of 7.8 µM, similar to GS441524. We also observed substantial diversity between cytotoxicity and efficacy against FIPV for the rest of the four drugs, such as Teriflunomide, Ruxolitinib, Molnupiravir, and Ritonavir. As a result, it can be said that GS441524 and Nirmatrelvir were found to be the most effective against FIPV among all six drugs.

6.4.6 Selectivity index quantification of six drugs

We calculated 50% cytotoxic concentration (CC_{50}), 50% effective concentration (EC_{50}), and selectivity indices (SI) for the six drugs (Figure 6.7). While GS441524 and Nirmatrelvir had the least deleterious effects on the CRFK cells with CC_{50} values of 260.0 µM and 279.1 µM respectively, Ritonavir was found highly toxic (CC_{50} 39.9 µM). When the selectivity index was calculated based on the ratio of CC_{50} and EC_{50}, the highest selectivity index value was found for GS441524 (SI 165.5) among the drugs tested against FIPV. The anti-FIPV selectivity of Nirmatrelvir was also found promising, with a selectivity index value of 113.7. Molnupiravir could also be considered a safe drug and found selective (SI = 29.3) against FIPV with EC_{50} 8.0 µM. The rest of the three drugs were found to be either cytotoxic (Ritonavir) or less effective against FIPV (Teriflunimide, Roxilitinib), hence not very selective to FIPV (SI 2.3, 0.4, 7.8 respectively). To summarize, GS441524 and Nirmatrelvir showed the promising result to be safe with higher anti-FIPV efficacy among all six drugs.

6.5 Discussion

Treatments with antiviral drugs targeting FCoV replication should ideally target the pathogen without affecting uninfected cells. In this case, viral enzymes necessary for replication may be excellent candidates for targets of antiviral drugs. Interest in antiviral drugs for infections has been much slower to develop when the host is an animal. However, FIPV infection without having a safe, approved vaccine could be the ideal candidate for antiviral drug development.

To identify drugs with anti-FIPV activity, six drugs, GS441524, Teriflunomide, Ruxolitinib, Molnupiravir, Rotinavir, and Nirmatrelvir were selected and used for *in vitro* assays based on the antiviral efficacy profiling and available data on SARS-CoV-2. These drugs belong

to different drug classes with various putative mechanisms of action, including nucleoside polymerase inhibitors, dihydroorotate dehydrogenase inhibitors, protease inhibitors, kinase inhibitors, and nucleoside analogs.

Based on the viral RNA inhibition (qRT-PCR) assay results, the most effective safe anti-FIPV drug identified is GS441524; then Nirmatrelvir and Molnupiravir with selectivity indices (SI) of 165.54, 113.67, and 29.27, respectively. The selectivity of Teriflunomide, Ruxolitinib, and ritonavir (SI of 2.7, 7.8, and 2.3, respectively) for FIPV was very low compared to the other three drugs in our study.

In previous studies, GS441524 was shown to be a safe and effective treatment for FIP (Murphy, Perron et al. 2018, Pedersen, Perron et al. 2019) with comparable EC_{50} (1.0 µM) and CC_{50} (>100 µM). In this study, GS441524 was also found to strongly inhibit FIPV in CRFK cells with an EC_{50} value of 1.57 µM and CC_{50} of 260.02 µM.

Nirmatrelvir, an FDA-approved oral SARS-CoV-2 protease inhibitor, exerts its action by altering the efficacy of intracellular protein (M^{pro}), affecting viral entry into the cell and SARS-CoV-2 replication (Sathish, Bhatt et al. 2022). Our study found Nirmatrelvir to be safe and efficacious (CC_{50} 279.1 µM and EC_{50} 2.5 µM) with a selectivity index value of 113.67 for FIPV. Other studies also reported a significant inhibitory effect (EC_{50} 2.52 µM) of Nirmatrelvir on FIPV when characterizing the mutation sites regarding the resistance of CoVs to M^{pro} inhibitors (Jiao, Yan et al. 2022). Based on the experience with closely related GC376, this drug can be an important oral treatment of FIP in the future (Kim, Liu et al. 2016, Pedersen, Kim et al. 2018).

Currently, Molnupiravir is considered an alternative to the GS441524, especially in FIPV resistance to GS441524 in cats, though found in the unapproved market. However, more experience with Molnupiravir is needed as the effective dosage recommendation is only based on

presumptions from published information on COVID-19 treatment (Wahl, Gralinski et al. 2021, Jayk Bernal, Gomes da Silva et al. 2022). In our study, Molnupiravir proved effective (EC_{50} 8.04 µM) against FIPV (Figure 6.7). We also determined that this drug is non-toxic to CRFK cells with a CC_{50} value of 235.35 µM. However, N4-deoxycytidine, an active metabolite of Molnupiravir, is a highly potent mutagen. As a result, the big unknown is whether this drug will be free from any greater chances of side effects as the treatment time for FIP is longer than for COVID-19 (Zhou, Hill et al. 2021). This factor suggests a need for additional investigation.

Paxlovid, an oral, bioavailable, novel drug, has been introduced to fight SARS-CoV-2 (Najjar-Debbiny, Gronich et al. 2022, Wen, Chen et al. 2022). This drug consists of Nirmatrelvir co-packaged with the HIV protease inhibitor Ritonavir. FDA authorized it in December 2021 to treat mild-to-moderate COVID-19 patients (https://www.fda.gov/drugs/news-events-human-drugs/fda-updates-paxlovid-health-care-providers, accessed June 20, 2022). However, neither Nirmatrelvir nor Paxlovid has been tested in cats with FIP, which may be an essential oral treatment for some FIPV forms.

The SARS-CoV and MERS-CoV epidemics and the current SARS-CoV-2 pandemic have highlighted the need to prepare for the emergence of novel coronaviruses in humans. As a result, developing antivirals for treating FIP and other veterinary coronaviruses will not only save the lives of many companion animals but also help reduce the impact of future coronavirus outbreaks.

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Table 6.1 Drugs with antiviral activity against animal and human coronaviruses (CoVs)

Drug Name	Drug category	Inhibition step	Investigated CoVs	References
GS441524	Adenosine nucleotide analog	Replication	FIPV, SARS-CoV-2, MERS-CoV,	(Murphy, Perron et al. 2018, Yan and Muller 2020, Li, Cao et al. 2022)
Nirmatrelvir	3C-like Protease inhibitor	Replication	FIPV, SARS-CoV-2	(Arbel, Wolff Sagy et al. 2022)
Molnupiravir	isopropyl ester cytidine analog	Replication	SARS-CoV-2, MERS-CoV	(Sheahan, Sims et al. 2020)
Ruxolitinib	Kinase inhibitor	Entry	SARS-CoV-2	(Neubauer, Johow et al. 2021)
Ritonavir	Antiretroviral protease inhibitor	Cleavage of viral polyproteins	FIPV, SARS-CoV-2	(Cao, Wang et al. 2020, Cook, Vogel et al. 2022)
Teriflunomide	dihydroorotate dehydrogenase inhibitor	Replication	SARS-CoV-2	(Maghzi, Houtchens et al. 2020)

Table 6.2 Oligonucleotide primers and probes used in FIP MN gene qRT-PCR

Primer/probe	**Sequence (5'-3')**
FIPV-Mgene_Up stream	GCYGGTGATTACTCAACAGAAGCACGTACTGA
FIPV_mRNA_Up stream	GCCTTGTGCTAGATTTGTCTTCGGACA
FIPV-Ngene_mRNA_Down stream	CCAATTTGTTGATCYTTATTACCTATTCCYTTGGGAAC
FIPV-Ngene_6FAM (Fluorescein probe)	ACGTCTTTTGGAAGGTTCATCTCCCCAGT-6FAM
FIPV-Ngene_TYE705 probe	TYE705-GACGCGTTGTCCCTGTGTGGCCAT-Phosphate

Table 6.3 Effect of GS441524 molecule on viral load of FIPV with the initial inoculum and incubation time in CRFK cells

Inoculum	48 hours		72 hours	
	No drug	**GS441524**	**No drug**	**GS441524**
2.50×10^3	5.91×10^6	2.76×10^3	4.67×10^6	3.24×10^3
2.50×10^2	1.17×10^7	3.48×10^2	5.18×10^6	2.09×10^2
2.50×10^1	1.23×10^7	4.60×10^1	9.05×10^6	2.36×10^1

(*) Fold reduction was calculated for the drug group compared to the control (no drug) group for each inoculum group to observe the inoculum-response antiviral efficacy of GS441524.

Table 6.4 Time of GS441524 molecule addition with the replication of FIPV in CRFK cells

Treatments*	Increase in viral load #					
	24 hrs	**Fold increase#**	**48 hrs**	**Fold increase#**	**72 hrs**	**Fold increase#**
-2 hpi	1.97×10^4	0.79	2.94×10^5	11	6.89×10^6	275
-2 & +1 hpi	2.47×10^4	0.99	1.03×10^4	0.41	1.62×10^4	0.65
0 hpi	2.58×10^4	1.03	1.97×10^4	0.79	2.04×10^4	0.82
+1 hpi	2.84×10^4	1.14	1.52×10^4	0.61	1.39×10^4	0.56
No drug	1.63×10^6	65	5.82×10^6	232	5.48×10^6	219

(*) hpi=hours post infection;

(#) Fold increase was calculated based on the initial FIPV inoculum (2.5×10^4 $TCID_{50}$) used in this experiment for all treatments

Table 6.5 Percent inhibition response by six drugs (serial 1:10 dilutions) against FIPV in CRFK cells with CPE

Drug Concentration (UM)	GS441524 (RNA copies)	Percent inhibition	CPE	Teriflunomide (RNA copies)	Percent inhibition	CPE	Ruxolitinib (RNA copies)	Percent inhibition	CPE	Molnupiravir (RNA copies)	Percent inhibition	CPE	Ritonavir (RNA copies)	Percent inhibition	CPE	Nirmatrelvir (RNA copies)	Percent inhibition	CPE
0.00	4.36×10^6	0.00	Yes	4.36×10^6	0.00	Yes	4.36×10^6	0.00	Yes	4.36×10^6	0.00	Yes	8.06×10^6	0.00	Yes	8.06×10^6	0.00	Yes
0.05	2.52×10^6	42.18	Yes	4.32×10^6	0.92	Yes	7.00×10^6	0.00	Yes	2.18×10^6	49.88	Yes	6.82×10^6	15.40	Yes	8.04×10^6	0.29	Yes
0.50	6.62×10^5	84.82	Yes	3.65×10^6	16.37	Yes	1.47×10^6	66.37	Yes	2.47×10^6	43.37	Yes	8.73×10^6	0.00	Yes	8.79×10^6	0.00	Yes
5.00	2.67×10^5	93.89	No	2.72×10^6	37.67	Yes	3.38×10^6	22.54	Yes	1.52×10^6	65.16	Yes	6.91×10^6	14.28	Yes	1.58×10^6	80.38	No
50.00	1.34×10^4	99.69	No	7.62×10^5	82.52	Yes	3.62×10^6	16.92	Yes	1.14×10^6	73.83	Yes	2.81×10^4	99.65	Yes	3.18×10^4	99.61	No
500.00	1.37×10^4	99.69	Yes	3.27×10^4	99.25	Yes	4.51×10^4	98.97	Yes	1.71×10^5	96.07	Yes	3.38×10^4	99.58	Yes	2.38×0^4	99.70	Yes

Table 6.6 Inhibition response by CRFK cells with CPE when treated with different concentrations with 1:2 dilution of six individual drugs

Concentration (UM)	GS441524 (RNA copies)	Percent inhibition	CPE	Teriflunomide (RNA copies)	Percent inhibition	CPE	Ruxolitinib (RNA copies)	Percent inhibition	CPE	Molnupiravir (RNA copies)	Percent inhibition	CPE	Ritonavir (RNA copies)	Percent inhibition	CPE	Nirmatrelvir (RNA copies)	Percent inhibition	CPE
0.00	3.36×10^6	0.00	Yes	3.36×10^6	0.00	Yes	3.36×10^6	0.00	Yes	3.36×10^6	0.00	Yes	3.36×10^6	0.00	Yes	3.36×10^6	0.00	Yes
0.98	2.60×10^6	22.50	Yes	2.27×10^6	32.27	Yes	3.48×10^6	0.00	Yes	3.47×10^6)	0.00	Yes	4.33×10^6	0.00	Yes	2.93×10^6	12.74	Yes
1.95	1.23×10^6	63.34	Yes	1.66×10^6	50.67	Yes	2.13×10^6	36.66	Yes	2.63×10^6	21.61	Yes	4.60×10^6	0.00	Yes	2.02×10^6	39.94	Yes
3.90	4.08×10^5	87.84	Yes	2.02×10^6	39.87	Yes	2.58×10^6	23.25	Yes	2.07×10^6	38.30	Yes	3.20×10^6	4.62	Yes	1.05×10^6	68.83	Yes
7.80	5.87×10^4	98.25	No	1.32×10^6	60.58	Yes	4.52×10^6	0.00	Yes	2.06×10^6	38.52	Yes	2.90×10^6	13.64	Yes	5.62×10^4	98.33	No
15.65	3.93×10^4	98.83	No	1.50×10^6	55.29	Yes	6.17×10^6	0.00	Yes	1.01×10^6	69.84	Yes	2.57×10^6	23.55	Yes	3.72×10^4	98.89	No
31.25	3.92×10^4	98.83	No	1.17×10^6	65.14	Yes	4.55×10^6	0.00	Yes	7.89×10^5	76.48	Yes	7.39×10^5	77.99	Yes	2.78×10^4	99.17	No
62.50	4.13×10^4	98.77	No	1.29×10^6	61.70	Yes	1.48×10^6	56.04	Yes	5.83×10^5	82.63	Yes	5.61×10^4	98.33	Yes	3.70×10^4	98.90	No
125.00	3.34×10^4	99.00	No	5.12×10^5	84.73	Yes	1.99×10^6	40.83	Yes	1.89×10^5	94.38	Yes	5.60×10^4	98.33	HT	3.24×10^4	99.04	No
250.00	6.32×10^4	98.12	Yes	3.33×10^5	90.09	Yes	2.40×10^6	28.61	Yes	6.08×10^4	98.19	Yes	5.61×10^4	98.33	HT	3.38×10^4	98.99	Yes

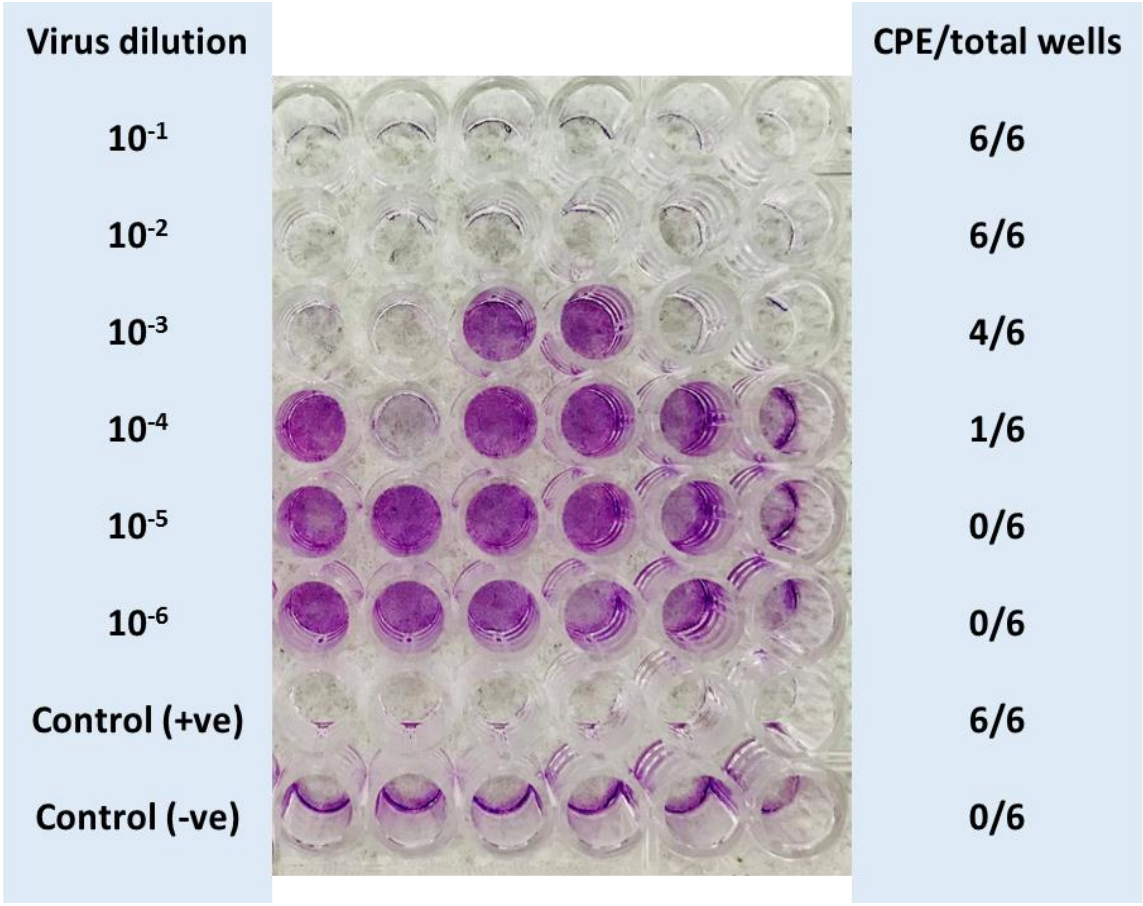

Figure 6.1 Visual representation of FIPV $TCID_{50}$ assay. CRFK cell cultures infected with FIPV-1146 show typical CPE and staining patterns. In the top six rows, the cells were infected with 10-fold dilutions of FIPV stock with six replicates per dilution, and CPE was recorded 3 days post infection. In positive control wells (row 7), cells were infected with undiluted FIPV whereas CRFK cells were uninfected in the negative control wells (row 8). The wells with crystal violet color indicate the presence of cells remaining intact and attached to the well surface as stained with crystal violet. On the other hand, loss of staining indicates cell loss due to cell death and detachment from the well surface. Based on total CPE scores per dilution, the FIPV titer was determined to be 2.5×10^4 $TCID_{50}$/mL for CRFK cells according to the Reed and Muench method.

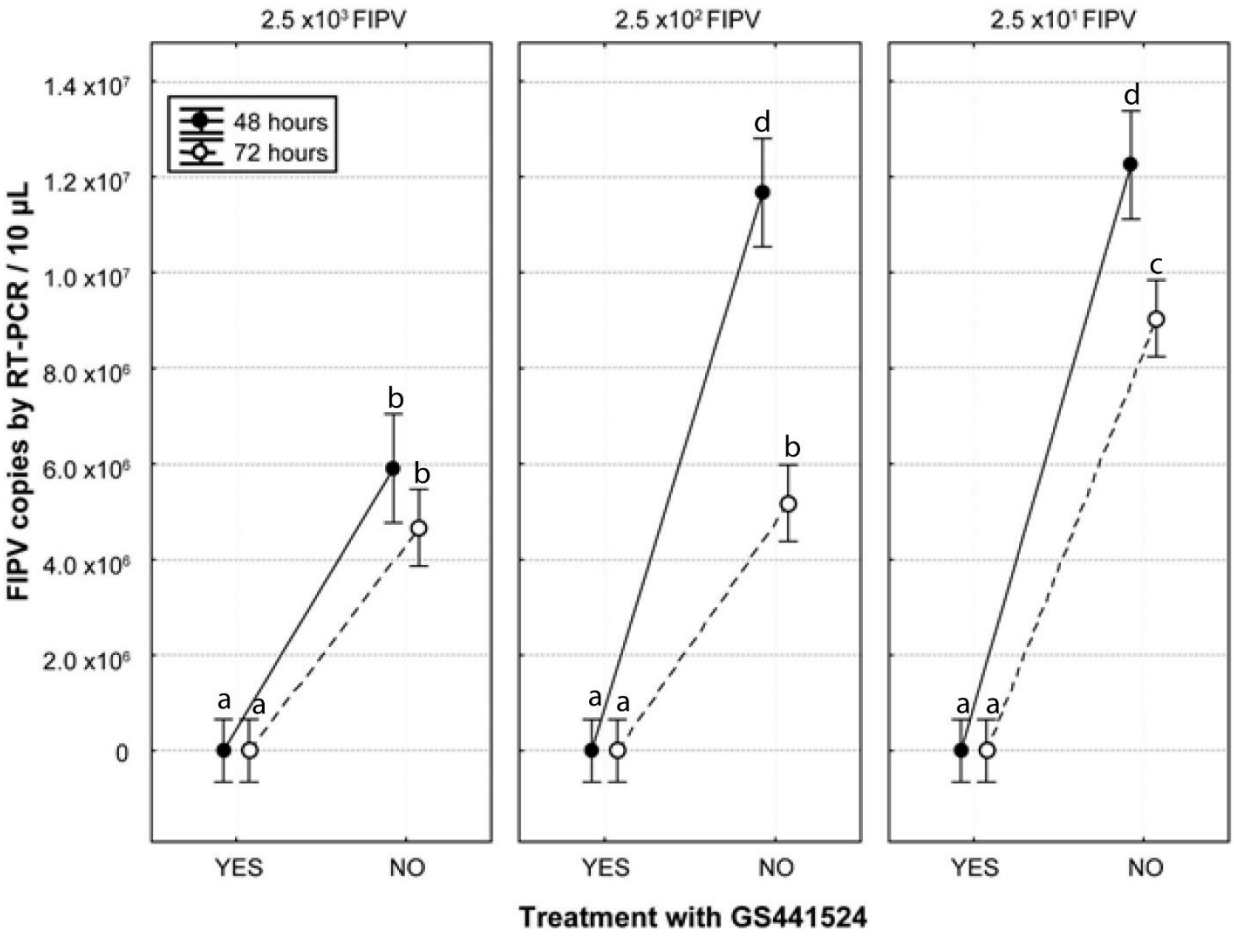

Figure 6.2 Anti-FIPV efficacy of GS441524 affected by initial inoculum and incubation time. Confluent monolayer of CRFK cells was infected with three different initial FIPV inocula (2.5×10^3, 2.5×10^2, 2.5×10^1 $TCID_{50}$). The cells were incubated in the presence or absence of GS441524 (25 µM) with 48 hours and 72 hours of incubation times. GS441524 molecule inhibited FIPV replication irrespective of initial inocula and incubation times.

The data is presented as an average ±95% confidence interval (CI). Different letters (a, b, c and d) indicate significant differences in FIPV RNA copies.

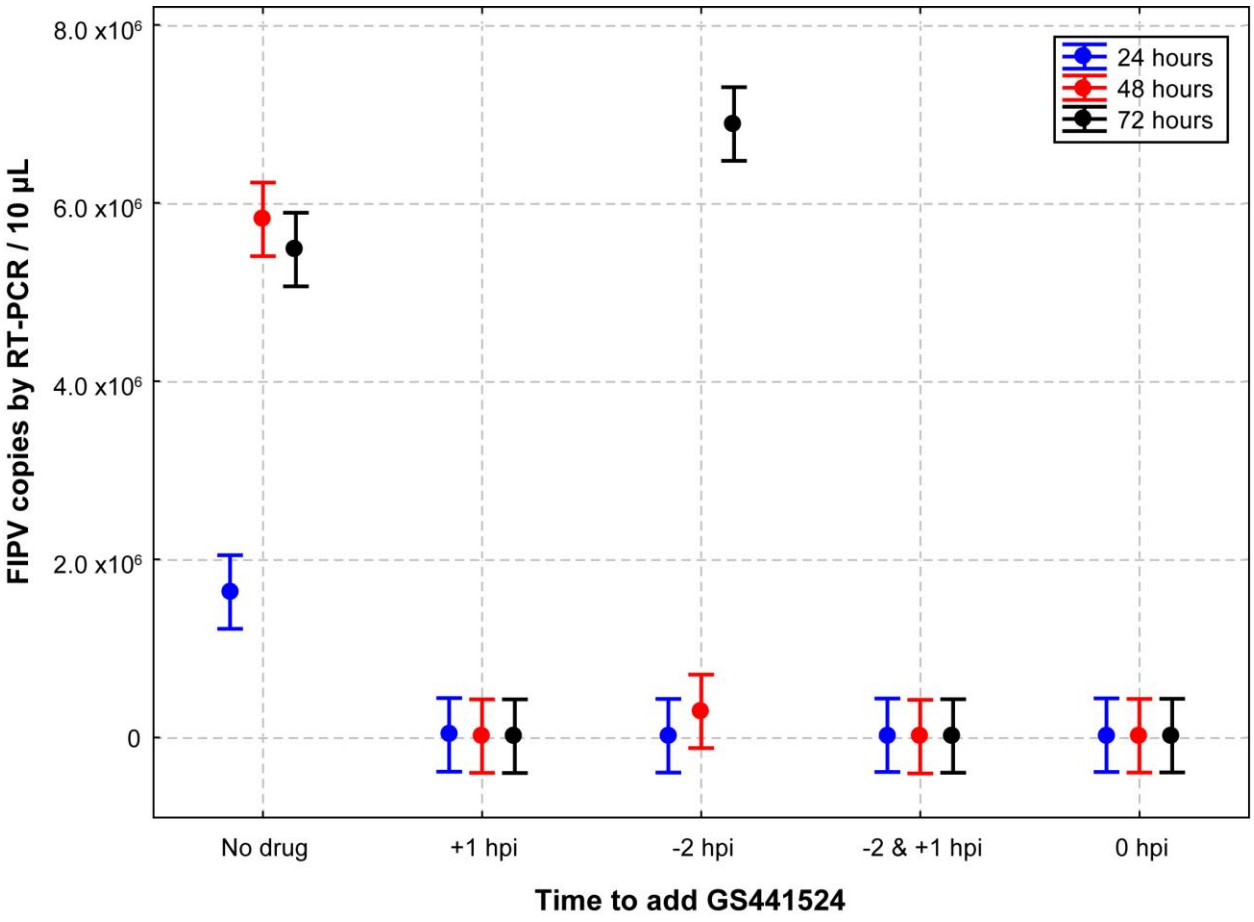

Figure 6.3 Effect of addition time and removal of GS441524 on its anti-FIPV efficacy.

CRFK cells infected with FIPV (2.5×10^4 $TCID_{50}$) received GS441524 (25 µM) with different times of addition and removal. No drug: no drug was applied; +1 hpi: the drug was applied 1-hour post-inoculation; -2 hpi: the drug was applied 2 hours before inoculation and was removed before virus inoculation; -2 & +1 hpi: the drug was applied 2 hours before infection and was removed before virus inoculation and applied again at 1 hpi; 0 hpi: the drug was applied at the time of inoculation and remained in the wells. The inhibition of FIPV replication in the presence of GS441524 in CRFK cells did not differ with different initial drug application time-point as long as 72 hours except for the -2 hpi well. In the -2 hpi wells, the FIPV RNA copies were significantly higher at 72 hours than at 24 and 48 hours. This data is presented as an average ±95% CI.

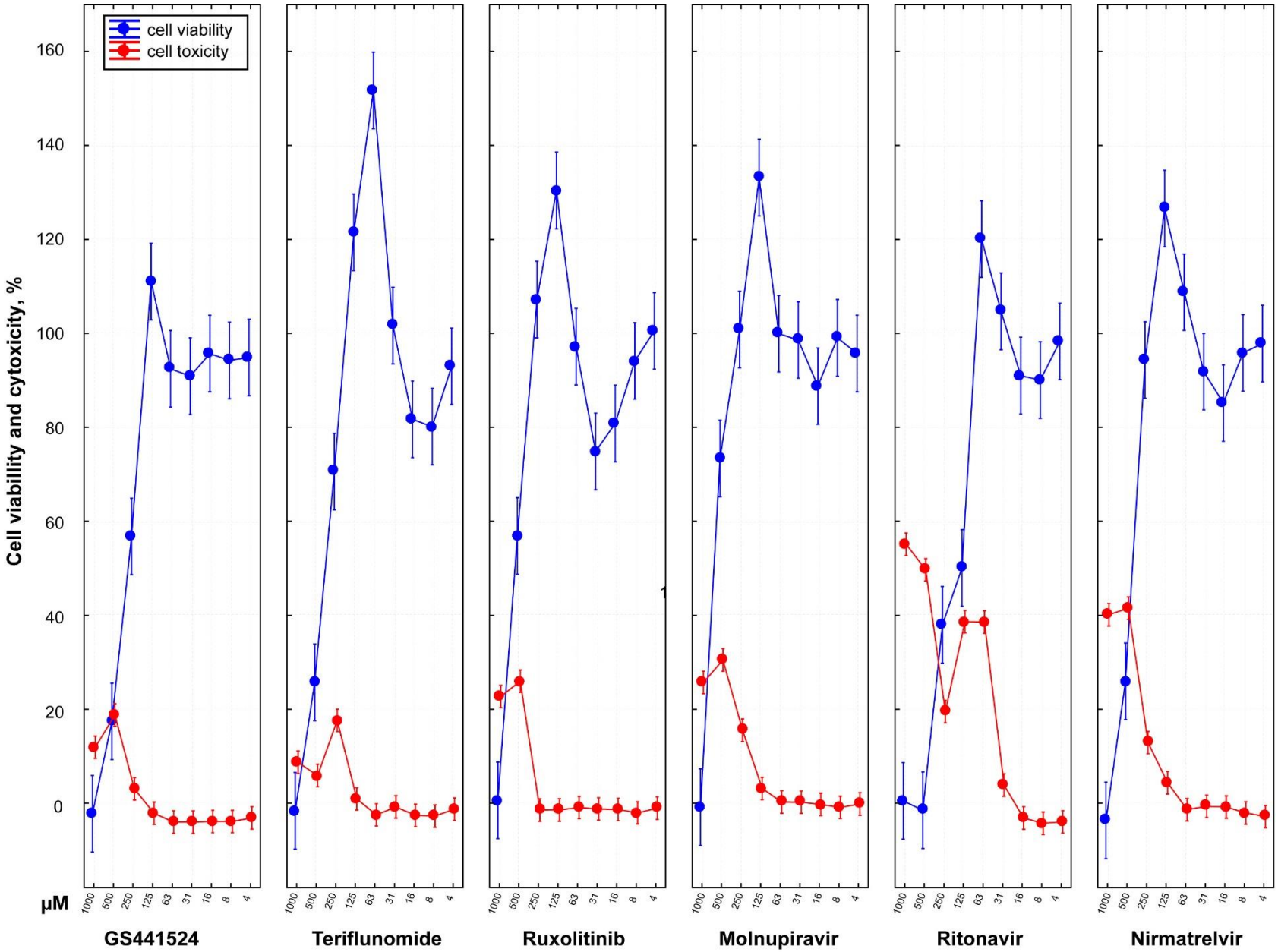
cell viability
cell toxicity
Cell viabillity and cytoxicity, %
160
140
120
100
80
60
40
20
0
μM
1000
500
250
125
63
31
16
8
4
GS441524
Teriflunomide
Ruxolitinib
Molnupiravir
Ritonavir
Nirmatrelvir

Figure 6.4 Dose-dependent cell viability and cytotoxicity profile of six antiviral drugs. In vitro analysis of cytotoxicity of different drugs was performed using commercially available CellToxTM Green assay for CRFK cells treated with serially two-fold diluted drugs (1,000, 500, 250,125, 63, 31, 16, 8, 4 µM) with four well replicates. At 48 hpi, drug cytotoxicity was calculated with a fluorescence intensity of 485-500nm$_{Ex}$/520-530nm$_{Em}$. In the graph, the y-axis presents the percent cytotoxicity determined by normalizing sample cytotoxicity to the positive toxicity control (set as 100% cytotoxicity) and untreated CRFK cells (set as 0% baseline cytotoxicity). The plotted data (red line) represent the average ± 95% CI of four replicates for each treated diluted drug concentration. All six antiviral drugs except Ritonavir demonstrated no cytotoxicity up to 63 µM. Ritonavir was found highly toxic to cells with CC_{50} of 39.85 µM, whereas Ruxolitinib showed a safer cytotoxicity profile undetectable at a high 250 µM concentration with CC_{50} of 338.05 µM compared to the other drugs. The value of CC_{50} of GS441524 had been determined as 260.02 µM. Cell viability assay was also performed using Cell Proliferation Kit I (MTT assay) for CRFK cells. Quantifying MTT staining of CRFK cells treated with varying concentrations of six drugs showed a dose-dependent loss of cell viability. Cell viability data inversely correspond with the drug cytotoxicity except at the highest drug concentration.

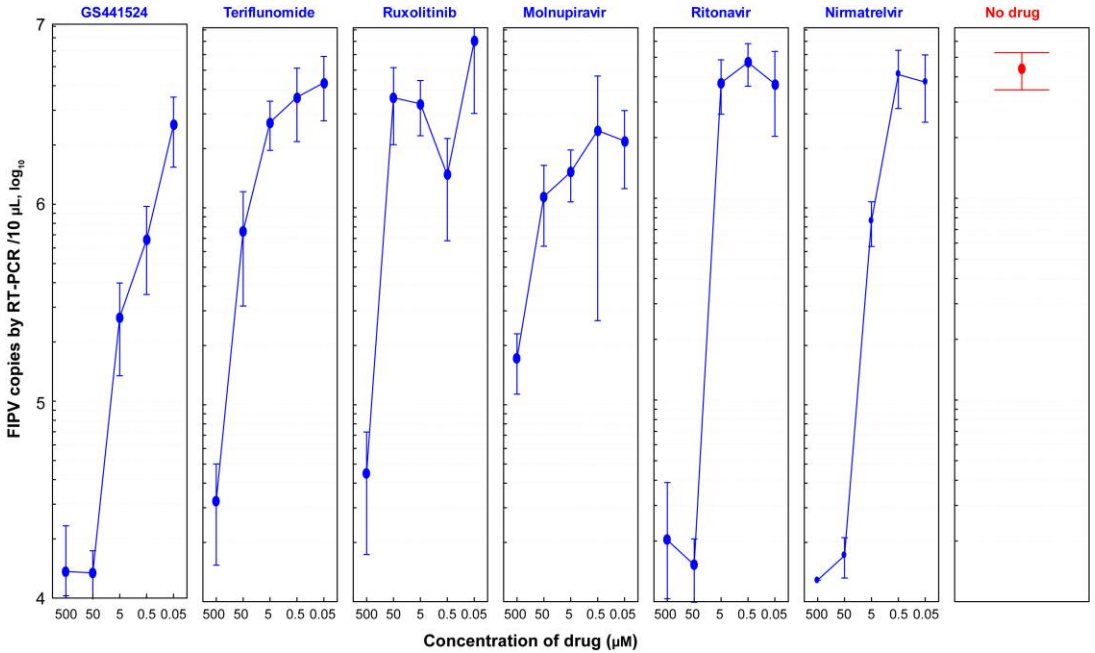

Figure 6.5 Dose-dependent anti-FIPV efficacy of antiviral drugs (1:10 dilutions). CRFK cells were infected with FIPV (2.5×10^4 $TCID_{50}$) and treated with 10-fold dilutions of six drugs (GS441524, Teriflunomide, Ruxolitinib, Molnupiravir, Ritonavir, and Nirmatrelvir). The viral load of FIPV was quantified by qRT-PCR at 48 hpi. Data represent an average ±95% CI of four replicates in the y-axis for each indicated concentration of six drugs. Compared to the no-drug group, all tested drugs showed anti-FIPV efficacy at a concentration as low as 5 µM. GS441524 molecule showed the most promising anti-FIPV efficacy at a concentration as low as 0.5 µM. Teriflunomide, Molnupiravir, and Nirmatrelvir harbored dose-dependent antiviral activities. Interestingly, Ritonavir showed a sudden drop in FIPV antiviral efficacy when the concentration changed from 50 µM to 5 µM

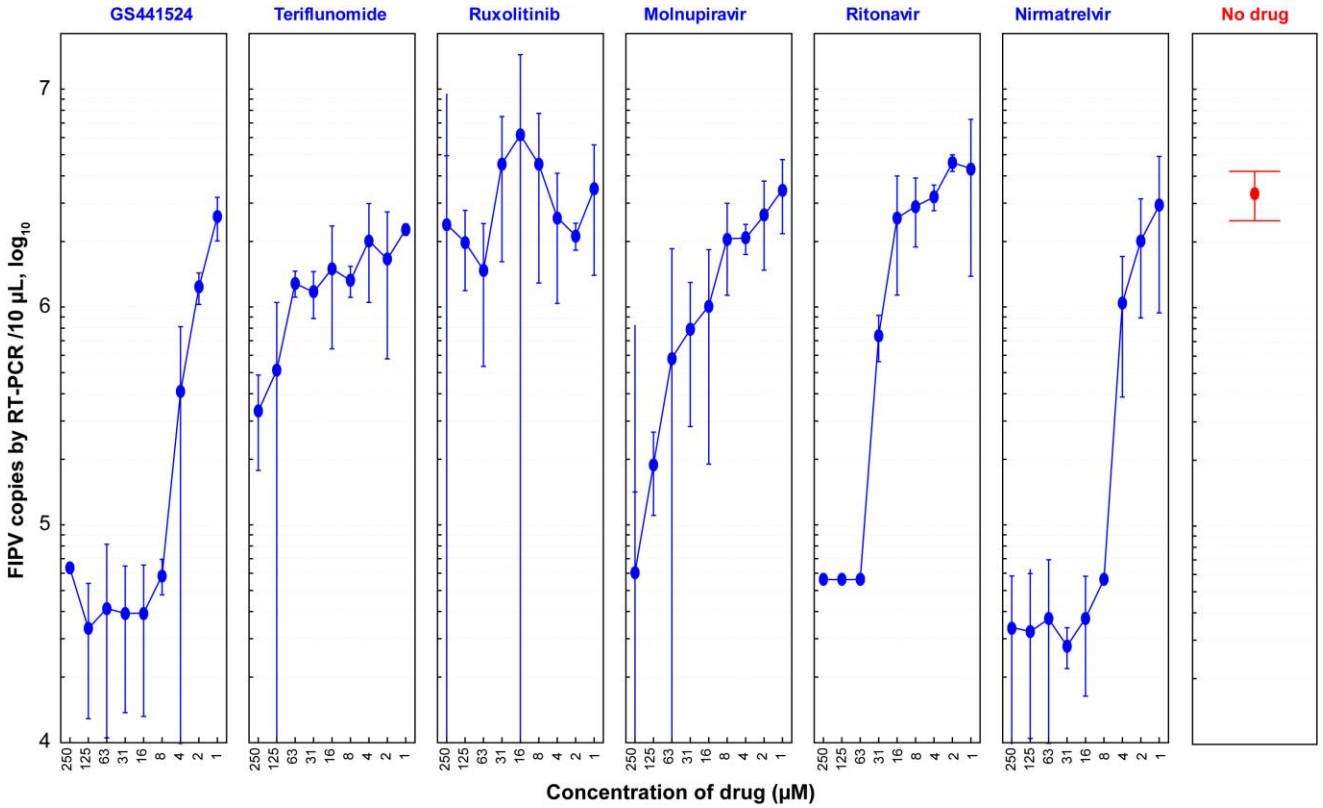

Figure 6.6 Reduction of FIPV RNA using drugs. CRFK cells were infected with FIPV (2.5×10^4 $TCID_{50}$/mL) and treated with two-fold dilutions of six drugs (GS441524, Teriflunomide, Ruxolitinib, Molnupiravir, Ritonavir, and Nirmatrelvir). At 48 hpi, the viral load of FIPV was quantified by qRT-PCR. In this graph, FIPV copies in the y-axis represent the average ±95% confidence interval (CI) of four replicates of samples for each diluted drug concentration. GS441524 and Nirmatrelvir showed significant anti-FIPV efficacy with EC_{50} values of 1.57 µM and 2.46 µM, respectively.

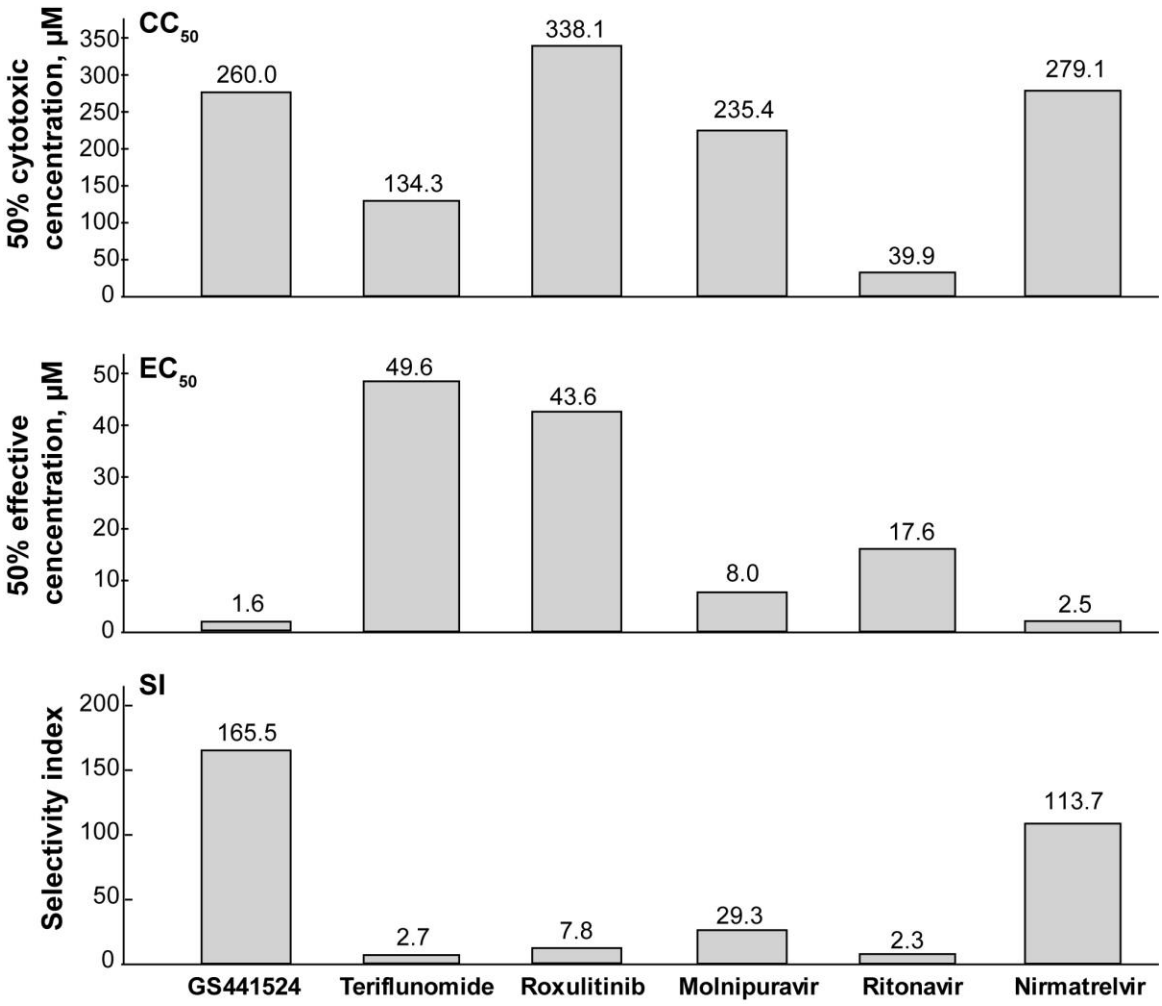

Figure 6.7 The CC_{50}, EC_{50} and selectivity index for six drugs. The half maximal cytotoxic concentration (CC_{50}) values are from four measurements of diluted drugs using MTT assay, in CRFK cells treated with drugs for 48 hours. The half maximal effective concentration (EC_{50}) values are from six measurements of diluted drugs against FIPV replication in CRFK cells for 48 hours. Based on the selectivity index (mean CC_{50})/(mean EC_{50}), GS441524 was found highly selective (SI 165.5) against FIPV among the drugs tested, and showed high efficacy (EC_{50} 1.6 μM) against FIPVwith less deleterious effect (CC_{50} 260.0 μM) on the cells. Nirmatrelvir also showed promising efficacy (EC_{50} 2.5 μM) and selectivity (SI 113.7) against FIPV. Ritonavir showed the highest toxicity level in the cells (CC_{50} 39.9).

Chapter 7 Conclusions and future research

Coronaviruses infect humans and a wide range of animals and cause predominantly respiratory and intestinal infections. Extensive knowledge in veterinary medicine about animal coronaviruses, their evolution, and pathobiology could help to forge a better understanding of the origin and spread of human coronaviruses such as SARS-CoV-2, and drive future research in human medicine towards the development of effective antiviral drugs.

SARS-CoV-2 VOCs are different in their transmissibility, clinical prognosis, response to vaccination, and therapy. As a result, the ability to distinguish between them is of ongoing interest. Although whole-genome sequencing is used for detailed genetic characterization, targeted nucleic acid amplification tests, such as RT FRET PCR, can be used as complementary approaches in local epidemiological variant surveillance and clinical settings as these tests are more rapid and accessible than sequencing.

Animals infected with SARS-CoV-2 causing COVID-19 have been reported around the world. Though the risk of spreading the virus from animal to person is low, people infected with SARS-CoV-2 can spread the virus to animals during close contact. As a result, early coordination and communication between public health and animal health are encouraged using a One Health approach to conduct epidemiological investigations for companion and wildlife animals with SARS-CoV-2 infection.

The SARS-CoV and MERS-CoV epidemics and the current SARS-CoV-2 pandemic have highlighted the need to prepare for the emergence of novel coronaviruses in humans. As a result, developing antivirals for treating FIP and other veterinary coronaviruses will not only save the lives of many companion animals but also help reduce the impact of future coronavirus

outbreaks. several antivirals have been tested for the treatment of FIP. Among those, nucleoside analog (GS441524) and protease inhibitor (GC376) showed promising antiviral activity. Interestingly, GS441524 is the parent molecule of Remdesivir, primarily employed as a potential antiviral against COVID-19. Veterinary medicine should thus support policymakers to adopt and strengthen sound measures for managing the environment and animals and advance the global 'One Health' movement.

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