COVID-19 in cats

– prevalence of antibodies in cats in Sweden, concern among owners and development of COVID-19 SIA as the analytical method

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Abstract

COVID-19 is a disease which has had great impact on the world in 2020. It has infected millions of people during the year, and been confirmed in animals such as cats, dogs and minks. The receptor ACE2 which is the target for the virus SARS-CoV-2 to use for attachment to the cell, is very similar between cats and humans. Therefore, there is a risk for cats to become infected with SARS-CoV-2. Studies have shown that cats can be infected by aerosol transmission, can show symptoms and can develop antibodies. Prevalence of SARS-CoV-2 antibodies in cats have been investigated in Wuhan, by using ELISA showing that 14.7% (15/102) of sampled cats were positive. However, there are no studies done on seroprevalence in Sweden.

The aim of this study was to investigate the prevalence of antibodies against SARS-CoV-2 in cats in four municipalities in Sweden. This by using a method previously not used for cats, but well tested for detection of SARS-CoV-2 antibodies in humans, called COVID-19 suspension immunoassay (SIA). The objective was to adapt the SIA to be able to detect antibodies against SARS-CoV-2 as well as against feline coronavirus (FCoV), which could have the capability to cross-react in the test. The goal was also to try and connect seroprevalence with different individual traits, environmental differences and whether the owner had been confirmed infected. The concern in cat owners regarding COVID-19 and their animals was to be simultaneously studied by a survey.

Blood samples were collected from both sick and healthy cats in Uppsala, Östhammar, Tierp and Halmstad. They were categorized into two groups, with 56 samples in the first (A) and 147 in the other (B). In group A, the samples were attached to a survey in which the owner answered 15 questions about their animal, its home environment, closeness to humans and other animals and if the owner had been sick in COVID-19. In group B, serum samples were collected from the university hospital for animals in Uppsala (UDS) with no survey administered.

Presence of SARS-CoV-2 antibodies in cat serum was analysed, using part of the spike protein (S1) as antigen. Six samples out of 203 turned out positive (preliminary cut off at >333 MFI), all in group B, which translates to a preliminary seropositivity of 3% in our population. Another two samples had doubtful results (MFI between 174-333), both in group A, why very little could be concluded from the survey regarding predisposing factors or symptoms. The two doubtfully positive cats did live very close to their humans, and one of the cats had a family with confirmed COVID-19. The COVID-19 SIA did unfortunately not work as well for FCoV despite efforts to try and solve a couple of possible reasons for failure, why it was not possible to examine the possibility for cross-reaction. However, previous studies have not shown any indications of this.

In conclusion, this study indicates that cats can produce antibodies against SARS-CoV-2, and that the seropositivity was 3% in the population used for the study. The SIA is reliable for detection of antibodies against SARS-CoV-2 in human sera and seems to have potential for analysis in cat sera, but is not yet successfully adapted for FCoV. Owners concern for COVID-19 in cats was low.

In the future, improvements and further studies could be done based on these results. A larger selection of cats, possibly from more areas of Sweden, should be included. All with information about their individual traits, environment, symptoms, closeness to humans and if they have been in contact with someone confirmed infected. A larger number of confirmed seronegative cats could aid in determining a more accurate cut off value for determining the results from the COVID-19 SIA.

This study was performed in parallel with another study, by Frida Österberg, which investigated the same subjects as this one, but in dogs.

Keywords: COVID-19, SARS-CoV-2, cats, antibodies, seroprevalence, SIA

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Abbreviations

ACE2	Angiotensin-converting enzyme 2
APN	Aminopeptidase N
COVID-19	Coronavirus disease 2019
ELISA	Enzyme-linked immunosorbent assay
FCoV	Feline corona virus
FECV	Feline endemic corona virus
FIP	Feline infectious peritonitis
FIPV	Feline infectious peritonitis virus
HR1/HR2	Heptad repeat 1/2 (part of S2)
Ig	Immunoglobulin
MERS	Middle eastern respiratory syndrome
MFI	Median fluorescent intensity
MHV	Murine hepatitis virus (coronavirus)
PCR	Polymerase chain reaction
PEA	Proximity extension assay
RBD	Receptor binding domain
RBM	Receptor binding motif
SARS	Severe acute respiratory syndrome
SARS-CoV-2	Severe acute respiratory syndrome- coronavirus- 2
SIA/SMIA	Suspension immunoassay/ suspension multiplex immunoassay
SLU	Sveriges lantbruksuniversitet (Swedish University of Agricultural Sciences)
SVA	Statens Veterinärmedicinska Anstalt (National Veterinary Institute)
S1/S2	Subunit 1/2 (on the S glycoprotein)
UDS	Universitetsdjursjukhuset (University Animal Hospital)
VNT	Virus neutralization test
WHO	World Health Organization

1. Introduction

1.1. Introduction

Coronavirus disease 2019 (COVID-19) is a newly emerged disease which so far has had great impact on the world. It has affected people all over the globe regarding health, economics, transportation, relations, and how to live life in general during 2020. By the middle of January 2021, over 95 million people have been confirmed with COVID-19 and more than 2 million deceased, and the pandemic seems to be far from over (WHO 2021).

It has been concluded that the origin of the virus causing COVID-19, the so-called severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is a coronavirus from an animal at a food market, most likely a bat or pangolin (Zhou *et al.* 2020b). Since the disease originated in animals it is not unthinkable that they could have a role in the transmission of the virus over the world and to humans. The animals living closest to us humans are our pets. And while most people have learned to keep distance from each other to reduce the risk of catching COVID-19, many still pet and interact with animals as usual. At the same time, more and more reports are being published of animals testing positive for antibodies against SARS-CoV-2. For example, outbreaks have been observed in mink farms, where new mutations in the genome have raised the concern for whether the zoonotic transmission could accelerate harmful or problematic new genotypes (ECDC 2020). However, no proof has yet been revealed to indicate transmission from pets to humans, and it is highly uncertain if animals get symptoms at all even if they develop antibodies. One animal which is generally close to humans and is often left to roam the neighbourhoods freely, is the cat. Therefore, they could have potential to meet many people or other cats and contribute to transmission. Cats have also tested positive for antibodies against SARS-CoV-2 in multiple studies (Shi *et al.* 2020; Segalés *et al.* 2020; Zhang *et al.* 2020).

More research is needed about animals and their role in this pandemic, since there are many uncertainties and questions left to be answered. This study is one step on the way to study seroprevalence of cats for SARS-CoV-2, by collecting samples

from cats in Sweden, and analysing them with an adapted method previously used for human samples. At the same time the owners concern for COVID-19, will be studied.

1.2. Aim of the study

- The aims of this study were to:
    - Through literature, research the role of animals in the COVID-19 pandemic, and compile studies about cats and SARS-CoV-2.
    - Study the prevalence of antibodies in cats
        - Modify the COVID-19 suspension immunoassay (SIA) method used for human samples to detect SARS-CoV-2 antibodies to work on cats as well
        - Explore the possibility for FCoV/FIPV to cross-react in this methodology, resulting in a false positive test for SARS-CoV-2 antibodies. Therefore, also make adaptations of the method to work for detection of FCoV/FIPV antibodies and work in suspension multiplex immunoassay (SMIA)
    - Examine if there is a higher probability for cats to have antibodies if they live closely with their humans, meet a lot of humans or other animals, or have met COVID-19-positive humans
    - Explore whether the positive cats had any symptoms
    - Investigate the concerns of the owners regarding COVID-19 and their animals

2. Literature review

2.1. Coronaviruses

Coronaviruses belong to the subfamily *Orthocoronavirinae* in the family *Coronaviridae*, which are spherical enveloped RNA viruses at a size of approximately 100nm in diameter. They are characterized by a round form with large protruding spikes. The replication occurs in the cytoplasm, where the RNA polymerase has a relatively high frequency of errors in the transcription. Combined with a large genome, corona viruses are prone to mutate (Holmes 1999). This large genome, which is positive-sense single-stranded non-segmented RNA at 27 to 32kb in size, is in fact the largest viral RNA genome known (Lai & Cavanagh 1997).

Coronaviruses are divided into four subgroups, called Alpha-, Beta-, Gamma- and Deltacoronavirus. Simplified, the first two groups consist of coronaviruses which infect mammals, and the last two primarily birds. Alpha- and Betacoronaviruses are believed to spring from a bat coronavirus while Gamma- and Deltacoronaviruses appear to have their origin in an avian coronavirus (Woo *et al.* 2012).

Here are some examples of the viruses in each subgroup according to "The Springer index of viruses" (Tidona & Darai 2011):

- *Alphacoronaviruses*: Feline coronavirus (FCoV), canine coronavirus (CCoV), transmissible gastroenteritis virus (TGEV, in pigs), human coronavirus 229E, human coronavirus NL63, porcine epidemic diarrhoea virus, and a number of bat coronaviruses.
- *Betacoronaviruses*: Severe acute respiratory syndrome-related coronavirus (SARS-CoV), canine respiratory corona virus (CRCoV), Middle East respiratory syndrome-related coronavirus (MERS-CoV), bovine coronavirus, human coronavirus OC43, human coronavirus HKU1, murine coronavirus, and even more bat coronaviruses.
- *Gammacoronaviruses*: Avian coronavirus, beluga whale coronavirus SW1
- *Deltacoronaviruses*: Bulbul coronavirus, porcine coronavirus HKU15

The most common health conditions caused by coronaviruses are respiratory or enteric diseases, but several corona infections can cause hepatitis, nephritis, myocarditis, peritonitis, neurological, reproductive or immunological disorders. Typically, the different coronaviruses cause disease in only one species, but there are exceptions. Disease caused by coronaviruses has been confirmed in many species, such as humans, cats, dogs, poultry, swine, cattle, mice, rabbits, camels, turkeys and more (Holmes 1999).

Coronaviruses are characterized by their big spikes, which are the reason they are called coronaviruses since the protrusions form a sort of crown or coronet around the envelope of the virus. They consist of proteins called Spike glycoproteins (S glycoproteins). These have the function of attaching to cells in the body of the host, and to instigate the fusion of the envelope of the virus to the membrane of the host-cell by binding to receptors. The S glycoproteins are also often the target for antibodies produced by the host to fend against the infection. This may be the reason the S glycoprotein is the structural protein with the most frequent genomic sequence variation within the same species, since immunity in hosts causes the variation to be a selective advantage (Collins *et al.* 1982).

The S glycoprotein consists of two subunits, S1 and S2. S1 determines the cellular tropism and the host range, while S2 mediates membrane fusion enabling the virus to enter the host cell. The S1 part of the S glycoprotein of the coronavirus contains a domain which binds to the receptor on the host cell, called receptor-binding domain (RBD). The affinity between the RBD and the receptor has been concluded to be the primary determinant for the host range. The outer part of the RBD, called receptor-binding motif (RBM), which is the part that binds to the surface of the targeted receptor, is an area of great interest when determining the affinity. The RBMs must match sufficiently to the receptor of the host cell to initiate infection, but it does not have to be a 100% match (Wan *et al.* 2020). In S2 there are two parts, heptad repeat 1 (HR1) and heptad repeat 2 (HR2) which create a helix-structure which is needed to bring the membranes close enough for merging. If the HR1 is inhibited, then the mechanism for fusion is also curbed (Xia *et al.* 2020). In short, when S1 protein recognizes its receptor on host cells, the HR1 and HR2 in the S2 interact with each other since the activated S1 expose both domains. The HR1 and HR2 then form a six-helix bundle (6-HB) to mediate membrane fusion between virus and target cell by bringing the membranes closer. If the HR1 and HR2 domains are unable to interact, for example due to an inhibitory peptide, the merging of the virus and cell is hindered (Chan *et al.* 2006).

The different coronaviruses target different receptors on the cells. For example, the porcine coronavirus TGEV use human or porcine aminopeptidase N (APN), FCoV uses the feline APN, while MHV (a murine coronavirus) uses murine biliary

glycoproteins (Holmes 1999). Even though S glycoproteins are important for attachment to target cells, there are more interactive parts imperative for the interaction with the cell. In human studies, there is proof that there are other mechanisms important for attachment than the target receptor on the host cell. They are not completely elucidated, but coronaviruses seem to make use of nonspecific receptors on the host cell as well. Sialic acid and C-type lectins are two which has been proven to facilitate the mechanism of entry. The receptor "dendritic cell-specific ICAM-3-grabbing nonintegrin" (DC-SIGN) has proven to promote entry by SARS-CoV into the cell (Regan & Whittaker 2008).

Then there is also the matter of tropism, which most animal coronaviruses have proved to have, and which is not yet fully understood. Many of the receptors needed for the virus to bind to the cell are also present in other parts of the body than the area of tropism. This indicates that a more cell-type specific molecule probably is needed for some of the coronaviruses to infect a cell. If a different species than the natural host species expresses the needed receptors (or receptors similar enough) on their cells, it may render them susceptible to infection with coronavirus virions. Therefore, interactions between coronavirus and the receptors on the host cell are an important determinant of the species specificity of coronavirus infection. Different coronaviruses have different target cells in the hosts. Macrophages and epithelial cells are common target cells. The virus can typically attach to the target cell even if the cell is immune, but during immunity the entry to the cell is stopped. Although erythrocytes are not a target cell, several coronaviruses can attach to erythrocytes and cause hemagglutination (Lai & Cavanagh 1997).

2.2. Immunology and serology

The body has several mechanisms to fend against viral infections. There are mainly three immunological reactions involved in the defence; antibody-mediated, interferon-mediated and cytotoxic cell-mediated response. Cytotoxic cells can directly kill infected cells to prevent or reduce the spreading of the virus. Interferons activate NK-cells, macrophages, and antigen presentation T-cells, while inhibiting the production of viral proteins (Channappanavar & Perlman 2017). These three immunological responses are all completely or partially part of the adaptive immunity, meaning they belong to the part of the immune system which learn to recognize and fend against infectants by encountering them. The antibody-mediated immune responses are of great importance for the body's defence, but also for determining if an individual has been infected or not. Antibodies are small molecules produced by the immune cells, which are designed to detect and attach to specific antigens. Antigens are recognizable parts of the infectant used as a fingerprint to identify it. Therefore, if a patient has antibodies against a virus, they have been

infected with that virus, unless there has been cross-reactivity. Some antigens are not unique for one specific virus or infectant, why the body will react and produce antibodies to destroy this threat even if it is not the exact same infectant it has encountered before. If the antigen is similar enough to cause the antibody to attach, it will not know the difference, and this is called cross-reactivity. Some infectious agents do however not result in a production of antibodies at all. The antigens have to be big, available, stable and foreign enough (Tizard 2013). Cross-reactivity has been proven between antigens from SARS-CoV-2 and a number of other similar coronaviruses. One study observed cross-reactivity with both the SARS-CoV S and S1 proteins, and to a lower extent with MERS-CoV (Middle Eastern respiratory syndrome corona virus) S protein, but not with the MERS-CoV S1 protein. This also indicates that the S2 protein is more similar between corona viruses then the S1 part, why then S1 is more specific than S as an antigen for SARS-CoV-2 serologic diagnosis (Okba *et al.* 2020).

Antibodies are produced by white blood cells in the body, B-lymphocytes and plasma cells. Antibodies can be made up by different proteins, classifying them into five main classes; Immunoglobulin (Ig) M, IgG, IgA, IgD and IgE. Microbial infections usually result in the production of IgM in the early stage of defence and IgG is generated in the next step as long term immunity and immunological memory. It takes some time for the body to developed antibodies against a virus, and when it has, the antibodies remain in the bloodstream for a time. This is the reason they cannot be used as a marker for acute infection, but can be used to determine whether an individual has been infected some time ago (Tizard 2013). According to Okba *et al.* (2020), the most easily detectable and applicable antibody against SARS-CoV-2 seems to be of the IgG type, but IgA and IgM also show potential so far in human studies. Ibarrondo *et al.* (2020) found that the half-life of the antibodies is between 26 and 60 days in people with relatively mild symptoms. No studies could be found on the half-life of feline antibodies against SARS-CoV-2.

When there is a need to confirm infection with a virus before the antibodies has reached a detectable concentration, another method is needed. When screening for active infection it is custom to search for the viral RNA, often through polymerase chain reaction (PCR) or nucleic acid hybridization techniques (Corman *et al.* 2020)

2.3. Coronavirus in cats

In cats, coronavirus is an infectious agent common in Sweden. It is known as feline coronavirus (FCoV) and belongs to the subgroup alphacoronaviruses. FCoV mainly

transmits through faecal-oral transmission since it is primarily found in the gastro-intestinal tract of the infected cats. In one study where 209 clinically healthy Swedish cats were tested, 31% (31/209) of them were seropositive to FCoV, with 65% (42/64) prevalence in purebred cats and 17% (24/142) in mixed breed cats. FCoV is seldom causing clinical signs of infection, but when cats do show clinical signs, it is commonly kittens with diarrhoea. However, the usually harmless virus has the tendency to occasionally mutate and cause feline infectious peritonitis (FIP), especially in young animals. This disease is often fatal. (Holst *et al.* 2006)

When the virus mutates to being able to cause FIP it is called feline infectious peritonitis virus (FIPV). Before the mutation, the FCoV which cannot cause FIP is often called feline enteric corona virus (FECV). The genetic difference in these two is that there are deleted parts in the genes from the FECV which are missing in the FIPV. It is not possible to distinguish between FECV and FIPV serologically (Vennema *et al.* 1998). The pathological differences are that the FECV targets the apical epithelial cells of the intestine, while FIPV mainly targets the macrophages. Clinical signs of FECV are diarrhoea, while FIP causes damage to the immune system. This results in lesions in various organs including liver, spleen, and kidney as well as a release of proinflammatory cytokines causing an inflammatory response in many parts of the body (Rottier *et al.* 2005).

The exact mechanism for the transformation in the virus from being able to cause no or mild enteric disease to FIP is not yet known. However there have been studies indicating that mutations in the S glycoproteins (Rottier *et al.* 2005), the membrane proteins (Brown *et al.* 2009) and non-structural protein 3c (Pedersen *et al.* 2009) are of significant importance to the change in pathology.

FCoV viruses can be divided into two different serotypes, I and II. The difference between these is the combination of amino acids constructing the genetic sequence of the spike proteins. Serotype I is the original type, and the predominant one. Serotype II is a recombinant of S glycoproteins from FCoV and canine coronavirus (CCoV) (Kipar & Meli 2014).

The FCoV mainly uses the receptor APN to enter the host cell in the cat, and this is the same receptor used by many of the viruses in the alphacoronavirus family (Jaimes & Whittaker 2018). It has also been confirmed that the FECV and FIPV use a number of nonspecific receptors for entry, such as C-type lectins and DC-SIGN, which are present in human cells and important for other coronaviruses such as SARS-CoV for cell binding (Regan & Whittaker 2008).

When cats have been experimentally infected with SARS-CoV-1, examination of the airways showed lesions associated with SARS (tracheo-bronchoadenitis), and antigens were also found in the respiratory tract. The target receptor angiotensin

converting enzyme (ACE2) was found mainly in type I and II pneumocytes, tracheo-bronchial goblet cells, serous epithelial cells of tracheo-bronchial sub-mucosal glands in the cats. None of the cats had clinical signs of infection even though they proved infected (Brand *et al.* 2008).

2.4. Coronavirus in humans

The virus causing COVID-19 is not the first human coronavirus. By 2004 there were four types of coronaviruses known to infect humans. They are called HCoV-229E, HCoV-OC43, HCoV-NL63, and SARS-CoV (van der Hoek *et al.* 2004). The first three are relatively harmless, mainly causing symptoms of the common cold, but SARS-CoV had a big impact on people's health when the breakout took place in 2003 (Drosten *et al.* 2003). It infected approximately 8000 people and killed 750, but then the epidemic faded off due to a relatively low infectivity (WHO 2003). SARS-CoV is believed to have originated in bats in China, and then evolving to infect humans, causing a disease called Severe acute respiratory syndrome (SARS) with symptoms as dry cough, dyspnoea, fever, headache and hypoxemia (Drosten *et al.* 2003). SARS-CoV has been proven to use the ACE2 to infect cells in humans (Zhou *et al.* 2020b).

In 2004 the endemic coronavirus HCoV-HKU1 was identified, and in 2012 an outbreak of Middle East respiratory syndrome (MERS) caused by MERS-coronavirus (MERS-CoV) started. The clinical signs of MERS are very similar to those of SARS, but the viral genetics are more closely related to bat coronaviruses HKU4 and HKU5. Despite this, more than 20% difference in the relevant open reading frame caused MERS-CoV to be classified as its own species within Betacoronaviruses. MERS is a zoonotic disease, spreading from dromedaries and camels to humans (Zaki *et al.* 2012). MERS spread to over 27 countries but only 858 deaths have so far been confirmed with infection with MERS-CoV as certain cause. It is still an actively spreading disease in Africa, the Middle East and South Asia (WHO 2020).

2.5. COVID-19

2.5.1. Outbreak and identification

The World Health Organization (WHO) was noticed about a cluster of cases of viral pneumonia in the Chinese city of Wuhan the 31st of December 2019. The 7th of January 2020, sequencing of the viral genome revealed it to be a new coronavirus. In a few days cases outside of China were confirmed, and even though Wuhan was

quarantined, the disease spread over the world. It was announced to be a pandemic in the beginning of mars 2020. Countries worldwide took measures to decrease the transmission, with adaptations to fit the own country's circumstances and choice of strategy. By the middle of January 2021, over 95 million cases were confirmed worldwide with over 2 million deceased (WHO 2021). The first case of COVID-19 in Sweden was detected 31st of January, a woman who had recently visited Wuhan. The first confirmed infection in a person in Sweden who had not travelled abroad was detected in early Mars. By the middle of January 2021, over 520,000 cases had been confirmed, with over 10,000 people dead (Folkhälsomyndigheten 2021).

The spread of infection has been traced back to a local food and animal market in Wuhan. When analysed, it was identified as a new type of Betacoronavirus. The full genome was obtained from humans infected in the early stage of the outbreak in a study by Zhou *et al.* 2020b, and it was almost completely identical between the patients. When compared to other genomes of coronaviruses it was found to have 79.6% sequence identity to SARS-CoV and was 96% identical at the whole-genome level to a bat coronavirus (RaTG13). It was also concluded that the virus causing COVID-19 does belong to the species of SARS-CoV since pairwise protein sequence analysis of seven conserved non-structural proteins domains matched those of this new virus. SARS-CoV and COVID-19-virus also proved to share the same cell entry receptor- ACE2. The virus causing COVID-19 was therefore named SARS-CoV-2, previously known as 2019-nCoV (2019 novel coronavirus). According to Xia *et al.* (2020), when the S glycoprotein was analysed, the HR2 was revealed to be 100% identical to SARS-CoV, but HR1 showed 38% difference due to multiple mutations.

2.5.2. COVID-19 in humans

Typical clinical symptoms of COVID-19 in human patients are fever, breathing difficulties (dyspnea), dry cough, pneumonia and headache. The disease may progress to cause alveolar damage and therefore progressive respiratory failure. In some cases, it can even result in death. However asymptomatic infections or mild upper respiratory illness has been observed as well, indicating a wide range in severity (Zhou *et al.* 2020b). The more severe cases are thought to be caused by an imbalanced immune response, where the Th1-response is over activated, resulting in an uncontrolled release of cytokines and chemokines. This causes a progression in the disease into acute respiratory distress, multiple organ failure and possibly death (Gozalbo-Rovira *et al.* 2020). A cohort study of patients from Wuhan has shown an increased risk of mortality in older people, and people with diseases as hypertension, diabetes and coronary heart disease. The duration of shedding of virus was also studied, with the median of 20 days and the longest duration observed was 37 days (Zhou *et al.* 2020a).

Transmission of SARS-CoV-2 is not fully understood yet, but studies have been conducted (van Doremalen *et al.* 2020; Riddell *et al.* 2020) to investigate the different possibilities. Person-to-person transmission has been confirmed so far, but it also seems to be able to travel in aerosol and survive a short while on surfaces. The aerosol is created by sneezes and coughs from infected people, when microscopic droplets containing virus particles are projected through the air. By using the Bayesian regression model, it is possible to estimate the decay rate of the virus in a certain environment. According to van Doremalen *et al.* (2020), SARS-CoV-2 does survive for at least 3 hours (h) in the air, 72h on plastic and 48h on stainless steel, but the infectious titre is moderately reduced. The viability was quickly decreased on cardboard but even more so on copper. In the study by Riddell *et al.* (2020), viable virus particles could survive much longer in dark laboratory environment than what was found in the study by van Doremalen *et al.* (2020) where the study was conducted in light during daytime. By using an initial viral load equivalent to what could be excreted by infectious patients with a high titre, in-fectious virus was isolated for up to 28 days at 20 °C from common surfaces such as glass, vinyl, stainless steel and both paper and polymer banknotes. In cotton cloth, viable virus was found up to 14 days after inoculation (van Doremalen *et al.* 2020).

2.6. Zoonotic aspects and risks

As previously discussed, the host range of coronaviruses is quite restricted and they usually only infect their natural host species, but cross-species infection does however occur occasionally (Lai & Cavanagh 1997). There are multiple pieces of evidence suggesting interactions between the genomes of coronaviruses from different species or another type of coronavirus within the same species. For example, the biotype FCoV type II has been naturally created as a recombinant between canine coronavirus and FCoV type I. Therefore there is a high risk for mutations, possibly allowing the virus to infect animals of another species (Holmes 1999). This alteration may happen in a series of passages through a heterologous cell line. This was seen in a study where the murine type of coronavirus (MHV) managed to evolve to infect and replicate in human, hamster and primate cells. When the genetic differences were analysed this was the conclusion;

> "These findings differ from the hypothesis that neutral changes are the predominant feature of molecular evolution and argue that changing ecologies actuate episodic evolution in the MHV spike glycoprotein genes that govern interspecies transfer and spread into alternative hosts" (Baric *et al.* 1997).

As proven, coronaviruses generally have the capacity to expand their range in hosts through evolution. When it comes to SARS-CoV-2, this must have happened since there is evidence it originated in bats but now infect humans. It has also infected several species except for humans. For example, in the US there are proof of tigers, lions, minks, cats and dogs with confirmed antibodies or nucleic acid for SARS-CoV-2 (Animal and Plant Health Inspection Service 2020).

The quote above is also supported by the recent spread of the virus and surfacing mutations in mink farms, in Denmark and multiple other countries. In Denmark, 214 human COVID-19 cases were reported in late November 2020 as infected with SARS-CoV-2 virus variants related to mink. Over 200 mink farms were reported as infected with SARS-CoV-2. The conclusion from investigations about the spread of disease is that humans introduced the virus to a few farms, where the virus replicated, mutated, and spread to other farms. The minks suffered from respiratory symptoms and many died due to this. A number of new genotypes have been recorded in the minks, where the one called cluster 5 has been lifted in official discussions as a potential health concern since the multiple mutations in the S-protein may hinder the effectiveness of upcoming vaccines. Almost all minks in Denmark were euthanized, resulting in millions of dead minks and huge economic losses. Demonstrably, the zoonotic aspects of the virus and its tendency to mutate, is a huge risk for human and animal health, animal welfare and economics (ECDC 2020).

According to one study where multiple species of animals were investigated, some of the animals living close to humans can get infected with SARS CoV-2. The replication of virus was poor in dogs, pigs, ducks and chickens. Species permissive to infections were cats and ferrets, where cats proved susceptible to airborne transmission. The replication only occurred in the upper airways in these species, but in cats there was evidence that viruses could also be found in the small intestine in one cat as well. However, the number of animals was small, which makes the scientific evidence weaker. All cats became seropositive for SARS-CoV-2. None of the swabs performed on the dogs showed any viruses in the airways or organs, but half of the dogs had seroconverted. Pigs, chickens and ducks did not seroconvert or show any positive swabs for viral RNA, indicating these species to be non-susceptible (Shi *et al.* 2020).

A case study of one cat which suffered from severe respiratory distress at the same time as several of its owners being sick in COVID-19 proved the cat to be both seroconverted and positive on the nasal swab. However, the only pathological findings in the autopsy turned out to be hypertrophic cardiomyopathy and severe pulmonary oedema and thrombosis, no histopathological lesions compatible with a viral infection could be detected (Segalés *et al.* 2020).

ACE2 has been proven to be the receptor which is targeted by both SARS-CoV and SARS-CoV-2 to initiate the infection. When simply comparing the genome sequence of the ACE2 between species, the animal most similar to the human sequence (except for primates) was domestic cat at 85.2%. Dogs had a similarity of 83.4% and ferrets at 82.6%. The match does not have to be 100% correct between the RBD and ACE2, but there are hot spots on the ACE2 which are critical to binding. In multiple studies, where the capacity of ACE2 from different animal species to bind to the SARS-CoV-2 RBD was evaluated, interactions were confirmed. It evidently interacted with the cells expressing ACE2 orthologs from primates, civets, rabbits, pangolins, horses, cat, fox, dog, raccoon dog, pig, wild Bactrian camel, bovine, goat and sheep. Rodents, hedgehogs and chickens did not have similar enough ACE2 to bind to the specific RBD. Notably, the ACE2 orthologs from five bat species exhibited varieties in ability to bind, with two displaying minimal fluorescent shift due to the SARS-CoV-2 RBD binding and three showed no detectable interaction at all (Wu *et al.* 2020; Wan *et al.* 2020; Stout *et al.* 2020).

In the time period between January and Mars 2020, 102 cats were sampled in Wuhan for a study. These cats came from private homes (15), shelters (46) and pet hospitals (41). When the blood was analysed for antibodies using ELISA for SARS-CoV-2 RBD-protein, 14.7% (15/102) of the cats were positive. No cross-reactivity was seen between type I or type II FIPV and SARS-CoV-2 RBD-protein. By performing a virus neutralization test (VNT), 10.8% of the cats proved to have neutralizing antibodies. The three cats with the highest neutralization titres were all owned by confirmed COVID-19 patients. No cross-reactivity to FIPV was seen in the VNT. By performing a western blot assay, the existence of SARS-CoV-2 specific IgG was confirmed in cat serum. Two cats were tested every 10 days, and antibodies were detectable until 110 days after the first sample was taken (Zhang *et al.* 2020).

3. Method and materials

3.1. In short

This study is based on 203 blood samples collected in a few areas of Sweden, from both sick and healthy cats, with data from surveys submitted by the owners. The samples were categorized into two groups, samples for group A and group B. Group A samples were collected prospectively together with a survey. Permission was always asked before collecting any blood. The blood and the surveys were marked with an identical unique code, enabling the owner and animal to be anonymous while the two datapoints could be paired. The samples for group B were collected from the laboratory in Universitetsdjursjukhuset (UDS) at Swedish University of Agricultural Sciences (SLU) from animals who had donated blood for general research purposes.

The blood was prepared and later analysed regarding prevalence of antibodies against SARS-CoV-2 and FCoV. For group A, seropositivity was then compared to the information in the surveys to try and find common conditions in the environment of the cats with or without antibodies. For group B, only seropositivity was studied.

This study had an ethical approval Dnr 5.8.18-101125/2020, and was performed in parallel with another study which investigated the same subjects as this one, but in dogs.

3.2. Sample collection

The population of cats for the study was chosen for practical purposes, as explained below.

Group A samples: Blood samples from 56 cats were collected in Östhammar, Tierp, Halmstad and Uppsala, between 1st of August to 4th of November. These are the areas where Jennifer Högberg Jeborn (the author of this study) and Frida Österberg (made the parallel study on COVID-19 in dogs) worked or lived during this time.

The participating cats were both sick and healthy cats, and the samples were gathered at veterinary clinics or in a few cases in the homes of the cats. All cats were cats which either came to the clinics for other health reasons, or which had owners who wanted their cat to participate in the study.

The blood was taken from the cephalic vein on the top of the front leg. It was gathered either with an open needle (no vacuum) or from a vein catheter, into a serum test tube. After a cool-off period of approximately 30 minutes it was then centrifuged at 5000 spins/min in 5 minutes. The serum was transferred to a smaller container which was put in the freezer (-20 °C) until analysis.

Group B samples: 147 serum samples collected during week 35 and 44 from cats were retrieved from the freezers of the UDS laboratory, or from group A where the owner failed to submit the survey during the whole period of collecting. The samples had been taken from patients at the small animal clinic with permission from the owners to save for science. In some cases, there was additional information such as age, breed and gender. These samples were collected in Uppsala, but patients may have travelled some distance to seek care why it is not possible to know the home municipality.

3.3. Survey

The survey was either submitted at the time of blood collecting or sent to the owner by e-mail to be submitted electronically after the sample was gathered. It consisted of 15 questions with information about the animal, its environment, its closeness to its owner and other people and animals, and whether it had been in contact with seropositive humans. The questions were compiled in an Excel document and evaluated whether there was a correlation between the data points. Please read Appendix 1 to see the full survey.

3.4. Analysis

The blood was prepared and analysed at the Zoonosis Science Centre at the Department of Medical Biochemistry and Microbiology at Uppsala University. It was analysed with a relatively new method, COVID-19 SIA (suspension immuno-assay). This method was used both in a singleplex (only using one antigen) and multiplex (using multiple antigens) manner. The method is then called SMIA (suspension multiplex immunoassay) when multiplex.

The COVID-19 SIA for detection of SARS-CoV-2 antibodies has so far only been used for analyses of human sera, why the method had to be adapted to animal samples. The antigen used for this study was S1 containing RBD from SARS-CoV-2 (SinoBiologiscals). When testing this method for antibodies against SARS-CoV-2, an attempt was made to try and conform it to also working on FIPV/FCoV. The antigen used was Feline Infectious Peritonitis virus antigen, strain WSU 79-1146 from the Native antigen company. The machines used to perform SIA/SMIA were Luminex Magpix and Luminex LX200.

A few samples were also tested using another new method, still in development at Uppsala University called proximity extension assays (PEA) (unpublished study). This method can shortly be described as incubating serum, with pairs of oligo-conjugated S1-parts from SARS-CoV-2 that will form homodimers via interaction with specific SARS-CoV-2 antibodies. The homodimers are then detected by real-time PCR. Thus, total antibodies against the S1-part are detected (both IgG, IgM, and IgA). This method was used only to compare the results from the SIA/SMIA and see if the results did tally, which could indicate a correct result.

3.4.1. About SIA/SMIA

SIA is a method where millions of small magnetic beads covered with carboxyl groups are coupled with an antigen, in this case the S1 part containing RBD of the SARS-CoV-2 S-glycoprotein. When the antibodies against a specific infectant (SARS-CoV-2) is added (by adding serum) they will bind to the antigens on the bead. Subsequently, biotinylated animal specific anti-antibodies are added and will bind to the SARS-CoV-2 antibodies. Lastly, a fluorescent signal molecule together with a binding molecule, Streptavidin Phycoerythrin (SA-PE), is added to bind to the end of the biotin (Figure 1). This signal molecule can then be read by a camera, where detection of the signal molecules confirms presence of the studied antibody (Figure 2).

Between every addition of a new type of molecule, the beads are secured with a magnet and rinsed from redundant molecules unable to bind. In all steps, PBS (phosphate-buffered saline, a water-based solution to maintain a stable pH) or PBS-T (PBS with added tween, which hinders unwanted bindings) are used to rinse the beads.

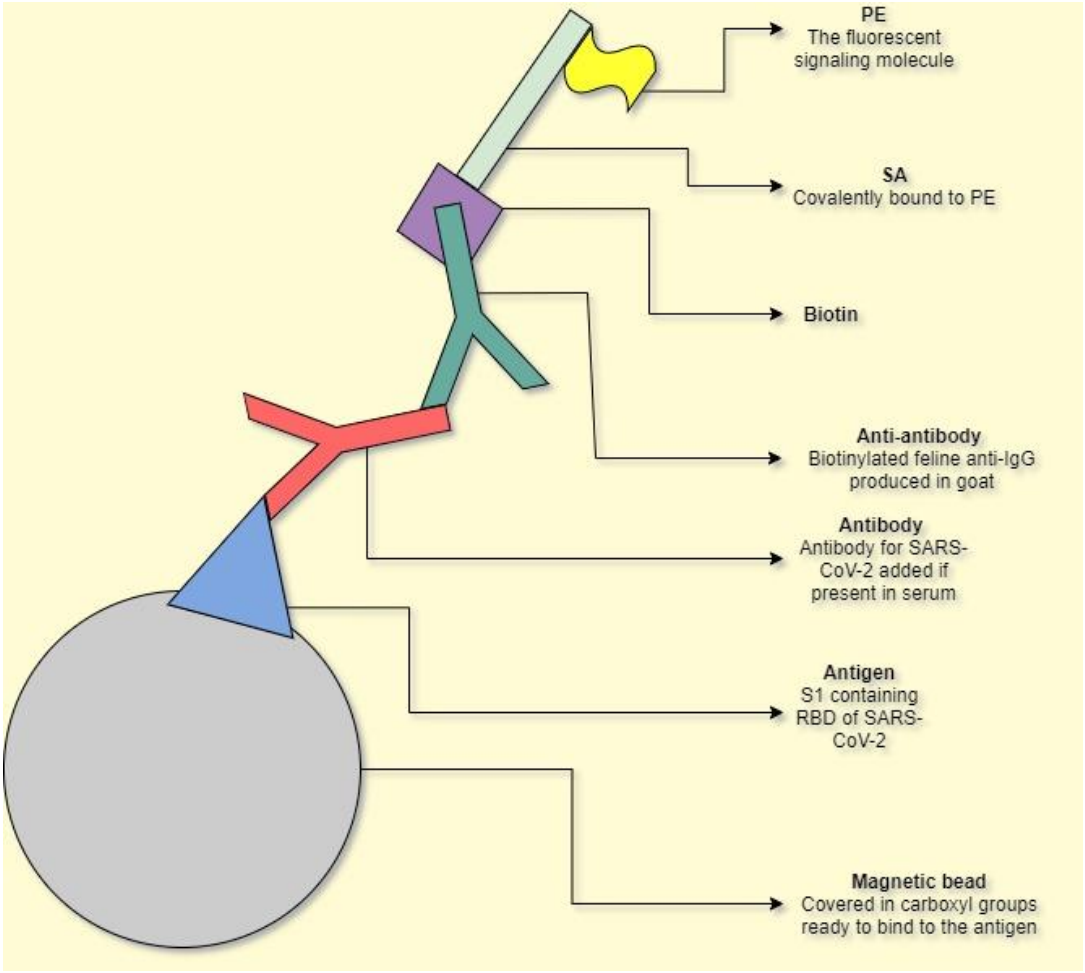

Figure 1. A schematic figure of how the method of suspension multiplex/singleplex immunoassay using MagPlex-C microspheres is constructed for detection of antibodies against SARS-CoV-2 in cats. Figure designed by the author.

It is possible to add more than one type of bead with antigen, to detect multiple types of antibodies, resulting in a multiplex immunoassay. In this study, the main goal was to study the prevalence of antibodies against SARS-CoV-2 in cats. However, the method was also adapted to analyse prevalence of antibodies against FCoV, both to examine the capacity of the multiplex method and to try and detect signs of cross-reactivity. One type of bead was not coupled with any antigens, acting as a measure of what values are detectable without any coupled proteins on the bead. Since the machine is able to distinguish between the types of beads, it is possible to read the positivity of antibodies against both SARS-CoV-2 and FCoV in the same sample. By analysing samples with known seroconversion status of both SARS-CoV-2 and FCoV, it is possible to try and detect cross-reactivity to the antigens, which would give a false positive result.

The two machines used to analyse the samples, the Luminex MagPix and Luminex LX200, uses two different methods to read the SA-PE on the beads (Figure 2). The Luminex MagPix uses a LED-based analysis and Luminex LX200 a flow cytometry-based analysis with laser. The MagPix uses a magnet to hold on to the beads while they are scanned by LED lights, while the Luminex LX200 uses two frequencies of laser while the beads are flowing through a fluid.

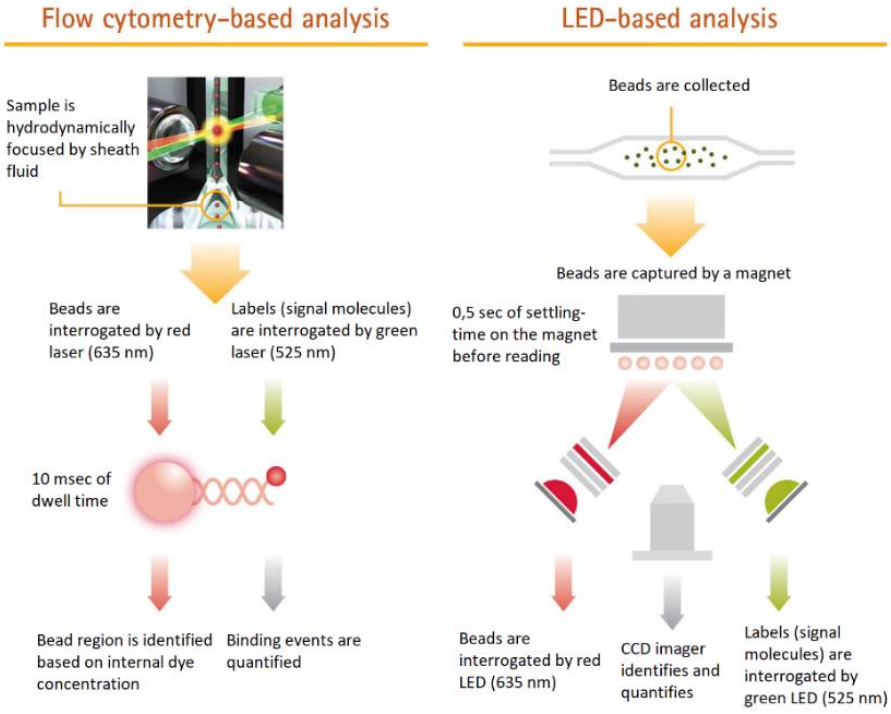

Figure 2. The differences between the reading method of Luminex LX200 and Luminex MagPix. The figure is modified from an informative picture created by Luminex.

The results are presented in numbers with median fluorescence intensity (MFI) as unit. This is the measure of how strong the signal transmitted by the fluorescent molecule (PE) is as a median from all the recognized beads in a well. There will never be a 0 as a result, since there is always a background signal even when there are no antigens attached to the beads or no beads at all.

3.5. Laboratory work

3.5.1. Materials

- Beads and antigens
    - #28, not coupled to any antigen, “blank” bead

    - #66, coupled to S1 containing RBD of SARS-CoV-2 (from Sino Biologicals)
    - #42, coupled to FIPV-antigen (Feline Infectious Peritonitis virus antigen, strain WSU 79-1146 from the Native antigen company)
- Anti-antigens and proteins
    - Biotinylated feline anti-IgG
    - Biotinylated protein G
- Positive controls (provided by the National Veterinary Institute [SVA])
    - 3 cat sera infused with feline antibodies against SARS-CoV-2, all FCoV-positive as well
    - 13 cat sera immunofluorescent-positive for FCoV antibodies
- Negative controls (provided by SVA)
    - 13 SARS-CoV-2-negative cat sera
    - 3 cat sera immunofluorescent-negative for FCoV antibodies
- Samples
    - 56 serum samples in group A (with survey)
    - 147 serum samples in group B (no survey)

3.5.2. Coupling of antigens to beads

See appendix 2 for full protocols for both coupling of antigens to the beads and serology.

The beads were conjugated with antigens, resulting in finished mixes with beads and antigens coupled together, which could later be used in our different steps of developing the method and analysing the samples. Bead #28 was not coupled to any antigen; it was used as a "blank" bead. Bead # 66 was coupled to S1 of SARS-CoV-2. Bead #42 was coupled to FIPV-antigen (Feline Infectious Peritonitis virus antigen, strain WSU 79-1146)

3.5.3. First test of positive and negative controls

See appendix 2 for full protocol for serology, and appendix 3 for exact protocol of amounts and placements.

The first run on the control samples was structured as such: One SARS-CoV-2-seropositive human sample and one seronegative sample was run in both a singleplex (#28 and #66) and a multiplex (with #28, #66, #42) manner with human protein G as anti-antibody. The 16 cat samples with both positive and negative controls for FCoV and SARS-CoV-2 were also run in singleplex manners (one batch with #28 and #42, one batch with #28 and #66) for both antigens and a multiplex manner (#28, #42 and #66). The anti-antibodies used for the cat samples were feline anti-IgG.

One additional batch with the 16 samples were run with singleplex method with #66 and #28, but using protein G instead of feline anti-IgG, to try and determine whether the antibodies added by SVA were human or feline.
The cat serum samples were diluted to 1:50.

3.5.4. Optimization of protocol regarding serum dilution

See appendix 2 for full protocol for serology. The protocol with amounts and placements was similar in layout to appendix 3, why it is not added as an appendix.

For understanding the optimal dilution of serum, serum was diluted 1:20 and 1:100, and then compared to previous 1:50. The dilution 1:50 yielded the best results in the assay, and therefore we used this concentration for the rest of the analyses.

The controls were again the 16 cat samples, this time only analysed in singleplex manners, for SARS-CoV-2 and FIP separately.

3.5.5. Optimization of protocol regarding concentration of anti-antibody and SA-PE

See appendix 2 for full protocol for serology. The protocol with amounts and placements was similar in layout to appendix 3, why it is not added as an appendix.

To determine the optimal concentration of biotinylated feline anti-IgG, a concentration of 2 µg/ml, 4 µg/ml, 8 µg/ml and 16 µg/ml were tested. Singleplex batches of both antigens were combined with the three different concentrations of anti-antibody solution, and also 2 µg/ml and 4 µg/ml of SA-PE were tested.

3.5.6. Analysing the collected samples

See appendix 2 for full protocol for serology, and appendix 4 for exact protocol of amounts and placements for the first samples. The rest of the samples used similar protocols as appendix 4, why they are not added as appendixes.

Samples from group A and group B were tested using a serum dilution of 1:50, with an IgG- and SA-PE solution of 2µg/ml each (same as original protocol). One negative and positive control was used. The first plate was run in a Luminex Magpix, but the other plates were analysed by using a Luminex LX200.

3.5.7. Rerun of interesting samples

See appendix 2 for full protocol for serology. The protocol with amounts and placements was similar in layout to appendix 4, why it is not added as an appendix.

Previously positive or inconclusive samples not yet tested on Luminex LX200, but on the Luminex MagPix and with the PEA-method, were run on Luminex LX200 to compare the results. Serum dilution was 1:50, with the IgG- and SA-PE solution of 2µg/ml each (same as original protocol). One negative and positive control was used.

3.5.8. Calculations of cut off value

This method has not been previously used for animals, why there was no stated cut-off (CO) values for positive results. In previous projects where this method has been used for detection of SARS-CoV-2 antibodies in human sera, all samples with an MFI >300 were categorized as positive but with a grey zone up to 900 MFI (un-published results). When deciding a cut-off MFI for this project a mean value of all the negative control samples was calculated, and by adding 6 standard deviations. Samples that were above the average + 3 standard deviations were classified as doubtful. The statistics for the results was descriptive, no significance could be calculated due to few positive results.

4. Results

4.1. SARS-CoV-2 seroprevalence in Swedish cats

A total of 203 cat samples were collected between August 1st and November 4th in four municipalities in Sweden (Figure 4). Out of these 203 serum samples, six tested positive and two doubtful (Table 2). This number was determined by the cut-off value which was preliminary set at 333 MFI after calculating the average (15,9) of the negative control samples and adding six standard deviations (52,9 x 6). The number of positive samples therefore became six, which equals 3% of all analysed samples. All the positive samples were in group B (Figure 3) why no calculations could be made using the data from the surveys. Values between average +3 standard deviations and average +6 standard deviations, 174-333 MFI, were classified as doubtful which corresponded to two samples in group A.

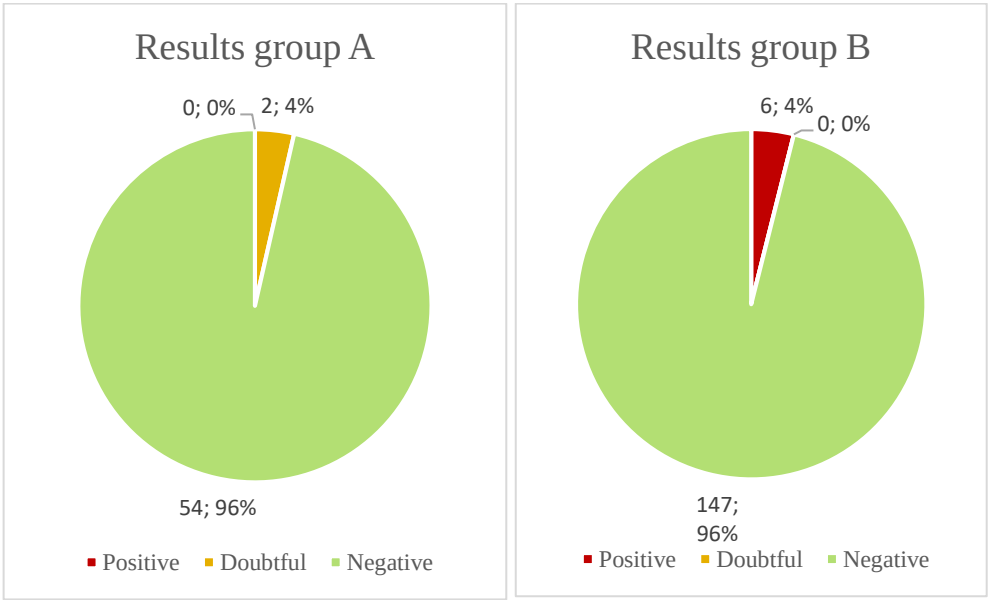

Figure 3. The prevalence of antibodies against SARS-CoV-2 in cat sera from group A and B respectively. In group A, 0% were positive and 3.6% (2/56) were doubtful. In group B, 4.1% (6/147) were positive. Group A=56. Group B=147.

When sorted from where they were collected, 4.1% (6 of 148) of the samples collected in Uppsala tested positive, while 0% and 4.8% (2 of 42) of the samples from Östhammar tested positive and doubtfully positive respectively (Table 1). All samples from Halmstad and Tierp were negative. This means all positive samples were collected in Uppsala.

Table 1. Seroprevalence in the cats categorized by where they were collected

Area of collecting	***Total samples collected***	***Serologically positive for SARS-CoV-2***	***Doubtfully positive for SARS-CoV-2***
Uppsala	*148*	*6*	*0*
Östhammar	*42*	*0*	*2*
Halmstad	*10*	*0*	*0*
Tierp	*3*	*0*	*0*

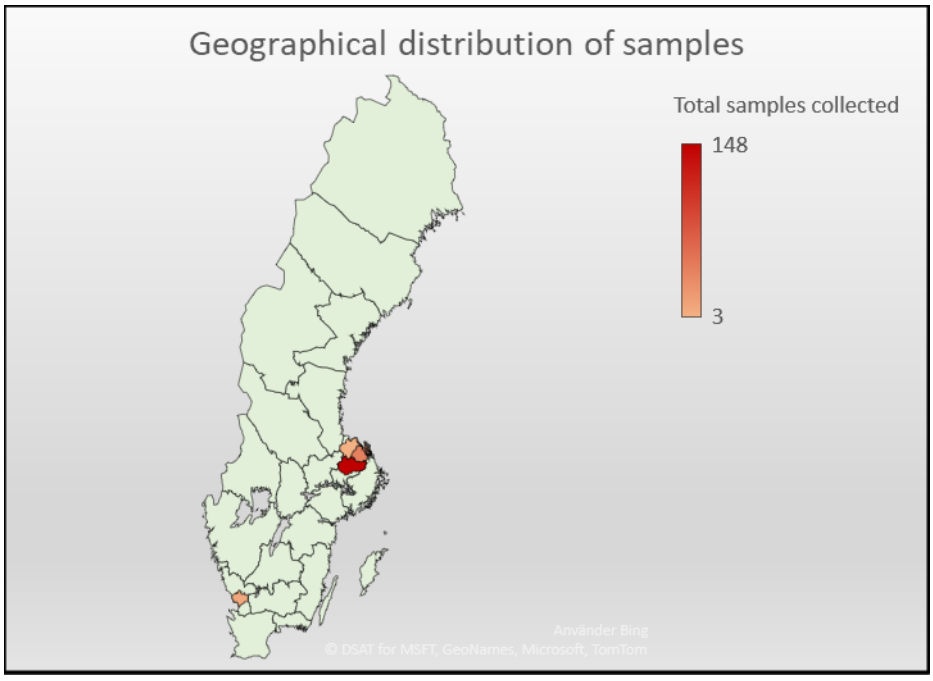

Figure 4. Map showing where the samples were collected in Sweden. 72.9% (148) were collected in Uppsala, 20.7% (42) in Östhammar, 4.9% (10) in Halmstad and 1.5% (3) in Tierp, Sweden. Map from Geonames Microsoft Tomtom. Microsoft product used for non-commercial purposes with permission from Microsoft Corporation. (http://mapsforenterprise.binginternal.com/en-us/maps/product/print-rights)

Table 2. Positive and doubtful results

Samples	MFI (corrected)*	Age (years)	Breed	Gender	Other information
XK29	397	17	House cat	Male, neutered	Strongly positive with PEA**
XK34	526.5	17	House cat		Strongly positive with PEA
XK41	469	16			Strongly positive with PEA
XK71	699	5	Ragdoll	Female, neutered	Strongly positive with PEA
XK107	1368.5	12	House cat	Male, neutered	Strongly positive with PEA
XK130	741	5	Ragdoll	Female, neutered	Strongly positive with PEA
K13	174	1	House cat	Male, neutered	Lives with four more cats where all human owners have been confirmed infected. Has very close contact with humans. No symptoms. Strongly positive with PEA
K29	178	0.5	British short hair	Male	Has very close contact with humans. No symptoms. Weakly positive with PEA.

Table showing data from the positive (red) and inconclusive (orange) samples from the analysis for SARS-CoV-2 antibodies. Cut off was calculated to 333 MFI.
**Median fluorescence intensity (MFI) of the SARS-CoV-2 antigen corrected by subtracting the MFI of the blank beads.*
***Results from PEA-test were graded negative, weakly positive or strongly positive.*

Samples with corrected MFI values over 100 were tested using the PEA-method (Table 2). All SIA-positive samples turned out positive with the PEA. Other than these, K13 was strongly positive, while K29 and 5 more samples were weakly positive. Note that sample XK71 and XK130 have the same information regarding age, gender and breed. It is not impossible that XK71 and XK130 were the same cat visiting the clinic in two different weeks, resulting in two donated samples from the same cat, but it cannot be confirmed.

4.2. Synopsis of the survey

Eight of 56 cats in group A had an owner with confirmed COVID-19, and two more cats had been in contact with people with confirmed COVID-19. Six of 56 cats had been in contact with someone the owner suspected had been ill in COVID-19. As seen in Table 3, only one of the confirmed previously infected owners turned out to have a possibly positive cat, but it was one with inconclusive seropositivity. This despite there being four more cats in the same home, which all tested negative in

this study, where all human members of the family had been seropositive, and where all cats had very close contact with their owners. The two cats from group A with doubtful serological result did have a very close contact with the owners (Table 2), but so did 37 of the seronegative cats.

Table 3. Correlation between seropositivity in owner and cat results

	Seronegative cats	Doubtfully positive cats
Owner not confirmed seropositive	47	1
Owner confirmed seropositive	7	1

Table showing the correlation of whether the owner had been tested seropositive in COVID19 or not and if antibodies were detected in the cat. Only cats from group A were included, why only doubtfully positive samples could be used.

None of the cats with doubtful seropositivity from group A had any symptoms which could be connected to infection with SARS-CoV-2. Because of there being only two samples from group A which were not clearly negative, there were no obvious connections between seropositivity and age, gender, breed or if the owners had tested positive for COVID-19 or not. Among 50 individual cat owners, three were confirmed seropositive and seven thought they had had COVID-19, leaving 40 which believed they had not been infected or were confirmed negative for COVID-19.

Generally, the owners did not worry about their pets getting sick in COVID-19, as seen in Figure 5, and regarding the own safety and health most owners felt no to only a slight concern. There was a higher concern for both family and friends and for the society. The owners who were worried about their animal, all had a very close or close contact with their animals, and all owners who did not have close contact felt no worry. These were the exact results regarding the concern for COVID-19 for;

- Myself: 52.7% (29) of owners did not worry, 41.8% (23) were a little worried, 3.6% (2) worried and 1.8% (1) quite worried.
- My family and friends: 9.1% of owners did not worry, 45.4% (25) were a little worried, 36.4% (20) worried and 9.1% quite worried.
- The society: 7.3% (4) of owners did not worry, 40% (22) were a little worried, 51% (28) worried and 1.8% (1) quite worried.
- My pets: 71% (39) of owners did not worry, 23.6% (13) were a little worried, 3.6% (2) worried and 1.8% (1) quite worried.

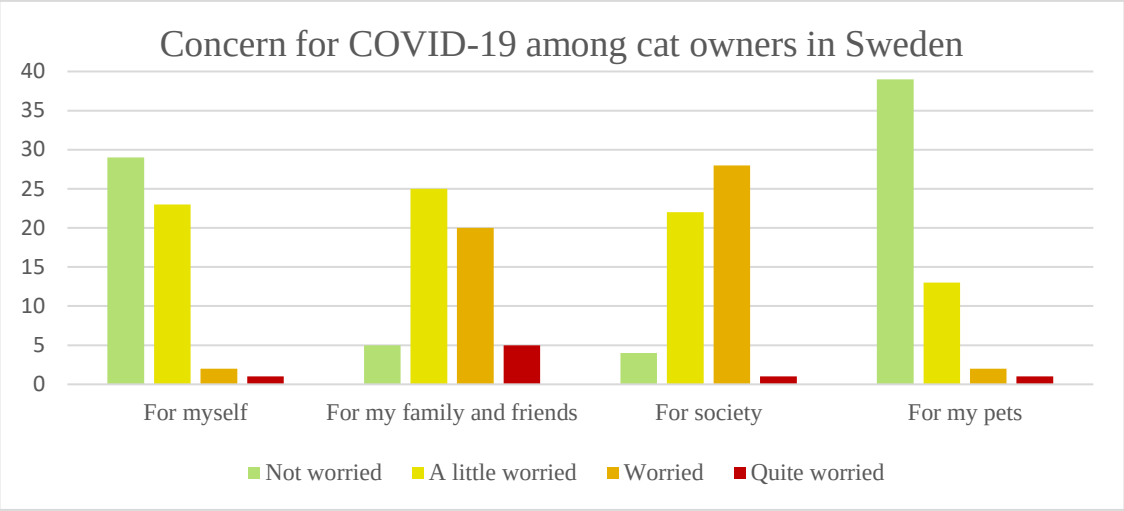

Figure 5. Diagram showing how many of the 55 cat owners who were concerned for themselves, family and friends, society and their pets regarding COVID-19 at the time of filling in the survey.

4.3. Results regarding development of the method

The first test of the protocol using antigens for both FCoV and SARS-CoV-2 and feline anti-IgG was on the positive and negative controls (Table 4). One sample (SVA16) was strongly suspected positive (with a remarkably higher MFI) for SARS-CoV-2 IgG, and one suspected positive (SVA 8) for FCoV IgG. None of the samples proved positive using protein G.

Table 4. Test of control samples

Sample	FIPV (#28)	(#42)	SARS-CoV-2 (#28)	(#66)	Both antigens (#28)	(#42)	(#66)	Correct answer from SVA
SVA1	107	74	164	131	80	141	57	FCoV titre 1:80
SVA2	61	82	362.5	185	69	99.5	37	FCoV titre 1:160
SVA3	78	94.5	72	36	60	56	32	FCoV titre 1:160
SVA4	61.5	79.5	145	71.5	62	101.5	32	FCoV titre 1:1280
SVA5	68	95	138	62	63	93.5	36	FCoV titre 1:1280
SVA6	89	161	85	65	76	154	63	FCoV titre 1:1280
SVA7	84	126.5	317	181	86	166	47	FCoV titre 1:1280
SVA8	88	287	148	141	152.5	697	161	FCoV titre 1:1280
SVA9	138.5	169	211	72	93.5	194	50	FCoV titre 1:640
SVA10	97.5	178	225	155	102	333.5	85	FCoV titre 1:640, SARS-CoV-2*
SVA11	72	116	185.5	91	145.5	382	87	FCoV titre 1:10
SVA12	87	153	108	123	101.5	234.5	112	FCoV Neg.
SVA13	83.5	105.5	175	366.5	114	299	139	FCoV Neg.
SVA14	93	123.5	210	78	61	97	32	FCoV titre 1:160, SARS-CoV-2*
SVA15	102.5	55	106.5	30	106	73	32	FCoV Neg.
SVA16	76	136	211.5	3849	60	101	950	FCoV titre 1:10, SARS-CoV-2*

Results of the analysis of the control samples from SVA. Both the blank bead and conjugated beads are shown. The samples were analysed by a Luminex MagPix, run in singleplex for each of FCoV and SARS-Cov-2, and multiplex with both of them. Red numbers are of extra interest regarding SARS-CoV-2 values, with a corrected value higher than the cut-off point of 333. Blue numbers are interesting values for FCoV, with no calculated cut off. The titre of FCoV should be interpreted as how many times the sample can be diluted but still be detected as positive by the method at SVA. FCoV was analysed using immunofluorescence at SVA.
** Samples spiked with feline antibodies against SARS-CoV-2.*

Since not all positive samples were detected, the dilution of the serum was altered to try to optimize the number of antibodies in the wells, since the dilution of antibodies added by SVA was unknown. There was no improvement in singleplex detection of antibodies against either SARS-CoV-2 or FCoV by using a dilution of 1/20 or 1/100. Therefore, the dilution of 1/50 was used in the later steps.

The next theory tested, that the concentration of biotinylated feline anti-IgG or SA-PE was too low to bind to the antibodies and therefore not allowing them to be detected, resulted in no improvement. The signals from both the blank beads and the conjugated beads greatly increased with higher concentration of both anti-antibodies and SA-PE. Conclusively, the original concentration of biotinylated feline anti-IgG and SA-PE was used from here on.

Further efforts to adapt the method for detection of FCoV antibodies were halted due to having to focus on SARS-CoV-2 to be able to finish the study in time.

5. Discussion

Antibodies against SARS-CoV-2 have previously been confirmed in cats, with or without symptoms. However, no known studies on cats have been conducted in Sweden, and no analysis have been done using SIA. Since COVID-19 is a newly erupted disease more data is needed, especially concerning the roles animals have in the transmission and development of the virus. Accurate methods for analysis are thus needed. This study indicates that the COVID-19 SIA, using S1 containing RBD from SARS-CoV-2 to detect IgG, can be used to analyse serum from cats. It also indicates that cats in Sweden can be infected with SARS-CoV-2 and as a result may develop antibodies. This result is supported by previous studies by Wu *et al.* (2020), Wan *et al.* (2020) and Stout *et al.* (2020), where the S1 containing RBD has been used as antigen in other analysis-methods than SIA, and where cats have seroconverted. The seropositivity out of all 203 samples turned out to be 3%, with a local seropositivity of 4.1% in Uppsala, and a doubtful seropositivity of 4.8% in Östhammar. When compared to the study by Zhang *et al.* (2020) where 14.7% of sampled cats in Wuhan were seropositive, the number seems reasonable. The cats in this study comes from private homes distributed over multiple municipalities over 13 weeks, in comparison to the cats in the Wuhan-study. The level of transmission was also higher in Wuhan in January 2020. Since all cats in the study by Shi *et al.* (2020) seroconverted when they were infected, we know that infected cats tend to produce antibodies. However, we do not know how likely cats are to be infected in different conditions. The calculated seroprevalence in this study is only representative for the sampled population, which is limited to blood collected from sick cats or voluntary donations from a few municipalities in Sweden.

No conclusions could be made regarding which cats are predisposed to get infected based on breed, age, gender, other health issues or closeness to humans/other animals. All the positive samples lacked most additional information other than species, since they belonged to group B which had no attached survey. Therefore, it is also not possible to comment on symptoms connected to COVID-19. All positive samples were collected in Uppsala, which could indicate a higher seroprevalence there. However, UDS is one of few animal hospitals in the area, why patients may have travelled some distance to get treatment. There is no way of knowing where the patients live normally. To be able to find these answers, a larger selection of samples needs to be collected, where sufficient data is provided for each

individual. It would also be of great interest to collect samples and data from more areas of Sweden, getting a bigger geographical picture of the seroprevalence. Another approach which could be interesting to use in the future is to ask confirmed COVID-19 positive cat owners to test their cats using PCR. This way it may be possible to try and detect acutely infected cats and to screen for symptoms.

The SIA-method can be used for detection of antibodies against SARS-CoV-2 in cat serum, but the reliability should be subject to further studies. It only detected one out of three positive control samples provided by SVA, indicating that the SIA has a low sensitivity. It is also unknown how the samples were spiked with antibodies. The results using both Luminex machines did however suggest a low risk of false positive results since the suspected negative samples got a low signal while the positive samples had a remarkably higher signal, with few samples in between. The preliminary cut off value used was higher than expected after calculations, since 6 standard deviations were added to the average negative value, which is a very conservative approach, meant to eliminate risks of false positives. The high number of added standard deviations also strengthen the theory that COVID-19 SIA could have a low sensitivity and high specificity. After calculations, the cut-off point turned out to be similar compared to human analysis which has a cut off of at least 300 MFI, with a grey zone to up to 900 MFI. The cut off does however not have to be similar between species, why it is important to have plenty of data for calculating it correctly. There are no previously known studies where the cut off for SIA for SARS-CoV-2 antibodies in cat serum have been determined. A larger selection of animals would aid in calculating a more accurate cut off point for the SIA.

When compared to the PEA-method (unpublished data), all the positive samples turned out positive, but some of the samples we thought of as negative turned out to be weakly positive in the PEA. This further indicates that the SIA has a low sensitivity but high specificity, maybe because it needs a high concentration of antibodies to be able to detect them. The PEA also analyses two antigens (S1 and nucleocapsid protein) from SARS-CoV-2 in comparison to one with the COVID-19 SIA, which could make it more accurate with higher sensitivity. It must be said that the PEA is no gold standard, but a method in developing, which however succeeded better in correctly analysing the control samples. In this study, it is used only as a comparison.

The attempt to adapt the method for FCoV and try to multiplex with analysis for SARS-CoV-2 did not result in a reliable method, despite efforts to try and optimize the protocol to work for both SARS-CoV-2 and FCoV analysis. It is unclear which exact method was used at SVA to determine the titre, but our results were not consistent with theirs. The reason for this may be that the antigen was not

compatible with the SIA, why for example the conjugation of the beads did not succeed properly, the antibodies did not attach to the antigen, or that any other step failed in the preparation of the wells. The antigen was the whole (inactivated) virus, in contrast to only the S-protein (recombinant) for SARS-CoV-2 analysis. It is also possible that the conjugation of the antigen to the beads failed, not actually deeming the antigen a failure. No indications of this could be found when recalculating dilutions and comparing protocol to laboratory notes. Further studies could be conducted regarding cross-reactivity, but since the FCoV and SARS-CoV-2 belong to two different subgroups (Alpha- and Betacoronaviruses) with different target receptors, the risk seems quite small. Especially since a couple of studies already have been made without finding indications of cross-reactivity.

The cat owners generally expressed a higher concern regarding COVID-19 for acquaintances and the society than for themselves or their pets. It was a bit surprising, finding that 52.7% of the cat owners in group A did not worry at all for themselves, and only 3 of 55 people feeling more than a little worried. One could speculate whether this feeling of security could result in unnecessary recklessness resulting in further transmission of infection, but no such results can be drawn from this study. The concern for their pet was low with 71% of owners feeling no worry, which is a good thing. It was interesting that the owners who were worried about their animal, all had a very close or close contact with their animals, and all owners who did not have close contact felt no worry. This could indicate that living closely to your cat results in a higher tendency to feel concern for it regarding COVID-19. However, the general concern for the pets was still very low. At the time when the surveys were being filled in, very few reports of sick or infected animals had been discussed publicly in media. The concern may have increased since the outbreak of SARS-CoV-2 infections in minks in Denmark, a phenomenon which has been discussed in media in Sweden. In these discussions, cats have been mentioned as possible hosts. However, it is not probable that a scenario like the one with the Danish minks would unfold in cats, since they are held in very different environments. If cats were kept as densely as minks in mink farms, it is not unthinkable a similar outbreak could occur. But even in catteries, it should not be the same level of infectiousness.

The transmission of virus between species is however a risk, since it promotes mutations which could result in genotypes with new traits. These new traits do not necessarily need to result in a more dangerous disease, but could for example change the parts of the virus which are targeted by antibodies, as seen in the cluster 5 mutation in minks. This could render vaccines ineffective and make the virus unrecognizable for the body's defence mechanisms. Therefore, the zoonotic aspects of the virus and its tendency to mutate, is a huge risk for human and animal health, animal welfare and economics. These reasons promote further studies on COVID-

19 in animals, especially species with a higher risk of infection and transmission, should be conducted.

References

Animal and Plant Health Inspection Service, U.S.D.O.A. (2020). *Cases of SARS-CoV-2 in Animals in the United States*. https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/sa_one_health/sars-cov-2-animals-us [2020-11-24]

Baric, R.S., Yount, B., Hensley, L., Peel, S.A. & Chen, W. (1997). Episodic evolution mediates interspecies transfer of a murine coronavirus. *Journal of Virology*, vol. 71 (3), ss. 1946–1955

Brand, J.M.A. van den, Haagmans, B.L., Leijten, L., Riel, D. van, Martina, B.E.E., Osterhaus, A.D.M.E. & Kuiken, T. (2008). Pathology of experimental SARS coronavirus infection in cats and ferrets: *Veterinary Pathology*, SAGE PublicationsSage CA: Los Angeles, CA. DOI: https://doi.org/10.1354/vp.45-4-551

Brown, M.A., Troyer, J.L., Pecon-Slattery, J., Roelke, M.E. & O'Brien, S.J. (2009). Genetics and pathogenesis of feline infectious peritonitis virus. *Emerging Infectious Diseases*, vol. 15 (9), ss. 1445–1452

Chan, W.-E., Chuang, C.-K., Yeh, S.-H., Chang, M.-S. & Chen, S.S.-L. (2006). Functional characterization of heptad repeat 1 and 2 mutants of the spike protein of severe acute respiratory syndrome coronavirus. *Journal of Virology*, vol. 80 (7), ss. 3225–3237

Channappanavar, R. & Perlman, S. (2017). Pathogenic human coronavirus infections: causes and consequences of cytokine storm and immunopathology. *Seminars in Immunopathology*, vol. 39 (5), ss. 529–539

Collins, A.R., Knobler, R.L., Powell, H. & Buchmeier, M.J. (1982). Monoclonal antibodies to murine hepatitis virus-4 (strain JHM) define the viral glycoprotein responsible for attachment and cell-cell fusion. *Virology*, vol. 119 (2), ss. 358–371

Corman, V.M., Landt, O., Kaiser, M., Molenkamp, R., Meijer, A., Chu, D.K., Bleicker, T., Brünink, S., Schneider, J., Schmidt, M.L., Mulders, D.G., Haagmans, B.L., van der Veer, B., van den Brink, S., Wijsman, L., Goderski, G., Romette, J.-L., Ellis, J., Zambon, M., Peiris, M., Goossens, H., Reusken, C., Koopmans, M.P. & Drosten, C. (2020). Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Euro Surveillance: Bulletin Europeen Sur Les Maladies Transmissibles = European Transmittable Disease Bulletin*, vol. 25 (3)

van Doremalen, N., Bushmaker, T., Morris, D.H., Holbrook, M.G., Gamble, A., Williamson, B.N., Tamin, A., Harcourt, J.L., Thornburg, N.J., Gerber, S.I., Lloyd-Smith, J.O., de Wit, E. & Munster, V.J. (2020). Aerosol and surface stability of SARS-CoV-2 as compared with SARS-CoV-1. *The New England Journal of Medicine*,. DOI: https://doi.org/10.1056/NEJMc2004973

Drosten, C., Günther, S., Preiser, W., van der Werf, S., Brodt, H.-R., Becker, S., Rabenau, H., Panning, M., Kolesnikova, L., Fouchier, R.A.M., Berger, A., Burguière, A.-M., Cinatl, J., Eickmann, M., Escriou, N., Grywna, K., Kramme, S., Manuguerra, J.-C., Müller, S., Rickerts, V., Stürmer, M., Vieth, S., Klenk, H.-D., Osterhaus,

A.D.M.E., Schmitz, H. & Doerr, H.W. (2003). Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *The New England Journal of Medicine,* vol. 348, ss. 1967-1976. DOI: https://doi.org/10.1056/NEJMoa030747

ECDC- European Centre for Disease Prevention and Control. (2020). *Rapid Risk Assessment: Detection of new SARS-CoV-2 variants related to mink*. https://www.ecdc.europa.eu/en/publications-data/detection-new-sars-cov-2-variants-mink [2020-11-27]

Folkhälsomyndigheten (2021). *Bekräftade fall i Sverige — Folkhälsomyndigheten*. http://www.folkhalsomyndigheten.se/smittskydd-beredskap/utbrott/aktuella-utbrott/covid-19/statistik-och-analyser/bekraftade-fall-i-sverige/ [2021-01-17]

Gozalbo-Rovira, R., Gimenez, E., Latorre, V., Francés-Gómez, C., Albert, E., Buesa, J., Marina, A., Blasco, M.L., Signes-Costa, J., Rodríguez-Díaz, J., Geller, R. & Navarro, D. (2020). SARS-CoV-2 antibodies, serum inflammatory biomarkers and clinical severity of hospitalized COVID-19 patients. *Journal of Clinical Virology*, vol. 131, s. 104611

Holmes, K.V. (1999). Coronaviruses (Coronaviridae). *Encyclopedia of Virology*, ss. 291–298

Holst, B.S., Englund, L., Palacios, S., Renström, L. & Berndtsson, L.T. (2006). Prevalence of antibodies against feline coronavirus and Chlamydophila felis in Swedish cats. *Journal of Feline Medicine and Surgery*, vol. 8 (3), ss. 207–211

Ibarrondo, F.J., Fulcher, J.A., Goodman-Meza, D., Elliott, J., Hofmann, C., Hausner, M.A., Ferbas, K.G., Tobin, N.H., Aldrovandi, G.M. & Yang, O.O. (2020). Rapid decay of anti–SARS-CoV-2 antibodies in persons with mild covid-19. *New England Journal of Medicine*, vol. 383 (11), ss. 1085–1087 Massachusetts Medical Society.

Jaimes, J.A. & Whittaker, G.R. (2018). Feline coronavirus: Insights into viral pathogenesis based on the spike protein structure and function. *Virology*, vol. 517, ss. 108–121

Kipar, A. & Meli, M.L. (2014). Feline infectious peritonitis: still an enigma? *Veterinary Pathology*, vol. 51(2), pp. 505-526. SAGE Publications. Los Angeles, CA. DOI: https://doi.org/10.1177/0300985814522077

Lai, M.M.C. & Cavanagh, D. (1997). The molecular biology of coronaviruses. *Advances in Virus Research*, vol. 48, ss. 1–100

Okba, N.M.A., Müller, M.A., Li, W., Wang, C., GeurtsvanKessel, C.H., Corman, V.M., Lamers, M.M., Sikkema, R.S., de Bruin, E., Chandler, F.D., Yazdanpanah, Y., Le Hingrat, Q., Descamps, D., Houhou-Fidouh, N., Reusken, C.B.E.M., Bosch, B.-J., Drosten, C., Koopmans, M.P.G. & Haagmans, B.L. (2020). Severe acute respiratory syndrome coronavirus 2-specific antibody responses in coronavirus disease patients. *Emerging Infectious Diseases*, vol. 26 (7), ss. 1478–1488

Pedersen, N.C., Liu, H., Dodd, K.A. & Pesavento, P.A. (2009). Significance of coronavirus mutants in feces and diseased tissues of cats suffering from feline infectious peritonitis. *Viruses*, vol. 1 (2), s. 166 Multidisciplinary Digital Publishing Institute (MDPI).

Regan, A.D. & Whittaker, G.R. (2008). Utilization of DC-SIGN for entry of feline coronaviruses into host cells. *Journal of Virology*, vol. 82 (23), ss. 11992–11996

Riddell, S., Goldie, S., Hill, A., Eagles, D. & Drew, T.W. (2020). The effect of temperature on persistence of SARS-CoV-2 on common surfaces. *Virology Journal*, vol. 17 (1), s. 145

Rottier, P.J.M., Nakamura, K., Schellen, P., Volders, H. & Haijema, B.J. (2005). Acquisition of macrophage tropism during the pathogenesis of feline infectious peritonitis is determined by mutations in the feline coronavirus spike protein. *Journal of Virology*, vol. 79 (22), ss. 14122–14130

Segalés, J., Puig, M., Rodon, J., Avila-Nieto, C., Carrillo, J., Cantero, G., Terrón, M.T., Cruz, S., Parera, M., Noguera-Julián, M., Izquierdo-Useros, N., Guallar, V., Vidal, E., Valencia, A., Blanco, I., Blanco, J., Clotet, B. & Vergara-Alert, J. (2020). Detection of SARS-CoV-2 in a cat owned by a COVID-19-affected patient in Spain. *Proceedings of the National Academy of Sciences of the United States of America*, vol. 117(40), pp. 24790-24793

Shi, J., Wen, Z., Zhong, G., Yang, H., Wang, C., Huang, B., Liu, R., He, X., Shuai, L., Sun, Z., Zhao, Y., Liu, P., Liang, L., Cui, P., Wang, J., Zhang, X., Guan, Y., Tan, W., Wu, G., Chen, H. & Bu, Z. (2020). Susceptibility of ferrets, cats, dogs, and other domesticated animals to SARS–coronavirus 2. *Science (New York, N.Y.)*, vol. 368 (6494), ss. 1016–1020

Stout, A.E., André, N.M., Jaimes, J.A., Millet, J.K. & Whittaker, G.R. (2020). Coronaviruses in cats and other companion animals: Where does SARS-CoV-2/COVID-19 fit? *Veterinary Microbiology*, vol. 247, 108777

Tidona, C. & Darai, G. (2011). *The Springer Index of Viruses*. 2nd ed. New York: Imprint

Tizard, I.R. (2013). *Veterinary Immunology*. 9th ed. Texas: Elsevier.

Vennema, H., Poland, A., Foley, J. & Pedersen, N.C. (1998). Feline infectious peritonitis viruses arise by mutation from endemic feline enteric coronaviruses. *Virology*, vol. 243 (1), ss. 150–157

Wan, Y., Shang, J., Graham, R., Baric, R.S. & Li, F. (2020). Receptor recognition by the novel coronavirus from Wuhan: an analysis based on decade-long structural studies of SARS coronavirus. *Journal of Virology*, vol. 94 (7). DOI: https://doi.org/10.1128/JVI.00127-20

WHO (2003). *Severe Acute Respiratory Syndrome (SARS)*. https://www.who.int/westernpacific/health-topics/severe-acute-respiratory-syndrome [2020-09-22]

WHO (2020). *Middle East respiratory syndrome coronavirus (MERS-CoV)*. https://www.who.int/health-topics/middle-east-respiratory-syndrome-coronavirus-mers#tab=tab_1 [2020-09-23]

WHO (2021). *Timeline: WHO's COVID-19 response*. https://www.who.int/emergencies/diseases/novel-coronavirus-2019/interactive-timeline?gclid=CjwKCAiAouD_BRBIEiwALhJH6B0_5Z2YXCC0vkyL-9TJhuOWbO58AI1lqnkj8KGRM5zGKlmONb_KEhoCF74QAvD_BwE#event-115 [2021-01-17]

Woo, P.C.Y., Lau, S.K.P., Lam, C.S.F., Lau, C.C.Y., Tsang, A.K.L., Lau, J.H.N., Bai, R., Teng, J.L.L., Tsang, C.C.C., Wang, M., Zheng, B.-J., Chan, K.-H. & Yuen, K.-Y. (2012). Discovery of seven novel mammalian and avian coronaviruses in the genus Deltacoronavirus supports bat coronaviruses as the gene source of Alphacoronavirus and Betacoronavirus and avian coronaviruses as the gene source of Gammacoronavirus and Deltacoronavirus. *Journal of Virology*, vol. 86 (7), ss. 3995–4008

Wu, L., Chen, Q., Liu, K., Wang, J., Han, P., Zhang, Y., Hu, Y., Meng, Y., Pan, X., Qiao, C., Tian, S., Du, P., Song, H., Shi, W., Qi, J., Wang, H.-W., Yan, J., Gao, G.F. & Wang, Q. (2020). Broad host range of SARS-CoV-2 and the molecular basis for SARS-CoV-2 binding to cat ACE2. *Cell Discovery*, vol. 6 (1), ss. 1–12 Nature Publishing Group.

Xia, S., Zhu, Y., Liu, M., Lan, Q., Xu, W., Wu, Y., Ying, T., Liu, S., Shi, Z., Jiang, S. & Lu, L. (2020). Fusion mechanism of 2019-nCoV and fusion inhibitors targeting HR1 domain in spike protein. *Cellular and Molecular Immunology*, ss. 1–3

Zaki, A.M., van Boheemen, S., Bestebroer, T.M., Osterhaus, A.D.M.E. & Fouchier, R.A.M. (2012). Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *The New England Journal of Medicine*, 367:1814-1820. http://dx.doi.org/10.1056/NEJMoa1211721.

Zhang, Q., Zhang, H., Gao, J., Huang, K., Yang, Y., Hui, X., He, X., Li, C., Gong, W., Zhang, Y., Zhao, Y., Peng, C., Gao, X., Chen, H., Zou, Z., Shi, Z.-L. & Jin, M. (2020). A serological survey of SARS-CoV-2 in cat in Wuhan. *Emerging Microbes & Infections*, vol. 9 (1), ss. 2013–2019 Taylor & Francis.

Zhou, F., Yu, T., Du, R., Fan, G., Liu, Y., Liu, Z., Xiang, J., Wang, Y., Song, B., Gu, X., Guan, L., Wei, Y., Li, H., Wu, X., Xu, J., Tu, S., Zhang, Y., Chen, H. & Cao, B. (2020a). Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet (London, England)*, vol. 395 (10229), s. 1054. Elsevier.

Zhou, P., Yang, X.-L., Wang, X.-G., Hu, B., Zhang, L., Zhang, W., Si, H.-R., Zhu, Y., Li, B., Huang, C.-L., Chen, H.-D., Chen, J., Luo, Y., Guo, H., Jiang, R.-D., Liu, M.-Q., Chen, Y., Shen, X.-R., Wang, X., Zheng, X.-S., Zhao, K., Chen, Q.-J., Deng, F., Liu, L.-L., Yan, B., Zhan, F.-X., Wang, Y.-Y., Xiao, G.-F. & Shi, Z.-L. (2020b). A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature*, vol. 579 (7798), ss. 270–273

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Populärvetenskaplig sammanfattning

COVID-19 är en sjukdom som haft stor inverkan på världen under 2020. I mitten på januari 2021 hade 95 miljoner sjukdomsfall och över 2 miljoner dödsfall rapporterats. Det virus som orsakar COVID-19 är ett coronavirus, därav namnet som är en förkortning för "coronavirus disease 2019". Själva viruset heter SARS-CoV-2 som står för "severe acute respiratory syndrome- corona virus 2". Detta virus upptäcktes först i januari 2020, och har spårats tillbaka till en mat och djur-marknad i den kinesiska staden Wuhan. När generna i viruset analyserats har man sett att det har likheter med både SARS-CoV, som är ett annat coronavirus som gav ett utbrott hos människor år 2003, och fladdermus-coronavirus. Det troligaste är därför att viruset muterat via fladdermöss för att skapa en version av viruset som kan smitta människor och göra oss sjuka.

Coronavirus är en stor samling virus som finns hos flera olika djurslag, där det oftast inte smittar till andra arter. Mest troligen har SARS-CoV-2 smittat från ett djur till människor, och då väcks frågan huruvida andra även djurslag kan smittas och eventuellt överföra smitta. Man har hittat antikroppar hos flera arter, som katt, hund, mink, mm. Ett djur som lever nära människan och dessutom har vissa egenskaper som kan göra det mottagligt för viruset är katten. Studier har visat att den del (kallad ACE2) som viruset binder till på cellen för att ta sig in är väldigt likartad hos både människa och katt. I experiment har man också sett att katter kan smittas via luftsmitta, och att katter som infekterats har bildat antikroppar och även påvisat virustillväxt genom analys av svabbprover. I en studie där katter i Wuhan testades, hade 14,7 % (15/102) av katterna bildat antikroppar.

Denna studie har gjorts i form av ett examensarbete för att ge inledande data, som även kan öppna upp för framtida mer djupgående studier.

Målet med studien var att undersöka förekomsten av antikroppar (IgG) mot viruset som orsakar COVID-19 hos katter i fyra kommuner i Sverige, genom att använda en ny metod, suspension immunoassay (SIA), som tidigare använts för detta syfte hos människor. Därför behövde metoden anpassas till att fungera för djurslaget katt. Samtidigt anpassades den till att även kunna analysera antikroppar mot kattens coronavirus (FCoV) för att undersöka om det finns någon risk att ett positivt test orsakas av förekomst av FCoV-antikroppar istället för SARS-CoV-2-antikroppar.

Genom att kattägare fått fylla i en enkät undersöktes även om risken är större för en katt som bor med eller träffat COVID-sjuka människor att ha antikroppar, samt om graden av närhet till djurägarna kan ha en betydelse. Genom enkäten kunde även data om djuret, dess symptom sedan februari och levnadsförhållanden undersökas för att se om något av detta ger en ökad risk för att katten bildar antikroppar. Även djurägarnas oro gällande COVID-19 undersöktes. Parallellt med denna studie genomfördes en liknande studie på hundar och deras ägare.

För denna studie samlades 203 blodserumprover in från både friska och sjuka katter, från kommunerna Uppsala, Östhammar, Tierp och Halmstad. 56 av dessa hade en enkät med 15 frågor som fyllts i av djurägaren, och kallades grupp A. De 147 resterande proverna kallades grupp B och saknade ytterligare information förutom djurslag, och i vissa fall ålder, kön och ras. Dessa samlades in från Universitetsdjursjukhuset (UDS) i Uppsala, eller i syfte att användas till grupp A men där djurägaren ej fyllde i enkäten i tid.

Metoden som användes för att analysera blodproverna kallas Suspension Immuno-Assay (SIA) och går ut på att man fäster hela eller delar av viruset på magnetiska kulor, där antikroppar mot det viruset fastnar om de finns i blodserumet från en patient. I och med att man sätter signalmolekyler på dessa kan en maskin läsa av resultatet. En fördel är att man kan analysera för antikroppar mot flera virus på samma gång från samma serumprov.

Efter att ha analyserat alla prover samt 16 kontrollprover (både positiva och negativa) från SVA (Statens Veterinärmedicinska Anstalt) kunde resultaten beräknas. Av 203 prover blev sex positiva, två fick tveksamma resultat, och resterande 195 var negativa. Därför blev seroprevalensen, alltså andelen katter med antikroppar mot SARS-CoV-2, 3 % (se figur 3). Alla sex preliminärt positiva prover samlades in Uppsala inom grupp B, och de två tveksamma resultaten var båda från Östhammar inom grupp A (se tabell 1). Detta gör att det inte går att säga så mycket om den data som samlades in via enkäterna då inga av de positiva djuren hade någon enkät. Att samtliga positiva prover samlats in i Uppsala kan antyda att det finns ett högre smittryck där, men UDS är ett av få djursjukhus i området varför patienter kan ha färdats från andra områden för att få vård. Gällande de två tveksamma proverna så, hade bägge mycket nära kontakt med sina djurägare och den ena katten bor tillsammans med fyra andra katter i en familj där samtliga människor testats positiva för COVID-19. Detta kan tyda på att risken för smitta och infektion är relativt låg både från människa till katt och även mellan katter under normala levnadsförhållanden.

Dessvärre blev inte resultaten tillförlitliga för kattens coronavirus FCoV. Därför kunde inga slutsatser dras gällande korsreaktivitet, alltså huruvida en katt med

FCoV kan visa positivt på COVID-19-test. Tidigare studier har dock inte visat någon korsreaktivitet.

Gällande oron hos djurägarna var det tydligt att oron för sitt djur och sig själv var betydligt lägre än för samhället eller släkt och vänner (se figur 5). De djurägare som angav att de inte var oroliga alls för sitt djur gällande COVID-19, hade alla även sagt sig inte ha nära kontakt med katten. En tendens till att närmre närhet med katten gav något mer oro kunde ses.

Sammanfattningsvis kan katter bilda antikroppar mot SARS-CoV-2 och för studien provtagna katter hade en preliminär antikroppsförekomst på 3 %. COVID-19 SIA bör fortsätta optimeras ytterligare för att analysera antikroppar mot SARS-CoV-2 hos katter, och det finns flera förslag på förbättringar och vidare studier som kan göras med data från denna som grund. Ett större urval av katter vore bättre, med fördel från fler delar av Sverige, där samtliga katter har komplett data gällande egenskaper, levnadsförhållanden, symptom, närhet till människor och djur samt om de varit i kontakt med bekräftat COVID-19-positiva människor. Man skulle även kunna ta svabbprover för att se om de har levande virus i svalg, vilket skulle kunna ge misstanke om att katter eventuellt kan sprida smitta vidare. Vidare utredning om korsreaktivitet skulle kunna göras, men det är inte så troligt då SARS-CoV-2 och kattens coronavirus FCoV hör till två olika grupper av coronavirus som infekterar olika delar av kroppen.

Appendix 1, The survey

Kod:
Station/klinik:

Enkät och godkännande för provtagning och studiedeltagande (Questionnaire and approval of participation)

Godkänner du att ditt djur donerar en liten mängd blod till denna studie och att den information du delger i detta dokument används anonymt i studien? (Do you approve of your animal donating a small amount of blood and for the information in this document to be used anonymously in the study?)

Ja jag godkänner (Yes I approve) ☐

Vill du ha en länk efter avslutad studie där resultat publiceras? Fyll i så fall i din mailadress nedan. Genom att skriva din mailadress godkänner du att adressen sparas enbart för detta syfte och sedan raderas.
(Would you like to receive a link with the results after the study is finished and published? If so, write your email address below. By writing your email address you approve your address is saved and used only for this purpose, and is then deleted)

E-mail: _____

Information om ditt djur (Information about your animal):

1. Djurslag (Type of animal): Katt (Cat) ☐ Hund (Dog) ☐
2. Ras (Breed):
3. Kön (Sex):
4. Ålder (Age):
5. Kommun (Municipality):
6. Tror du att ditt djur har haft COVID-19? Och i så fall varför? (Do you think that your animal has had COVID-19? If so why?)

   _____

   _____

7. Har ditt djur haft några av följande symptom utan diagnos på annan sjukdom sedan mars 2020? (Has your animal had any of the following symptoms without confirmed cause since mars 2020?)

Symptom	Nej (No)	Mild	Medium	Allvarligt (Severe)
Feber (Fever)				
Hosta (Cough)				
Nedsatt allmäntillstånd (Feeling low)				
Symptom på förkylning (Cold symptoms)				
Svårt att andas (Difficulty breathing)				
Diarré (Diarrhea)				
Aptitlöshet (No appetite)				

8. Har ditt djur varit i kontakt med någon som har COVID-19 (Have your animal been in contact with anyone that had COVID-19)?

Ja, bekräftat med prov Yes, laboratory confirmed	Ja, någon jag misstänker har haft det Yes, someone I suspect had it	Nej, jag tror inte det No, I don't think so

9. Träffar ditt djur många andra djur (does your animal meet many other animals)?

_____

10. Träffar ditt djur många andra människor (does your animal meet many other people)?

_____

11. Har ditt djur några sjukdomar (does your animal have any health problems/sicknesses)?

_____

12. Har du nära kontakt med ditt djur? (Do you have close contact with your animal)

Ja mycket nära ex sover i sängen, slickar/gnider sig mot mitt ansikte (yes very close, eg sleeps in the bed, licks or rubs my face)	Ja nära Ex vistas i möbler i huset, vidrörs dagligen (yes close, eg uses furniture or is touched daily)	Ja ganska nära Ex bor i huset men bara i vissa rum/på golvet (yes, quite close, eg lives in the house but only on the floor or certain rooms)	Inte så nära Ex är mycket utomhus, hanteras ibland av människor (Not so close, eg stays outside much of the time or is handled only sometimes by people)	Inte nära Ex bor utomhus och är i stort sett aldrig i huset eller nära människor (Not close, eg is seldom in the house or close to people)

Information om djurägare:

1. Tror du att du har haft COVID-19 (have you had COVID-19)?

Ja, bekräftat med prov (Yes, laboratory confirmed)	Ja, jag tror det men inte bekräftat (I think so but not confirmed)	Nej, jag tror inte det (No, I don't think I have had it)

2. Har du fler djur hemma, om ja hur många och vilka djurslag (Do you have other animals, if yes how many and of what kind)?

   _____

3. Hur orolig är du för COVID-19? (How worried are you about COVID-19)

	Inte orolig (not worried at all)	Lite orolig (a bit worried)	Rätt så mycket (quite worried)	Mycket orolig (Very worried)
För mig själv (For me)				
För släkt och vänner (for friends and family)				
För samhället (for the society)				
För mitt djur (for my animal)				

Datum:

Jag ger härmed mitt godkännande för blodprovstagning, användande av blodet till studien och användning av information som givits i denna enkät till samma studie:

Signatur

_____

Namnförtydligande

_____

Appendix 2, Protocols for laboratory work

Coupling of antigens using MagPlex-C microspheres:

1. Gently invert **bead stocks** for 1-2 min, then immediately transfer 200 µl (2.5 x 10^6 beads) of the stock microspheres (containing 1.25 x 10^7 beads per ml) to a **2-ml Micro tube with cap (Sarstedt; 72.694.007)**.
2. Wash beads once with 200 µl of **100 mM monobasic sodium phosphate (MSP) (Sigma; S3139), pH 6.2**, using a **magnetic tube separator**.
3. Resuspend the bead pellet in 80 µl MSP and then add 10 µl of freshly made **sulfo-N-hydroxy-succinimide (Sulfo-NHS) (ThermoFisher Scientific; 24510) (50 mg/ml H_20)** and 10 µl of **1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC) (Sigma; 03449-1G) (50 mg/ml H_20)**.
4. Incubate the suspension on a **rocking mixer** for 20 min at room temperature in the dark.
5. Wash beads with 250 µl of **50 mM 2-(N-morpholino)-ethanesulfonic acid (MES) sodium salt (Sigma; 71119-23-8), pH 5**, using the magnetic tube separator.
6. Resuspend the beads in 100 µl MES.
7. Add 10 µg of **antigen (S1 containing RBD of SARS-CoV-2 or Feline Infectious Peritonitis virus antigen, strain WSU 79-1146)**, and then more MES up to 500 µl.
8. Mix gently and incubate on a rocking mixer for 2 h at room temperature in the dark.
9. After the coupling procedure, the beads are washed in 0.5 ml of **PBS, pH 7.4, containing 0.5 ml/l Tween 20 and 50 mM Tris (PBSTT)** to block unreacted carboxyl groups with primary amines.
10. The beads are then washed with 0.5 ml **StabilGuard (SurModics; SG01-1000)**.
11. The final pellet is resuspended in 400 µl of StabilGuard. This creates a bead mixture consisting of 6250 beads/µl. The coupled beads are stored at 4ºC in the dark.

Serology using MagPlex-C microspheres:

1. The **S**uspension **M**ultiplex **I**mmuno**A**ssay (SMIA) is carried out in a **round bottom 96-well microtiter plate (Greiner bio-one; 650101)**.
2. Add **PBST** to the appropriate wells, according to protocol.
3. Add **sample** and **control** to the appropriate wells.
4. Resuspend **the working microsphere mixture** (for **96 wells** use a total volume of **6 ml** for easy pipetting → **48 µl of each set** x 6250 beads/µl = 300000 beads → 300000 beads/6000 µl **PBST** = **50 beads of each set/**µl PBST) by vortex and sonication for approximately 20 seconds.
5. Add 50 µl of the working microsphere mixture to each well.
6. Cover the plate and incubate (1^{st}) for 60 minutes at room temperature on a **plate shaker** at 400 rpm.
7. During this incubation period, dilute **biotinylated protein G (Pierce/ThermoFisher Scientific; 29988) (0.5 mg/ml)** (for human IgG analysis) or **biotinylated feline anti-IgG (0,5mg/ml)** to a final concentration of 2 µg/ml PBST.
8. After 60 min of incubation, wash beads once in 100 µl **PBS** using a **magnetic plate separator (Invitrogen/ThermoFisher Scientific; A14179)**.
9. Add 100 µl of the diluted biotinylated reagents to the appropriate wells.
10. Incubate (2^{nd}) in the dark for 30 minutes at room temperature on a plate shaker at 400 rpm.
11. During this incubation period, start **the Luminex analyzer** and dilute **the SA-PE conjugate (Invitrogen/ThermoFisher Scientific; SA10044)** to 2 µg/ml in PBST (6 µl 4 mg/ml SA-PE + 12 ml PBST → ≈ 12 ml 2 µg/ml).
12. After 30 min of incubation, wash beads once in 100 µl PBS using the magnetic plate separator.
13. Add 100 µl of the diluted SA-PE to each well.
14. Cover the plate and incubate (3^{rd} and final) for 15 minutes at room temperature on a plate shaker at 400 rpm.
15. After 15 minutes of incubation, wash wells once in 100 µl PBS using the magnetic plate separator.
16. Bring final volume of each reaction to 100 µl with PBS.
17. Mix the reactions briefly on a plate shaker at 400 rpm.
18. Analyze 75 µl on the Luminex analyzer according to the system manual

Appendix 3, First test of controls

SARS-CoV-2-SIA/SMIA Veterinary project, test run with controls FIP (cat) IgG 201105

Protokoll; MagPix, SARS-CoV-2 Vetproj cat dog 201105 LK

	1	2	3	4	5	6	7	8	9	10	11	12
A	PC *KMB9, predil*	PC/NC *SVA 1*	PC/NC *SVA 9*	PC/NC *SVA 1*	PC/NC *SVA 9*	PC/NC *SVA 1*	PC/NC *SVA 9*	PC/NC *SVA 1*	PC/NC *SVA 9*			
B	NC BD 4	PC/NC *SVA 2*	PC/NC *SVA 10*	PC/NC *SVA 2*	PC/NC *SVA 10*	PC/NC *SVA 2*	PC/NC *SVA 10*	PC/NC *SVA 2*	PC/NC *SVA 10*			
C	PC *KMB9, predil*	PC/NC *SVA 3*	PC/NC *SVA 11*	PC/NC *SVA 3*	PC/NC *SVA 11*	PC/NC *SVA 3*	PC/NC *SVA 11*	PC/NC *SVA 3*	PC/NC *SVA 11*			
D	NC BD 4	PC/NC *SVA 4*	PC/NC *SVA 12*	PC/NC *SVA 4*	PC/NC *SVA 12*	PC/NC *SVA 4*	PC/NC *SVA 12*	PC/NC *SVA 4*	PC/NC *SVA 12*			
E	PBS	PC/NC *SVA 5*	PC/NC *SVA 13*	PC/NC *SVA 5*	PC/NC *SVA 13*	PC/NC *SVA 5*	PC/NC *SVA 13*	PC/NC *SVA 5*	PC/NC *SVA 13*			
F	PBS	PC/NC *SVA 6*	PC/NC *SVA 14*	PC/NC *SVA 6*	PC/NC *SVA 14*	PC/NC *SVA 6*	PC/NC *SVA 14*	PC/NC *SVA 6*	PC/NC *SVA 14*			
G	PBS	PC/NC *SVA 7*	PC/NC *SVA 15*	PC/NC *SVA 7*	PC/NC *SVA 15*	PC/NC *SVA 7*	PC/NC *SVA 15*	PC/NC *SVA 7*	PC/NC *SVA 15*			
H	PBS	PC/NC *SVA 8*	PC/NC *SVA 16*	PC/NC *SVA 8*	PC/NC *SVA 16*	PC/NC *SVA 8*	PC/NC *SVA 16*	PC/NC *SVA 8*	PC/NC *SVA 16*			

IgG

Samples:

Green; Positive control (PC);

SARS-CoV-2-positive serum KMB9 (predil 1/100(from 24/9/20) (BoA) (human)

Orange; Negative control (NC);

Blood donor serum 4 from Uppsala 2018 (BoA) (human)

Green; Positive control (PC)/ Orange; Negative control (NC);

FIP+/-, 1-15 (unknown number of samples spiked with SARS CoV-2 Abs) (cat)

Beads

Light green (single plex); #28 #66(SARS-CoV-2 S1 Sino Biological 40591-V08H 2020-10-30 LK. *Note*; ca 11 ug antigen) human (biot protein G)

Dark green (multiplex); #28 #66 #42 (biot protein G)

Light blue (single plex); #28 #42(FIPV, cat, 2020-10-30. *Note*; 10 ug antigen) cat (biot anti cat)

Dark blue (multiplex); #28 #66 #42 (biot anti cat)

Purple (single plex); #28 #66 (biot protein G)

Turquise (multiplex); #28 #66 (biot anti cat)

Serum dilution 1/25 (final dilution 1/50) for NC **(human, cat):**

- IgG: 2 µl serum + 48 µl PBSTT

Serum dilution 1/500 (final dilution 1/1000) for PC **(human)** predil 1/100**:**

- IgG: 10 µl predil serum + 40 µl PBSTT

Serum dilution 1/25 (final dilution 1/50) for PC **(cat):**

- IgG: 2 µl serum + 48 µl PBSTT

Bead mixture (50 µl of the working microsphere mixture to each well)**:**

12 µl of each bead set into 3 ml PBSTT → 25 beads beads of each set/µl
(Mix or use already mixed ones for these beads)

- Light green, Purple, Turquoise (single plex);
  #28(blank 2020-10-30 LK)
  #66 (SARS-CoV-2 S1 Sino Biological 40591-V08H 2020-10-30 LK. *Note*; ca 11 ug antigen)

4 µl of each bead set into 1 ml PBSTT → 25 beads of each set/µl

- Light blue (single plex);
  #28
  #42 (FIPV, cat, 2020-10-30. *Note*; 10 ug antigen)

4 µl of each bead set into 1 ml PBSTT → 25 beads of each set/µl

- Dark green, Dark blue (multiplex);
  #28
  #66
  #42

Biotinylated detection reagent (100 µl of the diluted biotinylated reagent to each well)**:**

Light green, Dark green- human, Turquoise

- Biotin Protein G (SARS-CoV-2): 12 µl of 0.5 mg/ml into 3 ml PBSTT → ≈ 3 ml 2 µg/ml

Light blue, Dark blue- cat, Purple-cat

- Biotin anti-cat IgG: 24 µl of 0.5 mg/ml into 6 ml PBSTT → ≈ 6 ml 2 µg/ml

SA-PE conjugate (100 µl of the diluted SA-PE conjugate to each well)
6 µl of 4 mg/ml into 12 ml PBSTT → ≈ 12 ml final concentration 2 µg/ml

Deviations from protocol during laboratory work: When adding the bead mix in the dark green wells, the mix with #28 + #66 (50µl) was added instead of #28 + #66 + #42. To compensate, 50 µl of the mix with #28 and #42 was added.

Appendix 4, Analysis of samples

SARS-CoV-2-SIA Veterinary project, 1:50, (cat), K, UK, HK, XK1-XK32 IgG 201111

Protokoll; MagPix, SARS-CoV-2 Vetproj cat dog 201105 LK

	1	2	3	4	5	6	7	8	9	10	11	12
A	SVA16 (1 µl)	K6	K14	K22	K30	K38	K46	UK5	HK5	XK5	XK13	XK21
B	SVA16 (2 µl)	K7	K15	K23	K31	K39	TK1	UK6	HK6	XK6	XK14	XK22
C	SVA 4	K8	K16	K24	K32	K40	TK2	UK7	HK7	XK7	XK15	XK23
D	K1	K9	XK29	K25	K33	K41	TK3	UK8	HK8	XK8	XK16	XK24
E	K2	K10	XK30	K26	K34	K42	UK1	HK1	XK1	XK9	XK17	XK25
F	K3	K11	XK31	K27	K35	K43	UK2	HK2	XK2	XK10	XK18	XK26
G	K4	K12	XK32	K28	K36	K44	UK3	HK3	XK3	XK11	XK19	XK27
H	K5	K13	K21	K29	K37	K45	UK4	HK4	XK4	XK12	XK20	XK28

IgG

Samples:

Green; Positive control (PC)

SVA16 (spiked with SARS CoV-2 Abs) (cat) 1 µl

SVA16 (spiked with SARS CoV-2 Abs) (cat) 2 µl

Orange; Negative control (NC)

SVA 4 SARS –

Black; Samples for analysis

K1-K16, K21-K46, TK1-TK3, UK1-UK8, HK1-HK8, XK1-XK32

Serum dilution 1/25 (final dilution 1/50) for PC SVA16 (1ul):

- IgG: 1 µl serum + 49 µl PBSTT

Serum dilution 1/25 (final dilution 1/50) for B1-12H:

- IgG: 2 µl serum + 48 µl PBSTT

Bead mixture (50 µl of the working microsphere mixture to each well)**:**

24 µl of each bead set into 6 ml PBSTT → 25 beads of each set/µl

#28(blank 2020-10-30 LK)

#66 (SARS-CoV-2 S1 Sino Biological 40591-V08H 2020-10-30 LK. *Note*; ca 11 ug antigen)

Biotinylated detection reagent (100 µl of the diluted biotinylated reagent to each well)**:**

- Biotin anti-cat IgG: 24 µl of 0.5 mg/ml into 12 ml PBSTT → ≈ 12 ml 2 µg/ml

SA-PE conjugate (100 µl of the diluted SA-PE conjugate to each well)

6 µl of 4 mg/ml into 12 ml PBSTT → ≈ 12 ml final concentration 2 µg/ml

Deviations from the protocol during laboratory work: When serum was added to well B2, the amount of sample added was less than it should have been, but at least 1 µl.

Also, when the last wash with PBS was conducted in the last step before adding the final amount of PBS, column nr 8 was washed with the double amount of PBS.