Covid-19 and dogs

– Seroprevalence in dogs in Sweden, concerns among their owners and development of the method COVID-19 SIA

Covid-19 och hundar - förekomst av antikroppar hos hund i Sverige, oro hos deras ägare, samt utveckling av analysmetoden COVID-19 SIA

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Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the third coronavirus to cause an epidemic or pandemic in the 21st century. In the beginning of January 2021 almost 88 million cases of covid-19 have been reported to World Health Organization (WHO) and more than 1.8 million people have died. The world is currently waiting for a vaccine and in the meantime, scientists worldwide continue to investigate the features of the virus. According to current literature it seems improbable that dogs would serve as reservoirs, but cats and other felines might be possible intermediate hosts, as well as minks.

In this project a serological method called COVID-19 Suspension Immunoassay (SIA), earlier used for both humans and animals, was further developed to study the prevalence of SARS-CoV-2 antibodies in dogs. In total, 443 dogs from five municipalities participated in the study and donated blood. Eighty-three (18.7%) of them lived with owners who also participated in a survey study. The dogs were divided into two groups, group A for dogs with completed owners' questionnaires and group B for anonymous blood donors. Since the method had never been used for this purpose before, the limit for positive results was not determined when the project started. Depending on where the cut-off was set, results differed from 12 to 16 antibody positive samples. A preliminary cut-off point of 300 median fluorescence intensity (MFI) was determined, resulting in a study prevalence of 2.7%. The purpose of the survey performed in group A was to investigate whether there might be a higher probability for dogs to have SARS-CoV-2 antibodies if they lived with owners who were seropositive and how closely the dogs lived with their owners. The questionnaires also addressed concerns among the dog owners, if they worried about covid-19 regarding themselves, their friends and families, the society, and their pets. Results showed that there was an indication but not a statistically significant higher probability for the dogs to develop antibodies if they lived with owners who had been confirmed with covid-19. All seropositive dogs lived close or very close with their owners. The results indicated that owners in general worried more about their friends, family, and the society than for themselves. Concerns about their pets getting sick from covid-19 were very small.

Further studies with a greater quantity of data would give more reliable results for the cut-off point and consequently also for the seroprevalence and the probability of dogs developing antibodies if their owners have been confirmed with covid-19.

Keywords: covid-19, SARS-CoV-2, coronavirus, dogs, zoonosis, SMIA

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Abbreviations

ACE2	Angiotensin-converting-enzyme 2
APN	Aminopeptidase N protein (also called CD13)
CIRD	Canine infectious respiratory disease
CoV	Coronavirus
CCoV	Canine coronavirus
COVID-19	Coronavirus disease 2019
CRCoV	Canine respiratory coronavirus
FECV	Feline enteric coronavirus
FIP	Feline infectious peritonitis
FIPV	Feline infectious peritonitis virus
HCoV	Human coronavirus
HR1	Heptad repeats 1
HR2	Heptad repeats 2
MERS	Middle Eastern respiratory syndrome
MERS-CoV	Middle Eastern respiratory syndrome coronavirus
MFI	Median fluorescence intensity
SARS	Severe acute respiratory syndrome
SARS-CoV	Severe acute respiratory syndrome coronavirus
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SIRS	Systemic inflammatory response syndrome
SMIA	Suspension multiplex immunoassay

1. Introduction

In March 11th 2020, covid-19 was declared a pandemic by the World Health Organization, WHO (WHO, 2020a). The virus causing the disease was named SARS-CoV-2 and have changed the lives of millions of people ever since. The virus origin is not yet known but is suspected to have been transmitted to humans from bats through an intermediate host at a food market in China (Leitner & Kumar 2020). Which species constituted the intermediate host is not completely clear yet but pangolins have been suggested (Leitner & Kumar 2020).

Coronaviruses are known to cause mainly respiratory and enteric symptoms in both humans and animals, some being more severe than others (Rota *et al*. 2003). Clarifying the zoonotic aspects of covid-19 is of eminent relevance since many coronaviruses have been shown to spread between species. Twice before, coronaviruses have caused outbreaks that resulted in thousands of deaths, severe acute respiratory syndrome (SARS) 2002 and Middle Eastern respiratory syndrome (MERS) 2012 (Chen *et al*. 2020)

No studies so far have indicated that common pets as cats and dogs spread SARS-CoV-2 but recent studies have showed that SARS-CoV-2 can replicate in and spread from minks, posing them as a potential threat to human health but also resulting in the culling of millions of animals (WHO, 2020c). Misconceptions about the risk of whether pets are contagious could lead to panic and euthanizing of pets, therefore studies generating and spreading accurate information about the virus in pets is highly important.

This study was performed in parallel with another study which investigated the same question as in this one, but in cats.

1.1. Aims of this study

Aims of this study were to:

- Conduct a literature review of the importance of pets in the epidemiology of covid-19
- Develop the method of Suspension Immunoassay (SIA) in animal samples for covid-19

- Study prevalence of antibodies to SARS-CoV-2 in dogs in Sweden and whether positive dogs have had any symptoms
- Examine if the prevalence of animals with antibodies are higher among dogs that live closely with their owners, meet a lot of other people and dogs or have had contact with people confirmed with covid-19
- Investigate the concerns dog owners have regarding covid-19 for themselves and their animals

2. Literature review

2.1. Coronavirus

2.1.1. Covid-19

Covid-19 sometimes causes no signs of sickness at all, but infected humans can also suffer from various symptoms. Some only experience mild symptoms like coughing, anosmia, sore throat, and fever, while others undergo longer periods of fever, shortness of breath, muscle pain and even death (Esakandari *et al*. 2020).

On December 31st 2019, WHO was alerted about cases of pneumonia, caused by an unknown virus, in Wuhan, People's Republic of China. (WHO, 2020a). On January 11th China reported its first case of death caused by the virus, according to WHO, and on January 13th 2021 there have been 90,335,008 reported cases of covid-19 whereof 1,954,336 deaths according to WHO (WHO, 2020b). Europe had its first culmen of cases in March and April and reported cases then decreased over the summer, whereas American and Asian epidemiological curves remained constant. During fall 2020 a second wave struck the world and the number of cases are still increasing though the European Centre for Disease Prevention and Control (ECDC) posted a report in the last week of 2020 that many countries in Europe had started to observe a flattening of the epidemiological curve or even decreases of cases (ECDC, 2020).

In January 31st 2020 Folkhälsomyndigheten (The Public Health Agency of Sweden, FoHM) in Sweden announced that the country had confirmed its first case of covid-19. A young woman who had just arrived home from a visit in Wuhan was experiencing cough and tested positive for covid-19 (Folkhälsomyndigheten, 2020). At the day of writing, January 7th, 2021, there have been 506,866 confirmed cases of covid-19 with 9,667 deaths in Sweden. An epidemic curve of reported cases in Sweden can be seen in figure 1. The neighbouring countries have experienced less cases according to the reports from WHO; Finland 39,011 cases and 602 deaths, Denmark 183,801 cases and 1,623 deaths and Norway 56,614 cases and 509 deaths (WHO, 2020b).

Two times during the year FoHM has studied the prevalence of antibodies against SARS-CoV-2 in volunteering blood donors. In the end of April 2020, the prevalence was 0,5% and in the middle of June 2020 it had increased to 7.1%. Further studies on prevalence of antibodies continued during fall 2020 but the results have yet not been published (*Folkhälsomyndigheten, 2020*).

Figure 1: Epidemic curve over reported cases of covid-19 in Sweden from March 2020 to December 2020. The data for the last bar may be incomplete. The figure is downloaded from World Health Organization (WHO coronavirus disease (COVID-19) dashboard. Geneva: World Health Organization, 2020. Available online: https://covid19.who.int/ [2020-01-15])

2.1.2. Coronaviruses in general

SARS-CoV-2 is a coronavirus, order *Nidovirales*, family *Coronaviridae* and subfamily *Coronavirinae*. Coronaviruses infect both animals and humans and cause mainly enteric or respiratory diseases, depending on which virus it is. They are enveloped, positive-stranded RNA viruses with about 30,000 nucleotides, which makes them the largest RNA viruses that has yet been found (Rota *et al*. 2003). *Coronavirinae* is divided into four genera: alpha-, beta-, gamma- and deltacorona-virus. The first two only infect mammals whilst the latter two mainly infect birds, but some gamma- and deltacoronaviruses can infect mammals too. All known coronaviruses that infects humans originate from animals (Cui *et al.* 2019). The novel coronavirus SARS-CoV-2 belongs to the genera of betacoronaviruses and is suspected to be a spill-over from bats, just as the former known related SARS-CoV (Tan *et al*. 2020).

Coronavirus got its name from the crown-like shape seen in electron microscope (corona = crown in Latin). The structures forming the spikes of the crown are one of two major glycoproteins of the virus envelope; the spike (S) glycoproteins that is important for the attachment of the virus to the cell. The S protein consists of two subunits, S1 and S2. S1 attaches to a receptor at the host cell's surface and S2 integrate with host cell's membrane, making it merge with the virus membrane. The other important glycoprotein at the surface is membrane (M) protein which together with the envelope (E) protein have important roles in mediating virus entry into host cells by endocytosis (Li 2016).

SARS-CoV uses the receptor angiotensin-converting-enzyme 2 (ACE2) to bind to host cells. Recent studies have shown this to be the case for SARS-CoV-2 as

well, but also that its binding efficiency to ACE2 is 10 to 20 times higher than SARS-CoV. Subunit S1 attaches to ACE2 at the surface of cells in the respiratory tract of the host, using a sort of key called receptor binding domain (RBD). Virus fusion with the cell is then mediated by subunit S2, which uses heptad repeats 1 and 2 (HR1, HR2) and is allowed entry. The virus then utilizes the mechanisms of the host cells to replicate and new viruses are released through the host cell's membrane by budding (Guo *et al*. 2020b).

2.1.3. Coronaviruses in humans

In 2018 there were six known human coronaviruses (HCoVs). Two alpha, HCoV-NL63 and HCoV-229E, and four beta, HCoV-OC43, HCoV-HKU1, SARS-CoV and MERS-CoV. MERS-CoV and SARS-CoV were found to be more fatal than the rest of the HCoVs, but all viruses might cause respiratory and gastrointestinal symptoms. The origins of HCoVs are still not entirely mapped but can all be traced back to animals. Bats are suspected to be reservoirs of most alpha- and beta-CoVs, with palm civets being the intermediary hosts for SARS-CoV and dromedary camels for Middle Eastern respiratory syndrome (MERS)-CoV (Yin *et al.* 2018). In 2019 the new coronavirus, SARS-CoV-2, was detected which is the third coronavirus responsible for causing an epidemic or pandemic. The two other are SARS-CoV that caused the outbreak of SARS and MERS-CoV, causing the outbreak of MERS (Gorbalenya *et al*. 2020). The outbreak of SARS 2002-2003 spread to more than 25 countries, confirmed cases were around 8,000 and almost 800 of them died (CDC, 2017). The first cases of MERS were reported in September 2012 and since then through 31st of May 2019 just over 2,400 were infected and about 800 of them had died (Donnelly *et al*. 2019). SARS-CoV-2 therefore seems to be more contagious but has a lower mortality rate, 4.2%, compared to SARS-CoV 11% and MERS 34% (Hu *et al*. 2020). SARS-CoV-2 resembles SARS-CoV in several ways. They are both betacoronaviruses lineage B and use ACE2 at the host cell as receptor to access entry to the cell whereas MERS is a betacoronavirus C and uses dipeptidyl peptidase 4 (DPP4, also known as CD26) (Yin *et al.* 2018).

2.1.4. Coronaviruses in various animals

Coronaviruses circulate throughout the animal population and may cause mild to severe symptoms. Alpha- and betacoronaviruses infect only mammals whereas delta- and gammacoronaviruses mostly infects birds, but a few have been found in mammals. In general, coronaviruses in animals tend to cause gastrointestinal problems more than respiratory symptoms (Cui *et al.* 2019).

Livestock can get infected with several different coronaviruses, for example porcine transmissible gastroenteritis virus (TGEV), porcine enteric diarrhoea virus

(PEDV) and the swine acute diarrhoea syndrome coronavirus (SADS- CoV) in pigs (Cui *et al.* 2019). In cattle, younger animals can suffer from diarrhoea whilst it's more common for older animals to get respiratory symptoms from bovine corona virus (BCoV) (Saif 2010). Canine coronavirus (CCoV) and feline coronavirus (FCoV) are all alphacoronaviruses, however the canine respiratory coronavirus (CRCoV) is a betacoronavirus (Le Poder 2011). FCoV have two serotypes, type I and type II and both can have two clinical forms or biotypes: feline enteric coronavirus (FECV) and feline infectious peritonitis virus (FIPV). FECV causes an often-harmless infection, located in the enteric tract. FECV can however mutate, change its virulence, and turn into FIPV that in almost every case leads to death. FIPV itself is thought to not be able to transmit between animals but FECV can, suggesting the outbreaks that have occurred with FIP (feline infectious peritonitis) is due to repeated infections with FECV in different cycles that have pushed the virus to mutate into FIPV. FCoV type II utilizes the cellular receptor feline aminopeptidase N (fAPN) to gain access into the host cell. What receptor FCoV type I uses is still unclear (Jaimes & Whittaker 2018). Subclinical infection with FCoV is common in Sweden, according to Holst *et al.* (2006) the prevalence of antibodies in Swedish cats was 31%. No studies in the subject have been done after 2006.

2.1.5. Coronavirus in dogs

There are three known CCoVs, two of them are classified as alphacoronaviruses (CCoV type I and II) and one to betacoronaviruses (CRCoV). Sequencing of CCoV and FCoV have shown that FCoV type I and both CCoV originate from the same virus, again pointing at the high tendency for mutation of coronaviruses. Dogs infected with CCoV may have both virus types and CCoV II is more common than type I, 44% respectively 19%. Both types can cause an enteric form of infection, or a pantropic form that affects various tissue. Aminopeptidase N (APN) is the cellular receptor for CCoV type II but the receptor for CCoV type I has not yet been found. Due to similarities in genome it is suspected that CCoV I uses the same receptor as FCoV I, though not yet proven (Le Poder 2011).

Alpha-CCoVs typically cause an infection with clinical symptoms in the enteric system with diarrhoea and vomiting but there have been cases with a certain strain of CCoV type II, CB/05, that have caused systemic disease with fatal outcome in younger dogs. A study performed in 2010 stated that dogs that have undergone an infection with the enteric form of the virus did not have immunity to experimental infection with CB/05 (Decaro *et al*. 2010).

CRCoV is part of the disease complex CIRD, which stands for canine infectious respiratory disease, commonly known as kennel cough. CIRD often causes mild respiratory syndromes but bronchopneumonia may be developed. CRCoV has a

higher genetical similarity to BCoV and HCoV than the other CCoVs (97.3% respectively 96.9%). Both CRCoV and BCoV use the same receptors for host cellular entry, human leukocyte antigen class I (HLA-1) and sialic acids as attachment molecules (Szczepanski *et al*. 2019). According to a publication in 2020, 14.7% of the dogs in Sweden diagnosed with CIRD were positive for CRCoV (Wille *et al*. 2020a).

2.1.6. Zoonotic aspects

Coronaviruses are, similar to most RNA-viruses, highly susceptible to mutations, and spillover events from animals have caused three epidemics in two decades. Therefore it is interesting to monitor outbreaks of coronaviruses in populations of both human and animals, to find out what species might be affected and track possible zoonoses (Hartenian *et al*. 2020).

SARS-CoV-2 is spread mainly through droplets and virus have been shown to survive for up to 72 hours at some surfaces. Theoretically these droplets could land at a pet and be transmitted to another person's mucosa if that person pets the animal and then touches for example their face. However, this applies to any surface and the animals' fur is not more contagious than any other surface (van Doremalen *et al*. 2020).

Diseases caused by coronaviruses are not only important in a perspective of zoonoses that infect and make humans sick, but also for the animals themselves and economical aspects. For example, a big outbreak of acute diarrhoea in pigs in China 2016 ended in euthanizing over 24,000 pigs in four farms within a few weeks. The isolated virus was suspected to have originated from the same bat-related virus as a human coronavirus, possible transmitted to the pigs from animal caretakers (Zhou *et al*. 2018). Recent studies have showed that SARS-CoV-2 can transmit from humans to mustelids, including ferrets and minks, and cause severe cases of pneumonia and systemic disease leading to death of thousands of animals (Molenaar *et al.* 2020; Oreshkova *et al.* 2020). The American newspaper *The Guardian* reported a press release from The United States Department of Agriculture's (USDA) about the death caused by covid-19 in almost 10,000 minks in mink farms in Utah, either as a direct cause of the virus or euthanizing due to disease control (The Guardian 2020).

Concern about whether SARS-CoV-2 can transmit from humans to companion animals and then further to other people has been of big interest since the pandemic started. It should be considered when searching for information about covid-19 that the disease is new, caused by a novel virus and therefore many of the current studies have been performed during a short period of time and with few objects examined. Media frequently reports about animals suspected to have been infected with SARS-CoV-2. For example Agriculture, Fisheries and Conservation Department

(AFCD) in Hong Kong made several press releases in Mars 2020 that dogs had tested positive by both polymerase chain reaction (PCR) and serology under a short period of time. These dogs were owned by people confirmed with covid-19 but the dogs did not have any symptoms (AFCF, 2020). AFDC however stressed the fact that there was no evidence that the dogs were sick from covid-19 or that they would be contagious to other pets or humans. In April 2020, USDA reported that National Veterinary Services Laboratories had tested lions and tigers with respiratory symptoms in a zoo in New York for SARS-CoV-2. The laboratory confirmed that one tiger was infected with SARS-CoV-2, probably transmitted from an employee. USDA too underlined that the evidence for zoonotic risks were lacking (USDA, 2020). Suspicions about pets and other animals being possible sources for infection might lead to decrease in animal welfare. Reports about abandoned or even killed pets have been released, without any evidence of them being contagious (Parry 2020).

A recent study from India performed by Dutta *et al.* (2020) showed through relative synonymous codon usage (RSCU) that it is most unlikely that SARS-CoV-2 could survive and replicate in dogs. Until the day of writing, December 2020, there are no evidence of dogs being susceptible to SARS-CoV-2 infections or transmission (Almendros & Gascoigne 2020; Shi *et al.* 2020). Cats, however, have been found to be both susceptible to infection with SARS-CoV-2 and able to transmit the virus to other cats. In a study from China, cats were inoculated with the virus and viral RNA was detected both in faeces from living cats and the upper and lower respiratory tract from cats that had died or been euthanized. Some young cats that had been inoculated with the virus, or infected from an airborne transmission from other cats, showed severe lesions in both nasal and tracheal mucosa epithelium and lungs. The same study investigated ferrets which also showed a high susceptibility for infection and transmission to animals of the same species but not the same tendency of getting sick as the cats did. Antibodies against SARS-CoV-2 were detected in both cats and ferrets (Shi *et al*. 2020). In a case report from Spain where a cat was euthanized due to severe respiratory symptoms, both viral RNA from SARS-CoV-2 and antibodies against the virus were found post mortem. The cat was not considered to have died from covid-19 though, but from cardiorespiratory failure developed from hypertrophic cardiomyopathy and secondary thromboembolism. This cat together with the household's second cat had been in close contact with people with confirmed covid-19 and people who were suspected to be infected. The other cat also had SARS-CoV-2 RNA and antibodies but did not develop respiratory symptoms (Segalés *et al*. 2020). Antibodies against SARS-CoV-2 have been found in several cats that have been in close contact with people confirmed with covid-19, although, there is at present no evidence of cats transmitting SARS-CoV-2 to humans (Zhang *et al*. 2020).

2.2. Responses of the immune system to coronavirus infections

SARS-CoV-2 infects primarily pneumocytes type II in the lungs, causing the immune system to start an inflammatory response. Except the direct damage the virus has on the lungs with oedema and tissue lesions, it also activates the release of inflammatory mediators to the rest of the body, among others interleukin-6 (IL-6). This is the cytokine that in particular has been seen in increased levels in covid-19 patients who have died from the disease. Instead of engaging an immune system response that only neutralizes the virus, a so-called cytokine storm is induced causing systemic inflammatory response syndrome (SIRS) with acute respiratory distress syndrome (ARDS) and blood clots in multiple organs as a result (Hojyo *et al.* 2020; Hanidziar & Bittner 2020). Excessive immune responses are seen in infections with other coronavirus as well, for example the wet form of FIP where B cells are overactivated and produce more antibodies than necessary (Mustaffa-Kamal *et al*. 2019).

2.2.1. Antibodies against SARS-CoV-2

A normal immune response against SARS-CoV-2 would start with reacting to the released damage-associated molecular patterns (DAMP) which are released when an infected cell undergoes pyroptosis due to infection of the virus. These proteins signal infection of a foreign intruder to nearby cells and they in their turn further activate the inflammatory system by pro-inflammatory cytokines and chemokines. Monocytes, macrophages, and T-cells are recruited to eliminate the virus and B-cells are presented for the antigen and start to produce antibodies. The antibodies' primary site for neutralization is in SARS-CoV by blocking the binding of RBD to ACE2, assumingly the same for SARS-CoV-2 (Tay *et al*. 2020). SARS-CoV-2 enters the body via mucosal tissue in the eyes, mouth and respiratory and therefore the mucosa-associated lymphoid tissue (MALT) encounters the virus first, responding with immunoglobulin (Ig) A defence (Paces *et al.* 2020; Yu *et al.* 2020). IgM and IgG rise in a couple of days (4-12 in different literature) (Zhao *et al.* 2020; Guo *et al.* 2020a). Zost *et al.* (2020) found two particularly interesting antibodies in their study about potently neutralizing and protective human antibodies, COV2-2196 and COV2-2130, that both bind to a trimeric S ectodomain (S2Pecto) which is important in the process where RBD binds to ACE2. This could be of value in producing a vaccine or treatment against covid-19.

2.2.2. Possible cross-reactivity with different coronaviruses

When the immune system reacts to a foreign pathogen there might be cross-reactivity. This happens when the host, instead of reacting with a primary response

like the pathway explained briefly above, recognizes the antigen as something it has been in contact with before. The protein that works as an antigen is similar enough to a former antigen which trigger a memory response. Instead of, or at the same time, producing a new sort of antibodies, the B-cells manufactures antibodies against something they have encountered before (Frank 2002).

A study performed by Wang *et al.* (2020) showed that one monoclonal neutralizing antibody against SARS-CoV-2 also can bind to SARS-CoV. The genomes for the spike protein are very similar, according to Wang *et al.* SARS-CoV-2 and SARS-CoV spike protein are 77.5% identical and in a report from Khan *et al.* (2020) the nucleotide sequences shows 82% similarity. This might be useful information for potential treatments but also evokes interest for possible cross-reactivity with other coronaviruses. Cross-species cross-reactivity was investigated in a study from 2019 by Zhao *et al.* (2019). They found that antibodies for FCoV type 1 and 2 could bind to each other's S1 protein and that FCoV type 1 cross-reacted with PEDV. One serum confirmed with FCoV type 1 and 2 was also seropositive for HCoV-229E but not with any other coronaviruses.

Since Covid-19 is caused by a novel virus and so far only seem to make humans sick, few studies have been made on animals. Zhang *et al.* (2020) collected 102 blood samples from cats after the outbreak of covid-19 and screened them with enzyme linked immunosorbent assay (ELISA) of which 15 cats (14.7%) were positive for antibodies. The same study also tested the cats for possible cross-reactions with FIPV and found none.

3. Material and methods

3.1. Data collection, blood samples

Blood samples were collected during 2020 from a total of 443 dogs whose owners volunteered to participate in the study. The study has two groups: 83 samples in group A (with completed questionnaires) and 351 samples in group B (without questionnaires). Dogs were not randomly selected or categorized by for example breed or gender; all dogs were welcomed to contribute.

Dogs in group A were both patients that visited veterinary clinics during June to October 2020 due to health issues but also volunteers who had found out about the study from the researchers, social media or from announcements at clinics. A post about the study was posted on Facebook in several groups for veterinary students as well as on the author's private account that were later shared several times. Announcements were also hung up at the veterinary clinics the author worked at (Distriktsveterinärerna Halmstad/Torup) and at Distriktsveterinärerna Östhammar were the author of the cat study worked.

Blood samples in group B were donated from the clinical lab at the University Animal Hospital (UDS) in Uppsala. Samples were there collected when the dogs visited the hospital due to different health issues during June to August 2020 and serum was then saved in -20ºC for further studies with the owners' permission. The authors did not have access to medical records about the dogs and the samples were only run for seroprevalence.

Blood was collected from a peripheral venous catheter or a needle designed for blood sampling into a serum tube. The tube was then left in upright position for at least 30 minutes to let the blood clot and then centrifuged for 10 minutes in 4,500 RPM to separate the serum from the rest of the blood. With a pipette, the serum was transferred to an Eppendorf tube and then put in the freezer at -20ºC. Every sample was given a code, a letter that indicated which clinic it was from and a number. For group A; H-x for samples collected in Halmstad, Ö-x for Östhammar, U-x for Uppsala and Z-x for samples not fitting into any other code. X-x for group B.

This study had an ethical approval Dnr 5.8.18-101125/2020.

3.2. Survey study

Together with the blood sample, every dog owner in category A was given a questionnaire to fill in (see appendix 1). The questions asked were about the dog's and owner's health and if any of them had met someone with covid-19, if the dog usually meets other dogs and people and the worries the owner had about the disease. The questions were compiled in an Excel document and evaluated whether there was a correlation between seropositive animals who have had close contact with people with or without symptoms of covid-19 and confirmed or not confirmed disease.

3.3. Analyses

3.3.1. Laboratory work

Laboratory work was performed at the Zoonoses Science Centre (ZSC), Department of Medical Biochemistry and Microbiology, Uppsala University. Protocols for the analyses were originally designed for human SARS-CoV-2 projects and modified for this project. The conjugation of antigen to beads was performed with the protocol presented in appendix 2 and for the serology immunoassay the protocol in appendix 3 was followed. ZSC provided the project with controls, one human serum samples confirmed negative and one positive for SARS-CoV-2. Feline and canine serum samples, some cat samples positive for FCoV or SARS-CoV-2, were provided from National Veterinary Institute (SVA) and Swedish University of Agricultural Sciences (SLU). They worked as positive and negative controls for the method while it was developed for animal tests. Canine samples collected prior to 2019 acted as negative control for SARS-CoV-2, but there was no known confirmed SARS-CoV-2 positive control for dogs, therefore test optimization for the most accurate dilution of antigen and antibody was only performed with cat samples.

All tests were run together with a laboratory assistant and security measures were taken, such as the use of gloves, glasses and lab coats, working under a microbiological safety cabinet class II, while handling human samples confirmed with SARS-CoV-2 and disposing possible contagious materials in special containers. Due to the current pandemic with covid-19, social distancing was practiced to lab-workers who were not in the same project, masks were used when close contact to other members of the project was necessary and hands were washed regularly.

3.3.2. Suspension Immunoassay (SIA)

The protocol used was originally developed for serology testing in humans, a serological method called Suspension Immunoassay (SIA) or Suspension Multiplex Immunoassay (SMIA). SMIA if two or more for example agents or antibodies are investigated or SIA if there is only one of interest. This method has been used previously for other viruses in both humans and animals (Rönnberg *et al*. 2017; Albinsson *et al*. 2018; Lindahl *et al*. 2019). The method is designed to detect proteins and nucleic acids from samples by fluorescence from color-coded magnetic beads. For this project antigens specific to the antibodies of investigation were first conjugated to the beads, SARS-CoV-2 S1 (Sino Biological, 40591-V08H) (bead #66) and CCoV (Native antigen company) (bead #74). There was also a blank bead (#28) included. Subsequently, serum was added to the bead mix followed by addition of a canine biotinylated anti-antibody and Streptavidin Phycoerythrin (SA-PE). Phycoerythrin is a fluorescent macromolecule isolated from red algae and cyanobacteria and is the component which will signal detection of an antibody at the surface of the bead.

Two different instruments from Luminex® were used, MAGPIX® and LX-200®. The MAGPIX was the primary instrument for the study until technical issues forced the project to change its analyses to LX-200. The instruments use the same technique with microspheres but MAGPIX is a LED-based analysis while LX-200 is a flow cytometry-based analysis. In MAGPIX, the microspheres are being lit up by LED-lights and then captured by the camera inside the instrument. MAGPIX® will identify the illuminated beads based on their fluorescent pattern and create a picture which will be processed by the software. A median fluorescent intensity (MFI) value is calculated, where the signals of the blank bead is withdrawn from the signal of the bead with the antigen of investigation. The corrected MFI is then used to define positive samples from negative. The LX-200 instead uses a flow cytometry-based analysis with laser with two different wavelengths, 635 nm (red) and 525 nm (green). The red laser or LED will interrogate the bead and identify them while the green laser scans for the label and in this way the software can count the beads that have the protein of interest attached to them.

To optimize the protocol, different concentrations of the feline secondary antibody (1:10, 1:100, 1:1000) and the sample (1:20, 1:50, 1:100) were evaluated. There were also trials to see if it was possible to multiplex the assay by adding beads conjugated with CCoV. In the final optimized protocol that was used for the remaining batches, the dilution for the secondary antibody was the original 1:500 and the serum dilution was 1:50.

3.3.3. Possible cross reactions with CCoV

In one run at the end of the laboratory work, an experiment with potential cross-reactions between CCoV and SARS-CoV-2 was performed. Bead #74 was conjugated with antigen from CCoV and used together with bead #66 for SARS-CoV-2. Two samples worked as positive controls for SARS-CoV-2 as they had been positive several times, one was negative control (blood sample collected before 2019) and five samples were randomly selected. No positive controls with samples confirmed with antibodies for CCoV were available. The antigen used was for CCoV and the anti-antibody was the same as for SARS-CoV-2.

4. Results

4.1. Cut-off value for positive results

In summary, the preliminary cut-off value for canine samples was set to 300 MFI. Samples with an MFI-value between 200-300 were classed as doubtful. Samples with an MFI <200 were classed as negative.

The explanation to this is since this method has not been used for animals before there were no positive dog controls or stated values for positive results. In previous project where this method has been used for human sera, all samples with an MFI >300 were categorized as positive (unpublished results). When stating a preliminary cut-off value for this project, a mean value of all the samples with a corrected MFI greater than 0 but below 200 was calculated and with 6 standard deviations. Corrected MFI means that the value for the blank bead has been subtracted from the value for the bead with SARS-CoV-2, to correct for unspecific signals, resulting in a reliable value for how strongly positive (or negative) the sample is. The limit at 200 was determined since the curve for corrected MFI value began to rise steep at that point (see figure 2). The mean value with the added standard deviations was 135 and theoretically all samples above 135 could therefore be categorized as positive but then with many false positive as a result. Experience from human research have shown that a cut-off at 300 is reliable and therefore all samples with MFI above 200 but under 300 were categorized as doubtfully positive in the present study and all above 300 as positive. Statistical analyses included logistic regression and Chi2 test in STATA 14.2 (STATACorp Ltd).

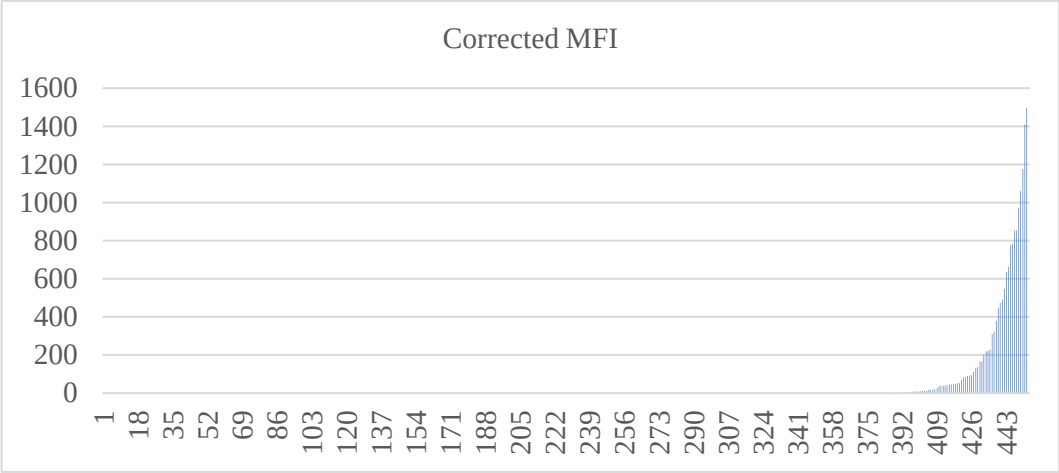

Figure 2: Corrected mean fluorescence intensity (MFI) for all samples, meaning the value for the blank bead has been subtracted from the value for the bead with SARS-CoV-2. The curve is beginning to rise steeper at ~200. The study had totally 443 samples and some samples of interest were run again, why there are 451 samples in this diagram. MFI value at x-axis and number of samples at y-axis.

4.2. Seroprevalence

Twelve of 443 dogs tested positive for antibodies against SARS-CoV-2, giving a study seroprevalence of 2.7 % (see figure 3). Four of 83 (4.8%) samples tested positive in group A (see figure 4) while eight of 351 (2.3%) samples tested positive in group B (see figure 5). Additionally, results from four dogs were doubtful.

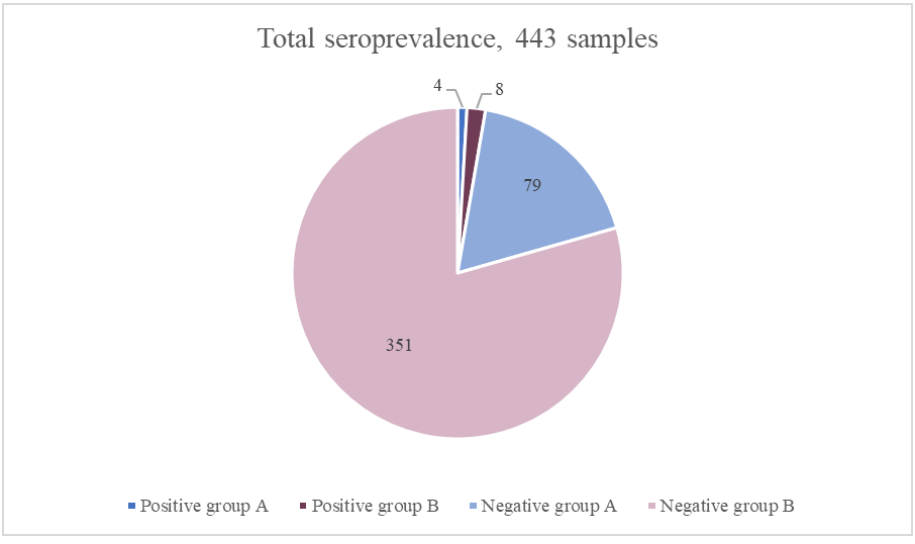

Figure 3: Total seroprevalence of SARS-CoV-2, twelve of 443 (2.7%).

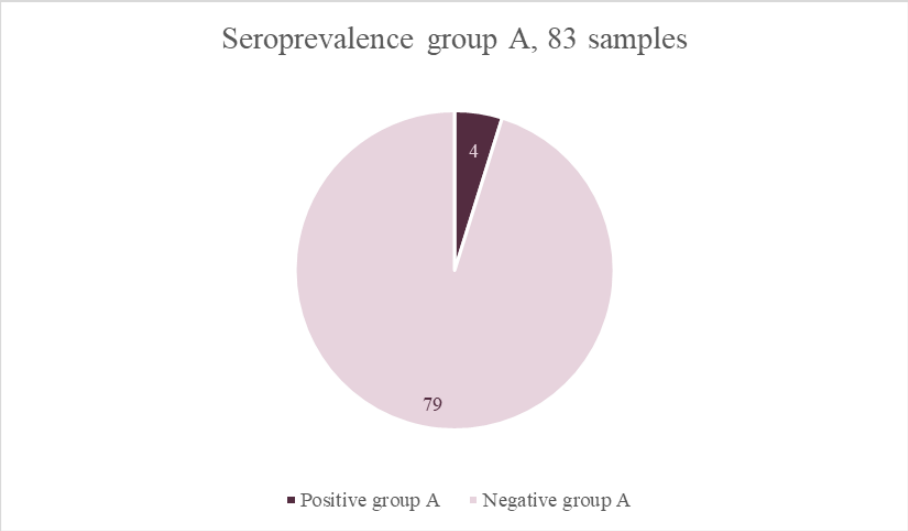

Figure 4: Seroprevalence of SARS-CoV-2 in group A:4 of 83 (4,8%).

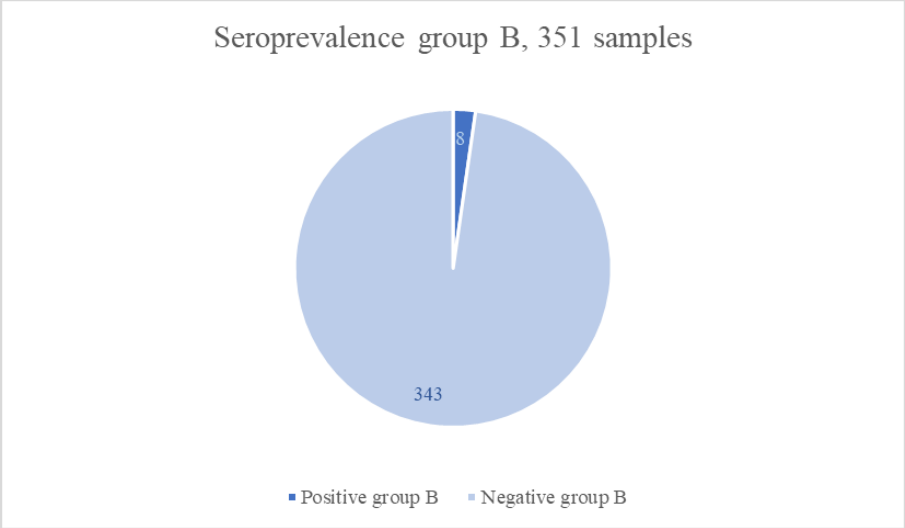

Figure 5: Seroprevalence of SARS-CoV-2 in group B:8 of 351 (2.3%).

Four samples with MFI 200-300 were categorized as "doubtful". One lived in a household with owners who were confirmed with covid-19 and three were in group B. See table 1.

Table 1: Doubtfully positive samples, between 200-300, and if they had owners positive for covid-19.

Code	**MFI**	**Covid-19 positive owner**
XH81	200	Unknown owner
XH26	218	Unknown owner
XH94	221	Unknown owner
UH10	229	Yes

4.3. Features of seropositive dogs

In group A, there were three male dogs and one bitch that were seropositive. Ages one, four, five and ten years old. In group B, one dog was female, one male and six of unknown sexes. Ages six, seven, eight years and three dogs of the age of 12 years. Two dogs are of unknown age. See table 2.

Table 2: Breed, sex, age, and gender of seropositive dogs if known. Dogs in group B are of unknown owners.

Code	Breed	Sex	Age	Symptoms	Collected in:	Owner confirmed with covid-19	Additional information
H16	Kleiner münsterländer	Male	10 years	Mild diarrhoea, coughing	Halmstad	No, but suspected to have been infected	One other dog in household, seronegative
H21	Golden retriever	Female	1,5 years	-	Halmstad	Yes, antibodies	-
Z2	Mix breed	Female	4 years	-	Gävle	Yes	Two cats in household, not participating in the study
Ö4	Icelandic sheepdog	Female	5 years	-	Östhammar	No	Two dogs and one cat in the household, not participating
X23	-	Female	6 years	-	Uppsala	-	-
X51	-	-	-	-	Uppsala	-	-
X195	-	-	8 years	-	Uppsala	-	-
X287	-	-	12 years	-	Uppsala	-	-
X304	-	-	7 years	-	Uppsala	-	-
X337	-	-	-	-	Uppsala	-	-
X345	-	-	12 years	-	Uppsala	-	-
X351	Dachshund	Male	12 years	-	Uppsala	-	-

4.4. Survey study

The questionnaire (see appendix 1) was filled in by 83 of the 91 (87.3%) dog owners whose dogs' blood was originally in group A. Questionnaires were filled in during sampling or emailed afterwards. Eight blood samples could not be coupled to a questionnaire since the owners did not respond to the email that was sent out. Those samples were still analysed and counted in the total prevalence as group B.

In figure 6, locations of collection of blood samples in group A are shown. Samples were collected from five municipalities: 29 dogs in Halmstad, 13 in Uppsala, 47 in Östhammar, one in Tierp and one in Gävle.

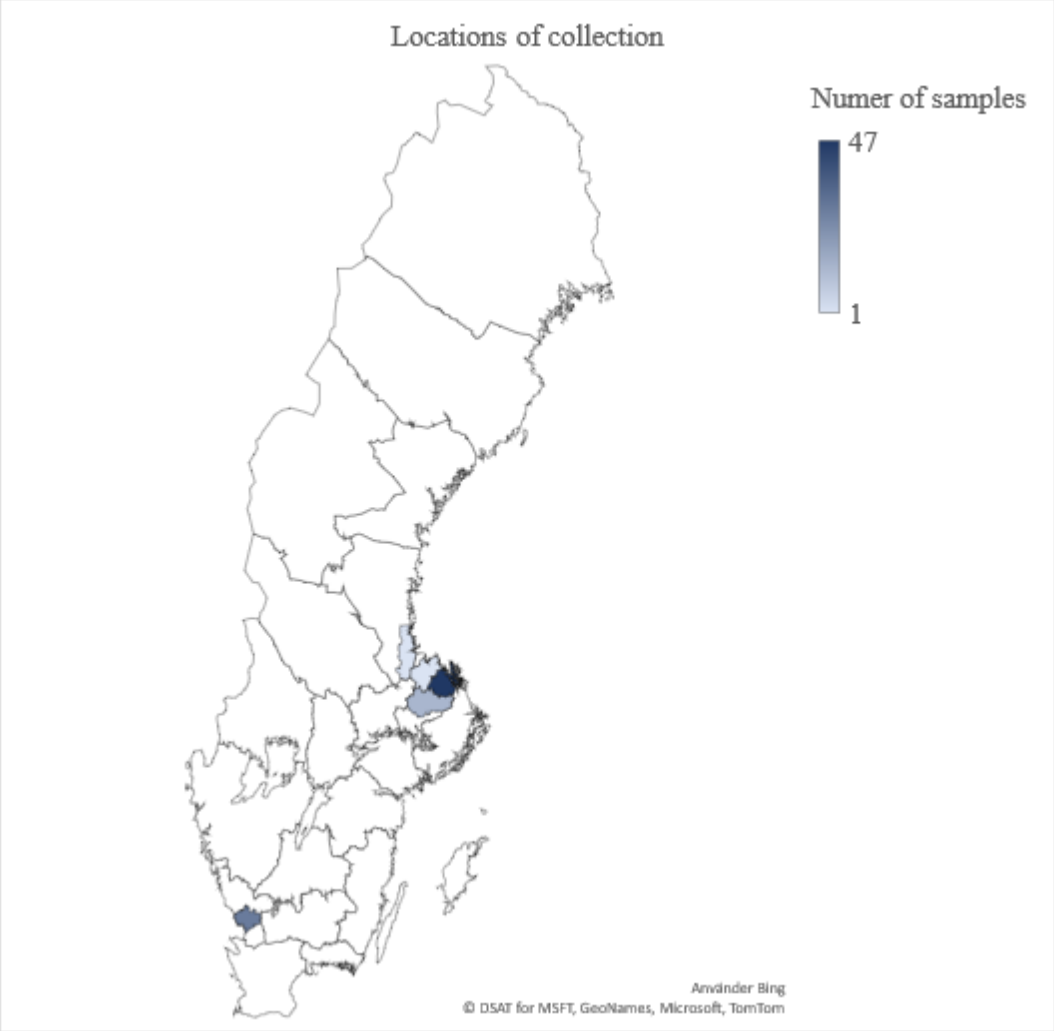

Figure 6: Locations of collection of blood samples from dogs: 29 dogs in Halmstad, 13 in Uppsala, 47 in Östhammar, one in Tierp and one in Gävle.

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).

4.4.1. Correlations between confirmed covid-19 in owners, seropositive dogs and symptoms

Out of the four positive samples categorized as group A, two dogs with different owners had been confirmed with covid-19, either by PCR or serology. Consequently, the dogs had met people infected with SARS-CoV-2. One dog owner answered that they believed they had had covid-19 but had not yet been tested. This dog suffered from a mild diarrhoea, mild coughing, and general impaired condition, as well as the other dog in the family who tested negative for antibodies. The last seropositive dog's owner did not suspect herself to have been infected and the dog did not meet a lot of other people and dogs. In this household there were two other dogs, one cat and several horses. Neither of these animals participated in this study.

Three of the seropositive dog owners answered that their dogs either lived "very close" e.g., were being petted a lot, were allowed in couches, slept in the owners' beds and touched their faces. One answered "close", meaning the dog was e.g., being petted a lot and allowed in couches but does did not sleep in their owners' bed or touch their faces.

Twelve seronegative dogs lived with people who were confirmed with covid-19. Three of those dogs had mild diarrhoea during a period sometime between March and November 2020, all in the same family. One dog had intermittent coughing during February and April 2020 and mild diarrhoea some time not defined during this period.

The odds ratio for a dog to be seropositive if the owner was confirmed to have had covid-19 was 5.5 (95% confidence interval 0.71-42.9, p=0.1), and the difference in proportion between the two categories (see table 3) was not significant (chi-square statistic 3,29, p=0.07) in Chi-square test.

Table 3: Serological results of dogs in group A, cut-off >300. The chi-square statistic is 3.29. The p-value is 0.07, the result shows it was not a statistically significant higher risk for the dogs to be seropositive if the dog owner was confirmed with covid-19.

	Seropositive	Seronegative	Total
Owner confirmed with covid-19	2 (14.3%)	12 (85.7%)	14
Owner not confirmed with covid-19	2 (2.9%)	67 (97.1%)	69
Total	4	79	83

If the doubtful positive sample in group A with the highest MFI (MFI 229) would be categorized as positive, the risk would be statistically significant higher. The chi-square statistic there was 7.1 and the p-value is 0.008. The odds ratio was 9.14 (see table 4).

Table 4:Serological results of dogs in group A if the cut-off is >200. The chi-square statistic is 7.1 and the p-value is 0.008 which means the probability is statistically significant higher for the dogs to have antibodies.

	Seropositive	Seronegative	Total
Owner confirmed with covid-19	3 (21.4%)	11 (78.6%)	14
Owner not confirmed with covid-19	2 (2.9%)	67 (97.1%)	69
Total	5	78	83

4.4.2. Concern among dog owners

Generally, the dog owners were more concerned about their friends and family and the society than themselves and their pets. Not one was “very much worried” about covid-19 regarding themselves. Ninety-five percent (79/83 answers in both categories) answered that they were “not at all” (“myself” 39 answers + “my pet” 65 answers”) or “a bit” (“myself” 40 + “my pet” 14) worried about covid-19 concerning both themselves and their pets, while 36% (30/83) were “quite much” (27) or “very much” (three) in question of their family and friends and 34% (28/83) considering the society (“quite much” 26/83 + “very much” 2/83). See figure 7.

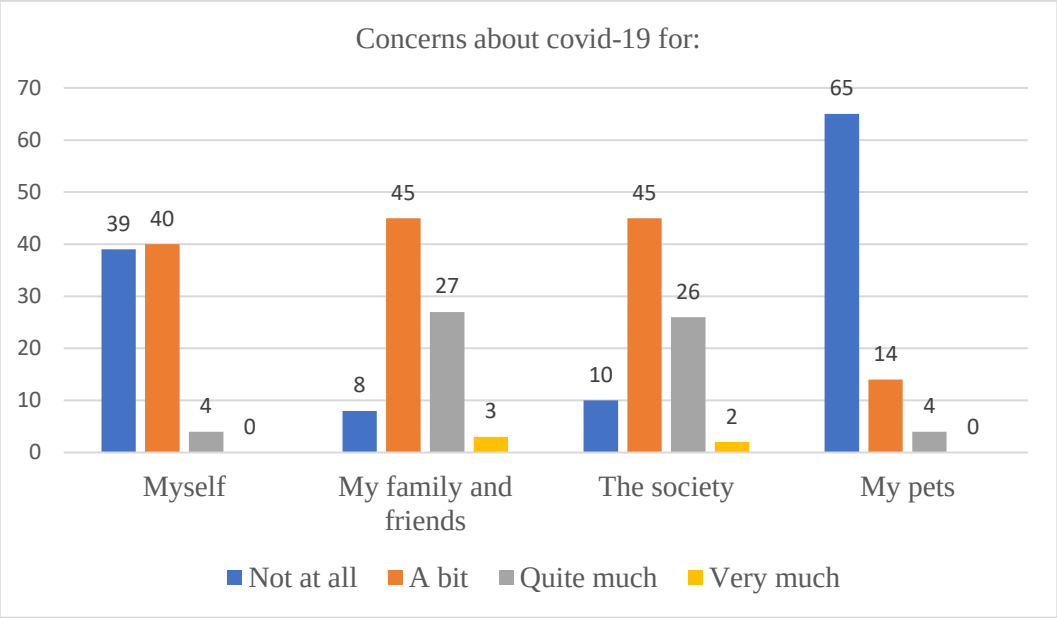

Figure 7: Concerns in dog owners. Number of people that answered each alternative.

4.5. Cross reactions with CCoV

No samples with SARS-CoV-2 antibodies reacted with antigen for CCoV (bead #74). Therefore, no samples indicated cross reactions between CCoV and SARS-CoV-2 with this method.

5. Discussion

Previous studies performed during 2020 have shown that animals can seroconvert for SARS-CoV-2, but no one has yet investigated the seroprevalence in pets in Sweden. SARS-CoV-2 is a novel virus hence not much is known about it and methods for analysing samples and evaluating data are still a work in progress. The main purpose of this study was to develop the SIA-method for SARS-CoV-2 in animals and investigate the seroprevalence of SARS-CoV-2 in dogs in chosen parts of Sweden. The method has been used for human analyses but not yet for animals and this project was successful in showing the usefulness of the method for this purpose. The seroprevalence for positive dogs (2.7%) is realistic considering the seroprevalence for humans (0.5% in April 2020 and 7.1% in June 2020) in Sweden. This project though, studied samples collected during a later period, from June 2020 to October 2020, compared to those studies FoHM performed in humans, as their samples were collected up to June 2020. The periods did not occur during the same time in the course of the pandemic, which in Sweden had its first culmen in the end of spring 2020. Fewer cases were then reported from June to September before the number of cases started to rise again. Considering this, there is a possibility that our results could have been different if we had the same collecting period. Worth taking into account is also that SARS-CoV-2 is a human coronavirus and therefore the prevalence is expected to be higher in humans.

In previous projects at Uppsala University where SIA has been used for SARS-CoV-2, the cut-off for positive results was set to 300 MFI based on the use of a large number of pre-covid-19negative samples, collected in 2018 (unpublished results). Therefore, as described in the results, samples between 200-300 were classed as doubtful. The reason for adding six standard deviations was to be certain of not getting too many false positive results, however, we might have missed some samples as false negative instead. A cut-off of 135 MFI could have been used but with experiences from human studies in consideration, this cut-off was considered too low. More data needs to be analysed to get clearer results and determine a proper cut-off value.

Four samples were doubtful, with MFI between 200 and 229. One of them was in group A, the one with the highest MFI, and had owners who were confirmed with covid-19. This dog showed symptoms like the ones seen in humans with covid-19, coughing and diarrhoea, between February and April and never got a diagnose. It

would have been interesting to run this sample again, since some of the samples that were run twice showed slightly different MFIs. Sample HH7 for example, had an MFI of 89 in the first run and when it was run again and got 168, XH23 was run four times and got MFI between 310-1496. Because of the big variation in MFI and the reasoning described above, we classed samples under 200 as negative. Differences in MFI could depend on the person pipetting, if the samples have not been mixed enough, small changes in incubations times or possibly many other reasons. To avoid most of these errors, the samples were corrected against the MFI for the blank bead, but there may still be reasons for varying results remaining.

In this study, 351 samples were collected from a sample bank with anonymous dogs. Therefore, there is a risk that some samples are doublets. Some dog owners in group A had two dogs or more and this could also be a factor that changes the statistics from the answers from the questionnaire. Eighty-three of 443 dog samples (18.7%) had questionnaires, and out of the four positive results in that group one had showed symptoms that were consistent with symptoms of covid-19 (plus the one doubtfully positive dog with symptoms). When the dog had symptoms and when the antibodies were formed is not known, but it is likely that these symptoms were from another disease. Hence, nothing about if dogs get sick can be concluded from the data presented in this study. Results from other studies have however implied that dogs do not get sick from SARS-CoV-2 and the same is indicated in this study. Interesting investigations for future studies at the subject could be trying to isolate virus in dogs and cats in Sweden who live with family who currently are infected with confirmed covid-19, and to investigate the prevalence there.

There was no significant higher probability for dogs to develop antibodies if they lived with covid-19 positive owners. From a chi-square test the p-value was 0,07, which is slightly above a significance level of 0.05. Results with a p-value <0.05 would mean that the probability would be significant higher for dog to develop antibodies if they lived with owners who were confirmed positive, which means the results are close to the limit. The odds ratio of 5.5 indicates that there is a correlation between owners with confirmed covid-19 and seropositive dogs but according to chi-square-statistics it is not significant. However, if the doubtful positive sample with the highest MFI (229) is categorized as positive, the p-value was 0.008, meaning the risk would be statistically significant higher. Consequently, further studies to decide the cut-off with higher certainty would benefit the reliability of studies like this one.

Previous studies performed during 2020 have shown that animals can seroconvert for SARS-CoV-2, but no one has yet investigated the seroprevalence in pets in Sweden. Due to practical reasons, samples in this study were collected from a limited area of Sweden. A greater quantity of samples from a bigger geographic area should have given more reliable results representing the seroprevalence in

Sweden. Comparations between densely and sparsely populated areas have not been made but could also be of interest for future studies.

From the questionnaires the conclusions could be made that the dog owners generally were not very worried about getting infected with covid-19 themselves. This is a bit concerning, since lack of fear for getting sick could lead to people being more reckless. However, more dog owners worried about their friends, family, and the society overall, arising optimism about them following restrictions and recommendations. Whether they do, will be the subject for other studies. A majority was on the other hand positively not worried at all about their pets. Fear of zoonotic diseases could in worst case lead to unwarranted euthanizing of animals. The authors of these studies were careful not to awake concerns when meeting with dog owners for blood sampling and referred to former studies where it has been indicated that dogs do not get sick from or spread SARS-CoV-2.

When testing for possible cross-reactions between CCoV and SARS-CoV-2 no samples were found positive for CCoV and no cross reactions between SARS-CoV-2 and CCoV could be found. However, we had no positive controls for CCoV, meaning that all the samples could be negative for CCoV and therefore no cross reactions were shown. When preparing for this study only antigen for CCoV was available, no CRCoV. The probability for cross-reactions would likely be higher with CRCoV, since two betacoronaviruses are more genetically similar to each other than an alphacoronavirus compared with a betacoronavirus, though the expectations for cross-reactions were overall small. SARS-CoV-2's target cell receptor is ACE2, CCoV type II uses APN and CRCoV utilizes HLA-1. Previous studies have indicated that cross reactions between SARS-CoV-2 and canine coronaviruses are not likely. In future studies further research for cross reactions with different animal coronaviruses, betacoronaviruses in particular, and SARS-CoV-2 would be interesting for how the virus is spread and how the animals react to it.

In summary, the results of this study further elaborate the indications made in former studies that dogs can form antibodies against but that do not get sick from SARS-CoV-2. In this study the prevalence of seropositive dogs was 2.7%, which is not representative of the entire country since there was no random selection.

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Populärvetenskaplig sammanfattning

Våren 2020 drabbades världen av ett nytt virus, det så kallade SARS-CoV-2-viruset som orsakar sjukdomen covid-19. Viruset är ett coronavirus som misstänks ha spridits till människor från djur på en marknad i Kina. Coronavirus är en vanlig virustyp som finns i olika former hos både människor och djur och oftast ger sjukdomstecken i luftvägarna, lungorna och mag/tarm-systemet. Det finns fyra olika grupper av coronavirus: alfa, beta, delta och gamma. De flesta infektioner med coronavirus är ofarliga och kan te sig som till exempel en vanlig förkylning. Andra coronavirusinfektioner, till exempel FIP (felin infektiös peritonit) hos katt, är nästan alltid obotlig och leder i de flesta fall till döden. Hundar drabbas i regel av två olika coronavirus som för det mesta inte är livshotande. Det första är ett alfacoronavirus som ger mag/tarm-relaterade sjukdomstecken och kallas canint coronavirus (CCoV). Det andra, canint respiratoriskt coronavirus (CRCoV), är ett betacoronavirus som ger respiratoriska (luftvägsrelaterade) symptom (sjukdomstecken). Coronaviruset som nu spridits bland människor, SARS-CoV-2, är ett betacoronavirus och nära besläktat med det virus som orsakade utbrottet av SARS i delar av Asien 2002–2003, SARS-CoV. SARS-CoV-2 är ett mer smittsamt virus än SARS-CoV men har en lägre mortalitet, det vill säga det är inte lika dödligt. Covid-19 är en så kallad droppsmitta, där viruset sprids genom att människor nyser, hostar eller talar. Små droppar med viruspartiklar skvätter då ut och kan hamna i kroppen genom inandning eller att personen tar sig runt munnen eller ögon med händer som kommit i kontakt med dropparna på ytor. På så vis skulle smitta kunna överföras från en infekterad person till ett husdjurs päls och vidare till en annan människa genom att denne klappar djuret och sedan vidrör sin mun, näsa eller ögon. Risken för detta är dock inte större än att bli smittad från vilken yta som helst. Forskning har visat att vissa djur, t ex kattdjur och minkar, kan få symptom och bilda antikroppar vid en pågående infektion av SARS-CoV-2 men det finns inga tecken på att de skulle föra smittan vidare till människor. Inga liknande resultat har presenterats för hundar, de verkar enbart bilda antikroppar mot SARS-CoV-2. Bildandet av antikroppar innebär endast att djurets immunsystem har reagerar på viruset och bildat ett försvar.

Syftet med denna studie var främst att genom blodprov testa hundar och se hur stor andel av de provtagna som hade antikroppar. Metoden som användes för att detektera antikroppar är sedan tidigare utvecklad för mänskliga studier av covid-19

men inte testad för djur. En del av projektet innebar därför även att utveckla metoden för att ge tillförlitliga resultat även för djurprover. Proverna delades in i två grupper, grupp A och B. Till grupp A hörde hundar som antingen kom in till klinikerna där författarna till den här och en motsvarande studie på katt arbetade. En del av hundarna var sjuka och sökte vård, om ett blodprov då skulle tas fick djurägaren frågan om de tillät att blodet även användes till denna studie. Andra var friska hundar vars ägare frivilligt ställde upp i studien. Som ett komplement till blodproverna i grupp A skickades även enkäter ut till hundägarna. Denna enkät innehöll frågor om hundarnas ras, kön och ålder, hur nära de levde sina djurägare och om ägarna haft bekräftad eller misstänkt covid-19. Även frågor om huruvida ägarna var oroliga för covid-19 gällande sig själva, familj och vänner, samhället och sina husdjur ingick. Blodproverna från hundarna i grupp B kom från Universitetsdjursjukhuset i Uppsala. Där sparas blodprover från patienter i en frys om djurägarna fyllt i ett medgivande att djurens prov tillåts användas till forskning. De proverna är därför anonyma, inte heller författarna vet ifrån vilka djur proverna kommer, på vilket sätt de varit sjuka eller varifrån i landet djuren kommer, bara att de är provtagna i Uppsala. De här proverna har därför följaktligen inga enkätsvar.

Tekniken som användes kallas SMIA (förkortning för Suspension Multiplex Immunoassay) och går ut på att antigen (delar av ett smittämne, här SARS-CoV-2) fästs på en mycket liten magnetisk kula. Kulorna läggs sedan i små brunnar på en platta tillsammans med serum (blod där själva blodkropparna separerats från resten av blodet) från hundarna i studien som potentiellt har antikroppar i sig, en brunn för varje hund. Om antikroppar finns i proverna kommer de binda till antigenet och därigenom kulan. En lösning tillförs, denna innehåller anti-antikroppar som kommer att fästa till antikropparna om det finns några. Till anti-antikropparna fästs ett ämne som fluorescerar, alltså lyser i starka färger och fungerar som ”flaggor”. Hela blandningen ställs undan en stund under omrörning för att alla delar ska få tid på sig att binda till varandra och sätts sedan på en magnet. När blandningen sedan sköljs kommer kulorna ha fastnat på magneten. Alla brunnar innehållande kulor med antikroppar på kommer nu även ha flaggan. Plattan med brunnarna innehållande kulorna körs igenom en maskin som läser av alla proverna och märker i ett datorprogram ut dem som har flaggor. Hur stark signalen från dem blir beror på hur många kulor som antikropparna har fastnat på.

Vid projektets början fanns inget fastställt värde för var gränsen för positivitet gick. Hos människor går denna vid 300 MFI (median fluorescence intensity), ett värde som beskriver hur stark signalen var. För att bestämma var gränsen för positiva resultat gick räknades ett medelvärde ut av de resultaten som hade ett lågt MFI, mellan 0 och 200. Då kurvan för MFI började stiga brant vid ca 200, tolkades detta som en indikation på positiva resultat och därför valdes just 200. Medelvärdet med sex standardavvikelser (ett statistiskt mått på felmarginaler) gav ett värde på 135. Teoretiskt sett skulle gränsen för positivt test kunna sättas där men av erfaren-

het från forskning på humansidan, variation av MFI på samma prov på en del som kördes flera gånger och att kurvan blev mycket brantare efter 300 bestämdes det att gränsen för positivitet var 300. Prover mellan 200 och 300 MFI kategoriserades därför som tveksamt positiva och de över 300 MFI som positiva.

Resultaten bekräftade det tidigare studier indikerat, att hundar kan utveckla antikroppar mot SARS-CoV-2. Av totalt 443 hundar var 12 seropositiva, alltså hade antikroppar i blodet. Fyra av dem ingick in grupp A och åtta i grupp B. Prevalensen (andelen av de undersökta) blev därför 2,7 %. Fyra prover låg på gränsen, mellan 200 och 300 MFI. Enkätsvaren visade på att det inte var en högre statistiskt signifikant sannolikhet för att ägare som bekräftats med covid-19 hade hundar med utvecklade antikroppar men att det finns indikation på ett samband mellan dem. Totalt 14 hundägare svarade att de fått positiva provsvar för covid-19 och två av dem ägde hundar som utvecklade antikroppar. Enkätstudien visade även att alla ägarna till de seropositiva hundarna levde nära ihop med sina hundar, majoriteten av dem tillät hundarna att slicka dem i ansiktet och sova i deras sängar. Angående oron hos djurägare indikerade resultaten på att människorna i studien generellt var mer oroade för sina vänner, sin familj och samhället än för sig själva. Mycket få djurägare var oroliga för sina husdjur och covid-19.

För att få ett mer tillförlitligt resultat, både för gränsen för positiva prover och prevalensen i sig, skulle vidare studier med ett större antal hundar behöva genomföras. Hundarna i den här studien kom från enbart tre olika län och en större geografisk spridning skulle ge en mer rättvis bild av hur läget ser ut i Sverige. Även jämförelser mellan tätbefolkade orter och glesbygd hade varit intressant för vidare forskning. Detta var inte genomförbart på grund av praktiska skäl men vore lämpligt för framtida studier. En större andel i grupp A, alltså prover med tillhörande enkäter, skulle göra att sambandet mellan infekterade ägare och hundar kan undersökas med större tillförlitlighet. Betydelsen av potentiella faktorer för utvecklandet av antikroppar, t.ex. ålder, ras, kön och tidigare sjukdomar hos hundarna skulle då kunna undersökas närmre.

Studien undersökte även ifall det fanns indikationer för korsreaktioner i djurens immunförsvar. En korsreaktion innebär att kroppen bildar antikroppar mot ett visst virus som sedan fäster in på ett annat, liknande virus. Det virus som jämfördes med SARS-CoV-2 i denna studie var CCoV som är ett alfacoronavirus, till skillnad från CRCoV som är ett betacoronavirus precis som SARS-CoV-2. Anledningen till att det var CCoV som undersöktes och inte CRCoV var att det enbart gick att beställa antigen för CCoV vid tiden för projektets början. Resultatet visade inte på någon indikation för korsreaktioner mellan de två virustyperna, vilket även varit resultatet i tidigare forskning under 2020.

Resultatet av denna och tidigare studier indikerar att hundar inte utgör en risk för spridning av SARS-CoV-2 och att djurägare inte behöver oroa sig för att göra sina djur sjuka eller bli smittade av dem.

Appendices 1-3

Appendix 1: Questionnaire and approval for participation
Appendix 2: Coupling of antigens to MagPlex-C microspheres
Appendix 3: Serology using MagPlex-C microspheres

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)				

Appendix 1, Questionnaire and approval for participation, page 2

1. 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

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

_____

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

_____

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

_____

5. 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)

Appendix 1, Questionnaire and approval for participation, page 3

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)?

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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)				

Appendix 2: Coupling of antigens to MagPlex-C microspheres

Coupling of antigens to 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 **protein**, 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 (PBST)** 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 4°C in the dark.

Appendix 3: Serology using MagPlex-C microspheres

Serology using MagPlex-C microspheres (v5)

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.
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 **RT** on a **plate shaker** at 600 rpm.
7. During this incubation period, dilute **biotinylated anti-dog IgG (SAB3700117, Sigma-Aldrich) (0.5 mg/ml)** (for IgG analysis) 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 RT on a plate shaker at 600 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 RT on a plate shaker at 600 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 600 rpm.
18. Analyze 75 µl on the Luminex analyzer according to the system manual.