Development of novel Competitive Enzyme-linked immunosorbent assays to detect SARS-CoV-2-specific antibodies in animals

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ABSTRACT

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the COVID-19-causing virus, is a zoonotic pathogen. There is concern about the virus spilling over from humans into wildlife species, which may then serve as reservoirs for future infection of humans and other animals. Furthermore, the level of exposure of potentially susceptible wildlife species is currently not known. There is, therefore, an urgent need to develop a single test that could be used for the serosurveillance of multiple wildlife species for exposure to SARS-CoV-2. Although there are serological techniques to detect the exposure of humans to the virus, few assays have the capacity to detect antibodies in a wide variety of species. Here, I describe the development of a competitive enzyme-linked immunosorbent assay (cELISA) to detect SARS-CoV-2 antibodies in mammals for which species-specific reagents are not available. Therefore, cELISAs were developed to detect SARS-CoV-2 spike S1 and S2 domains and nucleocapsid (N) specific antibodies and were validated using sera from experimentally infected hamsters. We further validated our cELISA by comparing it with results obtained from the surrogate virus neutralization test (cPASS, GenScript) and indirect ELISA using anti-hamster horse radish peroxidase (HRP) conjugated reagents. Our initial cELISA was based on the ability of test antibodies to displace the binding of commercially obtained rabbit antibodies against viral proteins coated on the ELISA plate. Rabbit antibody reagents are expensive and anti-rabbit detection antibody may cross-react with other mammalian antibodies. Therefore, I explored the use of antibodies produced in hen eggs (IgY) as a substitute for rabbit sera. Hens were immunized against SARS-CoV-2 antigens: S1, S2 and N. IgY antibodies were purified from egg yolk, and the assay was optimized to use specific antibody and antigen combinations. Among S1, S2 and N-IgYs, only the S2-IgY based cELISA was specific and comparable with both the rabbit anti serum based cELISA and the surrogate virus neutralization test (cPASS). This assay will be a valuable tool which can be implemented in surveillance programs investigating exposure to and transmission of SARS-CoV-2 in multiple domestic, captive, or wildlife species.

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DEDICATION

I would like to dedicate my thesis to my late maternal grandfather! I wish you were here.

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LIST OF ABBREVIATIONS

ACE2- Angiotensin Convertase Enzyme 2

cDNA- Complementary DNA

cELISA- Competitive ELISA

COVID-19- Coronavirus Infectious Disease-2019

ECCC- Environment and Climate Change Canada

Ig- Immunoglobulin

LIPS- Luciferase Immunoprecipitation System

N- Nucleocapsid

NML- National Microbiology Laboratory

NSERC- Natural Sciences and Engineering Research Council of Canada

PCR- Polymerase Chain Reaction

RBD- Receptor Binding Domain

S1- Spike 1

S2-Spike 2

SARS-CoV-2- Severe Acute Respiratory Syndrome Coronavirus 2

TMPRSS2- Transmembrane Protease, Serine 2

VIDO- Vaccine and Infectious Disease Organization

WCVM- Western College of Veterinary Medicine

Chapter 1

Literature Review

Introduction

Beginning in December 2019, health officials reported a cluster of pneumonia-like cases in humans connected to the Wuhan City seafood and wet market in Central China[1]. Since then, Severe Acute Respiratory Syndrome Coronavirus type 2 (SARS-CoV-2) has infected over 750 million people (https://covid19.who.int/) all over the world, causing Coronavirus Infectious Disease-2019 (COVID-19) with a death toll of over 6 million people, at time of writing. SARS-CoV-2, a single stranded positive sense RNA virus, belongs to the genus *betacoronavirus*, a highly promiscuous group of zoonotic coronaviruses which are capable of mutating and propagating across species[2].

Researchers have found that SARS-CoV-2 most likely originated in the horseshoe bat (*Rhinolophus affinis*) with a possible intermediate animal host before being transmitted to humans[3]. In human-to-human transmission, SARS-CoV-2 is transmitted via fomites, droplets, and aerosols[4], manifesting a range of symptoms from fever, cough, and shortness of breath to severe pneumonia and even death. Although COVID-19 is largely a human disease, there is a concern that the virus may infect and establish in wildlife or domestic animal species. These animals could then serve as reservoir hosts in which the virus may mutate and/or subsequently spill back into humans. Therefore, recent interest has been concentrated on identifying which species could act as potential reservoirs. There is also interest in understanding which species are susceptible to infection, which can shed virus and thus serve as sources of infection, and which are susceptible to disease if infected by SARS-CoV-2. The initial *in vitro* studies based on the ability of SARS-CoV-2 to use angiotensin convertase enzyme-2 (ACE2) receptor suggested that other than humans (*Homo sapiens)*, civets (*Paradoxurus hermaphroditus*), chinese horseshoe bats (*Rhinolophus sinicus*) and pigs (*Sus domesticus*) were susceptible[2]. However, natural, and experimental infections have since been reported in tiger (*Panthera tigris*), lion (*Panthera leo*), dog (*Canis lupus familiaris*), western lowland gorilla (*Gorilla gorilla gorilla*), Asian small clawed ottler (*Aonyx cinerea*), black-tailed marmoset (*Callithrix melanura*), giant anteater (*Myrmecophaga tridactyla*), Canada lynx (*Lynx canadensis*), cougar (*Puma concolor*), snow leopard (*Panthera uncia*), binturong (*Arctictis binturong*), mule deer (*Odocoileus hemionus*), hippo (*Hippopotamus amphibius*), cats (*Felis catus*), ferrets (*Mustela putorius furo*), minks (*Mustela lutreola*), white-tailed deer (*Odocoileus virginianus*), pangolin (*Manis pentadactyla*), and Syrian hamster

(*Mesocricetus auratus*) etc[5-9] (Table 1.1). Acknowledging the presumed zoonotic origin of SARS-CoV-2 and presence of highly similar orthologues of the human ACE2 receptor in certain animal species, it is important to recognize potentially susceptible animal species and to assess SARS-CoV-2 prevalence among animals to mitigate further virus spillover events[10-12].

Currently, reverse transcriptase polymerase chain reaction (RT-PCR) is being used to detect viral nucleic acid (RNA) from infected patients and/or animals[13]. Even though nucleic acid amplification is a highly sensitive and specific method to detect infection, experimental field data revealed that animals test positive only during a very short window (for two-six weeks) once the virus starts replicating in the host[14]. A serological test may provide evidence of exposure by detecting antibodies, long after viral nucleic acid is no longer detectable. Serological tests have been used to detect antibodies in exposed wildlife. Amongst several other serological tools, Enzyme Linked Immunosorbent Assay (ELISA) is often preferred because it is simple, objective, quantitative, and has high specificity and sensitivity. Different ELISAs have been developed for screening both human and non-human animal serum to detect SARS-CoV-2 specific antibodies[8,16,27,38]. Most of these assays include species-specific enzyme conjugates to detect antibody. This approach is limited to the detection of antibodies in species for which species-specific conjugates are readily available and cannot be used for broad multispecies screening. Multispecies antibody detecting conjugates can be used but these are not widely available. The surrogate virus neutralization assay[35], commercialized as the 'cPASS test kit' (Genscript™) has also been used to detect antibodies to SARS-CoV-2[2]. The test detects neutralizing antibodies to the receptor binding domain (RBD) of the spike S1 domain (S1) from an earlier virus isolate and some recent variants of the virus, but not the entire S1 or S2 or nucleocapsid (N) proteins. The luciferase immunoprecipitation system (LIPS) targets S and N antigens in their native conformation and detects antibody directed to their linear and conformational epitopes[13]. However, LIPS is a complex technique, and it may not accurately detect antibodies against the more conserved SARS-CoV-2 proteins, such as N protein[1].

To overcome some of the limitations of techniques currently used for wildlife surveillance of SARS-CoV-2 exposure, my goal was to develop a competitive ELISA (cELISA) that could detect antibodies from different mammalian species, like hamsters, minks, deer, ferrets, non-human primates etc. The assay would only require a mechanism for detecting the "competed" (known) antibody and not the "competing" test antibody. In addition to detection of antibodies

against the viral RBD within the S1 domain, we also included the structural proteins S2 and N. S2 and N proteins are less likely to be subjected to selective pressure in a new host than the RBD and S1, and therefore are more likely to detect exposure to an adapted virus.

Table 1.1: Animals naturally infected with SARS-CoV-2 as of January 16, 2023

Family	**Animals**
Callitrichidae	Black-tailed Marmoset
Canidae	Dog Fox
Cebidae	Squirrel Monkey
Cercopithecidae	Mandrill
Cervidae	White-tailed deer Mule deer
Cricetidae	Hamster
Felidae	Cat Fishing cat Lion Lynx Puma Snow leopard Tiger
Hippopotamidae	Hippopotamus
Hominidae	Gorillas
Hyaenidae	Spotted Hyena
Mustelidae	American mink (farmed and wild) Asian small-clawed otters Ferret
Myrmecophagidae	Giant anteaters
Procyonidae	Coati
Trichechidae	West Indian manatee
Viverridae	Binturong

Source: WOAH: World Organisation for Animal Health; USDA APHIS; One Health-SARS-

CoV-2 in Animals.

1.1. SARS-CoV-2 in non-human animals

Coronaviruses infect a wide variety of species including humans and other animals. Three recently discovered human coronaviruses are thought to have a zoonotic origin[15]: Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV), Middle East Respiratory Syndrome Coronavirus (MERS-CoV) and the latest, SARS-CoV-2[16-18]. Huanan South China Seafood market was presumed to be the site of the index case of SARS-CoV-2[19]. Such live-animal markets usually escalate inter-species transmission of virus between animals, and human beings. Since SARS-CoV-2 and a coronavirus identified from intermediate horseshoe bats share a high degree of genetic similarity, it is possible that SARS-CoV-2 originated from bats[20]. However, there is 4% genetic difference between the SARS-CoV-2 and the closest bat-CoV that has been found[20,21], suggesting that there may have been a bridge host which aided the cross species transmission of virus from bats to humans. Therefore, it is suspected that SARS-CoV-2 may have undergone adaptive genetic recombination within a bridge host, followed by spillover of virus to the human population[22,23]. SARS-CoV-2 has now been found in domestic animals, wildlife species and experimental animal models, and the current dynamics of virus transmission (See fig 1.1) necessitates further detailed investigations concerning the potential for zoonosis and reverse zoonosis[17,23-26]. Understanding SARS-CoV-2 circulation in animals and at the animal-human interface requires targeted surveillance to inform dynamic risk assessments and development of effective prevention measures to reduce the spread of SARS-CoV-2 and related coronaviruses. Domestic species such as cats, cows, buffalo, goats, sheep also appear to share a homologous spike protein receptor enzyme known as angiotensin converting enzyme-2 (ACE2) that make them susceptible hosts[27]. Therefore, research is urgently needed to track the extent of SARS-CoV-2 prevalence and susceptibility in animals in Canada.

1.1.1. Susceptible wildlife hosts for SARS-CoV-2 virus and potential reservoirs:

A natural reservoir is the population of host organisms or the environment in which an infectious pathogen naturally lives and replicates, or upon which the pathogen primarily depends for its survival, as defined by infectious disease ecology and epidemiology. Coronaviruses are known for their ability to jump the 'species barrier': i.e., cross-species transmission due to innate genetic plasticity sets up a broad host range[19,28]. Moreover, species bearing human orthologues

of ACE2 receptor have higher potential to be infected with SARS-CoV-2[29]. Therefore, potential hosts for SARS-CoV-2 can be predicted by understanding the conservation of ACE2 receptor and its expression pattern across different animal species[11]. Three-dimensional modelling of host-virus protein-protein interactions on ACE2 homology suggested that bat-to-human direct transmission of SARS-CoV-2[30] was unlikely. While a certain number of animals were defined as susceptible host according to this *in silico* analysis[31], results were not always supported by *in vivo* and epidemiological analysis[32]. For example, snakes were predicted to be susceptible hosts, but there was no evidence of infection[33-35].

The ACE2 proteins of three cervid species - white-tailed deer (*Odocolieus virginianus)*, reindeer (*Rangifier tarandus)*, and the Pere David's deer (*Elaphurus davidianus*) showed high propensity for binding the SARS-CoV-2 S RBD[36]. Being social animal, the geographic distribution of white-tailed deer over North America leads to investigate the potential for intraspecies transmission in North American deer population. Indeed, SARS-CoV-2 experimental infection of white-tailed deer showed that not only do deer develop sub-clinical infections, but also shed virus in nasal secretions and feces, and can transmit the virus to healthy deer[8]. SARS-CoV-2 specific antibodies have also been detected in sera from wild white-tailed deer collected in 2020 and 2021[7]. Recently, a report came out finding divergent SARS-CoV-2 variants emerging in white-tailed deer in Ontario, Canada and speculated about possible deer-to-human virus transmission.[37]

Apart from ACE2 receptor, Transmembrane Protease, Serine 2 (TMPRSS2) plays a role where it cleaves at the proteolytic cleavage site of spike protein, which mediates SARS-CoV-2 entry into the host cell[38,39]. Molecular analysis of ACE2 and TMPRSS2 suggested Savanna Monkeys are susceptible hosts to the virus. Moreover, this study revealed the possibility of virus transmission from Savanna monkeys to human[39]. In addition to ACE2 and TMPRSS2 homology, it was established that the RBD of pangolin-lineage CoV is almost identical to SARS-CoV-2-RBD[40]. However, there is no evidence of direct transmission from pangolins to humans.

1.1.2. Pets:

Pet animals live in close contact with humans and often they share a common living place. Close interaction between humans and pets increases the risk of transmission of zoonotic pathogens[41]. Early in the pandemic, Wuhan was the site of the first surveillance for SARS-CoV-2

in pet animals. By using ELISA, antibodies against SARS-CoV-2 were detected in 15 (14.7%) of 102 randomly selected cat serum samples. 11 of the 15 samples were also positive by a virus neutralization test. [42]. In a comparable survey in Italy, neutralizing antibodies were detected in 15 out of 451 (3.3%) dogs and 11 out of 191 cats (5.7%)[43]. Surveillance studies in Germany and France revealed seropositivity in dogs and cats[44,45]. Overall, these initial surveillance studies show that infection of cats and dogs with SARS-CoV-2 can occur, but there is little evidence that they are epidemiologically significant in spreading the virus to each other or to people.

1.1.3. Livestock and farmed animals:

There were concerns that farmed livestock species may be involved in viral transmission when the SARS-CoV-2 epidemic quickly spread to become a worldwide pandemic and the pathogen's zoonotic origin became clear. Furthermore, both small-scale agricultural and concentrated livestock-producing systems allow contact between people and farm animals. This interaction is thought to be a major contributor to the emergence of zoonotic illnesses, with viral infections[46-48]. Despite the initial link of COVID-19 to a sea food market in China[49], terrestrial mammalian and avian species were thought to be susceptible. Concerns about the potential involvement of cattle in the SARS-CoV-2 transmission were raised due to the close relationship between bovine coronaviruses and human HCoV-OC43[50-52], as well as the similarity of ACE2 receptor between cattle and human[33,36]. In an *in vivo* study, experimental SARS-CoV-2 infection of 6-month-old Holstein-Friesian dairy calves resulted virus replication in the upper respiratory tract. However, transmission to co-mingled cattle did not occur. Despite viral replication and seroconversion, clinical disease was not observed[53]. Furthermore, no serological cross-reactivity of antibodies against bovine coronavirus and SARS-CoV-2 was recorded[53]. In addition to cattle, horses were also discovered to have asymptomatic SARS-CoV-2 infections from their carer, who had been identified as having the Delta strain. Swine, not being a compatible host for SARS-CoV-2, virus is able to replicate in porcine cell lines. Even swine can be experimentally infected and transmit virus to co-housed pigs[54]. However, like cattle, no clinical signs in swine were observed.

Mink are also hosts for SARS-CoV-2. The first case of SARS-CoV-2 in a mink farm was reported from the Netherlands at the end of April 2020 during the first wave of the COVID-19 pandemic[55]. Furthermore, there are reports of back-and-forth transmission of SARS-CoV-2 between farmed mink, cats, and people who were associated with those farms. Whole genome analysis of viruses from these infected humans matched with animal sequences, showing that

most likely the human-to-animal followed by animal-to-human virus transmission had happened[56]. Moreover, a new spike gene mutation had been found in SARS-CoV-2 of mink origin, referred to as cluster V mutations[57,58] and this variant was reported as less sensitive towards neutralizing antibodies[59].

1.2. Transmission of SARS-CoV-2

Increasing evidence shows that the primary route of transmission of SARS-CoV-2 between individuals is respiratory. Also, there is a speculation about wastewater mediated transmission of SARS-CoV-2[60]. Most cases associated with human infections are mainly due to close contact with infected patients, initially assumed from droplets but with the potential to be spread by aerosols as well[61]. Along with human-to-human transmission, some reports revealed and/or speculated about human-to-animal transmission[56,62], animal-to-animal transmission[56,63] or animal-to-human transmission[37,56] of SARS-CoV-2 (Figure 1.1).

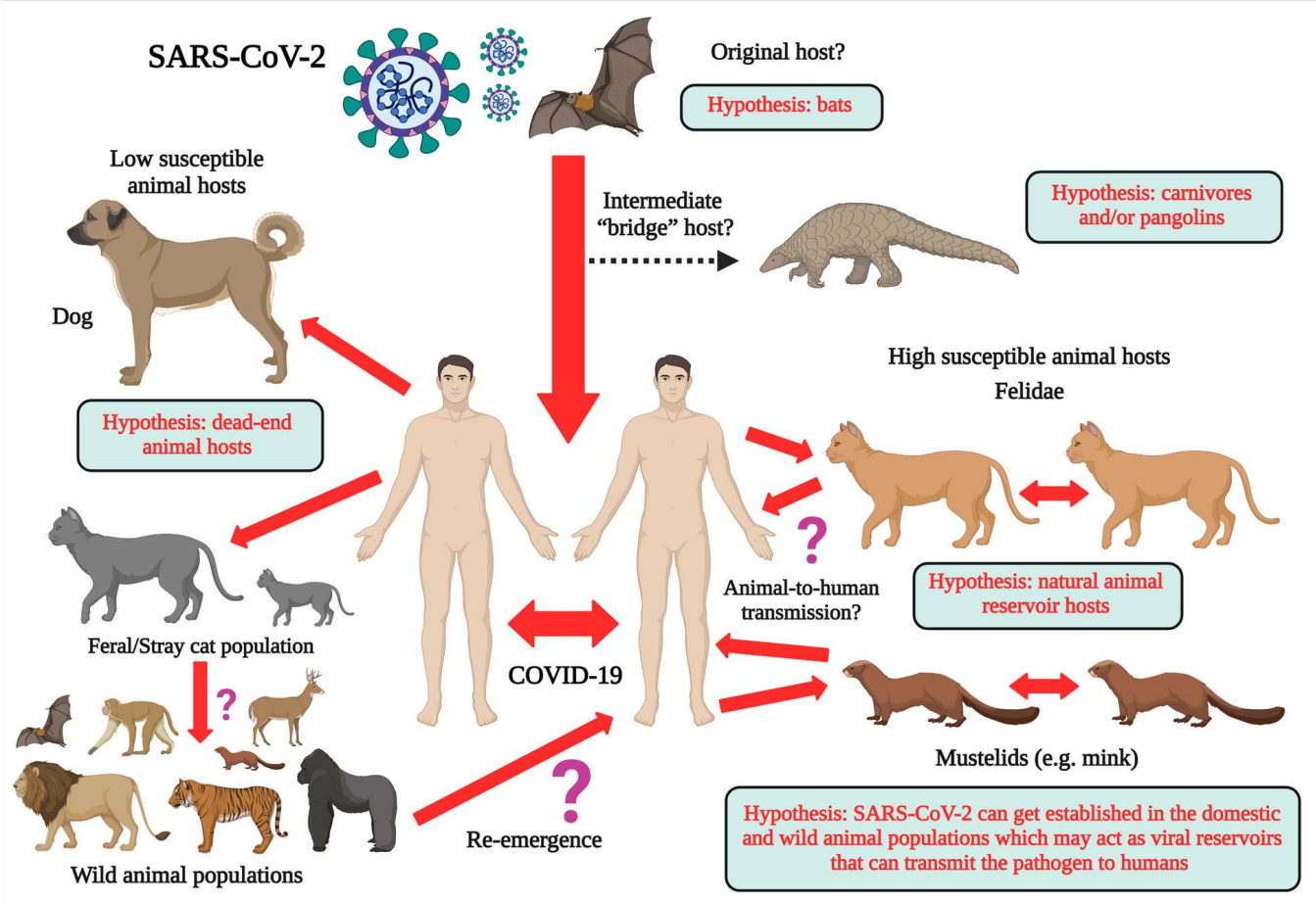

Figure 1.1: Potential hosts and transmission of SARS-CoV-2 virus

1.3. Different methods to detect exposure of SARS-CoV-2:

Rapid identification of the infected and/or exposed animals is the way to track virus transmission. Different diagnostic techniques have been developed to detect SARS-CoV-2 based on serological, molecular and nanotechnological approaches. Viral nucleic acid is frequently detected by high-throughput sequencing, reverse-transcription-polymerase chain reaction (RT-PCR), RT-loop-mediated isothermal amplification (RT-LAMP), and quantitative real-time PCR (qPCR)[64,65], where qPCR is recommended as the most effective method by the WHO (World Health Organization). Serological methods like detection of SARS-CoV-2 specific antibodies can reveal recent or previous exposures[66,67].

1.3.1 Molecular approaches in the detection of SARS-CoV-2:

Globally, several labs and companies have developed diagnostic tools for this single-stranded, positive-sense RNA virus. RT-PCR is now the gold standard method for detecting SARS-CoV-2 in samples from the upper respiratory tract. It successfully amplifies minute quantities of viral genetic material in a mixture of other nucleic acid sequences. In this procedure, the reverse transcriptase first transforms the RNA viral genome into DNA using a short DNA sequence primer before producing complementary DNA (cDNA). A fluorescent dye or a fluorescent-labelled sequence-specific DNA probe tracks DNA amplification in real time. Following several amplification rounds, a fluorescent or electrical signal displays the viral cDNA[68]. Several SARS-CoV-2 genomic areas, such as ORF1b and ORF8, as well as the nucleocapsid (N), RNA-dependent RNA polymerase (RdRP), spike (S) protein, and envelope (E) genes, have been employed in molecular diagnosis of the virus using RT-PCR technology[69,70]. Although RT-PCR has been used most extensively in the detection of viral nucleic acid, obstacles such as high cost, expertise, sample quality and storage issues have led to delayed or compromised test results.

For each cycle of RT-PCR, several temperature changes are needed, requiring specialized thermal cycling equipment.[71] Isothermal nucleic acid amplification is such a strategy that allows amplification at a constant temperature and subtracts the need for a thermocycler. Therefore, several methods were developed on this concept, such as Reverse Transcription Loop-mediated Isothermal Amplification (RT-LAMP) as time-effective and low-cost techniques[65]. Depending on isothermal nucleic acid amplification, two other techniques have also been developed: Transcription-Mediated Amplification and CRISPR-based assays. However, all molecular techniques solely depend on the viability of viral nucleic acids and timing of the test. For molecular diagnostics, the samples are usually collected from upper and lower respiratory specimens such as nasal, nasopharyngeal, oropharyngeal swabs, sputum, and lower respiratory tract aspirates. However, there is evidence of variable biodistribution of virus particles in different specimens.[72] Moreover, false-negatives may occur due to inappropriate sample collection time with respect to the onset of symptoms or technical inefficiencies in sampling procedures.[73]

1.3.2. Serological approaches in the detection of SARS-CoV-2:

Serological studies can be implemented to collect epidemiological data on the seroprevalence of SARS-CoV-2. Furthermore, in cases of COVID-19 undetected by RT-PCR, the serological assays should be considered as a supplementary diagnostic tool, especially from the second week of illness when the sensitivity of the current molecular tests decreases[74,75]. Therefore, developing serological assays is critical to detect the presence of antibodies against SARS-CoV-2 in the population as an indicator of an ongoing or previous infection. The serological test of COVID-19 reveals information about the type and titer of various immunoglobulins (IgA, IgM and IgG) produced due to infection by SARS-CoV-2.

Coronavirus spike proteins are variable because most neutralizing antibodies are directed against the RBD and surrounding regions[76]. In addition to Spike, coronavirus virions contain structural proteins (including the "N" or nucleocapsid protein) that induce protective immunity[77]. Antibodies against N or S2 proteins do not neutralize virus infectivity and therefore lack selective pressure. Therefore, a mutation in the S1 region might be a key factor in generating escape mutants and those anti-S1 antibodies may not be detected by standard competitive ELISAs. The detection of antibodies against S2 and N could be more useful in this case. Also, different types of antibodies are produced following the onset of symptoms. Previous studies show that IgM and IgA require 3-7 days and IgG requires 7-18 days to develop after primary infection[78,79]. According to another study, after two weeks of onset of symptoms, anti-SARS-CoV-2-IgA and IgM antibodies were detected in 100% of infected individuals, whereas IgG antibodies were detected in only 60% of the same individuals. IgG became more dominant over IgM as days increase from onset of symptomatic infection.

Widespread human vaccination for SARS-CoV-2 has made it more difficult to interpret serological results relying on spike antibodies, and the relevance of antibody detection must be considered in the context when it is employed and its limitations. The most common method used to detect antibodies is ELISA. Apart from different types of ELISA, virus neutralization tests, fluorescent microparticle immunoassays[80] and lateral flow immunoassays[67,81,82] are also considered in serology. The sensitivity and specificity of different serological assays do vary. Thus, careful attention should be drawn towards designing of the studies, their limitations, and whether the conclusions derived from these evaluations are justified. For example, luciferase-based immunoassay is complex to perform and less sensitive to detect N-specific antibodies[83].

Surrogate virus neutralization test (cPASS) is limited to detect only S1-RBD specific neutralizing antibodies but not whole S1 and other viral antigens[84].

1.4. 'ELISA'- Serological tool to detect exposure in animals:

ELISA is a sensitive immunoassay that is used to detect and/or quantify antibodies, antigens, proteins, glycoproteins, and hormones. ELISAs can be used in different settings, including pathogen specific antibody detection, autoimmune disease detection, forensic toxicology, and many other diagnostic settings[85,86]. According to CDC, like other serological tools, ELISA could be used for epidemiological and surveillance studies to determine SARS-CoV-2 seroprevalence and exposure in a population.

Serological tests to detect antibodies in humans are rapidly being developed and deployed; however, very few assays can detect past viral exposure in a wide variety of animal species. RT-PCR is highly sensitive and specific, but experimental and field data suggest that animals test positive only during a very short time interval (two weeks to six weeks). Therefore, a multi-species ELISA would be useful to detect and quantify antibody levels against SARS-CoV-2. An indirect ELISA has been developed using a conjugate specific to Schmallenberg virus for detection of multi-species antibody directed to receptor binding domain (RBD) of spike 1 (S1) protein from SARS-CoV-2[87]. However, importation of multispecies conjugate Schmallenberg virus milk is not allowed in many countries including Canada. There are indirect ELISAs[87-90] to detect antibodies against SARS-CoV-2; however, enzyme conjugated secondary detection antibody is not available against every wild species. In contrast to indirect ELISA, competitive ELISA (cELISA) relies on the ability of the test serum to compete with the binding of known amounts of antigen specific antibodies from a common species for which reagents are readily available (Fig 1.2).

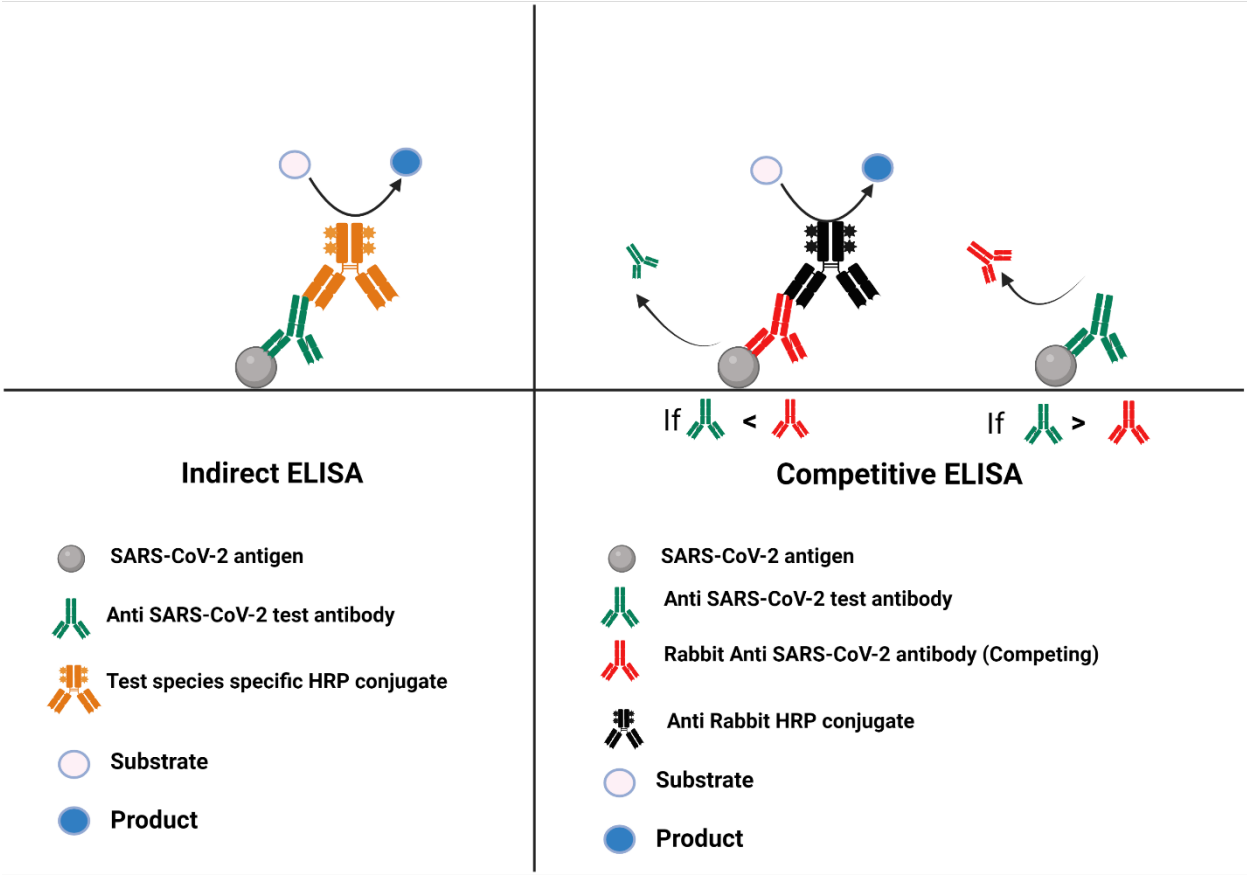

Figure 1.1: Difference between indirect and competitive ELISA. Indirect ELISA includes HRP-conjugated species-specific secondary conjugate while competitive ELISA detects test sample antibody by competing with known rabbit antibodies, which are then measured with anti-rabbit HRP-conjugated antibody

1.5. Egg immunoglobulin (IgY) as diagnostic tool:

Chicken egg yolk immunoglobulin, also known as immunoglobulin Y (IgY), possesses numerous advantages over mammalian IgG in terms of high yield, low cost and convenience, making it preferable over the mammalian counterpart[91] (Fig 1.3). IgY is accumulated in the chicken egg yolk to protect offspring from harmful pathogens[92]. IgY possesses special qualities that are being investigated from several angles to use it for research, diagnosis, and treatment. Therefore, numerous IgYs have been developed against a variety of antigens, including parasites.

IgY is structurally different from mammalian IgG (Fig 1.4). Unlike mammalian

immunoglobulin, IgY molecules have an extra heavy chain constant domain and unique oligosaccharide side chains while lacking a well-defined hinge region[93]. The sequence analysis of IgY and IgG suggests that CH3 and CH4 domains of IgY are closely related to the CH2 and CH3 domains of IgG[94]. Unlike mammalian IgG, IgY does not interact with mammalian complement and Fc receptors, which can avert triggering antibody-dependent enhancement (ADE) of disease and complement mediated adverse inflammatory responses[95]. Therefore, administering neutralizing IgY may be useful for limiting clinical effects in severely symptomatic SARS-CoV-2 infected patients[96]. More importantly, the phylogenetic distance between birds and mammals may allow a stronger immune response against conserved mammalian proteins[97].

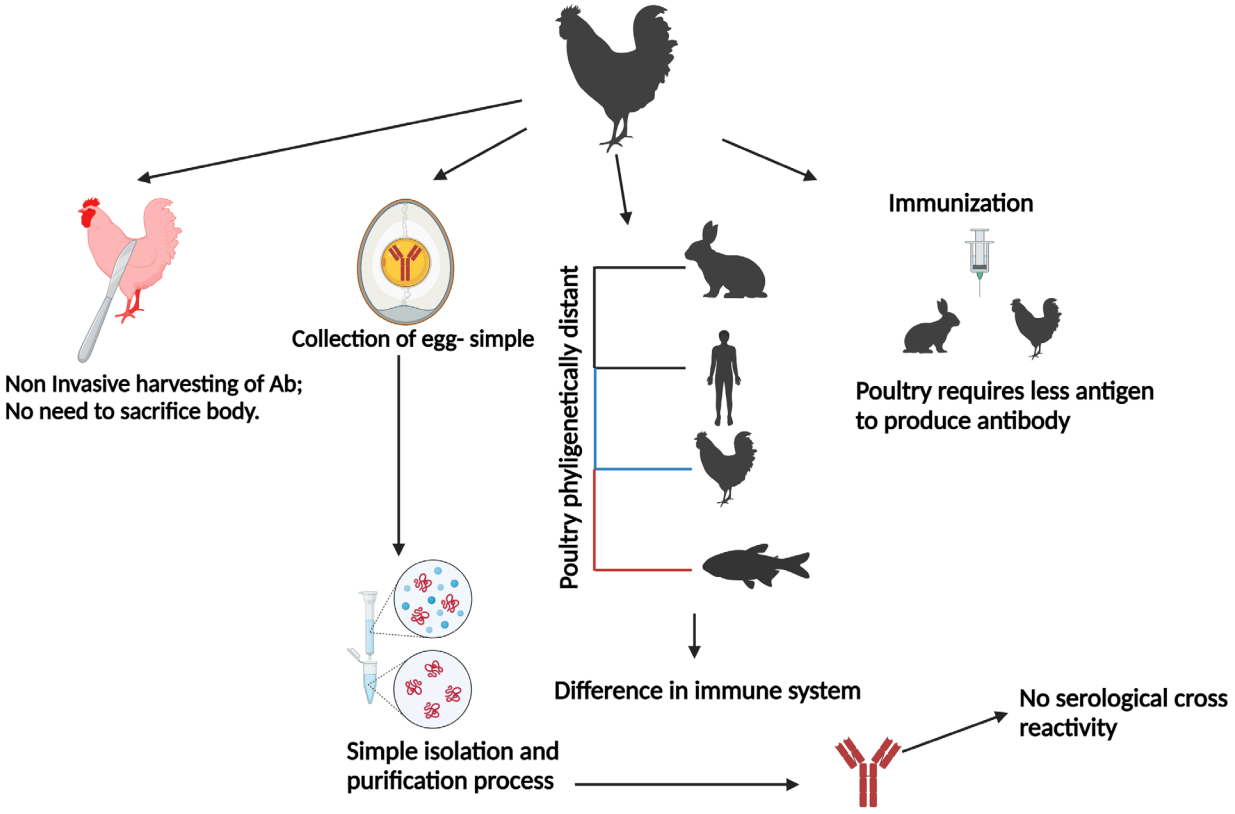

Figure 1.2: Use of hen egg immunoglobulin (IgY)

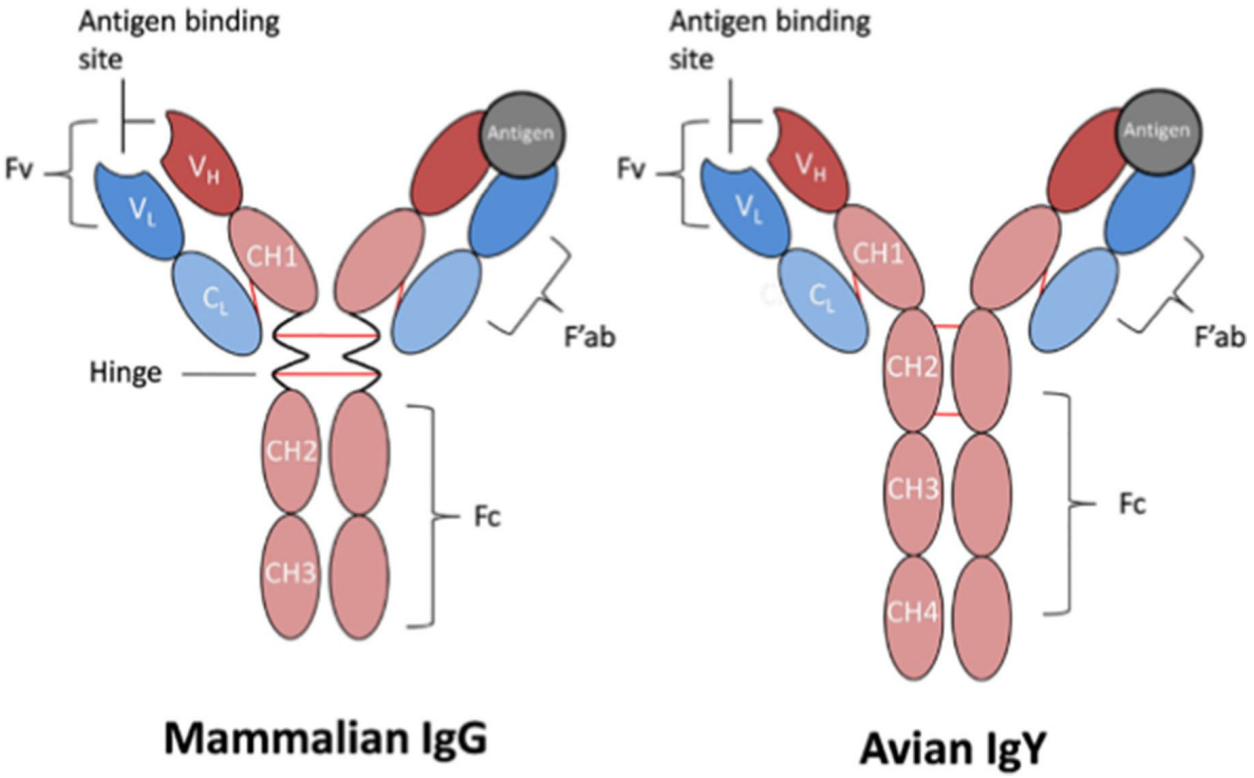

Figure 1.4: Structural differences and similarities between mammalian IgG and avian IgY.

Applying IgY as an alternative source of antibodies for diverse parasitic antigens has already been suggested, due to the ability to recover large quantities of IgY's without the need to bleed animals[98]. In 1985, scientists developed IgY technology to produce *Echinococcus granulosus* specific IgY for use in the serological diagnosis of cystic echinococcosis[98] for the first time. Later, a Chinese research group developed IgY based immune-magnetic bead ELISA to detect circulating schistosomal antigen in sera of mice infected with *S. japonicum*[99,100]. Similarly, another group of scientists developed anti-*S. japonicum*-IgY polyclonal and IgM monoclonal antibodies sandwich ELISA to detect schistosomal antigen and evaluated its sensitivity and specificity against serum samples from patients infected with *S. japonicum*[101]. Besides detecting parasitic exposure, IgY has been used to detect virus exposure. IgY against canine parvovirus virus like particles were used in ELISA in dog fecal samples[102]. E2 protein of bovine viral diarrhea virus specific IgY has been used in ELISA to detect this pathogen in cattle[103]. Furthermore, IgY based direct or competitive ELISAs were developed to detect the exposure of ebola virus[104] and avian influenza virus[72,105]. The main advantage of using IgY in ELISA to detect mammalian antibodies is that the distant phylogenetic relationship between avian and mammals reduces the serological cross reactivity,

leading to increased specificity of developed assays.

Chapter 2
Rationale and Objectives

Rationale and Objectives:

An assay that can be used to detect previous SARS-CoV-2 infection in a wide range of animal species is still not widely available, even though serological assays to detect viral antibodies in humans who have been exposed to the virus were developed early in the pandemic. Therefore, my first objective was to develop and validate a multi-species competitive ELISA to identify and measure SARS-CoV-2 antibody levels in several animal species. Moreover, the newly developed competitive ELISA would target SARS-CoV-2-specific S1, S2 and N antigen specific antibodies. Coronavirus S1 proteins, that contain the receptor binding domain RBD are variable as most neutralizing antibodies are directed against the RBD and surrounding regions. Antibodies directed against the Spike proteins are therefore specific to epidemiologically related viral isolates. By virtue of this nature of Spike proteins to be virus specific, we can differentiate SARS-CoV-2 infection from other closely related sarbecoviruses or betacoronaviruses. In addition to S1, coronavirus virions contain other proteins (including the "N" and "S2" proteins) that induce protective immunity. Antibodies against these proteins do not neutralize virus infectivity and therefore are less likely to be subjected to strong selective pressure. Mutations in S1 region might be a key factor in generating escape mutants and those anti S1 antibodies would be less likely to be detected in antigen-based assays. Therefore, inclusion of whole S protein (S1 and S2) with N would strengthen the ELISA assay in detecting SARS-CoV-2 and its variant specific antibodies in animals.

My second objective was to compare two different types of antigen specific competing antibodies, commercially obtained purified immunoglobulin raised in rabbits (IgG), and in-house purified hen egg yolk immunoglobulins (IgY).

Chapter 3

Development of a novel competitive ELISA to investigate exposure of animals to SARS-CoV-2.

Development of a novel competitive ELISA to investigate exposure of animals to SARS-CoV-2

Authors:

Shubham Dutta, Noreen Rapin, Catherine Soos, Emily Jenkins, Vikram Misra

Authors' contributions:

Shubham Dutta, University of Saskatchewan, Veterinary Microbiology, Method development, testing and screening, procurement of agents for assay development, statistical analysis.

Noreen Rapin, University of Saskatchewan, Veterinary Microbiology, Study design, Method development, testing and screening, procurement of agents for assay development, statistical analysis.

Catherine Soos, Ecotoxicology and Wildlife Health Division, Science & Technology Branch, Environment and Climate Change Canada, Sample procurement, study design, editorial review.

Emily Jenkins, University of Saskatchewan, Veterinary Microbiology, study design, sample procurement, editorial review.

Vikram Misra, University of Saskatchewan, Veterinary Microbiology, supervisor, study design, method development support, statistical analysis, editorial review.

3.1 Abstract:

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus causing COVID-19, is a zoonotic pathogen. There is concern that it may spillover into wildlife species which may then serve as reservoirs for future infection of humans, domestic animals, or other wildlife species. Furthermore, impacts of the virus on potentially susceptible wildlife species are currently not entirely known. There is, therefore, an urgent need to develop a single test that could be used for the serosurveillance of multiple wildlife species for exposure to SARS-CoV-2. Despite having serological techniques to diagnose human patients, few assays have the capacity to detect antibodies in a wide variety of species. Here, we describe the development of a competitive enzyme-linked immunosorbent assay (cELISA) to detect SARS-CoV-2 antibodies in animals for which species-specific reagents are not available. This cELISA was developed to detect SARS-CoV-2 spike 1 (S1), spike 2 (S2) and nucleocapsid (N) specific antibodies and was validated using sera from experimentally infected hamsters. We further validated our cELISA by comparing it with results obtained from the surrogate virus

neutralization test (cPASS, GenScript) and indirect ELISA using anti-hamster horseradish peroxidase (HRP) conjugated reagents. This cELISA will have broad applications in screening potential animal reservoirs for SARS-CoV-2, and uses multiple targets, including more conserved structural proteins which are subjected to less selective immunological pressure. This would allow detection of exposure to variants missed by conventional assays that target antibodies against the viral receptor binding domain. This assay will be a valuable tool which can be implemented in surveillance programs investigating evidence of exposure to SARS-CoV-2 in multiple domestic, captive, or wild animal species.

Keywords: Competitive ELISA, Indirect ELISA, Screening, cPASS, SARS-CoV-2.

3.2 Introduction:

As of February 2023, the COVID19 pandemic has caused 6.8 million human deaths and 600 million confirmed cases ('WHO COVID Dashboard'- https://covid19.who.int/). The virus that causes COVID-19, Severe Acute Respiratory Syndrome coronavirus-2 (SARS-CoV-2) resembles SARS-CoV, which caused a pandemic in 2002-2003, and the Middle East Respiratory Syndrome coronavirus (MERS-CoV), which has caused outbreaks in several countries and is currently a pandemic threat[16,106,107]. All three viruses belong to the genus betacoronavirus[2,108]. The presence of similar viruses in other species suggests that the pandemic viruses might have originated in animals.

Based on the ability of the receptor-binding domain (RBD) on SARS-CoV-2 spike protein to bind host angiotensin-converting enzyme receptor 2 (ACE2) of various species, the virus is predicted to have the capacity to infect several mammalian and avian species[11,12,21,36,109-111]. These predictions are supported by more elaborate computer modelling that includes other factors, such as ecological and biological traits[31,112].

SARS-CoV-2 has been incidentally transmitted from humans to numerous domestic, captive, or wild animals, including commercial mink in numerous countries in Europe, the USA, and Canada[55,63,113,114], captive and free-ranging white-tailed deer (*Odocolieus virginianus*)[8,115,116], free-ranging mule deer (https://cahss.ca/cahss-tools/sars-cov-2-dashboard), domestic cats[5], ferrets, dogs[5,117] and many species in zoos and aquaria (https://www.woah.org/app/uploads/2022/04/sars-cov-2-situation-report-11.pdf,https://www.aphis.usda.gov/aphis/dashboards/tableau/sars-dashboard). Introduction of the virus into non-human animals has led to efficient intra-species transmission with spread in

captive mink[56] and captive and free-ranging white-tailed deer populations[7,8,115,116]. These observations suggest that SARS-CoV-2 may establish in wildlife, leading to adaptive changes with the potential spillback of a virus with novel characteristics into humans. The Omicron variant of the virus may represent such a spillback from mice, supporting this concern[29].

Surveillance of wildlife for evidence of exposure to SARS-CoV-2 and the establishment of potential reservoirs for the virus is challenging. Polymerase chain reaction (PCR) has been used to detect the virus in non-human species[13]. Despite being highly sensitive and specific, experimental data from human samples demonstrate that the window for PCR positivity extends much earlier than two weeks[14]. Serological tests have also been used to detect antibodies in exposed wildlife[7]. Different ELISAs have been established for screening both human and non-human animal serum to detect SARS-CoV-2 specific antibodies[87,88,118,119]. Most of these tests are indirect or indirect sandwich ELISAs[87,88]. This approach is limited to the detection of antibodies in species for which species-specific conjugates are available and cannot be used for most wildlife. While conjugates capable of detecting antibodies from many species, such as the reagent developed to detect exposure to Schmallenberg virus, have been used[87], these reagents are not widely available. The surrogate virus neutralization assay[84], commercialized as 'cPASS test kit' (Genscript™) has also been used to detect exposure to SARS-CoV-2[7]. The test detects neutralizing antibodies to the receptor binding domain (RBD) of the spike 1 (S1) protein from an earlier virus isolate and some of the recent variants of the virus but not the entire S1 or spike 2 (S2) or nucleocapsid (N) proteins. The luciferase immunoprecipitation system (LIPS) targets S and N antigens in their native conformation and detects linear and conformational epitope-specific antibodies[83]. Despite being highly sensitive, LIPS is time consuming and may not accurately detect antibodies against the more conserved SARS-CoV-2 proteins, such as N protein[120].

To overcome some of the limitations of techniques currently used for wildlife surveillance of SARS-CoV-2 exposure, our goal was to develop a competitive ELISA (cELISA) that could detect antibodies from all species. The assay would only require a mechanism for detecting the "competed" (known) antibody and not the "competing" test antibody. In addition to detection of antibodies against the viral RBD, included in the S1, we included the structural proteins S2 and N. These proteins are less likely than the RBD and S1to be subjected to selective pressure in a new host and therefore remain effective targets to detect exposure to an adapted virus.

In this article, we describe the optimization of the test and its validation using serum from hamsters experimentally infected with SARS-CoV-2.

3.3 Methods and Materials:

Sera from uninfected and SARS-CoV-2 infected hamsters were used to optimize and validate the cELISA. Viral proteins (S1, S2 and N) as well as rabbit antisera against the proteins were obtained from commercial sources.

3.3.1 Sample collection:

Hamster serum samples from previous experimental infection studies (Table 1) were obtained from the National Microbiology Laboratory (NML), (Public Health Agency of Canada, Winnipeg). Hamsters were experimentally infected by Wuhan strain of SARS-CoV-2 virus at NML, and their blood was collected on days 0, 56, 81, and 140 post infection (dpi) (Table 1). Samples received included six paired samples from individual hamsters (, *i.e.,* 0 and 56 dpi), and two pooled samples (i.e., 81 dpi and 140 dpi).

Table 3.1: List of hamster serum samples used in developing cELISA

Species	Sample number	Volume	Time of collection
Hamster	31	0.1 mL	Infected (0 dpi†)
Hamster	32	0.1 mL	Infected (0 dpi)
Hamster	33	0.1 mL	Infected (0 dpi)
Hamster	34	0.1 mL	Infected (0 dpi)
Hamster	35	0.1 mL	Infected (0 dpi)
Hamster	36	0.1 mL	Infected (0 dpi)
Hamster	31	0.1 mL	Infected (56 dpi)
Hamster	32	0.1 mL	Infected (56 dpi)
Hamster	33	0.1 mL	Infected (56 dpi)
Hamster	34	0.1 mL	Infected (56 dpi)
Hamster	35	0.1 mL	Infected (56 dpi)
Hamster	36	0.1 mL	Infected (56 dpi)
Hamster	Pooled	0.5 mL	Infected (81 dpi)

Hamster	Pooled	0.5 mL	Infected (140 dpi)

† *(dpi: day/s post infection)*

3.3.2 Optimization of ELISA parameters by Indirect ELISA:

Immulon 4HBX plates (Thermo Scientific 3855) were coated overnight at 4^0C with 50 µL/well of coating buffer (3.03 g Na_2CO_3 + 6.0 g $NaHCO_3$ in 1000 mL distilled water and pH 9.6) containing SARS-CoV-2-S1 protein (Invivogen, #his-SARS2-s1; A soluble fusion protein generated by fusing the full length spike S1 subunit to a C-terminal poly-histidine tag with a 3 amino acid linker) at three different concentrations: 2 µg/mL, 1 µg/mL and 0.5 µg/mL or recombinant Human coronavirus SARS-CoV-2 Spike Glycoprotein S2 (Abcam, #ab274366; expressed in mammalian system with Fc-C terminus tag having >80% purity) at three different concentrations: 0.4 µg/mL, 0.2 µg/mL and 0.1 µg/mL or soluble SARS-CoV-2-N protein fused to a poly-histidine tag (Invivogen, #his-SARS2-n; A soluble fusion protein generated by fusing the full length nucleocapsid subunit to a C-terminal poly-histidine tag with a 3 amino acid linker) at concentrations of 0.25 µg/mL, 0.12 µg/mL and 0.06 µg/mL. The next day, wells were washed three times with 300 µL/well wash buffer (PBS-Tween) (phosphate buffered saline (PBS) with 0.05% Tween-20) using Bioplex ProII plate washer (BioRad) and blocked for 1 hour at 37^0C with 200 µL/well PBS containing 1% BSA (Fisher BP1600-100 Fraction V) + 0.05% Tween-20. Following three PBS-Tween washes, anti-SARS-CoV-2 spike protein S1 rabbit polyclonal antibody (ThermoFisher PA5114528; stock concentration: 1 mg/mL), anti-SARS-CoV-2 spike protein S2 rabbit monoclonal antibody (Abcam, #ab283913; stock concentration: 1 mg/mL) and anti-SARS-CoV-2 protein N rabbit polyclonal antibody (ThermoFisher PA581794; stock concentration: 1 mg/mL) diluted in blocking solution (See Figure 1) were added (100 µL/well) and incubated for 1 hour at 37^0C. The plates were then washed three times with PBS-Tween and anti-SARS-CoV-2 antibodies were detected using horse radish peroxidase (HRP) conjugated goat anti-rabbit IgG (Abcam, #ab7090) diluted in blocking solution (1: 50000), 100 µL/well and incubated for 1 hour at 37^0C. Following an additional three PBS-Tween washes, wells were incubated with 100 µL of tetramethylbenzidine substrate (TMB) (ThermoFisher P134021) for 15 min at room

temperature. Enzyme reactions were terminated by adding 100 µL 2 N sulfuric acid. Optical density (OD) was measured at 450 nm using a Varioskan microplate reader (ThermoFisher Scientific, USA). The optimal parameters were decided by evaluating the ratio of absorbance of specific (absorbance from the binding of S1, S2 and N antigen to anti-S1, anti-S2 and anti-N antibodies respectively) to non-specific (absorbance from the binding of S1 to anti-S2 and anti-N, S2 to anti-S1 and anti-N, N to anti-S1 and anti-S2) antigen-antibody binding.

3.3.3 Development of competitive ELISA:

As described above, plates were coated overnight at 4^0C with 1 µg/mL of soluble SARS-CoV-2 -S1 protein, 0.4 µg/mL recombinant SARS-CoV-2 spike glycoprotein S2- and 0.06 µg/mL soluble SARS-CoV-2-N protein diluted in coating buffer. The next day, wells were washed three times using 300 µL/well wash buffer and blocked for 1 hour at 37^0C using blocking solution. Following three washes, hamster serum samples diluted in blocking solution (1: 20; Dilution of hamster serum was optimized by another cELISA where hamster serum was serially diluted from 1:10 to 1:10240), were added (100 µL /well) and incubated for 1 hour at 37^0C. The plates were then washed three times and SARS-CoV-2-S1 rabbit polyclonal antibody, SARS-CoV-2-S2 rabbit polyclonal antibody and SARS-CoV-2-N rabbit monoclonal antibody diluted in blocking solution (1: 4000, 1: 20000 and 1: 5000 respectively) 100 µL /well was added to corresponding wells for 1 hour at 37^0C. The plates were then washed three times and known anti-SARS-CoV-2 antibodies were detected using HRP conjugated goat anti-rabbit IgG diluted in blocking solution (1: 50000, 100 µL/well) for 1 hour at 37^0C. Following an additional three washes, wells were incubated with 100 µL of TMB substrate for 15 min at room temperature. Enzyme reactions were terminated adding 100 µL/well 2 N sulfuric acid. Optical density (OD) was measured at 450 nm using a Varioskan microplate reader (Thermo Scientific).

3.3.4 Surrogate virus neutralization test:

The level of virus neutralizing antibodies was estimated using a commercial kit (cPASS, Genscript) according to the manufacturer's instruction and OD was measured at 450 nm using a Varioskan plate reader (Thermo Fisher Scientific, USA).

3.3.5 Indirect ELISA:

As described above for cELISA, plates were coated, blocked, and washed. Test serum was added (1:20) and antibody binding was detected using HRP conjugated goat anti-hamster IgG

(Abcam, #ab6892).

3.3.6 Statistical Methods:

Calculation of percent inhibition for cELISA by the formulae: *(average absorbance of negative sample – average absorbance of test sample)/ average absorbance of negative sample *100.*The cut-off value of percentage inhibition for positive/negative discrimination was determined by the following formula: *mean of known negative 0 dpi samples + (3 x standard deviation of known negative day 0 samples)*[121].

3.4 Results and Discussion:

Optimum concentrations of plated antigen and primary antibody were established by incubating various amounts of all three proteins with increasing dilutions of the three rabbit antisera (Figure 3.1). Antibody binding was detected using HRP conjugated anti-rabbit IgG. Combinations of antigen and antibody that gave the highest ratio of specific to non-specific binding were selected for further use in the cELISA. For S1, N and S2, optimum coating concentrations were determined as 1 µg/mL, 0.06 µg/mL, and 0.4 µg/mL respectively (Figure 3.1 A, B, C). Similarly, S1, N and S2 specific antibody dilutions were standardized at 1:4,000, 1:5,000 and 1:20,000 respectively (Fig 3.1 A, B & C). Serial dilution of hamster serum sample showed dilution effect in measuring inhibition against N (from 1: 20) and S1-specific antibodies. S2-specific inhibition could not be tested at different dilution of hamster serum as S2-antigen was commercially not available during that experiment. For the cELISA test serum was diluted 1/10 or 1/20 (Fig 3.1 D)

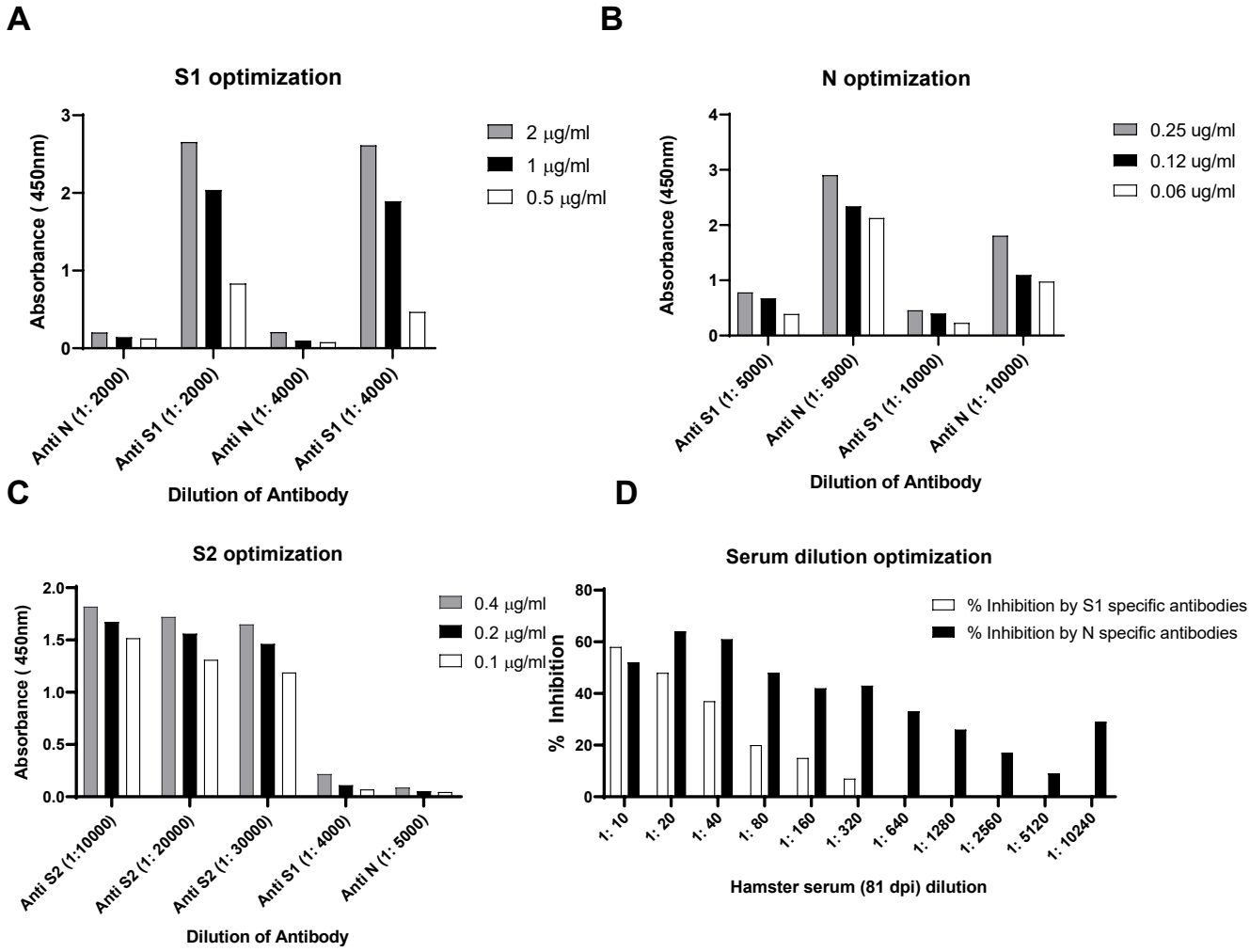

Fig 3.1: *Optimization of antigen, rabbit antibody, and sample dilution (A) Optimal SARS-CoV-2-S1 coating antigen concentration was determined by Indirect ELISA to be 1 µg/mL with 1: 4,000 dilution of rabbit anti S1 antibody. (B) Optimal SARS-CoV-2-N coating antigen concentration was determined by indirect ELISA to be 0.06 µg/mL with 1: 5,000 dilution of rabbit anti N antibody. (C) Optimal SARS-CoV-2-S2 coating antigen concentration was determined by Indirect ELISA to be 0.4 µg/mL with 1: 20,000 dilution of known rabbit anti S2 antibody. (D) Optimal test antisera (experimentally infected hamster antisera) dilution was determined by cELISA to be 1: 20..*

Six paired individual (0 and 56 dpi) and two pooled (81 and 140 dpi) hamster serum samples were tested by cELISA for the presence of antibodies to inhibit the binding of rabbit antibody against S1, N and S2 proteins. All sera collected at 56 dpi, as well as the pooled sera collected

at 81 and 140 dpi, inhibited binding of rabbit antibodies to S1 (Figure 3.2A). While four of the six sera collected at 56 dpi inhibited binding of rabbit antibodies to N beyond the cut-off (Figure 3.2B), the degree of inhibition was low (< 20%). However, samples collected 81 and 140 dpi displayed high levels of inhibition. All 56 dpi samples, as well as the positive control (pooled 81 and 140 dpi) inhibited binding of rabbit antibodies to S2 (Figure 3.2C).

These data indicate that the cELISA can reliably detect antibodies against S1 and S2 in infected hamsters 56 dpi. High levels of antibodies against N may be delayed until later in infection.

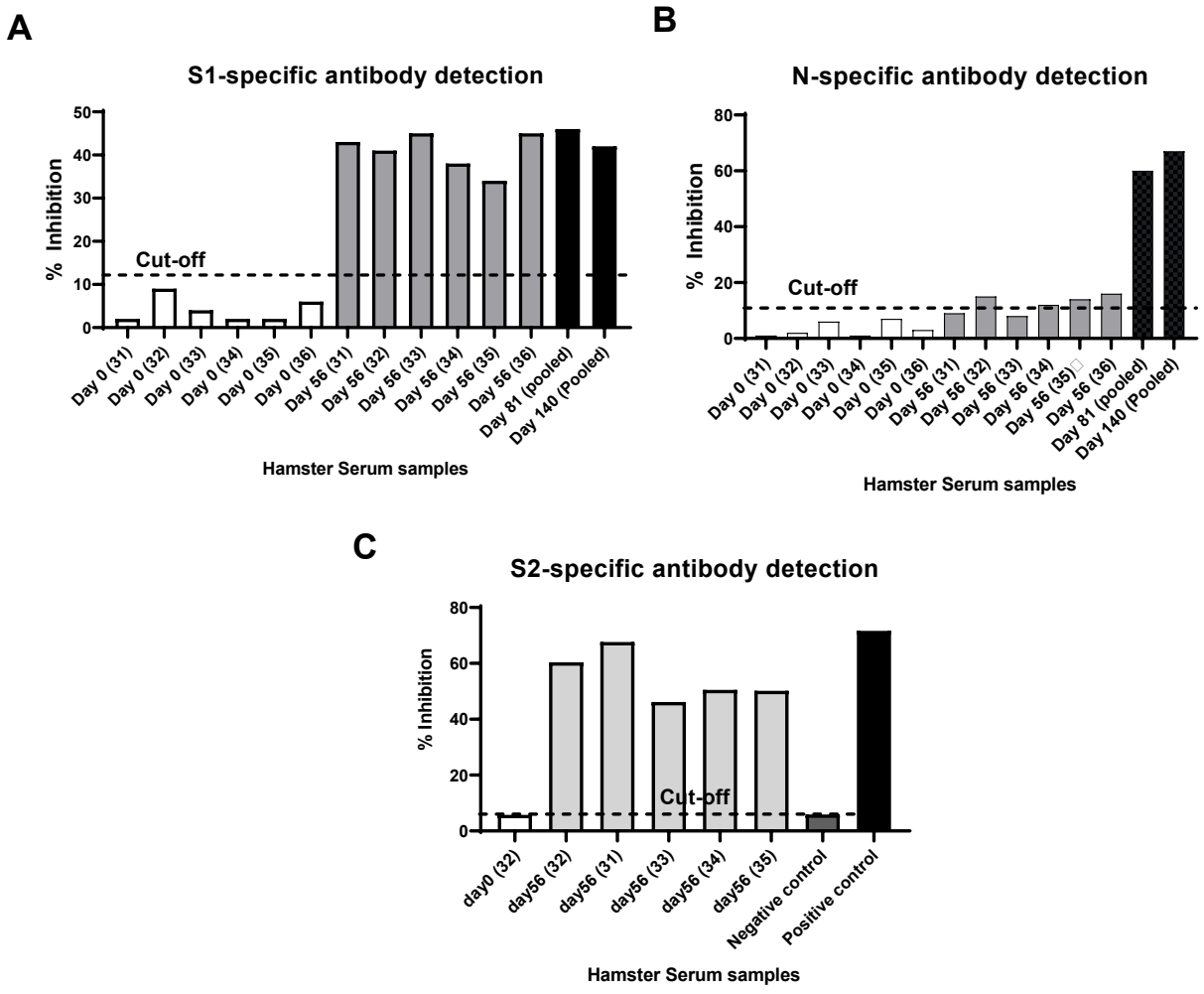

Fig 3.2: Competitive ELISA to detect S1, S2 and N specific antibodies in experimentally infected hamster serum. *In all three figures, the black dotted line denotes cut-off percent inhibition. (A) SARS-CoV-2-S1 specific antibodies were detected in samples from 56, 81 and*

140 dpi, but not dpi 0. (B) SARS-CoV-2-N specific antibodies were detected in samples collected at 81 and 140 dpi, but not reliably at dpi 56. (C) SARS-CoV-2-S2 specific antibodies were detected in samples collected at dpi 56 and pooled samples collected at dpi 81 and 140 denoted as "positive control". Negative control is pooled dpi 0 hamster samples. normal hamster serum.

The virus neutralization assay is the "gold standard" in serology[122]. However, performing this technique would require a higher biosafety level lab facility (BSL-3 or BSL-3 advanced) than most labs are classified. The surrogate virus neutralization assay, cPASS, allows more flexibility to work within BSL-2 labs. However, the original version of the kit only measures antibodies against the RBD in S1 of earlier isolates and could miss antibodies against later viral variants.

The results of the S1 specific cELISA data corroborated those of the cPASS test (Fig 3.3A) as well as indirect ELISA (Fig 3.3 B), where convalescent samples (dpi 56, 81 and 140) showed relatively higher percent inhibition than dpi 0 samples by cPASS and higher absorbance by indirect ELISA using hamster specific enzyme conjugate as detection antibody. The cELISA results for S2 also corroborated those of the indirect ELISA (Fig 3.3C). In contrast, while the cELISA detected only low levels of antibodies against N at 56 dpi, the indirect ELISA detected antibodies in these samples at levels like later collection dates (Fig 3.3D). Possibly, this non-agreement between cELISA and indirect ELISA to detect N-specific antibodies in dpi 56 samples might be the consequence of a lack of epitope specific competition between test and known antibodies. In indirect ELISA, anti-hamster HRP-conjugate will detect all antibodies that interact with multiple epitopes of N-antigen, whereas, in cELISA only detects antibody that binds to the same epitopes as the rabbit antibody. If, during the immune response the antibody specificity repertoire evolves to be more like the rabbit repertoire, then percent inhibition will increase.

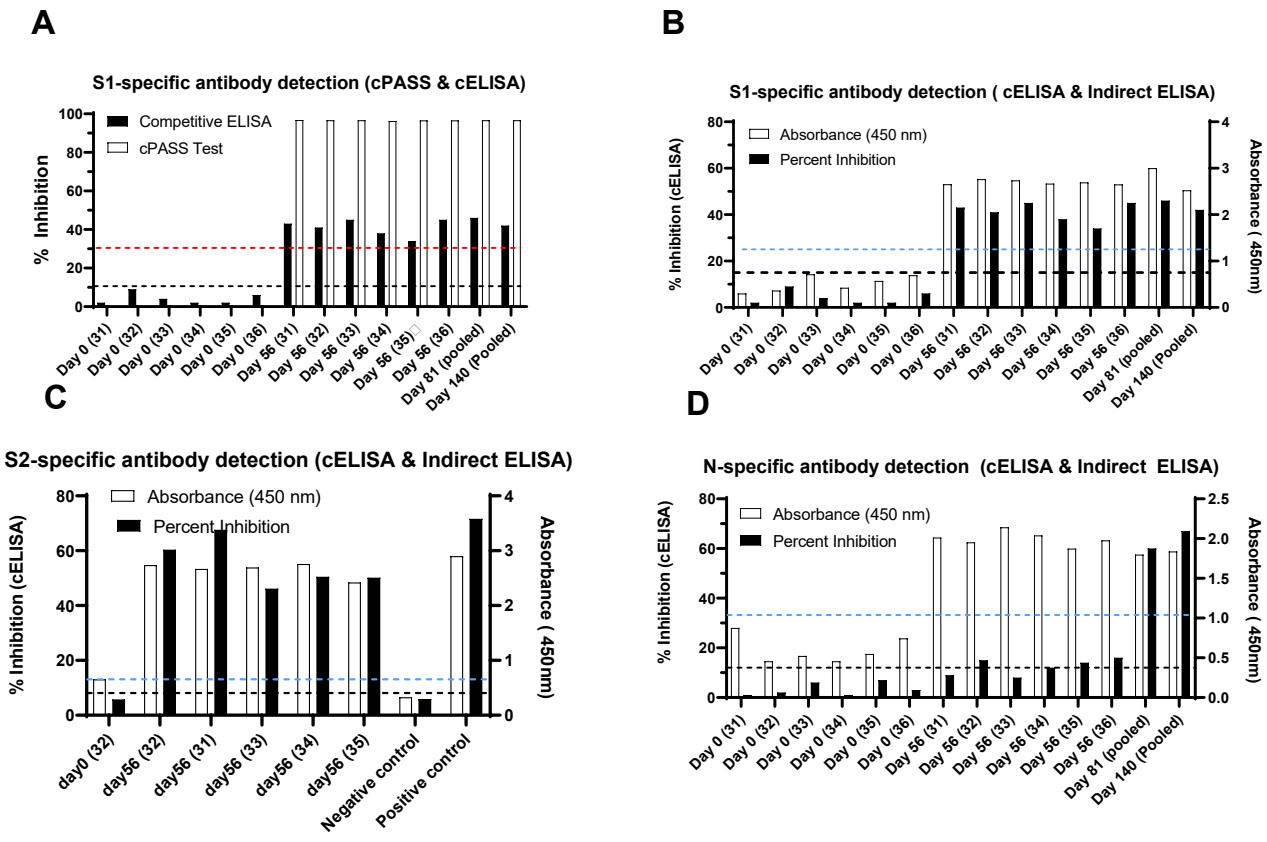

Fig 3.3. *Comparison of the SARS-CoV-2 S1, S2 and N specific cELISA to other antibody detection assays using experimentally infected hamster sera. For the cELISA, cutoff is indicated as the black dotted line in all figures. (A) Comparison of the S1 cELISA with cPass. Red dotted line indicates the manufacturer recommended 30% inhibition cutoff for cPass. (B) Comparison of cELISA for S1 with an indirect ELISA. (C) Comparison of cELISA for S2 with indirect ELISA. (D) Comparison of cELISA for N with indirect ELISA. In B, C and D, the indirect ELISA absorbance cutoff is indicated as blue dotted line. Here, 'Negative control' denotes pooled dpi 0 samples, and 'Positive control' denotes pooled dpi 81 and dpi 140 samples.*

This study describes a novel competitive ELISA developed and validated using known positive and negative hamster sera. However, to establish the specificity and sensitivity of the test, a greater number of known positive and negative samples should be tested. Based on the limited hamster antisera screening data, specificity and sensitivity of the test were determined to be 100 % for S1 and S2 cELISA. While anti-N antibody was detected with 60-70%

specificity and sensitivity. We propose to further validate the test in epidemiologic investigations and surveillance of wildlife populations for exposure and/or establishment of SARS-CoV-2 and its variants.

The major limitation of this study was the limited sample size of hamster serum that restricted from running the greater number of biological replicates to get more accurate inter-assay statistical significance. Work is underway to further validate the assay and determine cut-off points, sensitivity, and specificity for multiple species of domestic and wild animals, using sera from experimentally infected, naturally infected, or vaccinated animals. We are currently validating the assay for use on other types of samples commonly collected from live or dead wildlife, including plasma, filter paper strips soaked in blood (e.g., Nobuto strips), thoracic fluid, and heart blood, to broaden the utility of this assay. This assay will be a valuable tool to be implemented in surveillance programs investigating evidence of exposure to SARS-CoV-2 in multiple domestic, captive (zoo), or wild animal species, in studies investigating impacts of SARS-CoV-2 on wildlife populations, and in studies examining wildlife populations for the potential to become reservoirs for SARS-CoV-2.

3.5 Ethics statement:

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. Animal studies at the National Microbiology Laboratory were conducted following the Canadian Council of Animal Care guidelines and in accordance with an animal use document approved by the Canadian Science Centre for Human and Animal Health's institutional animal care and use committee. Potentially infectious materials were inactivated according to approved procedures for subsequent analysis. Studies at the University of Saskatchewan were conducted under biosafety permit no. VMB-03.

3.6 Conflict of interest:

None

3.7 Acknowledgement:

This study was funded by an Alliance-COVID-19 grant from the Natural Sciences and

Engineering Research Council (NSERC) of Canada awarded to VM, EJ, Darryl Falzarano and Christy Morrissey, with support from Environment and Climate Change, Canada, and Public Health Agency of Canada. Serum samples from SARS-CoV-2 infected hamsters were kindly provided by Drs. Robbin Lindsay, David Safronetz and members of the One Health Section of the National Microbiology Laboratory of Canada, Winnipeg. The authors also acknowledge the editorial and other assistance by Dr Sonu Subudhi.

Transition Statement

Having developed and applied a new cELISA in Chapter 3, in Chapter 4, I address my second thesis objective, to further optimize these assays by using IgY instead of rabbit IgG. I am presuming that hen IgY would increase the cELISA specificity due to long phylogenetic distance between avis and mammals.

This chapter is presented as a manuscript intended for publication. This manuscript was developed by all below said authors in which SD and NR are the joint first author and VM, NR and SD contributed to designing experiments; NR and SD did the sampling, lab work, and data analysis; VM, NR and SD interpreted the data and SD wrote the manuscript that was edited by NR, VM, EJ and CS. SG and SP helped us by arranging hens for immunization.

Chapter 4

Development of a chicken egg yolk IgY antibody based competitive ELISA to detect SARS-CoV-2-specific antibodies.

Development of a chicken egg yolk IgY antibody based competitive ELISA to detect SARS-CoV-2-specific antibodies

Authors:

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Authors' contributions:

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Catherine Soos, Ecotoxicology and Wildlife Health Division, Science & Technology Branch, Environment and Climate Change Canada, Sample procurement, study design, editorial review.

Emily Jenkins, University of Saskatchewan, Veterinary Microbiology, study design, sample procurement, editorial review.

Shelly Popowich, University of Saskatchewan, Veterinary Pathology, chicken immunization study support

Susantha Gomis, University of Saskatchewan, Veterinary Pathology, Chicken immunization study support

Vikram Misra, University of Saskatchewan, Veterinary Microbiology, supervisor, study design, method development support, statistical analysis, editorial review.

4.1. Abstract:

As the COVID-19 pandemic spreads through human populations with devastating effects, there is increasing evidence that the virus can infect and establish in free living wildlife. These animals could then serve as a reservoir, from which animal adapted mutations of the virus may subsequently spill back into humans. The ability to identify exposure to severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) or related coronavirus infection and define the acquired immune response across non-human animal population is essential for understanding virus

transmission. Therefore, to detect virus-specific antibodies across a wide variety of animal species, we have developed a competitive ELISA (cELISA) using rabbit sera against three antigens: spike 1 (S1), spike 2 (S2) and nucleocapsid (N) of SARS-CoV-2. However, rabbit antibody is expensive and invasive to obtain. In addition, there is a possibility of cross-reactivity between the anti-rabbit detection antibody with other mammalian antibodies. Therefore, we explored the use of antibodies produced in hen eggs (IgY) as a substitute for rabbit sera. Hens were immunized against SARS-CoV-2 antigens: S1, S2 and N. IgY antibodies were purified, and the assay was optimized for sensitive and specific detection of antiviral S2 antibodies in animals. Among S1, S2 and N-IgYs , only S2-IgY based cELISA was specific and compared well with both rabbit anti serum based cELISA and surrogate virus neutralization test (cPASS).
Keyword: COVID-19, Competitive ELISA, IgY, Antigens, cPASS

4.2. Introduction:

Coronavirus infectious disease-19 (COVID-19) has had a serious impact on the global population and economy[123] as global Gross Domestic Product (GDP) declined by 6.7 percent during the pandemic according to a survey in 2020 (https://www.statista.com/statistics/1240594/gdp-loss-covid-19-economy/). To date, there have been more than 600 million confirmed human cases with 6 million deaths worldwide. (https://covid19.who.int/). Control measures have been taken to reduce mortality, including the development of safe and effective vaccines [124,125]. However, the COVID-19 pandemic continues to persist in human populations with the introduction of new variants of concern and devastating effects. While the virus appears to be established in humans, there are incidents of intra-species transmission among captive and wild mink[55,56,63] and white-tailed deer [7,8,115,116]. Animals can serve as sources of virus infection to human population. Moreover, the virus can mutate and spill back into humans[18,56]. In addition, mutation of the virus in any reservoir species can increase the emergence of neutralization escape variants[126,127] which means viral variants, emerged through specific mutations, could evade neutralization antibodies. There is circumstantial evidence suggesting that the omicron variant may have evolved in mice before introduction to humans[29].

Molecular and serological surveillance of different animal species to identify potential reservoir populations for SARS-CoV-2 and related coronaviruses is urgently needed in this and future pandemics. Quantitative polymerase chain reaction (q-PCR) is the most accurate way to

detect viral RNA from exposed patients or animals[13]. However, viral RNA in a host is usually detectable for a short period of time[14]. Therefore, serological tests have been used to detect antibodies as a measure of exposure in wildlife due to the long persistence of antibody[128]. Most of the serological assays are indirect ELISA or indirect sandwich ELISA that rely upon species-specific enzyme conjugates[87,88]. These approaches are limited to the detection of antibodies in species for which species-specific conjugates are available. A reagent was developed to detect antibodies in multiple species by indirect ELISA[87] but is not broadly available. In addition, other assays such as surrogate virus neutralization assay[84] and luciferase immunoprecipitation system[83] have limited utility to detect evolving variants, as they target the highly variable receptor binding domain of S1 or have less sensitivity to detect the more conserved N protein of SARS-CoV-2. To overcome some of these limitations, we developed a competitive ELISA (cELISA) that could detect antibodies from a wide range of animal species. Unlike indirect ELISAs, cELISAs do not require 'test species'-specific secondary conjugate, which simplifies the screening procedure. Even our newly developed cELISA has some limitations, however, as detecting mammalian antibodies using a mammalian conjugate might reduce the specificity of the assay. Also, developing commercial rabbit antibodies is costly. Therefore, we explored the utility of chicken egg yolk immunoglobulins (IgY) in developing a cELISA[96,129-133].

Egg yolk antibodies may be generated without significant suffering or discomfort to the animal. Collecting eggs is a non-invasive process compared to collecting serum from animals. Unlike mammalian immunoglobulin, IgY molecules have an extra heavy chain constant domain and unique oligosaccharide side chains while lacking a well-defined hinge region. These structural differences from mammalian IgG make IgY less cross-reactive with mammalian test antibodies[93].

Therefore, we explored the use of IgY in our cELISA to detect antibodies from mammals to three viral structural proteins: 2 spike protein subunits (S1 and S2) and the nucleocapsid (N) protein. S1 has the receptor binding domain (RBD) which contains most of the neutralizing epitopes [134]. Since there are structural constraints on the RBD, neutralizing epitopes in adjacent regions in S1 are likely to be under selective pressure. Tests that detect antibodies to S2 and N, which are less likely to be subjected to selective pressure than S1, are more likely to detect exposure to viral variants. Here, we developed an IgY-based cELISA directed against S1, S2, and N, and compared it with a rabbit serum based cELISA (Rabbit cELISA) and the commercial cPass test for detecting SARS-CoV-2 antibodies in several mammalian species.

4.3. Methods and Materials:

4.3.1. Immunization of hens:

Nine 'Ross-308' broiler breeder hens (three hens per pen, per immunogen) from Aviagen, were immunized with 10 µg of SARS-CoV-2- N (Invivogen, #his-SARS2-n), SARS-CoV-2-S1 (Invivogen, #his-SARS2-S1) or SARS-CoV-2-S2 (Abcam, #ab274366) antigen mixed with 20 % Emulsigen adjuvant (Phibro Animal Health Corporation, USA, #PVO-ADJV-0020). Hens were first immunized when they were 13 weeks old, and they were revaccinated with the same antigen-adjuvant complex 4 weeks and 9 weeks post original vaccination. During first boost (4^{th} Week after immunization), before second boost (8^{th} week after immunization), during second boost (9^{th} week after immunization), after second boost (12^{th} week after immunization) and during egg laying period (20^{th} week post immunization and 29^{th} week post immunization), we collected blood and separated serum by centrifugation before storing the serum at -20^0 C until analyzed. Eggs were collected from pens of immunized hens after they started laying eggs at 20 weeks post immunization.

4.3.2. Extraction, purification, and detection of egg yolk IgY:

IgY antibodies were extracted from more than 80 (including all S1, S2 and N-immunized eggs) immunized chicken egg yolks using Pierce™ Chicken IgY Purification Kit (Thermofisher Scientific #44918). After separating the yolk from the egg white using an egg separator, yolks were rinsed in distilled water and transferred to a paper towel to remove the remaining egg white. The yolk sac membrane was punctured over a 100 mL beaker and the contents of the yolk were mixed with five times the egg yolk volume of delipidating buffer (from kit) for 2 min with a magnetic stirrer followed by overnight incubation at 4^0 C. The yolk/buffer mixture was then centrifuged at 10000 x g for 15 min at 4°C. The supernatant was transferred to another beaker before adding an equal volume of precipitation buffer (from kit) followed by 2 hours of incubation at 4^0 C temperature. The solution was centrifuged at 10000 x g for 15 min at 4^0 C. The supernatant was discarded, and the pellet was dissolved in a volume of phosphate-buffered saline (PBS) equivalent to yolk volume. A sample was diluted in blocking buffer at 1: 20 , and IgY concentration was calculated by measuring the absorbance at 280 nm using a NanoDrop spectrophotometer

(INSERT SOURCE).using the formula (according to kit): A_{280}/1.4 X 20. Purified IgY antibody was aliquoted and stored at -80^0 C. The protein bands of the purified IgY were separated using 12% SDS-PAGE gel electrophoresis (10 µg/lane), visualized by Coomassie blue staining and compared to a commercial IgY protein (Abcam, #ab50579) along with delipidated yolk solution.

4.3.3. Detection of antigen specific IgY by indirect ELISA:

After assessing the purity of the purified IgY preparations, we did indirect ELISA to determine the specificity of purified S1, S2 and N-IgYs to S1, S2 and N antigen respectively. Immulon 4HBX plates (Thermofisher scientific,#3855) were coated overnight at 4^{0}C with 50 µL/well of coating buffer (3.03 g Na_2CO_3 + 6.0 g $NaHCO_3$ in 1000 mL distilled water and pH 9.6) containing either SARS-CoV-2-S1 protein fused to a poly-histidine tag (Invivogen, #his-SARS2-s1) at 2 µg/mL, 1 µg/mL, 0.5 µg/mL and 0.25 µg/mL, or recombinant Human coronavirus SARS-CoV-2 Spike Glycoprotein S2 (His tag) (Abcam, #ab274366) at 0.4 µg/mL, 0.2 µg/mL, 0.1 µg/mL and 0.05 µg/mL. Also, SARS-CoV-2-N protein fused to a poly-histidine tag (Invivogen, #his-SARS2-n) was coated at 0.06 µg/mL for an indirect ELISA experiment (Fig 4.3). The next day, wells were washed three times with 300 µL/well wash buffer [phosphate buffered saline (PBS) with 0.05% Tween-20] using Bioplex ProII, BioRad plate washer (BioRad, CA, USA) and blocked for 1 hour at 37^{0}C with 200 µL/well PBS containing 1% BSA (Fisher BP1600-100 Fraction V) + 0.05% Tween-20. Following three PBS-Tween washes, purified IgY specific to S1, S2 and N diluted in blocking solution was added, washed and incubated 100 µL/well and incubated for 1 hour at 37^{0}C. The plates were then washed three times with PBS-Tween and anti-SARS-CoV-2 IgYs were detected using horseradish peroxidase (HRP) conjugated rabbit anti-chicken IgY (Abcam, #ab6753) diluted in blocking solution (1: 50000), 100 µL/well and incubated for 1 hour at 37^{0}C. Following an additional three PBS-Tween washes, wells were incubated with 100 µL of tetramethylbenzidine substrate (TMB) (Fisher P134021) for 15 min at room temperature in the dark. Enzyme reactions were terminated by adding 100 µL 2N sulfuric acid. Optical density (OD) was measured at 450 nm using a Varioskan microplate reader (ThermoFisher Scientific, Waltham, Massachusetts, USA). The optimal parameters were determined based on the highest signal (or absorbance) ratio from specific to non-specific antigen-antibody binding.

4.3.4. Determination of optimal working concentrations of antigen and antibody for

developing IgY based cELISA:

To establish optimum amounts of antigen and antibody to use in cELISAs, we assessed various combinations of antigen and IgY combinations. The goal was to determine combinations of antigen and antibody concentrations that gave the highest ratio of specific to non-specific binding on indirect ELISAs. As described above, plates were coated overnight at 4^0C with 1 µg/mL of soluble SARS-CoV-2 -S1 protein, 0.4 µg/mL recombinant SARS-CoV-2 spike glycoprotein S2, and 0.06 µg/mL soluble SARS-CoV-2-N protein diluted in coating buffer. The next day, wells were washed three times using 300 µL/well wash buffer and blocked for 1 hour at 37^0C using blocking solution. Following three washes, known positive hamster and/or human serum (previously exposed to virus) was diluted in blocking solution (1: 20) at 100 µL /well and incubated for 1 hour at 37^0C. Purified IgY specific to S1, S2 and N diluted in blocking solution (antibody concentration: 1 µg/mL) were added to plate, washed and incubated. The plates were then washed three times and known anti-SARS-CoV-2 antibodies were detected using HRP conjugated anti-chicken IgY (diluted in blocking solution (1:50000, 100 µL/well) for 1 hour at 37^0C. Following an additional three washes, wells were incubated with 100 µL of TMB substrate for 15 min at room temperature. Enzyme reactions were terminated by adding 100 µL/well 2N sulfuric acid. Optical density (OD) was measured at 450 nm using a Varioskan microplate reader.

4.3.5. IgY cELISA

Plates were coated overnight at 4^0C with 1 µg/mL of soluble SARS-CoV-2 -S1 protein and 0.4 µg/mL recombinant SARS-CoV-2 spike glycoprotein S2 antigen, diluted in coating buffer. The next day, wells were washed three times using 300 µL/well wash buffer and blocked for 1 hour at 37^0C using blocking solution. Following three washes after blocking, experimentally infected hamster serum samples were serially diluted in blocking solution from 1: 10 to 1: 160. Diluted hamster samples were added (100µL/well) and incubated for 1 hour at 37^0C. After three times wash, SARS-CoV-2 protein S1-specific chicken egg yolk (IgY) antibodies and SARS-CoV-2 protein S2-specific chicken IgY antibodies which were diluted in blocking solution at a final concentration of 1µg/mL, were added (100 µL /well) before incubating the plates for 1 hour at 37^0C. The plates were then washed three times and known anti-SARS-CoV-2 antibodies were detected using HRP conjugated goat anti-rabbit IgG diluted in blocking solution (1: 50000, 100 µL/well) for 1 hour at 37^0C. Following an additional three washes, wells were incubated with 100 µL of TMB substrate for 15 min at room temperature. Enzyme reactions were terminated adding

2 N sulfuric acid (100 µL/well). Optical density (OD) was measured at 450 nm using a Varioskan microplate reader (Thermo Scientific,USA). Percentage of inhibition from cELISA was calculated by the following formula: *(mean absorbance of no serum control)- (mean absorbance of test sample)/mean absorbance of no serum control x 100.*

4.3.6. Comparison of the IgY-based cELISA to the rabbit cELISA for detection of antibodies to S2

We compared the IgY based cELISA with the rabbit antibody-cELISA (described in Chapter 3) using sera from various species that were either negative or positive for antibodies against SARS-CoV-2 using the commercial cPass test which detects antibodies against the receptor binding domain on the S1 spike protein. The cPASS kit was used according to the manufacturer's (Genscript^{TM}) instruction and OD was measured at 450 nm using a Varioskan plate reader. Sera used were from controlled experiments, clinical human samples, or wildlife surveillance studies (Table 4.1). The cut off was set to 30% inhibition by including the cPASS negative samples' intra-assay standard deviation and inhibition percentage, implied to Classen's formula[121] : for calling a sample positive or negative. Classen's formula includes the percentage inhibition of known negative samples. Variable sample size of negative sera in different assays might change the cut-off percentage.

4.3.7. Time dependent comparison of antibody response in chicken serum

Out of all nine chickens, three chickens were allocated in single pen, immunized by SARS-CoV-2-S1, S2 or N antigen with adjuvant. Taking the first immunization as zeroth day, time dependent antibody response was observed in the serum those were collected at certain timepoints (See Fig 4.1 A) by cELISA. Furthermore, specificity ratio for each immunogen specific immune response was determined (See Fig 4.1 B).

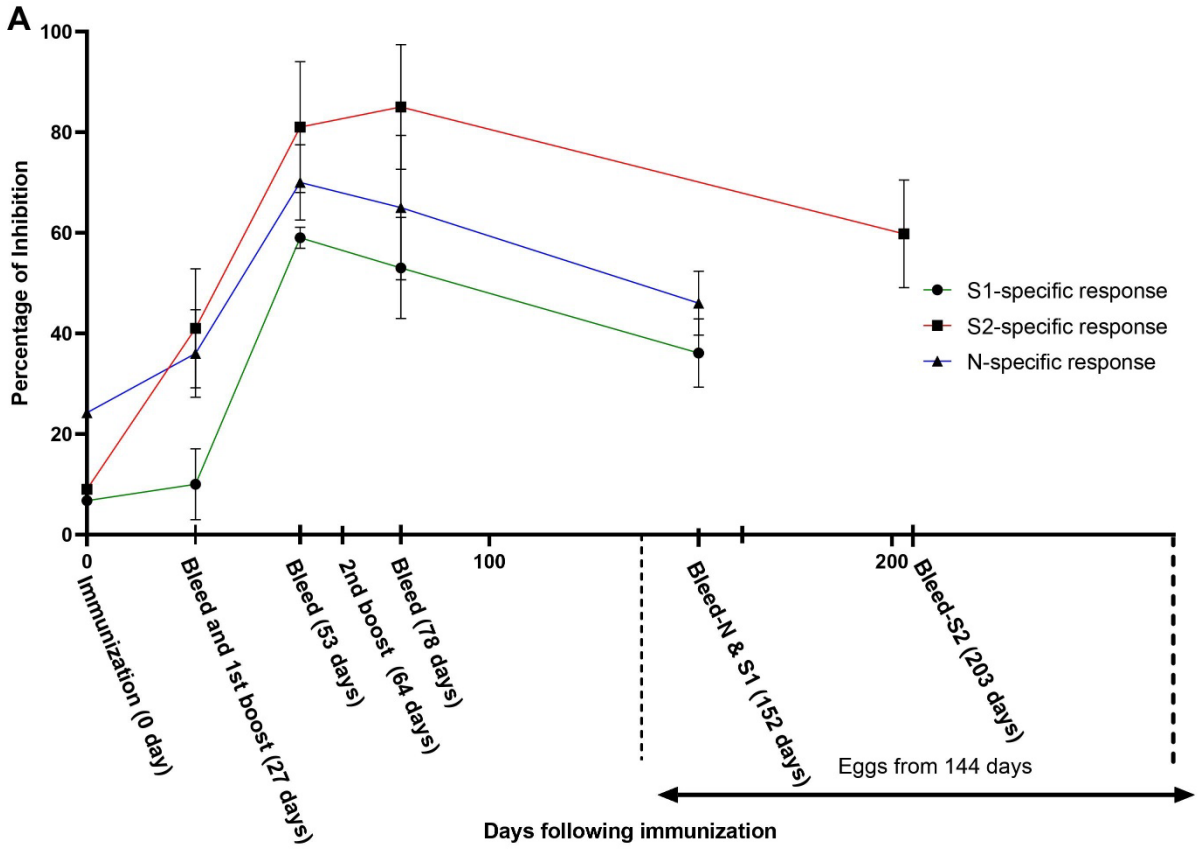
4.3.8. Statistical Methods:

Each serum sample was tested in triplicate and the mean value was calculated in Excel (Microsoft)). The data were analysed with Graphpad Prism Software (La Jolla, CA, USA) and Excel for determining statistical significance (T-test) and standard deviation between same or different sets of samples. T-test was used to compare results from rabbit and IgY-based cELISAs with P value <0.05 taken for significance.

4.4. Results:

4.4.1. Antibody responses in hens immunized with SARS-CoV-2 antigens:

We immunized hens with one of three SARS-CoV-2 antigens: S1, S2 or N. Prior to the collection of eggs, we monitored the serum antibody levels of the hens at various times after immunization.



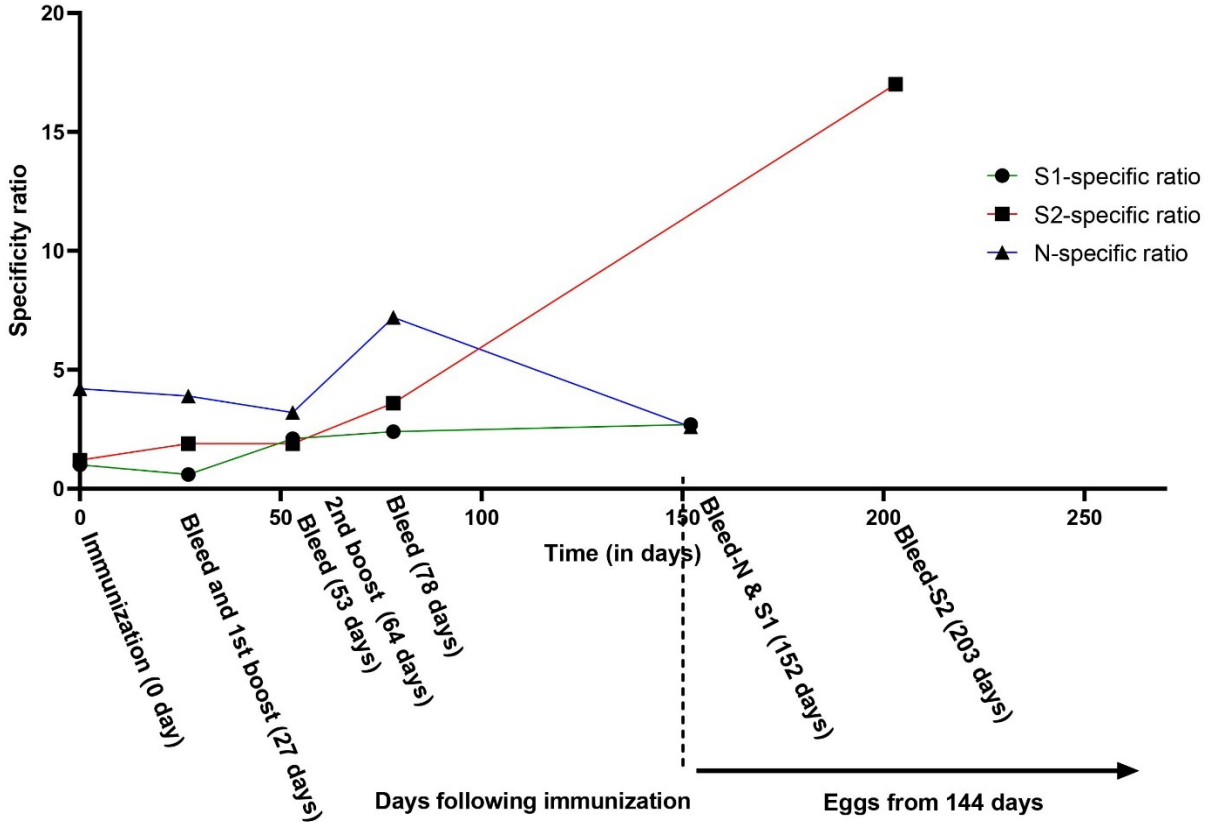


Fig 4.1: Antibody responses by IgY based cELISA in immunized hen serum: S1 (circle), S2 (square) and N (triangle) specific immune responses were measured in serum samples collected at different timepoints following immunization. Each data point is the mean percentage of inhibition from 3 hens that were immunized by same antigen (Fig 4.1 A). The bars at data point represent standard deviation. In addition, specificity ratios (average of specific percent inhibition/ average of non-specific inhibition; Here, non-specific inhibition means 'Competing' antibody competes with different antibodies than immunogen-specific ones) were plotted against different timepoints (Fig 4.1B).

Pre-bleed hens did not exhibit immunity against any of the SARS-CoV-2 antigens, while after boosting there was a spike in specific immunity against corresponding antigens (S1, S2 and N). Antibody levels against S1 and N antigen declined after day 53 *i.e.,* after the second boost. When eggs were laid (after day 144), the levels of antibodies were lower than at day 78. In addition,

the specificity of N antibody dropped down drastically after 78 days.

4.4.2. Purification of egg yolk IgY

IgYs were extracted and purified from individual SARS-CoV-2 immunized chickens using a commercial kit and 1-4 mg/mL of purified IgY could be yielded. As, the purified IgY pellet from each egg (after last centrifugation) was suspended in equal volume (to initial egg yolk volume) of PBS, final volume was varied depends on individual egg. To assess the purity of IgY from eggs, the samples were analysed by SDS-PAGE, followed by staining of the separated proteins by Coomassie Blue (Fig 2). The banding pattern was compared with that of delipidated yolk and a commercially obtained pure IgY isotype control.

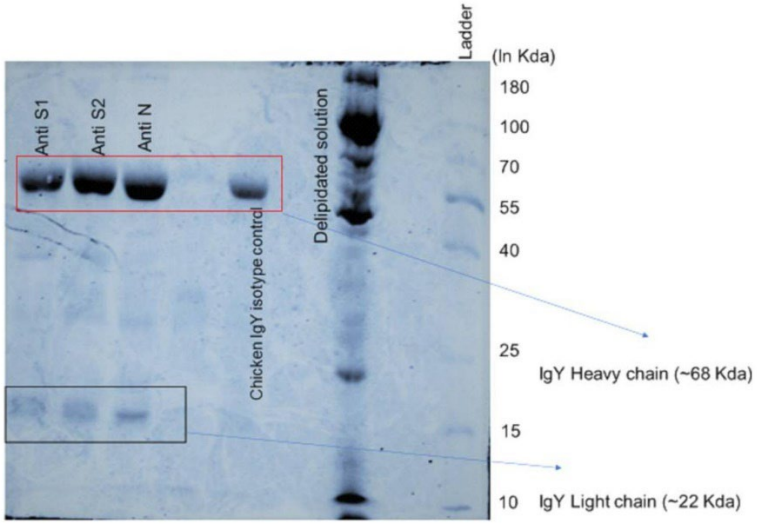


Fig 4.2: Protein analysis of purified IgY from SARS-CoV-2-S1, S2 and N immunized hens compared to a commercial chicken IgY isotype control and delipidated yolk solution.

While delipidated egg yolk displayed several bands, the purified IgY samples contained prominent bands at molecular weights of 68,000 and 25,000 respectively (Fig 4.2). These bands likely correspond to IgY heavy and light chains. There were additional minor bands which may be minor impurities or products of degradation[135]. The same banding pattern of the purified IgY samples was also observed in commercially obtained purified IgY control.

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4.4.3. Exclusion of N-IgYs from IgY-based cELISA development:

After assessing the purity of the purified IgY preparations, we performed indirect ELISA to determine the specificity of purified S1, S2 and N-IgYs to S1, S2 and N antigen respectively (Fig 3).

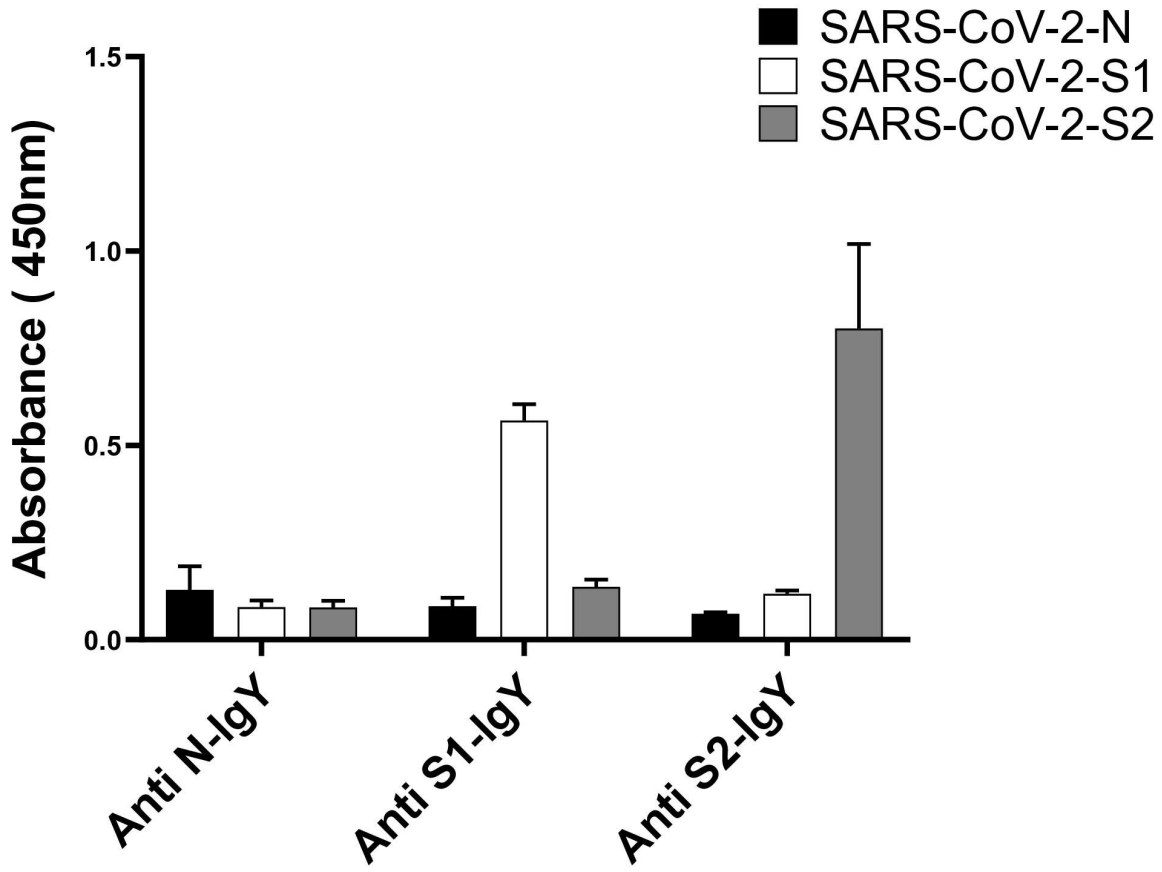

Fig 4.3: Assessment of the specificity of purified anti N, S1 and S2-IgY: In the same plate, 1µg/mL S1, 0.4 µg/mL S2 and 0.06 µg/mL N was coated and IgYs (specific and non-specific to corresponding antigens) were added to compare the specificity of antigen-antibody binding. The columns represent average values of three replicates and the bars represent the standard deviation from the mean. Black, white, and grey columns are representing absorbance (450 nm) of IgYs against N, S1 and S2 antigens respectively.

Anti S1 and anti S2 IgY bound specifically to S1 and S2 antigens respectively. However, antibody binding to the N-antigen was not detected using the Anti-N-IgY preparation. (Fig 4.3). Therefore, anti-N-IgY was excluded from the development of IgY based cELISA.

4.4.4. Determination of optimal working concentrations of antigen and antibody for IgY based cELISA:

For S1 and S2, optimum coating concentrations were determined as 1µg/mL and 0.4 µg/mL respectively, which had the highest specificity ratio when antigen specific IgY concentration was standardized at 1µg/mL (Fig 4.4). These optimal coating concentrations were then used to determine the optimal Anti-S1-IgY and anti-S2-IgY concentrations.

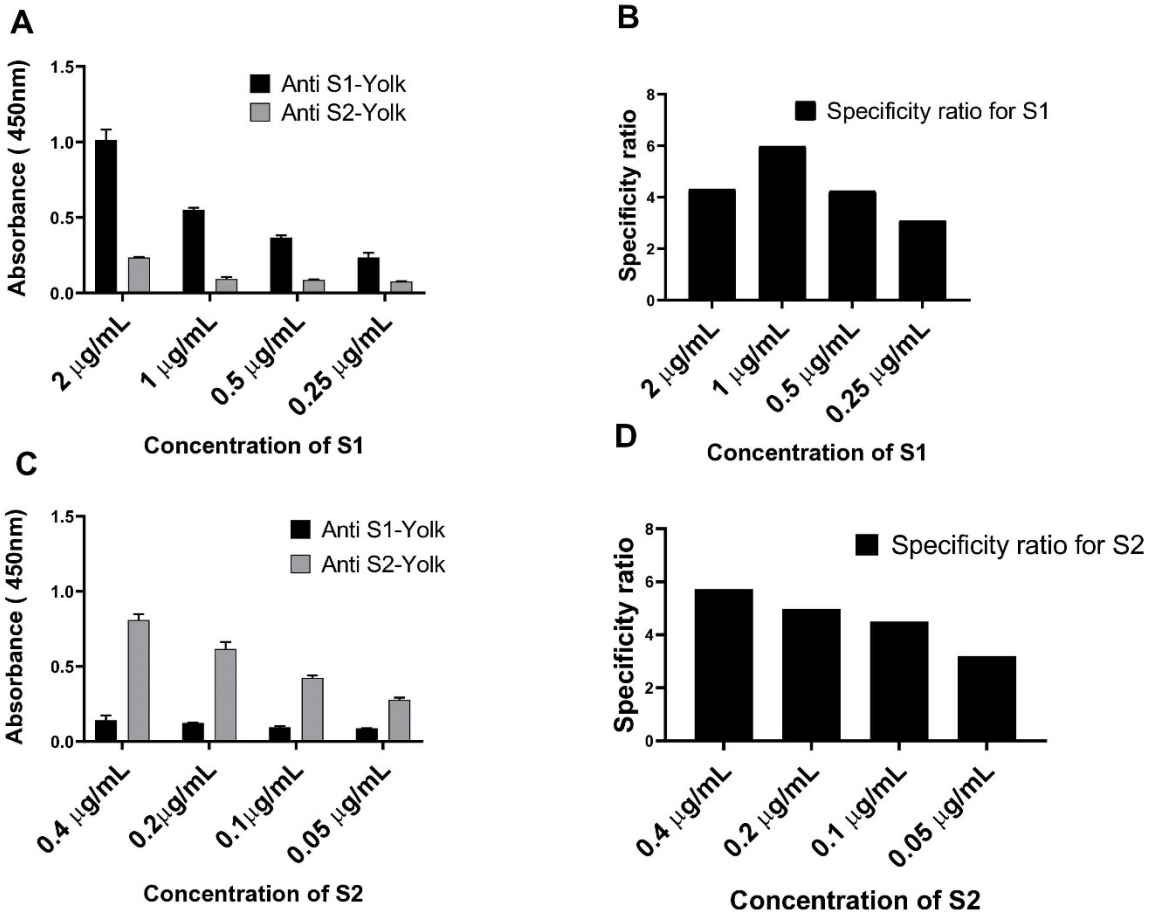

Fig 4.4: Optimization of SARS-CoV-2-S1 and SARS-CoV-2-S2 antigen concentration by indirect ELISA: ELISA plates were coated with different concentrations of S1 (Fig 4.4A) and S2 (Fig 4.4C).Anti-S1 and anti-S2 were added to both antigens coated plates at 1 ug/ml. Black columns indicate the mean absorbance (at 450 nm) of three replicates for anti S1-IgY , while grey columns indicate binding by anti S2-IgY. Error bars represent the standard deviation from mean. The

specificity ratio (Fig 4.4B, 4.4D) was calculated as: specific antigen-antibody absorbance (at 450 nm)/ non-specific antigen-antibody absorbance (at 450 nm).

S1 and S2-antigen concentration was optimized as 1 and 0.4 µg/mL respectively.

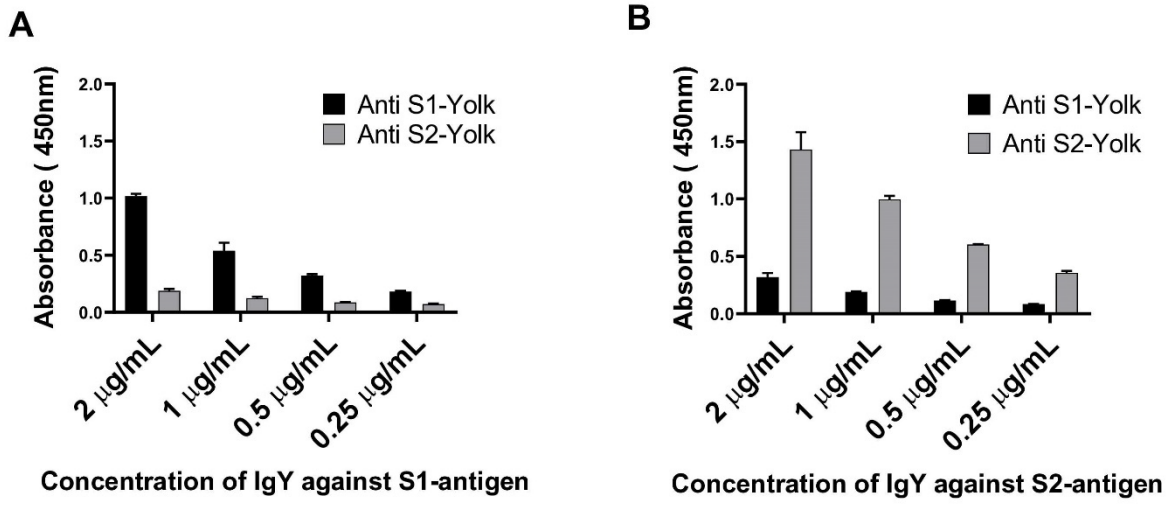

Fig 4.5: *Optimization of anti-SARS-Cov-2-S1 and anti-SARS-CoV-2-S2 yolk antibody concentration. Purified egg yolk IgY preparations, starting at 2 ug/ml with two-fold dilution, were tested by indirect ELISA. Plates were coated with 1 µg/mL S1 (Fig 4.5A) or 0.4 µg/mL S2 (Fig. 4.5B). Columns represent the mean absorbance (at 450 nm) of three replicates for anti S1-IgY (black) and anti S2-IgY (grey). Error bars represent the standard deviation from the mean.*

Anti-S1-IgY and anti-S2-IgY concentrations were optimized as 1 µg/mL. We chose 1 µg/mL rather than 2 µg/mL as there was no significant difference of the specificity ratio (data not shown) and using the higher concentration might give rise to non-specific background, i.e. there might be excess binding by the indicator antibody which would not be blocked by antigen specific binding of the test antibody, would reduce the competitive readout.

We next determined if cELISA using IgY directed against S1 and S2 proteins could detect antibodies against the proteins in serum from an animal experimentally infected with SARS-CoV-2. Serum from an infected hamster (See table 4.1), previously shown by the rabbit cELISA as well as the cPass test to have antibodies against S1, was serially diluted and tested by the IgY-cELISA (Fig 4.6). In two independent experiments (Fig 4.6A and 4.6B) serial dilution of the serum decreased inhibition of the S2 IgY binding. The level of inhibition of S1 IgY binding was low and serial dilution had no effect, indicate that the IgY assay against S1 may not inhibit binding of anti S1 hamster antibodies. We therefore concentrated on further assessing the IgY based assay for

detecting antibodies only against S2.

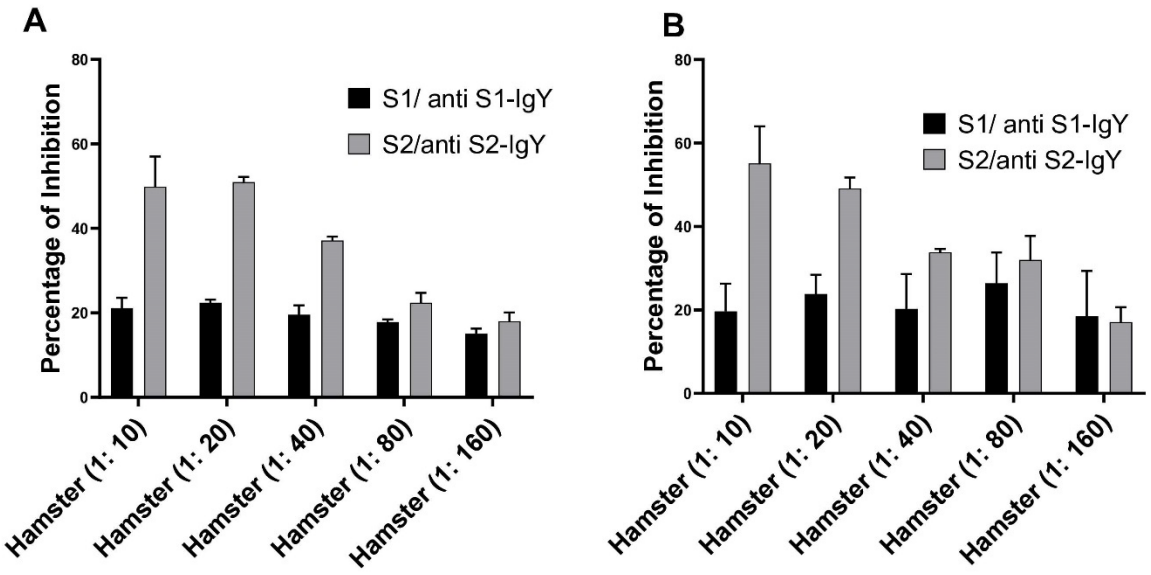

Fig 4.6: Inhibition of S1 and S2 antigen specific IgY binding by experimentally infected hamster serum in cELISA s: The columns represent the mean percentage inhibition, and the bars represent the standard deviation for three replicates at each test serum dilution. Fig 4.6A and 4.6B are the results from two independent assays.

4.4.5. Validation of the anti-S2 IgY-based cELISA:

The results of the IgY-based S2 assay was validated favourably with the rabbit-based cELISA as well as the cPass test. In addition, a paired T-test between the two cELISAs did not demonstrate a significant difference between the assays (p=0.47).

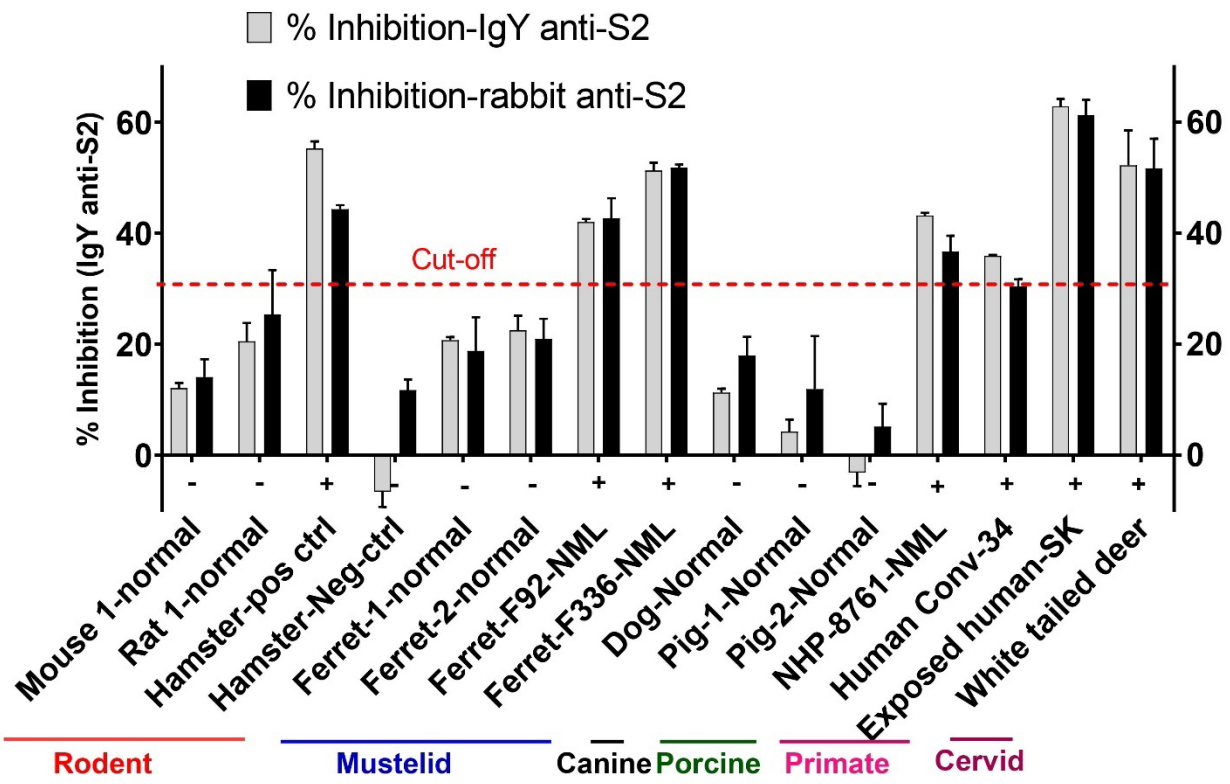

Fig 4.7: **Comparison of -IgY based cELISA and rabbit based cELISA to detect S2-specific antibodies in different animal sera.** Sera from multi species samples with known exposure status to SARS-CoV-2 (Table 4.1) were tested using both a competitive ELISA assay with IgY anti-S2 antibodies (grey columns) and a rabbit anti-S2 based cELISA (black columns). The columns represent mean percent inhibition of three replicates and the error bars indicate the standard deviation from mean. The horizontal red dotted line at 30% (depending upon the statistical analysis on known negatives) represents "cut-off" for calling a sample "positive". Samples with standard deviations that span the line are regarded as "negative". Percent inhibition results were not significantly different between assays.

Table 4.1: Sera from different species of known exposure status used to compare S2-IgY based cELISA and rabbit Anti-S2 based cELISA.

Group	Species	Designation	Source	Information	CPASS status
Rodent	mouse	Mouse-1-Normal	Health Science	Unknown	Neg
	rat	Rat-1-Normal	Health Science	Unknown	Neg
	hamster	Hamster-Pos-Ctrl	VIDO	Experimentally infected	Pos
	hamster	Hamster-Neg-Ctrl	NML	Not infected	Neg
Mustelid	ferret	Ferret-1-Normal	ACU	Unknown	Neg
	ferret	Ferret-2-Normal	ACU	Unknown	Neg
	ferret	Ferret-F92-NML	NML	Experimentally infected/vaccinated	Pos
	ferret	Ferret-F336-NML	NML	Experimentally infected/ Vaccinated	Pos
Canine	dog	Dog-Normal	ACU	Unknown	Neg
Porcine	pig	Pig-1-Normal	ACU	Unknown	Neg
	pig	Pig-2-Normal	ACU	Unknown	Neg
Cervid (*Provided as nobuto strips which were eluted in PBS with*	white tail deer	White tailed deer	Sask. Wildlife management Zone	Unknown	Pos

1:20 dilution)					
Primate	non-human	NHP-8761-NML	NML	Experimentally infected/ Vaccinated	Pos
	human	Human conv.-34	Mt. Sinai	Exposed/convalescent	Pos
	human	Exposed human serum	Saskatoon	Exposed	Pos

ACU: Animal Care Unit, USask ; NML: National Microbiology Laboratory, Winnipeg. Experimentally infected/vaccinated: Animals were infected by live virus/ immunized by virus or viral antigen.; Exposed: Naturally infected by virus

4.5. Discussions:

This study addresses an urgent need for a validated, affordable serological test that can be used for surveillance of non-human mammalian species for SARS-CoV-2. Here we demonstrate that IgY extracted from eggs from hens, immunized withSARS-CoV-2 S2 antigen, is a non-invasive technique to produce robust quantities of antibodies, and that our competitive ELISA assay with IgY anti-S2 antibodies has strong potential for detecting antibodies to a conserved protein of SARS-CoV-2 in a variety of mammalian species. The optimal S2 antigen coating concentration was 0.4 μg/mL and the anti-S2-IgY concentration was optimized at 1 μg/ml, and the assay performed well in sera samples from a range of mammalian species of known exposure status, including those most important for SARS CoV2 epidemiology: mustelids, cervids, and rodents[7,120].

According to our knowledge, this is the first time in which an IgY based ELISA has been used to detect SARS-CoV-2-specific antibodies in animals.We focused on the S2 target for the following reasons. Comparative studies with rabbit antibodies revealed that anti-S1 IgY is less able to compete with mammalian antibodies against S1 compared to anti-rabbit antibodies. A possible explanation behind this difference is that IgY (detected by anti-chicken HRP) might recognize different epitopes on SARS-CoV-2-S1 antigen from those detected by rabbit anti-S1 antibodies. So, there is less competition from IgY showing less inhibition. N-specific IgY was not detectable in the purified egg yolk preparation (Fig 4.3). N-specific antibodies were detected in

hen sera (Fig 4.1); however, titres declined after 63 days post vaccination, potentially explaining why minimal or no N-IgY was detected in egg yolks since laying commenced at day 144 post immunization. IgY is exclusively transferred to the yolk by a receptor-mediated process and the amount of IgY transferred is related to the IgY serum concentration[136,137]. Therefore, another possibility is that, IgY concentration in N-immunized egg yolks are very low. Regardless, anti-N-IgY was excluded from further development of IgY based cELISA.

This S2-specific ELISA assay offers another utility in differentiating infected from vaccinated animals (DIVA). Most human vaccines for SARS CoV2 utilize the S1 protein[138-146], and CPASS[84], the most commonly available commercial serological assay, also targets anti S1 whereas our assay detects anti S2. Compared to humans, very few animals have been vaccinated, although vaccines have been piloted in mink and captive felids. DIVA strategies are very important for epidemiological studies and to monitor efficacy of interventions such as vaccination. Our findings suggest that IgY based cELISA methods could be deployed as an additional tool in surveillance programs investigating SARS-CoV-2, and indeed other pathogens, in animals across a broad species range. This is critical to managing the current pan zoonotic of SARS-CoV-2, and to rapidly respond to other emerging viruses of pandemic potential.

4.6. Ethics statement:

Animal studies at the National Microbiology Laboratory were conducted following the Canadian Council of Animal Care guidelines and in accordance with an animal use document approved by the Canadian Science Centre for Human and Animal Health's institutional animal care and use committee. Potentially infectious materials were inactivated according to approved procedures (heating to 60°C for 30 minutes) for subsequent analysis. Studies at the University of Saskatchewan were conducted under biosafety permit VMB-03 and VMB-12. Exposed human samples were collected and processed under biomedical research ethics ID 3369. Experimentally infected hamster serum from VIDO is under animal research ethics approval no. 20210079 and wildlife samples are under ethics approval number 20220023 and exemption of animal-based activity ID 013Exempt2021.

4.7. Conflict of interest:

None

4.8. Acknowledgement:

This study was funded by an Alliance-COVID-19 grant from the Natural Sciences and Environmental Research Council of Canada (NSERC) awarded to VM, EJ, Darryl Falzarano and Christy Morrissey, as well as a grant and contribution from from Environment and Climate Change, Canada, and Public Health Agency of Canada. Serum samples from ferrets and non-human primates were kindly provided by Drs. Robbin Lindsay, David Safronetz and members of the One Health Section of the National Microbiology Laboratory of Canada, Winnipeg. Heat inactivated serum samples from SARS-CoV-2 infected hamsters were kindly provided by Dr. Daryl Falzarano from VIDO. The wild deer sample was kindly provided by Iga Stasiak and her team from the 'Saskatchewan Environment'. The authors also acknowledge the statistical, editorial, and other assistance by Dr Sonu Subudhi.

Chapter 5
General Discussions and Conclusions

5.1. General Discussion and Conclusions

ELISA is a useful technique to detect the prevalence of exposure (antibodies) to pathogens in animal and human populations. Developing a competitive ELISA test to identify SARS-CoV-2 specific antibodies in animals plays a crucial role in comprehending the involvement of animals in the spread and transmission of the virus. However, direct or indirect ELISAs require species-specific reagents. In contrast, competitive ELISAs can detect pathogen-specific antibodies in sera of all species, critically for a panzootic virus like SARS-CoV-2. At the onset, competitive ELISA was being developed utilizing rabbit-derived polyclonal IgG antibodies as competing antibodies. However, in the subsequent phase of the project, chicken egg yolk antibodies (IgY) were included instead of the rabbit-generated ones. Rabbit IgG based cELISA could detect S1 and S2 antigen specific antibodies in hamster (experimentally infected with SARS-CoV-2) while it was only possible to develop and validate an IgY-based competitive ELISA for the detection of S2-specific antibodies. IgG based cELISA showed less sensitivity in detecting N antigen specific hamster antibody than S1 and S2. The amount of S1 and S2-specific antibodies at dpi 56 were relatively higher in hamster serum than N (Chapter 3: Fig 3.2 A, B and C), but, at dpi 81 and 140, N-specific antibodies were at similar level compared to S1 and S2. This observation suggests that SARS-CoV-2-N-specific antibodies might take longer period to generate than that of S1 and S2. Depending on the limited availability of known samples and using Classen's Method (1987), I determined the threshold cut off for cELISA in hamster sera as 10% inhibition. In addition, a larger number of known positive and negative samples should be analyzed to determine the specificity and sensitivity of the test more accurately in other species. Therefore, future work should validate this newly developed cELISA in multiple species (such as mink, white-tailed deer, mule deer etc) and determine species-specific cut-off values, which may vary among different species.

Along with the rabbit antibody based cELISA developed in chapter 3, in the chapter 4, I demonstrated the development of SARS-CoV-2-S2 antigen specific hen IgY based cELISA. IgY-based cELISA could be a better alternative because avian antibodies would be least cross reactive

in competing against mammalian antibodies to bind with coated antigen, thereby increasing the specificity of cELISA. To the best of my knowledge, this is the first time where IgY based ELISA has been used to detect SARS-CoV-2-specific antibodies in animal populations. As the work developed, it became evident that S2-specific IgY was the logical focus for our IgY based cELISA among the three potential immunogens considered: S1, S2 and N. S2-specific IgY competed with test serum S2 antibodies at a similar level as of S2-specific IgG, showing comparable percentage of inhibition (Chapter 4, Fig. 4.7) Both cELISAs comparing anti S1- IgY to rabbit anti-S1 antibodies in detecting SARS-CoV-2 specific antibodies in exposed human serum, showed that the yolk antibodies are less able to compete with mammalian antibodies against S1. This was unexpected and may be explained by the possibility that IgY (detected by anti-chicken HRP) and rabbit anti-S1 antibodies identify distinct epitopes on the SARS-CoV-2-S1 antigen. Therefore, IgY is less of a competitor and its inhibition is lower. Alternative possibility behind less competition from S1-IgY might be the range of test serum dilution (1:10- 1:40) falls under saturation level. From the data acquired from indirect ELISA, it is shown that hen IgYs are not detectable for N-specific antigen-antibody binding. N-antibodies were initially found in hen sera, but began to decline 9 weeks after immunization, which may account for the low or absent levels of N-IgY in egg yolk. According to earlier investigations[147,148], IgY is only transported to the yolk through a receptor-mediated mechanism, and the amount transferred correlates with the blood IgY levels.

In influenza virology, differentiating infected from vaccinated animals (DIVA) strategy utilizes an inactivated oil emulsion vaccine that includes the same haemagglutinin (H) subtype as the virus being challenged, but with a different neuraminidase (N) to differentiate infected from vaccinated animals[149]. Here implying DIVA strategy similarly, S1 and S2-specific antibody detecting IgG based cELISA along with S2-specific antibody detecting IgY-based cELISA could differentiate naturally infected animals from vaccinated ones. Together, these results and the effort on creating an IgY-based cELISA described here improve the strategy for monitoring SARS-CoV-2 exposure even in a fully vaccinated human population.

In conclusion, using both (rabbit and IgY) based cELISAs, S1 and S2 specific antibodies would be detected in wildlife animal sera, but also would be beneficial to discriminate vaccinated animals to exposed ones. Both assays will be a valuable tool to be implemented in surveillance programs investigating evidence of exposure to SARS-CoV-2 in multiple domestic, captive (zoo), or wild animal species, in studies investigating impacts of SARS-CoV-2 on wildlife populations,

and in studies examining wildlife populations for the potential to become reservoirs for SARS-CoV-2.

5.2. Limitations of this study and Future prospects

This was a high-risk master's thesis, working at the forefront of new tests and a newly emerged and highly mutable pathogen. The main limitation of this study was the sample size and limited access to known experimentally infected serum samples (which has to occur under level 3 conditions which were not available to us). Therefore, next steps will be to validate both cELISAs using known positive and negative serum samples of white-tailed deer that were experimentally infected by multiple variants of SARS-CoV-2 at United States Department of Agriculture (USDA) laboratory. Moreover, we are trying to accumulate other animal serum samples from different species whose serological status is unknown. Acquiring a larger sample size would quantitate sensitivity and specificity of the assay more accurately and might alter the cut-off percentage of inhibition of cELISAs. While PCR results reveal the recent infection in animals, newly developed ELISAs would detect recent or previous exposure to SARS-CoV-2 or its variants. In conjunction to PCR results, seroprevalence reinforces potential sources for virus transmission and even spillover events. Therefore, newly developed cELISAs are valuable tools that could be incorporated in surveillance programs for SARS-CoV-2 in wildlife. Ultimately, this project has a wider aspect to be considered under the umbrella of 'One Health-COVID' approach to deal with further outbreaks among farmed, companion, wild and livestock animal populations. Enhanced surveillance should be prioritized and quantifying disease prevalence in a susceptible animal population would give the leverage to understand virus transmission followed by proactive measurements to restrict another outbreak.

6. References

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