Seroprevalence of SARS-CoV-2 across diverse animal species due to viral exposure

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Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a single-stranded, enveloped RNA virus belonging to the *Coronaviridae* family, genus *Betacoronavirus*. Emerging in late 2019, SARS-CoV-2 spread rapidly leading to the global COVID-19 pandemic. Since its emergence, several species of domestic and wild animals have been found susceptible to SARS-CoV-2 infection through experimental inoculation and surveillance activities by detecting a virus in animals. The possibility of zoonotic transmission poses a significant public health concern, particularly in densely populated animal farming operations. The infection of wildlife species raises concerns about the virus's impact on biodiversity and the potential for creating new reservoirs in nature. Therefore, accurate detection of SARS-CoV-2-specific antibodies is crucial for surveillance and epidemiological studies. Cases of SARS-CoV-2 infection have been reported in large cats, hippopotamuses, and gorillas. However, the susceptibility of bison, elk, and other wildlife species to SARS-CoV-2, as well as natural infection in sheep, goats, and cattle, remains poorly understood.

Two experiments were conducted to assess the seroprevalence of SARS-CoV-2 antibodies in cattle, sheep, goats, bison, elk, and other wildlife species from several geographically distinct regions in the United States using two commercially available ELISAs targeting the nucleocapsid (N; N-ELISA) and spike (RBD; sVNT) proteins of SARS-CoV-2 and conventional virus neutralization test (VNT). Additionally, we provide an assessment of the performance of these serological detection methods for identifying antibodies against SARS-CoV-2 in these animal species.

The first study indicated that 6/691 bovine, 18/707 goats, and 13/698 sheep serum samples were positive for the sVNT, whereas 25/691 bovine, 18/707 goats, and 27/698 sheep

serum samples tested positive for the N-ELISA. SARS-CoV-2 positivity rates were slightly higher in goats and sheep compared to bovine using sVNT, while higher seropositivity was observed in bovine and sheep than goat using N-ELISA. However, none of the sVNT-positive cattle, sheep, or goat serum samples showed detectable neutralizing activity against the SARS-CoV-2 using VNT. These results demonstrated a low level of SARS-CoV-2 seroprevalence in cattle, sheep, and goats in the U.S, indicating that these species have low susceptibility to SARS-CoV-2 infection.

The second study demonstrated that 7/575 of bison, 4/180 of elk, and 6/147 of the other wildlife species serum samples were seropositive in the sVNT, whereas 24/575 of bison and 6/180 of elk, and 2/147 of the other wildlife species serum samples tested positive by the N-ELISA. Among the sVNT serum samples, 2 samples from bison, 1 sample from elk, and 5 serum samples from other wildlife species (1 cheetah, 1 gorilla, 2 lions, and 1 hippopotamus) had neutralizing antibody titers in the VNT. The presence of neutralizing antibodies in these species indicates their susceptibility to SARS-CoV-2 infection.

Overall, our results indicated the necessity of ongoing surveillance of SARS-CoV-2 prevalence in animal species that interact closely with humans, as well as in other wildlife species inhabiting natural environments and zoological settings. This approach enables the assessment of the reservoir potential of diverse animal populations for SARS-CoV-2 and facilitates the implementation of preventive measures to mitigate the risk of virus transmission.

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Dedication

To my parents and beloved sister

Thank you for all of your unwavering love and support

Chapter 1 - Literature Review

The majority of emerging infectious in humans are caused by zoonotic pathogens originating from animals, specifically wildlife [1, 2]. Wildlife have the potential to serve as important reservoirs for many pathogens detrimental to the health and production of livestock [3]. Therefore, the increasing interactions between humans and animals through wet markets, exotic pet trade, hunting, and farm animals, have led to the greatest increase in cross-species virus infection and zoonotic transmission [4, 5]. Approximately 60% of emerging infectious diseases are zoonotic in origin and 71.8% of these diseases originate from wildlife [1]. Therefore, zoonotic diseases pose one of the greatest threats to human and animal (wildlife and livestock) health and socioeconomic conditions. It is crucial to identify susceptible animal species, either wild or domestic, to infections such as SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2) and to determine the role of animals in the epidemiology of the disease to prevent further spread, reduce the impact of the disease, and manage other novel viruses in the future.

Detection of antibodies and viruses plays a key role in identifying the natural reservoir hosts and intermediate hosts for emerging zoonotic diseases, as well as in assessing seroprevalence and the durability of protective immunity [6]. Levels of virus-specific antibodies (IgM, IgA, and IgG) are crucial measurements for estimating population and herd immunity against SARS-CoV-2. Some antibodies may have cross-reactivity with other coronaviruses. Therefore, effective and rapid serological assays for SARS-CoV-2 variants, with sufficient sensitivity and specificity, are necessary to identify and control outbreaks of SARS-CoV-2. Moreover, accurate detection of SARS-CoV-2 virus-specific antibodies after an immune response is important for surveillance, epidemiological purposes, and understanding the protective effects of the immune response. Antibodies to SARS-CoV-2 antigens are typically evaluated using various methods, including ELISAs [7] and protein microarrays [8] serum neutralization assay, etc., although ELISA is the most commonly used classical serological test [9]. The variability in diagnostic test performance among enzyme-linked immunosorbent assays (ELISA) can be attributed to the type of antigen used, SARS-CoV-2 spike (S) or receptor-binding domain (RBD), the whole virus, and monoclonal proteins (M) or SARS-CoV-2 nucleocapsid proteins (N) [10].

Coronaviruses: historically perspectives

Coronaviruses (CoVs) are the largest group of viruses (27-32 Kilobytes (kb)) [11], belong to the family of Coronaviridae, classified in the order Nidovirales [12]. Based on phylogeny, CoVs divided into four genera *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus* [11, 13]. Alpha- and Beta CoV originate from bats and mainly infect mammals, while Gamma- and Delta CoV include viruses isolated from birds and infect both birds and various mammalian species [14]. Coronaviruses can adapt to new environments through mutation and recombination, resulting in alterations to host range and tissue tropism, allowing for rapid global spread [13]. As a consequence, coronaviruses have the potential to cause respiratory, gastrointestinal, neurologic, or systemic diseases across a broad spectrum of hosts, including humans, wildlife, and domestic animals [15, 16].

During the last two decades, seven zoonotic coronaviruses causing disease in humans have been identified: 2 Alpha CoVs (229E and NL63) and 5 Beta CoVs (HKU1, OC43, SARS-CoV (severe acute respiratory syndrome coronavirus), MERS-CoV (Middle East respiratory syndrome coronavirus), and SARS-CoV-2 (Severe acute respiratory syndrome coronavirus 2) [17, 18]. SARS-CoV, MERS-CoV, and SARS-CoV-2 were first identified in Guangdong, China, in 2002-2003; in Middle Eastern countries, particularly Saudi Arabia in 2012; and in Wuhan, China, in December 2019, respectively [19-21]. Bats appear to be the natural hosts of SARS-CoV, MERS-CoV, and SARS-CoV-2 [22]. Intermediate hosts such as civet cats, dromedary camels, and other yet unknown species can play a significant role in the transmission of SARS-CoV, MERS-CoV, and SARS-CoV-2 to humans [23]. While SARS-CoV, MERS-CoV, and SARS-CoV-2 can cause severe respiratory infections, it should be noted that SARS-CoV-2, has high viral titer in the respiratory system in the early phases of infection before symptom onset, and is less pathogenic and more transmissible than SARS-CoV [24, 25].

Overview of SARS-CoV-2

SARS-CoV-2 is a novel human coronavirus that belongs to the same family as SARS-CoV and MERS-CoV, and is the seventh coronavirus known to infect humans [26, 27]. SARS-CoV-2, a zoonotic pathogen and cause of coronavirus disease-19 (COVID-19) in humans, is a single-stranded, enveloped RNA virus belonging to the Reoviridae family, genus *Betacoronavirus* [11]. Genetic analysis confirmed that SARS-CoV-2 shares 96.2% genome homology with the bat coronavirus RaTG13 [28]. In addition, genome sequencing revealed that SARS-CoV-2 has 79% genomic similarity to SARS-CoV and 50% with MERS-CoV [29].

The genetic makeup of SARS-CoV-2 is composed of 13-15 open reading frames (ORFs) and contains approximately 30,000 nucleotides [30], and encodes 9860 amino acids [31]. The genome contains a 5′ untranslated region (UTR; 265 nucleotides long), which is a leader sequence and plays critical role in RNA replication, transcription, and immune evasion. Additionally, there is a 3′ poly (A; 337 nucleotides long) tail, essential for initiating replication, synthesizing viral RNA, and forming virions [11, 32]. Two-thirds of the viral RNA genome (5′ untranslated region (UTR)) consists of two large open reading frames (ORF1a and ORF1b). The

ORF1ab and ORF1a are translated into polypeptides 1a or 1ab to form sixteen nonstructural proteins. The remaining one-third of the genome at the 3′ end encodes accessory proteins and four structural proteins [19, 33-35].

All coronaviruses share a similar genomic structure. SARS-CoV-2 contains four major structural proteins, which are common features in all coronaviruses: spike protein (S), membrane protein (M), envelope protein (E), and nucleocapsid (N). Additionally, there are several accessory proteins (ORF3a, ORF3b, ORF6, ORF7a, ORF7b, ORF8, ORF9b, ORF9c, and ORF10) [8, 36, 37]. The S protein mediates viral attachment to specific cell receptors and the fusion of the viral membrane with the host cell membrane. Moreover, it serves as the primary target for the induction of neutralizing antibodies. Therefore, the S protein plays an important role in viral entry into host cells and interactions with the host's immune system [30]. The E protein is responsible for viral morphogenesis, viral assembly and budding, as well as altering host cell membrane permeability by forming oligomeric cation-selective ion channels and activating the host inflammasome, which may be important for viral infection and enhancing the host antiviral response [30, 38, 39]. The M protein or matrix protein, is the most abundant protein in the virus envelope. It serves as the central organizer for the assembly of viral particles and gives the virus its distinctive shape by interacting with all the other structural proteins [30, 40]. The SARS-CoV-2 M protein showed 90.5% sequence identity with the M protein of SARS-CoV [41].

The N protein is involved in coating the viral RNA genome, participates in viral replication and assembly by interacting with the viral membrane protein (M), and modulates viral RNA synthesis [19, 30]. Nevertheless, the functions of many accessory proteins are still not completely understood. It has been described that accessory proteins may play important roles in the interaction between coronaviruses and their hosts (specifically in the interaction with the innate immune system), the virulence of SARS-CoV, and the modulation of interferon signaling [42, 43]. Moreover, the RNA genome size of SARS-CoV-2 is approximately 30 kb in length [35], which encodes 16 nonstructural proteins, 4 structural proteins, and several accessory proteins [35].

Spike glycoprotein and nucleocapsid protein

Entry of SARS-CoV-2 into host cells is mediated by the S glycoprotein. Viruses enter cells and initiate infection through the interaction between the S1 receptor-binding domain (RBD) and the peptidase domain (PD) of the angiotensin-converting enzyme 2 (ACE2) receptor on the surface of host cells. After binding to the host receptor and undergoing conformational changes, proteolytic cleavage of the S protein is cleaved by the host transmembrane serine protease 2 (TMPRSS2) to initiate the fusion of viral and cell membranes [44, 45]. Accordingly, the spike glycoprotein or S protein, is a main target for neutralizing antibodies due to its high immunogenicity and vaccine design efforts. The S protein is a surface-located homotrimeric class I fusion glycoprotein and is composed of two functionally distinct subunits (S1 and S2) [46]. The surface subunits, S1 and S2, are composed of 672 and 588 amino acids, respectively [47]. The transmembrane S2 domain contains the N-terminal hydrophobic fusion peptide (FP), heptad repeat (HR) regions 1 and 2 (HR1 and HR2), central helix (CH), connector domain (CD), transmembrane domain (TM), and cytoplasmic tail (CT) [48].

The surface-exposed S1 possesses two major domains: an amino (N)-terminal domain (NTD) and a carboxy (C)-terminal domain (the receptor binding domain (RBD)), and two subdomains (SD1 and SD2) [47]. Both SARS-CoV-2 and SARS-CoV bind to ACE2 via RBD in CTD [49], suggesting ACE2 is the primary target of deactivating antibodies. It should be noted that, depending on the virus, one or both of the two major domains of S1 may function as the RBD [50]. Additionally, NTD appears to facilitate the viral binding to the sugar-based receptors, whereas CTD mediates the binding between the virus and protein-based receptors [49]. SARS-CoV Spike glycoprotein and SARS-CoV-2 Spike glycoprotein have high homology (76% amino acid sequence identity). Moreover, SARS-CoV-2 and SARS-CoV RBD domains reveal 74% identity in the amino-acid sequences [50], indicating that the main factor for SARS-CoV2 to enter the host cell is ACE2 receptor. Of note, although the NTD of SARS-CoV-2, because of the S1-RBD, is critical region and the main target antigen for neutralizing antibodies, but mutations and deletions in the NTD might lead to resistance to neutralizing antibodies in newly formed variant [45].

The spike protein's receptor-binding domain is the most divergent region. Amino acid residues in the RBD have been demonstrated to play a crucial role in binding to ACE2 receptors and in identifying the host range of SARS-CoV. Six amino acids of the RBD are critical for binding to ACE2; five out six amino acid residues were different between SARS-CoV-2 and SARS-CoV [51]. The dissociation constant (KD) of SARS-CoV-2 to bind to human ACE2 was 14.7, while the KD for SARS-CoV was 325.8 nM (10–20 times higher) [52]. It has been demonstrated that the binding affinity of SARS-CoV-2 to ACE2 was higher than SARS-CoV RBD [52, 53], suggestive of higher transmissibility of SARS-CoV-2 than SARS-CoV. On the other hand, the entire SARS-CoV-2 S protein can be equal or lower ACE2-binding affinity than SARS-CoV S [54]. Therefore, it is reasonable to conclude that amino acid sequence and structure of the receptor-binding domain (RBD) within the spike (S) protein are important factors in identifying potential hosts for SARS-CoV-2.

N protein is another important structure protein after spike proteins. It showcases various activities essential in genome replication, the cell signaling pathway, and suppressing host innate immune response [55, 56]. Nucleocapsid protein has a molecular weight of 45.6 kD [57]. It is a phosphoprotein [55], a multi-domain structural protein [57], encoded by the ninth ORF of the virus, and consists of 419 amino acids [58]. Nucleocapsid proteins contain three domains: N terminal RNA-binding domain (NTD; amino acid residues 44–176), a C-terminal dimerization domain (CTD; amino acid residues 248–369), which are flanked by N-arm and C-tail, and three intrinsically disordered regions (IDRs) at the N- (amino acid residues 1–43) and C- (amino acid residues 370–419) termini and between the NTD and CTD [59, 60].

Nucleocapsid protein is one of the most abundant viral proteins during infection [61], which can be used as an immunodominant antigen to stimulate immune system to produce antibodies. Nucleocapsid protein has an important role in association with binding to the genomic RNA and encapsulation of the viral RNA to form helical ribonucleoprotein (RNP) complex to protect the viral RNA from degradation by host factors [62].

Nucleocapsid protein of SARS-CoV-2 has exhibited high homology (~90%) with SARS-CoV [30] and 48% with MERS-CoV [63]. The N and S proteins, because of having highly antigenic properties, are the main immunogens and can act as serological markers [58]. Therefore, it is reasonable to consider that high homology between SARS-CoV-2 and SARSCoV-1 can induce cross-reactivity in antibody binding to the N protein.

Immune response to SARS-CoV-2 infection

Innate and adaptive immunity

In viral infections, the host's innate immune system, along with biological barriers such as skin and mucous membranes, provides a critical first line of defense against pathogen infection to prevent viral invasion or replication [64]. Cytokine (pro-inflammatory cytokines, anti-inflammatory, chemokines) [65], dendritic cells (DCs), macrophages, and natural killer cells (NK cells) are the most important cells associated with innate immune responses [66, 67]. Innate immune responses are induced by pattern recognition receptors (PRRs), which Toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) are two important receptors for activation of antiviral immunity [68]. The activation of PRRs leads to the production of multiple interferons (IFNs) and proinflammatory cytokines [69]. There are three major types of IFNs, type I, type II, and type III. In most nucleated cell types, activation of PRR leads to production of type I and III IFNs, while production of type II IFN is mainly regulated by activated T cells and natural killer cells [42, 70]. The type I IFNs (IFNα and IFNβ) not only are the most important inflammatory cytokines but also play critical role in antiviral functions early during infection [71]. It is noteworthy that although the innate immune system is the first line of defense against viral invasion to protect the host from infection, viruses continuously develop new evasion strategies to escape from host antiviral defense programs. Most viruses antagonize the host's innate immune response by using their proteins to target viral sensors or block antiviral signaling molecules. Accordingly, coronavirus uses various viral proteins to suppress host innate immunity and facilitate invasion [64].

Upon stimulation by SARS-CoV-2 antigens, mature B cells become activated and differentiate into 1) antibody-secreting plasma cells, which can neutralize or assist in eliminating antigens from the body, and 2) memory B cells, with a lifespan ranging from months to years [72].

Cross-Reactive immunity

The timing and type of antibody and antigen are important factors that affect the accuracy of antibody tests for SARS-CoV-2. Therefore, identifying antibody targets is crucial for improving assay performance. Spike (S) and nucleocapsid (N) proteins are widely used in serologic assays due to their immunogenicity. A meta-analysis [73] reported that IgG-IgM based ELISA tests provide accurate overall diagnostic results. In terms of sensitivity, IgM-IgG combined antibody tests appear to be a better choice in comparison to antibody measurements alone.

The presence of anti-spike or anti-nucleocapsid antibodies from prior infection not only can be associated with protective immunity against reinfection but also shows antibodies' longevity and functionality. Although it should be noted that SARS-CoV-2 antibodies response to S and N proteins were sensitive during the acute phase of COVID-19, the earlier reduction in anti-N antibody responses was observed in the post-infection phase compared with S protein which was persistent and slowly declined over time [74-76]. IgG antibodies that target S protein are more specific than those targeting N, which are more sensitive. The higher sensitivity of the anti-N antibody response reflects the high sequence similarity among the N protein of SARS-CoV-2 and other betacoronavirus strains [77, 78]. Observational evidence indicated that SARS-CoV-2 exposure estimates may be underestimated by 30% to 45% based on anti-N antibody responses compared with anti-S antibodies [74]. Virus neutralizing antibodies (NAbs) block the binding of virus to ACE2 on host cells and neutralize the virus [79]. As detailed above, the sequence similarity of the SARS-CoV-2 with SARS-CoV and MERS-CoV may lead to elicit antibodies that cross-react with other betacoronaviruses, which increases the potential for cross-reactivity among these three emerging coronaviruses. Additionally, bovine coronaviruses

(BCoVs) are widespread globally, infecting cattle and wild ruminants and causing respiratory and gastrointestinal diseases [80]. They belong to the genus Betacoronavirus, which also includes significant human pathogens such as SARS-CoV, MERS-CoV, and SARS-CoV-2. These viruses share structural similarities, particularly in their spike (S) protein, nucleocapsid (N) protein, membrane (M) protein, and envelope (E) protein, which are crucial for the virus's ability to infect host cells and evade the immune system [81]. Several strains of CoVs have demonstrated biological, antigenic, and genetic similarities with Bovine coronaviruses (BCoV), thereby classified as bovine-like CoVs [82]. Therefore, the similarities in sequence and structural proteins among various coronaviruses suggest potential for cross-reactive immune responses between SARS-CoV-2 and other coronaviruses.

Strong conservation, associated with the functions of the S and N proteins in viral replication, was identified in short regions of these proteins as cross-reactive epitopes [83]. Based on sequence analysis of the S2-associated immunogenic epitopes, it is apparent that epitopes are located in the S2 subunit can be the main target of IgG and cross-reactive antibodies [84]. Most SARS-CoV- Neutralizing antibodies (SARS-CoV-nAbs) that target the RBD of SARS-CoV do not bind the SARS-CoV-2 RBD, which can reduce cross reactivity in antibody binding to the RBD regions in the spike protein in human [85]. Monoclonal antibodies have a critical impact on the cross-reactivity of neutralizing antibodies. Developing novel monoclonal antibodies that specifically recognize and bind to the SARS-CoV-2 receptor-binding domain (RBD) is essential. Therefore, it's important to note that many antibodies that broadly react with the S protein may not exhibit high levels of neutralizing activity against the RBD [86]. The presence of antibodies to the S protein and surrogate Virus Neutralization Test (sVNT) may indicate exposure to SARS-CoV-2 infection [86, 87]. Another study found that the cross-reactive antibodies target both RBD and non-RBD regions following SARS-CoV-2 infection. While a high tendency for cross-reactivity of antibody binding to the spike protein was observed, cross-neutralization between live SARS-CoV and SARS-CoV-2 viruses was shown to be relatively rare [88]. It should be noted that using N protein-based assays could exhibit cross-reactivity between human alpha and betacoronaviruses [89, 90].

Notable recent observations were the finding that SARS-CoV-2-specific CD4+ (help B cells to produce antibodies) and CD8+ T cells were determined in responding to SARS-CoV-2 infection in human [91, 92]. Researchers have found that CD4 and CD8 T cells have potential to recognize multiple regions of the N protein in individuals who recovered from SARS [91, 92]. Therefore, previous infection can generate T cells that cross-react against SARS-CoV-2 due to epitope-specific T cells within different SARS-CoV proteins [93].

To investigate pre-existing cross-reactive SARS-CoV-2 antibodies in pre-COVID-19 pandemic samples, [94] Anderson (2021) conducted a study to determine antibody levels in pre-pandemic serum samples by standard ELISA from individuals infected with SARS-CoV-2 and individuals (control group of pre-pandemic samples) who did not infect with SARS-CoV-2. They indicated that non-neutralizing antibodies that cross-reacted with SARS-CoV-2 spike and nucleocapsid proteins were found in 20% of human serum samples before SARS-CoV-2 infection [94]. In addition, data showed that 4.2%, 0.93%, and 16.2% of samples possessed pre-pandemic antibodies reactive to the SARS-CoV-2 full-length S protein, S-RBD, and N protein, respectively. These data showed that pre-pandemic cross-reactive antibodies reacted with the N protein (16.2% seropositive) are more prevalent than those directed against the S protein (4.2% seropositive) [94].

A study by [91] Grifoni et al. demonstrated SARS-CoV-2-specific CD4+ T cell targets ORFs spike, M and N proteins. These antigens accounted for 27%, 21%, and 11% of the total CD4+ T cell response in COVID-19 cases, respectively. The data also indicated SARS-CoV-2-reactive CD4+ T cells were identified in 40 to 60% of unexposed healthy controls, suggesting some degree of SARS-CoV-2 pre-existing immune reactivity existed between common cold coronaviruses and SARS-CoV-2. Accordingly, spike protein is a first target of CD4+ T cells.

Long et al. [95] indicated the absence of cross-binding to the S1 subunit of the SARS-CoV spike antigen in serum samples from patients with COVID-19. However, some cross-reactivity of serum samples was observed with nucleocapsid antigens of SARS-CoV. In a similar study, SARS-CoV-2 rNPs ELISA, with the exception of SARS-CoV, showed no cross-reactivity with other CoVs [96]. Furthermore, it is noteworthy that S or RBD antigens provide better diagnostic results and greater specificity than whole virus, M, or N proteins, which exhibit close similarity among the virus family. Strong cross-reactivity was observed between SARS-CoV-2 N proteins and SARS antibodies in patient sera; therefore, N protein may not be suitable for detecting virus-specific antibodies [6]. In the Netherlands, 500 domestic cat and 500 dog samples were collected during April–May 2020 and assessed for antibodies against SARS-CoV-2 using S1-, RBD-, and N-ELISA, as well as virus neutralization assays with SARS-CoV-2 spike pseudotyped virus [97]. The results indicated that 1.2% (6/500), 2.4% (12/500), and 0.4% (2/500) of cat samples tested positive for SARS-CoV-2 S1, SARS-CoV-2 RBD, as well as SARS-CoV-2 pseudotyped virus, respectively. Among the samples positive for SARS-CoV-2 S1 or RBD by ELISA, only two demonstrated neutralizing ability against SARS2-VSV infection. In the case of 500 dogs, 1.8% (9/500), 0.2% (1/500), and 0.2% (1/500) of samples were positive for SARS-CoV-2 S1, RBD, and pseudotyped virus. This study also tested the serum of a dog (n=1) from a COVID-19-positive household and serum samples from 44 stray cats exposed to SARS-CoV-2 near positive mink farms. A strong correlation (R = 0.95) was observed between the OD values obtained from SARS-CoV-2 S1 and RBD ELISAs, both of which strongly correlated with VNT (R = 0.87). However, a weak correlation (R = 0.57) was found between the OD values from N protein ELISA and VNT. The authors concluded that the correlations between the results of the N protein ELISA and the VNT and the S1 and RBD ELISAs were weak [97].

In addition, an antibody reacting with the SARS-CoV-2 N protein was observed in SARS-CoV-2-free cats infected with feline coronavirus (FCoV), indicating that anti-SARS-CoV-2 N protein antibodies can be cross-reactive with FCoV. Therefore, the authors suggested that N protein-based antibody assays for SARS-CoV-2 are not appropriate for screening samples from cats and dogs [97].

The study conducted by Hancock et al. [98], serum samples from pre-pandemic animals (cows, n=33; wild tigers, n=9; and feline, n=128) and post-pandemic animals (dogs, n=36; wild elk, n=12; and wild white-tailed deer, n=22) were collected to assess cross-reactivity and the presence of antibodies against SARS-CoV-2's RBD using an in-house ELISA. Among the six species tested, 97% (35/36) of post-pandemic dogs, 52% (67/128) of pre-pandemic feline sera, and 9% (2/22) of post-pandemic deer sera samples were positive for antibodies against SARS-CoV-2 RBD. However, the authors noted potential cross-reactivity of pre-pandemic feline samples with the RBD of SARS-CoV-2. Additionally, it's worth mentioning that 19 out of 128 feline sera samples had a known FCoV serological status, while the status of the remaining 109 was unknown [98]. The researchers suggested various factors that could contribute to cross-reactivity, including exposure to different pathogens/antigens leading to the production of cross-reactive antibodies against SARS-CoV-2's RBD, infection with common coronavirus strains such as feline coronavirus, or prior exposure of animals to SARS-like coronaviruses before the pandemic [98].

In a study conducted by Yilmaz et al. [99], a total of 30 and 125 cat serum samples were obtained from before and during COVID-19 to investigate the presence of SARS-CoV-2-specific antibodies by S- and RBD ELISA in turkeys. The results indicated a seroprevalence of 26.45% (41/155; in-house S-ELISA), 28.38% (44/155; in-house RBD-ELISA), and 21.9% (34/155; the S- and RBD-ELISA) in the 155 cats evaluated. Twenty-seven of the samples were also positive for the presence of antibodies to feline coronavirus (FCoV). Thirty cat sera from before the pandemic were tested for antibodies to SARS-CoV-2. The results showed that 20% (6/30) of cats and 22.4% (28/125) of cats were positive before and during COVID-19 by both the S- and RBD-ELISAs. Five out of six and 22 out of 28 SARS-CoV-2 seropositive cats had antibodies to FCoV. The authors suggested that seropositive cats sampled before the emergence of COVID-19 may be due to cross-reactivity between FCoV and SARS-CoV-2, leading to the generation of cross-reactive antibodies against SARS-CoV-2's N and/or a SARS-CoV-2-like virus [99].

Kinetics of neutralizing antibody response to SARS-CoV-2

Neutralizing antibodies against SARS-CoV-2 can persist for at least 6–8 months, and in some cases, for more than 12 months in humans [100-104] and up to 10 months in some animals [105]. Based on some of the findings from previous experimental studies, cats developed and either maintained or increased neutralizing antibody responses by 7–42 days after infection [106, 107] and dogs exhibited detectable antibodies by 14 days post-infection [108]. Van Elslande et al. [109] reported that severe COVID-19 patients (exhibiting clinical signs and/or requiring hospitalization) had higher levels of IgG antibodies against S and N proteins than non-severe patients. Additionally, the median time to seronegativity for anti-S and anti-N was reported to be more than 2 years and less than one year, respectively, with similar half-lives [109].

The antibody response against SARS-CoV-2 usually develops 7 to 14 days after symptom onset [110]. Sterlin et al. [111] described a positive correlation between IgA and SARS-CoV-2 neutralization than IgM or IgG in the first weeks after symptom onset. IgA serum concentrations, particularly anti-RBD IgA, might be detect earlier than IgG, but their levels significantly reduced one month after symptom onset. This suggests that the persistence of IgA (against RBD) is markedly shorter than that of IgG [111] (Sterlin et al., 2021). Additionally, it is also possible that IgA may exhibit cross-reactivity against SARS-CoV-2, SARS-CoV, MERS-CoV, and MERS-OC43 [112]. Furthermore, Post et al. [113] reported that the median or average time for IgG seroconversion after the onset of symptoms ranged between 12 and 15 days, with wide variations from 4 to 73 days in the first to last IgG detection after symptom onset. For IgM and IgA, the mean or median time to seroconversion ranged from four to 14 days and from four to 24 days after symptom onset, respectively. Additionally, a positive correlation was observed between neutralization ability and anti-S IgG or anti-RBD IgG [113]. In another study [114] the peak median Relative Light Units (RLU) of RBD-specific IgA occurred during 16–20 days following disease onset and then started to decline. However, the level of IgA antibodies decreased but remained fairly high until 31–41 days. However, Padoan et al. [115] reported that average levels of IgM and IgA antibodies were found between 6 and 8 days from COVID-19 onset, with peak levels observed at 20–22 days and 10–12 days for IgA and IgM, respectively.

The longevity of neutralizing antibodies may correlate with protection against infection; therefore, the duration of immunity can be a critical factor following SARS-CoV-2 infection. The sensitivity of nucleocapsid-dependent ELISA for rapid COVID-19 diagnosis can be improved not only by using the highest purified dimeric form of the N protein [116] but also sensitivity may increase in samples collected near the time of symptom onset [117]. The different seroconversion times of antibodies against SARS-CoV-2 may be related to the severity of the disease [117]. In addition, lower or undetectable antibody levels may be observed in asymptomatic or mildly symptomatic patients [118]. Therefore, sensitivity of the assays can be influenced by the timing of blood collection and the severity of the disease [117]. Anti-S IgG levels can be used as a reliable marker for previous infection when anti-N IgG assays and/or PCR testing are unavailable. A positive antibody test result may indicate a past exposure to one or several antigenic epitopes of the pathogen and does not necessarily imply an active infection. Serological assays utilizing RBD and S proteins to assess neutralizing antibodies are considered the most reliable antigens for the SARS-CoV-2 virus [119].

Burbelo et al. [120] highlighted that the sensitivity of luciferase immunoprecipitation assay systems (LIPS) for detecting antibodies against the nucleocapsid and spike proteins was 100% and 91%, respectively, while specificity for antibodies targeting the nucleocapsid and spike protein at more than 14 days after symptom onset in patients was 100%. Additionally, it was observed that antibodies against the nucleocapsid and spike proteins were detectable between days 8 and 14 after the onset of initial symptoms [120]. The results showed that antibodies against nucleocapsid protein appear earlier than spike antibodies [120]. A prospective cohort study [121] indicated a higher rate of viral clearance was observed in patients with a low IgG response compared to those with a high IgG response, and this was associated with disease severity [121]. This suggests a correlation between stronger antibody response, disease severity, and late viral clearance [121]. A study investigated virus-specific antibody detection by testing serum samples from 285 COVID-19 patients using magnetic chemiluminescence enzyme immunoassay (MCLIA) [95]. The seropositive rates for IgG and IgM reached 100% and 94.1% around 17–19 days and 20–22 days after the onset of symptoms, respectively. Plateau levels for both IgG and IgM were observed within 6 days after seroconversion. These findings indicated that the seroconversion time for IgG antibodies can occur simultaneously with that for IgM against SARS-CoV-2 [95].

Some serological studies reported that anti-SARS-CoV-2 IgM antibodies were detectable between day 8 and 12 after infection, peaking at approximately 20-22 days, and then began to decline by week 12. Meanwhile, anti-SARS-CoV-2 IgG antibodies appeared on day 14, were detectable for around 3 months, and lasted even longer [122-124]. Nevertheless, another study demonstrated that the time of appearance of IgM, IgA, and IgG antibodies to SARS-CoV-2 after the onset of symptoms was 5 days (range 3-6 days) for IgM and IgA, and 14 days (range 10-18 days) for IgG, suggesting that the development time for antibodies varies based on different methods [96, 122].

In a study conducted by Sun et al. [125], blood samples from 38 COVID-19 patients were collected, totaling 130 samples, to evaluate IgM and IgG responses against SARS-CoV-2 N and S proteins (N-IgM, N-IgG, S-IgM, S-IgG) after symptom onset by ELISA. The results showed that N- and S-specific IgM and IgG were detected in 75% of SARS-CoV-2-infected patients in the first week. In the third week after onset of symptoms, 73.7% of patients were seropositive for N-IgM or S-IgM, while 100% were seropositive for N-IgG and S-IgG. As well, in an experiment conducted by To et al. [126] seropositivity rates for anti-NP IgG (n=15), anti-NP IgM (n=14), anti-RBD IgG (n=16), and anti-RBD IgM (n=15) were reported as 94%, 88%, 100%, and 94%, respectively. It should be noted, however, that blood samples were collected at 14 days or later after symptom onset [126].

Iyer et al. [127] demonstrated that the median time to seroconversion was 10.7 days for IgG and 11.7 days for IgA after symptom onset in patients. In another study [128], humoral memory responses to SARS-CoV-2 were observed in patients 1.3 and 6.2 months after infection. Furthermore, a reduction in anti-RBD IgM (53%), anti-RBD IgG (32%), anti-RBD IgA (15%), and anti-N IgG (22%) was observed between 1.3 and 6.2 months after infection. Wheatley et al. [129] observed faster decay in N-specific IgG than S and RBD-specific IgG. It should be noted that factors such as gender, viral load, co-morbidities, patient age, and disease severity may affect the dynamics of IgM, IgG, and IgA antibody distribution and variation [130].

Serological surveys of SARS-CoV-2 exposure in different species

According to natural infection history and experimental findings, it has been shown that some wildlife and domestic animals can be infected with SARS-CoV-2, either naturally or through experimental infection. Under some experimental conditions, cats appear more susceptible to SARS-CoV-2 and develop relatively higher antibody titers than dogs [106, 107]. In addition, hamsters and ferrets appear to be highly susceptible to SARS-CoV-2 infection [107, 117, 131-134]. The absence of susceptibility in swine and poultry to SARS-CoV-2 indicates that these species are not prone to SARS-CoV-2 infection and/or productive viral replication, and sustained viral replication could not occur through virus inoculation. In contrast dogs, sheep, and cattle exhibit limited susceptibility [106, 133, 135-137].

While the binding site residues are highly conserved (84%) in cows, the ACE2 protein in cows shows lower homology (78.8%) compared to that in many other vertebrates (over 80%) [138]. ACE2 in humans, cats, and hamsters showed a higher binding affinity to the SARS-CoV-2 spike RBD, indicating their susceptibility to coronavirus infection [138]. The sequence alignment of immunogen sequence of human ACE2 with different species showed that it has

100% homology with western gorilla, 94% for horse, ferret and American mink, 89% for little brown bat, least horseshoe bat, domestic and wild felids (tiger and cheetah), dog and sheep, 83% for cattle,78% for pig and common marmoset, and 67% for hamster. This suggests that all these species have highly conserved amino acid sequences [139]. Researchers recently found that horses, because of binding affinity between ACE-2 and the receptor binding domain of the S protein [140] and presence of conserved pattern of residues in ACE2 proteins of horses (Equus caballus), are apparently susceptible to SARS-CoV-2 [141]. Although horses can be infected with SARS-CoV-2 through contact with COVID-19 individuals, they typically exhibit subclinical symptoms. Therefore, they are unlikely to serve as significant sources of the spread of SARS-CoV-2 [142]. A recent study [143] showed that close contact with a COVID-19-positive individual during the pandemic period led to the detection of SARS-CoV-2 antibodies (IgG) in the younger of two mares 7 days post-COVID-19 diagnosis of the owner, using an RBD protein-ELISA and the plaque reduction neutralization test (PRNT). This study indicated that horses can be asymptomatically infected with SARS-CoV-2 through close contact with infected humans. Therefore, to avoid spreading the disease, it is recommended that COVID-19-positive individual avoid close contact with horses and other animals during their illness [143]. Nonetheless, continuous surveillance is necessary to monitor potential SARSCoV-2 transmission among equids.

Cat and Dog

Colitti et al. [144] performed serologic survey via flow cytometry-based system among dogs and cats housed with SARS-CoV-2–Infected owners in Italy. COVID-19 N-Protein ELISA Kit (Eradikit COVID19-IgG) was used to detect SARSCoV-2 IGg antibodies in feline and canine serum samples. Diagnostic specificities of 96.5% and 100.0% for dog and cat serum were obtained, respectively. The results indicated that SARS-CoV-2 seroprevalence was higher among cats (16.2%) compared with dogs (2.3%), which showed cats are more susceptible than dogs. These results suggested that higher rates of seropositivity can be associated with interaction and the amount of time pets and SARS-CoV-2–positive owners spent together [144]. Similar observations have been witnessed when SARS-CoV-2 S1-specific IgG of two cats and one dog (from 10 cats and 9 dogs) were strongly positive. In addition, samples showed neutralization titers 1:240, 1:240, and 1:120 using PRNT [131]. According to a survey conducted in Portugal, 15 out of 69 (21.74%) cats and 7 out of 148 (4.73%) dogs tested positive for IgG antibodies by SARS-CoV-2 RBD ELISA. The study revealed that 5 out of 15 (33.33%) cats were infected via cat-to-cat transmission. Overall, 22 out of 217 (10.14%) pets tested positive for SARS-CoV-2, with 11 out of 22 (50.00%) possibly infected through human-to-animal transmission [145].

Similar results were observed in a study conducted by Sailleau et al. [146] who consider putative infection by SARS-CoV-2 in 22 cats and 11 dogs, which have been exposed to owner with suspected or confirmed COVID-19, through genome sequence analysis and serological analysis (a microsphere immunoassay (MIA) and ID screen® SARS-CoV-2-N IgG indirect ELISA (ID vet) in France. Samples taken from the dogs were tested negative for SARS-CoV-2 by ELISA and MIA. Serological analysis by ELISA and MIA detected SARS-CoV-2 antibodies in two cat serum samples taken 10 days apart. Antibody responses displayed cross-reactivity between N SARS-CoV-2 and N SARS-CoV antigen, indicating that a high degree of sequence identity can be considered in increased cross-reactivity [146].

Further some experimental studies regarding cats and dog living with SARS-CoV-2 infected humans or in geographic areas affected by COVID-19 reported that SARS-CoV-2 neutralizing antibodies were detected in 3.4% (1/29) of dogs and 20% (2/10) of cats via PRNT90 in Brazil [147]. In another study, 2103 dogs and 1112 cats were tested for SARS-CoV-2 antibodies using indirect N-ELISA (ID.Vet) in Thailand. Thirty-five dogs (1.66%) and four cats (0.36%) were positive. Neutralizing antibodies were not detected in either the positive or suspect sera using the surrogate virus neutralization test (sVNT), which has a sensitivity of 95-100% and a specificity of 99.93% [148].

Bienzle et al. [86] developed an S-based IgM and IgG ELISA and used a commercial kit (sVNT; GenScript) to detect antibodies in cats and dogs (household pets, shelters, and low-cost neuter clinics). Seropositivity among neuter-clinic and shelter samples were 16% (35/221) and 9.3% (7/75) by S-based IGg ELISA, respectively. Results also indicated 41% (24/59) of dog serum samples and 52% (25/48) of cat serum samples from household pets tested positive for SARS-CoV-2 IgG or IgM antibodies, respectively [86]. In addition, 112 dog and cat serum samples were tested with sVNT to compare S protein ELISA and sVNT results. The correlation between S protein ELISA and sVNT results was significantly greater for cats ($P < 0.0001$) than for dogs ($P = 0.05$). Some serum samples generated S antibodies with weak neutralizing activity, suggesting many antibodies that are broadly reactive with S protein are non-neutralizing RBD antibodies. It is important to note that host age, immunocompetence, and comorbidities can affect the development of antibodies against SARS-CoV-2 [86].

Jairak et al. [149] evaluated SARS-CoV-2 antibodies in 35 dogs and 9 cats from 17 households using indirect ELISA (with the anti-SARS-CoV-2 nucleocapsid antibodies IgG Kit IDVET) and the Pass™ SARS-CoV-2 Neutralization Antibody Detection Kit (GenScript) during the COVID-19 pandemic in Thailand. The authors reported that 3 out of 39 dogs and 1 out of 9 cats were positive by indirect ELISA and/or sVNT. Dog-A, tested 20–21 days after the owner tested positive, and cat-B, tested 35 days after the owner tested positive, developed anti-N IgG antibodies. Dog-A and cat-B also produced neutralizing antibodies 17 and 35 days after the owner tested positive, respectively. Furthermore, neutralizing antibodies were detected in dog C and dog D, 20 and 27 days after the owner tested positive, respectively. This indicates that transmission may have occurred from humans to pets via living with SARS-CoV-2-infected humans [149].

Additional work performed by Dileepan et al. [150] with 239 cats and 510 dogs in the early phase of the COVID-19 pandemic in Minnesota, USA. This study was conducted to determine the SARS-CoV-2 exposures through the recombinant SARS-CoV -2 nucleocapsid (N) protein and the receptor-binding-domain (RBD) of the spike protein to detect IgG antibodies in serum. The average seroprevalence of N- and RBD-specific antibodies were 8% (19/239) and 3% (7/239) for cat, respectively. These authors stated that N-based ELISA has higher levels of sensitivity in comparison with RBD-based ELISA to detect SARS-CoV-2 antibodies (IgG) in cats. Moreover, SARS-CoV-2 neutralizing activity was detected in 15 out of 19 N-seropositive cat sera using a virus neutralization assay. Evaluation of cross-reactivity between SARS-CoV-2 and Feline Coronavirus (FCoV) showed that 78.9% (15/19) of the SARS-CoV-2 N-seropositive samples were seropositive for FCoV, while 21.1% (4/19) were seronegative [150]. Opposite to cat results, the average seroprevalence of N- was approximately 1% (4/510), and no neutralizing activities were observed in dogs. None of the N-seropositive dog's sera were RBD-seropositive, indicating lower seroprevalence rates for SARS-CoV-2 in dogs. These results suggested that cats are more susceptible to natural SARS-CoV-2 infections than dogs. Additionally, the N-based ELISA (IgG) demonstrates greater specificity and sensitivity compared to the RBD-based ELISA [150].

Bosco-Lauth et al. [106] used ELISA that recognizes antibodies against the RBD, full-length spike, and N protein in cat and dog experimental infections, and it showed that all animals were seronegative at the onset of the study. All cats reached or exceeded neutralizing antibodies (titers; 1:2560) by day 14, and the titers remained stable or continued to increase between days 28 and 42 post-infection (DPI). In dogs, neutralizing antibodies responses were observed at 14 DPI, and the maximum neutralizing antibody responses were observed at 21 DPI, with titers between 1:40 and 1:80 [106]. In experimentally inoculated cats, IgG antibody response against the S protein (RBD and complete spike glycoprotein) and N protein were identified at 7 DPI. The spike protein showed the fastest and strongest seroconversion, and the response to RBD was more specific and stronger than to the N protein. By day 14, seroconversion of anti-RBD and spike IgG antibodies appeared in dogs. In cats, the response of serum IgM antibodies against RBD was detected on days 7 and 14, but it did not persist until day 28, indicating that the IgG response is stronger and more persistent than the IgM response. Overall, a higher correlation between ELISAs and PRNT results was observed for cats compared to dogs [106].

On the other hand, Deng et al. [151], who evaluated the specificity of SARS-CoV-2 double-antigen sandwich ELISA (based on recombinant S1 protein) through a serological survey of SARS-CoV-2 in 35 animal species (1,914 serum samples) in China, demonstrated that SARS-CoV-2 antibodies were not detected in all the samples. Notably, cats (n = 87; 66 pet cats and 21 street cats) and dogs (n = 487, 90 beagle dogs, 147 pet dogs, and 250 street dogs) showed serological negative to SARS-CoV-2 [151]. Similarly, an experiment conducted by Temmam et al. [108] to evaluate the antibody response in a cluster of 21 domestic pets (9 cats and 12 dogs) that had close contact with their owners (n= 11/18; showed clinical signs and n= 2/11; positive for COVID-19 and) by luciferase immunoprecipitation assay (based on S1 domain of the SARS-

CoV-2 S spike protein and C-terminal part (residues 233–419) of the SARS-CoV-2 N nucleoprotein). Results indicated that antibody responses were not detectable in sera one month after COVID-19 Diagnosis, suggesting that cats and dogs, through close contact with persons infected with COVID-19, cannot be easily infected. Therefore, a low transmission rate of SARS-CoV-2 between humans and pets can occur in natural conditions [108].

In Italy, a total of 603 dogs and 316 cats, living in households with confirmed SARS-CoV-2 cases or in affected geographic areas, were sampled and analyzed by plaque reduction neutralization test [152]. Fifteen dogs (3.3%, 15/451) with titers between 1:20 to 1:160 and 11 cats (5.8%, 11/191) with titers between 1:20 to 1:1280 were seropositive to SARSCoV-2. It has been reported that 12.8% (6 out of 47) of dogs and 4.5% (1 out of 22) of cats in households with COVID-19 patients have been infected. In households with suspected COVID-19-positive individuals, 14.3% (1 out of 7) of dogs and 0% (0 out of 3) of cats were infected. Furthermore, in COVID-19-negative households, 1.5% (2 out of 133) of dogs and 2.6% (1 out of 38) of cats showed SARS-CoV-2-neutralizing antibodies. The author suggested that the seroprevalence was found to be higher in dogs from a known COVID-19-positive household than in dogs from COVID-19-negative households. Furthermore, cats had higher antibody titers than dogs, but there is a need for further investigation regarding the susceptibility of dogs to SARS-CoV-2 Infection [152].

A study was conducted in Poland between June 2020 and February 2021 to investigate the prevalence of SARS-CoV-2 in domestic animals [153]. The study included 279 cats, 343 dogs, and 29 rabbits. The ELISA assay (ID Screen® SARS-CoV-2 Double Antigen Multi-species, IDVet) was used to detect antibodies against SARS-CoV-2. The results showed that

1.79% (5/279) of cats and 1.17% (4/343) of dogs had antibodies against SARS-CoV-2. However, all of the rabbit samples tested negative for the SARS-CoV-2 antibodies [153].

Barua et al. [154] used both nucleocapsid-ELISA (IDVet) and Surrogate Virus Neutralization Test Kits (GenScript) to monitor SARS-CoV-2 Antibodies to SARS-CoV-2 in serum samples from 1336 dogs and 956 cats in USA. Out of 956 cats tested with the I.D. Screen ELISA, six cats tested positive and two cats tested doubtful, while 948 cats were negative. Out of 1336 serum samples from dogs, 5 tested positive and 5 were doubtful using the I.D. Screen ELISA, while the remaining 1326 samples tested negative [154]. In order to confirm the antibody status and assess the level of neutralizing antibodies, 11 positive, seven doubtful, and six negative samples, along with 434 randomly selected negative samples, were tested with the sVNT. According to the findings, four out of eleven samples from cats had neutralizing antibodies for SARS-CoV2 through sVNT (GenScript sVNT) and VNT testing (titers ranged between 1/32 and 1/256). On the other hand, none of the serum samples from dogs, which were tested through sVNT and VNT, showed any positive result (0/1336). These authors also reported that differences in IDVet Screen ELISA and sVNT results can be attributed to exposure to other animal coronaviruses and antigenic cross-reactivity [154].

In Croatia, samples were collected from 656 dogs and 131 cats admitted to three veterinary facilities and tested by in-house microneutralisation test (MNT) for neutralization activity. Then, 172 randomly selected dog sera were tested using a commercial ELISA with S and N protein antigens, exhibiting 93.53% specificity and 100% sensitivity [155]. Neutralizing antibodies were found in 0.76% of cats and 0.31% of dogs, while 7.56% (13/172) of dogs tested positive via ELISA. Therefore, ELISA showed more positive results than MNT, which may be due to the severity of infection, immune system response, or inappropriate timing of sample collection (early or late in the course of the infection) [155].

Through experimental infection and the application of indirect ELISA along with a microneutralization assay [156], the response of virus neutralizing antibodies (ranging from 1:20 to 1:320) were detectable in sera from all principal infected at 7, 10, 14 and 21 days and sentinel cats at 10, 14 and 21 days post-challenge [156]. Antibodies to N and S protein in principal and sentinels cats were detected at 5 days post infection and 13 days post co-mingling, respectively. The higher infectious dose in some of the principal infected cats compared to the sentinel cats could lead to the development of an early antibody response [156].

In Germany, 0.69% (6/920) cats tested positive for SARS-CoV-2 antibodies using RBD-ELISA and indirect immunofluorescence assay (iIFA) from April to September 2020. No cross-reactivity with FCoV-specific antibodies was observed [157]. Another study by Michelitsch et al. [158] indicated seroprevalence of SARS-CoV-2 in domestic cats was 1.36% (16/1173) using RBD detection ELISA and indirect immunofluorescence assay (iIFA) from September 2020 to February 2021. However, 31.25% (5/16) of serum samples showed measurable SARS-CoV-2 neutralizing antibodies via virus neutralization test (VNT).

In an experiment conducted by Schulz et al. [159] to evaluate SARS-CoV-2 seroprevalence among 1,136 cats in Germany, 331 in the United Kingdom, 333 in Italy, and 360 in Spain using RBD-ELISA and plaque-reduction virus neutralization test in Europe during and after the first COVID-19 pandemic. Results showed that seroprevalence of RBD-specific antibodies was 4.2% in Germany, 3.3% in the United Kingdom, 4.2% in Italy, and 6.4% in Spain. Sensitivity of 90.6% and specificity of 99.8% were observed for RBD-ELISA compared with VNT. Overall, among the 2,160 cats included in this study, 4.4% (96 cats) and 4.3% (92 cats) tested positive by VNT and RBD-ELISA, respectively, indicating positive correlation between VNT and RBD-ELISA [159].

In a study conducted in northern Italy, the N-ELISA (ID-Screen® ELISA; IDvet) was used to assess the prevalence of SARS-CoV-2 infection in a cohort of stray colony and shelter cats during both the pre-pandemic (136 samples) and pandemic (105 samples) periods. The results showed that 1% of 105 cats were positive and none of the pre-pandemic serum samples tested positive for SARS-CoV-2 antibodies. Also, antibody cross-reactivity to SARS-CoV-2 antigens was not observed [160].

Natale et al. [161] investigated the cat's specific serological response against SARS-CoV-2 by using two ELISA commercial kits (ID.vet (nucleocapsid (N) protein) and ERADIKITTM), an electrochemiluminescence immunoassay (ECLIA) and plaque reduction neutralization test (PRNT) at Days 14 and 31. The commercial kit (ID.vet), ECLIA, and PRNT indicated the presence of antibodies against SARS-CoV-2 from day 14. These results indicated that N-based ELISA for SARS-CoV- 2 antibodies (except for early stage of infection) had a positive correlation with S-based ELISA and neutralization test (PRNT50) on day 31 [161].

Zhao et al. [162] conducted a serological study of SARS-CoV-2 infection in dogs (910 dog serum samples) to analyze the infection rates through ELISA (SARS-CoV-2-RBD-specific IgG) and plaque reduction neutralization test (PRNT) in Wuhan, China, between January and September 2020. Results indicated that 1.75% (16/910) of dogs tested positive by ELISA. Neutralization activity was detected in 10 out of 16 samples with titres ranging from 1:20 to 1:180 by PRNT. Serologic cross-reactivity between SARS-CoV-2 and canine coronavirus (CCV) was not detected by performing cross-reaction test. In addition, Zhao et al. [162] observed a reduction in serum antibodies against SARSCoV-2 in dogs with passing time, suggesting that the reduction in serum antibodies could be due to the decreased infection source and/or the longevity of antibodies being relatively short [162].

Perisé-Barrios et al. [163] evaluated SARS-CoV-2 infection in 40 dogs with pulmonary pathologies and 20 healthy dogs from COVID-19-positive households by SARS-CoV-2 Spike S1 protein ELISA Kit between April and June 2020 in Spain. The results showed that 25% (5/20) healthy dogs in COVID-19-positive households and 5.88% (1/17) pathological ones had antibodies to SARS-CoV-2. All Six dogs with detected antibodies against SARS-CoV-2 showed a negative result for virus detection using RT-qPCR [163].

A recent serological study was performed using RBD-based ELISA (strain Wuhan-Hu-1spike protein) on 78 dogs from SARS-CoV-2-positive owners and 1069 dogs from Veterinary Hospital in Croatia [164]. Neutralizing antibodies were detected in 25.64% of dogs from positive owners and 14.69% of dogs from Veterinary Hospital. The absence of neutralizing antibodies in some samples could be attributed to the amount of time that has passed since virus exposure, which may not have been sufficient to develop enough antibodies [164].

Overall, findings from naturally or experimentally infected cats and dogs suggest that both species are susceptible to SARS-CoV-2 infection and can develop immunological responses against SARS-CoV-2. It is important to note that seropositivity was significantly higher among pets from COVID-19-positive owners than those with owners of unknown status [152, 165].

Ruminant

SARS-CoV-2 replicates in bovine respiratory tissues (lung and trachea) [166]. However, it has been reported that expression of ACE2 gene was higher in the liver and kidney than in the lung in cows [169]. Bovine ACE2 contains most of the key residues for RBD of SARS-CoV-2 [167, 168]. Farm animals such as buffaloes, goats, sheep, horses, rabbits, hens, pigs, and cows that had been in contact with their SARS-CoV-2-positive breeder for at least 2 weeks tested negative for SARS-CoV-2 infection [169]. The geographic distribution of cervid wildlife species such as white-tailed deer shows that white-tailed deer are a free-living wild animal species widespread in North America and live in close proximity to human dwellings [170]. Due to the social nature of deer, contact patterns among deer can facilitate intraspecies transmission of multiple pathogens [171]. In addition, ACE2 receptors of three Cervid species, Odocoileus virginianus (white-tailed deer), Rangifer tarandus (reindeer), and Elaphurus davidianus (Pere David´s deer), showed a high similarity to human ACE2 receptors [172], which show white-tailed deer are highly susceptible to SARS-CoV-2 infection [173]. SARS-CoV-2 transmission within the deer population may have occurred through various routes, including direct contact with people, wildlife tourism, hunting, wildlife rehabilitation, supplemental feeding, contaminated water sources [171], and vertically from doe to fetus [174]. Some studies have documented that the seroprevalence of SARS-CoV-2 in white-tailed deer (Odocoileus virginianus) was approximately 30-40% across Illinois, Michigan, New York, Pennsylvania [171], and Texas [175]. Domestic livestock ruminants such as cattle, sheep, and goats are farmed around the world due to their economic importance. These livestock animals can come into contact with humans during normal farming activities. There have been reports of natural SARS-CoV-2 infection in cattle, but studies suggest that they are unlikely to serve as reservoirs because they are low susceptibility to the virus and virus is not transmitted between species [176].

To investigate the susceptibility of common domestic livestock to SARS-CoV-2, a study was performed in which 3 cattle, 4 sheep, 3 goats, 2 alpaca, 4 rabbits, and 1 horse were inoculated with the SARS-CoV-2 virus strain 2019-nCoV/USA-WA1/2020 [177]. Low-level neutralizing antibodies were observed in cattle, sheep, goat, alpaca, and rabbit within 14 days of infection, and the majority of seronegative animals were observed on day 28 post-infection. These results demonstrated that domestic livestock species are poorly hosts to transmit infection to other animals [177].

Wernike et al. [178] evaluated the presence of SARS-CoV-2 antibodies in serum samples from 1000 cattle collected in late 2021 in Germany using RBD-based multispecies ELISA, indirect immunofluorescence assay (iIFA), and surrogate Virus Neutralization Test (GenScript Kit). In addition, 100 samples that were collected across Germany in 2016, and all of them tested negative, were considered as a control group. In total, 11, 4, and 10 samples tested positive by the RBD-ELISA, sVNT, and iIFA, respectively. These authors stated that a low number of seropositive cattle might occasionally become infected with the virus due to exposure to keepers who were positive for SARS-CoV-2 [178].

In another study, six out of nine dairy calves were intranasally inoculated with 1×105 50% tissue culture infectious dose of SARS-CoV-2 (strain 2019_nCoV Muc-IMB-1) [179]. Serum samples were collected on days -1, 6, 12, and 20 and tested by RBD-ELISA, indirect immunofluorescence assay, and virus neutralization test. The ELISA results showed an increase in the number of seropositive samples in one out of the six calves from day 12 onward, with another calf showing a slight increase on day 20. Serological cross-reactivity of antibodies against bovine coronavirus and SARS-CoV-2 was not observed. Moreover, all serum samples tested negative for both the indirect immunofluorescence assay and virus neutralization test on day 20, indicating that cattle have low susceptibility to SARSCoV-2 infection [179].

Ulrich et al. [179] results are consistent with those of Falkenberg et al. [180], who inoculated three bull calves via the intratracheal or intravenous routes with the SARS-CoV-2 strain, TGR1/NY/20. Neutralizing antibodies titers of 8 and 4 were detected in the intratracheal and intravenous inoculated calves by day 7, respectively. Although, SARS-CoV-2 antibodies were not detectable by 21 days post-challenge. The results from this study failed to show productive replication of SARS-CoV-2 in inoculated calves [180].

According to a study conducted by [181] Dias et al. in Brazil between 2020 and 2021, SARSCoV-2 antibodies were detected in 4 out of 367 samples using the ID Screen® SARS-CoV-2 Double Antigen (N-ELISA) test. Cool et al. [182] also identified SARS-CoV-2/Delta RBD-specific antibodies at 14 days DPC using Indirect ELISAs. Neutralizing antibodies against the SARS-CoV-2 Delta variant were detected at 10 DPC in one calf; however, no neutralizing antibodies were observed against the Omicron BA.2 variant.

In an experiment conducted by Fiorito et al. [183], a total of 24 lactating cows were sampled from a farm with 150 animals which 13 of the 20 farmworkers showed COVID-19-like symptoms. using the Elecsys® Anti-SARS-CoV-2 test (an electrochemiluminescence immunoassay based on anti-SARS-CoV-2 N antibodies), the Roche Elecsys® Anti-SARS-CoV-2 S test for the SARS-CoV-2 spike RBD, and the Microneutralisation Test (MTN) [183]. The results showed that 46% (11/24) and 58% (14/24) of the cows had antibodies for the SARS-CoV-2 nucleocapsid protein and the SARS-CoV-2 spike protein, respectively. Neutralizing antibodies, with titers varying from 1:20 to 1:160, were found in 54% (13/24) ot the tested cows. In this study age of the cows showed a significant correlation with anti-S and neutralizing antibodies against SARS-CoV-2 [183]. Older cows tend to have higher antibody titers compared to younger cows, likely due to the presence of preexisting antibodies and memory T and B cells [184]. Since ACE2 of cattle exhibits high sequence identity to human ACE2, and cattle and humans share four of the five hotspot residues, this may facilitate the interaction between the spike protein of SARS-CoV-2 and ACE2 [139, 185].

Eight of ten male sheep, approximately 6 months of age, inoculated orally and intranasally with a 1:10 titer ratio of lineage A WA1 and the alpha VOC B.1.1.7 strains [186]. Two uninfected sheep held in a dedicated clean room and then cohoused with infected sheep. Sera collected from sheep and tested for reactivity against the bovine coronavirus spike antigen prior to SARS-CoV-2 challenge by in-house ELISA (RBD and N protein). Serum samples were negative for BCoV infection. Anti-SARS-CoV-2 antibodies to N and RBD protein were detected in all infected sheep at 10, 14, 17, and 21 post infection. Only one of the sheep developed low level of neutralizing antibodies with a titer of 1:20 at 10 and 21 DPC. Anti-RBD and anti-N antibodies were not detectable in sentinel sheep [186].

In a study by Villanueva-Saz et al. [187], 90 and 336 sheep serum samples were collected from the pre-pandemic and pandemic (June 2020 to March 2021) period, respectively, to evaluate SARS-CoV-2 antibodies in sheep having close contact with humans through in-house IgG ELISA with RBD. Sheep did not develop a significant specific antibody response; therefore, despite close contact with humans, the absence of exposure in all sheep suggests that sheep might not be susceptible to SARS-CoV-2 [187].

Happi et al. [188] used indirect IgG ELISA to assess the prevalence of natural SARS-CoV-2 infections in 3 cattle, 130 sheep, and 223 goats sampled between May and August 2021 and January and October 2022. The results indicated that SARS-CoV-2 RNA was detected in all of these species. However, the development of antibodies targeting both the S-RBD and N-Nterm SARS-CoV-2 antigens was observed in only 4 out of 223 goats.

Furthermore, Bae and colleagues [189] evaluated the prevalence of SARS-CoV-2 infection among Korean native cattle and Korean native black goats. The results indicated a 4.60% (54/1174) positivity rate in Korean native cattle and 2.56% (16/624) in Korean native black goats using an indirect ELISA kit (ID Screen® SARS-CoV-2 Double Antigen). Virus-neutralizing antibodies were detected in 4.34% (51/1174) of Korean native cattle and 2.24% (14/624) of goats. An additional finding of this study was that (humans) animal handlers on farms where seropositive cattle and goats were reported had previously been infected with SARS-CoV-2, suggesting a potential reverse zoonotic transmission event(s) of SARS-CoV-2 from humans to livestock in Korea [189].

A recent study [190] conducted on sheep and goats from 24 different farms in Italy demonstrated that 3.48% (17/488) of sheep and 4.83% (6/124) of goats tested positive for ELISA using the ID Screen SARS-CoV-2 Double Antigen Multi-Species ELISA Kit. Additionally, one sheep exhibited a neutralizing antibody titer of 1:20, indicating a potential susceptibility of these animals to natural infection by SARS-CoV-2 [190].

An experimental inoculation [191] with the B.1.351/Beta variant in domestic goats showed antibody development at 10 and 18 days post-infection (dpi) using the RBD Inhibition ELISA assay (GenScript cPass™ kit). Additionally, a live virus neutralization assay detected neutralizing antibodies at 7, 10, and 18 dpi, indicating seroconversion starting from 7 days post-infection [191].

A total of 624 pre-pandemic and post-pandemic wild white-tailed deer (Odocoileus virginianus) serum samples from four US states, Michigan (n = 113), Pennsylvania (n = 142), Illinois (n = 101), and New York (n = 29), and 239 wild WTD serum samples from five state, Michigan (n = 37), Pennsylvania (n = 104), Illinois (n = 16), New Jersey (n = 8), and New York (n = 74), were tested for SARS-CoV-2 exposure through surrogate virus neutralization test (sVNT, Genscript cPass) [171]. Antibodies to SARS-CoV-2 were detected in 40%, 3%, and 2% of 2021, 2020, and 2019 samples, respectively. High percent inhibition values (80 to 100%) were observed in sample from 2021, while relatively low percent inhibition values (30.03% to 43.72%) were for samples collected from 2019 to 2020. These authors reported that low percent inhibition could be attributed to fluctuating immunity, nonspecific antibody binding, or cross-reactivity [171]. Seroprevalence varied by county and state and was lowest in Illinois (7%), intermediate in New York (31%) and Pennsylvania (44%), and highest in Michigan (67%). Overall, antibodies for SARS-CoV-2 were observed in 40% of white-tailed deer from various regions in the United States [171].

In a study conducted by Cool et al. [174], four of six female white-tailed deer (WTD) that were approximately 2 years old, inoculated orally and intranasally with a 1:1 titer ratio of lineage A WA1 and the alpha VOC B.1.1.7 strains. Two uninfected deer held in a dedicated clean room and then cohoused with infected deer starting one day after inoculation. Neutralizing serum antibodies were evaluated for all deer by microneutralization assay and in-house ELISA (RBD and N protein) [174]. Except one principal infected deer that developed antibodies to N protein at 7-, 14- and 18-days post infection, three remaining principal infected deer and the sentinels showed weakly positive antibody levels against the N-protein over the 18-day experimental period. Two principal infected deer were positive at 7 days post infection and sentinel deer showed weak positive or even negative for the SARS-CoV-2 RBD antigen over the 18-day experimental period. Principal infected deer and sentinel deer demonstrated significant levels of neutralizing antibody titers on days 7 and 10 of post infection, respectively. In addition, similar neutralization titers (approximately 1:1280) were observed for both the principal infected and sentinel deer at 14 and 18 DPC. Among sera from both infected and sentinel deer, all samples exhibited levels similar to negative bovine control sera when tested using indirect ELISA [174].

In another study, four of six white-tailed deer fawns, approximately 6 weeks of age, inoculated intranasally with a virus suspension containing $10^{6.3}$ $TCID_{50}$ .ml-1 of SARS-CoV-2 tiger isolate TGR/NY/20 [192]. Serum samples collected on days 0, 7, 14, and 21 days post inoculation and were tested using a Luminex (S and N protein) and in-house virus neutralization (VN) assay. For evaluation of SARS-CoV-2 transmission via indirect contact, two of six white-tailed deer fawns were maintained as noninoculated animals. Results indicated that seroconversion against SARS-CoV-2 occurred in all inoculated and indirect contact animals. On day 7 post infection, neutralizing antibodies against S-RBD were detected and increased on days 14 and 21 post infection. Antibodies against the N protein not only had lower titers but also observed at later time points (days 14 to 21). Therefore, authors suggested that white-tailed deer are susceptible to SARS-CoV-2 infection and transmission of the virus to other white-tailed deer can occur through indirect contact [192].

Palermo et al. [175] reported results of serological survey performed on white-tailed deer serum samples collected in Travis County, Texas, during 2018 and prior to and during the pandemic in 2021. From January to February 2021, a total of 54 blood samples were collected from hunter-harvested deer, along with another subset of 40 deer serum samples collected in 2018. Some factors such as gender and age were recorded as well [175]. Serological test was done by a standard plaque reduction neutralization assay. Among 54 deer sera samples evaluated, 37% (20/54) were positive for SARS-CoV-2 neutralizing antibody. None of the samples (n=40) that were collected during 2018 or the pre-pandemic period were positive, which means SARS-CoV-2 and other coronavirus infections, which cause serological cross-reactivity, did not occur during this period. Seroprevalence rates of SARS-CoV-2 between males and females were 50% (15/30) and 21% (5/24), respectively. Additionally, 1.5 years age group had highest SARS-CoV-2 seroprevalence rate (82% (9/11)) [175].

To evaluate the presence of antibodies against SARS-CoV-2 and initial exposure in deer, samples from 21 non-pregnant adult female deer in Texas were tested in September 2021, including 19 seropositive deer, tested by plaque reduction neutralization test (PRNT90), and 2 seronegative deer [193]. While all samples tested seronegative in November and December 2020, samples tested positive by PRNT90 antibody test in January 2021, indicating that the infection and seroconversion occurred between 16 December 2020 and 27 January 2021 [193]. The result showed that the neutralizing antibodies were detected in 90.5% (n=19) of deer from initial seroconversion (January 27, 2021) to the end of the study (March 4, 2022). These data suggested antibody responses to SARS-CoV-2 remained detectable for around 13 months [193].

Roundy et al. [194] investigated the seroprevalence of SARS-CoV-2 in three captive cervid facilities in central and southern Texas. Between September 15, 2021 and November 29, 2021, serum of 80 deer (Odocoileus virginianus, Axis, and Dama dama) were collected and tested by neutralization assay (PRNT90). At a single captive cervid facility, 94.4% (34 out of 36) of adult white-tailed deer tested positive for SARS-CoV-2 neutralizing antibodies, with endpoint titers reaching up to 1280. In the other hand, all the tested deer at two other captive cervid facilities were seronegative. These authors stated that confined environment might lead to transmission and circulation of SARS-CoV-2 among deer in one of three captive cervid facilities [194].

Another serological survey was conducted by Holding et al. [195] in the United Kingdom that screened a total of 1748 deer serum samples from 6 species and a cross-bread species. The sera of 654 and 1094 deer collected from January to the end of March 2020 and October 2020 to

May 2021, respectively, from 48 counties/Unitary Authorities. Antibodies to SARS-CoV-2 were determined by anti-spike and anti-nucleocapsid serology assays. Positive samples that were positive on both the S and N assays were further tested using the Gen Scriptc Pass SARS-CoV-2 surrogate virus neutralization test (sVNT). Fifty-four and nine deer serum samples were positive on the S and N assays, respectively; while the positive results were not observed when tested with the sVNT. Indeed, agreement among results of S and N antigen assay and neutralizing antibodies to SARS-CoV-2 was not observed. They found no evidence of exposure and spill-over infections of SARS-CoV-2 occurred from the human population to deer or among deer. These authors reported that the higher seroprevalences in WTD across Northern America in comparison with United Kingdom can be attributed to varying sequence differences in the virus-binding residues in different species [195]. Along these lines, in a study conducted by Moreira-Soto et al. [196], Sera from German and Austrian deer, including red deer (Cervus elaphus, n = 67), roe deer (Capreolus capreolus, n = 97), and fallow deer (Dama dama, n = 68), were collected between January 2020 and December 2021 to evaluate exposure to SARS-CoV-2 during the pandemic period using the SARS-CoV-2 surrogate virus neutralization test (sVNT; GenScript). No SARS-CoV-2 antibodies were detected in pandemic deer sera [196].

In another study by Martins et al. [197], six fawns (8 months old) were inoculated intranasally with 5 ml of virus suspension containing $10^{\wedge 6.38}$ $TCID_{50}$/ml of SARS-CoV-2 isolate NYI67-20 (lineage B1), and six fawns were maintained as contact animals to evaluate deer-to-deer transmission. Apart from a slight, short-term rise in body temperature in 5 out of 6 inoculated fawns on day 1 and in 1 out of 6 fawns on day 2 post-infection (pi), none of the fawns (both uninoculated and inoculated) exhibited clinical signs. Nasal and oral secretions of inoculated animals showed viral RNA between days 2 and 22 pi. It was observed that all inoculated animals exhibited detectable antibodies by virus neutralization assay (VNA) on days 7, 14, and 22 [197].

A study assessed the seroprevalence of SARS-CoV-2 in 472 free-ranging white-tailed deer serum samples collected from 10 sites in northeastern Ohio between November 2020 and March 2021 using an in-house S-ELISA [198]. The findings revealed that 17.2% (81/472) of the deer had antibodies against the SARS-CoV-2 spike protein, with titers ranging from 1:10 to 1:10,240 [198]. To evaluate the seroprevalence of SARS-CoV-2 in free-ranging white-tailed deer, 1164 serum samples from 1522 white-tailed deer were collected across 83 of Ohio's 88 counties between October 2021 and March 2022 [199]. The results showed that SARS-CoV-2-neutralizing antibodies were detected in 274 WTD (23.5%; 274/ 1164) using the GenScript SARS-COV-2 Surrogate Virus Neutralization Test (sVNT) [199].

In a study where seven North American elk calves and seven adults were intranasally infected with the ancestral Wuhan-like variant of SARS-CoV-2 (USAWA1/2020), neutralizing antibodies were detected in both calves and adults 7 days post-infection and persisted for 21 days by the RBD Inhibition ELISA assay (sVNT, GenScript Biotech) [200]. The highest titers were found in one calf at 14 days post-infection (1:256) and one adult at 7 days post-infection (1:16) via virus neutralization (VN) tests, suggesting that calves exhibited a stronger neutralization response. These findings imply that the lower antibody responses in adult elks may be due to pre-existing immunity, which could limit the amount of antigen available for new antibody responses [200]. In another study by Porter et al. [201] weanling elk and mule deer were inoculated with the Delta variant of SARS-CoV-2, B.1.617.2, via intranasal routes. The findings indicated that mule deer generated higher neutralizing antibody responses, with peak titers surpassing 1:1,280 at 21 dpi, whereas elk showed peak neutralizing titers of only 1:20 at the same time point. This investigation highlighted the differing susceptibility to the Delta variant of SARS-CoV-2 between the two species, with mule deer displaying high susceptibility and elk exhibiting low susceptibility [201].

Pigs, chickens, and ducks

Shi et al. [107] conducted an experimental infection research using animals (ferrets, cat, dog, pigs, chickens, and ducks) to detect antibodies against SARS-CoV-2 by ELISA and a neutralization assay. As a result, antibodies against SARS-CoV-2 were detected in all cats and ferrets on days 14 and 13 post-infection, increasing substantially by 20 days post-infection, by ELISA kit and neutralization assay; while except for two dogs, the other dogs and animals were seronegative for SARS-CoV-2 on day 14. These data support the idea that ferrets and cats are the most susceptible, dogs have low susceptibility, and pigs, chickens, and ducks have no susceptibility to SARS-CoV-2.

The study, which involved nine fruit bats, ferrets, pigs, and 17 chickens inoculated with SARS-CoV-2, was designed to measure SARS-CoV-2 reactive antibodies using indirect immunofluorescence assay (IIFA) and virus neutralization tests [133]. SARS-CoV-2-reactive antibodies were observed in all inoculated bats between days 8 and 21, with titers ranging from 1:16 to 1:64. In ferrets, antibodies were detected by day 8, with titers ranging from 1:64 to 1:8192, using IIFA. Neutralizing antibodies were observed in all inoculated bats with titers up to 1:64 and in ferrets on day 21 using VNT. SARS-CoV-2-reactive antibodies were not detected in pigs or chickens [133]. In a study [202] involving the infection of four groups of conventional piglets (5- to 6-weeks-old) via intranasal (IN), intratracheal (IT), intramuscular (IM), and intravenous (IV) routes, all animals in the IM and IV groups showed low levels of SARS-CoV-2 antibodies against the Spike protein in an in-house ELISA at 14 and 22 days post-infection.

Neutralizing antibodies were also observed at 22 dpi via a virus neutralization assay. Additionally, all IV inoculated animals, and one out of three IM inoculated animals exhibited low antibody levels against SARS-CoV-2 N proteins in in-house ELISAs at the end of the experiment. Consequently, seroconversion indicates the possibility of using pigs in SARSCoV-2 immunogenicity studies [202]. In another study [135] nine pigs (five weeks of age) were inoculated orally, intranasally, and intratracheally with SARS-CoV-2, while three uninfected pigs were maintained as contact animals to evaluate pig-to-pig transmission. The inoculated pigs did not develop neutralizing antibodies or IgG and IgM antibodies against SARS-CoV-2 using Indirect RBD and N ELISAs. One sentinel contact pig exhibited transient IgM reactivity on days 3 and 5 DPC and isolated IgG reactivity on day 3 DPC against the N and RBD proteins; however, sentinel contact pigs did not develop neutralizing antibodies against SARS-CoV-2 [135].

These findings indicate that neither pigs nor chickens were susceptible to SARS-CoV-2, which supports other study findings [107]. Although, it should be considered that ACE2 receptor binding sites of pigs, ferrets, cats, orangutans, monkeys, and humans likely can be recognized by 2019-nCoV RBD [203].

Ferret, Mink, Hamster, and rabbit

In a recent study, Giner et al. [204] assessed the detection of SARS-CoV-2 antibodies in serum samples from 127 household ferrets (Mustela putorius furo) in Spain, utilizing an in-house ELISA designed for IgG antibodies targeting the RBD. In this study, a total of 194 samples were collected: 37 from the first quarter of 2020, 77 from the second quarter, 44 from the third quarter, and the remaining 36 serum samples from October and November. The results indicated that

1.57% of ferrets tested positive for anti-RBD SARS-CoV-2 antibodies, which persisted for at least 129 days after initial detection.

Using the ferret model, Kim et al. [132] used ELISA to detect neutralizing antibodies against SARS-CoV-2 in infected ferrets. Results of this study indicated that neutralizing antibodies appeared within 2–3 weeks post-infection, with titers ranging from 32 to 128 [132]. Another ferret experimental model showed that neutralizing antibodies found by day 21 after the infection or exposure by RBD-ELISA in infected ferrets [205].

Another study of 98 Mink serum samples, which were collected from mink farm in Spain, showed that 89% (82/92) of samples contained antibodies against SARS-CoV-2 by ID Screen SARS-CoV-2- ELISA, indicating that virus has spread rapidly among farmed mink [206]. A serological survey of SARS-CoV-2 in Danish mink farms was conducted to characterize the spread of the disease in infected farms using the SARS-CoV-2 Ab ELISA [207]. The results indicated that 69% (111/169 farms) exhibited a seroprevalence of 100%. Specifically, nearly 72% of farms with clinical signs and 45% of farms without clinical signs demonstrated a 100% prevalence of antibodies to SARS-CoV-2 at the initial sampling. Some dogs and cats tested positive for antibodies among the various species of animals residing on or near the infected farms. In contrast, chickens, rabbits, and horses in the vicinity of the infected farms tested negative. Consequently, it has been suggested that minks are highly susceptible to SARS-CoV-2 infection [207]. In an experiment reported by Greenhorn et al. [208], 219 samples, which were collected between June 2020 and May 2021, across three species (141 raccoons, 36 striped skunks, 42 minks) were tested by RBD-ELISA (GenScript cPass™) for the presence of neutralizing and not neutralizing antibodies. Serum samples showed no evidence of neutralizing antibodies response.

A study by Villanueva-Saz et al. [10] investigated the presence of SARS-CoV-2 in 127 European mink (Mustela lutreola) and 35 American mink (Neogale vison) from Spain between January 2020 to February 2022 via in-house indirect ELISA (RBD). None of the samples tested positive for SARS-CoV-2-specific antibodies.

A study conducted by Moreno et al. [209], who evaluated SARS-CoV-2 in a mink farm in Italy by several tests, including N-ELISA (ID Vet); recombinant N protein ELISAs (Eradikit™ and in-house IZSLER (DAS-N ELISA)); virus neutralisation test (VNT); two different surrogate virus neutralization tests (GenScript and Proteogenix), and double-antigen ELISA (Wantai SARS-CoV-2 total antibody ELISA; Ig M, Ig G and IgA), to detect antibodies against different SARS-CoV-2 antigens [209]. Seventy-four blood samples were collected during the culling procedures from positive farms, and 44 blood samples were collected from other negative farms. Based on antibodies against the N protein, the serum samples tested negative for SARS-CoV-2-specific IgG antibodies (IDvet). However, 62/69 and 59/66 positive samples were detected using in-house IZSLER (with sensitivity of 89.39%) and Eradikit™ (with sensitivity of 86.15%). Based on Neutralizing antibodies, positive results were identified via the VNT assay with different titers. All sera from the infected farm were positive by the GenScript sVNT while negative controls (44 sera collected from negative farms) were negative. Proteogenix kit did not identify any serum as positive. The Wantai SARS-CoV-2 total antibody ELISA showed positive results for the infected farm and negative results for negative farms [209]. These authors stated that: 1) a high serological prevalence indicates extensive virus spread within the farm; 2) the antibody response to the RBD of the S protein was stronger than to the N protein, indicating that highly sensitive tests are more effective at detecting anti-RBD antibodies than anti-N antibodies; and 3) most commercial serology kits for measuring anti-SARS-CoV-2 antibodies are developed and validated for human samples, and less is known about their diagnostic performance with animal samples in detecting antibodies. Therefore, it is necessary to compare the effectiveness of different diagnostic tests in animals [209].

An antibody survey was conducted among approximately 15,000 farmed mink in Denmark, including virus-infected animals, to evaluate antibody levels during the first and second waves of infection using RBD-ELISA and serum neutralization assays [210]. Significant variations were observed in the levels of antibodies detected by ELISA in the serum samples collected at different time points. Higher antibody levels were evident during the period of 'free-testing' compared to the initial sampling, and substantially elevated levels were observed following reinfection in comparison to the earlier tests. Indeed, levels of anti-SARS-CoV-2 antibodies significantly elevated after the second wave of infection, indicating a heightened antibody response and immune boost following reinfection [210].

The experimental infection of golden Syrian hamsters, where three donor hamsters were inoculated intranasally and naïve hamsters were exposed, has demonstrated that serum neutralizing antibodies were detected using the PRNT assay in the donor hamsters (titers of 1:640 for all) by 14 days post-infection and in the co-housed hamsters (titers of 1:160, 1:320, and 1:160) by 13 days post-contact [134]. Chan et al. [211] reported that infected golden Syrian hamsters developed neutralizing antibodies by day 7 post-infection using microneutralization assay and passive immunization. The mean serum neutralizing antibody titers were 1:480 or higher at 7 days post-infection and 1:427 or higher at 14 days post-infection. Imai et al. [212] used ELISA and virus neutralization assays for detection of antibodies in SARS-CoV-2–infected hamsters. The findings demonstrated that infected hamsters produced antibodies against SARS-CoV-2 by day 19 following the initial infection, indicating that primary exposure to SARS-CoV-

2 enhances the production of neutralizing antibodies and protect animals from reinfection. In an experiment conducted by Lee et al. [213], golden Syrian hamsters were orally inoculated with 105 PFUs of the virus and intranasally with different doses of SARS-CoV-2 (102, 103, 104, and 105 plaque-forming units [PFUs]. The results indicated that serum neutralizing antibodies were detected in animals inoculated both orally (titer = 10–40) and intranasally (titer = 20–40) at 7 DPI. By 14 DPI, higher levels of neutralizing antibodies were observed in hamsters inoculated intranasally (titer = 40–160) [213].

In another study [214], four groups of three New Zealand white rabbits were selected, and three groups were infected intranasally with 10^4, 10^5, or 10^6 $TCID_{50}$ SARS-CoV-2. Serum samples were collected on day 21 from animals infected with 10^6 $TCID_{50}$ SARS-CoV-2 to be tested by PRNT50. All animals seroconverted and developed neutralizing antibodies by day 21 with titers ranging from 1:40, 1:320, and 1:640 [214].

To evaluate the seroprevalence of SARS-CoV-2 on four French mink farms, in late 2020, a total of 1912 blood samples were collected to determine the percentage of SARS-CoV-2 seropositivity by N-ELISA (IDVet (SARS-- CoV-2 Double Antigen Multi-species) and seroneutralization assays [215]. The results indicated the presence of SARS-CoV-2 antibodies against the N protein in 96.6% of serum samples from the first farm, and in 0.8%, 0.3%, and 1.1% of samples from the other three farms. SARS-CoV-2 neutralizing antibodies were detected in positive samples by ELISA from the first farm, ranging from 81 to above 2239, while no SARS-CoV-2 neutralizing antibodies were detected in samples from the other three farms [215].

Non-human primates, large captive animals, and other species

The susceptibility of animals to SARS-CoV-2 varies; different species exhibit varying levels of susceptibility. Various animal species, including captive felines like pumas, cougars, snow leopards, lions, and tigers, as well as other wildlife in captivity such as gorillas and otters, have demonstrated susceptibility to SARS-CoV-2.

Additionally, natural infections by SARSCoV-2 have been reported in hippopotamuses at a zoo in Hanoi, Vietnam [216] and Belgium [217].

In Belgium [217], a study investigated the potential spread of SARS-CoV-2 in two adult Hippopotamus amphibius using ELISA Wantai (RBD-ELISA) and ID Screen® SARS-CoV-2 Double Antigen (N-ELISA). Wantai (16.79%) and ID Screen (60.33%) confirmed the presence of neutralizing antibodies against SARS-CoV-2, suggesting the susceptibility of hippos to the virus [217].

Another study [216] documented the death of a hippopotamus in a Hanoi zoo from SARS-CoV-2 infection, occurring 17 days after the onset of clinical symptoms. This event underscores the close genetic relationship between the SARS-CoV-2 strain found in the hippopotamus and three human SARS-CoV-2 strains identified in Vietnam [216].

Following the detection of SARS-CoV-2 in hippopotamuses, a subsequent study [218] aimed at monitoring SARS-CoV-2 in mammals potentially exposed indirectly to the infected hippopotamuses was conducted in two zoos in Belgium during 2020-2021. Among the 50 serum samples from 26 mammal species, all tested negative for SARS-CoV-2 using sVNT (GenScript cPass™) [218] .

A serosurvey of 204 red foxes (Vulpes vulpes) conducted in Croatia from June 2020 to February 2021 revealed that 2.9% (6/204) tested positive for anti-SARS-CoV-2 antibodies against the N protein (ID.Vet, France). However, none of the foxes developed humoral immune responses against SARS-CoV-2 as assessed by the surrogate virus neutralization test (sVNT; GenScript) [219].

In an experimental infection study [220], juvenile (3–5-monthold), 6 mixed sex red foxes (3 female, 3 male) and 4 coyotes (3 female, 1 male) were infected with the SARS-CoV-2 strain WA1/2020WY96 via their nares. Virus-neutralizing antibodies were detected in all red foxes at 7 days post-infection (dpi), peaked at 1:80 or higher at 14 dpi, and were sustained until 28 dpi, using plaque reduction neutralization assays. None of the remaining animals seroconverted [220].

In February 2021, at the Prague Zoological Garden, western lowland gorillas, Asiatic lions, Sumatran and Malayan tigers, and Amur leopards were confirmed positive for the SARS-CoV-2 B.1.1.7 variant via PCR testing. Prior to the animal outbreak, one gorilla keeper and two cat keepers, tested positive for COVID-19, indicating a likely transmission pathway through direct contact with infected humans [221].

African green monkeys were used as a non-human primate model of SARS-CoV-2 infection. In a study conducted by Cross et al. [222], six African green monkeys (AGMs) were infected with a target dose of 3.0 × 106 PFU of SARS-CoV-2 (SARS-CoV-2/INMI1-Isolate/2020/Italy) through the intranasal and intratracheal routes. At 5 dpi, three animals were euthanized (peak stage of virus replication) and the remaining infected AGMs were euthanized at 34 dpi. Nucleoprotein ELISA kits were used to measure SARS-CoV-2 IgG antibodies, revealing that antibodies against the N-Protein developed by day 15 in two animals. Low levels of neutralizing titers were detected in all animals as early as 5 dpi, but by 34 dpi, these titers had gradually increased to a range of 1:16 to 1:128 [222].

In an experimental study by Munster et al. [223], eight adult rhesus macaques were inoculated with the SARS-CoV-2 isolate nCoV-WA1-2020 and assessed for development of IgG against the SARS-CoV-2 spike protein by ELISA. Four macaques had seroconverted to the

SARS-CoV-2 spike protein by 10 dpi and neutralizing antibody responses (IgG) were detected at around 7–10 dpi.

In another study, Porter et al. [224] evaluated 2 wild canid species, red foxes (Vulpes vulpes) and coyotes (Canis latrans), for susceptibility to SARS-CoV-2. A Juvenile (3–5 month old), mixed sex red foxes (3 female, 3 male), and coyotes (3 female, 1 male) were experimentally infected with the SARS-CoV-2 strain WA1/2020WY96 through instilling into the nares of each animal. Blood samples collected weekly from the remaining animals [(red fox; until 28 dpi) or (coyote until 30 dpi)] and tested via PRNT80. Neutralizing antibodies were detected in all of the red foxes at 7 dpi, reached a peak at 14 dpi (1:80 or higher), and continued to be present until 28 dpi. None of the coyotes seroconverted. Therefore, red foxes can be susceptible to infection with SARS-CoV-2 because of consuming other species [224].

The study developed by Fernández-Bellon et al. [225] included four lions with natural SARS-CoV-2 infection at the Barcelona Zoo. Serological assays were performed to detect antibodies against SARS-CoV-2, using the serum neutralization test (SNT), the surrogate virus neutralization technique (sVNT; Genescript), and an in-house N-ELISA. Four lions were positive by SNT and sVNT. Although initially high levels of neutralizing antibodies (nAbs) were detected using both SNT and sVNT assays, a decline was observed over a 4-month period following infection. In this study, two tigers exhibited low levels of anti-N antibodies, while one tiger failed to produce detectable levels of neutralizing antibodies against the N protein. Notably, only one tiger demonstrated a significant increase in neutralizing antibodies against the N protein 4 months post-SARS-CoV-2 infection [225].

Furthermore, Tewari et al. [226] reported lions, tigers, and lynx exhibited a robust neutralizing antibody response against the viral spike protein, indicative of exposure to SARS-

CoV-2. In contrast, cheetahs did not display any clinical signs of infection, and antibody responses were undetectable using the surrogate virus neutralization assay. This lack of detectable antibodies suggests either an absence of exposure to SARS-CoV-2 or resistance to the infection in cheetahs.

A serological study conducted by Borkakoti et al. [227] to evaluate the prevalence of SARS-CoV-2 infection among wildlife species examined 126 leopards, 96 Asiatic lions, and 98 Bengal tigers across eight Indian states. The findings revealed detectable levels of SARS-CoV-2 neutralizing antibodies in 15% (48/320) serum samples tested, distributed among 24 lions, 14 tigers, and 10 leopards. The antibody titers varied, ranging from 2.1 to greater than 2.4, thereby highlighting potential exposure and the dynamics of immune response within these populations [227].

A study [228] conducted in Thailand between 2020 and 2021 found that 6.5% of captive tigers had neutralizing antibodies against the Wuhan Hu-1 and Delta variants of SARS-CoV-2 using PRNT. The probable source of SARS-CoV-2 exposure for these tigers is close interactions with their handlers, who had recently contracted the virus. Only one tiger showed low levels of neutralizing antibodies against the Omicron BA.2 subvariant [228].

Conclusion

Serological assays are critical tools in the evaluation of a defined population to determine virus exposure and estimate seroprevalence. The performance of different serological assays can vary in detecting antibody levels, and the sensitivity and specificity of different assays may vary significantly. This makes the interpretation of assays more difficult. Determining the susceptibility of species to infection with SARS-CoV-2 and identifying immunological cross-reactivity between SARS-CoV-2 and other coronaviruses are crucial. Anti-RBD IgM and IgA contribute most to neutralization in the early phase of infection, while the IgG antibody can persist much longer in animals. Investigating the kinetics of the antibody response to SARS-CoV-2 is essential for establishing long-term protection and sustained immunity against reinfection with the same viral strains. Nonetheless, preexisting cellular and humoral immunity (cross-reactive antibodies) from exposure to different seasonal coronaviruses (sCoVs) and the apparent similarities between betacoronaviruses can cause cross-reactivity to SARS-CoV-2. It is reasonable to conclude that cross-reactive antibodies, which lack neutralization activity and are elicited by different coronaviruses, cannot prevent SARS-CoV-2 infections or reduce clinical symptoms. However, it is important to consider that the timing, composition, and magnitude of the adaptive immune response can affect the immune response to SARS-CoV-2. Overall, the observed differences in immune responses among various species highlight the variability in susceptibility and immune defense mechanisms.

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Chapter 2 - Bovine, sheep, and goat humoral immune responses against SARS-CoV-2

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Running title: Seroprevalence to SARS-CoV-2 in cattle, sheep and goats

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Following the emergence of SARS-CoV-2, various animals have been found susceptible. This study collected serum samples from 691 cattle, 698 sheep, and 707 goats from several regions in the United States (2019-2022) to test for SARS-CoV-2 antibodies using ELISA assays. Approximately 1% of cattle, 2% of sheep, and 2.5% of goats tested positive with the SARS-CoV-2 surrogate virus neutralization test, and 4% of cattle and sheep, and 2.5% of goats with the double antigen ELISA. None showed neutralizing antibody activity against the SARS-CoV-2 USA/WA1/2020 isolate. Results indicate low seropositivity and the importance of monitoring for SARS-CoV-2 prevalence in animal species.

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spread rapidly through human-to-human transmission, resulting in the global COVID-19 pandemic, initiating a prolonged public health crisis and interruptions to global economics. The latest epidemiological data from the World Organization for Animal Health (WOAH) reports 775 outbreaks in animals globally, impacting 23 animal species across 36 countries [1]. Impacted species include companion animals (dog, cat, ferret, and hamster), zoo animals (large cats, otters, and gorillas), farmed animals (mink), and wildlife (white-tailed deer) [1]. Experimental infections of animals have revealed that several species are highly susceptible to the virus, including non-human primates, hamsters, ferrets, cats, and white-tailed deer, whereas dogs, sheep, and cattle exhibit limited susceptibility, and swine as well as avian species such as chickens and ducks demonstrate resistance to infection [2]. The receptor binding domain (RBD) is the primary target for neutralizing antibodies and used as an immunogen to induce immunity against SARS-CoV-2 due to its high immunogenicity [3]. The nucleocapsid (N) is a highly conserved structural protein of SARS-CoV-2 [4] and induces a specific immune response in the host which makes for an ideal diagnostic target in infected animals. Since SARS-CoV-2 and bovine coronavirus (BCoV) both belong to the betacoronavirus family [5], there exists the potential for cross-reactivity with the similar antigens present in these betacoronaviruses, resulting in false positives. The enzyme-linked immunosorbent assay (ELISA) serves as a rapid and cost-effective serological test [6]. The variability in diagnostic test performance among ELISAs can be attributed to the type of target antigen used, e.g., SARS-CoV-2 S, RBD, M or N proteins, or the whole virus [7].

Experimental and natural infection studies show cattle can seroconvert but have low SARS-CoV-2 antibody titers [8-11]. Overall, experimental and natural infection studies in cattle, sheep, and goats indicate that these animals are unlikely to serve as reservoir species for SARS-CoV-2, since they show limited susceptibility to the virus [12-16]. However, there has been very limited evidence of natural infections with SARS-CoV-2 in these animals. The main objectives of this study were to investigate the seroprevalence of SARS-CoV-2 antibodies in cattle, sheep, and goats from several geographically distinct regions in the United States using two commercially available ELISAs targeting the nucleocapsid (N) and spike (S) proteins of SARS-CoV-2. Additionally, we provide an assessment of the performance of these serological detection methods for antibodies against SARS-CoV-2 in these animal species. Serum samples, provided by the Virology and Serology section of Kansas State Veterinary Diagnostic Laboratory, were collected from 691 bovines across fifteen states [Oregon (OR), Kansas (KS), Nebraska (NE), Arizona (AZ), Kentucky (KY), Texas (TX), Minnesota (MN), Pennsylvania (PA), Tennessee (TN), Indiana (IN), Illinois (IL), West Virginia (WV), Montana (MT), Missouri (MO), and Colorado (CO)], 698 sheep across fourteen states [Kansas (KS), Massachusetts (MA), California (CA), Missouri (MO), Texas (TX), Georgia (GA), Oregon (OR), Tennessee (TN), Colorado (CO), Indiana (IN), North Dakota (ND), New Jersey (NJ), Oklahoma (OK), and Nebraska (NE)], and 707 goats across fifteen states [Kansas (KS), Nebraska (NE), Iowa (IA), Illinois (IL), Missouri (MO), Florida (FL), Oklahoma (OK), California (CA), Georgia (GA), Indiana (IN), Virginia (VA), Massachusetts (MA), Tennessee (TN), Montana (MT), and Minnesota (MN)] between 2019 and 2022 in the United States, with the majority of samples originating from Kansas (**Figure 2.1**). Upon arrival at the laboratory, aliquots of the serum were frozen at -80 °C.

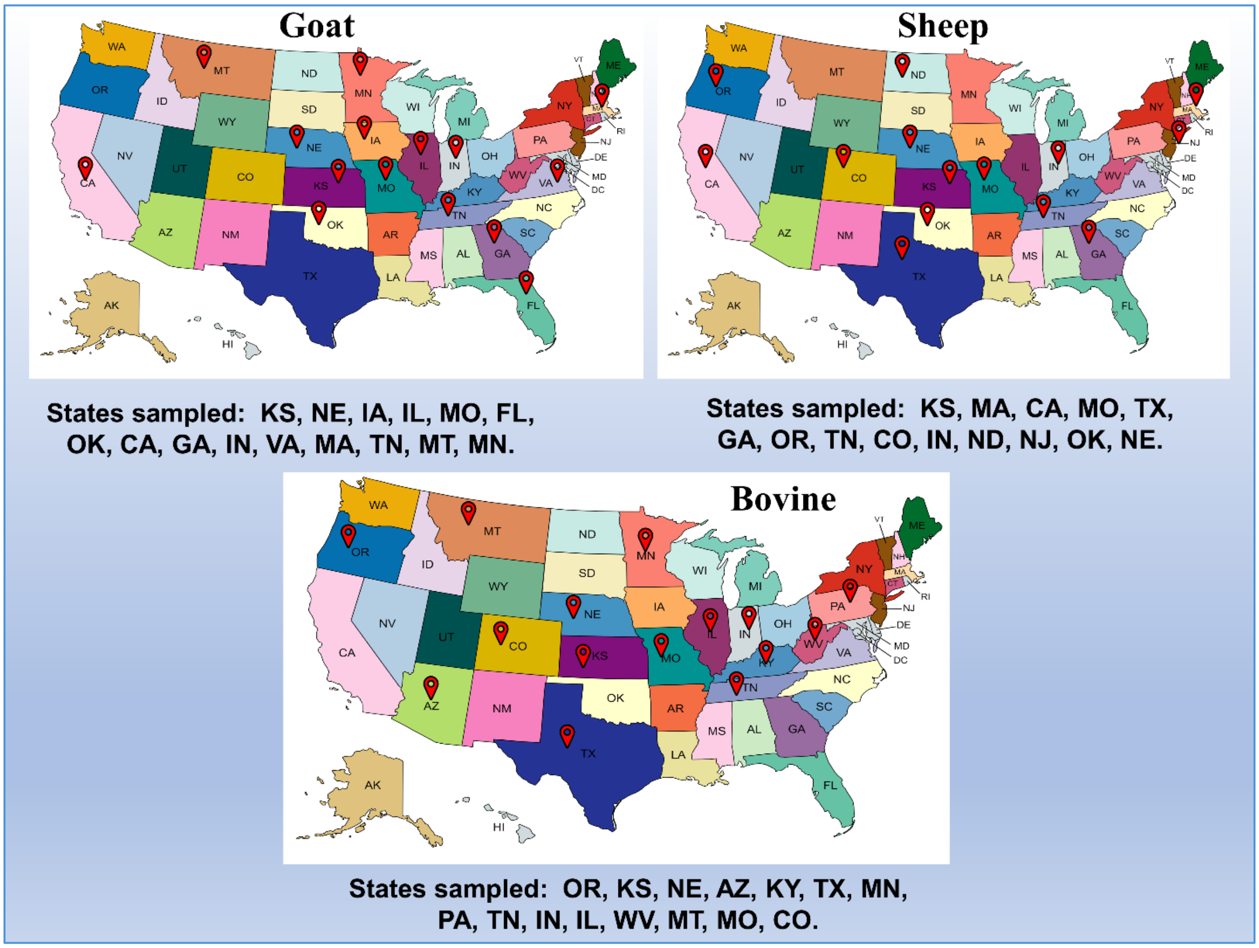

Figure 2.1. Map showing the distribution of goat, sheep, and bovine samples collected and tested across the United States. (Created with mapchart.net)

To determine if serum samples contained SARS-CoV-2 N-specific antibodies, the commercially available ID Screen® SARS-CoV-2 Double Antigen Multi-species (Innovative Diagnostics, Grabels, France), containing a purified recombinant N protein of SARS-CoV-2, referred to as N-ELISA below, was tested using according to the manufacturer's instructions. The optical density (OD) was measured at 450 nm using an ELISA microplate reader (BioTek

Cytation5; Agilent, CA, US). Samples' OD calculated as S/P%. S/P% ≥ 60% = positive, 50-60% = suspect, ≤ 50% = negative.

The SARS-CoV-2 surrogate virus neutralization test (sVNT; GenScript L00847, NJ, USA) was used for detection of neutralizing antibodies against the interaction between the virus RBD and the ACE2 cell surface receptor. The test was conducted according to the manufacturer's instructions. Absorbance at 450 nm was measured using an ELISA microplate reader (BioTek Cytation5; Agilent, CA, US). Samples with ≥ 30% inhibition were considered positive; < 30% were negative.

A classic neutralization assay was used to determine if neutralizing antibodies against bovine coronavirus were present. Serum samples were heat-inactivated at 56°C for 30 minutes and were diluted 1:8 with Dulbecco modified Eagle medium (DMEM; Gibco™ 11965092) containing Trypsin (1000) and Antibiotic-Antimycotic (100X; CORNING 30-004-Cl, VA, USA). Subsequently, 100µl of each serum dilution was mixed with 100µl of supplemented media on 96-well plates and subjected to 2-fold serial dilutions from 1:8 to 1:2560. The BCoV virus stock was diluted to 100TCID$_{50}$/100µl, 100µl of diluted virus added to 100µl of the sera dilutions, and incubated for 1 hour at 37°C. The virus-serum mixtures were transferred to 96-well plates containing human rectal tumor (HRT) cell monolayers, incubated for 48 hours at 37°C for infection. Wells were washed twice with PBS-T, fixed with 80% acetone, and dried under a fume hood for 5 hours. After rehydration with PBS-T, BCoV-specific primary antibody Z3A5 (diluted 1:10) was added and incubated for 1 hour at 37°C. After two washes with PBS-T, Anti-Mouse IgG (H + L) secondary antibody (diluted 1:75) was added and incubated for 1 hour at 37°C. Plates were washed twice with PBS-T and observed under a fluorescence microscope

(Nikon ECLIPSE TE2000-U) to determine the endpoint titer of BCoV based on the presence or absence of viral replication.

The purpose of the SARS-CoV-2 neutralization assay was to confirm the presence or absence of SARS-CoV-2 neutralizing antibodies in samples that were positive or were chosen as negative (pre-pandemic) tested using the sVNT. SARS-CoVCoV-2 neutralizing antibodies in sera were determined using a microneutralization assay as previously described [17]. Briefly, heat-inactivated (56°C/30 min) serum samples were diluted and then subjected to 2-fold serial dilutions starting at 1:8 and tested in duplicate. SARS-CoV-2 virus stocks (USA/WA1/2020; BEI NR: 52281) were diluted to 100 $TCID_{50}$ in 100 μL DMEM culture media (1000 $TCID_{50}$/mL) and added 1:1 to 100 μL of the sera dilutions. The virus/sera dilutions were then incubated for 1 hour at 37°C. The mixture was subsequently transferred to 96-well plates seeded with a confluent monolayer of Vero-E6 cells stably expressing the transmembrane serine protease 2 (Vero-E6/TMPRSS2). The neutralizing antibody titer was recorded as the highest serum dilution at which at least one of the wells showed complete virus neutralization based on the absence of CPE observed under a light microscope at 96 hours post infection. Positive control sera and back-titrations of diluted virus stock were used to monitor assay performance and consistency.

Out of 691 bovine sera screened, 4.5% (15/330) and 2.8% (10/361) tested positive for N-ELISA (ID.Vet) in 2020 and 2021. However, the sVNT ELISA targeting the RBD (GenScript) resulted in lower seropositivity of 0.3% (1/330) and 1.4% (5/361) in 2020 and 2021, respectively (**Figure. 2.2A**). Of the 707 goat serum samples, N-ELISA results indicated 1.3% (1/76) in 2019, 2.7% (15/566) in 2020, 2% (1/50) in 2021, 6.7% (1/15) in 2022 tested positive. When assayed with the sVNT assay, SARS-CoV-2 positivity rates in goats were 3.9% (3/76) in 2019, 2.1% (12/566) in 2020, 4% (2/50) in 2021, 6.7% (1/15) in 2022 (**Figure 2.2B**). Of the 698 sheep serum samples, 3.9% (2/51) in 2019, 3.8% (5/131) in 2020, 2.5% (6/239) in 2021, and 5.1% (14/277) in 2022 showed antibodies for SARS-CoV-2 nucleocapsid protein (N-ELISA). The sVNT assay indicated that antibodies against the RBD were detected in 3.9% (2/51) in 2019, 3.1% (4/131) in 2020, 1.3% (3/239) in 2021, and 1.4% (4/277) in 2022 (Fig. 2C). These results demonstrate weak agreement between anti-S and anti-N antibodies, as detailed in **Table 2-1**.

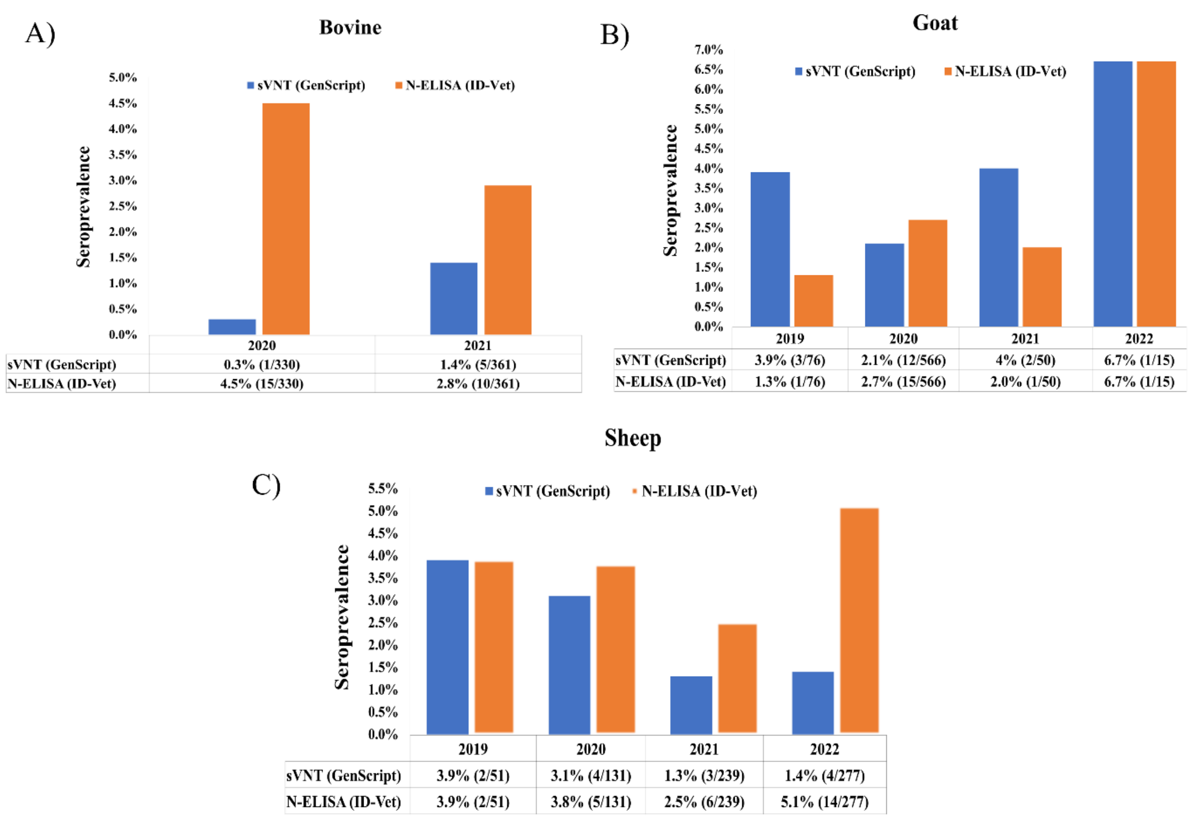

Figure 2.2. Detection of SARS-CoV-2 antibodies against the N and S (RBD region) proteins in A) bovine, B) goat, and C) sheep using both N and sVNT IgG ELISA. The X-axis represents the year of sample collection and the number and percentage of serum samples that resulted in positive detection of the sVNT and N-ELISAs from various geographically distinct regions in the United States, while the Y-axis represents the percentage of SARS-CoV-2 antibody seropositivity.

Table 2-1. The detection of SARS-CoV-2 positive samples among bovine, goat, and sheep using sVNT, N-ELISA, and their agreements.

species	Total sera	Positivity for sVNT (n)*	Positivity for N-ELISA (n)	Positivity for sVNT and N-ELISAs (n)
Bovine	691	6	25	ND**
Goat	707	17	18	1
Sheep	698	13	27	1

* n: Number

** ND: Not detected

In this study, neutralizing activity (<1:8) was not observed in any of the sVNT positive cattle, sheep, or goat serum samples when assayed via VNT using the USA/WA1/2020 isolate. Our results from sVNT (GenScript) and VNT assays indicated a lack of agreement between GenScript and VNT. Of the 6 bovine serum samples that were positive for sVNT, neutralization activity against the BCoV was also observed, with titers ranging between 1:256 and 1:4096. The sera from goats and sheep that tested positive for antibodies to the RBD were not further examined to determine the presence of neutralizing antibodies against BCoV.

A majority of information regarding the susceptibility of bovine, sheep, and goats to SARS-CoV-2 originates from experimental infections and suggests these ruminant species are mostly resistant to SARS-CoV-2 infection. To date, there has been limited research on natural infection to SARS-CoV-2 in cattle, sheep, and goats, likely due to their lower susceptibility to this infectious disease. There have been reports of natural SARS-CoV-2 infection in cattle, but studies suggest that they have a low susceptibility to the virus and are unlikely to serve as reservoirs. In Germany, a study conducted on 1,000 cattle from 83 farms indicated that 11 samples tested positive using the RBD-ELISA, and 4 samples tested positive using the sVNT (GenScript Kit), suggesting that low number of seropositive cattle may be become virus-infected through exposure to SARS-CoV-2-positive keepers [11]. Another study on 24 lactating cows in Italy found antibodies for SARS-CoV-2 nucleocapsid and spike proteins in 11 and 14 cows, respectively, with neutralizing antibodies in 13 cows [10]. In Brazil, a recent report of natural

SARS-CoV-2 infection in bovine indicated that 4/367 were antibody-positive using ID Screen® SARS-CoV-2 Double Antigen (N-ELISA) [18]. In a study infecting six cattle intranasally, only one showed low SARS-CoV-2 antibody levels from day 12 onward via indirect ELISA. However, significant neutralizing antibodies and cross-reactivity between bovine coronavirus and SARS-CoV-2 were not detected [8]. Cool et al. [12] also reported detection of SARS-CoV-2/Delta RBD specific antibodies SARS-CoV-2 at 14 DPC using Indirect ELISAs and neutralizing antibodies against SARS-CoV-2 Delta, but not against the Omicron BA.2 virus, at 10 DPC in one of eight calves.

In Sheep, a recent study showed that close contact with humans during the pandemic period demonstrated that SARS-CoV-2 antibodies were not detectable in sheep when using an in-house IgG ELISA targeting the RBD. This suggests that sheep may have resistance to natural SARS-CoV-2 infection [16]. In contrast, experimentally inoculated sheep with a mixture of wildtype SARS-CoV-2 and the alpha VOC exhibited detectable antibodies in 10 infected sheep by Indirect RBD and N ELISAs. However, only one showed low level of neutralizing antibody titer (1:20) at 10 and 21 DPC [15]. In an additional experimental inoculation study, low levels of neutralizing antibodies were observed in cattle, sheep, and goats within 14 days of infection, and the majority of animals were observed to be seronegative on day 28 post-infection [19]. In Nigeria, natural infection of SARS-CoV-2 was confirmed in goats (4/223), but not cattle or sheep, through the detection of antibodies targeting both the S-RBD and N-Nterm SARS-CoV-2 antigens [20]. In Korea, SARS-CoV-2 antibodies were found in 4.60% of Korean native cattle and 2.56% of Korean native black goats using an indirect ELISA kit. Virus-neutralizing antibodies were detected in 4.34% of Korean native cattle and 2.24% of goats, suggesting possible reverse zoonotic transmission of SARS-CoV-2 from previously infected farm handlers to livestock [21]. A recent study from 24 Italian farms demonstrated that 3.48% of sheep and 4.83% of goats tested positive with the N-ELISA kit. Additionally, one sheep exhibited a neutralizing antibody titer of 1:20, suggesting that sheep and goats can naturally be infected by SARS-CoV-2 [14]. A recent experimental inoculation with B.1.351/Beta variant in domestic goats indicated antibodies developed at 10 and 18 days post-infection (dpi) by RBD Inhibition ELISA assay (GenScript cPassTM kit). However, the neutralizing antibodies were observed at 7, 10, and 18 dpi by the live virus neutralization assay [13]. Evidence suggests seroconversion occurs from 7 days post-infection onwards. It is important to note that the challenge dose used in the study may exceed the natural exposure dose encountered by goats [13].

In this study, SARS-CoV-2 positivity rates were slightly higher in goats (~2.5%) and sheep (~2 %) than bovine (~1%) using sVNT, while higher seropositivity was observed in bovine and sheep (~4%) than goat (~2.5%) using N-ELISA. Overall, these results exhibited the presence of SARS-CoV-2 antibodies in cattle, goat, and sheep with sVNT and N-ELISA. Additionally, we observed a higher rate of seropositivity against SARS-CoV-2 when samples were evaluated using the N-ELISA compared to the sVNT (**Table 2-1**). Importantly, no serum samples in our study exhibited neutralizing activity (<1:8) against the SARS-CoV-2 USA/WA1/2020 isolate. In our study, the higher number of SARS-CoV-2 seropositive samples detected by the N-ELISA may be partially attributed to the conservation of the N protein among coronaviruses, potentially leading to cross-reactions with other coronaviruses that may have infected these animals prior to the time of sampling [20, 22].

Taken together, the results obtained from this study, as well as natural infection studies by other groups, suggest that these species may occasionally become infected with SARS-CoV-2 but are likely dead-end hosts for SARS-CoV-2 variants in circulation at the time this study was conducted. Despite low seropositivity to SARS-CoV-2 in cattle, sheep, and goats across the U.S., the results support that it is still important to monitor these animal species that share contact with humans and other susceptible animal species.

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Chapter 3 - Bison, Elk, and other captive wildlife species humoral immune responses against SARS-CoV-2

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Running title: Seroprevalence to SARS-CoV-2 in bison, elk, and other wildlife species

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Simple Summary

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus responsible for COVID-19, primarily spreads among humans, but there have been cases of transmission between humans and animals, as well as some instances of animal-to-animal transmission. Several zoos have reported cases of large cats such as tigers and lions, gorillas, and other animal species testing positive for SARS-CoV-2, presumably due to contact with humans. White-tailed deer (WTD) are highly susceptible to SARS-CoV-2 and are prevalent throughout the United States with a high population density. Separately, there is limited research and reporting on the susceptibility of bison, elk, and other wildlife species to SARS-CoV-2. This study determines the SARS-CoV-2 seroprevalence for various captive wildlife, elk and bison, across different regions in the United States, using several serological tests. The presence of neutralizing antibodies to SARS-Cov-2 virus in cheetahs, gorillas, lions, hippopotamuses, elk, and bison indicates that they are susceptible to SARS-CoV-2 infection. This highlights the importance of continuous monitoring of the prevalence of SARS-CoV-2 antibodies in various species that come into close contact with humans.

Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus responsible for COVID-19, has been found to infect various domestic and wild animal species. The presence of antibodies serves as a reliable indicator of viral exposure. In this study, convenience serum samples from 575 bison, 180 elk, and 147 samples from various wildlife species collected between 2020 – 2023 from several regions in the United States were analyzed for the presence of SARS-CoV-2 antibodies. Two commercial ELISA assays based on the inhibition of the SARS-CoV-2 receptor-binding domain (sVNT) or the nucleocapsid protein (N-ELISA) of SARS-CoV-

2 were used. Positive samples from the sVNT were additionally evaluated using a conventional virus neutralization test (VNT). Our results indicated that 1.2% of bison, 2.2% of elk, and 4.1% of the other wildlife species serum samples were seropositive in the sVNT, whereas 4.2% of bison and 3.3% of elk, and 1.4% of the other captive wildlife species serum samples tested positive by the N-ELISA. Among the sVNT serum samples, 2 samples from bison, 1 sample from elk, and 5 serum samples from other wildlife species (1 cheetah, 1 gorilla, 2 lions, and 1 hippopotamus) had neutralizing antibody titers in the VNT, indicating these species are susceptible to SARS-CoV-2 infection.

Keywords: Bison, Elk, wildlife species, SARS- CoV-2, Surveillance, ELISA

1. Introduction

Zoonotic pathogens account for 75% of emerging infectious diseases [1] and represent a significant threat to the global economic stability and the public health of the. Humans, animals, and the environment greatly influence the emergence and transmission of various infectious diseases. Over recent decades, newly emerged diseases in humans have originated from animals due to the consumption of animal derived food products [2]. Wild animals serve as reservoirs for numerous infectious diseases with zoonotic potential. A significant percentage (72%) of zoonotic infections originate from wildlife [3]. Humans face increased risks of contracting these diseases due to heightened interaction with wild animals [1]. Accordingly, an increase in cross-species virus infection and zoonotic transmission can be a result of growing human-animal interaction through wet markets, exotic pet trade, hunting, and farmed animals [4, 5]. Therefore, it is necessary to identify those pathogens and the host species and determine the circumstances most likely to be the source of create reservoirs for infection.

Three major outbreaks of coronaviruses have been reported in the past two decades. The first coronavirus outbreak in 2002, severe acute respiratory syndrome (SARS-CoV), was followed by Middle Eastern respiratory syndrome (MERS) in 2013, and the most recent one in 2019 was caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [6]. Evidence suggests that SARS-CoV-2 is less pathogenic in humans than SARS-CoV but is more transmissible among humans, resulting in global dissemination and persistent human to human transmission [7]. Rapid mutations in SARS-CoV-2 have contributed to the persistent transmission via immune evasion and have become a major threat due to mutations that occurred as part of its natural evolution. These mutations have resulted in alterations to the characteristics of the virus, including increased transmissibility and its capacity to evade the host immune response. Accordingly, these changes have posed challenges in vaccine development, making it more difficult to effectively prevent the spread of the virus [8, 9].

The vast majority of animal SARS-CoV-2 infections result from human sources, especially among domestic and captive animal populations. Transmission to free-living wildlife is less common and less understood due to fewer opportunities for direct contact [10]. However, certain activities like animal rescue, conservation efforts, and wildlife tourism can elevate the risk of transmission. Indirect transmission pathways may also exist, including human contamination of the environment (anthropogenic food waste) and supplemental food used for hunting and/or wildlife viewing [10].

SARS-CoV-2 is a single-stranded, positive-sense, enveloped RNA virus belonging to the Coronaviridae family, genus *Betacoronavirus* [11]. Coronavirus (CoVs) divided into one of four genera, *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus* [12]. Infections caused by Alpha and Beta CoVs are mainly observed in mammals and were initially identified in bats, while Gamma and Delta CoV, identified in birds, have been found to infect both birds and mammals [13]. The coronavirus virion consists of four major structural proteins: spike protein (S), membrane protein (M), envelope protein (E), and nucleocapsid protein (N). Virions are assembled and released by E proteins, while M proteins maintain the virus's structure, stabilize the nucleocapsid (N) protein, and assist in viral budding and assembly [14, 15]. The S protein helps viral attachment and entry into host cells [15]. Viral entry is initiated by the binding of the receptor-binding domain (RBD) of the S protein to the angiotensin-converting enzyme 2 (ACE2) receptor on the surface of host cells [16]. Analyzing the interaction/specificity between (S) RBD:ACE2 has made it possible to predict potential susceptible host species for SARS-CoV-2 infection, predicting a wide range of mammals [10]. The high immunogenicity of the RBD contributes to the induction of cellular immunity against SARS-CoV-2, making it a key target for neutralizing antibodies [17, 18].

The N protein plays an important role in encapsulating the viral RNA within the ribonucleoprotein (RNP) and virus particle assembly [19]. The N protein is one of the main immunogenic proteins of SARS-CoV-2, making it an ideal target for detection in serological assays. However, the high conservation of the N protein among related coronaviruses [19] could result in cross-reactions and false positives in diagnostic tests [20].

The high transmission rate of SARS-CoV-2 requires rapid, accurate, and reliable serological assays for detecting SARS-CoV-2-specific antibodies. Validation of these assays for use in diverse species is crucial for accurate reporting the prevalence of antibodies within domestic and wild animal populations and studying the dynamics of infection spread. To identify neutralizing antibodies against viruses, plaque reduction neutralization tests (PRNTs) and conventional virus neutralization tests (cVNTs) are both considered the gold standard.

Alternatively, the enzyme-linked immunosorbent assay (ELISA) also serves as a rapid and cost-effective serological test [21]. Notably, results of serological assays provide information regarding prior exposure to a pathogen, but are not able to provide information regarding the presence of active infections. Therefore, they can provide more sensitive or specific results regarding pathogen-specific antibodies to determine if an individual has been infected [22]. Therefore, ELISAs may not be effective tests for early COVID-19 detection because antibodies emerge later in infection, typically around the second week, leading to a higher likelihood of negatives during the early stages of the disease [23]. Additionally, the type of target antigens, such as SARS-CoV-2 S, RBD, M, or N proteins, or the entire virus, used in diagnostic tests, can lead to variability in performance among ELISAs [24].

Various antigens were assessed as targets for SARS-CoV-2 serological assays, and cross-reactivity among different coronaviruses was observed in whole-virus or N-based serological assays [25, 26]. Cross-reactive immune responses may occur between SARS-CoV-2 and other related CoVs due to high conservation in some genomic sequences and subsequent homology of certain structural proteins in these pathogens. Several strains of CoVs have demonstrated biological, antigenic, and genetic similarities with bovine coronaviruses (BCoV) and are named as bovine-like CoVs [27]. Bovine coronaviruses (BCoVs) also belong to the genus *Betacoronavirus*, and cause respiratory and enteric infections in cattle and wild ruminants [28], including sheep, musk oxen, elk, sambar deer, goats, dromedaries, camels, alpacas, giraffes, and wisents [29].

The susceptibility of animals to SARS-CoV-2 varies, and different species have different levels of susceptibility. Captive cats (including pumas, cougars, snow leopards, lions, and tigers), white-tailed deer, and various other wildlife in captivity, such as gorillas and otters, have been affected [30]. As of June 2023, the epidemiological data from the World Organization for Animal Health (WOAH) indicate 775 reported animal outbreaks worldwide, affecting 29 species in 36 countries [31]. These species include domestic pets (such as dogs, cats, ferrets, and hamsters), zoo inhabitants (including large cats, otters, and gorillas), farmed animals (such as mink), and wildlife (such as white-tailed deer) [31]. SARS-CoV-2 variants of concern (VOCs) have exhibited alterations in their host range, necessitating continued virus surveillance in animal populations [32]. Several animal species, such as non-human primates, white-tailed deer, ferrets, Syrian golden hamsters, cynomolgus macaques, and raccoon dogs, have been shown to be susceptible to SARS-CoV-2 following experimental inoculation, and they are capable of shedding and/or transmitting the virus to other animals [33-39]. Dogs, sheep, and cattle exhibit limited susceptibility, and swine as well as avian species such as chickens and ducks demonstrate resistance to infection [39]. Factors such as adequate cell receptors, body temperature, population density, group behavior, and human-animal interactions play crucial roles in determining the potential for animals to host and spread SARS-CoV-2 [40].

There are several reports that provide evidence of a reverse zoonosis with SARS-CoV-2 between mink and humans in mink farm workers in the Netherlands [41] and hamster to human [42] . Rapid virus transmission within the mink population has led to the emergence of a new mink-associated variant of SARS-CoV-2, which has been identified in both humans and minks [41]. More commonly, SARS-CoV-2 has spilled over from infected humans into animals. In January 2021, several gorillas at the San Diego Zoo Safari Park tested positive for SARS-CoV-2 [43]. The gorillas likely contracted the virus from an asymptomatic staff member, who tested positive for the virus. The affected gorillas exhibited symptoms consistent with a respiratory infection, such as coughing, congestion, and mild lethargy, similar to those seen in humans [43].

This incident raised concerns about the susceptibility of great apes, including gorillas, orangutans, and chimpanzees to the virus. Following that, SARS-CoV-2 was also detected in gorillas in a zoo in Prague, Czech Republic [44] and Rotterdam Zoo, Netherlands. Researchers have suggested that nonhuman primates such as gorillas and big cats can be infected with SARS-CoV-2, develop clinical signs, and effectively spread the disease [44].

The abundance and geographic distribution of cervid species, such as the white-tailed deer, demonstrates that they are free-living wild animals widespread in Northern and Eastern regions of North America, particularly due to their close proximity to human dwellings [45]. Cervids such as white-tailed deer have identified as potentially susceptible to SARS-CoV-2 infection due to the high similarity between their ACE2 sequence and that of humans [46]. A recent experimental study conducted by Porter et al. [47] investigated the susceptibility of weanling elk (Cervus canadensis) and mule deer (Odocoileus hemionus) to SARS-CoV-2. The study revealed weanling elk showed low susceptibility to infection with the Delta variant of SARSCoV-2. However, they did develop low-level antibody responses. Conversely, mule deer were found to shed infectious virus capable of infecting naïve mule deer and developed high-level antibody responses. This indicates that mule deer have the potential to play a role in SARS-CoV-2 epidemiology [47]. Another experimental study demonstrated that North American elk, including elk calves and adults, not only developed virus-neutralizing antibodies but also had viral RNA detected in the medial retropharyngeal lymph node [48], suggesting that North American elk are susceptible to infection with the ancestral Wuhan-like variant of SARS-CoV-2 (USAWA1/2020) [48].

Bison (Bison bison) and buffalo (Bubalus bubalis) are a known reservoir species for some coronaviruses, such as BCoV, [49], however, the susceptibility of bison or buffalo to

SARS-CoV-2 and the potential consequences are not well-documented. In a systematic surveillance study, Asian buffaloes tested positive for SARS-CoV-2 RNA, suggesting that buffaloes are susceptible to SARS-CoV-2 infection [50]. Ahmed et al. [51] identified a potential risk of SARS-CoV-2 infection among cattle, buffalo, goats, and sheep, which are the main species in family farming systems in South Asia.

Zoos provide a unique environment to document reverse zoonotic transmission events for observing and studying human-animal interactions due to the close proximity between a wide variety of wildlife species and animal caretakers. These settings increase the chances for sporadic cross-species transmission and raise concerns about the potential for widespread infection among animals that interact closely with humans. Natural infections of captive feline species in zoos have revealed that they are highly susceptible to SARS-CoV-2 [52]. Natural infection in captive cats has been demonstrated in Asia, Europe, and the Americas [39]. In March 2020, the first natural infections by SARS-CoV-2 were reported in tigers (Panthera tigris) and lions (Panthera leo), exhibiting only mild respiratory signs, at the Bronx Zoo in New York City, USA, following exposures to zookeepers [53]. Further reports SARS-CoV-2 infections in tigers at Knoxville Zoo in Tennessee (USA) [54] and Malayan tigers at a zoo in Virginia, USA [55], Amur tigers, Canadian lynx, and African lions at the Pittsburgh Zoo, USA [56], Asiatic lions, Bengal tigers, and leopards in India [57], African Lion at a zoo in Indiana, USA [58], lions at the Barcelona Zoo, Spain [59] and Rotterdam Zoo, Netherlands [60], Asiatic lions and Sumatran and Malayan tigers in a zoo in Prague, Czech Republic [44], and snow leopards at the Louisville Zoo in Louisville, Kentucky, USA [61]. Additionally, natural infections by SARSCoV-2 have been reported in hippopotamuses at a zoo in Hanoi, Vietnam [62] and Belgium [63].

Following the emergence of SARS-CoV-2 in humans, likely originating from an animal host, there has been considerable interest in exploring the potential of animals as reservoirs for SARS-CoV-2. Experimental infection studies and surveillance efforts (molecular and serological) suggest a broad host range of SARS-CoV-2, particularly those in close contact with humans, have been infected with SARS-CoV-2. Although extensive studies on the virus's susceptibility in bison and elk have not been conducted, it is crucial to consider the possible risks, particularly in situations where there is human-animal interaction, such as in captive settings or areas where animals and humans coexist closely. The primary aims of this research were to investigate the seroprevalence of SARS-CoV-2 antibodies in bison, elk, and other wildlife species across different regions of the United States using two commercially available ELISAs designed to detect antibodies to the nucleocapsid (N) and spike (S) proteins of SARS-CoV-2. Additionally, we evaluate the performance of serological detection methods for identifying SARS-CoV-2 antibodies in these animal populations.

2. Materials and Methods

2.1. Sample Collection

Convenience serum samples, provided by Kansas State Veterinary Diagnostic Laboratory, consisted of 575 bison serum samples collected between 2020 – 2022 from two states [Kansas (KS) and Montana (MT)]; 199 elk samples collected in 2016, 2022, and 2023 from two states [Indiana (IN) and Kansas (KS)]; and 147 serum samples from several captive species (elephants, gray foxes, foxes, llamas, camel, koala, alpaca, rhinoceros, lions, addax, giraffes, pandas, tigers, Goral , porcupines, oryx, cheetah, zebras, hippopotamus, wallaby, cockatiel, macaw, bearcat, eagle, lynx, chevrotain, mick, sea lion, hylobates lar, antelope, caribou, bobcat, hyena, impala, primate, ferret, bear, buck, and gorilla), collected between 2020 –

2023 from sixteen U.S. states [Kansas (KS), California (CA), Ohio (OH), Texas (TX), Iowa (IA), Missouri (MO), Indiana (IN), New York (NY), Wisconsin (WI), Pennsylvania (PA), Massachusetts (MA), Florida (FL), Tennessee (TN), Oklahoma (OK), Washington (WA), Georgia (GA)]. These samples were analyzed for the presence of SARS-CoV-2 antibodies (**Figure 3.1**; **Table 3-1**). Upon arrival, all samples were cataloged according to the owner's location. Samples lacking owner location data were cataloged based on location of the veterinary clinic instead.

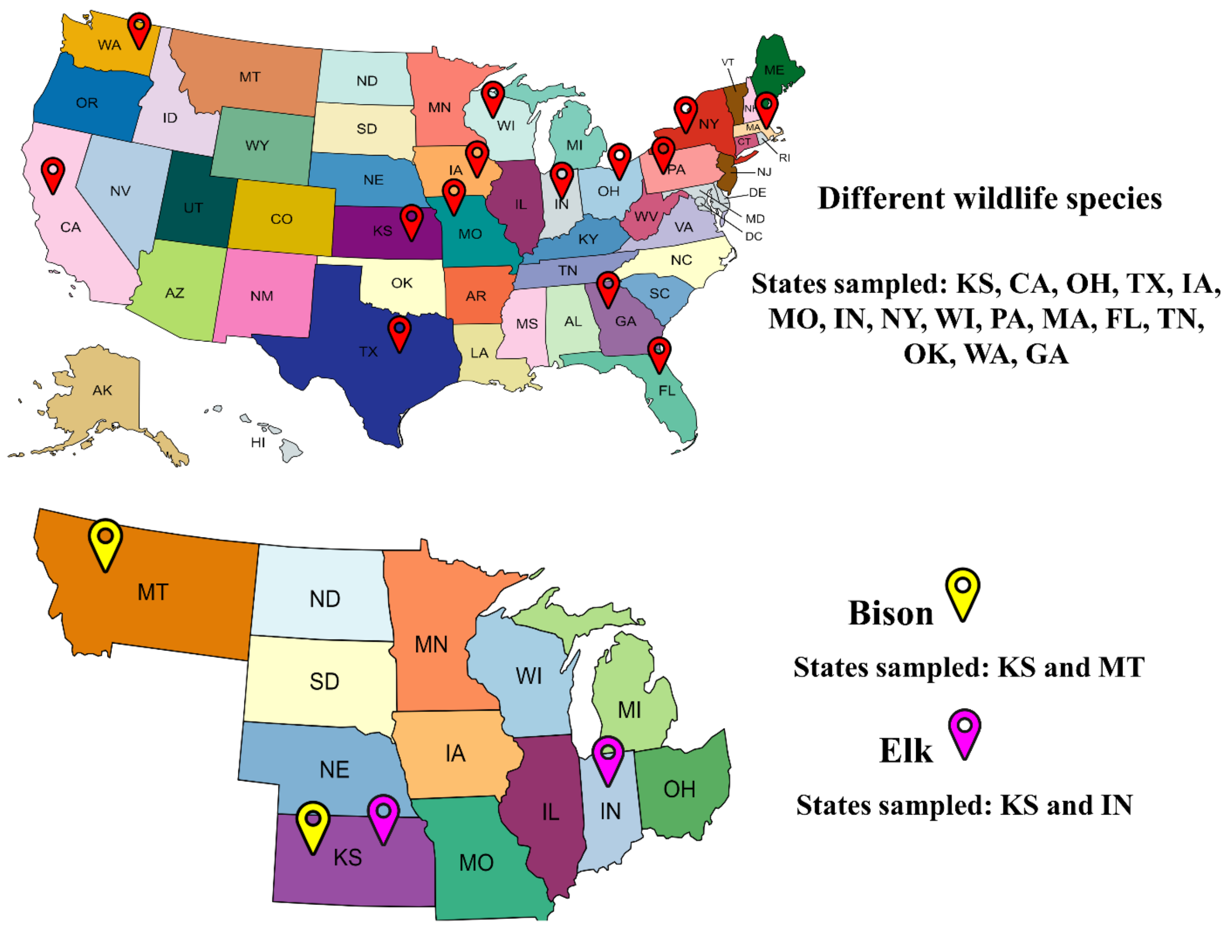

Figure 3.1. Map showing the distribution of serum samples collected from bison, elk, and different captive wildlife species (as mentioned in Materials and Methods) across the United States. (Created with mapchart.net)

2.2. Detection of SARS-CoV-2 antibodies by ELISAs

SARS-CoV-2 double antigen ELISA for detection of N-specific antibodies (N-ELISA)

Serum samples were tested for SARS-CoV-2 N-specific antibodies using the commercially available ID Screen® SARS-CoV-2 Double Antigen Multi-species ELISA (Innovative Diagnostics, Grabels, France) according to the manufacturer's instructions, and referred to here as the N-ELISA. Briefly, serum samples were heat-inactivated at 56° C for 30 minutes. Each well of the ELISA plate then received 25 µl of dilution buffer, followed by the addition of 25 µl of serum. Positive and negative assay controls were included with each run. The mixture was then incubated for 45 minutes at 37°C. After incubation, each well was washed 5 times with 300 µl of wash solution. Following the wash, 100 µl of N protein recombinant antigen horseradish peroxidase (HRP) conjugate was added and incubated at room temperature (RT) for 30 minutes. Subsequently, the wells were washed 5 times with 300 µl of wash solution. After this, 100 µl of substrate solution was added to each well and incubated for an additional 20 min at RT before the reaction was stopped by addition of 100 µl stop solution. The optical density (OD) was measured at 450 nm using an ELISA microplate reader (BioTek Cytation5; Agilent, CA, US) immediately afterward. The OD of each sample was calculated as the S/P percentage (S/P%). Serum with S/P% ≥ 60% was considered positive, while serum with S/P% 50−60% was defined as 'suspect,' and serum with S/P% ≤ 50% was considered negative.

SARS-CoV-2 surrogate virus neutralization test (sVNT)

The SARS-CoV-2 surrogate virus neutralization test (sVNT; GenScript L00847, NJ, USA) was used for detection of neutralizing antibodies against the RBD of the virus S protein.

The test was conducted according to the manufacturer's instructions. Briefly, serum samples were heat-inactivated at 56°C for 30 min. Serum samples, and the positive and negative assay controls were each diluted 1:10 in sample dilution buffer. Subsequently, these diluted samples were mixed with an equal volume of HRP-conjugated RBD, which was diluted at 1:1000. The mixture was then incubated at 37°C for 30 min. After incubation, 100 µl of each mixture was added to a plate that was precoated with human ACE2 protein, which binds with the viral RBD. Following incubation at 37°C for 15 min, the plate was washed four times with 260 µl of wash solution. Subsequently, 100 µl of tetramethylbenzidine substrate (TMB) was added to each well and incubated at RT for 15 min before the reaction was stopped by addition of 50 µl stop solution. The absorbance was read at 450 nm (OD450) using an ELISA microplate reader (BioTek Cytation5; Agilent, CA, US) immediately afterward. The OD of each sample was calculated as the inhibition percentage (% inhibition). For the expression of the results, % inhibition ≥ 30% was considered positive, and % inhibition < 30% was considered negative.

2.3. Virus neutralizing antibodies

SARS-CoV-2 Neutralization Assay (VNT)

The purpose of the SARS-CoV-2 neutralization assay was to confirm the presence or absence of SARS-CoV-2 neutralizing antibodies in samples that were positive or were chosen as negative (pre-pandemic) by the sVNT.

SARS-CoVCoV-2 neutralizing antibodies in sera were determined using a microneutralization assay as previously described [64]. Briefly, heat-inactivated (56°C/30 min) serum samples were diluted and then subjected to 2-fold serial dilutions starting at 1:8 and tested in duplicate. SARS-CoV-2 virus stocks (USA/WA1/2020; BEI NR: 52281) were diluted to 100 $TCID_{50}$ in 100 µL DMEM culture media (1000 $TCID_{50}$/mL) and added 1:1 to 100 µL of the sera dilutions. The virus/sera dilutions were then incubated for 1 hour at 37°C. The mixture was subsequently transferred to 96-well plates seeded with a confluent monolayer of Vero-E6 cells stably expressing the transmembrane serine protease 2 (Vero-E6/TMPRSS2). The neutralizing antibody titer was recorded as the highest serum dilution at which at least one of the wells showed complete virus neutralization based on the absence of CPE observed under a light microscope at 96 hours post infection. Positive control sera and back-titrations of diluted virus stock were used to monitor assay performance and consistency.

BCoV Neutralization Assay

A classic neutralization assay was used to determine if neutralizing antibodies against bovine coronavirus were present. Serum samples were first heat-inactivated at 56°C for 30 min and were diluted 1:8 with Dulbecco modified Eagle medium (DMEM; Gibco™ 11965092) containing Trypsin (1000) and Antibiotic-Antimycotic (100X; CORNING 30-004-Cl, VA, USA). Subsequently, 100 µl of each serum dilution was combined with 100µl of supplemented media on 96-well plates and subjected to 2-fold serial dilutions starting from 1:8 to 1:2560. In addition, back titration was performed for each viral dilution. The BCoV virus stock was diluted to 100 $TCID_{50}$/100 µl and then 100 µl of diluted virus in DMEM was added to 100 µl of the sera dilutions and incubated for 1 h at 37°C. Following incubation, 100 µl of the virus–serum mixtures were transferred to 96-well plates containing confluent monolayers of human rectal tumor (HRT) cells and incubated for 48 h at 37°C to allow for the infection of the cells. After incubation, the wells were washed two times with 0.01M phosphate-buffered saline (PBS; pH 7.4) (PBS-T) and fixed with 80% acetone for 10 minutes. After acetone fixation, the plates were left under a fume hood for 5 h to dry. Following fixation, samples were rehydrated with PBS-T, and 50 µl of BCoV-specific primary antibody, Z3A5 (developed in-house), a monoclonal antibody that targets the spike protein subunit of BCoV, was diluted 1:10, added to each well and incubated for 1 h at 37°C. After 1 h, plates were washed two times with PBS-T, and 50 µl of Anti-Mouse IgG (H + L) secondary antibody (Jackson ImmunoResearch, Code: 115-095-003), diluted 1:75, was added to each well and incubated for 1 h at 37 ◦C. Following incubation, plates were washed two times with PBS-T and observed under a fluorescence microscope (Nikon ECLIPSE TE2000-U) to determine endpoint titer to BCoV based on the presence or absence of viral replication. Samples with known BCoV-neutralizing antibodies were used as a positive control and to monitor consistency between assays.

3. Results

Of the 575 bison sera screened via sVNT, 14.3% (1/7) in 2020, 1.9% (4/212) in 2021, and 0% (0/205) in 2022 from Kansas, and 1.3% (2/151) in 2022 from Montana resulted as positive for detection of SARS-CoV-2 RBD. When the same samples were evaluated for the presence of SARS-CoV-2 nucleocapsid protein (N-ELISA) antibodies were detected in 0% (0/7) of bison sera collected in 2020, 2.4% (5/212) in 2021, and 5.4% (11/205) in 2022 from Kansas, and 5.3% (8/151) in 2022 from Montana, indicating higher seropositivity when evaluated with the N-ELISA compared to sVNT (**Figure 3.2 A**). Sera from elk collected in 2016, before the COVID-19 pandemic, showed no antibodies against the RBD (sVNT) and nucleocapsid protein (N-ELISA). Out of the 180 elk serum samples by both sVNT and N-ELISA, none of the 8 samples from 2022 in Kansas tested positive. However, in 2023, the SARS-CoV-2 positivity rates in elk from Kansas were 2.3% (4/172) and 3.5% (6/172) for sVNT and N-ELISA, respectively (**Figure 3.2B**).

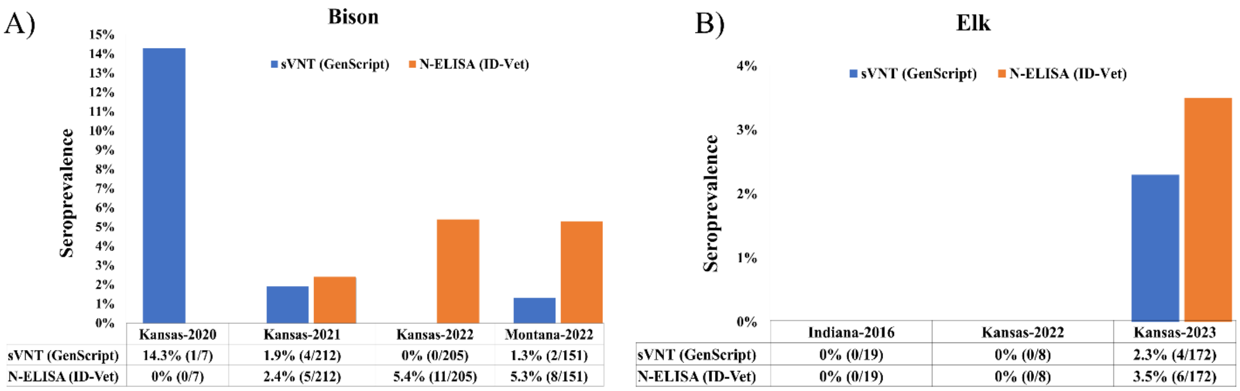

Figure 3.2. Detection of SARS-CoV-2 antibodies against the N and S (RBD) proteins in A) bison and B) elk using commercial N (ID-Vet; orange bars) and sVNT (GenScript; blue bars) IgG ELISAs. The X-axis represents the number and percentage of serum samples by collection year and location that were positive by the sVNT and N-ELISAs; the Y-axis represents the percentage of SARS-CoV-2 antibody seropositivity.

Out of the 147 serum samples collected from various wildlife species, 4.1% (6/147) tested positive for the sVNT assay. Among the sVNT-positive samples, positive antibody titers were identified in 1 cheetah from Massachusetts, 1 gorilla from Massachusetts, 2 lions from Kansas, and 1 hippopotamus from Massachusetts. The highest neutralization titers were found in lions (1:2048), followed by the cheetah (1:512), the gorilla (1:128), and the hippopotamus (1:8) (**Table 3-1**). Antibodies against SARS-CoV-2 nucleocapsid protein (N-ELISA) were only detected in 1 gorilla and 1 goral, indicating a lower seropositivity rate of 1.4% (2/147) (**Table 3-1**). Notably, sera from different animal species that tested positive for antibodies to the RBD were not further examined to determine the presence of neutralizing antibodies against BCoV.

Table 3-1. Results of serological testing (sVNT, N-ELISAs, and VNT) of captive wild species

Species	Total sample tested	Geographical location	Number of positive Samples			
			sVNT	N-ELISA	VNT	VNT Titer
Elephant	24	TN, CA, TX, OK	1			

Gray fox	19	IN				
Fox	10	IA				
Llama	16	KS, MO				
Camel	13	KS				
Koala	7	CA, OH				
Alpaca	6	KS, MA				
Rhinoceros	5	FL, MA, KS				
Lion	4	WI, KS	2		2	1:2048
Addax	3	KS, MA				
Giraffe	3	KS, MA				
Panda	3	GA				
Tiger	3	FL				
Goral	2	KS		1		
Porcupine	2	MO, KS				
Oryx	2	MA, KS				
Cheetah	2	MA, KS	1		1	1:512
Zebra	2	MA				
Hippopotamus	1	MA	1		1	1:8
Wallaby	1	KS				
Cockatiel	1	NY				
Macaw	1	MA				
Bearcat	1	KS				
Eagle	1	MO				
Lynx	1	KS				
Chevrotain	1	TX				
Mick	1	KS				
Sea lion	1	PA				
Hylobates lar	1	KS				
Antelope	1	FL				
Caribou	1	MO				
Bobcat	1	KS				
Hyena	1	MA				

Impala	1	MA				
Primate	1	CA				
Ferret	1	WA				
Bear	1	GA				
Buck	1	FA				
Gorilla	1	MA	1	1	1	1:128

Concordance among SARS-CoV-2 sVNT and VNT, and BCoV test in bison and elk

Among the 11 sVNT-positive samples, 2 samples from bison (titers 1:8 and 1:32) and 1 sample from an elk (titer 1:16) showed SARS-CoV-2 neutralizing antibodies via VNT using the USA/WA1/2020 isolate. Of the 7 bison and 4 elk serum samples that were positive for sVNT, neutralization activity against BCoV was observed in 6 bison (range 1:8-1:2048) and 4 elk (range1:8-1:128). However, among the 4 sVNT and BCoV -positive samples in elk, only 1 elk (titer 1:128) tested also positive by VNT (**Table 3-2**). In bison, among the 7 sVNT-positive samples and 57 randomly selected VNT- and sVNT-negative samples, 44 samples tested positive for BCoV. Higher seropositivity was observed when BCoV was positive, and both sVNT and VNT results were negative (**Table 3-2**).

Table 3-2. Comparison and concordance of Elk and Bison serums evaluated by sVNT, VNT, and BCoV tests

Species		**Testing Results**		
	sVNT	**VNT**	**BCoV**	**Total (Numbers)**
Elk	Pos*	Pos	Pos	1
	Pos	Neg**	Pos	3
	sVNT	**VNT**	**BCoV**	**Total (Numbers)**
Bison	Pos	Pos	Pos	1
	Neg	Neg	Neg	19
	Neg	Neg	Pos	38
	Pos	Pos	Neg	1
	Pos	Neg	Pos	5

*Pos: Positive; **Neg: Negative

4. Discussion

The epidemiology of SARS-CoV-2, as well as its symptoms, morbidity, and mortality, has a wide range of variations around the world. When faced with an outbreak of novel zoonotic pathogens, such as SARS-CoV-2, it is critical that rapid diagnostic tools are developed, validated, and widely distributed to help inform policy decisions relating to animal and public health. There are several diagnostic methods available to evaluate the exposure of animals to SARS-CoV-2, including assays for the detection of antibodies, antigens, and molecular detection methods. It is important to note that there exists a range of diagnostic sensitivity and specificity in commercial assays. occasionally resulting in false negatives that can lead to a lack of effective intervention, possibly allowing for increased disease spread [65]. Moreover, false positives due to cross-reactivity pose significant challenges in diagnostic testing, as they can lead to incorrect diagnoses. Since SARS-CoV-2 and bovine coronavirus (BCoV) belong to the betacoronavirus family [66], there is a potential for cross-reactivity with the similar antigens of these betacoronaviruses, resulting in false positives. Therefore, it is essential to validate and verify the performance characteristics of diagnostic tests for species of interest and specificity to the pathogen of interest in order to minimize the risk of false positives and ensure the accuracy of the test results.

Infection with SARS-CoV-2 induces the host to produce detectable levels of IgG antibodies, which results in the development of protective immune responses and plays a crucial role in long-lasting immunity by eliciting a robust B cell response [67]. An individual hosts immune response can be influenced by various factors, including previous exposure to infections and vaccinations, the severity of infections, and the other co-morbidity factors (weight, immunocompromised, age, pulmonary conditions) [68, 69]. The identification of clinical symptoms in captive animals in zoos, such as coughing, nasal discharge, lack of appetite, lethargy, and subsequent diagnostic investigations, has led to the identification several species susceptible to natural infection [70, 71]. However, subclinical infection of SARS-CoV-2 infections may remain undetected because many animals can carry pathogens without showing any clinical signs of illness. Additionally, wildlife and animals in inaccessible areas are less likely to be routinely monitored for clinical signs, allowing infections in these populations to go unnoticed and potentially spill over to domestic animals or humans. These infections may only be identified through laboratory tests or post-mortem examinations.

Currently, there is limited information available on the susceptibility of bison, elk, and other captive/wildlife species to SARS-CoV-2. The present study aimed to assess the seroprevalence of SARS-CoV-2 antibodies in bison, elk, and different captive species from several geographically distinct regions in the United States to better understand the occurrence of natural infection by SARS-CoV-2 in these species. Additionally, this study assessed the diagnostic performance of two commercial ELISA kits in detecting antibodies against the SARS-CoV-2 N and S proteins in several species. A VNT was used as a reference test to confirm the results obtained from the sVNT. There have been reports of natural SARS-CoV-2 infection in captive wildlife species such as captive cats (tiger, lion, etc), semi-aquatic mammals (hippopotamuses), and other non-human primates (gorillas), suggesting that they are highly susceptible to the virus.

In Belgium [63], a study screened for the potential circulation of SARS-CoV-2 in two adult Hippopotamus amphibius using ELISA Wantai (RBD-ELISA), ID Screen® SARS-CoV-2 Double Antigen (N-ELISA), and polymerase chain reaction (PCR). Nasal discharge was observed in both hippopotamuses. The results showed the presence of SARS-CoV-2 RNA in 53% of pool water samples and 27% of the combined nasal and fecal samples, which was suspected to have been transmitted to the hippopotamuses by asymptomatic zookeepers. Neutralizing antibodies against SARS-CoV-2 were detected with Wantai (16.79%) and ID Screen (60.33%), suggesting that hippos had developed specific immune responses after an active infection [63]. Following the detection in hippopotamus, additional studies [72] were conducted to evaluate possible SARS-CoV-2 infections in mammals, including those that could have been in indirect contact with the hippopotamuses, in two Flemish zoos between 2020 and 2021. Out of 50 collected serum samples from 26 mammal species and 1,523 fecal samples from 103 mammal species, none of the samples tested positive by sVNT (GenScript cPass™) and PCR [72]. Another study reported the death of a hippopotamus due to SARS-CoV-2 infection 17 days after the onset of clinical symptoms in a zoo in Hanoi, which can be attributed to the close relation between the SARS-CoV-2 strain in hippopotamus and 3 human SARS-CoV-2 strains in Vietnam. Tissue samples taken from the lung, spleen, liver, and intestine tested positive by PCR [62]. In line with previous studies, we detected the presence of SARS-CoV-2 antibodies in hippopotamus serum samples. Together these results indicate that hippopotamuses are susceptible to SARS-CoV-2 and should be among the species monitored for infection.

Incidence of SARS-CoV-2 infections have been reported in non-human primates and large cats in captivity at several locations across Europe and North America since 2020. Many of these cases have been linked to SARS-CoV-2 infected human caretakers. Both molecular and serological diagnostic methods have been successful in monitoring these events. In February 2021, at the Prague Zoological Garden, western lowland gorillas, Asiatic lions, Sumatran and Malayan tigers, and Amur leopards tested positive for the SARS-CoV-2 B.1.1.7 variant by RT-qPCR [44]. Almost all cases were symptomatic, presenting with clinical signs such as cough and nasal discharge. Among the zookeepers, one gorilla handler and two cat handlers tested positive for COVID-19 shortly before the outbreak in the animals, suggesting that SARS-CoV-2 transmission likely occurred via direct contact with infected humans [44]. Another multispecies animal outbreak occurred at Rotterdam Zoo in the Netherlands in November 2021. Fecal and nasal samples from seven western lowland gorillas living in the same enclosure and four Asiatic lions that developed symptoms were confirmed SARS-CoV-2 positive by RT-qPCR [60]. In October 2020, three Malayan tigers (Panthera tigris jacksoni) at a Tennessee zoo tested positive for SARS-CoV-2 by RT-qPCR, potentially linked to positive zookeepers. All the tigers exhibited symptoms and clinical signs [54]. Similarly, another study reported the presence of viral RNA in nasal swabs, nasal turbinates, lung tissue, and intestinal tissues in an African lion in Indiana zoo, USA [58]. In Barcelona Zoo in November 2020 [59], four lions showed respiratory clinical signs, and SARS-CoV-2 RNA was detected in nasal and fecal samples from the infected lions. Subsequent serological tests revealed that all four lions developed neutralizing antibody responses. Although high levels of neutralizing antibodies (nAbs) were initially observed with both SNT and sVNT, these levels decreased over a 4-month period following infection. When the serum was evaluated for the presence of SARS-CoV-2 N protein, low levels of anti-N antibodies were detected in three of four tigers [59]. A study at the Virginia Zoo in the USA found that three Malayan tigers exhibiting respiratory symptoms were infected with the SARS-CoV-2 Alpha (B.1.1.7) variant. A unique finding in this report was that no employees had tested positive for SARS-CoV-2 or exhibited any clinical symptoms in the four weeks prior to the detection in tiers [55]. Furthermore, Tewari et al. [56] reported five African lions, four Amur tigers, and a Canadian lynx at the Pittsburgh Zoo, USA, exhibited respiratory clinical signs and tested positive for viral RNA in feces. It was shown that lions were naturally infected with the alpha variant (B.1.1.7 lineage), and tigers and lynx tested positive for the delta variant (AY.25.1

lineage). A strong neutralizing antibody response against the viral spike protein was observed in lions, tigers, and lynx. However, none of the cheetahs showed any clinical signs, and antibody responses were not detected by surrogate virus neutralization assay, indicating the absence of exposure to SARS-CoV-2 or resistance to infection [56]. A serological study evaluated the prevalence of SARS-CoV-2 infection among 126 leopards, 96 Asiatic lions, and 98 Bengal tigers from eight Indian states. The results indicated the presence of SARS-CoV-2 neutralizing antibodies in 48 out of 320 serum samples, including 24 lions, 14 tigers, and 10 leopards, with titers (as the log of the reciprocal of the dilution in the range of < 1.5, 1.8, 2.1, and > 2.4) ranging from 2.1 to more than 2.4 [57]. Moreover, SARS-CoV-2 RT-qPCR positive results were reported in 2 out of 18 and 1 out of 20 Asiatic lions at two different locations in India [73]. A study conducted in Thailand during 2020–2021 [74] showed that 6.5% of captive tigers had SARS-CoV-2 neutralizing antibodies against the Wuhan Hu-1 and Delta variants via the plaque reduction neutralization test (PRNT). The possible exposure of SARS-CoV-2 to tigers could be attributed to close interactions with their caretakers, who had a recent history of SARS-CoV-2 infection. However, only one seropositive tiger had a low level of neutralizing antibodies against the Omicron BA.2 subvariant [74].

In our study, SARS-CoV-2 positivity rates among the 147 captive wildlife species were 4.1% (6/147), including 1 Elephant, 1 hippopotamus, 1 gorilla, 2 lions, and 1 cheetah, for sVNT and 1.4% (2/147), including 1 gorilla and 1 goral, for N-ELISA (**Table 3-1**). Lower seropositivity was observed when the same samples were evaluated with the N-ELISA. Samples that were positive for sVNT were subsequently evaluated using the VNT, and we observed that 4/6 sVNT-positive samples, including 1 gorilla, 2 lions, and 1 cheetah, were also positive for VNT.

The increases in SARS-CoV-2 seropositivity by the sVNT can be attributed to the decrease in levels of anti-nucleocapsid antibodies within 5-15 months of infection, whereas anti-spike antibody levels persist and gradually decline over time, indicating time as a strong influential factor [75]. So far, natural SARS-CoV-2 infection has not been reported in cheetahs. Seropositivity in cheetahs can be associated with their very low genetic diversity, which reduces the population's resilience to environmental changes and increases susceptibility to diseases [76].

Limited information is available about the susceptibility of bison and elk to SARS-CoV-2. The high incidence of SARS-CoV-2 amongst white-tailed deer suggest that evaluating the susceptibility of other cervid species to SARS-CoV-2 could benefit disease management and intervention strategies. In a systematic surveillance study conducted in India, 33.33% (13/39) of buffaloes tested positive for SARS-CoV-2 RNA (delta B.1.1.617.2 variant) in nasal and/or rectal swab samples using RT-qPCR, suggesting that buffaloes are susceptible to SARS-CoV-2 [50]. Two experimental infection studies have evaluated the susceptibility of elk to ancestral Wuhan-like and the Delta variant of SARS-CoV-2. A study conducted by Boggiatto et al. [48] infected seven North American elk calves and seven adults intranasally with ancestral Wuhan-like isolate of SARS-CoV-2 (USAWA1/2020). Serum was evaluated using the RBD Inhibition ELISA assay (sVNT, GenScript Biotech) and showed that both calves and adult elk developed neutralizing antibodies as early as 7 days post-infection (dpi) and persisted for 21 days using. Results were confirmed when serum was tested using a traditional VNT. Notably, the highest titers were detected in elk calfs (1:256) at 14 dpi, whereas lower titers were observed in adult elk (1:16) at 7 dpi, indicating that the elk calves had a stronger neutralization response. The authors suggest that the lower neutralizing antibody responses in adult elk (older animals) might relate to pre-existing immunity from infection with related coronaviruses, which may explain a more limited response and a reduction in the amount of antigen available for de novo antibody responses. Additionally, viral RNA was detectable throughout the medial retropharyngeal lymph node in both calves and adult elk via RT-qPCR [48]. In a separate study by Porter et al. [47], inoculated weanling elk and mule deer with the Delta variant of SARS-CoV-2, B.1.617.2 via intranasal routes. This study demonstrated that inoculated mule deer shed infectious virus both orally and nasally. However, inoculated elk did not exhibit clinical signs, shed the virus, or transmit the virus to an in-contact elk. Neutralizing antibody responses were higher in mule deer, with peak neutralizing titers exceeding 1:1,280 at 21 dpi, compared to elk, which had peak neutralizing titers of 1:20 at 21 dpi. This experimental infection study demonstrated that elk have low susceptibility and mule deer have high susceptibility to the Delta variant of SARS-CoV-2 [47].

Consistent with previous studies, our results indicated that 1.2% (7/575) of bison and 2.2% (4/180) of elk were seropositive in the sVNT, whereas 4.2% (24/575) of bison and 3.3% (6/180) of elk serum samples tested positive by the N-ELISA. The N protein is relatively conserved among coronaviruses, therefore higher seropositivity in N-ELISA compared to sVNT may result from potential cross-reactivity between SARS-CoV-2 and other betacoronavirus found in cervid species. In this study, two bison samples and one elk sample contained virus-neutralizing antibodies via VNT, suggesting that bison and elk are susceptible to SARS-CoV-2. The seropositivity of BCoV in the bison and elk serum samples in our study were 7.7% (44/575) and 2.2% (4/180), respectively. When comparing results obtained from each assay, serum from one elk and one bison exhibited neutralizing antibodies in sVNT, VNT, and BCoV tests (**Table 3-2**). Although, 3 elk and 5 bison had tested positive for both the sVNT and BCoV tests. The was likely due to antigenic cross-reactions that might occur between BCoV and SARS-CoV-2. The high similarity between the spike protein epitopes of SARS-CoV-2 and bovine BCoV suggests that BCoV is one of the viruses most similar to SARS-CoV-2 [77].

5. Conclusions

Taken together, this study was one of the first to provide evidence of potential natural SARS-CoV-2 infection in bison, elk, and cheetahs in the USA. The presence of neutralizing antibodies in cheetahs, gorillas, lions, hippopotamuses, elk, and bison indicates their potential susceptibility to SARS-CoV-2 infection. It is crucial to monitor the impact of SARS-CoV-2 in bison and elk, both in the wild and in zoos, as well as other captive wildlife species. This will help better assess the reservoir potential of various animal populations for SARS-CoV-2, the risk of transmission in different settings, and the implications for both animal and human health.

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Chapter 4 - Research Implications

The challenge of managing pathogenic diseases like SARS-CoV-2 in animals involves a range of complex issues. Pathogens that have zoonotic potential, such as SARS-CoV-2, can undergo interspecies transmission, making their control particularly difficult. The genetic diversity and rapid mutation rates of these pathogens can lead to new variants that may spread more easily or evade existing vaccines and treatments. Wildlife reservoirs further complicate control efforts, as these reservoirs can maintain and spread the virus to other animals and humans. Accurate serological assessment for SARS-CoV-2 remains a significant global challenge. To effectively manage and contain outbreaks, there is a critical need for advanced serological assays that offer rapid, reliable detection of various SARS-CoV-2 variants. To identify neutralizing antibodies against viruses, plaque reduction neutralization test (PRNT) and conventional virus neutralization tests (cVNTs) are considered the gold standard. Enzyme-linked immunosorbent assays (ELISAs) are rapid and cost-effective serological tests; therefore, they are widely used for detecting and quantifying antibodies and antigens. ELISA is used to screen large number of samples in a short period of time. But they are no assay exists without their limitations and concerns.

The effective use of serological tools for detecting SARS-CoV-2 in animals presents several challenges. Several factors contribute to the complexities of using serological tools for SARS-CoV-2 in animals. One critical aspect is the variability in immune responses among different animal species. Animals may mount different types and magnitudes of antibody responses to SARS-CoV-2, influenced by factors such as species-specific immune mechanisms, age, and health status. Another challenge is the potential for serological assays to produce unclear results due to the presence of low levels of antibodies or cross-reactive antibodies from prior infections with related viruses. Furthermore, the continuous evolution of the SARS-CoV-2 virus and the emergence of new variants may affect the performance of existing serological tests. Regular updates and validation of tests against circulating variants are essential to maintain their effectiveness.

However, several limitations and concerns regarding ELISA assays should be considered. One major limitation is the assay's sensitivity and specificity. Although ELISA is generally sensitive, it may exhibit lower specificity compared to other methods. In ELISA methods no specific binding can occur for several reasons. This can result in false positives and false negatives, especially if the assay conditions are not optimized or if the antigen or antibody used lacks high specificity. Furthermore, the variability in immune responses among different animal species can also impact test sensitivity and specificity. Another limitation of ELISA is its potential for cross-reactivity, which can lead to false results in SARS-CoV-2 testing. This issue arises when antibodies or antigens bind to structures similar to the intended target, resulting in erroneous outcomes. Therefore, it is essential to validate and verify the performance characteristics of diagnostic tests for species of interest and specificity to the pathogen of interest in order to minimize the risk of false positives and ensure the accuracy of the test results.

In scientific research, unexpected or negative results can provide valuable insights and open new avenues for further study. As the epidemiological role and susceptibility of bovine, sheep, goats, bison, elk, and captive wildlife to natural SARS-CoV-2 infection are still not fully understood and remain unclear, two projects (Chapters 2 and 3) were undertaken to investigate the seroprevalence of SARS-CoV-2 antibodies in various animal species across geographically distinct regions in the United States and to evaluate the efficacy of serological detection methods. These methods included two commercial ELISAs targeting the receptor-binding domain (RBD; sVNT GenScript) and nucleocapsid protein (N; ID-Vet Screen® ELISA), a conventional virus neutralization test (VNT), and bovine coronavirus neutralization assays, for detecting antibodies against SARS-CoV-2 and bovine coronavirus in these animal species.

In Chapter 2, our results indicate that ~1% of cattle, ~2% of sheep, and ~2.5% of goat serum samples were positive when using the sVNT, whereas ~4% of cattle and sheep, and 2.5% of goat serum samples tested positive with the N-ELISA. A higher rate of seropositivity against SARS-CoV-2 was observed when samples were evaluated using the N-ELISA compared to the sVNT. None of the sVNT positive cattle, sheep, or goat serum samples had detectable neutralizing antibody activity against the SARS-CoV-2 USA/WA1/2020 isolate by the VNT. We also observed that, of the six bovine serum samples positive for sVNT, neutralization activity against BCoV was detected.

In Chapter 3, our results indicated that 1.2% of bison, 2.2% of elk, and 4.1% of the other wildlife species serum samples were seropositive in the sVNT, whereas 4.2% of bison and 3.3% of elk, and 1.4% of the other captive wildlife species serum samples tested positive by the N-ELISA. Among the sVNT serum samples, 2 samples from bison, 1 sample from elk, and 5 serum samples from other wildlife species (1 cheetah, 1 gorilla, 2 lions, and 1 hippopotamus) had neutralizing antibody titers in the VNT. Additionally, of the 7 bison and 4 elk serum samples that were positive for sVNT, 2 samples from bison and 1 sample from an elk showed SARS-CoV-2 neutralizing antibodies via VNT using the USA/WA1/2020 isolate and neutralization activity against BCoV was observed in 6 bison and 4 elk.

A higher rate of seropositivity against SARS-CoV-2 was observed in two experiments when samples were evaluated using the N-ELISA compared to the sVNT. According to the company's explanation, the common conserved region of the N protein was removed to ensure specificity to SARS-Cov-2 specific antibodies. The increased seropositivity rate observed with the N-ELISA assay can be ascribed to several factors. First, antigenic cross-reactions between SARS-CoV-2 and other coronaviruses may lead to elevated seropositivity, as antibodies produced in response to one coronavirus may bind to antigens from another. Second, non-specific binding, where antibodies interact with non-target antigens or the ELISA plate itself, can contribute to higher observed rates of seropositivity. Third, the gradual decline in anti-nucleocapsid antibodies within 5-15 months post-infection can also account for the observed seropositivity rates. In contrast, anti-spike antibodies tend to persist longer and exhibit a more gradual decline over time, suggesting that temporal factors play a significant role in shaping the dynamics of antibody levels.

The Multi-Species IDvet Kit (according to company information on assay validation) is designed for use with a variety of animal species, including minks, ferrets, cats, dogs, cattle, sheep, goats, horses, and other susceptible species to SARS-CoV-2 sera such as bats, agoutis, opossums, ocelots, jaguars, camels, and howler monkeys. The average specificity of the ELISA for these species is reported by the company to be 99.1%. However, the sensitivity is not mentioned due to the limited availability of documented positive sera for these animal species. The sVNT (Surrogate Virus Neutralization Test) Kit by GenScript uses a recombinant SARS-CoV-2 Spike Protein Receptor Binding Domain (RBD) and ACE2 receptor protein to mimic the virus's entry mechanism into host cells.

RBD region of SARS-Cov-2 used in the study will detect and react with antibodies to SARS-1 (according to company validation data). This meant detection of antibodies to SARS-Cov-2 is broad and can detect antibodies to multiple viral strains of SARS-Cov-2 even data was not available at the time of assay validation. It should be noted that sVNT has been validated primarily for human serum samples, and their performance in animals such as sheep, bovine, and goat may not be well-established. Although the kit is designed to detect antibodies in any species (species independent), it is also applicable for studying SARS-CoV-2 infection in some animals such as rats, mice, ferrets, and rabbits. Without proper validation, there is a risk of relying on assays that do not accurately reflect the serological status of animals.

A significant limitation of our study is that these two ELISAs were not validated for all the species we tested or all the coronaviruses specific antibodies. Consequently, the performance (sensitivity and specificity) of these ELISAs for each species remains unknown, necessitating the use of multiple assays to assess agreement. Another limitation is the potential for cross-reactivity between different coronaviruses, such as SARS-CoV-2 and bovine coronavirus, within the ELISA assay, which can result in false-positive results and complicate the interpretation of serological data.

We detected that among the sVNT-positive serum samples in both experiments, several exhibited neutralizing antibody titers in both the VNT (USA/WA1/2020 isolate) and the BCoV Neutralization Assay. The high similarity between the spike protein epitopes of SARS-CoV-2 and BCoV suggests that BCoV is one of the viruses most similar to SARS-CoV-2.

In this study, serum samples exhibiting high-level neutralizing antibodies (1:1256 or higher) when tested using a virus neutralization (VN) assay against SARS-CoV-2 do not demonstrate any cross-reactivity, as no neutralizing antibodies were detected for the BCV viral strain utilized in the assay. Therefore, the principal limitation of such studies is the unknown diversity of circulating coronaviruses in animal populations that could potentially cross-react in ELISA assays. Additionally, ELISA methods may produce non-specific false-positive results at certain levels, thereby precluding the specificity from reaching 100% for any ELISA method.

To evaluate the specificity of serum samples, cross-reactivity testing can assess their reactivity with various coronaviruses. A strategy to mitigate cross-reactivity in ELISA assays for SARS-CoV-2 antibodies involves pre-incubating samples with BCoV antigens. This approach aims to reduce cross-reactivity by blocking or absorbing antibodies that may bind to both BCoV and SARS-CoV-2 antigens.

Additionally, to minimize the impact of cross-reactivity and false results in SARS-CoV-2 testing, several strategies should be implemented: 1) evaluating serum samples using the virus neutralization (VN) assay with various SARS-CoV-2 strains to enhance strain-specific detection, and 2) incorporating confirmatory tests, such as Western blotting, in-house ELISA specific for the receptor-binding domain (RBD), immunoblot bead assays, and plaque reduction neutralization tests (PRNT), 3) Antigen-specific assays [design of unique epitopes (epitope mapping and/or recombinant proteins] and synthetic peptides to validate and corroborate findings.

In conclusion, while serological testing, particularly through ELISA, is a critical tool for detecting and managing SARS-CoV-2 in animal populations, several challenges must be addressed to ensure it. Cross-reactivity remains a significant concern. This underscores the importance of developing highly specific assays to minimize cross-reactivity and improve the accuracy of results. Additionally, the sensitivity of serological tests, including ELISA, must be carefully calibrated to ensure they can detect low levels of antibodies, thereby reducing the likelihood of false negatives. High sensitivity and specificity are essential for reliable epidemiological surveillance, vaccine efficacy assessments, and effective disease management. By refining these aspects, serological testing can provide invaluable insights into the spread and control of SARS-CoV-2, ultimately contributing to better public health outcomes and enhanced understanding of the virus in animal hosts.