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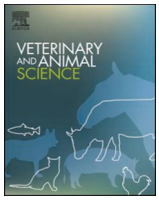

Cattle, sheep, and goat humoral immune responses against SARS-CoV-2

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ABSTRACT

Following the emergence of SARS-CoV-2 in late 2019, several species of domestic and wild animals have been found to be susceptible to SARS-CoV-2 infection through experimental inoculation and animal surveillance activities. Detection of SARS-CoV-2 specific antibodies in animals is an important surveillance tool since viral shedding in animals can only be detected for a short period of time. In this study, convenience serum samples were collected from 691 cattle, 698 sheep, and 707 goats from several regions in the United States, between 2019 and 2022. The samples were evaluated for the presence of SARS-CoV-2 specific antibodies using two commercial enzyme-linked immunosorbent assays (ELISA); one based on the inhibition of the SARS-CoV-2 receptor-binding domain (sVNT) and the other based on the nucleocapsid protein (N-ELISA) of SARS-CoV-2. Positive samples from the sVNT were additionally evaluated using a conventional virus neutralization test (VNT) employing the Wuhan-like SARS-CoV-2 USA/WA1/2020 isolate. Our results indicate that ~1 % (6/691) of cattle, ~2 % (13/698) of sheep, and ~2.5 % (18/707) of goat serum samples were positive when using the sVNT, whereas ~4 % of cattle (25/691) and sheep (27/698), and 2.5 % (18/707) of goat serum samples tested positive with the N-ELISA. None of the sVNT positive cattle, sheep, or goat serum samples had detectable neutralizing antibody activity (<1:8) against the SARS-CoV-2 USA/WA1/2020 isolate by the VNT. Our results indicate low seropositivity in cattle, sheep, and goats in the U.S., indicating the importance to continue monitoring for SARS-CoV-2 prevalence in animal species that are in close contact with humans.

1. Introduction

Several anthropogenic factors have been associated with an increase in the incidence of emerging zoonotic infectious diseases originating from animals, specifically wildlife (Frazzini et al., 2022; Jones et al., 2008). The increase in cross-species transmission may be attributed to several activities that promote human-animal interaction, such as changes in land use (deforestation), live animal wet markets, exotic pet trade, hunting, and close contact with domesticated livestock (Bengis et al., 2004; Piret & Boivin, 2021). In the last two decades, there have been three major outbreaks of coronaviruses that affected humans. The first of which was severe acute respiratory syndrome (SARS) coronavirus in 2002, followed by Middle Eastern respiratory syndrome (MERS) coronavirus in 2013, and most recently, the emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in 2019 (Cerino et al., 2021; da Costa et al., 2020). SARS-CoV-2, the causative agent of coronavirus disease 2019 (COVID-19), was first identified in December 2019 in the city of Wuhan, Hubei province, China (Cui et al., 2022). 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. According to the World Health Organization (WHO), COVID-19 has affected over 775 million people, resulting in approximately 7 million deaths as of March 31, 2024 (WHO, 2024). A distinctive characteristic of SARS-CoV-2 that has contributed to its persistence is the rapid evolution and emergence of SARS-CoV-2 variants of concern (VOCs). Some VOCs have been associated with changes in host range, warranting extensive surveillance of SARS-CoV-2 in animal populations to identify potential reservoir hosts (Su et al., 2016). The latest epidemiological data from the World Organization for Animal Health (WOAH, 2023) reports 775 outbreaks in animals globally, impacting 29 animal species across 36 countries (WOAH, 2023). 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) (WOAH, 2023).

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The broad host range of SARS-CoV-2 poses concern for the establishment of reservoir species that could serve as a source for future outbreaks of animal adapted variants that could circumvent current mitigation strategies. The potential establishment of SARS-CoV-2 reservoir species has received significant interest worldwide, promoting enhanced surveillance networks that rely on rapid diagnostic methods. The susceptibility of animals to SARS-CoV-2 varies, and different species have different levels of susceptibility (Meekins et al., 2021; Ulrich et al., 2020). Several computational, experimental, and diagnostic methods have been employed to examine the susceptibility of animal species to SARS-CoV-2 including: (*i*) computational modeling (in silico), (*ii*) in vitro experiments conducted outside a living organism, (*iii*) in vivo studies in a living organism, and (*iv*) analysis of epidemiological data (Rutherford et al., 2022). 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 (Meekins et al., 2021). SARS-CoV-2 transmission has been observed to occur via direct contact with infected animals, aerosol and droplets, contaminated surfaces and objects, and environmental factors (water, soil, air, etc.) (Fang et al., 2023; Kwon et al., 2021, 2023a, 2023b).

SARS-CoV-2 is a single-stranded, positive sense, enveloped RNA virus belonging to the *Coronaviridae* family, genus *Betacoronavirus* (White & Razgour, 2020). Coronaviruses (CoVs) are divided into four genera: *Alphacoronavirus, Betacoronavirus, Gammacoronavirus*, and *Deltacoronavirus* (Kirtipal et al., 2020). Alpha and Beta CoV mainly infect mammals and originate from bats, while Gamma and Delta CoV have been identified in birds and infect both birds and mammals (Woo et al., 2012). Four major structural proteins, common to all coronaviruses, have been described: spike protein (S), membrane protein (M), envelope protein (E), and nucleocapsid protein (N). The E protein helps in the assembly and release of the virions, and the M protein plays a crucial role in the structural integrity of the virus, stabilization of the N proteins, and in virus budding and assembly (Astuti & Ysrafil, 2020; Naqvi et al., 2020).

The receptor binding domain (RBD) of the S protein is involved in viral attachment and entry into host cells (Naqvi et al., 2020). SARS-CoV-2 entry is facilitated through the interaction between the receptor-binding domain (RBD) on the S1 subunit and the peptidase domain (PD) of the angiotensin-converting enzyme 2 (ACE2) receptor on the surface of host cells (Jackson et al., 2022). Accordingly, 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 (Zost et al., 2020). Mutations in the RBD are associated with immune escape, as observed in newly emerging virus variants (Jackson et al., 2022), and have also been implicated with increased transmissibility and/or changes in virulence (pathogenicity) (Rodrigues et al., 2023). The interaction between the spike protein and ACE2 is the main factor in determining the host range of SARS-CoV-2 (Lean et al., 2022).

The nucleocapsid (N) is a highly conserved structural protein of SARS-CoV-2 involved in packaging the viral RNA genome into aribonucleoprotein (RNP) complex, facilitating viral assembly (Song et al., 2023; Wu et al., 2023). The nucleocapsid protein is one of the most abundant viral proteins during infection (He et al., 2004) and induces a specific immune response in the host which makes for an ideal diagnostic target in infected animals.

To effectively monitor SARS-CoV-2 infections in domestic and wild animal species, rapid and accurate diagnostic methods must be available. The plaque reduction neutralization test (PRNT) or a conventional virus neutralization test (cVNT) are considered the gold standard for identifying neutralizing antibodies for SARS-CoV-2. Alternatively, the enzyme-linked immunosorbent assay (ELISA) serves as a rapid and cost-effective serological test (Cordero-Ortiz et al., 2023). 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 (Villanueva-Saz et al., 2022). False positives may occur when conserved antigens present in related pathogens are targeted (Caradonna & Schmidt, 2021). Accurate detection of SARS-CoV-2 specific antibodies after an immune response is important for surveillance, epidemiological studies, and for understanding the protective effector molecules of the immune response. Therefore, effective and rapid serological assays for SARS-CoV-2 and its variants, which have high sensitivity and specificity, are necessary to identify outbreaks of SARS-CoV-2 and guide public health interventions.

Historically, domestic ruminants such as cattle, sheep, and goats have been an important resource for food production and have developed into a significant component of global economics. In order to maintain herds of cattle, sheep, and goats, intensive human care is required. In silico modeling has predicted that the SARS-CoV-2 RBD is capable of interacting with the sheep ACE2 receptor, suggesting sheep are susceptible to SARS-CoV-2 infection (Damas et al., 2020). Di Teodoro et al. (2021) demonstrated that *ex vivo* respiratory organ cultures of sheep and cattle respiratory tissues (tracheal and lung) were permissible to infection with SARS-CoV-2. Importantly, when sheep were experimentally infected with SARS-CoV-2, RNA was detectable throughout the respiratory tract and lymphoid tissues, but only low levels of virus were shed for a short period of time, suggesting that sheep are unlikely to maintain SARS-CoV-2 transmission within herds (Gaudreault et al., 2022). Furthermore, Bae et al. (2023) found that domesticated Korean native cattle and native black goats are susceptible to SARS-CoV-2, and they can contract the virus from humans. In a recent experimental study by Cool et al. (2024) cattle were found to be more susceptible to infection with SARS-CoV-2 Delta VOC compared to Omicron BA.2. Additionally, the study revealed limited seroconversion and no evidence of transmission to sentinel calves, suggesting that cattle may not act as reservoir hosts for currently circulating SARS-CoV-2 variants. Other experimental and natural infection studies show cattle can seroconvert but have low SARS-CoV-2 antibody titers (Falkenberg et al., 2021; Fiorito et al., 2022; Ulrich et al., 2020; Wernike et al., 2022). 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 (Cool et al., 2024; Fernandez-Bastit et al., 2022; Fusco et al., 2023; Gaudreault et al., 2022; Villanueva-Saz et al., 2021). However, there has been very limited evidence of natural infections with SARS-CoV-2 in these animals.

As the epidemiological role and susceptibility of cattle, sheep, and goats to different variants of SARS-CoV-2 infection are still not fully understood and remain unclear, it is important to investigate these matters due to their constant contact with humans and other susceptible animal species. Therefore, the main objectives of this study were to investigate the seroprevalence of SARS-CoV-2 specific 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.

2. Materials and methods

2.1. Sample collection

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 (Fig. 1). Upon arrival at the laboratory, aliquots of the serum were frozen at -80°C until analysis. All samples were initially registered based on the owner's location. However, some samples were not associated with an owner's location information and were subsequently registered based on their clinical location.

2.2. Detection of antibodies by ELISAs

2.2.1. SARS-CoV-2 double antigen ELISA

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. Briefly, Serum samples were heat-inactivated at 56 °C for 30 min. Each ELISA plate well received 25 µl of dilution buffer and 25 µl of serum, with positive and negative controls included. After 45 min of incubation at 37 °C, wells were washed 5 times with 300 µl of wash solution. Then, 100 µl of N protein recombinant antigen horseradish peroxidase (HRP) conjugate was added and incubated for 30 min at room temperature (RT). Wells were washed 5 times with 300 µl of wash solution, followed by adding 100 µl of substrate solution and incubating for 20 min at RT. The reaction was stopped with 100 µl of 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% $\geq$ 60 % was considered positive, while serum with S/P% 50−60 % was defined as 'suspect,' and serum with S/P% $\leq$ 50 % was considered negative.

2.2.2. 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 interaction between the virus RBD and the ACE2 cell surface receptor. The test was conducted according to the manufacturer's instructions. Briefly, serum samples were heat-inactivated at 56 °C for 30 min. Serum samples, 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. 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, incubated at room temperature for 15 minutes, and the reaction was stopped with 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

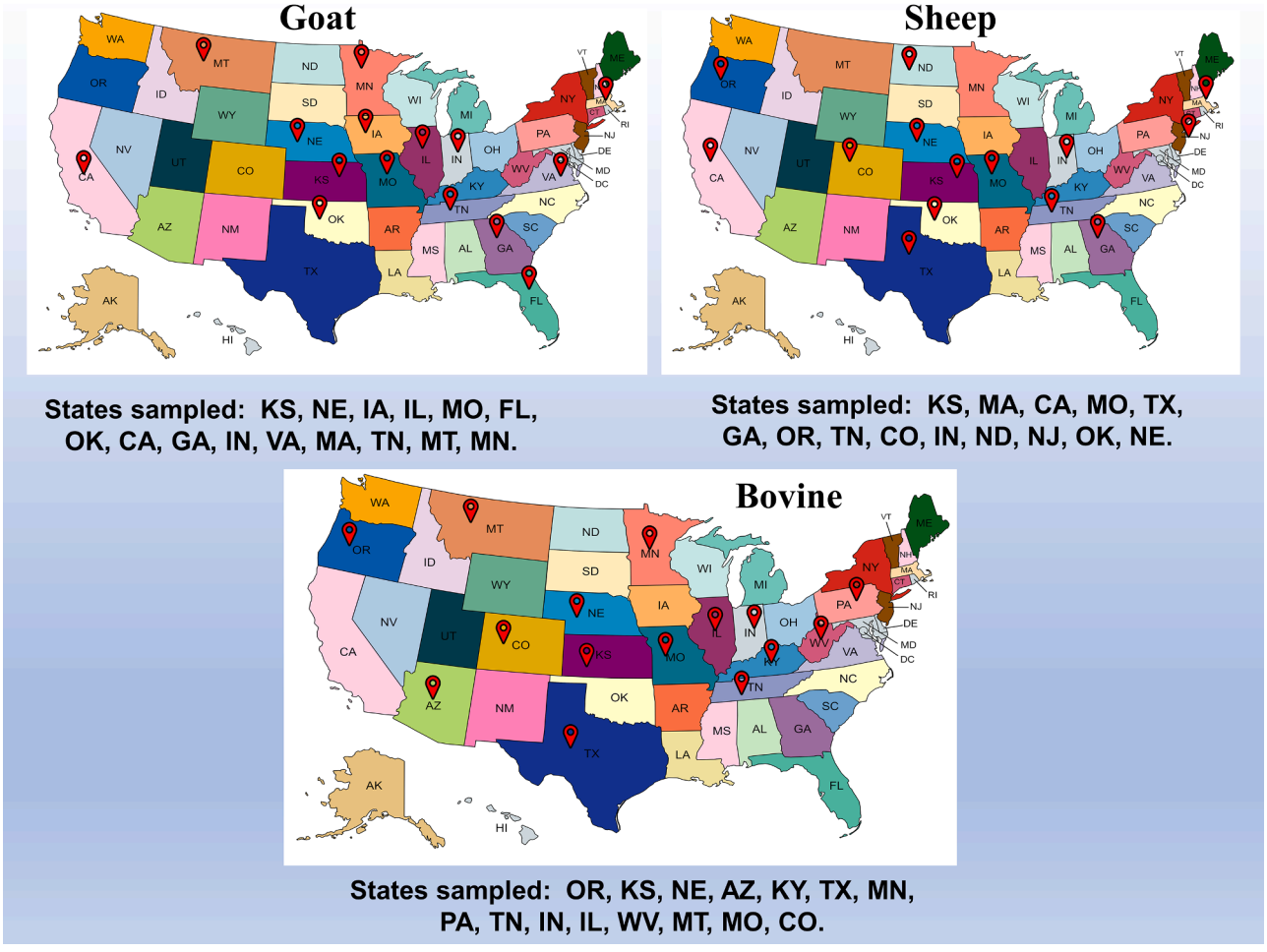

Fig. 1. Map showing the distribution of goat, sheep, and bovine samples collected and tested across the United States. (Created with mapchart.net).

percentage (% inhibition). For the expression of the results, % inhibition $\geq$ 30 % was considered positive, and % inhibition $<$ 30 % was considered negative.

2.3. Virus neutralizing antibodies

2.3.1. 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 100 µl of supplemented media on 96-well plates and subjected to 2-fold serial dilutions starting from 1:8 to 1:2560. The BCoV Mebus strain (Mebus CA, et al.,1973; Benfield et al.,1990) virus stock was diluted to 100TCID50/ 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 seeded 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 PBS-T and fixed with 80 % acetone for 10 min. 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 Zhang et al., 1997 et al), a monoclonal antibody that targets the spike protein subunit of BCoV, diluted 1:10, was 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 BCV 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.

2.3.2. 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) tested using the sVNT.

SARS-CoVCoV-2 neutralizing antibodies in sera were determined using a microneutralization assay as previously described (Gaudreault et al., 2020). 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 h 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 h post infection. Positive control sera and back-titrations of diluted virus stock were used to monitor assay performance and consistency.

3. Results

3.1. Seroprevalence using different methods

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 (Fig. 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 (Fig. 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 1.

3.2. Concordance among sVNT, VNT, and BCoV

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.

4. Discussion

The global epidemiology of SARS-CoV-2, along with its associated symptoms, morbidity, and mortality, exhibits significant variability across different regions. In the context of outbreaks involving novel zoonotic pathogens, the development, validation, and widespread dissemination of rapid diagnostic tools are crucial for informed policy decisions concerning both animal and public health. Although there are several techniques available to evaluate the exposure of animals to SARS-CoV-2, such as the detection of antibodies, antigens, and molecular detection methods, the use of broad serological detection assays are considered the most effective method for surveillance. Traditional virology (culturing) and molecular diagnostic methods alone may not be sufficient to determine the full extent and spread amongst animal populations due to the narrow window of time to capture animals when actively shedding virus, as reported by WOAH (Fusco et al., 2023). It is important to note that there exists a range of diagnostic sensitivity and specificity in commercial assays, occasionally resulting in undetected cases and ineffective responses (Taylor et al., 2021). This inconsistency highlights the need for careful selection and validation of testing methods to ensure reliable detection and timely intervention, which are essential to preventing the further transmission of the disease. Determining the levels of virus-specific antibodies are crucial measurements for estimating population and herd immunity against SARS-CoV-2 (Jiang et al., 2022). Infection with SARS-CoV-2 stimulates the host's immune system to produce detectable levels of IgG antibodies, which are critical for establishing protective immune responses. This process is crucial for the development of long-term immunity, primarily through the activation and maturation of a robust B cell response (Li et al., 2023). It should be considered that the antibody levels can be influenced by various factors, including previous infections, the severity of infection, and the time point of sample collection (Dhar, 2022; Wei et al., 2021).

SARS-CoV-2 and bovine coronavirus (BCoV) belong to the betacoronavirus family. Phylogenetically, BCoV is closely related to human coronavirus (HCoV) OC43, classified under Betacoronavirus 1 (Wensman & Stokstad, 2020). This may provide opportunity for potential for cross-reactivity with the similar antigens present in these betacoronaviruses, resulting in false positives.

A majority of information regarding the susceptibility of bovine,

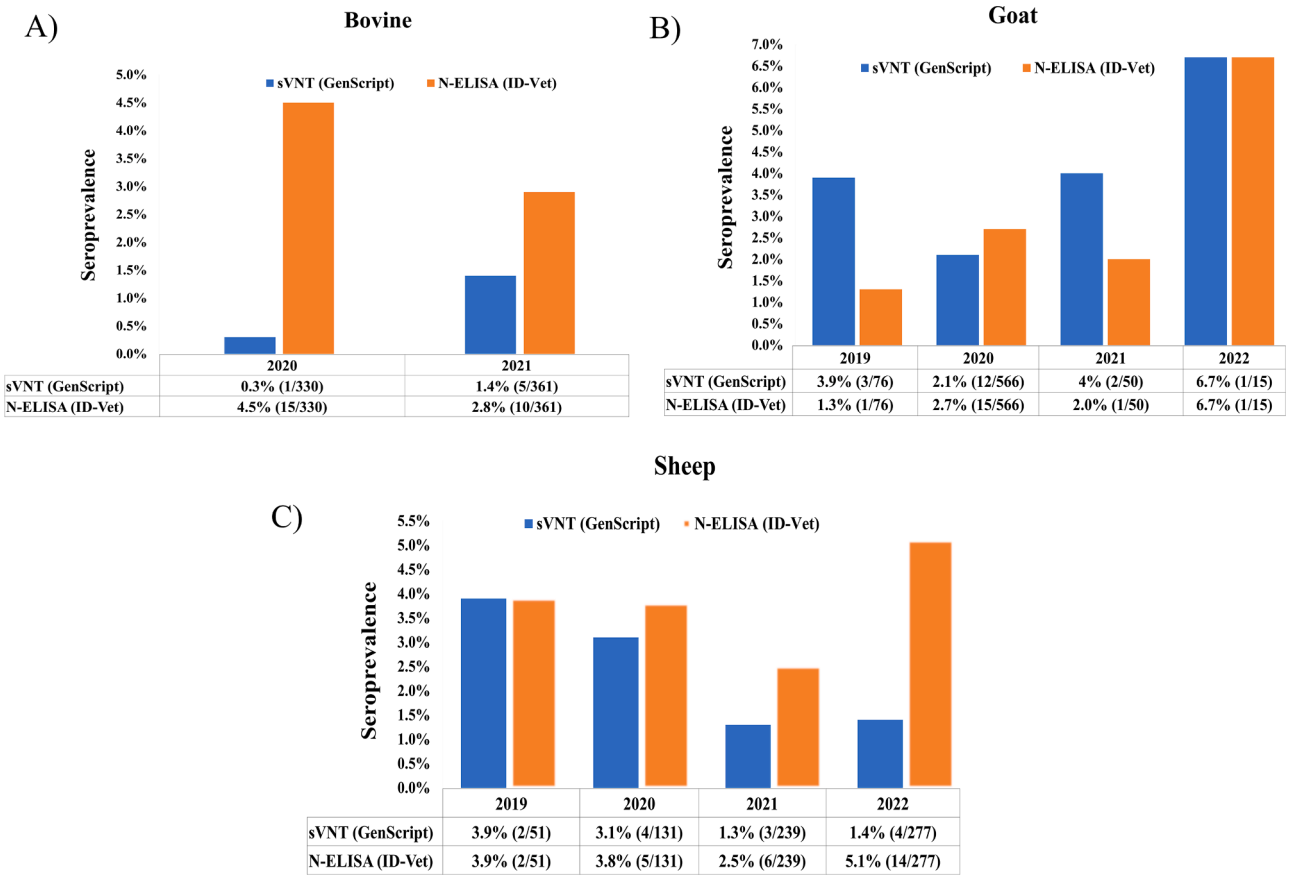

Fig. 2. Detection of SARS-CoV-2 specific antibodies against the N and S (RBD region) proteins in A) bovine, B) goat, and C) sheep using both N and sVNT IgG ELISA. The X-axis represents the year of sample collection and the number and percentage of serum samples that resulted in positive detection of 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 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 (0.9 %)	25 (3.6 %)	ND**
Goat	707	17 (2.4 %)	18 (2.5 %)	1
Sheep	698	13 (1.9 %)	27 (3.9 %)	1

* n: number
** ND: not detected

sheep, and goats to SARS-CoV-2 originates from experimental infections and suggests these ruminant species are mostly resistant to SARS-CoV-2 infection. It is important to note that most cattle, sheep, and goats included in these studies were infected with a high dose of SARS-CoV-2 through intranasal and oral inoculation, which is not a perfect model for potential exposure to SARS-CoV-2 in natural conditions. 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. The present study evaluated the diagnostic performance of two different commercial ELISA in detecting antibodies against N and S proteins of SARS-CoV-2. A VNT was used as a reference test to confirm results of ELISA assay in cattle, sheep, and goat serum samples. A second aim of this study was to report on the seroprevalence of SARS-CoV-2 antibodies in sheep, goats, and cattle from several geographically distinct regions in the United States.

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 was conducted on 1000 cattle from 83 farms using RBD-based multispecies ELISA and surrogate Virus Neutralization Test (sVNT) (Wernike et al., 2022). The results indicated that 11 samples tested positive by the RBD-ELISA and 4 samples tested positive by the sVNT (GenScript Kit), suggesting that low number of seropositive cattle may be become virus-infected through exposure to SARS-CoV-2-positive keepers (Wernike et al., 2022). Another study conducted on 24 lactating cows from a farm in Italy discovered that 11/24 and 14/24 cows showed antibodies for SARS-CoV-2 nucleocapsid protein and SARS-CoV-2 spike protein, respectively. Neutralizing antibodies against SARS-CoV-2 were also detected in 13/24 tested cows (Fiorito et al., 2022). 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) (Dias et al., 2023). 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 (Ulrich et al., 2020). The results obtained by Ulrich et al. (2020) were in line with those reported by Falkenberg et al. (2021), who inoculated bull calves with the SARS-CoV-2 strain, TGR1/NY/20 via intratracheal or intravenous routes. All calves exhibited neutralizing antibodies at 7 days post challenge (DPC) but were not detectable by 21 DPC (Falkenberg et al., 2021). Cool et al. (2024) 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.

A systematic surveillance study was conducted using RT-qPCR on samples collected from various regions of Gujarat state, including nasal and rectal swab samples from 64 cattle, 39 buffalo, and 19 sheep (Kumar

et al., 2022). The results showed the presence of SARS-CoV-2 RNA in 23.43 % (15/64) of cattle and 33.33 % (13/39) of buffaloes, respectively, suggesting that both cattle and buffaloes can be susceptible to infection by the virus (Kumar et al., 2022).

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 (Villanueva-Saz et al., 2021). In contrast, when sheep were experimentally inoculated with a mixture of wildtype SARS-CoV-2 and the alpha VOC, it was observed that among 10 infected sheep, all exhibited detectable antibodies by Indirect RBD and N ELISAs. However, only one of them developed a low level of neutralizing antibodies with a titer of 1:20 at 10 and 21 DPC (Gaudreault et al., 2022). An additional experimental inoculation study examining the susceptibility of cattle, sheep, and goats, low levels of neutralizing antibodies were observed within 14 days of infection, and the majority of animals were observed to be seronegative on day 28 post-infection (Bosco-Lauth et al., 2021). 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 (Happi et al., 2023). Furthermore, Bae and colleagues (Bae et al., 2023) 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. A recent study 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 (ID Screen SARS-CoV-2 Double Antigen Multi-Species 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 (Fusco et al., 2023).

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 (Fernandez-Bastit et al., 2022). According to the findings, domestic goats are less prone to getting infected by the SARS-CoV-2 B.1.351/Beta variant due to the limited amount of viral genome and antigen found in their tissues. Additionally, 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 (Fernandez-Bastit et al., 2022).

In this study, we demonstrated that 6 cattle, 18 goats, and 13 sheep serum samples were positive for the sVNT, whereas 25 cattle, 18 goats, and 27 sheep serum samples tested positive for the N-ELISA. 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 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 seropositivity samples by the N-ELISA may partially be attributed to the cross-reactivity of SARS-CoV-2 antibodies with other betacoronaviruses. The N protein is relatively conserved among coronaviruses that infect animals and humans, potentially leading to cross-reaction with other coronaviruses that may have infected these animals prior to the time of sampling (Di et al., 2021; Happi et al., 2023).

5. Conclusion

Our results demonstrate a low level of seropositive cattle, sheep, and goats in the United States when evaluated with commercially available ELISAs. Serum samples that were positive when evaluated with the sVNT did not show any neutralizing antibody response (<1:8) when assayed with classic VNT. The results obtained from our study align with observations from previously published surveillance efforts, suggesting 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 levels in cattle, sheep, and goats across the U.S. at the time of our study, the dynamic situation of SARS-CoV-2 variant emergence would suggest that it is still important to monitor for the presence of SARS-CoV-2 viral infection in animal species that may be in close contact with humans. A limitation of this study was that samples from sheep and goats testing positive for antibodies to the RBD were not assessed for the presence of neutralizing antibodies against BCoV. This limitation could be addressed in future research.

Disclaimer

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Institutional Review Board Statement: This study was performed under Kansas State University Institutional Biosafety Committee-approved protocol (#IBC1462 and #IBC-1695).

Ethical statements

The manuscript entitled “Cattle, sheep, and goat humoral immune responses against SARS-CoV-2 “ was prepared according to all the ethical standards required by the Journal of “Veterinary and Animal Science”.

CRediT authorship contribution statement

Mehrnaz Ardalan: Writing – review & editing, Writing – original draft, Software, Resources, Investigation, Funding acquisition, Formal analysis, Data curation. **Konner Cool:** Writing – review & editing, Methodology, Data curation. **Natasha N. Gaudreault:** Writing – review & editing, Supervision, Data curation. **Dashzeveg Bold:** Writing – review & editing, Formal analysis, Data curation. **Anna Mannix:** Methodology, Data curation. **Gregg A. Hanzlicek:** Writing – review & editing, Formal analysis, Conceptualization. **Juergen A. Richt:** Writing – review & editing, Investigation, Funding acquisition. **Roman M. Pogranichniy:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation,

Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Astuti, I., & Ysrafil. (2020). Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2): An overview of viral structure and host response. *Diabetes & Metabolic Syndrome, 14*, 407–412. https://doi.org/10.1016/j.dsx.2020.04.020

Bae, D. Y., Yang, J. H., Moon, S. H., Kim, W. H., Yoo, D. S., Park, C. K., Shin, Y. K., Kang, H. E., Tark, D., Oh, Y., & Cho, H. S. (2023). Demonstration of SARS-CoV-2 exposure in Korean native cattle and Korean native black goats in Korea. *Animals : An Open Access Journal from MDPI, 13*, 3498. https://doi.org/10.3390/ani13223498

Benfield, D. A., & Saif, L. J. (1990). Cell culture propagation of a coronavirus isolated from cows with winter dysentery. *Journal of Clinical Microbiology, 28*, 1454–1457. https://doi.org/10.1128/jcm.28.6.1454-1457.1990

Bengis, R. H., Leighton, F. A., Fischer, J. R., Artois, M., Mörner, T., & Tate, C. M. (2004). The role of wildlife in emerging and re-emerging zoonoses. *Revue Scientifique Et Technique-Office International Des Epizooties, 23*, 497–511.

Bosco-Lauth, A. M., Walker, A., Guilbert, L., Porter, S., Hartwig, A., McVicker, E., Bielefeldt-Ohmann, H., & Bowen, R. A. (2021). Susceptibility of livestock to SARS-CoV-2 infection. *Emerging Microbes & Infections, 10*, 2199–2201. https://doi.org/10.1080/22221751.2021.2003724

Caradonna, T. M., & Schmidt, A. G. (2021). Protein engineering strategies for rational immunogen design. *NPJ Vaccines,, 6*, 154. https://doi.org/10.1038/s41541-021-00417-1

Cerino, P., Buonerba, C., Brambilla, G., Atripaldi, L., Tafuro, M., Di Concilio, D., Vassallo, L., Lo Conte, G., Cuomo, M. C., Maiello, I., D'Auria, J., Cardinale, D., Viscardi, M., Rofrano, G., Gallo, A., Brusco, P., Pizzolante, A., Cicalese, V., Galdi, P., Galdi, L., De Vita, S., Volzone, P., Di Vuolo, G., Coppola, A., Pierri, B., & Fusco, G. (2021). No detection of SARS-CoV-2 in animals exposed to infected keepers: Results of a COVID-19 surveillance program. *Future Science Oa7:FSO711*. https://doi.org/10.2144/fsoa-2021-0038

Cool, K., Gaudreault, N. N., Trujillo, J. D., Morozov, I., McDowell, C. D., Bold, D., Kwon, T., Balaraman, V., Assato, P., Madden, D. W., Mantlo, E., Souza-Neto, J., Matias-Ferreyra, F., Retallick, J., Singh, G., Schotsaert, M., Carossino, M., Balasuriya, U. B. R., Wilson, W. C., Pogranichniy, R. M., Garcia-Sastre, A., & Richt, J. A. (2024). Experimental co-infection of calves with SARS-CoV-2 delta and omicron variants of concern. *Emerging Microbes & Infections, 13*, Article 2281356. https://doi.org/10.1080/22221751.2023.2281356

Cordero-Ortiz, M., Resendiz-Sandoval, M., Dehesa-Canseco, F., Solis-Hernandez, M., Perez-Sanchez, J., Martinez-Borges, C., Mata-Haro, V., & Hernandez, J. (2023). Development of a multispecies double-antigen sandwich ELISA using N and RBD proteins to detect antibodies against SARS-CoV-2. *Animals : An Open Access Journal from MDPI, 13*, 3487. https://doi.org/10.3390/ani13223487

Cui, S. J., Liu, Y. M., Zhao, J. C., Peng, X. M., Lu, G. L., Shi, W. X., Pan, Y., Zhang, D. T., Yang, P., & Wang, Q. Y. (2022). An updated review on SARS-CoV-2 infection in animals. *Viruses, 14*, 1527. https://doi.org/10.3390/v14071527

da Costa, V. G., Moreli, M. L., & Saivish, M. V. (2020). The emergence of SARS, MERS and novel SARS-2 coronaviruses in the 21st century. *Archives of Virology, 165*, 1517–1526. https://doi.org/10.1007/s00705-020-04628-0

Damas, J., Hughes, G. M., Keough, K. C., Painter, C. A., Persky, N. S., Corbo, M., Hiller, M., Koepfli, K. P., Pfenning, A. R., Zhao, H., Genereux, D. P., Swofford, R., Pollard, K. S., Ryder, O. A., Nweeia, M. T., Lindblad-Toh, K., Teeling, E. C., Karlsson, E. K., & Lewin, H. A. (2020). Broad host range of SARS-CoV-2 predicted by comparative and structural analysis of ACE2 in vertebrates. *Proceedings of the National Academy of Sciences of the United States of America, 117*, 22311–22322. https://doi.org/10.1073/pnas.2010146117

Dhar, B. C. (2022). Diagnostic assay and technology advancement for detecting SARS-CoV-2 infections causing the COVID-19 pandemic. *Analytical and Bioanalytical Chemistry, 414*, 2903–2934. https://doi.org/10.1007/s00216-022-03918-7

Di, D., Dileepan, M., Ahmed, S., Liang, Y., & Ly, H. (2021). Recombinant SARS-CoV-2 nucleocapsid protein: Expression, purification, and its biochemical characterization and utility in serological assay development to assess immunological responses to SARS-CoV-2 infection. *Pathogens (Basel, Switzerland), 10*, 1039. https://doi.org/10.3390/pathogens10081039

Dias, J. M., Costa, M. O., De Souza, A. O., Faria, M. L., Golcman, D. H., Selim, M. B., Medici, E. P., Haga, G. S. I., Romaldini, A. H.d. C. N., & Okuda, L. H. (2023). Serological assay for SARS-CoV-2 in domestic and wild animals in middle and southeast regions in Brazil. *Preprints*. https://doi.org/10.20944/preprints202307.0375.v1

Di Teodoro, G., Valleriani, F., Puglia, I., Monaco, F., Di Pancrazio, C., Luciani, M., Krasteva, I., Petrini, A., Marcacci, M., D'Alterio, N., Curini, V., Iorio, M., Migliorati, G., Di Domenico, M., Morelli, D., Calistri, P., Savini, G., Decaro, N., Holmes, E. C., & Lorusso, A. (2021). SARS-CoV-2 replicates in respiratory ex vivo organ cultures of domestic ruminant species. *Veterinary Microbiology, 252*, Article 108933. https://doi.org/10.1016/j.vetmic.2020.108933

Falkenberg, S., Buckley, A., Laverack, M., Martins, M., Palmer, M. V., Lager, K., & Diel, D. G. (2021). Experimental inoculation of young calves with SARS-CoV-2. *Viruses, 13*, 441. https://doi.org/10.3390/v13030441

Fang, R., Yang, X., Guo, Y., Peng, B., Dong, R., Li, S., & Xu, S. (2023). SARS-CoV-2 infection in animals: Patterns, transmission routes, and drivers. *Eco Environment & Health, 3*, 45–54. https://doi.org/10.1016/j.eehl.2023.09.004

Fernandez-Bastit, L., Roca, N., Romero-Durana, M., Rodon, J., Cantero, G., Garcia, O., Lopez, C., Perez, M., Lopez, R., Carrillo, J., Izquierdo-Useros, N., Blanco, J., Clotet, B., Pujols, J., Vergara-Alert, J., Segales, J., & Lorca-Oro, C. (2022). Susceptibility of domestic goat (Capra aegagrus hircus) to experimental infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) B.1.351/beta variant. *Viruses, 14*, 2002. https://doi.org/10.3390/v14092002

Fiorito, F., Iovane, V., Pagnini, U., Cerracchio, C., Brandi, S., Levante, M., Marati, L., Ferrara, G., Tammaro, V., De Carlo, E., Iovane, G., & Fusco, G. (2022). First description of serological evidence for SARS-CoV-2 in lactating cows. *Animals : An Open Access Journal from MDPI, 12*, 1459. https://doi.org/10.3390/ani12111459

Frazzini, S., Amadori, M., Turin, L., & Riva, F. (2022). SARS CoV-2 infections in animals, two years into the pandemic. *Archives of Virology*, 2503–2517. https://doi.org/10.1007/s00705-022-05609-1

Fusco, G., Cardillo, L., Levante, M., Brandi, S., Picazio, G., Napoletano, M., Martucciello, A., Fiorito, F., De Carlo, E., & de Martinis, C. (2023). First serological evidence of SARS-CoV-2 natural infection in small ruminants: Brief report. *Veterinary Research Communications, 47*, 1741–1748. https://doi.org/10.1007/s11259-022-10044-3

Gaudreault, N. N., Cool, K., Trujillo, J. D., Morozov, I., Meekins, D. A., McDowell, C., Bold, D., Carossino, M., Balaraman, V., Mitzel, D., Kwon, T., Madden, D. W., Artiaga, B. L., Pogranichniy, R. M., Roman-Sosa, G., Wilson, W. C., Balasuriya, U. B. R., Garcia-Sastre, A., & Richt, J. A. (2022). Susceptibility of sheep to experimental co-infection with the ancestral lineage of SARS-CoV-2 and its alpha variant. *Emerging Microbes & Infections, 11*, 662–675. https://doi.org/10.1101/2021.11.15.468720

Gaudreault, N. N., Trujillo, J. D., Carossino, M., Meekins, D. A., Morozov, I., Madden, D. W., Indran, S. V., Bold, D., Balaraman, V., Kwon, T., Artiaga, B. L., Cool, K., Garcia-Sastre, A., Ma, W., Wilson, W. C., Henningson, J., Balasuriya, U. B. R., & Richt, J. A. (2020). SARS-CoV-2 infection, disease and transmission in domestic cats. *Emerging Microbes & Infections, 9*, 2322–2332. https://doi.org/10.1080/22221751.2020.1833687

Happi, A. N., Ayinla, A. O., Ogunsanya, O. A., Sijuwola, A. E., Saibu, F. M., Akano, K., George, U. E., Sopeju, A. E., Rabinowitz, P. M., Ojo, K. K., Barrett, L. K., Van Voorhis, W. C., & Happi, C. T. (2023). Detection of SARS-CoV-2 in terrestrial animals in Southern Nigeria: Potential cases of reverse zoonosis. *Viruses, 15*, 1187. https://doi.org/10.3390/v15051187

He, Y., Zhou, Y., Wu, H., Kou, Z., Liu, S., & Jiang, S. (2004). Mapping of antigenic sites on the nucleocapsid protein of the severe acute respiratory syndrome coronavirus. *Journal of Clinical Microbiology, 42*, 5309–5314. https://doi.org/10.1128/JCM.42.11.5309-5314.2004

Jackson, C. B., Farzan, M., Chen, B., & Choe, H. (2022). Mechanisms of SARS-CoV-2 entry into cells. *Nature Reviews. Molecular Cell Biology, 23*, 3–20. https://doi.org/10.1038/s41580-021-00418-x

Jiang, R., Dou, X., Li, M., Wang, E., Hu, J., Xiong, D., & Zhang, X. (2022). Dynamic observation of SARS-CoV-2 IgM, IgG, and neutralizing antibodies in the development of population immunity through COVID-19 vaccination. *Journal of Clinical Laboratory Analysis, 36*, e24325. https://doi.org/10.1002/jcla.24325

Jones, K. E., Patel, N. G., Levy, M. A., Storeygard, A., Balk, D., Gittleman, J. L., & Daszak, P. (2008). Global trends in emerging infectious diseases. *Nature, 451*, 990–993. https://doi.org/10.1038/nature06536

Kirtipal, N., Bharadwaj, S., & Kang, S. G. (2020). From SARS to SARS-CoV-2, insights on structure, pathogenicity and immunity aspects of pandemic human coronaviruses. *Infection, Genetics and Evolution : Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases, 85*, Article 104502. https://doi.org/10.1016/j.meegid.2020.104502

Kumar, D., Antiya, S. P., Patel, S. S., Pandit, R., Joshi, M., Mishra, A. K., Joshi, C. G., & Patel, A. C. (2022). Surveillance and molecular characterization of SARS-CoV-2 infection in non-human hosts in Gujarat, India. *International Journal of Environmental Research and Public Health, 19*, 14391. https://doi.org/10.3390/ijerph192114391

Kwon, T., Gaudreault, N. N., Cool, K., McDowell, C. D., Morozov, I., & Richt, J. A. (2023a). Stability of SARS-CoV-2 in biological fluids of animals. *Viruses, 15*, 761. https://doi.org/10.3390/v15030761

Kwon, T., Gaudreault, N. N., & Richt, J. A. (2021). Environmental stability of SARS-CoV-2 on different types of surfaces under indoor and seasonal climate conditions.

Pathogens (Basel, Switzerland), 10, 227. https://doi.org/10.3390/pathogens10020227

Kwon, T., Osterrieder, N., Gaudreault, N. N., & Richt, J. A. (2023b). Fomite transmission of SARS-CoV-2 and its contributing factors. *Pathogens (Basel, Switzerland), 12*, 364. https://doi.org/10.3390/pathogens12030364

Lean, F. Z. X., Nunez, A., Spiro, S., Priestnall, S. L., Vreman, S., Bailey, D., James, J., Wrigglesworth, E., Suarez-Bonnet, A., Conceicao, C., Thakur, N., Byrne, A. M. P., Ackroyd, S., Delahay, R. J., van der Poel, W. H. M., Brown, I. H., Fooks, A. R., & Brookes, S. M. (2022). Differential susceptibility of SARS-CoV-2 in animals: Evidence of ACE2 host receptor distribution in companion animals, livestock and wildlife by immunohistochemical characterisation. *Transboundary and Emerging Diseases, 69*, 2275–2286. https://doi.org/10.1111/tbed.14232

Li, Q., Chen, L., Li, F., & He, A. (2023). Long-term evaluation of the seroprevalence of SARS-CoV-2 IgG and IgM antibodies in recovered patients: a meta-analysis. *BMC Infectious Diseases, 23*, 444. https://doi.org/10.1186/s12879-023-08425-3

Mebus, C. A., Stair, E. L., Rhodes, M. B., & Twiehaus, M. J. (1973). Neonatal calf diarrhea: Propagation, attenuation, and characteristics of a coronavirus-like agent. *American Journal of Veterinary Research, 34*, 145–150.

Meekins, D. A., Gaudreault, N. N., & Richt, J. A. (2021). Natural and experimental SARS-CoV-2 infection in domestic and wild animals. *Viruses, 13*, 1993. https://doi.org/10.3390/v13101993

Naqvi, A. A. T., Fatima, K., Mohammad, T., Fatima, U., Singh, I. K., Singh, A., Atif, S. M., Hariprasad, G., Hasan, G. M., & Hassan, M. I. (2020). Insights into SARS-CoV-2 genome, structure, evolution, pathogenesis and therapies: Structural genomics approach. *Biochimica et Biophysica Acta Molecular basis of Disease, 1866*, Article 165878. https://doi.org/10.1016/j.bbadis.2020.165878

Piret, J., & Boivin, G. (2021). Pandemics throughout history. *Frontiers in Microbiology, 11*, Article 631736. https://doi.org/10.3389/fmicb.2020.631736. ARTN 631736.

Rodrigues, G. M., Volpato, F. C. Z., Wink, P. L., Paiva, R. M., Barth, A. L., & de-Paris, F. (2023). SARS-CoV-2 variants of concern: Presumptive identification via sanger sequencing analysis of the receptor binding domain (RBD) region of the S gene. *Diagnostics (Basel, 13*, 1256. https://doi.org/10.3390/diagnostics13071256

Rutherford, C., Kafle, P., Soos, C., Epp, T., Bradford, L., & Jenkins, E. (2022). Investigating SARS-CoV-2 susceptibility in animal species: A scoping review. *Environmental Health Insights, 16*, Article 11786302221107786. https://doi.org/10.1177/11786302221107786

Song, W., Fang, Z., Ma, F., Li, J., Huang, Z., Zhang, Y., Li, J., & Chen, K. (2023). The role of SARS-CoV-2 N protein in diagnosis and vaccination in the context of emerging variants: Present status and prospects. *Frontiers in Microbiology, 14*, Article 1217567. https://doi.org/10.3389/fmicb.2023.1217567

Su, S., Wong, G., Shi, W., Liu, J., Lai, A. C. K., Zhou, J., Liu, W., Bi, Y., & Gao, G. F. (2016). Epidemiology, genetic recombination, and pathogenesis of coronaviruses. *Trends in Microbiology, 24*, 490–502. https://doi.org/10.1016/j.tim.2016.03.003

Taylor, S. C., Hurst, B., Charlton, C. L., Bailey, A., Kanji, J. N., McCarthy, M. K., … Knight, V. (2021). A New SARS-CoV-2 dual-purpose serology test: Highly accurate infection tracing and neutralizing antibody response detection. *Journal of Clinical Microbiology, 59*, e02438–20. https://doi.org/10.1128/JCM.02438-20

Ulrich, L., Wernike, K., Hoffmann, D., Mettenleiter, T. C., & Beer, M. (2020). Experimental infection of cattle with SARS-CoV-2. *Emerging Infectious Diseases, 26*, 2979–2981. https://doi.org/10.3201/eid2612.203799

Villanueva-Saz, S., Giner, J., Fernandez, A., Lacasta, D., Ortin, A., Ramos, J. J., Ferrer, L. M., Ruiz de Arcaute, M., Tobajas, A. P., Perez, M. D., Verde, M., Marteles, D., Hurtado-Guerrero, R., Pardo, J., Santiago, L., Gonzalez-Ramirez, A. M., Macias-Leon, J., Garcia-Garcia, A., Taleb, V., Lira-Navarrete, E., Pano-Pardo, J. R., & Ruiz, H. (2021). Absence of SARS-CoV-2 antibodies in natural environment exposure in sheep in close contact with humans. *Animals : An Open Access Journal from MDPI, 11*, 1984. https://doi.org/10.3390/ani11071984

Villanueva-Saz, S., Giner, J., Palomar, A. M., Gomez, M. A., Podra, M., Aranda, M. D. C., Jimenez, M. L. A., Lizarraga, P., Hernandez, R., Portillo, A., Oteo, J. A., Ruiz-Arrondo, I., Perez, M. D., Tobajas, A. P., Verde, M., Lacasta, D., Marteles, D., Hurtado-Guerrero, R., Santiago, L., Ruiz, H., & Fernandez, A. (2022). No evidence of SARS-CoV-2 infection in wild mink (Mustela lutreola and Neogale vison) from Northern Spain during the first two years of pandemic. *Animals : An Open Access Journal from MDPI, 12*, 1971. https://doi.org/10.3390/ani12151971

Wei, J., Matthews, P. C., Stoesser, N., Maddox, T., Lorenzi, L., Studley, R., Bell, J. I., Newton, J. N., Farrar, J., Diamond, I., Rourke, E., Howarth, A., Marsden, B. D., Hoosdally, S., Jones, E. Y., Stuart, D. I., Crook, D. W., Peto, T. E. A., Pouwels, K. B., Walker, A. S., Eyre, D. W., & team, C.-I. S (2021). Anti-spike antibody response to natural SARS-CoV-2 infection in the general population. *Nature Communications, 12*, 6250. https://doi.org/10.1038/s41467-021-26479-2

Wensman, J. J., & Stokstad, M. (2020). Could naturally occurring coronaviral diseases in animals serve as models for COVID-19? A review focusing on the bovine model. *Pathogens (Basel, Switzerland), 9*, 991. https://doi.org/10.3390/pathogens9120991

Wernike, B. J., Amelung, S., Albrecht, K., Gärtner, T., Donat, K., & M, B. (2022). Serological screening suggests single SARS-CoV-2 spillover events to cattle. *BioRxiv : the Preprint Server for Biology*. https://doi.org/10.1101/2022.01.17.476608

White, R. J., & Razgour, O. (2020). Emerging zoonotic diseases originating in mammals: A systematic review of effects of anthropogenic land-use change. *Mammal Review, 50*, 336–352. https://doi.org/10.1111/mam.12201

WHO. (2024). Coronavirus disease (COVID-19) weekly epidemiological update and weekly operational update. https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports. Accessed 12 April 2024.

WOAH. SARS-CoV-2 in Animals -Situation Report 22. SARS-CoV-2 in Animals. https://www.woah.org/app/uploads/2023/07/sars-cov-2-situation-report-22.pdf.

Woo, P. C., Lau, S. K., Lam, C. S., Lau, C. C., Tsang, A. K., Lau, J. H., Bai, R., Teng, J. L., Tsang, C. C., Wang, M., Zheng, B. J., Chan, K. H., & Yuen, K. Y. (2012). Discovery of seven novel mammalian and avian coronaviruses in the genus deltacoronavirus supports bat coronaviruses as the gene source of alphacoronavirus and betacoronavirus and avian coronaviruses as the gene source of gammacoronavirus and deltacoronavirus. *Journal of Virology, 86*, 3995–4008. https://doi.org/10.1128/JVI.06540-11

Wu, W., Cheng, Y., Zhou, H., Sun, C., & Zhang, S. (2023). The SARS-CoV-2 nucleocapsid protein: Its role in the viral life cycle, structure and functions, and use as a potential target in the development of vaccines and diagnostics. *Virology Journal, 20*, 6. https://doi.org/10.1186/s12985-023-01968-6

Zhang, Z., Andrews, G. A., Chard-Bergstrom, C., Minocha, H. C., & Kapil, S. (1997). Application of immunohistochemistry and in situ hybridization for detection of bovine coronavirus in paraffin-embedded, formalin-fixed intestines. *Journal of Clinical Microbiology, 35*, 2964–2965. https://doi.org/10.1128/jcm.35.11.2964-2965.1997

Zost, S. J., Gilchuk, P., Case, J. B., Binshtein, E., Chen, R. E., Nkolola, J. P., Schafer, A., Reidy, J. X., Trivette, A., Nargi, R. S., Sutton, R. E., Suryadevara, N., Martinez, D. R., Williamson, L. E., Chen, E. C., Jones, T., Day, S., Myers, L., Hassan, A. O., Kafai, N. M., Winkler, E. S., Fox, J. M., Shrihari, S., Mueller, B. K., Meiler, J., Chandrashekar, A., Mercado, N. B., Steinhardt, J. J., Ren, K., Loo, Y. M., Kallewaard, N. L., McCune, B. T., Keeler, S. P., Holtzman, M. J., Barouch, D. H., Gralinski, L. E., Baric, R. S., Thackray, L. B., Diamond, M. S., Carnahan, R. H., & Crowe, J. E., Jr. (2020). Potently neutralizing and protective human antibodies against SARS-CoV-2. *Nature, 584*, 443–449. https://doi.org/10.1038/s41586-020-2548-6