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DETECTION OF SARS-COV-2 NEUTRALIZING ANTIBODIES IN RETROPHARYNGEAL LYMPH NODE EXUDATES OF WHITE-TAILED DEER (*ODOCOILEUS VIRGINIANUS*) FROM NEBRASKA, USA

Korakrit Poonsuk[1,6], **Duan Loy**[1], **Rachael Birn**[2,3], **Bryan Buss**[2,4], **Matthew Donahue**[2], **Todd Nordeen**[5], **Kylie Sinclair**[5], **Luke Meduna**[5], **Bruce Brodersen**[1], **John Dustin Loy**[1]

[1]University of Nebraska–Lincoln, Nebraska Veterinary Diagnostic Center, School of Veterinary Medicine and Biomedical Sciences, 4040 E. Campus Loop N, Lincoln, Nebraska 68503, USA

[2]Division of Public Health, Nebraska Department of Health and Human Services, 301 Centennial Mall S, Lincoln, Nebraska 68508, USA

[3]Council State and Territorial Epidemiologists, 2635 Century Pkwy NE no. 700, Atlanta, Georgia 30345, USA

[4]Division of State and Local Readiness, Center for Preparedness and Response, Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, Georgia 30329, USA

[5]Nebraska Game and Parks Commission, 2200 N. 33rd St., Lincoln, Nebraska 68503, USA

Abstract

Disease surveillance testing for emerging zoonotic pathogens in wildlife is a key component in understanding the epidemiology of these agents and potential risk to human populations. Recent emergence of SARS-CoV-2 in humans, and subsequent detection of this virus in wildlife, highlights the need for developing new One Health surveillance strategies. We used lymph node exudate, a sample type that is routinely collected in hunter-harvested white-tailed deer (WTD, *Odocoileus virginianus*) for surveillance of chronic wasting disease, to assess anti-SARS-CoV-2 neutralizing antibodies. A total of 132 pairs of retropharyngeal lymph nodes collected from Nebraska WTD harvested in Nebraska, US, in 2019 (pre–SARS-CoV-2 pandemic) and 2021 (post–SARS-CoV-2 pandemic) were tested for SARS-CoV-2 with reverse transcription PCR. Thereafter, exudates obtained from these same lymph nodes were tested for SARS-CoV-2 neutralizing antibodies using a surrogate virus neutralization test. Neutralizing antibodies were detected in the exudates with high diagnostic specificity (100% at proposed cutoff of 40% inhibition). Application of this testing approach to samples collected for use in other disease surveillance activities may provide additional epidemiological data on SARS-CoV-2 exposure, and there is further potential to apply this sample type to detection of other pathogens of interest.

[6]Corresponding author (poonsuk@unl.edu).

SUPPLEMENTARY MATERIAL

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Keywords

Lymph node; neutralizing antibody; SARS-CoV-2; serology

INTRODUCTION

Natural infections with SARS-CoV-2 have been reported in dogs (*Canis lupus familiaris*), cats (*Felis catus*), mink (*Mustela lutreola*), tigers (*Panthera tigris*), lions (*Panthera leo*), snow leopards (*Panthera uncia*), gorillas (*Gorilla gorilla*), otters (*Lontra canadensis*), spotted hyena (*Crocuta crocuta*), mule deer (*Odocoileus hemionus*), and white-tailed deer (WTD, *Odocoileus virginianus*) in several regions, including the Netherlands, England, China, Hong Kong, and the US (Gibbons 2021; McAloose et al. 2020; Oreshkova et al. 2020; Sit et al. 2020; Wang et al. 2020; Chandler et al. 2021; Holding et al. 2022; Palermo et al. 2022; Qiu et al. 2022). Among these species, WTD have demonstrated susceptibility to infection with transmissibility both in experimental conditions (Palmer et al. 2021) and among the wild WTD population (Kuchipudi et al. 2022). Previous studies have hypothesized that these deer may act as reservoirs and may play a role in driving the variation in SARS-CoV-2 strains (Du et al. 2022; Mallapaty 2022). Deer-to-human transmission of SARS-CoV-2 has been confirmed (Pickering et al. 2022), further emphasizing the need to study possible wildlife reservoirs, including WTD, to better understand the ecology of the virus. Therefore, surveillance of SARS-CoV-2 infections in WTD is a priority for proactive detection of possible reverse zoonosis, given the identification of multiple circulating lineages and detection rates as high as 40% in some studies (Chandler et al. 2021).

While molecular-based methods are widely used to identify emerging and re-emerging SARS-CoV-2 in wildlife, antibody-based serological testing is used primarily to detect historical infections, due to an extended period of circulating antibodies detectable in serum (Mercer and Salit 2021). In captive WTD, SARS-CoV-2–specific antibodies were detectable for up to 13 mo postexposure (Hamer et al. 2021). Thus, serological testing provides a viable alternative for surveillance of SARS-CoV-2 in deer populations to assess exposure, as detection of antibodies indicating prior infection may persist longer than detection of the virus itself.

Serum was the specimen of choice for SARS-CoV-2 serological surveillance conducted by the United State Department of Agriculture (USDA)/Animal and Plant Health Inspection Service (APHIS)/Wildlife Services National Wildlife Disease Program (NWDP) in 2021 (Chandler et al. 2021). Although serum can be collected from blood of farmed deer or zoo animals, this specimen type is difficult to collect from post-mortem samples, such as hunter-harvested animals. Blood clotting and hemolysis occur soon after death, making serum specimen collection from a carcass unfeasible. Surveillance programs already exist in many states that use lymph node sampling to detect presence of prion proteins in WTD populations and thus provide a sample option with demonstrated use for PCR-based virus detection. Alternative specimens, e.g., meat exudate (meat juice), have been used for antibody-based serosurveillance of several viral diseases (Fabisiak et al. 2013; Ivanova et al. 2015; Poonsuk et al. 2018; Onyilagha et al. 2021). Similar to meat exudate, lymph node

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exudate contains water, enzymes, amino acids, intracellular fluid, extracellular fluid, blood, and lymph. Concentrations of antibodies within these exudates are probably lower than in serum, but they are detectable and provide sufficient diagnostic sensitivity and specificity. In addition, a previous study detected SARS-CoV-2 nucleic acids in retropharyngeal lymph nodes (RLNs) of WTD, indicating that such specimens may be used for SARS-CoV-2 surveillance to expand disease surveillance beyond just chronic wasting disease (CWD; Kuchipudi et al. 2022). The objective of this study was to validate the detection of SARS-CoV-2 neutralizing antibodies in lymph node exudates from WTD as a viable surveillance method.

MATERIALS AND METHODS

Sample source and collection

Retropharyngeal lymph node (RLN) samples were collected from free-ranging, hunter-harvested WTD as part of annual CWD surveillance organized by the Nebraska Game and Parks Commission (NGPC) during the 2019 and 2021 November firearm hunting seasons. Sample collection sites were established throughout the state of Nebraska, US. Sample collectors wore appropriate personal protective equipment, including gloves, face mask or properly fitted filtering facepiece respirator, and disposable laboratory coats, during the collection of the specimens. The RLNs, located in the upper part of the neck behind the pharynx, were removed from submitted WTD by trained NGPC staff who coded them with an identification number and documented location of animal harvest. A pair of RLNs from each animal were placed in a plastic bag, refrigerated, and sent to the University of Nebraska–Lincoln, Nebraska Veterinary Diagnostic Center, Lincoln, Nebraska. From the submitted lymph nodes, 200 mg were cut off for the CWD ELISA. The leftover portions of the pairs of lymph nodes were kept frozen at −80°C. A process chart of this study is shown in Figure 1.

RNA extraction and RT-qPCR for SARS-CoV2 detection

For each deer, 2–3 punches were taken from a RLN using a 6 mm disposable dermal biopsy punch (Medline, Northfield, Illinois, USA) and placed into a sterile 2 mL tube containing 1 mL of phosphate-buffered saline. Stainless steel beads were added to the tube, and tissue was disrupted using a sample disrupter (TissueLyser II, Qiagen, Hilden, Germany) for 2 min at 18 Hz. Then homogenate was transferred to a 1.5 mL tube and centrifuged at 1,000 × G for 2–3 min to clarify the homogenate. We extracted samples using the Mag-MAX™ Pathogen RNA/DNA Kit (ThermoFisher Scientific, Waltham, Massachusetts, USA) in 96 well plates using a benchtop automated extraction instrument (KingFisher Flex, Thermo Fisher Scientific, Vantana, Finland) using the manufacturer's recommended high-volume extraction protocol.

We used a multiplexed real-time reverse transcription (RT-PCR) method based on the 2019-nCoV CDC primer and probe sequences targeting the N1 and N2 genes, slightly modified and validated for use in animal samples (CDC 2019). Modifications included the N1 probe being labeled with FAM dye and BHQ1 quencher (Integrated DNA Technologies, Coralville, Iowa, USA). The N2 probe was labeled with Cy5 dye and BHQ2 quencher

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(Integrated DNA Technologies). The VetMAX Xeno Internal Positive Control (IPC)–VIC (ThermoFisher Scientific) was used as an internal control per manufacturer's instructions. The assay included 25 µL master mix, which contained 7.75 µL of nuclease-free water, 6.25 µL TaqMan™ Fast Virus 1-Step Master Mix (ThermoFisher Scientific), 1 µL of each primer and probe mix, and 8 µL of the template. The multiplex reactions contained N1 and N2 primers and probes at final concentrations of 0.3 and 0.1 µM, respectively. The multiplex assay was performed using an ABI 7500 FAST Realtime PCR system (ThermoFisher Scientific). The RT-qPCR cycling conditions were 50 C for 5 min and 95 C for 20 s, followed by 45 cycles at 95 C for 3 s and 55 C for 30 s. Samples were normalized using instrument settings based on the passive reference dye signal, and those with cycle threshold (Ct) values ≤37 for N1 and N2 were considered positive.

Retropharyngeal lymph node exudate collection

We randomly selected 131 pairs of RLNs, obtained from 40 WTD in 2019 and 91 WTD in 2021, for exudate collection. The 40 pairs of RLNs from WTD harvested in 2019 were considered SARS-CoV-2 antibody-negative specimens for diagnostic specificity evaluation. For lymph nodes collected in 2021, 52 and 39 pairs were randomly selected respectively from among the RT-PCR-positive and -negative groups to identify seroconversion and thus possible exposure to SARS-CoV-2 in the sampled WTD. The lymph nodes were subjected to two freeze-thaws at −80 C and room temperature (21–25 C), 2 h each cycle. Exudate that accumulated within the sample bag (approximately 0.5–1.5 mL per pair of lymph nodes) was collected using a sterile micropipette, transferred to a cryogenic tube (Nalgene Nunc, Rochester, New York, USA), and maintained at 4 C until use.

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

We tested the RLN exudate using a SARS-CoV-2 surrogate virus neutralization test (sVNT) kit (Genscript, Piscataway, New Jersey, USA). This test kit is a virus neutralization-based competitive ELISA designed to detect neutralizing antibodies against a receptor binding domain on the SARS-CoV-2 virus strain BJ01 (GenBank accession no. AY278488.2; Wang et al. 2020). The test was performed using the procedure described by the manufacturer with some minor modifications. In brief, 30 µl RLN exudate was aliquoted into each well of polypropylene predilution plates (Corning, Corning, New York, USA) and then mixed with 30 µL (1:1) of sample dilution buffer. The diluted lymph node extract samples and kit positive and negative controls were then mixed 1:1 with 1:1,000 diluted HRP-conjugated RBD solution, before being sealed and incubated at 37 C for 30 min to allow for the neutralization reaction. After the first incubation, the mixture was transferred to angiotensin converting enzyme 2-coated assay microtiter plates (Genscript, Piscataway, New Jersey, USA), sealed, and incubated at 37 C for 15 min. The assay plate was then washed four times with 260 µL 1X wash solution, then 100 µL of 3,3′,5,5′ tetramethylbenzidine solution was added to each well. After 5 min incubation at 25 C in the dark, 50 µL of stop solution was added to each well. Reactions were measured as optical density at 450 nm using an ELISA plate reader (Biotek Instruments, Winooski, Vermont, USA).

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The test cutoff was selected based on range of the 2019 SARS-CoV-2 negative samples. The SARS-CoV-2 surrogate virus neutralization test results were represented as percent inhibition calculated using a formulation as follows:

$$\% \text{ inhibition} = [1 - (\text{OD value of sample}/\text{mean OD value of negative control})] \times 100$$

Statistical analysis

Data distribution, percent positive of each group, and correlation coefficient of the RT-PCR Ct and sVNT percent inhibition were determined. All calculations were carried out using commercial statistical software (MedCalc 20.109, MedCalc Software, Ostend, Belgium).

RESULTS

The RLNs from 500 deer harvested in 2019 and 489 harvested in 2021 were sampled. The percentage inhibition of the 2019 RLN exudates collected before SARS-CoV-2 was detected in the US ranged between −3.43% and 39.49% with mean percent inhibition 15.91% (95% confidence interval [CI]=11.98–19.84%). A test cutoff at 40% was chosen to aim for 100% diagnostic specificity. Among the 52 PCR-positive samples chosen from among those collected from WTD in 2021, percent inhibition ranged from 0.83% to 94.56%, mean 72.74% (95% CI 64.99–80.48%). Although the SARS-CoV-2 RT-PCR results were correlated between N1 and N2 Ct values among positive lymph nodes (correlation coefficient r=0.99, P<0.0001), no correlation existed between the percent inhibition and either RT-PCR Ct among the RT-PCR-positive samples (N1 correlation coefficient r=−0.12, P=0.44 and N2 correlation coefficient r=−0.07, P=0.65). Based on the cutoff of 40% inhibition, 85% (44/52) of samples within this group were seropositive to SARS-CoV-2. Lymph node exudate specimens from the SARS-CoV-2 RT-PCR-negative samples collected in 2021 during the pandemic had percent inhibition that ranged between 14.08% and 94.08%, mean 43.68% (95% CI 35.30–52.06%). Of note, 36% (14/39) of these samples were seropositive to SARS-CoV-2 at the cutoff of 40%, potentially indicating high level of exposure in these populations (Fig. 2). Sample-specific information is provided in the Supplementary Table.

DISCUSSION

Our study reports serological evidence of pathogen-specific antibodies detected in lymph node exudates of WTD. Similar to meat exudate, the transudate produced as frozen lymph node undergoes the process of freeze-thawing contains intracellular fluid, extracellular fluid, blood, and lymph (Wallander et al. 2015). We found that the antibodies within these fluids were detectable using the SARS-CoV-2 sVNT with desirable diagnostic specificity. Although not tested in this study, it is worth noting that while the sVNT approach enables investigation of antibody neutralization at lineage specific levels using different surrogate virus receptor binding domains, there can be considerable cross-reactivity between lineages, which may limit the specificity of serologic variant typing. However, lymph nodes exudate might also be used to investigate neutralization potential across different lineages, which

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may enable further study of immune responses and diversity of SARS-COV2 in wildlife even in the absence of actively circulating virus detectable by RT-PCR.

Because the specimens used in this study were collected from wild, free-ranging WTD, the actual time of exposure to SARS-CoV-2 among the sample population is unknown. Our detection of antibodies in 85% of the RT-PCR-positive RLNs suggests that this method can be used to detect antibodies during active infection in WTD. In addition, as antibodies were detectable in the lymph node exudates collected from SARS-CoV-2 RT-PCR-negative samples, our results indicate SARS-CoV-2-specific antibody persistence after infection had been cleared. This finding is consistent with a previous report of antibodies being detectable in WTD for a long period (Hamer et al. 2021). However, there was nonspecific signal detected as percent inhibition as determined by the 2019 samples, and the suggested cutoff of 40% may not be applicable outside this sample set, which is limited in diversity across space and time. Because of resource constraints, a limited subset of samples was used for test development in our study. Although a larger sample size would have been ideal, the samples that we used were carefully selected and deemed sufficient for the objective of verifying the efficacy of the novel test method. Additional samples of known SARS-CoV-2 infection status should be tested to further establish the baseline cutoff to enhance accuracy.

Paired samples of serum and lymph node exudate from animals of known infection status would be needed to determine the performance of this assay in comparison with other immunodiagnostic techniques. Data from our study suggest that lymph node exudate specimens may be useful for SARS-CoV-2 immunosurveillance among WTD populations. Although not tested in this study, it is worth noting that the GenScript sVNT assay is isotype-independent, enabling detection of both IgG and IgM antibodies. This is an advantage over serological assays that focus solely on IgG antibodies, as IgM is generally more detectable at early time points in an infection.

White-tailed deer are one of the most abundant wildlife species in North America. As populations have expanded and more deer inhabit rural and urban human communities, possible spillback of SARS-CoV-2 infection from infected WTD to humans or transmission to other domestic and wildlife species has been theorized (Palermo et al. 2022). As a precaution to a possible SARS-CoV-2 zooanthroponosis to WTD, USDA/APHIS/NWDP have been conducting SARS-CoV-2 serosurveillance programs in wildlife and domestic animals (Chandler et al. 2021). As previously mentioned, serum samples are best collected from living animals, but collection of serum from live wild animals is time- and resource-intensive and either quite challenging or entirely precluded. Lymph nodes collected in routine, ongoing post-mortem–based disease surveillance in deer, particularly for CWD surveillance, offer a suitable sampling alternative. Lymph node exudates may be used concurrently with CDW surveillance for SARS-CoV-2 immunosurveillance within the same animal populations. Given the convenience, this approach should be given consideration for expanded post-mortem SARS-CoV-2 antibody testing in WTD. Use of lymph node exudates as a sample type should also be evaluated for broader antibody-based surveillance of other zoonotic wildlife pathogens, potentially enabling large numbers of samples to be tested serologically using existing surveillance programs where lymph nodes are collected.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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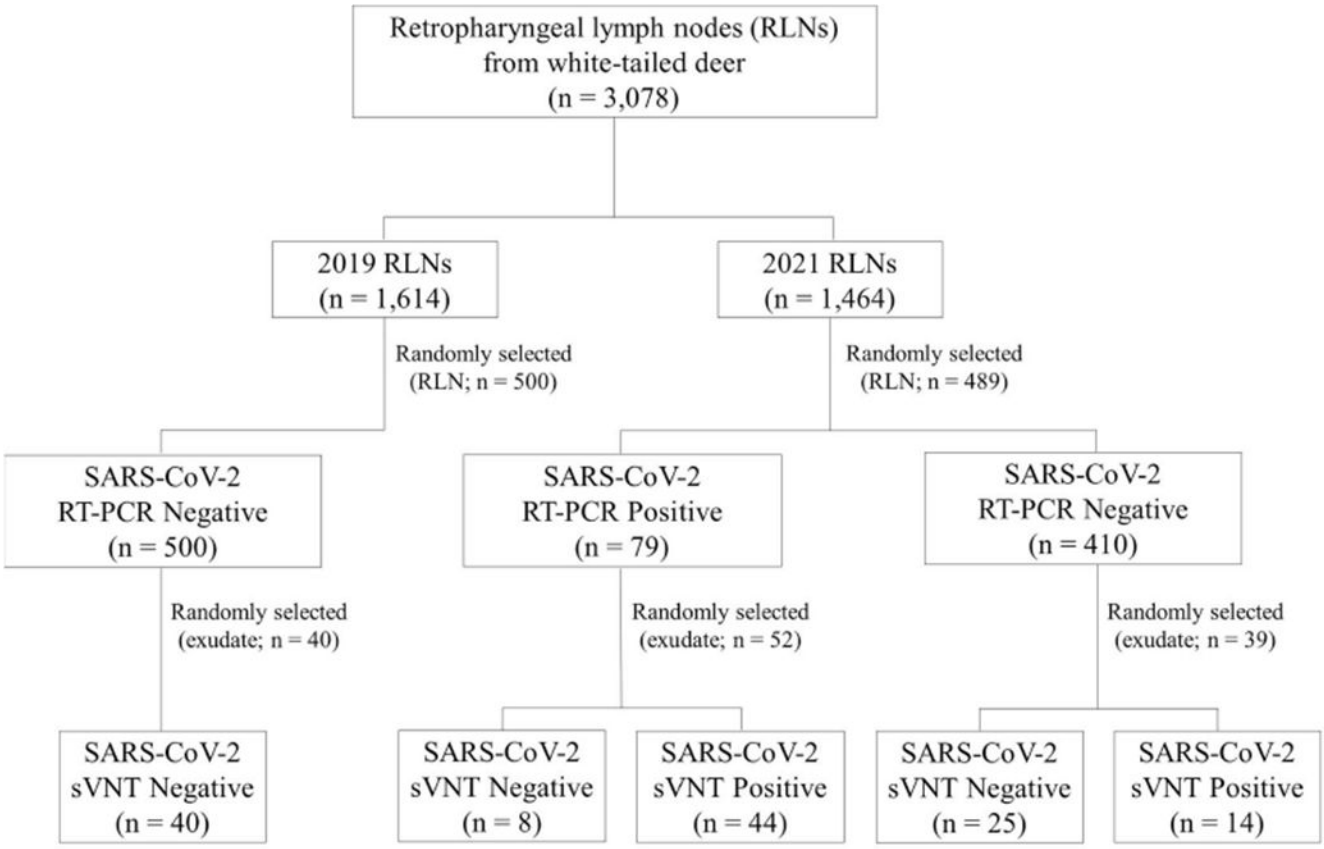

Figure 1.

Process chart of sample selection and testing performed to demonstrate the detection of SARS-CoV-2 antibodies in lymph node exudates from SARS-CoV-2 reverse transcription PCR-negative and -positive white-tailed deer (*Odocoileus virginianus*) samples collected in Nebraska, US, in 2019 to 2021.

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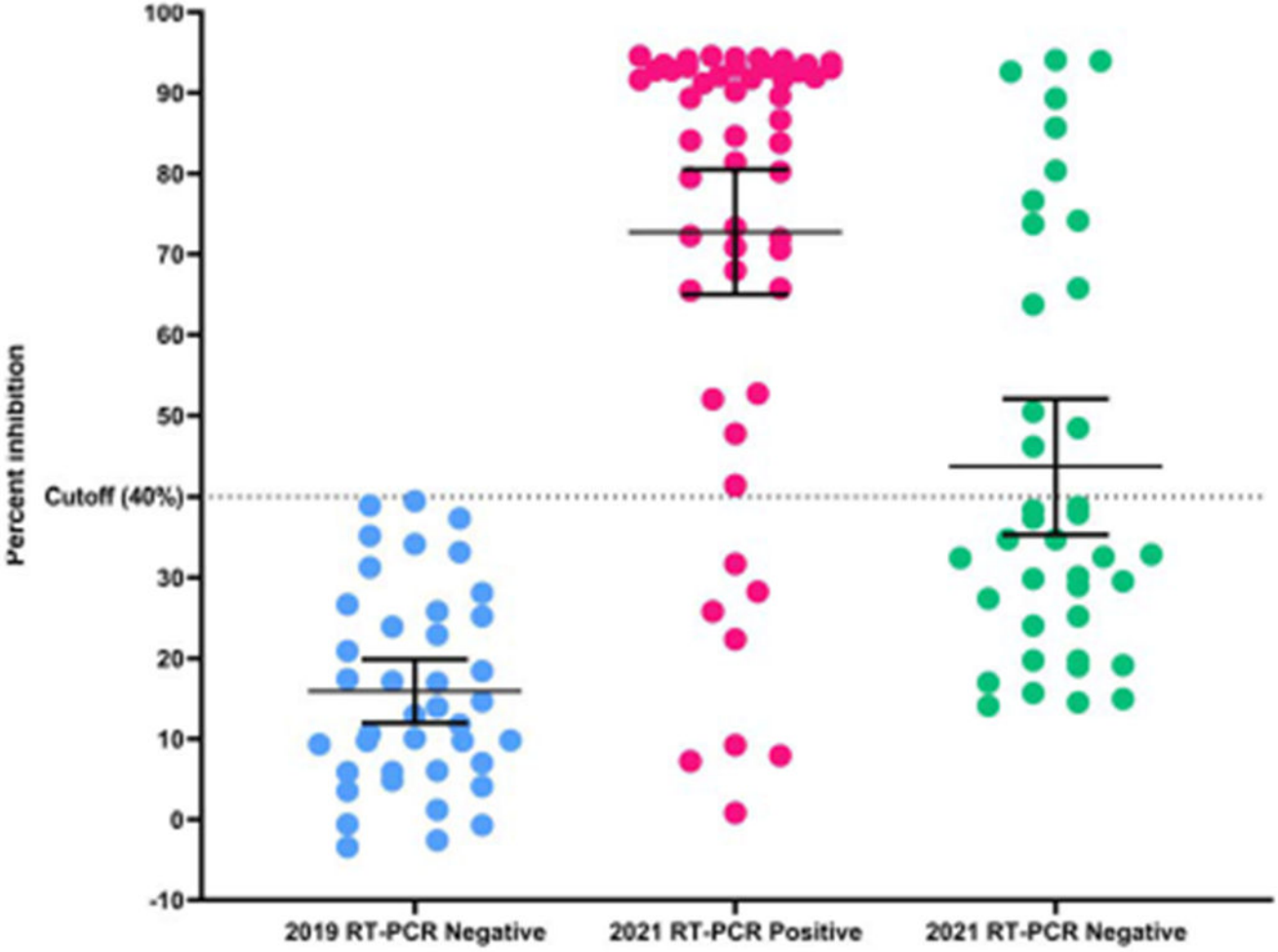

Figure 2.

Scatter plot of SARS-CoV-2 serological results for white-tailed deer (*Odocoileus virginianus*) lymph node exudates collected in Nebraska, US, in 2019 and in 2021 (both reverse transcription PCR (RT-PCR)-positive and RT-PCR-negative samples in 2021 and tested with the surrogated virus neutralization test (sVNT–a virus neutralization-based competitive ELISA). Each dot represents individual sample percent inhibition result; the horizontal bars show the mean (long bar) and upper and lower 95% confidence intervals (short bars).