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SARS-CoV-2 surveillance in a veterinary health system provides insight into transmission risks

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OBJECTIVE
To investigate the prevalence and seropositivity of SARS-CoV-2 in companion and exotic animals in a veterinary healthcare system.

SAMPLE
A total of 341 animals were sampled by a combination of oral and nasal swabs. Serum from whole blood was collected from a subset of animals (86 canines, 25 felines, and 6 exotic animals).

METHODS
After informed owner consent, convenience samples from client-owned animals and the pets of students and staff members associated with Colorado State University's Veterinary Health System were collected between May 2021 and September 2022. Study samples were collected by trained veterinarians, Veterinary Health System staff, and veterinary students.

RESULTS
SARS-CoV-2 RNA was detected by reverse transcription PCR in 1.6% (95% CI, 0.5% to 4.6%) of domestic canines and 1.1% (95% CI, 0.2% to 6.1%) of domestic felines. No RNA was detected in any of the exotic animal species tested (n = 66). Plaque reduction neutralization tests indicated that 12.8% (95% CI, 7.3% to 21.5%) of canines and 12.0% (95% CI, 4.2% to 30.0%) of felines had neutralizing antibodies against SARS-CoV-2.

CLINICAL RELEVANCE
This study provides insight regarding SARS-CoV-2 spillover in domestic companion and exotic animals and contributes to our understanding of transmission risk in the veterinary setting.

Keywords: SARS-CoV-2, animal health, exotic animals, zoonotic disease, surveillance

Wild and domestic animals are known to be susceptible to SARS-CoV-2, and transmission from infected animals to humans has been reported.[1] Companion animals (eg, domestic canines and felines and other exotic mammal species) have received notable attention as potential sources of transmission given their ubiquitous distribution and close peridomestic living conditions. A 2016 internet survey of more than 27,000 users (age ≥ 15) across 22 countries revealed that approximately 57% of respondents had pets in their household.[2] Canines and felines were reported in 33% and 23% of surveyed households, respectively. Pet ownership has increased in recent years, and it is currently estimated that there are likely over a billion pets worldwide.[3,4] Pet ownership increased drastically during the

COVID-19 pandemic, a phenomenon that may come to yield both positive (eg, increased companionship) and uncertain (eg, increased disease transmission risk) outcomes.[5,6]

Veterinary professionals have an increased risk of exposure to zoonotic disease, especially during infectious disease outbreaks.[7–9] In the case of SARS-CoV-2, concern for transmission was exceptionally high given that both humans and animals are susceptible and may shed infectious virus asymptomatically.[10,11] Although biosafety and biosecurity are commonplace in veterinary medicine, different circumstances necessitate various levels of personal protective equipment and adherence to standard operating procedures. Implementation of appropriate policy changes to combat novel pathogens requires epidemiological data to identify instances of increased transmission. Even now, after research effort has focused on the threat of SARS-CoV-2 in various population demographics, an incomplete understanding of the transmission risk at the human-animal interface remains.

To help address the occupational risk of exposure to SARS-CoV-2 for veterinary professionals, we conducted a case identification study with convenience sampling within Colorado State University's (CSU) Veterinary Health System (VHS) in Fort Collins, Colorado, including the small animal teaching hospital and clinical diagnostic services. Nasal and oral swabs were collected over a 17-month period from companion and exotic animals associated with the CSU VHS community. Serum from whole blood was also collected to determine seropositivity rates. The study results contribute to the overall understanding of SARS-CoV-2 risk in companion and exotic animals, including the risk of transmission from pets to humans in a veterinary setting.

Methods

Ethics statement

Methods in this study were approved by the CSU Veterinary Teaching Hospital Clinical Review Board (Protocol No. 1080: SARS-CoV2 Pet Surveillance). Informed consent was obtained from animal owners in person or over the telephone prior to sample collection. Owners were offered copies of the consent form as well as an informational handout for SARS-CoV-2 testing guidelines. All samples were deidentified prior to testing, and test results were not provided to animal owners.

Study population and design

In this case identification study with convenience sampling, samples were collected from companion and exotic animals that presented to multiple services or were associated with the CSU VHS in Fort Collins, CO, between May 2021 and September 2022. A power calculation using a 95% CI (Z), 5% precision (d), and a prevalence of 14% (P) in the formula $n = (Z^2) X P X (1 - P)/(d^2)$, estimated sample size (n) is 186. Prevalence was extrapolated from early SARS-CoV-2 surveillance and experimental studies.[12,13] Since prevalence is unknown in some of the species presenting to the hospital, sample number was maximized on the basis of available resources, leading to a total possible sample size (n) of 341 individual animals. Animals sampled encountered veterinary health professionals within the CSU VHS, including patients being seen for routine or specialized referral care and pet animals owned by VHS staff. Enrolled animals were not specifically presenting for complaints related to concerns for SARS-CoV-2 infection, as those pets were considered for individual diagnostic testing. Healthy and ill pets that presented to the hospital or VHS veterinarian for any other reason and without selection by clinical signs were enrolled with the goal of representing a pet population that would opportunistically interact with veterinary health professionals. Sample collection kits (see below) were provided ahead of time and stored at 4 °C until use. Individuals conducting sample collections were advised on best collection practices and instructed to keep samples in a supplied foam cooler with ice packs during storage and transport.

Animal sample collections

Sample collection kits containing the following items were distributed to trained staff members of several CSU VHS services and to consented veterinary students and VHS staff members: 1 FLOQSwab (COPAN Diagnostics) for nasal collections, 1 cotton spun swab (SteriPack) for oral collections, 2 (2-mL) vials of viral transport medium (VTM), 1 serum blood collection tube, a study consent form, and a sample collection form. VTM was prepared using Dulbecco's Modified Eagle Medium (Corning Inc) supplemented with 2% fetal bovine serum (Peak Serum), 1 mL/500 mL of L-glutamine (Corning), 50 U/mL penicillin (ATCC), and 50 g/mL streptomycin (ATCC). Aliquots of VTM were stored at –20 °C until use.

Samples were collected only if tolerated by the animal. Up to 3 samples (oral swab, nasal swab, and/or serum sample) were collected from each enrolled animal in the services of Community Practice, Oncology, Small Animal Internal Medicine, Blood Bank, Dentistry and Oral Surgery, Clinical Pathology, Dermatology and Otology, and Avian, Exotic, and Zoological Medicine. Veterinary students and VHS staff members from other services also provided swabs from their own animals. VHS staff were already competent in general biological sampling and handling and were given clear sampling instructions by a trained individual. A FLOQSwab was used to collect nasal samples by gently rubbing the tip of the swab on the exterior and most rostral portion of the animal's nose and nares. A cotton swab was used to collect an oral sample by gently rubbing the swab on the gingiva and buccal mucosa **(Figure 1)**. After sample collection, each swab was immediately inserted into a separate prelabeled screw-top vial containing VTM. The stem of each swab was broken off so that the vial could be closed securely with the swab inside. Opportunistic and residual blood samples from consented animals were collected if available

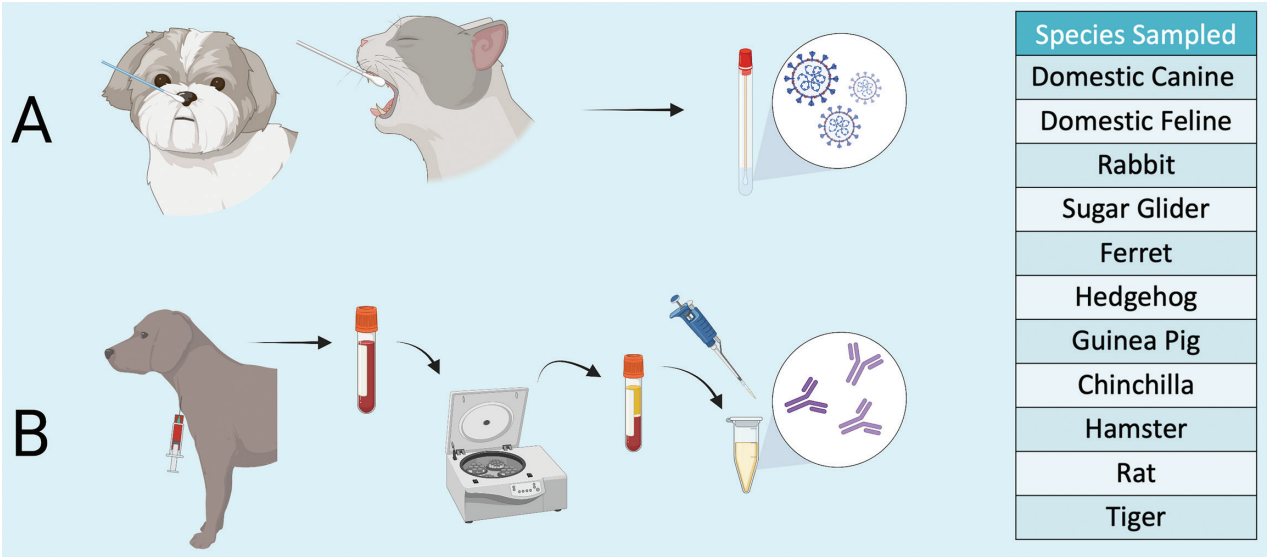

Figure 1—Illustration depicting sample collection and processing supplemented by a representative table of all species sampled in this study. A—Oral and nasal sample collection for viral RNA detection. B—Whole blood collection followed by processing and storage of serum-containing antibodies. Created with BioRender.com.

and tolerated by the animal. Collected samples were placed into the provided bag and kept refrigerated or on ice packs until they were deidentified and processed, usually within 12 hours of collection. Collected whole blood samples were centrifuged at 1,500 X *g* for 5 minutes, and the serum was then removed and aliquoted. All collected samples were stored at –80 °C until further testing.

SARS-CoV-2 RNA extraction and detection by reverse transcription PCR

Total RNA was extracted from nasal and oral swabs using a MagMAX Viral/Pathogen Nucleic Acid Isolation kit and KingFisher Flex magnetic particle processor (Thermo Fisher Scientific Inc) per the manufacturer's instructions. Reverse transcription PCR (RT-PCR) was carried out using the TaqPath 1-Step Multiplex Master Mix in combination with the TaqPath COVID-19 Combo kit (Thermo Fisher Scientific Inc), which includes assay primers and probes, a negative extraction control, and a SARS-CoV-2 positive amplification control. This kit was approved under Emergency Use Authorization and includes primers that detect genomic regions of the ORF1ab, N, and S genes. All reaction plates included a negative reaction control (no template control). RT-PCR reactions were conducted with an Applied Biosystems 7500 Fast thermocycler (Thermo Fisher Scientific Inc).

Amplification results were reviewed using the system's companion software and considered positive only when at least 2 targets produced a cycle threshold (Ct) value below 37.0 with a sigmoidal curve. Samples that produced positive amplification for 2 or 3 targets with at least 1 Ct value above 37.0 were classified as inconclusive. Samples with positive amplification for only 1 target, regardless of Ct value, were also considered inconclusive. Any samples that were positive by the above criteria or showed late Ct values for at least 1 target were retested for validity.

Antibody detection by plaque reduction neutralization test

The production of neutralizing antibodies was determined by a plaque reduction neutralization test (PRNT). Briefly, serum was first heat inactivated for 30 minutes at 56 °C in a water bath. Serum samples were diluted 2-fold in VTM beginning with a 1:5 dilution on a 96-well plate. An equal volume of SARS-CoV-2 virus (isolate USA-WA1/2020; BEI Resources NR-52281) equivalent to 100 plaque-forming units/well was added to the serum dilutions, and the sample-virus mixture was gently mixed. Plates were incubated for 1 hour at 37 °C. Following incubation, serum-virus mixtures were plated with Vero E6 cells as described for virus plaque assays.[14] Our protocol tested samples starting at a 1:10 dilution. Antibody titers detected at 1:10 or higher dilution were considered positive. Antibody titers were recorded as the highest dilution in which > 80% of virus was neutralized.

Next-generation sequencing and bioinformatic analysis

Two RT-PCR positive canine swabs (1 nasal and 1 oral) were selected for next-generation sequencing. The Superscript IV First Strand Synthesis System (Invitrogen; Thermo Fisher Scientific Inc) was used to synthesize cDNA from RNA. A reverse transcriptase reaction using random hexamers was performed on extracted RNA following the kit protocol. The incubation step for the annealed RNA with the reverse transcriptase reaction mix was modified to 50 °C for 30 minutes. Subsequent PCR amplification was performed per the kit protocol using the manufacturer's recommended cycle settings for low viral input. Libraries were prepared using the Swift Normalase Amplicon Panel kit

(Swift Biosciences Inc) and purified using AMPure XP beads (Beckman Coulter Inc). Paired-end sequencing (2 X 150 bp) was performed using a NextSeq 500/550 Mid Output kit version 2.5 (300 cycles) on a NextSeq 500 sequencer (Illumina Inc). Sequence analysis was conducted using the nf-core/viralrecon pipeline (version 2.5; nf-core) with default parameters.[15] Preprocessing in this pipeline includes quality assessment by FastQC (version 0.11.9), adapter trimming by Fastp (version 0.23.2), and primer removal by Cutadapt (version 3.5). De novo assembly was performed using SPAdes (version 3.15.4). Contigs were aligned to the Wuhan-Hu-1 reference strain (NC_045512.2) in Geneious (version 2023.0.4). Assembled sequences were assessed for lineage using the Nextclade tool (version 2.11.0; Nextclade) and cross-referenced using PanGUIlin (version 4.2; Pangolin).[16] Finally, mutational analysis was performed on assembled sequences using GISAID's CoVsurver tool (Freunde von GISAID eV).[17] Raw sequence data were uploaded to the National Center for Biotechnology Information's Sequence Read Archive under the reference PRJNA985764.

Results

Animal sample collections

A total of 341 animals were sampled during the study period. Of the 341 animals, 161 (47.2%) were animals owned by veterinary students and VHS staff members and 180 (52.8%) were client-owned animals. Domestic canines and felines comprised the greatest proportion of animals sampled (n = 186 and 89, respectively), and a total of 66 nasal or oral swabs were collected from various mammalian species presenting to the Avian, Exotic, and Zoologic Medicine Service (rabbit, n = 32; guinea pig, 13; ferret, 5; sugar glider, 5; chinchilla, 4; rat, 4; hedgehog, 1; hamster, 1; and tiger, 1). Serum samples were collected from 86 canines, 25 felines, and 6 exotic animals (1 tiger, 2 rabbits, 2 guinea pigs, and 1 rat).

Detection of SARS-CoV-2 RNA from nasal and oral swabs

SARS-CoV-2 RNA was detected by RT-PCR in 3 out of 186 domestic canines (1.6%; 95% CI, 0.5% to 4.6%) and 1 out of 89 domestic felines (1.1%; 95% CI, 0.2% to 6.1%; **Table 1**). Additionally, 2 canine and 3 feline samples were deemed inconclusive (see detection by RT-PCR methods). None of the samples collected from exotic animals tested positive or inconclusive for SARS-CoV-2. Including inconclusive samples, these results found a period prevalence of 2.7% (95% CI, 1.4% to 4.9%) for SARS-CoV-2 RNA in the animals sampled over 17 months.

Antibody detection by plaque reduction neutralization test

A total of 117 serum samples were tested for seropositivity via PRNT. Of the 6 samples collected from exotic mammals, none demonstrated neutralizing activity against the isolate of SARS-CoV-2 used for this assay. Eleven out of 86 (12.8%; 95% CI, 7.3% to 21.5%) canine samples and 3 out of 25 (12.0%; 95% CI, 4.2% to 30.0%) feline samples exhibited positive neutralizing antibodies, yielding a total seropositivity rate of 11.7% (95% CI, 7.7% to 20.1%) for the samples processed in this study. Detectable titers in canine samples ranged from 1:10 to 1:60, while feline samples ranged from 1:10 to 1:80.

Next-generation sequencing and bioinformatic analysis of positive canine swabs

Despite relatively high Ct values, sequencing of samples C-0208 and C-0353 yielded reads that mapped to the SARS-CoV-2 genome (NC_045512.2) with a median coverage depth of 12x and 889x, respectively. De novo genome assembly for C-0208 was 46.4% complete, while C-0353 was 99.2% complete **(Table 2)**.

Phylogenetic assessment using the Pangolin tool assigned sample C-0353 to the BA.5.1 (omicron) sublineage. Although genome coverage was too low for a lineage to be assigned by the Pangolin tool for sample C-0208, it was also designated as BA.5.1 by Nextclade inference. The Nextclade tool assigned both samples to the 22B Nextstrain clade. Mutational analysis of C-0353 conducted using GISAID's CoVsurver tool identified amino acid changes in each viral gene relative to the hCoV-19/Wuhan/WIV04/2019 reference sequence. Forty-three amino acid substitutions and 5 deletions were identified in the S gene of C-0353. Additional results of this analysis have been included elsewhere **(Supplementary**

Table 1—Cycle threshold values from reverse transcription PCR detection of SARS-CoV-2 in animals sampled for this study.

Sample ID	Collection date	Species	Source	Swab location	N protein	ORF1ab	S protein	Result
C-0113	4/25/2022	Canine	VHS staff	Nasal	—	38.75	38.31	Inconclusive
C-0208	6/29/2022	Canine	VHS Staff	Nasal	35.15	36.26	—	Positive
C-0232	5/18/2022	Feline	Oncology	Oral	—	35.52	—	Inconclusive
C-0260	5/18/2022	Canine	Oncology	Oral	36.9	37.37	36.96	Positive
C-0333	6/29/2022	Canine	VHS staff	Nasal	—	39.92	—	Inconclusive
C-0353	6/29/2022	Canine	VHS staff	Oral	28.06	28.21	—	Positive
C-0353	6/29/2022	Canine	VHS staff	Nasal	36.01	36.49	—	Positive
C-0356	6/29/2022	Feline	VHS staff	Nasal	35.97	37.17	—	Positive
C-0366	6/29/2022	Feline	VHS staff	Nasal	38.83	38.88	—	Inconclusive
C-0410	6/29/2022	Feline	VHS staff	Nasal	39.27	—	—	Inconclusive

VHS = Veterinary Health System.

Table 2—SPAdes de novo assembly statistics for samples analyzed in this study. Sample 208 was obtained from a canine nasal swab, and sample 353 was obtained from a canine oral swab.

Sample	Input reads	Trimmed reads	Contigs	Largest contig	Genome fraction	N50
C-0208	4,082,744	48,516	40	1,450	46.4%	815
C-0353	3,388,822	686,498	11	21,612	99.2%	21,612

Material S1). Mutational analysis was not performed for C-0208 given low genome coverage. The consensus sequence of sample C-0353 has been uploaded to GenBank and GISAID under the accession numbers OR165021 and EPI_ISL_17821928, respectively.

Discussion

We conducted a 17-month case identification study with convenience sampling to investigate the prevalence of SARS-CoV-2 in companion and exotic animal species within the CSU VHS community located in Fort Collins, CO. Our results support existing data indicating that domestic canines and felines are susceptible to SARS-CoV-2 and that RNA can be detected during infection. Additionally, neutralizing antibodies can be detected in domestic canines and felines given previous exposure to or infection by SARS-CoV-2.

From our data, we found that domestic canines and felines demonstrated approximately equal RT-PCR prevalence at approximately 1%. Domestic canines and felines additionally demonstrated approximately equal seropositivity at approximately 12%. We found that 1.6% of canines and 1.1% of felines were positive for SARS-CoV-2 RNA by RT-PCR. These findings are reflective of previously reported prevalence and seropositivity from multiple surveillance studies of differing designs.[18–20] Inconclusive results can occur from inadequate sample collection, sampling during early- or late-stage infection, or genomic changes that occur to RT-PCR targets.[21] Therefore, our 5 inconclusive samples may be representative of issues associated with sample collection, low infection burdens within our sample population, or intrahost pathogen evolution. We found that 11 of 86 domestic canine and 3 of 25 domestic feline samples included in this study exhibited neutralizing antibodies (detectable titers ranging from 1:10 to 1:80), demonstrating that overall seropositivity and detectable titer ranges were similar in both species. Additionally, we found a higher seroprevalence in canines and felines, but the titers were lower compared to studies conducted in Wuhan, China, Italy, Hong Kong, and Korea.[13,22,23] The range in titers could reflect that samples were taken at different time points of individual antibody responses or that each animal had a unique antibody response. Furthermore, samples were screened by PRNT using the USA-WA1/2020 strain, and samples may have a reduced neutralizing titer if the animal was instead exposed to SARS-CoV-2 variants such as delta and omicron. Evidence of neutralizing antibodies lasting up to 10 months postinfection in naturally infected companion animals has been documented, though studies addressing humoral immunity are generally lacking.[24]

Our study evaluated a single time point for each animal, so the time course and duration of immune response was not assessed. Additionally, deidentification of sampled animals resulted in the inability to collect information on variables such as age, breed, sex and medical conditions and whether they contributed to the likelihood that an animal would be seropositive for SARS-CoV-2. However, previous SARS-CoV-2 surveillance studies describe limited variables that were statistically significant as risk factors for seropositivity. Neutered animals, animals that were housed indoors, and animals that slept in the same bed as their owners had an increased likelihood of testing seropositive for SARS-CoV-2 antibodies.[25] Another study[26] found that male dogs had a higher risk of seropositivity, but sex was not a significant risk factor in cats. Due to limited data, variables such as breed and medical conditions have not been identified as significant risk factors in dogs or cats testing seropositive for SARS-CoV-2 antibodies.

Next-generation sequencing of 2 domestic canine samples obtained during this study revealed infection by SARS-CoV-2 subvariants of the BA.5.1 sublineage. These subvariants were also circulating in humans at the time and geographic location of our animal sampling.[27] A report generated by GISAID's CoVsurver tool identified amino acid mutations in C-0353 relative to the hCoV-19/Wuhan/WIV04/2019 reference strain (Supplementary Material S1). The BA.5 subvariant represents 1 of 5 main omicron lineages and contains 31 amino acid substitutions and 5 deletions in the S protein.[28] It is unknown at this time whether the additional mutations detected in our samples accumulated during human or canine infection, though previous studies have shown that rapid variant selection may occur during infection of companion animals.[29]

We acknowledge several limitations of this study related to patient sampling logistics. Namely, it was challenging to collect samples from patients within the veterinary hospital setting near the onset of the pandemic due to general logistical restrictions, staff burden, and social-distancing measures. As a result of these shortcomings, a majority of samples were collected between the months of March and August of 2022 and resulted in positive RT-PCR and PRNT tests occurring during this time. Ideally, consistent sampling would have been performed throughout the pandemic to determine whether variants circulating during different time periods could also be detected in companion animals. Additionally, client-owned and VHS staff– and student–owned animals were not intentionally sampled in proportion to the frequency of interactions with veterinary professionals, and therefore the number of animals sampled in each group may not be representative of the number

of animals veterinary professionals interact with day to day. Additionally, the deidentification of sampled animals prevented the collection and assessment of the influence of information such as age, breed, sex, and medical conditions on study results. Due to the uncertainty surrounding consequences of an animal testing positive for SARS-CoV-2 during the early phases of this study, the decision was made to deidentify all information pertaining to the sampled animals and their owners. This decision also made it difficult to accurately target specific groups within the study to achieve a sample population that was representative of the larger population.

Regarding sample collection for RT-PCR analysis, we swabbed the nares of sampled animals given size restrictions and the intolerance of most animals for adequate nasopharyngeal sampling without sedation. It is possible that sample collection in this manner could have led to decreased viral detection; however, paired oral samples did not appear to detect virus for cases in which nasal swabs failed to do so. To this point, we detected only 1 positive and 1 inconclusive oral sample without corresponding positive nasal swabs (Table 1). Our inclusion of a serology assay helps to elucidate companion animal exposure over time; however, our PRNT used only 1 SARS-CoV-2 reference strain (isolate USA-WA1/2020) for neutralizing antibody detection and results were not confirmed by an assay that quantifies antibodies directly (eg, ELISA). Finally, this study focused strictly on companion and exotic animals in a veterinary setting associated with a university in northern Colorado and may not be representative of all areas or veterinary settings.

In light of the COVID-19 pandemic, infectious disease research has increased focus on the concepts of pandemic preparedness, "Disease X," and the general importance of ongoing animal surveillance for zoonotic pathogens.[30,31] Specifically, we believe companion animal surveillance is critical to understanding fundamental aspects of community prevalence, seroprevalence, and transmission dynamics of multihost pathogens like SARS-CoV-2. Although documented companion animal infection and zoonotic transmission has occurred, further in-depth studies are needed to fully determine the risk and recommended preventative measures of SARS-CoV-2 transmission to veterinary health professionals. Additionally, more data need to be collected to understand seasonality, transmissibility of variants, and pathogenicity in our companion animal populations. Our study found supporting evidence of ongoing cross-species transmission of SARS-CoV-2 during later stages of the pandemic for dog and cat companion animals associated with an academic VHS in Colorado. A total seropositivity proportion of 11.7% for all dogs and cats was observed over 17 months of sample collection. Ten animals had detected or indeterminate results via PCR, sampled between May 18, 2022, and June 29, 2022, with 2 deep sequenced isolates most consistent with the BA.5.1 omicron subvariant. We highlight the utility and need for ongoing surveillance involving companion animals during prolonged cross-species pandemics. Future studies are needed that include collection of household information (human infection coincidence and risk factors, transmission risk between pets in the same household, number of veterinary health visits, etc), longitudinal surveillance, multiple locations and care settings, and increased animal numbers during cross-sectional studies.

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Supplementary Materials

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