

## Antibodies against severe acute respiratory syndrome coronavirus 2 in dogs and cats

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### Abstract

This study examined sera from companion animals in the Czech Republic to look for serological evidence of exposure to SARS-CoV-2 and to explore whether infection could relate to respiratory illness in dogs and cats. Between August 2023 and July 2024, specimens were obtained at the Small Animal Clinic of the University of Veterinary Sciences Brno from 283 dogs and 118 cats. Initial screening used an enzyme-linked immunosorbent assay (ELISA). Seropositivity to the nucleocapsid (N) antigen was observed in 6.01% of dogs (17/283) and 6.78% of cats (8/118). Samples classified as positive or doubtful were further analysed by virus neutralization test. Neutralizing antibodies with titres of 1:40, 1:80, and  $\geq 1:160$  were documented in five dogs and four cats. To date, no studies have addressed this topic in the Czech Republic. True prevalence was calculated with Working in Epidemiology, considering for the lower sensitivity of the selected enzyme-linked immunosorbent assay. Odds ratios were also computed for antibody occurrence in animals with respiratory signs compared to clinically healthy animals. Diseased animals showed a markedly higher prevalence of antibodies, suggesting a possible link between SARS-CoV-2 infection and respiratory illness. However, the small number of clinically affected animals limits the statistical validity of these findings.

*ELISA, virus neutralization test, companion animals, SARS-CoV-2*

Coronaviruses (family *Coronaviridae*) are long recognized in veterinary and human medicine as enveloped, positive-sense RNA viruses. A recently emerged member, Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), resulted in the worldwide COVID-19 pandemic in January 2020 (Weng et al. 2021). Since then, natural spillover from humans to animals has been documented in multiple species – including felids, canids, mustelids, primates, and hamsters – by international surveillance (World Organisation for Animal Health 2021). Most infected dogs and cats do not display clinical illness. However, individual reports describe disease compatible with infection, ranging from general malaise (lethargy, anorexia, fever) to respiratory signs (coughing, sneezing, ocular/nasal discharge), and, less frequently, gastrointestinal disturbances such as vomiting and diarrhoea (Gaudreault et al. 2020; Natale et al. 2021; Giraldo-Ramirez et al. 2021; Liew et al. 2023; Piewbang et al. 2024).

Given the widespread ownership of dogs and cats, this study evaluated sera or plasma from selected patients attending the Small Animal Clinic of the University of Veterinary Sciences Brno to assess serological evidence of exposure to SARS-CoV-2.

### Materials and Methods

#### Animals and sampling

Samples of plasma or serum collected from 283 dogs and 118 cats between August 2023 and July 2024 were analysed. The samples were originally collected as part of routine diagnostic procedures by veterinarians at

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the Clinic of Dog and Cat Diseases at the University of Veterinary Sciences Brno and processed at the Clinical Laboratory for Small Animals within the same institution.

Among dogs, 58% were female, whereas among cats, 58.5% were male. The age of animals ranged from 1.5 months to 18 years, with a mean age of 8 years in dogs and 6.5 years in cats. The canine cohort included 83 different breeds, most frequently mixed-breed dogs ( $n = 43$ ), followed by Yorkshire Terriers (22), Dachshunds (21), Jack Russell Terriers (9), Border Collies (7), Labrador Retrievers (7), Prague Ratters (6), and Chihuahuas (6). Among the cats, domestic shorthair cats predominated ( $n = 75$ ); the remaining animals belonged to various pure breeds.

Most animals originated from the South Moravian Region (81% of dogs and 96% of cats). Other dogs originated from the Vysočina Region (5%), Zlín Region (4%), and to a lesser extent from the Liberec, Moravian-Silesian, Olomouc, Plzeň, and Central Bohemian Regions.

A retrospective review of veterinary medical records identified seven animals (four dogs and three cats) showing signs of respiratory disease at the time of sampling. Samples from animals showing signs of gastrointestinal disease were also included in the study. Although these animals were subsequently diagnosed with specific conditions (e.g. foreign body in the gastrointestinal tract, parvovirus, food allergy), the samples were retained for the potential detection of antibody response to past SARS-CoV-2 infection at a later stage.

Information on household COVID-19 status was unavailable.

#### Enzyme-linked immunosorbent assay

We screened 283 canine and 118 feline samples using the enzyme-linked immunosorbent assay (ELISA) with the commercial kit ID Screen® SARS-CoV-2 Double Antigen Multi-species (Innovative Diagnostics, Grabels, France). This ELISA test is designed to detect antibodies against the nucleocapsid protein (N protein) of SARS-CoV-2. Data validation and interpretation of results, including calculation of the sample-to-positive (S/P) ratio, were performed according to the manufacturer's instructions. Samples with S/P values above 60% were considered positive, those between 50–60% as doubtful, and values below 50% as negative.

#### Virus neutralization test

Samples identified as positive or doubtful by ELISA were subsequently retested using the virus neutralization test (VNT). Additionally, samples with borderline S/P % values – defined in this study as greater than 35% – were included in the testing. The VNT was performed at the Biosafety Level 3 laboratory of the Veterinary Research Institute in Brno, Czech Republic. The procedure followed the protocol described by Palus et al. (2013), using a SARS-CoV-2 Omicron variant BA.2.86 isolate from autumn 2023 and the Vero E6 cell line.

#### Statistical analysis

The online tool Working in Epidemiology (WinEpi) was used to calculate true prevalence of antibodies against SARS-CoV-2 in dogs and cats, which allows correction of the apparent prevalence based on the sensitivity and specificity of the diagnostic test used. The manufacturer of the ELISA kit used in this study provided specificity values separately for each species, 99.7% for dogs and 98.9% for cats. However, sensitivity values were not available from the manufacturer, so published estimates from two independent studies with differing results were used instead (Ratti et al. 2022; Diezma-Díaz et al. 2023). Diezma-Díaz et al. (2023) reported species-specific sensitivity estimates separately as 36% and 63% for dogs and cats, respectively. In contrast, Ratti et al. (2022) reported a single overall sensitivity value of 23% for the double-antigen ELISA, without separate estimates for the two species. Therefore, for dogs, sensitivity values of 36% and 23% were used, while for cats the corresponding values were 63% and 23%. The true prevalence was therefore calculated twice for each species using both sensitivity values.

WinEpi was also used to calculate the odds ratio in case-control studies for each species separately. For each analysis, animals were divided into exposed and non-exposed groups according to the presence or absence of the assessed factor, and ELISA-positive animals were compared with ELISA-negative animals. The assessed risk factors included the presence of clinical signs of respiratory disease, clinical signs of gastrointestinal disease, age, and sex. For sex-based analysis, male sex was defined as the exposed category in both species. Odds ratios (OR) were calculated without applying a continuity correction.

## Results

### ELISA

Specific antibodies against the nucleocapsid (N) protein of SARS-CoV-2 were detected in 6.01% (17/283) of canine and 6.78% (8/118) of feline serum samples. These samples were classified as positive. One feline sample (1/118) was considered doubtful. All remaining samples were classified as negative.

A total of seven samples – four from dogs and three from cats – were obtained from animals that exhibited respiratory clinical signs during anamnesis or physical examination. One of the canine samples was classified as positive. This sample belonged to a 12-year-old male Yorkshire Terrier (ID 17 in Table 1) presenting with dyspnoea, bronchial

breathing, mucosal cyanosis, and moist rales on pulmonary auscultation. Radiographic and ultrasonographic examinations confirmed a non-cardiogenic pulmonary oedema.

One feline sample from a cat with respiratory symptoms was also classified as positive (ID 8 in Table 1). This was a seven-year-old domestic shorthair female showing wheezing, anorexia, polypnoea, and bronchovesicular breathing. Mucous membranes were pink, lymph nodes were symmetrical, non-enlarged, and non-painful. Radiographic examination did not reveal any focal changes in the pulmonary parenchyma.

### Virus neutralization test

A total of 33 samples were analysed by VNT, including 25 ELISA-positive samples, 1 doubtful sample, 5 borderline samples, and 2 serum samples used as negative controls. Virus-neutralizing antibodies were detected in nine samples, with titres of 1:40, 1:80, or  $\geq 1:160$ .

A comparison of ELISA S/P % values and VNT titres revealed differences between the two assays in some samples. Samples with high ELISA S/P % values and negative VNT results, as well as samples with lower ELISA S/P % values and detectable neutralizing titres, were observed. An overview of the virus neutralization titres in relation to the corresponding ELISA S/P % values is provided in Table 1.

Table 1. Identification of animals with a positive enzyme-linked immunosorbent assay result and corresponding virus neutralisation test result.

| ID | Species | Breed                          | Sex | Age* | Sampling date | ELISA S/P % | VNT          | Clinical signs |
|----|---------|--------------------------------|-----|------|---------------|-------------|--------------|----------------|
| 1  | Dog     | Beagle                         | F   | 10   | 11/09/2023    | 156.5       | Negative     | None           |
| 2  | Dog     | German Shepherd                | M   | 2    | 20/10/2023    | 80.8        | Negative     | None           |
| 3  | Dog     | American Staffordshire Terrier | F   | 13   | 27/10/2023    | 259.6       | Negative     | None           |
| 4  | Dog     | Yorkshire Terrier              | F   | 7    | 30/10/2023    | 371.8       | $\geq 1:160$ | None           |
| 5  | Dog     | Leonberger                     | M   | 9    | 03/11/2023    | 192.7       | Negative     | None           |
| 6  | Dog     | Weimaraner                     | M   | 8    | 03/11/2023    | 851.0       | 1:40         | None           |
| 7  | Dog     | Mixed breed                    | M   | 11   | 24/01/2024    | 205.9       | Negative     | None           |
| 8  | Dog     | White Swiss Shepherd           | F   | 5    | 30/01/2024    | 307.6       | Negative     | None           |
| 9  | Dog     | White Swiss Shepherd           | M   | 8    | 09/02/2024    | 307.6       | Negative     | None           |
| 10 | Dog     | Labrador Retriever             | F   | 11   | 07/03/2024    | 232.8       | Negative     | None           |
| 11 | Dog     | Miniature American Shepherd    | M   | 4    | 10/04/2024    | 61.1        | 1:80         | None           |
| 12 | Dog     | Belgian Shepherd               | M   | 8    | 19/04/2024    | 97.2        | $\geq 1:160$ | None           |
| 13 | Dog     | German Wirehaired Pointer      | M   | 2    | 08/07/2024    | 177.5       | 1:40         | None           |
| 14 | Dog     | Labrador Retriever             | F   | 11   | 22/01/2024    | 60.2        | Negative     | None           |
| 15 | Dog     | Mixed breed                    | M   | 8    | 18/01/2024    | 84.5        | Negative     | None           |
| 16 | Dog     | Yorkshire Terrier              | M   | 6    | 15/04/2024    | OVER        | Negative     | None           |
| 17 | Dog     | Yorkshire Terrier              | M   | 12   | 07/03/2024    | 71.9        | Negative     | RES            |
| 1  | Cat     | Domestic Shorthair             | M   | 3    | 22/04/2024    | 41.0        | Negative     | None           |
| 2  | Cat     | Domestic Shorthair             | F   | 13   | 02/10/2023    | 49.8        | $\geq 1:160$ | None           |
| 3  | Cat     | Domestic Shorthair             | M   | 2    | 22/01/2024    | 35.2        | Negative     | None           |
| 4  | Cat     | British Shorthair              | F   | 5    | 17/01/2024    | 35.9        | Negative     | None           |
| 5  | Cat     | Domestic Shorthair             | F   | 14   | 24/01/2024    | 41.8        | Negative     | None           |
| 6  | Cat     | Domestic Shorthair             | M   | 8    | 25/01/2024    | OVER        | 1:40         | None           |
| 7  | Cat     | Ragdoll                        | M   | 4    | 20/10/2023    | 67.4        | $\geq 1:160$ | None           |
| 8  | Cat     | Domestic Shorthair             | F   | 7    | 09/10/2023    | 259.8       | $\geq 1:160$ | RES            |

ID – Identification number of the dog or cat with a positive ELISA test result; \*age of the animal in years at the time of blood sample collection; ELISA – enzyme-linked immunosorbent assay; S/P % – sample to positive ratio; VNT – virus neutralization test; F – female; M – male; RES – respiratory clinical signs; OVER – value exceeded the measurable range

### True prevalence and odds ratio

The true prevalence of SARS-CoV-2 antibodies was estimated using the WinEpi tool. In dogs, the apparent seroprevalence was 6.01% (95% CI: 3.24–8.78). Using a sensitivity of 36%, the estimated true prevalence was 15.99% (95% CI: 11.72–20.26), and using a sensitivity of 23%, it was 25.14% (95% CI: 20.09–30.20). In cats, the apparent seroprevalence was 6.78% (95% CI: 2.24–11.32). Using a sensitivity of 63%, the estimated true prevalence was 9.18% (95% CI: 3.97–14.38), and using a sensitivity of 23%, it was 25.93% (95% CI: 18.03–33.84).

Case-control comparisons were subsequently performed to calculate odds ratios for selected factors. In animals with respiratory clinical signs, the odds of ELISA positivity were higher than in clinically healthy animals in both species, with an OR of 5.48 (95% CI: 0.54–55.69) in dogs and 8.43 (95% CI: 0.68–104.62) in cats. However, the confidence intervals were wide, and the results were not statistically significant.

As no ELISA-positive animals were identified among animals with gastrointestinal clinical signs, odds ratio analysis was not performed for this factor.

For sex, the odds of ELISA positivity were higher in males than in females in dogs (OR = 2.72; 95% CI: 0.98–7.59), whereas in cats they were lower in males than in females (OR = 0.69; 95% CI: 0.16–2.91). These associations were not significant in either species.

Age was also assessed as a potential risk factor; however, the analysis was inconclusive because some age categories contained only a small number of animals.

## Discussion

A widely used method for detecting antibodies against SARS-CoV-2 in dogs and cats is ELISA. The antibodies formed may be directed against various parts of the virion, such as the receptor-binding domain (RBD) on the spike (S) protein (Klaus et al. 2021; Espinosa-Gómez et al. 2023; Barroso-Arévalo et al. 2023), or against the nucleocapsid (N) protein (Barua et al. 2021; Jarrah et al. 2023).

The nucleocapsid (N) protein is conserved across coronaviruses. As species-specific coronaviruses circulate in dog and cat populations, cross-reactivity and false-positive results may occur when using ELISA assays targeting the N protein. Commonly occurring coronaviruses in these animals include canine enteric coronavirus (CECoV) and both variants of feline coronavirus (FECoV and FIPV). Dogs may also be infected with canine respiratory coronavirus (CRCoV), which, like SARS-CoV-2, belongs to the genus *Betacoronavirus* (Sharun et al. 2020). These differences may influence the antigenic structure of the N protein and the resulting antibody response. However, cross-reactivity between the most common coronaviruses in cats and dogs when using the same ELISA kit as in our study has been ruled out in several studies (Pomorska-Mól et al. 2021; Udom et al. 2022; Ratti et al. 2022). Nevertheless, this possibility should not be completely disregarded when interpreting serological results in dogs and cats, and a positive ELISA result should ideally be confirmed using another serological method, such as virus neutralization testing (Udom et al. 2022).

Our ELISA results for anti-N antibodies are similar to reports from countries that used the same commercial kit. In France, 5.5% (25/453) of dogs tested positive (Laidoudi et al. 2021). By contrast, in Thailand only 1.66% (35/2102) of dogs and 0.36% (4/1113) of cats tested positive (Udom et al. 2022). In the United States, seroprevalence was reported at 0.37% (5/1336) in dogs and 1.15% (11/956) in cats (Barua et al. 2021), while in Poland, 1.17% (4/343) of dogs and 1.79% (5/279) of cats tested positive (Pomorska-Mól et al. 2021). The lower seroprevalence reported in Thailand and the United States may reflect differences in study design – particularly the sampling frame and timing –

rather than sample size alone. Seroprevalence can also vary with sampling methodology, population structure, and inclusion criteria.

The findings of a study conducted in Brazil using a different double-antigen ELISA test, which also detects antibodies against the N protein in dogs and cats, are comparable to those of our study, with a seroprevalence of 4.3% (33/762) in dogs and 2.2% (4/182) in cats (Jarrah et al. 2023).

The molecular structure of the RBD on the spike (S) protein is highly specific to SARS-CoV-2 and differs substantially among coronavirus species. Different coronaviruses use different host cells for replication, which may express distinct surface receptors, contributing to the high variability of this protein (Masters 2006). Nevertheless, in China, antibodies against the RBD were detected by ELISA in a high proportion of cats, with a prevalence of 14.7% (15/102) (Zhang et al. 2020). This study was conducted at the onset of the pandemic. At that time, ELISA tests targeting RBD of the S protein were more suitable, as the differences between the spike proteins of viral variants and the antigen in the ELISA were minimal. In later antibody detection using RBD-based ELISA (in 2022), the prevalence of seropositive dogs was much lower, at 1.75% (16/910) (Zhao et al. 2022), most likely due to the accumulation of mutations within the RBD over time and the resulting altered antibody responses that the test could no longer effectively detect.

Using VNT, we also confirmed the presence of virus-neutralizing antibodies in plasma or serum samples from dogs and cats. Some samples that were classified as positive by ELISA and showed high S/P % values yielded negative VNT results. This included dog ID 17, in which the ELISA-positive result was not confirmed by VNT. One possible explanation is cross-reactivity with other canine and feline coronaviruses in ELISA (Pomorska-Mól et al. 2021; Udom et al. 2022; Ratti et al. 2022).

Household epidemiological status may also play an important role in seroprevalence. Studies focusing on animals living with COVID-19-positive owners have shown markedly higher seroprevalence rates. In France, researchers tested the same samples using four different methods and detected antibodies in 15.4% (2/13) of dogs and 3.5% (8/34) of cats (Fritz et al. 2020). Future studies should therefore focus on animals from households where the COVID-19 status of the owners is known. Incorporating this information would provide a deeper understanding of the potential for human-to-animal transmission and help clarify the dynamics of SARS-CoV-2 spread within domestic environments.

In the final part of this study, we compared the occurrence of SARS-CoV-2 antibodies between two groups of animals using the WinEpi tool. Antibodies were detected more frequently in symptomatic than in asymptomatic dogs and cats. The results are consistent with a possible role for SARS-CoV-2 in respiratory disease in these species. However, due to the small number of clinically affected animals included in the study, this conclusion remains speculative and warrants further targeted investigation. A limitation of this study is the clinic-based sampling design and the marked predominance of samples from the South Moravian Region (81% of dogs and 96% of cats), which should also be considered when interpreting the results.

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