

ORIGINAL ARTICLE

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection and humoral responses against different variants of concern in domestic pet animals and stray cats from North-Eastern Spain

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Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of the coronavirus disease 2019 (COVID-19) pandemic in humans, is able to infect several domestic, captive and wildlife animal species. Since reverse zoonotic transmission to pets has been demonstrated, it is crucial to determine their role in the epidemiology of the disease to prevent further spillover events and major spread of SARS-CoV-2. In the present study, we determined the presence of virus and the seroprevalence to SARS-CoV-2, as well as the levels of neutralizing antibodies (nAbs) against several variants of concern (VOCs) in pets (cats, dogs and ferrets) and stray cats from North-Eastern of Spain. We confirmed that cats and dogs can be infected by different VOCs of SARS-CoV-2 and, together with ferrets, are able to develop nAbs against the

Júlia Vergara-Alert and Joaquim Segalés contributed equally to this work.

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ancestral (B.1), Alpha (B.1.1.7), Beta (B.1.315), Delta (B.1.617.2) and Omicron (BA.1) variants, with lower titres against the latest in dogs and cats, but not in ferrets. Although the prevalence of active SARS-CoV-2 infection measured as direct viral RNA detection was low (0.3%), presence of nAbs in pets living in COVID-19-positive households was relatively high (close to 25% in cats, 10% in dogs and 40% in ferrets). It is essential to continue monitoring SARS-CoV-2 infections in these animals due to their frequent contact with human populations, and we cannot discard the probability of a higher animal susceptibility to new potential SARS-CoV-2 VOCs.

KEYWORDS

coronavirus disease 2019 (COVID-19), pets, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), stray cats, variants of concern (VOCs), zoonotic transmission

1 | INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is recognized as the causative pathogen of the current coronavirus disease 2019 (COVID-19) (Zhou et al., 2020). SARS-CoV-2 emerged in Wuhan (China) by the end of 2019, and rapidly spread worldwide causing 614 million infections and 6.5 million deaths so far (World Health Organization, 2022a). The high rates within the human population together with the moderate mutation rate of SARS-CoV-2 have facilitated the appearance of several variants over time during the pandemic, with significant impact on transmissibility, virulence and/or immune escape, which are designated as variants of concern (VOCs) (World Health Organization, 2022b). Since the beginning of the COVID-19 pandemic, at least five VOCs have been globally recognized, namely Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2) and Omicron (B.1.1.529) (World Health Organization, 2022b). All acquired mutations in multiple genes, being the most relevant ones those affecting the gene coding for the spike (S) glycoprotein, which mediates viral entry into target cells (Hoffmann et al., 2020). Consequently, these mutations may impact several spike functions, such as its affinity for binding to the angiotensin-converting enzyme 2 (ACE2), which is the main host cell receptor. Thereby, the infectivity, tropism and the host range of SARS-CoV-2 variants are under continuous evolution (Hoffmann et al., 2020; Tarrés-Freixas et al., 2022; Wang et al., 2021).

To date, it is strongly suggested that SARS-CoV-2 is a zoonotic virus that emerged from SARS-like coronaviruses from bats (Zhou et al., 2020). Although a direct ancestor has not been detected in the wild yet, the closest genome sequences have been identified in Horseshoe bats from South-East Asia (Lytras et al., 2022; Pekar et al., 2022; Temmam et al., 2022; Zhou et al., 2020). However, genomic analysis suggested that transmission from bat to humans likely occurred through an unidentified intermediate host (Ramasamy et al., 2020). On the other hand, the susceptibility to SARS-CoV-2 infection of different animal species (domestic, captive and wildlife) has been demonstrated through experimental and natural infections, suggesting their potential role in the epidemiology of the disease (Muñoz-Fontela et al., 2020;

Sharun et al., 2021). Of particular concern is the potential susceptibility of those animals that are frequently in contact with the human population, such as companion animals. Previous experimental *in vivo* studies performed in domestic cats, ferrets and golden Syrian hamsters demonstrated viral replication in respiratory and gastrointestinal tracts, as well as RNA shedding from the mentioned species (Bosco-Lauth et al., 2020; Shi et al., 2020; Sia et al., 2020). In addition, viral transmission between cats, ferrets and hamsters has also been shown experimentally (Bosco-Lauth et al., 2020; Shi et al., 2020; Sia et al., 2020). In contrast, dogs appeared to have a lower susceptibility to SARS-CoV-2 experimentally and no viral transmission to co-housed animals was observed (Bosco-Lauth et al., 2020; Shi et al., 2020). Besides, several natural cases of SARS-CoV-2 infection in pets have been reported in many different countries (World Organization for Animal Health, 2022). Interestingly, the majority of natural infections have occurred in animals in close contact with COVID-19-affected humans, suggesting a reverse zoonotic transmission of SARS-CoV-2 (Sharun et al., 2021). Importantly, the only well-characterized pet-to-human transmission occurred in Hong Kong, related to an outbreak in a pet shop, in which hamster-to-human transmission was evidenced by genomic analyses (Chan et al., 2022). More recently, a Thai veterinarian was diagnosed with COVID-19 after being sneezed on by a SARS-CoV-2-infected cat, strongly suggesting cat-to-human transmission (Sila et al., 2022). Besides, not only domestic pet cats but also stray cats have been demonstrated to be exposed to SARS-CoV-2, since a low proportion of them harboured neutralizing antibodies (nAbs) (Spada et al., 2021; Villanueva-Saz et al., 2022; Zhang et al., 2020). Furthermore, stray cats have been infected by other animal species, such as mink in the Netherlands (Van Aart et al., 2021). As stray cats usually live in colonies, their likelihood to become a potential reservoir for SARS-CoV-2 is not negligible.

For all above-mentioned facts, it is crucial to investigate the role of pets in the epidemiology of COVID-19 and to determine their susceptibility to infection by SARS-CoV-2 and its VOCs. According to the AIAC (Arxiu d'Identificació d'Animals de Companyia, 2021), a total of 1,531,002 pet animals were registered in 2021 exclusively in Catalonia, including 253,860 cats, 1,264,795 dogs and 5601 ferrets. Thus, the

present work aimed to determine the prevalence of SARS-CoV-2 infection and the seroprevalence of cats (stray and pet), dogs and ferrets from the North-Eastern of Spain (Catalonia and Valencian Community). The study comprised pets from COVID-19-positive households, pets with no evidence of exposure to COVID-19-affected owners and pets with no information about their COVID-19 environment. In addition, since the present work included samples from the beginning of the pandemic (April 2020) until January 2022, levels of nAbs against different recognized VOC to date were investigated for a first time in a large number of pet animals.

2 | MATERIALS AND METHODS

2.1 | Sample collection

A total of 1009 animals were included in the study: 564 dogs (*Canis familiaris*), 381 cats (*Felis catus*, 253 pet cats, 128 stray cats) and 64 ferrets (*Mustela putorius furo*). Nasopharyngeal or oropharyngeal swabs ($n = 987$), rectal swabs ($n = 929$) and serum samples ($n = 789$) were taken from most of these animals during the period April 2020 to January 2022. Samples were collected using sterile dry swabs or DeltaSwab Virus 2 ml contained in viral transport media (VTM) (Deltalab, S.L., Catalunya, Spain). At least one type of sample for each animal was obtained (Table S1). Such sampling was performed by veterinarians from multiple veterinary clinics (Catalonia and Valencian Community, North-Eastern Spain), from the *Hospital Clínic Veterinari* of the *Universitat Autònoma de Barcelona* (UAB, Barcelona, Spain), as well as from the Veterinary Pathology Diagnostic Service (SDPV) of the UAB. Lung tissue ($n = 236$) was also available from those animals necropsied at the SDPV. Samples from stray cats were obtained from veterinary clinics having permissions to work with these populations from the corresponding municipalities.

Pets were classified according to a questionnaire filled by the owners, emphasizing whether they had contact or not with a COVID-19-affected human. Thus, animals were divided within three main categories: (1) those from households with current or previous COVID-19-affected owners (COVID-19[+] group), (2) those pets with no evidence of contact with COVID-19-affected owners (COVID-19[-] group) and (3) pets from households from which no information on COVID-19 environment was available (Unknown COVID-19 group). In addition, the following data were recorded when possible: breed, gender (female/male), age and clinical signs (respiratory and digestive) if any.

All samples were obtained from veterinary clinicians using conventional sampling protocols in compliance with the guidelines by the Code of Research Ethics of IRTA. Samples from stray cats were obtained from two different veterinary clinics with the authorization of the local government from Palamós, Girona (reference 14869) and Barcelona city (project licence 21001495). Samples were subsequently sent to IRTA-CReSA for SARS-CoV-2 investigations by a transport company under the regulations stated in the UN3373 regulation. Owners/keepers were duly informed regarding the purpose of the study, the data protection policy and granted their consent for each pet.

2.2 | RNA extraction and detection of SARS-CoV-2 by RT-qPCR

A total of 992 out of 1009 animals were tested for the detection of SARS-CoV-2 RNA in at least one type of sample (Table S1): 380 cats (252 pet cats, 128 stray cats), 550 dogs and 62 ferrets. First, sterile dry oral/nasal and rectal swabs were transferred into cryotubes containing 500 μ l DMEM (Lonza, Basel, Switzerland) supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin and 2 mM glutamine (all from Gibco Life Technologies, Madrid, Spain) and finally vortexed. DeltaSwabs Virus with VTM was directly vortexed. Regarding lung tissue samples, a portion of approximately 0.2 mg was placed into cryotubes with 500 μ l of supplemented DMEM with a single zinc-plated, steel 4.5-mm beads. Tissues were mechanically homogenized at 30 Hz for 1 min using a TissueLyser II (QIAGEN GmbH, Hilden, Germany) and centrifuged for 3 min at 10,000 rpm. All samples were subjected to viral RNA extraction using the Indimag Pathogen Kit (Indical Biosciences, Leipzig, Germany) on a BioSprint 96 workstation (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Then, SARS-CoV-2 RNA was quantified by RT-qPCR using a previously described protocol, which targets the envelope protein (E)-encoding gene (Corman et al., 2020) with some modifications (Brustolin et al., 2021). Briefly, RT-qPCR was performed using AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystems, Life Technologies, Waltham, MA, USA) and amplification was achieved using a 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies). Samples with a Cq value <40 were considered positive for SARS-CoV-2 genomic detection. Positive samples were re-analysed by two different RT-qPCR assays, targeting the RNA-dependent RNA polymerase (RdRp) gene specific for SARS-CoV-2 and the nucleocapsid (N) gene (Corman et al., 2020), following a previously published protocol (Segalés et al., 2020).

2.3 | SARS-CoV-2 genome sequencing

Viral RNA from all positive samples was converted to cDNA with the PrimeScript™ RT reagent kit (Takara Bio Europe SAS, Saint-Germain-en-Laye, France), as previously described (Rodon et al., 2021). Samples were sequenced following a previously described procedure (Fernández-Bellón et al., 2021). Briefly, cDNA was used for DNA synthesis using the ARTIC-CoV v3 PCR protocol followed by Illumina sequencing (Pillay et al., 2020). Raw data analysis was performed by viralrecon pipeline (<https://github.com/nf-core/viralrecon>, accessed on 4 July 2022), while consensus sequence was called using samtools/ivar at the 75% frequency threshold. All high-quality genomic sequences were deposited in GISAID repository.

2.4 | Receptor binding inhibition ELISA

Blood samples were centrifuged at $1800 \times g$ for 10 min at 4°C, and the obtained sera were inactivated at 56°C for 1 h and stored at -20°C until further use. For the analysis, samples were previously thawed

and vortexed. nAbs targeting SARS-CoV-2 Receptor Binding Domain (RBD) were measured in available serum samples ($n = 789$; 444 dogs, 298 cats [170 pet cats, 128 stray cats] and 47 ferrets) using the GenScript cPass™ SARS-CoV-2 Neutralization Antibody Detection Kit (Genscript, the Netherlands), following the manufacturer's protocol. The percentage of inhibition of each sample was determined using the following formula: % Inhibition = $(1 - (\text{OD}_{450} \text{ sample} / \text{OD}_{450} \text{ negative control})) \times 100$. Samples and controls were included in duplicate ($\text{SD} \leq 10\%$). Inhibition of $\geq 30\%$ was considered as a positive neutralization.

2.5 | Neutralization assay of SARS-CoV-2 pseudoviruses expressing the spike protein of different VOCs

Serum samples that tested positive ($n = 40$) by the SARS-CoV-2 RBD inhibition ELISA were also analysed with a pseudovirus-based neutralization assay against different SARS-CoV-2 VOCs following a protocol described previously (Pradenas et al., 2022; Trinité et al., 2021). Briefly, HIV reporter pseudoviruses expressing SARS-CoV-2 S protein (from the ancestral virus and the Alpha, Beta, Delta and Omicron BA.1 VOCs) and luciferase were generated. Pseudoviruses expressing a VSV-G protein instead of the S protein were generated and used as control of specificity as previously described (Díez et al., 2021). For the neutralization assay, 200 TCID₅₀ (Median Tissue Culture Infectious Dose) of pseudovirus were preincubated with three-fold serial dilutions (1/20–1/43,740 for the Omicron BA.1 variant, and 1/60–1/43,740 for all the other variants) of heat-inactivated sera samples for 45 min at 37°C. Then, human ACE2-overexpressing HEK293T cells were added onto mixed samples. After 48 h, cells were lysed with Britelite Plus Luciferase reagent (Perkin Elmer, Waltham, MA, USA) and luminescence was measured for 0.2 s with EnSight multimode late reader (Perkin Elmer).

The neutralization capacity of the sera samples was calculated by comparing the experimental relative light unit (RLU) calculated from infected cells treated with each serum to the maximal RLUs (maximal infectivity calculated from infected untreated cells) and minimum RLUs (minimal infectivity calculated from uninfected cells), and expressed as percent neutralization: % Neutralization = $(\text{RLU}_{\text{max}} - \text{RLU}_{\text{experimental}}) / (\text{RLU}_{\text{max}} - \text{RLU}_{\text{min}}) \times 100$. ID₅₀ (Infectious Dose 50) values were calculated by plotting and fitting neutralization values and the log of plasma dilution to a four-parameter equation in Prism 9.0.2 (GraphPad Software, San Diego, CA, USA). All ID₅₀ values are reported as reciprocal dilution.

2.6 | Statistical analysis

Chi-square with Yate's correction test was used to compare differences in SARS-CoV-2 RNA detection and antibody prevalence among studied groups. The relationship between the antibody presence, households' conditions and gender was also analysed; p -value lower than .05 were

considered statistically significant. Relative risk (RR) ratios and 95% confidence intervals (CIs) were determined to evaluate the risk of exposure to SARS-CoV-2 in cats and dogs from positive and negative COVID-19 households.

Wilcoxon test with Bonferroni's correction test was used to compare titres of nAbs against different SARS-CoV-2 VOCs in each species. A Kruskal–Wallis test with Dunn's multiple comparison test was used to compare the titres of nAbs against different VOCs between different collection periods of the COVID-19 pandemic in all seropositive animal samples. On the other hand, comparison of the titres of nAbs against VOCs between species was also evaluated. Tests with P -values lower than 0.05 were considered as statistically significant in all tests.

Spearman's correlation test was used to evaluate the existence of a positive relationship between the RBD inhibition ELISA (% of Inhibition) and the pseudovirus-neutralization (ID₅₀) assays.

All results were analysed with GraphPad Prism 9.0 Software (La Jolla, CA, USA).

3 | RESULTS

3.1 | Sample data

The total number of pet cats, dogs and ferrets included in the study are displayed in Table 1, classified into three main categories (COVID-19[+], COVID-19[–] and unknown households). Besides, 128 stray cats were also included.

Both female ($n = 357$) and male ($n = 357$) animals were represented in the study. However, we did not obtain gender information from some of the animals ($n = 295$). Table S2 shows the total of samples analysed by RT-qPCR and by the RBD Inhibition ELISA according to the animal species and gender within animal species.

3.2 | SARS-CoV-2 RNA detection and SARS-CoV-2 sequencing

A total of 992 animals were analysed by RT-qPCR and only three of them tested positive for SARS-CoV-2 RNA: one pet cat (C1) (1/380; 0.26%) and two dogs (D1, D2) (2/550; 0.36%). No statistically significant differences (chi-square with Yates' correction, $p > .8832$) in RNA prevalence were observed between cats and dogs.

C1 was a 4-year-old male European $\times$ Persian crossbred cat, D1 corresponded to a male Schnauzer dog and D2 was a 13-year-old female Breton dog. Specific epidemiological and clinical investigations about the infection of C1 and D2 were described in previously published case reports (Fernández-Bastit et al., 2021; Segalés et al., 2020). All of them were living in a COVID-19-positive household with previous affected owners. C1 tested positive in nasal swab for the UpE ($C_q = 33.69$), RdRp ($C_q = 34.01$) and the N ($C_q = 35.1$) genes and resulted negative for all genes in rectal swab. D1 tested positive in nasal swab for the UpE ($C_q = 13.21$), RdRp ($C_q = 19.39$) and N ($C_q = 19.83$) genes, and in rectal swab for the UpE ($C_q = 24.68$), RdRp ($C_q = 29.73$) and N ($C_q = 30.92$)

TABLE 1 Pet cats, dogs and ferrets distributed in three main categories according to COVID-19 household environment conditions

Households	PET CAT	DOG	FERRET	Total
COVID-19 (+)	47 (18.58%)	196 (34.75 %)	6 (9.38 %)	249 (28.26%)
COVID-19 (-)	101 (39.92%)	218 (38.65 %)	42 (65.63 %)	361 (40.98%)
Unknown COVID-19	105 (41.50%)	150 (26.7 %)	16 (25.00 %)	271 (30.76%)
Total	253 (100%)	564 (100%)	64 (100%)	881 (100%)

Abbreviations: COVID-19 (+), pets that had contact with COVID-19-affected owners; COVID-19 (-), pets with no evidence of contact with COVID-19-affected owners; Unknown COVID-19, pets with unknown COVID-19 household environment.

genes. D2 tested positive in oral swab for the UpE (Cq = 34.36) and RdRP (Cq = 35.77) genes but negative for the N gene (Cq > 40) and for all the genes in the rectal swab. One week after the first positive result, both C1 and D2 tested negative by RT-qPCR; D1 was a dog received at the SDPV and the cause of euthanasia was renal failure due to an overdose of anti-inflammatory non-steroidal drugs. None of studied ferrets resulted positive by RT-qPCR.

SARS-CoV-2 genome sequencing was performed in all positive samples and a specific SARS-CoV-2 variant was identified in each case. C1 was infected with an early epidemic SARS-CoV-2 variant (B.1 pango lineage, D614G) (Global Initiative on Sharing Avian Influenza Data, GISAID acc. EPI_ISL_482820). Viral genome from nasal and rectal swabs of D1 was consistent with the Alpha (B.1.1.7) VOC (nasal swab: EPI_ISL_13608276, Rectal swab: EPI_ISL_13608277), while D2 was infected with the Delta (B.1.617.2) VOC (GISAID acc. EPI_ISL_6344510).

3.3 | Detection of SARS-CoV-2 nAbs targeting the RBD

A total of 789 serum samples were evaluated by a SARS-CoV-2 RBD Inhibition ELISA. RBD nAbs were detected in 16 out of 298 (5.36%) cats, 22 out of 444 (4.95%) dogs and two out of 47 (4.26%) ferrets (Table 2). Within the 16 positive samples from cats, 13 samples corresponded to pet cats (13/170, 7.65%) and three samples were from stray cats (3/128, 2.34%). From the total of 40 positive samples, 29 (72.5%) corresponded to pets from COVID-19(+) households, being specifically, 10 out of 16 (62.5%) cats, 17 out of 22 (77.27%) dogs and two out of two (100%) ferrets. However, nAbs were also detected in pet cats and dogs from the other groups (COVID-19[-] and Unknown COVID-19) (Table 2).

Along the positive feline serum samples, one corresponded to the C1 (No. 1 in Table S1), which exhibited a 96.24% of RBD inhibition 1 week after testing positive for RT-qPCR. C1 was living with another cat mate that also tested positive with an inhibition of 96.59%, although it tested negative by RT-qPCR (No. 2 in Table S1) (Segalés et al., 2020). On the other hand, D2 had RBD nAbs with an inhibition of 67.93% (No. 595 in Table S1) 21 days after the initial respiratory and digestive clinical signs and 67.60% two and a half months after the display of clinical signs (Fernández-Bastit et al., 2021). Unfortunately, it was not possible to obtain serum sample from the D1 since it was

sacrificed due to its clinical condition (severe necrosis of the bilateral renal papilla, mitral and tricuspid endocardiosis as well as of oedema and pulmonary congestion). Regarding the positive canine serum samples, one corresponded to a dog with an inhibition of 85.12% (No. 463 in Table S1), which was living with another dog mate that exhibited an inhibition of 30.62% (No. 462 in Table S1). They were included in the COVID-19-positive household group and were sampled at the same time point (June 2021). Finally, both seropositive ferrets were from the same household. No more animals came from the same household.

Cats from COVID-19-positive households (COVID-19[+]) were significantly more likely to seroconvert against SARS-CoV-2 (chi-square with Yates' correction, $p < 0.0001$) with a higher risk of SARS-CoV-2 exposure (RR = 11.67; 95% CI: 2.6703–50.9724), compared to those that did not have any contact with a COVID-19-affected human or no evidence was determined (COVID-19[-]) (Table 2). Similar results were observed in dogs (chi-square with Yates' correction, $p = 0.0030$; RR = 4.77, 95% CI: 1.6329–14.9179). Both positive ferret samples were living in a COVID-19-positive household.

In addition, the seroprevalence between females and males in each species was also compared. No significant link between seropositivity and the gender of animals, nor for cats (Chi-square with Yates' correction, $p = 0.6005$) or for dogs (chi-square with Yates' correction, $p = 0.3462$), was observed (Table 2). The two positive ferrets belonged to the male gender.

3.4 | Neutralizing responses against SARS-CoV-2 spike variants

Positive samples ($n = 40$) from the performed ELISA were then tested by the pseudovirus neutralization assay to evaluate their capacity for neutralizing ancestral (B.1), Alpha (B.1.1.7), Beta (B.1.351), Delta (B.1.617.2) and Omicron (BA.1) variants.

Almost all serum samples positive to the RBD Inhibition ELISA were able to neutralize all variants, except few of them from which no nAbs were detected ($ID_{50} < 60$ WH1, Alpha, Beta, Delta; $ID_{50} < 20$ Omicron BA.1) (Figure 1). Thus, D2, which was infected by the Delta (B.1.617.2) variant, was able to neutralize all the other variants (Fernández-Bastit et al., 2021). On the other hand, C1 demonstrated to neutralize the ancestral lineage, from which it was infected. However, it was not tested against the other variants due to a limited volume of sera. The cat mate of C1 was able to neutralize all variants (No. 2 in Table S1).

TABLE 2 Results obtained by the RBD inhibition ELISA assay

	Cats		Dogs	Ferrets	Total
	Pet cats	Stray cats			
Seroprevalence/households					
COVID-19(+) %	23.80 (10/42)	–	8.99 (17/189)	40.00 (2/5)	11.86 (28/236)
COVID-19(–) %	2.04 (2/98)	–	1.89 (4/212)	0.00 (0/35)	1.74 (6/345)
<i>p</i> -value	<i>p</i> < .0001		<i>p</i> = .0030		
Unknown COVID-19 (%)	3.33 (1/30)	–	2.32 (1/43)	0 (0/7)	2.50 (2/80)
Total population %	7.65 (13/170)	2.34 (3/128)	4.95 (22/444)	4.26 (2/47)	50.63 (40/790)
	5.36 (16/298)				
Seroprevalence/gender					
Female %	4.27 (5/117)		3.86 (8/207)	0.00 (0/17)	3.81 (13/341)
Male %	6.66 (8/120)		6.40 (13/203)	8.70 (2/23)	6.65 (23/346)
<i>p</i> -value	<i>p</i> = .6005		<i>p</i> = .3462		
Gender non-determined	4.91 (3/61)		2.94 (1/34)	0.00 (0/7)	3.92 (4/102)

Note: The table shows seroprevalence of each species (cat, dog and ferret) according to the COVID-19 environment household and gender; *p*-value determined by chi-square Yate's correction test to analyse the relationship between seroprevalence and household, and seroprevalence and gender within each species. COVID-19-positive households were associated with seropositivity of cats ($p < .0001$) and dogs ($p = .0030$), whereas gender was not associated with seropositivity against SARS-CoV-2 neither for cats ($p = .6005$) nor for dogs ($p = .3462$).

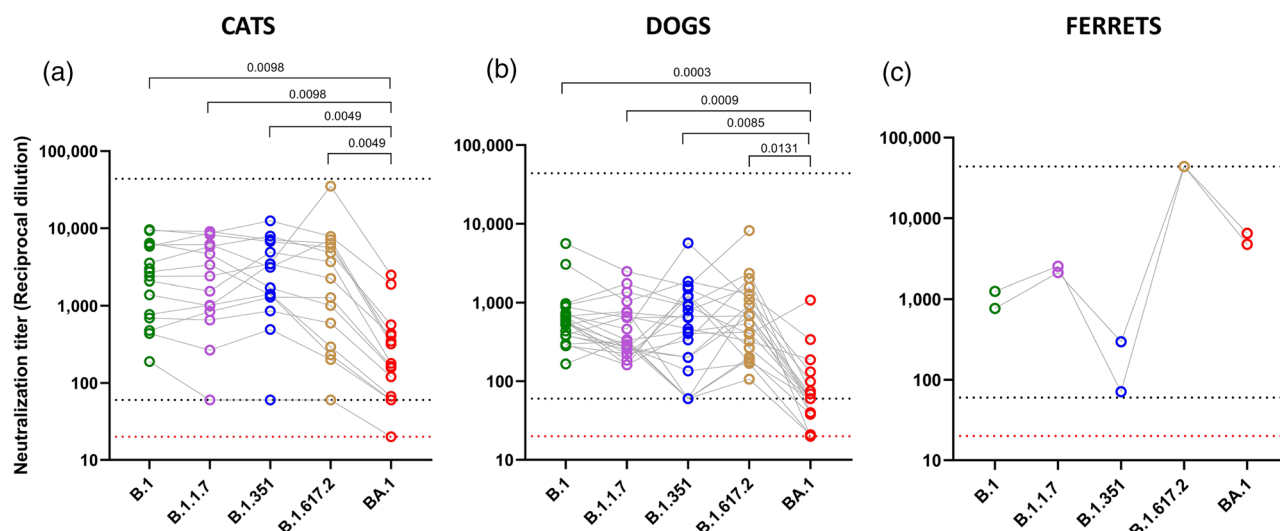


FIGURE 1 Neutralization titres for ancestral (B.1), Alpha (B.1.1.7), Beta (B.1.351), Delta (B.1.617.2) and Omicron (BA.1) variants of SARS-CoV-2 in (a) cats, (b) dogs and (c) ferrets. Three-fold serial dilutions of sera samples were performed to test all variants (1/20–1/43,740 BA.1 variant; 1/60–1/43,740 for the other variants). nAbs titres against different VOCs are represented as empty coloured circles. Grey lines connect the nAbs titres against different VOCs of individual samples. Black discontinuous lines indicate the maximum and minimum limits of quantification of the assay for all the variants; red discontinuous lines indicate the minimum limit of quantification for Omicron variant. Wilcoxon test with Bonferroni's correction test was used to compare titres of nAbs against the different variants in each species. Significant *p*-values ($< .05$) are indicated in each plot. Neutralization titres were expressed in ID₅₀ (reciprocal dilution).

No statistically significant differences were observed between titres of nAbs against ancestral (B.1), Alpha (B.1.1.7), Beta (B.1.351) and Delta (B.1.617.2) variants (according to the Wilcoxon test with Bonferroni's correction test) in either cats or dogs (Figure 1). In contrast, both cats and dogs had statistically lower titres against the Omicron (BA.1) variant compared to all the other variants. In the case of ferrets, statis-

tical analyses of humoral responses could not be performed since only two samples were positive.

Additionally, we compared titres of nAbs from all positive samples between three different periods established according to the main pandemic waves in Catalonia (Spain) (Troyano-Hernández et al., 2022) (Figure S1). The first period was established from March 2020 to

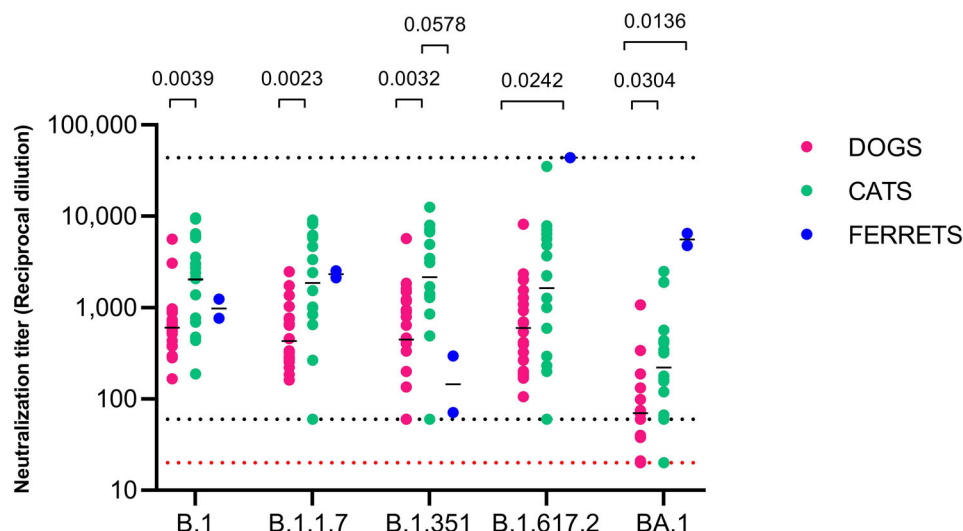


FIGURE 2 Titres of neutralizing antibodies for ancestral (B.1), Alpha (B.1.1.7), Beta (B.1.351), Delta (B.1.617.2) and Omicron (BA.1) in dogs ($n = 22$), cats ($n = 16$) and ferrets ($n = 2$). Discontinuous lines indicate the maximum and minimum limit of quantification of the assay for all the variants; red discontinuous lines indicate the minimum limit of quantification for Omicron variant. Bars indicate the geometric mean titre in each group; p -values show the significant differences of titres of nAbs among species (Kruskal–Wallis test with Dunn’s multiple comparison test).

December 2020, mainly dominated by the ancestral (B.1) variant; the second period was considered from January 2021 to July 2021, where the Alpha (B.1.1.7) variant was the most prevalent; finally, the third period was dated from June 2021 to January 2022 mainly dominated by the Delta (B.1.617.2) variant and also by the Omicron (BA.1) variant at the end (from November to December 2021 onwards). The second and third periods were overlapped since Alpha (B.1.1.7) and Delta (B.1.617.2) variants predominated together in Spain during June and July 2021. Significant higher titres of nAbs were observed against the ancestral variant in the second period than in the third period ($p = .0249$). No other significant differences were observed between periods.

Next, we compared titres of nAbs between species (Figure 2). Cats showed significantly higher neutralizing titres against all variants compared to dogs, except for the Delta (B.1.617.2). Ferrets showed significant lower titres for the Beta (B.1.351) VOC compared to cats and higher titres against the Delta (B.1.617.2) and Omicron (BA.1) VOCs compared to dogs.

Correlation analyses were performed using the results obtained from the RBD Inhibition ELISA (% Inhibition) and the results obtained from the pseudovirus-neutralization assays (ID_{50}) (Figure S2). A significant positive correlation between the percentage of inhibition and the neutralization titres was observed using all different pseudoviruses expressing the S protein of the ancestral (B.1, $r = 0.7775$), Alpha (B.1.1.7, $r = 0.7251$), Beta (B.1.351, $r = 0.7078$), Delta (B.1.617.2, $r = 0.6159$) and Omicron (BA.1, $r = 0.6253$) variants. A higher correlation of the RBD Inhibition ELISA assay with the pseudotype expressing the S protein from the B.1 variant (Figure S2) could be explained by the recombinant RBD used in the commercial kit, which has the sequence of the ancestral variant firstly detected in Wuhan (pango lineage A).

4 | DISCUSSION

Since the beginning of the COVID-19 pandemic, many studies have been performed to determine the incidence of infection and seroprevalence in pets, as well as to know their role in the epidemiology of the disease (Barroso-Arévalo et al., 2022; Hamer, Pauvolid-Corrêa, et al., 2021; Patterson et al., 2020). The present work stands out since it is the first large-scale study on SARS-CoV-2 infection in pets and stray cats performed in the North-Eastern of Spain. Additionally, this work included for the first time the study of the humoral immune response of a large series of stray cats and pet animals against different VOCs.

In our study, a very low percentage of SARS-CoV-2 actively infected animals was found (0.3%), corresponding to one pet cat (C1) and two dogs (D1 and D2). Interestingly, we determined that C1 was infected on April 2020 with the D614G variant (Segalés et al., 2020), D1 was infected on February 2021 with the Alpha (B.1.1.7) variant, whereas D2 was infected on July 2021 with the Delta (B.1.617.2) variant (Fernández-Bastit et al., 2021). The period in which animals were infected was in accordance with the period in which each variant causing infection was the predominant variant in Spain (Troyano-Hernández et al., 2022). Since there is evidence that all of them were living with COVID-19-affected owners, SARS-CoV-2 transmission from humans to the animals was strongly suspected (Fernández-Bastit et al., 2021; Segalés et al., 2020). These results are consistent with previous reports, since the majority of the natural infections in pets have been described in animals living in COVID-19-positive households (Garigliany et al., 2020; Ruiz-Arrondo et al., 2021; Sit et al., 2020). In some cases, human-to-pet transmission has been evidenced by genomic and sequencing analysis (Barrs et al., 2020; Hamer, Ghai, et al., 2021; Hosie et al., 2021), as the case of C1, included in this case series (Segalés et al., 2020). Reverse transmission has also been shown in other animal

species, such as in zoo animals as large felines and non-human primates (Fernández-Bellón et al., 2021) and farm minks (Munnink et al., 2021), and suggested in wild animals as white-tailed deer (Kuchipudi et al., 2022). Of note, evidence of SARS-CoV-2 adaptation and the appearance of new SARS-CoV-2 strains occurred in farm minks, which were subsequently transmitted to humans (Munnink et al., 2021), cats and dogs (Van Aart et al., 2021). RT-qPCR-positive dogs in the present study corresponded to pet animals that were not in contact with other animals, while only C1 was in contact with another cat mate, suggesting that no further spread of SARS-CoV-2 was possible.

Considering the large number of samples in our study, we confirmed a similar low incidence of SARS-CoV-2 infection in cats (0.26%) and dogs (0.36%), consistent with other references (Bienzle et al., 2022; Patterson et al., 2020). Although none of the ferrets tested positive by RT-qPCR in the present study, the detection of SARS-CoV-2 RNA has been already demonstrated under natural conditions in kept ferrets (Gortázar et al., 2021). Negative results in this species could be partially explained because ferrets do not have the closest contact with their owners as dogs or cats usually have. Another study performed in Spain by Barroso-Arévalo et al. (2022) showed higher viral RNA prevalence of infection in cats and dogs, albeit still low, with values of 1.63% and 2.59%, respectively. It is important to highlight that, in our study, a high number of animals got SARS-CoV-2 infection in the past based on serological results. This should not be surprising even for those animals living in COVID-19-positive households, since samples were usually collected days or even weeks after the direct contact between the animal and the owners due to the mandatory quarantines established for positive humans. Therefore, a large number of sampled animals that were positive to nAbs had already cleared the virus at the time of sampling, in agreement with the fact that a short RNA shedding period has been shown in previous studies (Neira et al., 2020). This also may partially explain the low viral load found in C1 and D2, since they could be overcoming the infection at the time samples were collected (Fernández-Bastit et al., 2021; Segalés et al., 2020). In contrast, high viral loads were found in nasal and rectal swabs of D1, although viral isolation was not successful. However, SARS-CoV-2 isolation has been achieved from swabs collected during natural and experimental infections of cats, dogs and ferrets, demonstrating infectious viral shedding in these species (Barroso-Arévalo et al., 2022; Gortázar et al., 2021; Hamer, Ghai, et al., 2021; Kim et al., 2020; Shi et al., 2020). Also, considering that both natural and experimental SARS-CoV-2 infections in pet animals have mostly caused subclinical infections so far (Hamer, Pauvolid-Corrêa, et al., 2021; Kim et al., 2020; Ruiz-Arrondo et al., 2021; Sánchez-Morales et al., 2022), it is rather difficult to suspect the right timing of active SARS-CoV-2 infection. In some cases, SARS-CoV-2 infections in pets caused mild clinical signs, mainly respiratory (coughing, sneezing) and digestive (diarrhoea, vomiting) (Fritz et al., 2021; Hamer, Ghai, et al., 2021; Kim et al., 2020), as we observed in the case of D2 (Fernández-Bastit et al., 2021). Although comorbidities contribute to the development of moderate or severe disease in humans (Yang et al., 2020), this has not been demonstrated in pets. In fact, C1 and D1 were sacrificed due to their worsening clinical status (Segalés et al., 2020), but apparently not associated with SARS-CoV-2

infection since no lesions attributable to the viral infection were found. However, infections caused by the Alpha (B.1.1.7) variant in dogs and cats have been tentatively associated with myocarditis (Ferasin et al., 2021). In any case, it is not clear whether SARS-CoV-2 infection in pets may worsen a previous disease or it is just a subclinical infection concomitant to pre-existing condition.

Due to abovementioned reasoning, serum sample collection was essential in this study, since presence of nAbs could support SARS-CoV-2 past infections of pet animals. Globally, we detected evidence of seroconversion in 7.65% of pet cats, 4.95% of dogs and 4.26% of ferrets, similar to other authors (Giner et al., 2021; Patterson et al., 2020; Zhang et al., 2020). We observed that seroprevalences were significantly higher in pets living in households with COVID-19-affected owners compared to those of COVID-19 negative households, which confirmed their major risk of virus exposure, as other authors have found (Barroso-Arévalo et al., 2022; Fritz et al., 2021; Hamer, Pauvolid-Corrêa, et al., 2021; Patterson et al., 2020; Zhang et al., 2020). Since groups of the study were classified from data provided by the owners, seroconversion observed in cats (2.04%) and dogs (1.89%) from COVID-19-negative households could be attributed to the pet exposure to SARS-CoV-2-infected asymptomatic or non-diagnosed owners. Besides, previous studies already demonstrated that stray cats can be exposed to SARS-CoV-2 infection, as evidenced by the detection of specific antibodies (Spada et al., 2021; Villanueva-Saz et al., 2022), as we observed in 2.35% of stray cats. These cats could have been in contact with SARS-CoV-2-contaminated environment or with infected humans who took care of them. SARS-CoV-2 transmission between cats has been demonstrated experimentally (Shi et al., 2020) and the probability of transmission in stray cats is high since they usually live forming colonies composed of hundreds of individuals. This led to consider them as a potential group of concern for the spread and maintenance of SARS-CoV-2; further studies would be needed to confirm this aspect. Noteworthy, Hancock et al. (2022) found seropositivity to the RBD in pre-pandemic feline samples, speculating cross-reactivity with some other etiological agent. However, there is evidence that the RBD is the main immunogenic target of SARS-CoV-2 that shows very low similarity with other coronaviruses (Premkumar et al., 2020). On the other hand, in the present study we did not observe correlation between the risk of infection and the gender of pets in agreement to the study of Pomorska-Mól et al. (2021) performed in Poland and also to what is observed in SARS-CoV-2 infection in humans (Scully et al., 2020).

As a novel insight of the present study, we demonstrated in a large series of sera that nAbs found in cats, dogs and ferrets can neutralize different VOCs of SARS-CoV-2. Our results indicated lower titres of nAbs against the Omicron (BA.1) variant and similar titres against the ancestral, Alpha, Beta and Delta variants within cats and dogs. The pseudoviral neutralization assay detected specifically nAbs against the whole S protein and previous phylogenetic analysis based on S genomic sequence evidenced that Omicron subvariants are the most distant variants in relation to the other SARS-CoV-2 variants (Yang et al., 2021). Furthermore, Omicron variants exhibit the mutation E484A which is associated with reduced recognition of nAbs, contributing to

immune scape (Marchi et al., 2022), which would help to explain the obtained results in animals as well. In addition, we must consider that most of the samples were collected before the Omicron wave started in Catalonia (November–December 2021); thus it is highly likely that they were not infected by this variant. Apparently, we observed a tendency of the positive sera to exhibit higher titres against the variant that predominated in Catalonia (Troyano-Hernández et al., 2022) at sampling, compared to other variants. Interestingly, both positive ferrets had highest titres against the Delta variant, which was the variant that predominated in Spain at the time that they were sampled (July 2021). These two ferrets were living at the same COVID-19-positive household, which could explain their similar capabilities of neutralizing responses against the different SARS-CoV-2 variants. Since ferret-to-ferret transmission has been demonstrated experimentally (Kim et al., 2020), we cannot confirm whether the viral transmission took place between positive ferrets or from the owners to each ferret. On the other hand, those dogs living together at the same COVID-19-positive household (No. 462 and No. 463 from the Table S1) exhibited different humoral responses against SARS-CoV-2, although they were sampled at the same time (June 2021). One of them showed high levels of nAbs, whereas its dog mate had low levels of nAbs against all the VOCs SARS-CoV-2. Interestingly, the animal with higher titres of nAbs against the Omicron (BA.1) variant corresponded to a cat which had direct contact with a positive owner on December 2021 and was sampled on January 2022, according to the period dominated by the Omicron variant among humans in Spain (Troyano-Hernández et al., 2022). Besides the previous comments, we cannot exactly confirm to which variant the seropositive animals were exposed since they tested negative in RT-qPCR, except for C1, D1 and D2. Importantly, the possibility that nAbs titres have been reduced, or even lost, in some animals cannot be discarded due to the time interval between the potential infection date and sample collection. At least in cats, a previous study showed that peak titres of nAbs are at 10 days after the infection and decreased to the limit of detection within 110 days (Zhang et al., 2020). Our study showed an overall higher capacity of SARS-CoV-2 neutralization in cats compared to dogs. In humans, higher levels of nAbs have been related to the severity of COVID-19 (Trinité et al., 2021). Although significant clinical disease seems to be sporadic in pets, different SARS-CoV-2 susceptibility at species level (Bosco-Lauth et al., 2020; Shi et al., 2020) may explain differences in humoral responses between species. Viral shedding and tissue tropism have been demonstrated in both cats and ferrets experimentally, whereas non-viral shedding and non-viral replication have been shown in dogs (Bosco-Lauth et al., 2020; Shi et al., 2020). Anyways, the differences in susceptibility to SARS-CoV-2 among animal species are not fully understood. Another related factor may be the presence and/or distribution of the ACE2 host cell receptor in these species, as well as the binding affinity between the spike of SARS-CoV-2 and their ACE2 receptors (Lean et al., 2021). Low ACE2 levels in the respiratory tract (lung, trachea and turbinate) from dogs could prevent efficient SARS-CoV-2 replication, whereas high levels of ACE2 in the respiratory tract from cats and ferrets (Zhai et al., 2020) may account for a more efficient viral replication in these species.

In summary, we confirmed direct and indirect evidence of SARS-CoV-2 infection by different VOCs in cats, dogs and ferrets from North-Eastern of Spain. Although the prevalence of active infection was low, the presence of nAbs in higher at-risk pets (from COVID-19 households) was relatively high (close to 25% in cats, 10% in dogs and 40% in ferrets). Our results highlight the importance to continue monitoring pet animals since we cannot discard the possibility that new potential SARS-CoV-2 variants could increase their susceptibility. A coordinated 'one health' approach should help in preventing the appearance of new animal reservoirs, new VOCs and a major spread of SARS-CoV-2 not only in humans and pets, but also in livestock and wildlife.

AUTHOR CONTRIBUTIONS

Benjamin Trinité, Julià Blanco, Júlia Vergara-Alert and Joaquim Segalés conceived and designed the study. Rosa Valle, Núria Roca and Jaume Martorell performed sample taking and processing. Leira Fernández-Bastit, Silvia Marfil, Edwards Pradenas, Rosa Valle, Núria Roca, Jordi Rodon, Mariona Parera and Marc Noguera-Julian performed the laboratory studies. Leira Fernández-Bastit, Lola Pailler-García, Benjamin Trinité, Marc Noguera-Julian, Nuria-Izquierdo-Useros, Jorge Carrillo, Bonaventura Clotet, Julià Blanco, Júlia Vergara-Alert and Joaquim Segalés analysed the data. Leira Fernández-Bastit wrote the paper. All authors discussed the results and commented on the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are already available in Table S1.

ETHICAL APPROVAL

The authors confirm that the ethical policies of the journal have been adhered to and the appropriate ethical review committee approval has been received.

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SUPPORTING INFORMATION

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