A cross-sectional serosurvey of SARS-CoV-2 and co-infections in stray cats from the second wave to the sixth wave of COVID-19 outbreaks in Spain

Sergio Villanueva-Saz[1,2,3] · Mariví Martínez[1,2] · Jacobo Giner[1,2] · Ana González[1,4] · Ana Pilar Tobajas[3,5] ·
María Dolores Pérez[3,5] · Erandi Lira-Navarrete[6] · Andrés Manuel González-Ramírez[6] · Javier Macías-León[6] ·
Maite Verde[1,3,4] · Andrés Yzuel[1] · Ramón Hurtado-Guerrero[6,7,8,9,10] · Maykel Arias[11,12] · Llipsy Santiago[11,12] ·
Jordi Aguiló-Gisbert[13] · Héctor Ruíz[2] · Delia Lacasta[2,3] · Diana Marteles[1] · Antonio Fernández[1,2,3]

Abstract

Severe Acute Respiratory Syndrome Coronavirus 2 is the causative agent of Coronavirus Disease 2019 in humans. Among domestic animals, cats are more susceptible to SARS-CoV-2 than dogs. The detection of anti-SARS-CoV-2 antibodies in seemingly healthy cats and/or infected cats which are in close contact with infected humans has been described. The presence of animals that tested positive by serology or molecular techniques could represent a potential transmission pathway of SARS-CoV-2 that can spill over into urban wildlife. This study analyses the seroprevalence variation of SARS-CoV-2 in stray cats from different waves of outbreaks in a geographical area where previous seroepidemiological information of SARS-CoV-2 was available and investigate if SARS-CoV-2-seropositive cats were exposed to other co-infections causing an immunosuppressive status and/or a chronic disease that could lead to a SARS-CoV-2 susceptibility. For this purpose, a total of 254 stray cats from Zaragoza (Spain) were included. This analysis was carried out by the enzyme-linked immunosorbent assay using the receptor binding domain of Spike antigen and confirmed by serum virus neutralization assay. The presence of co-infections including *Toxoplasma gondii*, *Leishmania infantum*, *Dirofilaria immitis*, feline calicivirus, feline herpesvirus type 1, feline leukemia virus and feline immunodeficiency virus, was evaluated using different serological methods. A seropositivity of 1.57% was observed for SARS-CoV-2 including the presence of neutralizing antibodies in three cats. None of the seropositive to SARS-CoV-2 cats were positive to feline coronavirus, however, four SARS-CoV-2-seropositive cats were also seropositive to other pathogens such as *L. infantum*, *D. immitis* and FIV (n = 1), *L. infantum* and *D. immitis* (n = 1) and *L. infantum* alone (n = 1).Considering other pathogens, a seroprevalence of 16.54% was detected for *L. infantum*, 30.31% for *D. immitis*, 13.78%, for *T. gondii*, 83.86% for feline calicivirus, 42.52% for feline herpesvirus type 1, 3.15% for FeLV and 7.87% for FIV.

Our findings suggest that the epidemiological role of stray cats in SARS-CoV-2 transmission is scarce, and there is no increase in seropositivity during the different waves of COVID-19 outbreaks in this group of animals. Further epidemiological surveillances are necessary to determine the risk that other animals might possess even though stray cats do not seem to play a role in transmission.

Keywords COVID-19, stray cats · ELISA · SARS-CoV-2 · Serology · VNT

Introduction

SARS-CoV-2 is a zoonotic betacoronavirus and the causative agent of a new coronavirus disease 2019 (COVID-19). This zoonotic coronavirus is capable of infecting different animals including domestic and wildlife animals such felids and mustelids (Haider et al. 2020; Shi et al. 2020; Tiwari et al. 2020).

Evidence from different sources confirms the susceptibility of cats to SARS-CoV-2 infection (Gaudreault et al. 2020; Barroso-Arévalo et al. 2021). Considering in vitro

---

Extended author information available on the last page of the article

findings using *in silico* analysis, cat angiotensin converting enzyme 2 (ACE 2), the natural receptor for SARS-CoV-2 cell colonization, showed an important affinity to SARS-CoV-2 spike protein (Piplani et al. 2021). Moreover, different studies indicate that SARS-CoV-2 is capable of infecting cats, showing a high infectivity capacity and the detection in some cases of lesions and signs of the disease (Opriessnig and Huang 2020; Shi et al. 2020). Detection of seropositive or/and infected cats have been described under natural conditions in different situations such as epidemiological studies and a compilation of case reports. Cat-to-cat transmission has been also demonstrated under experimental conditions (Bosco-Lauth et al. 2020; Shi et al. 2020). Generally, the absence of prominent clinical signs together with a high susceptibility to virus colonisation could have significant public health repercussions such as perpetuation of SARS-CoV-2 as potential animal reservoir with the possibility of new SARS-CoV-2 variants emerging, as well as the adaptation of the virus to a new host (Burkholz et al. 2021) or the transmission of the new variants from humans to cats (Curukoglu et al. 2021; Ferasin et al. 2021; Keller et al. 2021).

The detection of seropositive cats to SARS-CoV-2 in different regions of Europe and other countries including household and stray cats has been reported. The epidemiological role of domestic, stray and colony cats in Europe and other countries has been analyzed in different studies including: China (Deng et al. 2020; Zhang et al. 2020), France (Fritz et al. 2020), Italy (Spada et al. 2021), Spain (Villanueva-Saz et al. 2021a), Germany (Michelitsch et al. 2020), Switzerland (Kuhlmeier et al. 2022), Turkey (Yilmaz et al. 2021) and other european countries (Adler et al. 2022).

Among cats, strays are a type of animal that have an environmental impact due to the interaction with other urban and periurban animals including birds, small mammals, and other suburban native wildlife (Hall et al. 2016; Lilith et al. 2006). From the point of view of public health, human-animal interface occurs, and reports have confirmed human-to-cat SARS-CoV-2 transmission (Curukoglu et al. 2021; Pagini et al., 2021).

However, these cats do not receive the same veterinary and preventive cares as domestic cats, resulting in a serious health issue. In this sense, our hypothesis would be that stray cats with concomitant diseases associated to immunosuppressive status and/or chronic diseases could be more susceptible to SARS-CoV-2 and the number of SARS-CoV-2-seropositive cats should increase over time during the different waves of COVID-19 outbreaks due to an increase in the number of people infected by SARS-CoV-2 virus. The monitoring and evaluation of the impact of the different waves of SARS-CoV-2 infection in domestic animals should be conducted based on One Health approach,

Table 1 Number of samples collected and collection time

Number waves of COVID-19 outbreak	Collection time	Number of samples collected
Second wave	October 2020	14
	November 2020	21
	December 2020	15
	Total of samples	50
Third wave	January 2021	17
	February 2021	16
	March 2021	22
	Total of samples	55
Fourth wave	April 2021	15
	May 2021	18
	June 2021	20
	Total of samples	53
Fifth wave	July 2021	15
	August 2021	12
	September 2021	9
	October 2021	18
	November 2021	21
	Total of samples	75
Sixth wave	December 2021	9
	January 2022	12
	Total of samples	21
Total of samples		254

especially in those animals in close contact with people (owners and colony caregivers).

The aims of the present study were: (1) to evaluate the seroprevalence variation of SARS-CoV-2 in stray cats from the second to the sixth waves of outbreaks in Spain in a geographical area where previous seroepidemiological information of SARS-CoV-2 in stray cats was available; (2) to investigate if SARS-CoV-2-seropositive cats were exposed to other co-infections (*Toxoplasma gondii*, *Leishmania infantum*, feline calicivirus (FCV), feline herpesvirus type 1 (FHV-1), feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV), *Dirofilaria immitis*) causing an immunosuppressive status and/or a chronic disease that could lead to a SARS-CoV-2 susceptibility.

Materials and methods

Study area, sampling and data collection

The study was carried out in the city of Zaragoza (41° 38' 58.8948'' N and 0° 53' 15.7632'' W, Aragon region, Spain) from the second half of October 2020 to the first half of January 2022 (Table 1). The study population comprised 254 stray cats captured in urban areas of Zaragoza within a trap, neuter, and release sterilization program run locally

to control stray feline colonies. Samples from unvaccinated stray cats were collected based on previously published seroepidemiological study (Villanueva-Saz et al. 2021a).

Samples were obtained during the different waves of COVID-19 outbreak from the second to the sixth waves (Table 1) in Spain. This survey was included under Project License PI62/17 approved by the Ethic Committee for Animal Experiments for the University of Zaragoza.

Expression and purification of receptor binding domain (RBD) of spike

The DNA sequence encoding amino acid residues 319–541 (RVQPTESIVRFPNITNLCPFGEVFNATRFASVY-AWNRKRISNCVADYSVLYNSASFSTFKCYGVSPT-KLNDLCFTNVYADSFVIRGDEVRQIAPGQTGKI-ADYNYKLPDDFTGCVIAWNSNNLDSKVGGNYNY-LYRLFRKSNLKPFERDISTEIYQAGSTPCNGVEG-FNCYFPLQSYGFQPTNGVGYQPYRVVVLSFELL-HAPATVCGPKKSTNLVKNKCVNF) of the RBD was codon optimized and synthesized by Gen-Script (USA) for expression in HEK293 cells. The DNA, containing at the 5′-end a recognition sequence for KpnI, and at the 3′end a stop codon and a recognition sequence for XhoI, was cloned into a modified pHLSec containing after the secretion signal sequence a 12xHis tag, a superfolder GFP and a Tobacco Etch Virus (TEV) cleavage site, rendering the vector pHLSec-12His-GFP-TEV-SRBD. Both the synthesis of the RBD construct and the engineered pHLSec together with the cloning of RBD into pHLSec-12His-GFP-TEV were performed by GenScript. pHLSec-12His-GFP-TEV-RBD was transfected into HEK293F cell line (Thermo Fisher Scientific) as described below. Cells were grown in suspension in a humidified 37 °C and 8% CO2 incubator with rotation at 125 r.p.m. Transfection was performed at a cell density of 2.5×10^6 cell/mL in fresh F17 serum-free media with 2% Glutamax and 0.1% P188. For each 150 mL of culture, 450 μg of the plasmid (1 μg/μL) was diluted to 135 μL with sterilized 1.5 M NaCl. This mixture was added to each 150 mL cell culture flask and incubated for 5 min in the incubator. After that, 1.35 mg of PEI-MAX (1 mg/mL) was mixed to 135 μL with sterilized 1.5 M NaCl and added to the cell culture flask. Cells were diluted 1:1 with pre-warmed media supplemented with valproic acid 24 h post-transfection to a final concentration of 2.2 mM. Cells were harvested 6 days post-transfection by spinning down at 300 ×g for 5 min, after which the supernatants were collected and centrifuged at 4,000 ×g for 15 min. Supernatant was dialyzed against buffer A (25 mM TRIS pH 7.5, 300 mM NaCl) and loaded into a His-Trap Column (GE Healthcare). Protein was eluted with an imidazol gradient in buffer A from 10 mM up to 500 mM. Buffer exchange to 25 mM TRIS pH 7.5, 150 mM NaCl (buffer B) was carried out using a HiPrep 26/10 Desalting Column (GE Healthcare). TEV protease was then added in a ratio 1:50 (TEV:RBD) to the fusion construct in order to cleavage the His-GFP. After 20 h of reaction at 18 °C, the cleavage was satisfactorily verified through SDS-PAGE. TEV protease and GFP were removed from the solution using a His-TrapColumn (GE Healthcare), and the SRBD was collected from the flow-through. Quantification of protein was carried out by absorbance at 280 nm using the theoretical extinction coefficient, ε280 nm(RBD) = 33,350 M^{-1} cm^{-1}.

Detection of SARS-CoV-2 antibodies by in-house ELISA

Antibodies to SARS-CoV-2 were determined by an indirect ELISA for the detection of IgG specific for RBD (Giner et al. 2021; Villanueva-Saz et al. 2022a). Ninety-six–well plates were coated overnight, at 4 °C with 50 μl/well of RBD protein at 1 μg/ml in phosphate buffered saline (PBS). Subsequently, the coating solution was removed and the plate was washed three times with 200 μL per well of PBS containing 0.05% Tween 20 (PBST). After, 300 μl of PBST with 3% dry skimmed milk was added to each well as blocking solution. The plates were incubated with blocking solution for 1 h at 37 ºC in a moist chamber. 100 μl of cat sera, diluted 1:100 in PBST and 1% dry skimmed milk (PBST-M), was added to each well. The plates were incubated for 1 h at 37 °C in a moist chamber. After washing the plates for 30 s 6 times with PBST followed by 1 wash with PBS for 1 min, 100 μl/well of multi-species horseradish peroxidase conjugate (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was added per well. The plates were incubated for 1 h at 37 °C in the moist chamber and were washed again with PBST and PBS as described above. The substrate solution (ortho-phenylene-diamine) and stable peroxide substrate buffer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was added at 100 μl per well and developed for 20 ± 5 min at room temperature in the dark. The reaction was stopped by adding 100 μl of 2.5 M H_2SO_4 to each well. Absorbance values were read at 492 nm. in an automatic microELISA reader (Microplate Photometer Biosan Hipo MPP-96, Riga, Latvia). As a positive control, each plate included serum from a human patient diagnosed with COVID, confirmed by a molecular test and a commercial quantitative ELISA, and serum from a healthy, non-infected cat obtained prior to pandemic COVID-19 situation as negative control. The same positive and negative sera were used for all assays and plates, with a constant inter-assay variation of < 10%. Plates with an inter-assay variation of > 10% were discarded. All samples were run in duplicate. The cutoff was set to 0.30 Optical Density units (OD units) (mean + 3

standard deviations of values from 92 cats obtained prior the COVID-19 situation in 2015) and the results above this value were considered positive.

Micro-neutralization assay of SARS-CoV-2

SARS-CoV-2 virus used was isolated from a COVID-19 patient at the Hospital Clínico Lozano Blesa (Zaragoza, Spain). Virus identity was confirmed by real-time PCR, electron microscopy, and RNA sequencing and classified as B1.1 linage (Rambaut et al. 2020). The virus was titrated in serial 1 log dilutions to obtain the 50% tissue culture infectious dose (TCID50) per mL using VERO-E6 cultures in 96-well plates. The 50% endpoint titers were calculated according to the Ramakrishnan simple formula based on eight replicates per point for titration (Ramakrishnan 2016).

Serum samples were heat-inactivated for 30 min at 56 °C and two-fold serial dilutions, starting from 1:20 and were mixed with 500 TCID50 of SARS-CoV-2. The serum-virus mixture was incubated for 1 h at 37 °C and 5% CO2 and then 100 µl were added by duplicate to a 96-well plate containing a semi-confluent Vero E6 monolayer whose supernatant had been previously discarded. Positive and negative controls using the virus or serum/plasma alone, respectively, were used. Plates were incubated for 72 h at 37 °C and 5% CO2. Cytopathic effect (CPE) was studied using an inverted optical microscope. The neutralization ID50 was calculated as the highest dilution that protected more than 50% of the wells from CPE. This test was performed in serum samples that tested positive in the in-house ELISA for SARS-CoV-2 antibody detection as complementary technique.

Detection of T. gondii antibodies by in-house IFAT

For IFAT, the antigen was obtained as described previously (Goldman 1957). Briefly, purified tachyzoites were resuspended in 0.2% (v/v) formalin PBS and adjusted to a concentration of 10^7parasites/ml. Whole formalin-fixed tachyzoites were aliquoted and stored at $-20°C$ until use. For the detection of antibodies to *T. gondii* the sera diluted 1/32 and 1/64 in PBS. Briefly, 20 µl of each serum dilution was applied per well. The slides were incubated for 30 min at 37 °C in a moist chamber, and then washed twice with PBS for 5 min and once more with distilled water. After the washing procedure, 20 µl of goat anti-cat IgG-fluorescein isothiocyanate conjugate (SIGMA, Saint Louis, Missouri, USA) diluted 1:64 in 0.2% Evans blue was added to each well. The slides were incubated in a moist chamber at 37ºC for another 30 min in complete darkness and washed again as described above. After the second washing procedure, a few drops of mounting medium were placed on the cover slips. The slides were examined under a fluorescence microscope

(Leica DM750 RH; Leica Microsystems, Wetzlar, Germany) at 400× magnification and each well was compared to the fluorescence pattern seen in the positive (tachyzoites show a bright, sharp and clear, yellow-green fluorescence on their membranes) and negative controls (tachyzoites show a greyish-dark red colour lacking any clear fluorescence). Positive and negative controls were included on each slide. A positive control serum was obtained from a cat from Spain diagnosed with *T. gondii* in experimental condition, and a negative control serum was obtained from a healthy, non-infected indoor cat. The cut-off value for positive sera was 1:64.

Detection of L. infantum antibodies by in-house quantitative ELISA

The ELISA was performed on all sera as described previously (Villanueva-Saz et al. 2022b), with some modifications. Briefly, each plate was coated with 100 µl/well of 20 µg/ml antigen extracted from a sonicated *L. infantum* promastigote culture (MHOM/MON-1/LEM 75) in 0.1 M carbonate/bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C. Plates were then frozen and stored at -20 °C. 100 µl of cat sera, diluted 1:200 in PBST and 1% dry skimmed milk (PBST-M), was added to each well. The plates were incubated for 1 h at 37 °C in a moist chamber. After washing the plates for 3 min 3 times with PBST followed by 1 wash with PBS for 1 min, 100 µl of Protein A conjugated to horseradish peroxidase (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was added per well. The plates were incubated for 1 h at 37 °C in the moist chamber and were washed again with PBST and PBS as described above. The substrate solution (ortho-phenylene-diamine) and stable peroxide substrate buffer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was added at 100 µl per well and developed for 20 ± 5 min at room temperature in the dark. The reaction was stopped by adding 100 µl of 2.5 M H_2SO_4 to each well. Absorbance values were read at 492 nm in an automatic microELISA reader (Microplate Photometer Biosan Hipo MPP-96, Riga, Latvia). As a positive control (calibrator), each plate included serum from a cat from Spain diagnosed with FeL, confirmed by a positive *L. infantum* isolation using a NNN medium, and as a negative control, serum from a healthy, non-infected cat. The same calibrator serum was used for all assays and plates, with a constant inter-assay variation of <10%. Plates with an inter-assay variation of >10% were discarded. All samples and controls were run in duplicate. The results were quantified as ELISA units (EU) compared to the positive control serum used as a calibrator and arbitrarily set at 100 EU. The cut-off was established at 13 EU (mean+3 standard deviations of values from 50 indoor cats from northern Spain, considered

a non-endemic area) and the results above this value were considered positive.

Detection of D. immitis antibodies by in-house quantitative ELISA

The ELISA was performed on all sera as described previously (Villanueva-Saz et al. 2021), with some modifications. In brief, the plates were coated with 0.5 µg of D. immitis pepsin inhibitor Dit33 (DIT33) recombinant protein. Serum samples were prepared at 1/100. Anti-feline IgG antibody, horseradish peroxidase-labelled (Bethyl laboratories, Montgomery, USA), was applied at 1/20,000 dilution. The optical densities were measured in a microplate reader (ELISA Reader Labsystems Multiskan, Midland, Canada) at 450 nm. Each plate included a positive control, a cut-off control and a negative control. All samples and controls were analyzed in duplicate. The results were analyzed as OD450 (Optical Density 450) compared to the cut-off control used. For each sample a ratio has been calculated according to following formula:

Ratio = $(OD_{sample} - OD_{blank})/(OD_{cut-off} - OD_{blank})$.

Samples presenting a ratio greater than or equeal to 1.0 were classified as positive, less than 1.0 were considered negative. The in-house ELISA was validated using 12 sera infected by *D. immitis* from an experimental study. The sera were provided by TRS Labs (GA, USA), These samples contain a variable number of female and/or male worms. Moreover, they were evaluated for three different commercially available tests including two antigen tests: Uranotest Dirofilaria® (Urano Vet SL, Barcelona, Spain) and Filarcheck® (Agrolabo Spa, Scarmagno, Italy) and, one antibody test (Solo Step® FH) with a positive result for all tests. Each test used in our study was performed by a different researcher without knowledge of the results of the rest of the tests.

Detection of FCV antibodies by commercial IFAT

Indirect immunofluorescence tests for the detection of specific IgG antibody against feline calicivirus (MegaFLUO® FCV, Horbranz, Austria) were performed on sera following the instructions of the manufacturer. The slides were examined under a fluorescence microscope (Leica DM750 RH; Leica Microsystems, Wetzlar, Germany) at 400× magnification and each well was compared to the fluorescence pattern seen in the positive (the cytoplasm and membrane of the infected cells show a weak yellow-green fluorescence) and negative controls (there is no yellow-green fluorescence, or a weak red-greyish colour of the cells). Positive and negative controls were included on each slide. The cut-off value for positive sera was 1:40.

Detection of FHV-1 antibodies by commercial IFAT

The commercial IFAT (MegaFLUO® FVH, Horbranz, Austria) for detection of antibodies against this pathogen was performed on sera following the instructions of the manufacturer. The slides were examined under a fluorescence microscope (Leica DM750 RH; Leica Microsystems, Wetzlar, Germany) at 400× magnification and each well was compared to the fluorescence pattern seen in the positive (the cytoplasm and membrane of the infected cells show a weak yellow-green fluorescence) and negative controls (there is no yellow-green fluorescence, or a weak red-greyish colour of the cells). Positive and negative controls were included on each slide. The cut-off value for positive sera was 1:40.

Detection of FeLV antigens and FIV antibodies by immunochromatographic rapid test

The rapid test (Uranotest FeLV-FIV, URANOVET, Barcelona, Spain) was performed following the instructions of the manufacturer. All tests were stored at room temperature and were performed as described in the instructions supplied with the test kit.

Detection of Feline coronavirus (FCoV) antibodies by immunochromatographic rapid test

*FAST*est® FIP (MEGACOR Diagnostik, Hörbranz, Austria) is a rapid immunochromatographic test for the qualitative detection of antibodies against the FCoV in whole blood, plasma, serum and effusion of the cat. This rapid test was performed following the instructions of the manufacturer. All tests were stored at room temperature and were performed as described in the instructions supplied with the test kit. This test was performed in serum samples that tested positive in the in-house ELISA for SARS-CoV-2 antibodies detection.

Statistical analysis

Data collected for the entire population were analyzed using descriptive statistics. Univariate analysis of categorical data was performed to determine possible associations between SARS-CoV-2 positivity and the following variables: sex and seropositivity for *T. gondii*, *L. infantum*, *D. immitis*, FCV, FHV-1, FeLV, or FIV infection.

Equally, associations between variables (gender and pathogens detected) were analyzed. The significance of this difference was assessed using the Fisher's exact test. A $p \leq 0.05$ was considered significant. The SPSS v.22 software (SPSS Inc., Chicago, USA) was used.

Results

Epidemiological characterization of the animals

A total of 254 cats from an urban area were included in this study from the second half of October 2020 to the first half of January 2022 (Table 1). All of the tested cats were assessed as seemingly healthy, with no evident systemic signs found during the general physical examination prior to the surgical procedure. All animals were shorthaired type and more than one-year-old. One hundred and forty-two animals were females and the reaming 112 animals were males.

Serological prevalence of SARS-CoV-2 infection

The in-house quantitative ELISA revealed four positive samples (1.57%;

95% CI: 0.61–3.98), with OD units ranging from 1.35, 1.22, 0.62, and 0.48 (cutoff ≥ 0.30). The presence of antibodies anti-SARS-CoV-2 was detected in three males and one female cat. The seropositive samples were obtained at different waves of COVID-19 outbreaks: second (n = 1, November 2020), third (n = 2, February 2021 and March 2021), fourth (n = 1, April 2021) (Table 2). Among the four SARS-CoV-2 tested positive cats, three of them showed neutralizing antibodies (Table 3).

None of the seropositive to SARS-CoV-2 cats were positive to FCoV.

Serological prevalence of T. gondii infection

Thirty-five of the 254 cats tested were seroreactive by IFAT (13.78%; 95% CI: 10.08–18.56) including 15 males and 20 females (Table 2). The period of time with higher number of seropositive animals was during the third wave of COVID-19 outbreak (n = 13), followed by the fifth wave of COVID-19 outbreak (n = 10).

Serological prevalence of L. infantum infection

Among the 254 cats, 42 cats (22 males and 20 females) were positive by the in-house ELISA with a seroprevalence of 16.54% (95% CI: 12.47–21.60). Third and fifth waves of COVID-19 outbreak were the time periods with the highest number of seropositive cats, 20 and 10 animals, respectively.

Serological prevalence of D. immitis infection

The presence of antibodies against *D. immitis* was detected in 33 males and 44 females with a seroprevalence of 30.31% (95% CI: 21.60–34.99) with a variable number of seropositive animals among the different waves of COVID-19

outbreak with the lowest number during of sixth wave (n = 7) and the highest during the fifth wave (n = 30) (Table 2).

Serological prevalence of viral infection caused by respiratory pathogens FCV and FHV-1

Two hundred and thirteen (93 males and 120 females) of the 254 cats had a positive result for FCV with a seroprevalence of 83.86% (95% CI: 79.33–88.38). For FHV-1, 108 cats (48 males and 60 females) with a seroprevalence of 42.52% (95% CI:33.25–45.60). The presence of seropositive animals for respiratory viruses were detected among the different waves of COVID-19 outbreak (Table 2).

Serological prevalence of viral infection caused by FeLV and FIV

A total of 8 cats (4 males and 4 females) were seropositive by the immunochromatographic test for FeLV including a seroprevalence of 3.15% (95% CI: 1.60–6.09). In the case of FIV, 20 cats (15 males and 5 females) were positive to FIV test with a seroprevalence of 7.87% (95% CI: 5.15–11.85) (Table 2).

Co-infections detected

Among the 254 cats evaluated, 13 were seronegative for all pathogens analyzed, whilst, 86 cats were positive for one pathogen. The remaining 155 cats were positive to two or more pathogens (Table 4). Significant associations were observed between the variables detailed in the Table 5. A significant association was established between SARS-CoV-2 and *L. infantum* seropositivity (p = 0.018). The positivity results associated with the pathogen are listed in Table 4.

Discussion

To date, this is the first study that evaluates SARS-CoV-2 infection including serological analysis in stray cats during the different waves of COVID-19 outbreaks in a European city. Our findings suggest that there is no significant variation in SARS-CoV-2 seroprevalence in stray cats during the study period. Moreover, results are similar to previous study performed in the same region (Villanueva-Saz et al. 2022a). From January to October 2020, a seroprevalence of 3.51%, whilst, the next period of time analyzed in this study from October 2020 to January 2022, a seroprevalence of 1.57% was obtained in this study. During this period of time, different SARS-CoV-2 variants such as Delta and Omicron variant later, have been detected in Spain, a geographic area seriously affected by COVID-19. However, no increase of

Table 2 Data on stray feline populations investigated

| | | Sex | Classification | Waves of COVID-19 outbreak | | | | | |
				Second wave	Third Wave	Fourth wave	Fifth Wave	Sixth Wave	Total (%)
Pathogen	SARS-CoV-2	Male	Positive	1	1	1	0	0	3 (1.2%)
			Negative	21	27	27	21	13	109 (42.9%)
		Female	Positive	0	1	0	0	0	1 (0.4%)
			Negative	28	26	25	54	8	141 (55.5%)
		Overall	Positive	1	2	1	0	0	4 (1.6%)
			Negative	49	53	52	75	21	250 (98.4%)
	T. gondii	Male	Positive	3	5	2	3	2	15 (5.9%)
			Negative	19	23	26	18	11	97 (38.2%)
		Female	Positive	2	8	1	7	2	20 (7.9%)
			Negative	26	19	24	47	6	122 (48%)
		Overall	Positive	5	13	3	10	4	35 (13.8%)
			Negative	45	42	50	65	17	219 (86.2%)
	L. infantum	Male	Positive	1	7	4	6	4	22 (8.7%)
			Negative	21	21	24	15	9	90 (35.4%)
		Female	Positive	0	13	1	4	2	20 (7.9%)
			Negative	28	14	24	50	6	122 (48%)
		Overall	Positive	1	20	5	10	6	42 (16.5%)
			Negative	49	35	48	65	15	212 (83.5%)
	D. immitis	Male	Positive	6	10	6	8	3	33 (13%)
			Negative	16	18	22	13	10	79 (31.1%)
		Female	Positive	8	7	3	22	4	44 (17.3%)
			Negative	20	20	22	32	4	98 (38.6%)
		Overall	Positive	14	17	9	30	7	77 (30.3%)
			Negative	36	38	44	45	14	177 (69.7%)
	FCV	Male	Positive	18	26	21	17	11	93 (36.6%)
			Negative	4	2	7	4	2	19 (7.5%)
		Female	Positive	24	27	19	45	5	120 (47.2%)
			Negative	4	0	6	9	3	22 (8.7%)
		Overall	Positive	42	53	40	62	16	213 (83.9%)
			Negative	8	2	13	13	5	41 (16.1%)
	FHV-1	Male	Positive	8	17	8	9	6	48 (18.9%)
			Negative	14	12	20	12	7	65 (25.6%)
		Female	Positive	8	12	13	25	2	60 (23.6%)
			Negative	20	14	12	29	6	81 (31.9%)
		Overall	Positive	16	29	21	34	8	108 (42.5%)
			Negative	34	26	32	41	13	146 (57.5%)
	FeLV	Male	Positive	0	2	1	1	0	4 (1.6%)
			Negative	22	26	27	20	13	108 (42.5%)
		Female	Positive	1	0	0	3	0	4 (1.6%)
			Negative	27	27	25	51	8	138 (54.3%)
		Overall	Positive	1	2	1	4	0	8 (3.1%)
			Negative	49	53	52	71	21	246 (96.9%)
	FIV	Male	Positive	1	7	0	5	2	15 (5.9%)
			Negative	21	21	28	16	11	97 (38.2%)
		Female	Positive	1	0	0	3	1	5 (2%)
			Negative	27	27	25	51	7	137 (53.9%)
		Overall	Positive	2	7	0	8	3	20 (7.9%)
			Negative	48	48	53	67	18	234 (92.1%)

the number of seropositive cats was detected compared to previous results.

Epidemiological studies of cats performed in different countries report seroprevalences of SARS-CoV-2 ranging from 0 to 14.7% (Zhang et al. 2020; Stranieri et al. 2021). Surveys using different techniques to confirm SARS-CoV-2 infection have found that the prevalence of SARS-CoV-2 infection in cats in Spain varies from 2.1 to 6.4% (Schulz

Table 3 Results obtained by the in-house ELISA and micro-neutralization assay of SARS-CoV-2 in seropositive cats

Cat	SARS-CoV-2 ELISA (OD)	Classification by SARS-CoV-2 ELISA	SARS-CoV-2 micro-neutralization assay (ID50)	Classification by SARS-CoV-2 micro-neutralization
1	1.35	Positive	1/640	Positive
2	1.22	Positive	1/640	Positive
3	0.62	Positive	1/320	Positive
5	0.48	Positive	< 1/20	Negative

et al. 2021; Barroso-Arévalo et al. 2021) (Table 6). Several factors seem to have a direct influence of SARS-CoV-2 detection in cats such as the region where the study was performed considering SARS-CoV-2 prevalence in humans, lifestyle and cohabitation with COVID-19 positive contact, nature of the confirmatory technique to detect the presence of SARS-CoV-2 infection, type of sample or moment to perform the test (presence of suspected clinical signs or by contrast, seemingly healthy animal at the time of the test). Other important factor to consider is the difference between stray cat and pet cats. Stray cats, it can be defined as a cat who has been socialized with people at some point in its life, but has left or lost its indoor home, as well as most human contact and dependence. A stray cat can become a feral cat as its contact with people dwindles (Ogan and Jurek 1997). These are necessary variables to evaluate and be taken into when a comparison between studies is performed.

European epidemiological studies performed to analyse the potential epidemiological role of stray cats in Europe, confirm a low level of seroprevalence in this type of cats (Villanueva-Saz et al. 2022a). Although, the absence of SARS-CoV-2 RNA and anti-SARS-CoV-2 antibodies has also been also described (Stranieri et al. 2021).

In Europe, limited information is available during the different waves of COVID-19 outbreaks in susceptible animals such as dogs and cats in the same geographical area. A serological survey in Germany confirmed that 0.69% of cats had anti-SARS-CoV-2 antibodies detected by RBD-based ELISA (Michelitsch et al. 2021). More recently, another study performed mainly in Germany although some samples came from other European countries revealed a SARS-CoV-2 seropositivity from 0.5 to 1.9%, using RBD-based ELISA or nucleocapsid-based ELISA, respectively (Adler et al. 2022). In Poland, a total of 5 out of 279 owned cats were positive by nucleocapsid-based ELISA, obtaining a seroprevalence of 1.79% (Pomorska-Mól et al. 2021). In Italy, 5.8% of cats analysed were positive by serum viral neutralization test in a large-scale study (Patterson et al. 2020), whilst in another study in the Campania Region in Italy, a seroprevalence of 1.7% was detected by nucleocapsid-based ELISA (Cardillo et al. 2022).

In the case of other non-European countries such as Thailand, there is information about the evolution using the same serological methods used in the different studies at

different periods ncluding the presence of the first cases of SARS-CoV-2 as case series from April to May 2021 (Jairak et al. 2022a), the first wave (April 2020 to December 2020) (Udom et al. 2022), and finally, the second wave (December 2020 to February 2021) (Jairak et al. 2022b) in different owned animals. These studies revealed that the number of seropositive cats was very low with a feline seroprevalence of 0.36% during the first wave and the absence of seropositive cats during the second wave based on nucleocapsid-based ELISA test.

Different techniques to detect the presence of SARS-CoV-2 infection have been included such as serological techniques, molecular analysis, specific immunohistochemistry to detect SARS-CoV-2 antigen and virus isolation. Among serological techniques, ELISA with RBD as antigen is the most common test used for detecting anti-SARS-CoV-2 antibodies, followed by virus neutralization test. Differences in the detection of anti-SARS-CoV-2 antibodies between the different ELISAs techniques is due to the type of antigen and the technical methodology to obtain the results. In the case of ELISA techniques, other type of antigens coating the ELISA plate has been used based on Nucleocapside protein, Spike protein, S2 subunit has been described (Segalés et al. 2020).

The correlation of results by ELISA and virus neutralization test (VNT) has been evaluated in different studies. The spike-protein ELISA test correlates better with the neutralization assay that the Nucleocapsid ELISA (Okba et al. 2020). However, other studies confirm a good correlation among Nucleocapsid ELISA, Spike ELISA and virus neutralization test (Natale et al. 2021). A good correlation is also observed in the case of RBD ELISA and VNT (Schulz et al. 2021; Barroso-Arévalo et al., 2021). A study evaluated a total of 100 serum samples characterized using RBD ELISA and VNT. From these 100 samples, only 4 serum samples were considered negative result by the VNT but positive result by RBD ELISA, whilst, 1 serum sample was considered positive result by VNT but negative result by RBD ELISA (Barroso-Arévalo et al. 2021). A possible explanation of the positive result by the VNT but negative result by ELISA, is the fact VNT could identify a broader range of virus neutralizing antibodies including antibodies against Spike protein and other different domains, whilst, RBD ELISA is able to detect a single spike protein domain.

Table 4 List of stray cats with specific antibodies against different pathogens

SARS-CoV-2	L. infantum	T. gondii	D. immitis	FCV	FHV-1	FeLV	FIV	Number of seropositive cats
-	-	-	-	-	-	-	-	13
-	-	-	-	+	+	-	-	46
-	+	-	-	+	+	-	-	9
-	-	-	+	+	-	-	-	18
-	-	-	-	+	-	-	-	71
-	-	+	-	+	-	-	-	8
-	-	-	+	+	-	-	+	5
-	+	-	+	+	-	-	-	3
-	+	+	+	+	+	-	+	2
-	-	+	+	+	-	+	+	1
-	+	+	+	+	-	-	+	1
-	+	+	+	+	-	-	-	4
-	+	+	+	+	+	-	-	5
-	+	-	+	+	+	-	-	6
-	-	+	+	+	+	-	-	1
-	+	-	+	+	+	-	+	1
-	-	-	-	-	+	-	-	8
-	-	-	-	-	+	+	-	2
-	-	+	-	-	+	-	-	1
-	-	-	-	-	+	-	+	1
-	-	-	+	-	+	-	+	1
-	-	-	+	-	+	-	-	4
-	-	-	+	-	-	-	-	5
-	+	-	+	+	+	+	+	1
-	-	+	+	-	-	-	-	1
+	-	-	-	-	-	-	-	1
+	+	-	-	-	-	-	-	1
+	+	-	+	-	-	-	+	1
+	+	-	+	-	-	-	-	1
-	-	+	+	+	-	-	-	1
-	-	+	-	+	+	-	-	4
-	-	-	+	+	+	-	-	10
-	+	-	-	+	-	-	-	5
-	+	-	-	-	-	-	-	1
-	+	+	-	+	-	-	-	1
-	-	-	-	+	+	+	-	1
-	-	-	+	+	-	+	-	1
-	-	+	-	+	+	-	+	2
-	-	+	+	+	-	-	+	1
-	-	+	+	+	-	+	-	1
-	-	+	+	+	+	+	-	1
-	-	-	-	+	-	-	+	1
-	-	-	-	+	+	-	+	1
-	-	-	+	+	+	-	+	1
			Total of samples	254				

By contrast, the negative result obtained by the VNT but positive by RBD ELISA, could be justify due to a delayed production of neutralizing antibodies, situation that it has been described under experimental infection in ferrets (Schlottau et al. 2020) and in other epidemiological studies (Fritz et al. 2021; Yilmaz et al. 2021).

In our study, positive serum samples were detected by in-house ELISA. The presence of neutralizing activity was also detected in three of the four samples, being the negative sample by VNT, the lowest optical density detected by ELISA reader (OD = 0.48, cut-off = 0.30) in comparison to the remaining positive samples by ELISA and VNT

Table 5 Statistical associations between SARS-CoV-2-antibody and antibodies against other pathogens

Factor	P value							
	SARS-CoV-2 positivity	T. gondii positivity	L. infantum positivity	D. immitis positivity	FCV positivity	FHV-1 positivity	FeLV positivity	FIV positivity
Gender	0.323	0.999	0.188	0.891	0.863	0.999	0.734	0.004
ELISA positive (SARS-CoV-2)		0.999	0.018	0.587	0.999	0.999	0.999	0.281
IFAT positive (T. gondii)	0.999		0.001	0.002	0.084	0.715	0.083	0.012
ELISA positive (L. infantum)	0.018	0.001		0.001	0.005	0.017	0.635	0.1361
ELISA positive (D. immitis)	0.587	0.002	0.001		0.711	0.782	0.058	0.001
IFAT positive (FCV)	0.999	0.084	0.005	0.711		0.864	0.619	0.750
IFAT positive (FHV-1)	0.999	0.715	0.017	0.782	0.864		0.290	0.249
ICT positive (FeLV)	0.999	0.083	0.635	0.058	0.619	0.290		0.124
ICT positive (FIV)	0.281	0.012	0.1361	0.001	0.750	0.249	0.124	

Associations with a P value < 0.05 were considered as statistically significant

Table 6 Results of epidemiological surveys in cats of SARS-CoV-2 infections in Spain

Type of study	Number of cats included	Positive samples	Confirmatory techniques	Type of samples included	Clinical signs and laboratory findings	Origin of the positive animals	Dates	Reference
Epidemiological study	360	23	VNT	Serum	Not available	Laboratory samples for diagnostic purposes unrelated to suspicion of SARS-CoV-2 infection	April–June 2020	Schulz et al. 2021.
Case Series	1	1	RT-qPCR ELISA	Nasopharyngeal swab Serum	Yes	Domestic cat	December 2020 – June 2021	Miró et al. 2021.
Epidemiological study	114	4	ELISA	Serum	No	Stray cats	January 2020 - October 2020	Villanueva-Saz, et al.2021
Epidemiological study	753 184 selective 569 random	16 14: ELISA+VNT 2: RT-qPCR+VNT 2 RT-qPCR	RT-qPCR Virus isolation ELISA VNT	Whole blood, serum, nasopharyngeal swab, oropharyngeal swab, nasal swab and rectal swab	Yes (7 cats) No (9 cats)	Selective: domestic (cat or dog) living/in contact with confirmed COVID-19 positive people. Random: animals that visited veterinary clinics/ hospitals or were located in APCs with unknown COVID-19 positive contact and health status).	July 2020 - April 2021	Barroso-Arévalo et al., 2021.
Case series	2	2	RT-qPCR ELISA VNT Inmunohistochemistry to detect SARS-CoV-2 antigen	Serum, lung swabs, nasal swab, rectal swab, tissues for immunohistochemistriy and RT-qPCR	No (2)	Domestic cat	March 2020	Segalés et al., 2020
Case series	8	1	RT-qPCR	Oropharyngeal and rectal swabs	No	Domestic cat	April 8-May 4, 2020	Ruiz-Arrondo et al., 2021
Epidemiological study	254	4	ELISA VNT	Serum	No	Stray Cats	October 2020- January 2022	This study

ELISA: enzyme-linked immunosorbent assay; RT-qPCR: quantitative reverse transcription polymerase chain reaction; VNT: virus neutralization test

too (Table 3). These results agree with previous report of SARS-CoV-2 in cats that the serum samples with borderline results by ELISA, a negative result was also obtained by VNT (Michelitsch et al. 2021).

Among ELISA or VNT as serological methods for screening a large number of samples, ELISA is a test that can be run in a wider range of laboratories whilst, VNS requires the use of a specialized laboratory and reagents and equipment including the security of Biosafety level 3 laboratory associated to live SARS-CoV-2 virus manipulation.

Molecular analysis is not restricted to bodily fluid, and different type of *antemortem* samples such as nasopharyngeal, oropharyngeal, nasal, and rectal swabs (Barroso-Arévalo et al. 2021). Other authors have detected the presence of RNA material in *postmortem* samples such as lymph node or other respiratory tissues (Segalés et al. 2020). One common problem associated to molecular detection of SARS-CoV-2 infection is the difficulty to determine the best time of taking a sample in domestic animals, though it is easier with one´s pet that live with humans (Barroso-Arévalo et al. 2021) than shelter animals (van der Leij et al. 2021) and stray cats (Spada et al. 2021; Cardillo et al. 2022; Kuhlmeier et al. 2022; Stranieri et al. 2021).

The role of strays or shelter cats in epidemiology of SARS-CoV-2 virus, has been debated due to the risk of infection associated with the transmission to inter species bases on the existence shown in different transmission routes such as person to cat, cat to cat or mink to cat (Sharun et al. 2021; van Aart et al. 2021), making active surveillance programs for risk assessment necessary. In Europe, several studies have detected the presence of seropositive animals in different countries such Italy (Spada et al. 2021; Cardillo et al. 2022), Spain (Barroso-Arévalo et al. 2021; Villanueva-Saz et al. 2022a) or The Netherlands (van der Leij et al. 2021) detecting a very low seroprevalence result. By contrast, the presence of infected stray cats was not detected by other authors in a similar epidemiological study performed in Italy (Stranieri et al. 2021). In our study, a seroprevalence of 1.57% was detected in a sample of 254 cats. This result was very similar to the results obtained by other epidemiological studies with stray cats.

Active monitoring programme of stray cats provides early detection of a specific disease, situation specially interested in SARS-CoV-2 detection and other pathogens including both zoonotic and non-zoonotic pathogens that affect specifically cats (Spada et al. 2021; Villanueva-Saz et al. 2022a) considering a health approach.

In the same city, a serological study performed from January to the first half of October 2020 revealed 3.51% seropositivity stray cats for SARS-CoV-2, 16.67% for *L. infantum*, 12.28% for *T. gondii*, 4.39% for FeLV, and 19.30% for FIV (Villanueva-Saz et al. 2022a). Based on the

new results obtained in the present study, from the second half of October 2020 to the first half of January 2022, similar results were obtained for *L. infantum* (16.54%), while seropositivity level was slightly increased in some pathogens such as *T. gondii* (13.78%). By contrast, the seropositivity level decreased slightly for SARS-CoV-2 (1.57%) and for FeLV (3.15%), while the seroprevalence for FIV (7.87%) decreased even more compared to previous results. Information about the presence of anti-*Dirofilaria immitis* antibodies in stray cats from Zaragoza was evaluated in 250 stray cats from November 2017 to November 2019, obtaining a seroprevalence of 24.40% (Villanueva-Saz et al. 2022b), in comparison to the seroprevalence of the present study, 30.31%. Finally, epidemiological information about the seroprevalence of the viruses involved in the pathogenesis of Upper Respiratory Tract Disease (ABCD 2017a; ABCD 2017b) in stray cats in Spain is very limited. In our study, a high number of seropositive cats were detected, being the number of positive cats for FCV (213 cats) higher in comparison to FHV-1 (108 cats). Epidemiological studies performed in stray cats in different regions report seroprevalences of FCV ranging from 36.6% detected in Florida (DiGangi et al. 2012) to 100% detected in UK (Yamaguchi et al. 1996). In the case of FHV-1, seroprevalence varies from 10 – 11% in Galapagos (Levy et al. 2008) and Florida (DiGangi et al. 2011) from 100% in UK (Yamaguchi et al. 1996). Our findings are similar to other study recently published in similar region such as Italy with a seroprevalence of 85.4% for FCV and 37% for FHV-1 (Dall`Ara et al. 2019). However, our results are in agreement to previous studies suggesting widespread worldwide in the stray cat population (Dall`Ara et al. 2019).

The detection of antibodies against specific pathogens is an indirect evaluation of its presence. Co-infection can be suggested if there are antibodies against different agents in the same serum sample. We found that SARS-CoV-2 antibody positivity was associated with ELISA antibody positivity to *L. infantum* in cats but we did not find associations of SARS-CoV-2 seropositivity with the remaining pathogens included in this study. In dogs with clinical signs of *L. infantum* infection, a dysregulation of cytokine production and a reduction of cellular immune response was detected (Solano-Gallego et al. 2011). A similar pattern of humoral and cell-mediated adaptive immune response was detected in cats from endemic areas of *L. infantum* (Priolo et al. 2019). Other associations were detected including co-infection with *T. gondii*, *L. infantum*, *D. immitis*, FIV, FCV and FHV-1 (Table 5). In general, the presence of co-infections could be considered a risk factor with the progression of disease and causing chronic disease and facilitating an opportunistic infection. However, it would necessary longitudinal

clinical, serological and molecular evaluation of cats as an adequate way of data analysis.

Based on our results, stray cats are not adequate sentinel species for SARS-CoV-2 infection. However, stray cat as sentinels can be of great interest for other pathogens including feline viruses, *T. gondii* and vector-borne diseases, such as *L. infantum* and *D. immitis*. In the case of SARS-CoV-2 virus, cats have a low viral load and a short period of viral shedding, and particularly stray cats live outdoors. For thus, transmission implication in this context is very low. In the case of vector-borne diseases, the role these animals might play in the leishmaniosis and dirofilariosis cycles should not be underestimated, especially as the cats live close to humans in an endemic area. For feline retroviruses and *T. gondii*, the occurrence of these three pathogens and the rate of transmission of these diseases in stray cats is important to know the global health status of feline colonies from a city or a specific geographical area considering the possibility to harbour pathogens that produce disease in humans and animals (Taetzsch et al. 2018). In the case of SARS-CoV-2 infection, this condition might not be especially relevant in stray cats but it could be relevant for other zoonotic pathogens such as *T. gondii* and *L. infantum*.

The main limitations of this study are the retrospective nature, the lack of routine laboratory test such as complete blood count and biochemistry profile, and the absence of serological and molecular follow-up of SARS-CoV-2 seropositive cats. While the number of samples obtained from long period of the same region, together with the different serological tests to detect other pathogens or co-infections, the employment of the same serological technique to detect anti-SARS-CoV-2 and the subsequent evaluation of seropositive cats with the seroneutralization technique for SARS-CoV-2 virus are its strength.

In light of our results, it seems reasonable to believe that the impact of the different waves of COVID-19 outbreaks in stray cats is very limited. SARS-CoV-2 virus has infected some stray cats; however, lower prevalence is found in this group of cats in comparison to infected people or owned cat where human-to-cat transmission can happen. Although cats could be infected by people with SARS-CoV-2 infection, and experimental infection of domestic cats has been demonstrated. With our results, it can be considered that the probability of the virus was circulating in stray cats from a geographic area seriously affected by COVID-19 was very low. The presence of SARS-CoV-2-seropositive cats may be more likely associated to human-to-cat SARS-CoV-2 transmission instead of stray cat-to-cat transmission. Moreover, the presence of immunosuppressed cats or with chronic disease due to the pathogens included in this study is not a condition of susceptibility to SARS-CoV-2 infection.

Author's contribution Sergio Villanueva-Saz, Mariví Martínez and Jacobo Giner conceived and designed the experiments; Ana González and Maite Verde performed the sample collection; Ana Pilar Tobajas, María Dolores Pérez, Erandi Lira-Navarrete, Andrés Manuel González-Ramírez, Javier Macías-Leon, Llipsy Santiago and Maykel Arias did the laboratory examination; Jordi Aguiló-Gisbert, Héctor Ruíz and Delia Lacasta analyzed the data; Sergio Villanueva-Saz, Diana Marteles and Antonio Fernández wrote the manuscript; Sergio Villanueva-Saz, Ramón Guerrero-Hurtado, and Julián Pardo did the project management; Sergio Villanueva-Saz, Andrés Yzuel, and Antonio Fernández reviewed the manuscript; Diana Marteles and Antonio Fernández corrected the manuscript.

Data Availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Competing interests All authors have read and approved the final manuscript. Its contents are solely the responsibility of the authors. All authors declare that they have no competing interests.

Ethical approval This survey was included under Project License PI62/17 approved by the Ethic Committee for Animal Experiments for the University of Zaragoza. The care and use of animals were performed according to the Spanish Policy for Animal Protection RD 53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

Consent to participate Not applicable.

Consent to publish Not applicable.

References

ABCD - Advisory Board on Cat Diseases (2017a) Feline Calicivirus Infection. ABCD - Advisory Board on Cat Diseases. http://

www.abcdcatsvets.org/feline-calicivirus-infection-2012-edition/. Accessed 17 July 2022

ABCD - Advisory Board on Cat Diseases (2017b) Feline Herpesvirus Infection. ABCD - Advisory Board on Cat Diseases. http://www.abcdcatsvets.org/feline-herpesvirus/. Accessed 17 July 2022

Adler JM, Weber C, Wernike K, Michelitsch A, Friedrich K, Trimpert J, Beer M, Kohn B, Osterrieder K, Müller E (2022) Prevalence of anti-severe acute respiratory syndrome coronavirus 2 antibodies in cats in Germany and other European countries in the early phase of the coronavirus disease-19 pandemic. Zoonoses Public Health 69:439–450. https://doi.org/10.1111/zph.12932

Barroso-Arévalo S, Barneto A, Ramos ÁM, Rivera B, Sánchez R, Sánchez-Morales L, Pérez-Sancho M, Buendía A, Ferreras E, Ortiz-Menéndez JC, Moreno I, Serres C, Vela C, Risalde M, Domínguez L, Sánchez-Vizcaíno JM (2021) Large-scale study on virological and serological prevalence of SARS-CoV-2 in cats and dogs in Spain. Transbound Emerg Dis 69:e759–e774. https://doi.org/10.1111/tbed.14366

Bosco-Lauth AM, Hartwig AE, Porter SM, Gordy PW, Nehring M, Byas AD, VandeWoude S, Ragan IK, Maison RM, Bowen RA (2020) Experimental infection of domestic dogs and cats with SARS-CoV-2: Pathogenesis, transmission, and response to reexposure in cats. Proc Natl Acad Sci USA 117:26382–26388. https://doi.org/10.1073/pnas.2013102117

Burkholz S, Pokhrel S, Kraemer BR, Mochly-Rosen D, Carback RT 3, Hodge T, Harris P, Ciotlos S, Wang L, Herst CV, Rubsamen R (2021) Paired SARS-CoV-2 spike protein mutations observed during ongoing SARS-CoV-2 viral transfer from humans to minks and back to humans. Infect Genet Evol 93:104897. https://doi.org/10.1016/j.meegid.2021.104897

Cardillo L, de Martinis C, Brandi S, Levante M, Cozzolino L, Spadari L, Boccia F, Carbone C, Pompameo M, Fusco G (2022) SARS-CoV-2 Serological and Biomolecular Analyses among Companion Animals in Campania Region (2020–2021). Microorganisms 10:263. https://doi.org/10.3390/microorganisms10020263

Curukoglu A, Ergoren MC, Ozgencil FE, Sayiner S, Ince ME, Sanlidag T (2021) First direct human-to-cat transmission of the SARS-CoV-2 B.1.1.7 variant. Aust Vet J 99:482–488. https://doi.org/10.1111/avj.13109

Dall'Ara P, Labriola C, Sala E, Spada E, Magistrelli S, Lauzi S (2019) Prevalence of serum antibody titres against feline panleukopenia, herpesvirus and calicivirus infections in stray cats of Milan, Italy. Prev Vet Med 167:32–38. https://doi.org/10.1016/j.prevetmed.2019.03.010

Deng J, Jin Y, Liu Y, Sun J, Hao L, Bai J, Huang T, Lin D, Jin Y, Tian K (2020) Serological survey of SARS-CoV-2 for experimental, domestic, companion and wild animals excludes intermediate hosts of 35 different species of animals. Transbound Emerg Dis 67:1745–1749

Digangi BA, Gray LK, Levy JK, Dubovi EJ, Tucker SJ (2011) Detection of protective antibody titers against feline panleukopenia virus, feline herpesvirus-1, and feline calicivirus in shelter cats using a point-of-care ELISA. J Feline Med Surg 13:912–918. https://doi.org/10.1016/j.jfms.2011.07.009

Ferasin L, Fritz M, Ferasin H, Becquart P, Corbet S, Ar Gouilh M, Legros V, Leroy EM (2021) Infection with SARS-CoV-2 variant B.1.1.7 detected in a group of dogs and cats with suspected myocarditis. Vet Rec 189:e944. https://doi.org/10.1002/vetr.944

Fritz M, Rosolen B, Krafft E, Becquart P, Elguero E, Vratskikh O, Denolly S, Boson B, Vanhomwegen J, Gouilh MA, Kodjo A, Chirouze C, Rosolen SG, Legros V, Leroy EM (2021) High prevalence of SARS-CoV-2 antibodies in pets from COVID-19 + households. One health 11:100192. https://doi.org/10.1016/j.onehlt.2020.100192

Gaudreault NN, Trujillo JD, Carossino M, Meekins DA, Morozov I, Madden DW, Indran SV, Bold D, Balaraman V, Kwon T, Artiaga BL, Cool K, García-Sastre A, Ma W, Wilson WC, Henningson J, Balasuriya U, Richt JA (2020) SARS-CoV-2 infection, disease and transmission in domestic cats. Emerg Microbes Infect 9:2322–2332. https://doi.org/10.1080/22221751.2020.1833687

Giner J, Villanueva-Saz S, Tobajas AP, Pérez MD, González A, Verde M, Yzuel A, García-García A, Taleb V, Lira-Navarrete E, Hurtado-Guerrero R, Pardo J, Santiago L, Paño JR, Ruíz H, Lacasta D, Fernández A (2021) SARS-CoV-2 Seroprevalence in household domestic ferrets (*Mustela putorius furo*). Animals 11:667. https://doi.org/10.3390/ani11030667

Goldman M (1957) Staining *Toxoplasma gondii* with fluorescin labelled antibody: a new serologic test for antibodies to *Toxoplasma* based upon inhibition specific staining. J Exp Med 105:557–573

Haider N, Rothman-Ostrow P, Osman AY, Arruda LB, Macfarlane-Berry L, Elton L, Thomason MJ, Yeboah-Manu D, Ansumana R, Kapata N, Mboera L, Rushton J, McHugh TD, Heymann DL, Zumla A, Kock RA (2020) COVID-19-Zoonosis or Emerging Infectious Disease? Front Public Health 8:596944. https://doi.org/10.3389/fpubh.2020.596944

Hall CM, Adams NA, Bradley JS, Bryant KA, Davis AA, Dickman CR, Fujita T, Kobayashi S, Lepczyk CA, McBride EA, Pollock KH, Styles IM, van Heezik Y, Wang F, Calver MC (2016) Community attitudes and practices of urban residents regarding predation by pet cats on wildlife: An International Comparison. PLoS ONE 11:e0151962. https://doi.org/10.1371/journal.pone.0151962

Jairak W, Charoenkul K, Chamsai E, Udom K, Chaiyawong S, Bunpapong N, Boonyapisitsopa S, Tantilertcharoen R, Techakriengkrai N, Surachetpong S, Tangwangvivat R, Suwannakarn K, Amonsin A (2022a) First cases of SARS-CoV-2 infection in dogs and cats in Thailand. Transbound Emerg Dis 69:e979–e991. https://doi.org/10.1111/tbed.14383

Jairak W, Charoenkul K, Chamsai E, Udom K, Chaiyawong S, Hangsawek A, Waenkaew S, Mungaomklang A, Tangwangvivat R, Amonsin A (2022b) Survey of SARS-CoV-2 in dogs and cats in high-risk areas during the second wave of COVID-19 outbreak, Thailand. Zoonoses Public Health 69:737–745. https://doi.org/10.1111/zph.12907

Keller M, Hagag IT, Balzer J, Beyer K, Kersebohm JC, Sadeghi B, Wernike K, Höper D, Wylezich C, Beer M, Groschup MH (2021) Detection of SARS-CoV-2 variant B.1.1.7 in a cat in Germany. Res Vet Sci 140:229–232. https://doi.org/10.1016/j.rvsc.2021.09.008

Kuhlmeier E, Chan T, Klaus J, Pineroli B, Geisser E, Hofmann-Lehmann R, Meli ML (2022) A Pre- and Within-Pandemic Survey of SARS-CoV-2 RNA in Saliva Swabs from Stray Cats in Switzerland. Viruses 14:681. https://doi.org/10.3390/v14040681

Levy JK, Crawford PC, Lappin MR, Dubovi EJ, Levy MG, Alleman R, Tucker SJ, Clifford EL (2008) Infectious diseases of dogs and cats on Isabela Island, Galapagos. J Vet Inter Med 22:60–65. https://doi.org/10.1111/j.1939-1676.2007.0034.x

Lilith M, Calver M, Styles I, Garkaklis M (2006) Protecting wildlife from predation by owned domestic cats: application of a precautionary approach to the acceptability of proposed cat regulations. Austral Ecol 31:176–189. https://doi.org/10.1111/j.1442-9993.2006.01582.x

Michelitsch A, Schön J, Hoffmann D, Beer M, Wernike K (2021) The Second Wave of SARS-CoV-2 Circulation-Antibody Detection in the Domestic Cat Population in Germany. Viruses 13:1009. https://doi.org/10.3390/v13061009

Miró G, Regidor-Cerrillo J, Checa R, Diezma-Díaz C, Montoya A, García-Cantalejo J, Botías P, Arroyo J, Ortega-Mora LM (2021) SARS-CoV-2 Infection in One Cat and Three Dogs Living in COVID-19-Positive Households in Madrid, Spain. Front Vet Sci 8:779341. https://doi.org/10.3389/fvets.2021.779341

Natale A, Mazzotta E, Mason N, Ceglie L, Mion M, Stefani A, Fincato A, Bonfante F, Bortolami A, Monne I, Bellinati L, Guadagno C, Quaranta E, Pastori A, Terregino C (2021) SARS-Cov-2 natural infection in a symptomatic cat: Diagnostic, clinical and medical management in a one health vision. Animals 11:1640. https://doi.org/10.3390/ani11

Ogan CV, Jurek RM (1997) Biology and ecology of feral, free-roaming and stray cats. In: Harris JE, Ogan CV (eds) Mesocarnivores of Northern California: Biology, management and survey techniques, workshop manual, 1st edn. Humbolt State University, pp 87–92

Okba N, Müller MA, Li W, Wang C, GeurtsvanKessel CH, Corman VM, Lamers MM, Sikkema RS, de Bruin E, Chandler FD, Yazdanpanah Y, Le Hingrat Q, Descamps D, Houhou-Fidouh N, Reusken C, Bosch BJ, Drosten C, Koopmans M, Haagmans BL (2020) Severe Acute Respiratory Syndrome Coronavirus 2-Specific Antibody Responses in Coronavirus Disease Patients. Emerg Infect Dis 26:1478–1488. https://doi.org/10.3201/eid2607.200841

Opriessnig T, Huang YW (2020) Update on possible animal sources for COVID-19 in humans. Xenotransplantation 27:e12621. https://doi.org/10.1111/xen.12621

Pagani G, Lai A, Bergna A, Rizzo A, Stranieri A, Giordano A, Paltrinieri S, Lelli D, Decaro N, Rusconi S, Gismondo MR, Antinori S, Lauzi S, Galli M, Zehender G (2021) Human-to-Cat SARS-CoV-2 Transmission: Case Report and Full-Genome Sequencing from an Infected Pet and Its Owner in Northern Italy. Pathogens 10:252. https://doi.org/10.3390/pathogens10020252

Patterson EI, Elia G, Grassi A et al (2020) Evidence of exposure to SARS-CoV-2 in cats and dogs from households in Italy. Nat Commun 11:6231. https://doi.org/10.1038/s41467-020-20097-0

Piplani S, Singh PK, Winkler DA, Petrovsky N (2021) In silico comparison of SARS-CoV-2 spike protein-ACE2 binding affinities across species and implications for virus origin. Sci Rep 11:13063. https://doi.org/10.1038/s41598-021-92388-5

Pomorska-Mól M, Turlewicz-Podbielska H, Gogulski M, Ruszkowski JJ, Kubiak M, Kuriga A, Barket P, Postrzech M (2021) A cross-sectional retrospective study of SARS-CoV-2 seroprevalence in domestic cats, dogs and rabbits in Poland. BMC Vet Res 17:322. https://doi.org/10.1186/s12917-021-03033-2

Priolo V, Martínez-Orellana P, Pennisi MG, Masucci M, Prandi D, Ippolito D, Bruno F, Castelli G, Solano-Gallego L (2019) *Leishmania infantum*-specific IFN-γ production in stimulated blood from cats living in areas where canine leishmaniosis is endemic. Parasit Vectors 12:133. https://doi.org/10.1186/s13071-019-3386-y

Ramakrishnan MA (2016) Determination of 50% endpoint titer using a simple formula. World J Virol 5:85–86. https://doi.org/10.5501/wjv.v5.i2.85

Rambaut A, Holmes EC, O'Toole Á, Hill V, McCrone JT, Ruis C, du Plessis L, Pybus OG (2020) A dynamic nomenclature proposal for SARS-CoV-2 lineages to assist genomic epidemiology. Nat Microbiol 5:1403–1407. https://doi.org/10.1038/s41564-020-0770-5

Schlottau K, Rissmann M, Graaf A, Schön J, Sehl J, Wylezich C, Höper D, Mettenleiter TC, Balkema-Buschmann A, Harder T, Grund C, Hoffmann D, Breithaupt A, Beer M (2020) SARS-CoV-2 in fruit bats, ferrets, pigs, and chickens: an experimental transmission study. Lancet Microbe 1:e218–e225. https://doi.org/10.1016/S2666-5247(20)30089-6

Schulz C, Martina B, Mirolo M, Müller E, Klein R, Volk H, Egberink H, Gonzalez-Hernandez M, Kaiser F, von Köckritz-Blickwede M, Osterhaus A (2021) SARS-CoV-2-Specific Antibodies in Domestic Cats during First COVID-19 Wave, Europe. Emerg Infect Dis 27:3115–3118. https://doi.org/10.3201/eid2712.211252

Segalés J, Puig M, Rodon J, Avila-Nieto C, Carrillo J, Cantero G, Terrón MT, Cruz S, Parera M, Noguera-Julián M, Izquierdo-Useros N, Guallar V, Vidal E, Valencia A, Blanco I, Blanco J, Clotet B, Vergara-Alert J (2020) Detection of SARS-CoV-2 in a cat owned by a COVID-19-affected patient in Spain. Proc Natl Acad Sci USA 117:24790–24793. https://doi.org/10.1073/pnas.2010817117

Sharun K, Tiwari R, Natesan S, Dhama K (2021) SARS-CoV-2 infection in farmed minks, associated zoonotic concerns, and importance of the One Health approach during the ongoing COVID-19 pandemic. Vet Q 41:50–60. https://doi.org/10.1080/01652176.2020.1867776

Shi J, Wen Z, Zhong G et al (2020) Susceptibility of ferrets, cats, dogs, and other domesticated animals to SARS-coronavirus 2. Science 368:1016–1020. https://doi.org/10.1126/science.abb7015

Solano-Gallego L, Miró G, Koutinas A, Cardoso L, Pennisi MG, Ferrer L, Bourdeau P, Oliva G, Baneth G, The LeishVet Group (2011) LeishVet guidelines for the practical management of canine leishmaniosis. Parasit Vectors 4:86. https://doi.org/10.1186/1756-3305-4-86

Spada E, Vitale F, Bruno F, Castelli G, Reale S, Perego R, Baggiani L, Proverbio D (2021) A pre- and during Pandemic Survey of Sars-Cov-2 Infection in Stray Colony and Shelter Cats from a High Endemic Area of Northern Italy. Viruses 13:618. https://doi.org/10.3390/v13040618

Stranieri A, Lauzi S, Giordano A, Galimberti L, Ratti G, Decaro N, Brioschi F, Lelli D, Gabba S, Amarachi NL, Lorusso E, Moreno A, Trogu T, Paltrinieri S (2021) Absence of SARS-CoV-2 RNA and anti-SARS-CoV-2 antibodies in stray cats. Transbound Emerg Dis 69:2089–2095. https://doi.org/10.1111/tbed.14200

Tiwari R, Dhama K, Sharun K, Iqbal Yatoo M, Malik YS, Singh R, Michalak I, Sah R, Bonilla-Aldana DK, Rodriguez-Morales AJ (2020) COVID-19: animals, veterinary and zoonotic links. Vet Q 40:169–182. https://doi.org/10.1080/01652176.2020.1766725

Taetzsch SJ, Bertke AS, Gruszynski KR (2018) Zoonotic disease transmission associated with feral cats in a metropolitan area: A geospatial analysis. Zoonoses Public Health 65:412–419. https://doi.org/10.1111/zph.12449

Udom K, Jairak W, Chamsai E, Charoenkul K, Boonyapisitsopa S, Bunpapong N, Techakriengkrai N, Amonsin A (2022) Serological survey of antibodies against SARS-CoV-2 in dogs and cats, Thailand. Transbound Emerg Dis 69:2140–2147. https://doi.org/10.1111/tbed.14208

van Aart AE, Velkers FC, Fischer E, Broens EM, Egberink H, Zhao S, Engelsma M, Hakze-van der Honing RW, Harders F, de Rooij M, Radstake C, Meijer PA, Oude Munnink BB, de Rond J, Sikkema RS et al (2021) SARS-CoV-2 infection in cats and dogs in infected mink farms. https://doi.org/10.1111/tbed.14173. Transbound Emerg Dis Advance online publication

van der Leij W, Broens EM, Hesselink JW, Schuurman N, Vernooij J, Egberink HF (2021) Serological Screening for Antibodies against SARS-CoV-2 in Dutch Shelter Cats. Viruses 13:1634. https://doi.org/10.3390/v13081634

Villanueva-Saz S, Giner J, Verde M, Yzuel A, González A, Lacasta D, Marteles D, Fernández A (2021) Prevalence of microfilariae, antigen and antibodies of feline dirofilariosis infection (*Dirofilaria immitis*) in the Zaragoza metropolitan area, Spain. Vet Parasitol Reg Stud Reports 23:100541. https://doi.org/10.1016/j.vprsr.2021.100541

Villanueva-Saz S, Giner J, Tobajas AP et al (2022a) Serological evidence of SARS-CoV-2 and co-infections in stray cats in Spain. Transbound Emerg Dis 69:1056–1064. https://doi.org/10.1111/tbed.14062

Villanueva-Saz S, Giner J, Fernández A, Alcover MM, Riera C, Fisa R, Yzuel A, González A, Marteles D, Verde M (2022b) Serum protein electrophoretogram profile detected in apparently healthy cats infected with *Leishmania infantum* - Short communication. https://doi.org/10.1556/004.2021.00055. Acta Vet Hung Advance online publication

Yamaguchi N, Macdonald DW, Passanisi WC, Harbour DA, Hopper CD (1996) Parasite prevalence in free-ranging farm cats, *Felis silvestris catus*. Epidemiol Infect 116:217–223. https://doi.org/10.1017/s0950268800052468

Yilmaz A, Kayar A, Turan N, Iskefli O, Bayrakal A, Roman-Sosa G, Or E, Tali HE, Kocazeybek B, Karaali R, Bold D, Sadeyen JR, Lukosaityte D, Chang P, Iqbal M, Richt JA, Yilmaz H (2021) Presence of antibodies to SARS-CoV-2 in domestic cats in Istanbul, Turkey, before and after COVID-19 Pandemic. Front Vet Sci 8:707368. https://doi.org/10.3389/fvets.2021.707368

Zhang Q, Zhang H, Gao J, Huang K, Yang Y, Hui X, He X, Li C, Gong W, Zhang Y, Zhao Y, Peng C, Gao X, Chen H, Zou Z, Shi ZL, Jin M (2020) A serological survey of SARS-CoV-2 in cat in Wuhan. Emerg Microbes Infect 9:2013–2019. https://doi.org/10.1080/22221751.2020.1817796

Authors and Affiliations

Sergio Villanueva-Saz[1,2,3] · **Mariví Martínez**[1,2] · **Jacobo Giner**[1,2] · **Ana González**[1,4] · **Ana Pilar Tobajas**[3,5] · **María Dolores Pérez**[3,5] · **Erandi Lira-Navarrete**[6] · **Andrés Manuel González-Ramírez**[6] · **Javier Macías-León**[6] · **Maite Verde**[1,3,4] · **Andrés Yzuel**[1] · **Ramón Hurtado-Guerrero**[6,7,8,9,10] · **Maykel Arias**[11,12] · **Llipsy Santiago**[11,12] · **Jordi Aguiló-Gisbert**[13] · **Héctor Ruíz**[2] · **Delia Lacasta**[2,3] · **Diana Marteles**[1] ·

Antonio Fernández[1,2,3]

✉ Sergio Villanueva-Saz
svs@unizar.es

✉ Antonio Fernández
afmedica@unizar.es

1   Clinical Immunology Laboratory, Veterinary Faculty, University of Zaragoza, 50013 Zaragoza, Spain

2   Deparment of Animal Pathology, Veterinary Faculty, University of Zaragoza, Zaragoza, Spain

3   Instituto Agroalimentario de Aragón-IA2 (Universidad de Zaragoza-CITA), Zaragoza, Spain

4   Veterinary Teaching Hospital of the University of Zaragoza, Zaragoza, Spain

5   Department of Animal Production and Sciences of the Food, Veterinary Faculty, University of Zaragoza, Zaragoza, Spain

6   Institute for Biocomputation and Physics of Complex Systems (BIFI), University of Zaragoza, Edificio I+D, Campus Rio Ebro, Zaragoza, Spain

7   Aragon I+D Foundation (ARAID), Zaragoza, Spain

8   Laboratorio de Microscopías Avanzada (LMA), University of Zaragoza, Edificio I+D, Campus Rio Ebro, Zaragoza, Spain

9   Copenhagen, Denmark

10   Department of Cellular and Molecular Medicine, School of Dentistry, University of Copenhagen, Copenhagen, Denmark

11   Aragon Health Research Institute (IIS Aragón), Zaragoza, Spain

12   CIBER de Enfermedades Infecciosas, Instituto de Salud Carlos III, Madrid, Spain

13   Servicio de Análisis, Investigación, Gestión de Animales Silvestres (SAIGAS), Facultad de Veterinaria, Universidad Cardenal Herrera-CEU, Valencia, Spain