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

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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. 2022). Considering in vitro 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.

104 The monitoring and evaluation of the impact of the different waves of SARS-CoV-2
105 infection in domestic animals should be conducted based on One Health approach,
106 especially in those animals in close contact with people (owners and colony caregivers).

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

116

117 **Material and Methods**

118 *Study area, sampling and data collection*

119 The study was carried out in the city of Zaragoza (41° 38' 58.8948" N and 0° 53'
120 15.7632" W, Aragon region, Spain) from the second half of October 2020 to the first
121 half of January 2022 (Table 1). The study population comprised 254 stray cats captured
122 in urban areas of Zaragoza within a trap, neuter, and release sterilization program run
123 locally to control stray feline colonies. Samples from unvaccinated stray cats were
124 collected based on previously published seroepidemiological study (Villanueva-Saz et
125 al. 2021a).

126 Samples were obtained during the different waves of COVID-19 outbreak from the
127 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 (RVQPTESIVRFPNITNLCPFGEVFNATRFASVYAWNRKRISNCVADYSVLYNSA SFSTFKCYGVSPTKLNDLCFTNVYADSFVIRGDEVRQIAPGQTGKIADYNYKLP DDFTGCVIAWNSNNLDSKVGGNYNYLYRLFRKSNLKPFERDISTEIYQAGSTPC NGVEGFNCYFPLQSYGFQPTNGVGYQPYRVVVLSFELLHAPATVCGPKKSTNL VKNKCVNF) 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 × 106 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 (1µg/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 1µg/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 hour at 37 °C in a

moist chamber. After washing the plates for 30 seconds 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 hour 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 ℃ 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 ℃ and 5% CO2 and then 100µl 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 ℃ 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 hour 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 hour 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 1/20000 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:

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

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.

347

348 *Serological prevalence of SARS-CoV-2 infection*

349 The in-house quantitative ELISA revealed four positive samples (1.57%;
350 95% CI: 0.61–3.98), with OD units ranging from 1.35, 1.22, 0.62, and 0.48 (cutoff ≥
351 0.30). The presence of antibodies anti-SARS-CoV-2 was detected in three males and
352 one female cat. The seropositive samples were obtained at different waves of COVID-
353 19 outbreaks: second (n=1, November 2020), third (n=2, February 2021 and March
354 2021), fourth (n=1, April 2021) (Table 2). Among the four SARS-CoV-2 tested positive
355 cats, three of them showed neutralizing antibodies (Table 3).
356 None of the seropositive to SARS-CoV-2 cats were positive to FCoV.
357

358 *Serological prevalence of T. gondii infection*

359 Thirty-five of the 254 cats tested were seroreactive by IFAT (13.78%; 95% CI: 10.08–
360 18.56) including 15 males and 20 females (Table 2). The period of time with higher
361 number of seropositive animals was during the third wave of COVID-19 outbreak
362 (n=13), followed by the fifth wave of COVID-19 outbreak (n=10).
363

364 *Serological prevalence of L. infantum infection*

365 Among the 254 cats, 42 cats (22 males and 20 females) were positive by the in-house
366 ELISA with a seroprevalence of 16.54% (95% CI: 12.47–21.60). Third and fifth waves
367 of COVID-19 outbreak were the time periods with the highest number of seropositive
368 cats, 20 and 10 animals, respectively.
369

370 *Serological prevalence of D. immitis infection*

371 The presence of antibodies against *D. immitis* was detected in 33 males and 44 females
372 with a seroprevalence of 30.31% (95% CI: 21.60–34.99) with a variable number of
373 seropositive animals among the different waves of COVID-19 outbreak with the lowest
374 number during of sixth wave (n=7) and the highest during the fifth wave (n=30) (Table
375 2).
376
377 *Serological prevalence of viral infection caused by respiratory pathogens FCV and*
378 *FHV-1.*
379 Two hundred and thirteen (93 males and 120 females) of the 254 cats had a positive
380 result for FCV with a seroprevalence of 83.86% (95% CI: 79.33-88.38). For FHV-1,
381 108 cats (48 males and 60 females) with a seroprevalence of 42.52% (95% CI:33.25-
382 45.60). The presence of seropositive animals for respiratory viruses were detected
383 among the different waves of COVID-19 outbreak (Table 2).
384
385 *Serological prevalence of viral infection caused by FeLV and FIV.*
386 A total of 8 cats (4 males and 4 females) were seropositive by the
387 immunochromatographic test for FeLV including a seroprevalence of 3.15% (95% CI:
388 1.60-6.09). In the case of FIV, 20 cats (15 males and 5 females) were positive to FIV
389 test with a seroprevalence of 7.87% (95% CI: 5.15-11.85) (Table 2).
390
391 *Co-infections detected*
392 Among the 254 cats evaluated, 13 were seronegative for all pathogens analyzed, whilst,
393 86 cats were positive for one pathogen. The remaining 155 cats were positive to two or
394 more pathogens (Table 4). Significant associations were observed between the variables
395 detailed in the Table 5. A significant association was established between SARS-CoV-2

396 and *L. infantum* seropositivity (p=0.018). The positivity results associated with the
397 pathogen are listed in Table 4.
398

399 **Discussion**

400 To date, this is the first study that evaluates SARS-CoV-2 infection including
401 serological analysis in stray cats during the different waves of COVID-19 outbreaks in a
402 European city. Our findings suggest that there is no significant variation in SARS-CoV-
403 2 seroprevalence in stray cats during the study period. Moreover, results are similar to
404 previous study performed in the same region (Villanueva-Saz et al. 2022a). From
405 January to October 2020, a seroprevalence of 3.51%, whilst, the next period of time
406 analyzed in this study from October 2020 to January 2022, a seroprevalence of 1.57%
407 was obtained in this study. During this period of time, different SARS-CoV-2 variants
408 such as Delta and Omicron variant later, have been detected in Spain, a geographic area
409 seriously affected by COVID-19. However, no increase of the number of seropositive
410 cats was detected compared to previous results.

411 Epidemiological studies of cats performed in different countries report seroprevalences
412 of SARS-CoV-2 ranging from 0 to 14.7% (Zhang et al. 2020; Stranieri et al. 2022).
413 Surveys using different techniques to confirm SARS-CoV-2 infection have found that
414 the prevalence of SARS-CoV-2 infection in cats in Spain varies from 2.1 to 6.4%
415 (Schulz et al. 2021; Barroso-Arévalo et al. 2022) (Table 6). Several factors seem to have
416 a direct influence of SARS-CoV-2 detection in cats such as the region where the study
417 was performed considering SARS-CoV-2 prevalence in humans, lifestyle and
418 cohabitation with COVID-19 positive contact, nature of the confirmatory technique to
419 detect the presence of SARS-CoV-2 infection, type of sample or moment to perform the
420 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. 2022).

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. 2021).

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., 2022). 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. 2022). 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. 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 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. 2022). Other authors have detected the presence of RNA material in *postmortem* samples such as lymph node or other respiratory tissues (Segalés et al. 2021). 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. 2022) 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. 2022).

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. 2022; 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. 2022). 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. 2012) 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

544 agreement to previous studies suggesting widespread worldwide in the stray cat
545 population (Dall`Ara et al. 2019).

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

561

562 Based on our results, stray cats are not adequate sentinel species for SARS-CoV-2
563 infection. However, stray cat as sentinels can be of great interest for other pathogens
564 including feline viruses, *T. gondii* and vector-borne diseases, such as *L. infantum* and *D.*
565 *immitis*. In the case of SARS-CoV-2 virus, cats have a low viral load and a short period
566 of viral shedding, and particularly stray cats live outdoors. For thus, transmission
567 implication in this context is very low. In the case of vector-borne diseases, the role
568 these animals might play in the leishmaniosis and dirofilariosis cycles should not be

569 underestimated, especially as the cats live close to humans in an endemic area. For
570 feline retroviruses and *T. gondii*, the occurrence of these three pathogens and the rate of
571 transmission of these diseases in stray cats is important to know the global health status
572 of feline colonies from a city or a specific geographical area considering the possibility
573 to harbour pathogens that produce disease in humans and animals (Taetzsch et al. 2018).
574 In the case of SARS-CoV-2 infection, this condition might not be especially relevant in
575 stray cats but it could be relevant for other zoonotic pathogens such as *T. gondii* and *L.*
576 *infantum*.

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

585 In light of our results, it seems reasonable to believe that the impact of the different
586 waves of COVID-19 outbreaks in stray cats is very limited. SARS-CoV-2 virus has
587 infected some stray cats; however, lower prevalence is found in this group of cats in
588 comparison to infected people or owned cat where human-to-cat transmission can
589 happen. Although cats could be infected by people with SARS-CoV-2 infection, and
590 experimental infection of domestic cats has been demonstrated. With our results, it can
591 be considered that the probability of the virus was circulating in stray cats from a
592 geographic area seriously affected by COVID-19 was very low. The presence of SARS-
593 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.

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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.

Author´s contributions

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.

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.

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