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Serological evidence of SARS-CoV-2 and co-infections in stray cats in Spain

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Abstract

A new coronavirus known as SARS-CoV-2 emerged in Wuhan in 2019 and spread rapidly to the rest of the world causing the pandemic disease named coronavirus disease of 2019 (COVID-19). Little information is known about the impact this virus can cause upon domestic and stray animals. The potential impact of SARS-CoV-2 has become of great interest in cats due to transmission among domestic cats and the severe phenotypes described recently in a domestic cat. In this context, there is a public health warning that needs to be investigated in relation with the epidemiological role of this virus in stray cats. Consequently, in order to know the impact of the possible transmission chain, blood samples were obtained from 114 stray cats in the city of Zaragoza (Spain) and tested for SARS-CoV-2 and other selected pathogens susceptible to immunosuppression including *Toxoplasma gondii*, *Leishmania infantum*, feline leukaemia virus (FeLV) and feline immunodeficiency virus (FIV) from January to October 2020. Four cats (3.51%), based on enzyme-linked immunosorbent assay (ELISA) using the receptor binding domain (RBD) of Spike antigen, were seroreactive to SARS-CoV-2. *T. gondii*, *L. infantum*, FeLV and FIV seroprevalence was 12.28%,

16.67%, 4.39% and 19.30%, respectively. Among seropositive cats to SARS-CoV-2, three cats were also seropositive to other pathogens including antibodies detected against *T. gondii* and FIV ($n = 1$); *T. gondii* ($n = 1$); and FIV and *L. infantum* ($n = 1$). The subjects giving positive for SARS-CoV-2 were captured in urban areas of the city in different months: January 2020 (2/4), February 2020 (1/4) and July 2020 (1/4). This study revealed, for the first time, the exposure of stray cats to SARS-CoV-2 in Spain and the existence of concomitant infections with other pathogens including *T. gondii*, *L. infantum* and FIV, suggesting that immunosuppressed animals might be especially susceptible to SARS-CoV-2 infection.

KEYWORDS
concomitant infections, COVID-19, ELISA, SARS-CoV-2, serology, stray cats

1 | INTRODUCTION

Coronaviruses (Covs) are a group of zoonotic viruses classified into four different genera including alpha, beta, gamma and delta coronavirus (Tiwari et al., 2020), which affect humans and animals equally. In December 2019, a novel betacoronavirus pathogen called Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) was described in China as the causative agent of coronavirus disease 2019 (COVID-19). This new virus belongs to the same family and genus as SARS-CoV and MERS-CoV, sharing more genetic similarity with SARS-CoV, and it is considered a zoonotic agent mainly transmitted human to human (Petrosillo et al., 2020).

In animals, these viruses cause different clinical patterns. Two different types of feline coronaviruses have been described including type I and type II Covs that belong to alphacoronavirus, affecting both domestic and wild felines (Stout et al., 2020). The virus exists in a form that is responsible for the severe and frequently lethal disease named feline infectious peritonitis (FIP) (Paltrinieri et al., 2020). In experimental conditions, SARS-CoV-2 infection of cats occurs by respiratory droplets resulting in mild respiratory signs (Shi et al., 2020), but in most of the cases, cats remain asymptomatic (Bosco-Lauth et al., 2020; Ruiz-Arrando et al., 2020), capable of transmitting SARS-CoV-2 to other cats (Gaudreault et al., 2020). Nevertheless, SARS-CoV-2 and feline coronavirus are taxonomically distant viruses with different clinical and pathological features (Paltrinieri et al., 2020).

Coronaviruses enter into host cells through a combination of interactions between the viral Spike protein with the mammalian heparan proteoglycans and the angiotensin-converting enzyme 2 protein (ACE2) (Clausen et al., 2020). Among mammalian species, simian ACE2 is the closest homologue to human ACE2, followed by cat ACE2 with an 85.2% overall identity (amino acid) compared to human ACE2. Feline ACE2 differs only 4 out of a total of 20 contacting residues from human ACE2 (Stout et al., 2020). This variation can affect the efficiency of RBD binding to ACE2 and is the major determinant of a species' susceptibility to SARS-CoV-2 (Gryseels et al., 2020).

Stray cats in Europe and other world regions are a potential source of zoonotic diseases posing a risk for human health, they can be used as sentinels for the presence of infection in a given geographic area. The presence of SARS-CoV-2 in cats and other felids have been described in recent reports (Gryseels et al., 2020). Recently, in northern Italy, 3.9% of cats had measurable SARS-CoV-2 neutralizing antibody response that prevented re-infection to coronavirus (Patterson et al., 2020). Transmission of SARS-CoV-2 from humans to domestic cats, tigers and lions has also been detected and cat-to-cat transmission has been demonstrated in experimental infections (Bosco-Lauth et al., 2020; Shi et al., 2020). This situation should be taken into account as a possible warning towards the population of stray cats in urban areas. The role of domestic animals including pets and stray animals in the context of COVID-19 is not well determined and a human-to-animal zoonosis could be occurring (Hobbs & Reid, 2020; Leroy et al., 2020; Tiwari et al., 2020). However, the level of natural infections within this scope is largely unknown owing to a marked knowledge gap which requires urgent attention. The aim of this study is to contribute to the knowledge of the role played by stray cats in the context of SARS-CoV-2 and analyse if the presence of concomitant infections with other pathogens, including *Toxoplasma gondii*, *Leishmania infantum*, feline leukaemia virus (FeLV) and feline immunodeficiency virus (FIV), may predispose the SARS-CoV-2 infection in this species.

2 | MATERIAL AND METHODS

2.1 | 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 January to October 2020 (Table 1). The study population comprised 114 stray cats captured in urban areas of Zaragoza within a trap, neuter and release sterilization program run locally to control stray feline colonies. The procedure took place within 36 hr from the capture.

TABLE 1 Number of samples collected and collection time

Collection time	Number of samples collected	Sex		
		Male	Female	ND
January 2020	30	6	13	11
February 2020	11	5	6	
June 2020	21	11	10	
July 2020	12	6	6	
August 2020	10	3	5	2
September 2020	29	17	12	
October 2020	1	1		
Total of samples	114	49	52	13

Cats were anaesthetized by subcutaneous injection with a combination of dexmedetomidine (Dexdomitor®) 15 μg/kg), ketamine (Anaestamine®, 5 mg/kg) and methadone (Semfortan®, 0.3 mg/kg). Information about breed, age and gender was recorded and a complete physical examination was carried out before sampling.

Prior to collecting blood, the fur of the cats was trimmed around the jugular region. One ml of blood was collected aseptically by jugular venipuncture to obtain the serum. Blood and separated serum were stored at −20°C until processing. Routine laboratory tests such as a complete blood count and biochemical profile were not performed.

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.

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

The DNA sequence encoding amino acid residues 319–541 (RVQPTESIVRFPNITNLCPFGEVFNATRFASVYAWNRKR ISNCVADYSVLYNSASFSTFKCYGVSPTKLNDLCFTNVYADSF VIRGDEVRQIAPGQTGKIADYNYKLPDDFTGCVIAWNSNNLDS KVGGNYNYLYRLFRKSNLKPFERDISTEIYQAGSTPCNGV EGFNCYFPLQSYGFQPTNGVGYQPYRVVVLSFELLHA PATVCGPKKSTNLVKNKCVNF) of the RBD was codon optimized and synthesized by Gen-Script 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% CO_2 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 (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 hr post-transfection to a final concentration of 2.2 mM. Cells were harvested 6-day 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 hr of reaction at 18°C, the cleavage was satisfactorily verified through SDS-PAGE. TEV protease and GFP were removed from the solution using a HisTrapColumn (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 $M^{-1}cm^{-1}$.

2.3 | 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. 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). Later, 300 μl of PBST and 3% dry skimmed milk was added to each well as blocking solution. Plate was incubated with blocking solution for 1 hr at 37°C 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 hr at 37°C in a moist chamber. After washing the plates for 30 s six times with PBST followed by one wash with PBS for 1 min, 100 μl/well of multi-species horseradish peroxidase conjugate (Thermo Fisher Scientific) was added per well. The plates were incubated for 1 hr 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) were 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). 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 cut-off 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.

2.4 | 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) diluted 1:64 in 0.2% Evans blue was added to each well. The slides were incubated in a moist chamber at 37°C 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) 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.

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

The ELISA was performed on all sera as described previously (Villanueva-Saz et al., 2019), 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 containing 1% dry skimmed milk (PBST-M), was added to each well. The plates were incubated for 1 hr at 37°C in a moist chamber. After washing the plates for 3 min three times with PBST followed by one wash with PBS for 1 min, 100 µl of Protein A conjugated to horseradish peroxidase (Thermo Fisher Scientific) was added per well. The plates were incubated for 1 hr 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) 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). 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 + three 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.

2.6 | Detection of FeLV antigens and FIV antibodies by immunochromatographic rapid test

The rapid test (Uranotest FeLV-FIV, URANOVET) 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.

2.7 | Detection of feline coronavirus (FCoV) antibodies by immunochromatographic rapid test

*FAST*est® FIP (MEGACOR Diagnostik) 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.

2.8 | Statistical analysis

Data collected for the entire population were analysed 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 *L. infantum*, FIV, FeLV or *T. gondii* infection.

Equally, associations between variables (gender and pathogens detected) were analysed. The significance of this difference was assessed using the chi-square or Fisher's exact test. A $p \leq .05$ was considered significant. The SPSS version 22 software (SPSS Inc.) was used.

3 | RESULTS

3.1 | Characterization of the animals under study and their infection association with gender

All the tested cats (52 females, 49 males and 13 non-determined) were shorthaired type, adults (more than 1-year-old) and classified as apparently healthy, with no evident systemic signs found in the general physical examination.

No significant association (data not shown, $p > .05$) was detected between the positivity for anti-SARS-CoV-2 antibodies and gender as well as between the positivity for *T. gondii*, FeLV, FIV and *L. infantum*. A significant association was detected between gender and seropositivity for FIV ($p = .0117$), being higher percentages in males (75% higher than females, 25%).

3.2 | Serological prevalence of SARS-CoV-2 infection

The seroprevalence of SARS-CoV-2 infection was 3.51%. Among the 114 cats, four cats were seropositive by ELISA (Table 2), with OD units ranging from 0.36, 0.38, 0.44 and 0.61 (cut-off ≥0.30). The presence of antibodies against RBD was detected in two males, one female and one non-determined cat. The seropositive samples were obtaining at different time points: January 2020 ($n = 2$), February 2020 ($n = 1$) and September 2020 ($n = 1$).

None of the seropositive cats to SARS-CoV-2 were also positive to FCoV (Table 2). In this sense, no serological cross-reactivity was detected between the SARS-CoV-2 and FCoV.

3.3 | Serologic analyses for other infections

Seropositive results ranged from 16.67% (19/114) for *L. infantum*, 12.28% (14/114) for *T. gondii*, 4.39% (5/114) for FeLV, and 19.30%

TABLE 2 Serological results of cats with detected infections in the study

SARS-CoV−2	*L. infantum*	*T. gondii*	FeLV	FIV	Number of seropositive cats	Gender
+	+	-	-	+	1	Male: 1
+	-	+	-	+	1	Male: 1
+	-	+	-	-	1	Female: 1
+	-	-	-	-	1	Non determined
-	+	+	-	-	3	Male:3
-	-	+	-	+	3	Male:1 Female: 1 Non determined:1
-	-	+	-	-	6	Female 4 Male 2
-	+	-	-	-	8	Male 2 Female 6
-	+	-	-	+	5	Male 4 Female 1
-	+	-	+	-	1	Male
-	+	-	+	+	1	male
-	-	-	+	-	2	Female 2
-	-	-	+	+	1	Female 1
-	-	-	-	+	10	Female 2 Male:7 Non determined:1
-	-	-	-	-	70	Female 34 Male 26 Non determined: 10
Total of samples						114

(22/114) for FIV. Results of serologic analyses for *L. infantum*, *T. gondii*, FIV, FeLV and co-infections between infectious agents are reported in Table 2.

3.4 | Co-infections detected

Co-infection with SARS-CoV-2 and other pathogens were detected in three cats: one male co-infected with *T. gondii* and FIV, one male co-infected only with FIV and *L. infantum*, and a female co-infected only with *T. gondii*. The presence of other co-infections was detected including *T. gondii* and FIV ($n = 3$), *T. gondii* and *L. infantum* ($n = 3$), FeLV and FIV and *L. infantum* ($n = 1$), FeLV and FIV ($n = 1$), FeLV and *L. infantum* ($n = 1$), and FIV and *L. infantum* ($n = 5$). The positivity results associated with the pathogen are listed in Table 2.

4 | DISCUSSION

In this study, 114 stray cats in the city of Zaragoza, in Aragon Region (Spain), were evaluated for SARS-CoV-2 infection and co-infections with *T. gondii*, FIV and *L. infantum* based on serologic data. To our knowledge, this study demonstrates for the first time that stray cats are naturally exposed to SARS-CoV-2 infection in Spain. Likewise, this research evidences seropositivity for other feline pathogens in most of the positive SARS-CoV-2 tested cats.

Detection of anti-SARS-CoV-2 antibodies in human samples is mainly based on the ELISA technique that uses different types of antigens including the whole spike protein (S), the viral nucleocapsid (N) or membrane (M) proteins and RBD of spike (Beavis et al., 2020; Klumpp-Thomas et al., 2020; Tré-Hardy et al., 2020). However, differences in diagnostic results obtained among ELISA tests are influenced by the type of antigen, being S or RBD antigens the ones which provide better diagnostic results, giving more specificity in comparison with whole virus, M or N proteins because these proteins are closely similar among the virus family (Chia et al., 2020; Klumpp-Thomas et al., 2020). Therefore, it was decided to use RBD as antigen in the in-house ELISA described in the previous section. Furthermore, anti-RDB antibodies can be considered as neutralizing antibodies because in most cases they disrupt the RBD-ACE2 interaction (Barnes et al., 2020; Yuan, Liu, et al., 2020; Yuan, Wu, et al., 2020). This has been supported by studies in animal models of SARS-CoV-2 infection and in humans by the use of COVID-19 convalescent donors' serum, leading to a protection against SARS-CoV-2 (Fischer et al., 2020). Further studies are needed to investigate the immunological properties of the antibodies detected in animals as these would have important implications for public health and infection-control policies in animals.

In this study, most cats were seronegative to SARS-CoV-2 antibodies and only four cats were seropositive. Overall, this finding is significant of the potential exposition of stray cats to SARS-CoV-2 infection; however, no animals in this study presented evident SARS-CoV-2 clinical signs on physical examination performed before sampling. A similar situation has been described in infected cats by SARS-CoV-2 that never showed any clinical signs compatible with the infection (Bosco-Lauth et al., 2020; Deng et al., 2020; Halfmann et al., 2020; Ruiz-Arrando et al., 2020). Nevertheless, other animals such as farmed minks (*Neovison vison*) are susceptible to developing clinical signs associated exclusively to SARS-CoV-2 infection, and the presence of respiratory signs including laboured breathing, nasal exudates and other more unspecific symptoms such as stopped eating have been described (Molenaar et al., 2020).

Cats are susceptible to infection by SARS-CoV-2 and the possibility of SARS-CoV-2 infection could aggravate already existing health disorders. Besides, the presence of SARS-CoV-2 together with other type of infections might lead to a diminished immune response, potentially leading to rapid disease progression. Although this hypothesis has not been elucidated, the presence of concomitant disorders has been described in an infected cat by SARS-CoV-2 with the presence of hypertrophic cardiomyopathy and secondary thromboembolism not caused by the virus (Segalés et al., 2020).

The transmission is probably linked to exposure of the cats to asymptomatic SARS-CoV-2 infected humans (Hobbs & Reid, 2020), being these circumstances also described for tigers and lions living in zoos (McAloose et al., 2020). Although animal-to-human transmission of SARS-CoV-2 is not the main way of human transmission, which is usually produced by frequent encounters human-to-human, interspecies transmission should be considered especially between animal caretakers of feline colonies and stray cats. Anti-SARS-CoV-2 antibodies have been detected in stray cats living in SARS-CoV-2 infected mink farms in the Netherlands (Molenaar et al., 2020) and two different routes of transmission could have occurred, including cat-to-cat transmission or mink-to-cat transmission (Oreshkova et al., 2020), as well as animal (mink)-to-human transmission that has been also described (Oreshkova et al., 2020). Since SARS-CoV-2 is able to infect a wide range of mammal species and it is not possible to determine individual susceptibility for all mammal species, people interacting with any wild mammal species should take sanitary precautions to prevent transmission to wildlife (Gryseels et al., 2020). Also, people who are suspected or confirmed to be infected with SARS-CoV-2 and to prevent anthropogenic transmission should limit contact with animals altogether (Hobbs & Reid, 2020).

Among pets, cats are more likely than dogs to shed infectious virus, implying that they are more prone to transmitting SARS-CoV-2 cat-to-cat (Halfmann et al., 2020; Shi et al., 2020). In Wuhan, a survey identified that the seroprevalence of SARS-CoV-2 in stray cats was 14.7% (Zhang et al., 2020). On the other hand, our study reveals that 3.51% of the cats were seropositive for SARS-CoV-2, according to the in-house ELISA analysis. The differences in the results between these studies could be explained because, in Wuhan, samples were collected from January to March 2020 when probably the circulation of the virus was superior than in Zaragoza. It is important to remark that samples from most of the seropositive cats detected in our study were collected in January and February 2020, when the rapid outbreak of the disease was not detected yet in Spain. Therefore, it could be considered that

an important transmission could have happened in this period of time in Zaragoza. Recently, the presence of SARS-CoV-2 infection in Europe has been detected prior to pandemic time in Italy (La Rosa et al., 2020) and France (Deslandes et al., 2020) in December 2019. By contrast, other study performed in Italy detected the presence SARS-CoV-2 RBD-specific antibodies in individuals in September 2019 (Apolone et al., 2020). In Spain, SARS-CoV-2 RNA was detected in metropolitan wastewater samples taken in late February 2020 in the Region of Valencia (Randazzo et al., 2020) and Murcia being the samples collected from 12 March to 14 April 2020 (Randazzo, Truchado, et al., 2020). All these studies and our results could indicate that SARS-CoV-2 extended by European countries early 2020 and people could transmit infections to the cats in the moment of feeding or cuddling, kissing or being licked or sharing food with them.

We have evaluated the possible co-infection status of seropositive cats to SARS-CoV-2 by testing blood samples obtained from the cats of this study for important immunosuppressive pathogens such as FeLV, FIV, *T. gondii* and *L. infantum*, as well as co-infections with more than one pathogen. Two seropositive cats to SARS-CoV-2 were also positive to FIV, but none of FeLV positive cats were also positive to SARS-CoV-2. FIV and FeLV are retroviruses being the most common infectious diseases in cats. These two viruses cause an important decreased count of $CD4^+$ T-cells and as a consequence clinical signs of immunodeficiency can be observed, leading to secondary infections, for example neoplasias or immune-mediated diseases. The diagnosis of FIV is based on the detection of antibodies against the viral protein p24, whilst the identification of FeLV infection is focused on the detection of p27 antigen. The prevalence rate in our study for FeLV (4.39%) was slightly lower than that reported in previous studies in Catalonia (Spain) where prevalence of selected infectious disease agents in stray cats was 6% (Ravicini et al., 2016) and 8.5% in a previous study performed in Catalonia and Mallorca (Solano-Gallego et al., 2006). By contrast, the prevalence of FIV infection in 114 stray cats of our study was higher (19.3%), than the prevalence reported by previous studies performed in Spain, 2.6% in Catalonia and 7.4% in northeastern Spain. In this sense, our results are the first epidemiological information of FeLV and FIV infection in stray cats in Zaragoza.

Equally, in this study, the seroprevalence of *L. infantum* was performed. Little is known about the existence of *L. infantum* in cats in Zaragoza except for the congress communication (Zárate-Ramos et al., 2002) published including 50 domestic cats where 42% of these animals resulted seropositive to *L. infantum* by direct agglutination test and furthermore presenting some immune dysfunction. By contrast, the seroprevalence detected by ELISA in this study was 16.67%. The presence of *L. infantum* infection is associated with impaired immuno-competence based on immune exhaustion and associated with a predominant T helper 2 (Th2) and an impaired T helper 1(Th1) response. In our study, one cat was positive to FIV, *L. infantum* and SARS-CoV-2.

Toxoplasma gondii is a parasite that infects nearly all warm-blooded animals, including pets and humans. The seroprevalence of this parasite in our study was 12.28%. Different epidemiological surveys have been performed in different regions of Spain showing variable seroprevalence levels by IFAT from 25.5% to 54.4%. (Aparicio-Garrido et al., 1972; Miro et al., 2004). This type of serological analysis is important to be performed because the level of seroprevalence could be an alternative to measure the spread of *T. gondii* in the environment (Braga et al., 2012), being considered stray cats as sentinels of the exposure to oocysts of *T. gondii* from the environment and through ingestion of tissue cysts. The presence of *T. gondii* infection is more likely to occur in cats with suppressed immune systems, including young kittens and cats affected with FeLV or FIV (Sousa et al., 2014). In our study, co-infections were detected between *T. gondii* and other pathogens analysed, specifically four seropositive cats to both *T. gondii* and FIV and three seropositive cats to *T. gondii* and *L. Infantum*.

In general, seroprevalence studies including different detected pathogens vary depending on different factors including geographic location, type of lifestyle, age of animals and/or methods of serological analysis performed. The data of our study provide an estimation of exposure of stray cats to SARS-CoV-2 in Spain, as well as the prevalence of infections and co-infections regarding other important pathogens in stray cats in Zaragoza city (Spain), such as retroviruses, *T. gondii* and *L. infantum*. These findings should not have a negative impact on the cat's welfare and do not be an excuse from owners to abandon their pets because seropositive domestic cats could infect stray cats and extend the SARS-CoV-2 infection between them. Further prevalence surveys in stray cats, also using other diagnostic methods, are warranted to clarify the role of these hosts in the epidemiology of SARS-CoV-2 transmission as well as the potential of immunocompromised stray cats as a potential reservoir for SARS-CoV-2.

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ETHICAL APPROVAL

This survey was included under Project Licence PI62/17 approved by the Ethic Committee for Animal Experiments for the University of Zaragoza. The care and use of animals were performed according with the Spanish Policy for Animal Protection RD53/2013, which

meets the European Union Directive 2010/63 on the protection fo animals used for experimental and other scientific purposes.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

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

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