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Molecular detection of SARS-CoV-2 in domestic cats in close contact with positively-infected owners in Tehran, Iran in 2021

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Abstract

Objectives In 2019, COVID-19 emerged in China and has since spread worldwide. Owing to the virus's ability to adhere to specific receptors, cats are susceptible to infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The popularity of pet cats in Iran has sparked fears of human–cat–human transmission of the virus. This study aimed to identify positive cases in cats owned by people infected with SARS-CoV-2, to determine if they remained positive for >3 weeks and to examine the virus genome isolated from a number of cats and one of their owners.

Methods A total of 30 cats were sampled approximately 3 days after their owners tested positive (day 1), and 3 weeks later, in strict accordance with health regulations. Rectal and oropharyngeal samples were collected. All samples were subjected to a qualitative PCR and reverse transcription PCR. The *S*-gene region was partially sequenced in positive samples and the results were used to create a phylogenetic tree.

Results SARS-CoV-2 was detected in 7/30 (23.3%) cats examined. In the third week, every cat tested negative. The sequence data of positive cats and one of their owners revealed that the retrieved RNAs belonged to the alpha variation. The genetic distance between the samples and the reference sequence (20I/B.1.1.7: OM003849, MZ344997) was minimal, with a 99% similarity. Positive samples of cats had four mutations in gene *S*. Amino acid substitutions in the spike glycoprotein at positions N501Y, A570D, D614G and P681H were recorded in the isolates compared with 780 other sequences of Iranian strains.

Conclusions and relevance This study confirmed the presence of SARS-CoV-2-infected cats living in close contact with infected owners. Despite cats' susceptibility to COVID-19, the risk of severe infection in these animals is low, as evidenced by the lack of clinical signs in positive cats.

Keywords: COVID-19; SARS-CoV-2; sequencing; PCR; Iran

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Introduction

In December 2019, a large number of patients exhibiting symptoms of viral pneumonia were found in several health centres in Wuhan, China. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), later termed 2019-nCOV, was identified by unbiased sequencing in samples from patients with pneumonia.[1] Globally, by January 2023, 673.9 million cases of SARS-CoV-2 and 6.7 million deaths had been recorded. During this time in Iran, 7.5 million people have been infected and 144,000 people have died.[2]

Initially, SARS-CoV-2 binds to its receptor, the enzyme angiotensin convertase II (ACE2), which is expressed on the surface of upper respiratory epithelial cells, type I and II epithelial cells in the lung parenchyma, cardiac epithelial cells, endothelial cells, renal tubular epithelial cells, enterocytes and the pancreas.[3–6] In addition, ACE2 is mainly expressed in type II pneumocytes and serous epithelial cells of tracheobronchial submucosal glands in ferrets. Ferrets and cats differ by only two amino acids in the SARS-CoV-2 spike-contacting regions of ACE2. Cats are highly susceptible to SARS-CoV-2; dogs have a low susceptibility to SARS-CoV-2 owing to low levels of ACE2 in the respiratory tract.[7,8]

SARS-CoV-2 virological isolates from animals are scarcely represented in GenBank. The genomic sequence of a cat infected with SARS-CoV-2 in Wuhan, China, was remarkably similar to that of a bat and a pangolin. Human gene sample sequences were identical to those of dogs, cats and tigers.[6]

Owing to the incomplete understanding of the role of pets in virus transmission, this 3-week study investigated the infection of cats in contact with infected humans.

Materials and methods

Obtaining samples

In order to identify research-eligible cats in Tehran between February 2021 and July 2021, cat owners with positive COVID-19 PCR tests were invited to volunteer their pets to participate in the study. Recruited owners were then interviewed about their pets' clinical signs in the week preceding the testing of their cats. Rectal and oropharyngeal samples were collected using sterile swabs from 30 cats on the first day (1–3 days after the owners' positive PCR test) and 3 weeks later. Only 30 out of a possible 96 cats were recruited for the study. The sample size ($n = 96$) was estimated by the formula $n = Z^{2P}(1–P)/d^2$ ($Z = 1.96$, $P = 0.50$, $d = 0.1$). The ethical approval number for this study is IR.UT.VETMED.REC.1401.009.

RNA extraction and amplification of the SARS-Cov-2 genome

The RNJia Virus Kit (Roje Technologies Corporation) was used to extract RNA samples according to the manufacturer's instructions. The extracts were then stored at −20°C until PCR amplification was performed. The SARS CoV-2 genome was detected by the Sansure Novel Coronavirus (2019-nCoV) Nucleic Acid Diagnostic Kit (Ref S3102E SC2; Sansure Biotech) as described by the company. This kit is used for the qualitative detection of the *ORF1ab* and *N* genes of SARS-CoV-2 RNA.

Genome sequencing and phylogenetic analysis of the SARS-COV-2 genome

The positive samples were sent to the COVID-19 National Reference Laboratory at the Pasteur Institute of Iran for genetic analysis. Spike gene sequencing and mutation analysis were performed as previously described.[9] Briefly, partial spike gene amplification using a one-step RT-PCR Kit (biotechrabbit) and the PCR products were sequenced bidirectionally using the Sanger method on a Genetic Analyzer 3500 (Thermo Fisher Scientific).

The sequence information was then evaluated using the Bio Edit Version 7.0 Hall software. A phylogenetic analysis was carried out on the edited nucleotide by alignment of the spike sequence on the National Center for Biotechnology Information (NCBI) database using the BLAST program.

The phylogenetic tree was created using the MEGA program version X and the reference sequences from the Gen Bank (19A/B: NC_045512, 20I/B.1.1.7: OM003849, MZ344997). The Clustal W method was used to align the sequences of the examined isolates with the reference sequences, followed by the neighbour-joining nucleotide replacement pattern approach and the bootstrap test (1000 replicates).

Statistical analysis

The data were represented as absolute frequency and relative frequency percentage using SPSS software (version 26; IBM) for statistical analysis. The data were analysed using the χ^2 test and the kappa consistency coefficient, with $P \leqslant 0.05$ considered as significant.

Results

Molecular detection

Of the 30 cats, 16 (53%) were male and 14 (47%) were female. COVID-19 restrictions during the pandemic and limited financial resources reduced the number of cats able to be recruited. All the cats were located in the north, south, east and west of Tehran province, hundreds of kilometres apart.

Of the cats, seven (23%) tested positive (five pharyngeal samples only, one rectal sample only and one cat for both), including four (57%) male cats and three (43%) female cats among the positive samples. The cat with the positive rectal sample had vomiting about 10 days before testing, based on the history reported by the owner. Other SARS-CoV-2 positive cats had no clinical signs.

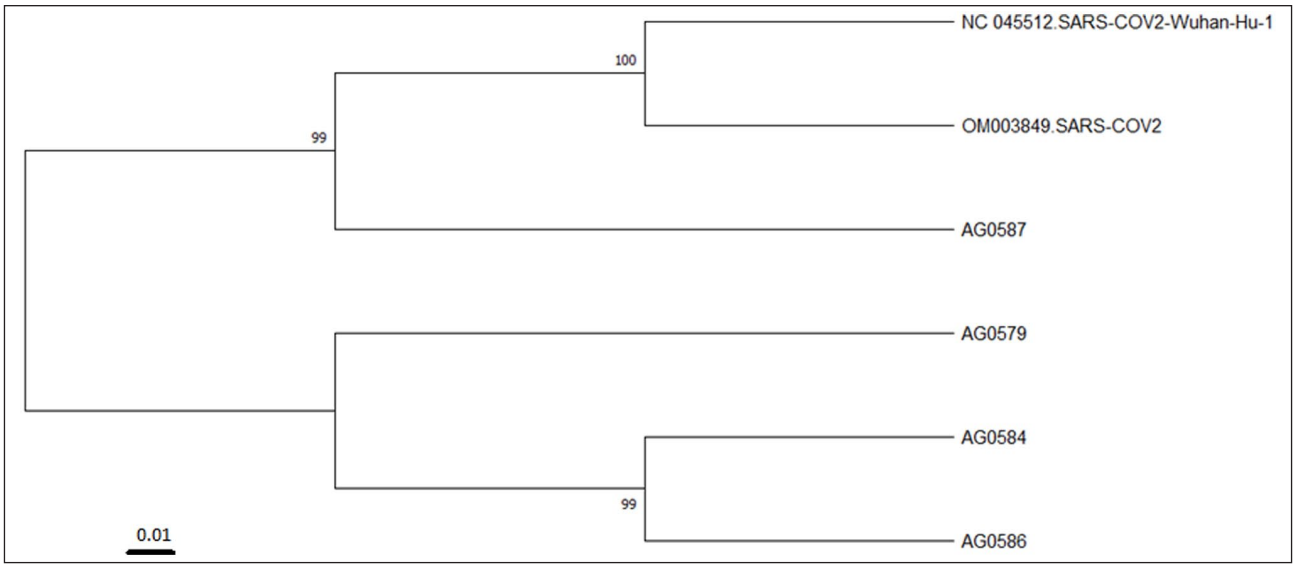

Figure 1 Neighbour-joining tree based on *S*-gene partial sequences of the four isolates described in this study. Bootstrap resampling was carried out using 1000 replicates of the data set, and the values are written as a percentile beside the appropriate branch. AG0587 belongs to the owner; AG0579, AG0584 and AG0586 belong to the cats

Table 1 Genetic distances between the studied samples and reference sequences (AG0587 belongs to the owner; AG0579, AG0584 and AG0586 belong to the cats)

	NC-045512.SARS-CoV2-Wuhan-Hu-1	OM003849.SARS-Cov2	AG0587 (owner)	AG0579 (cat)	AG0584 (cat)	AG0586 (cat)
NC-045512.SARS-CoV2-Wuhan-Hu-1						
OM003849.SARS-Cov2	0.001195077					
AG0587 (owner)	004882669	0				
AG0579 (cat)	0.004930822	0	0			
AG0584 (cat)	0.005030036	0	0	0		
AG0586 (cat)	0.138528429	0.148927363	0	0	0	

Of the 60 samples collected during the first week, eight (13%) were positive, with six (75%) pharyngeal samples and two (25%) rectal samples, with a Ct value in the range of 18 to 37. All 60 samples collected during the third week were negative.

Sequence analysis

All positive samples (eight from cats and one from one of the owners) from the virology lab's archives were forwarded to the COVID-19 National Reference Laboratory for sequencing. Four samples with a high viral load (Ct value of the cat's owner [AG0587] with *N* gene Ct value of 18, and the cats [AG0579, AG0584 and AG0586] with Ct values of 26, 19 and 29, respectively) successfully amplified by PCR of the sample were sequenced and evaluated in BLAST.

All detected samples belonged to the alpha variation based on the mutation. A phylogenetic tree was constructed using the GenBank nucleotide sequence identification numbers (NC045512 and OM003849) and MEGA (v. X) program, neighbour-joining method and 1000 bootstraps of this program (Figure 1). Table 1 displays the computed genetic distance between the samples in Figure 1's phylogenetic tree and their respective reference synonyms.

The genetic distance between the samples and the reference sequence was very small, as indicated by the phylogenetic tree, and their similarity was 99%. Only three pharyngeal samples from three cats and one sample from the owner were genetically sequenced out of six positive pharyngeal and two positive rectal samples. The remaining samples could not be sequenced, most likely owing to a low viral load.

All four sequenced isolates belong to the B.1.1.7 lineage, clade 19A, SARS-CoV-2. An analysis of the sequence homology revealed the relationship between these viruses with strains from the cities of Iran, South Korea and Slovakia. AG0587 showed a high genetic similarity with the Slovak strain OU819887 (Figure 2). In this study, positive samples were compared

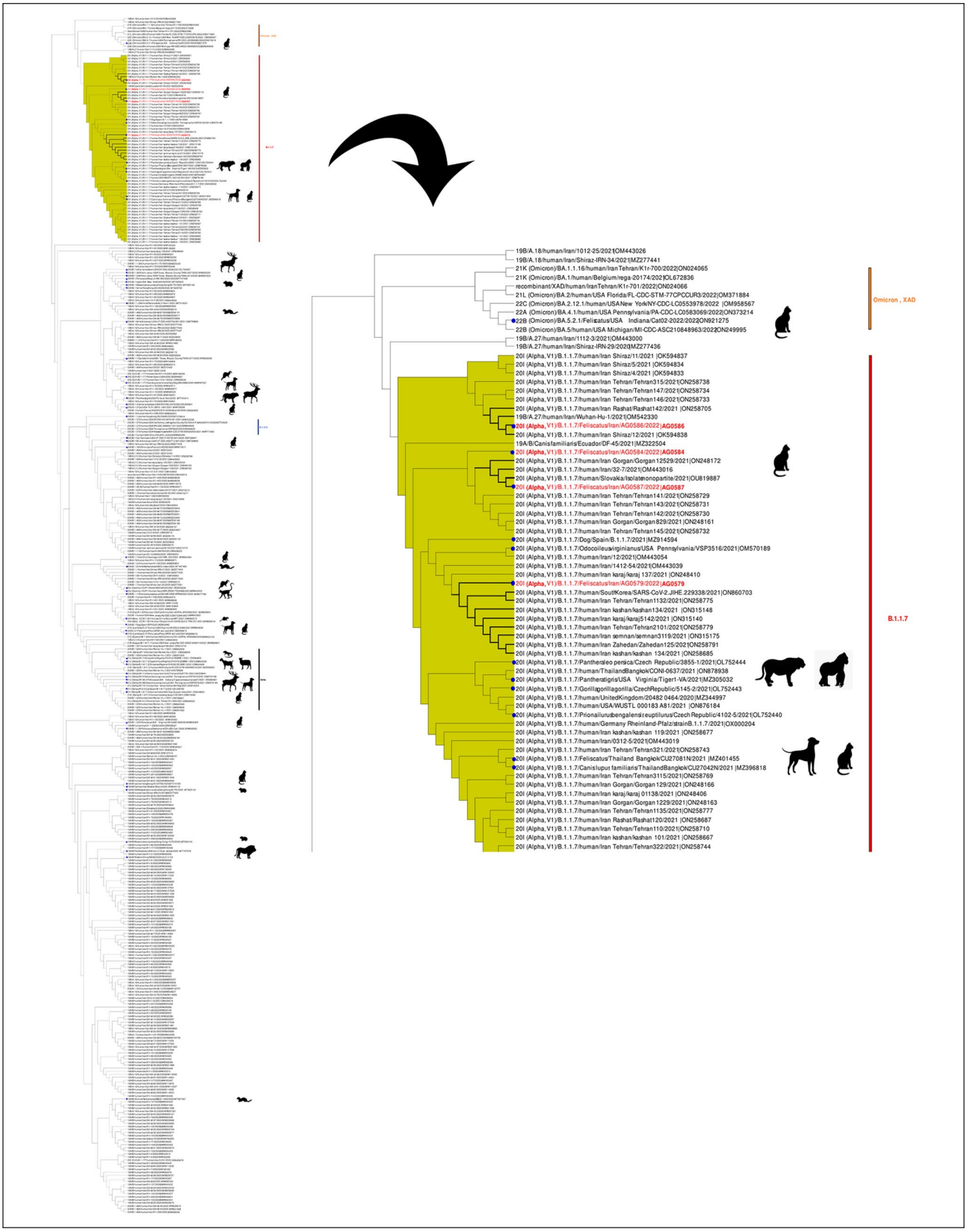

Figure 2 Phylogenetic tree showing the relationship of Iranian strains (red sequences) with other SARS-CoV-2 strains based on the *S* gene (sequences identified in animals are indicated by a blue dot). The analysis showed that the virus belongs to the B.1.1.7 lineage (green highlights). The construction was performed by the maximum likelihood (ML) method using GTR+F+I+G4 model in the program IQ-TREE version 1.6.12 with 1000 ultrafast bootstraps

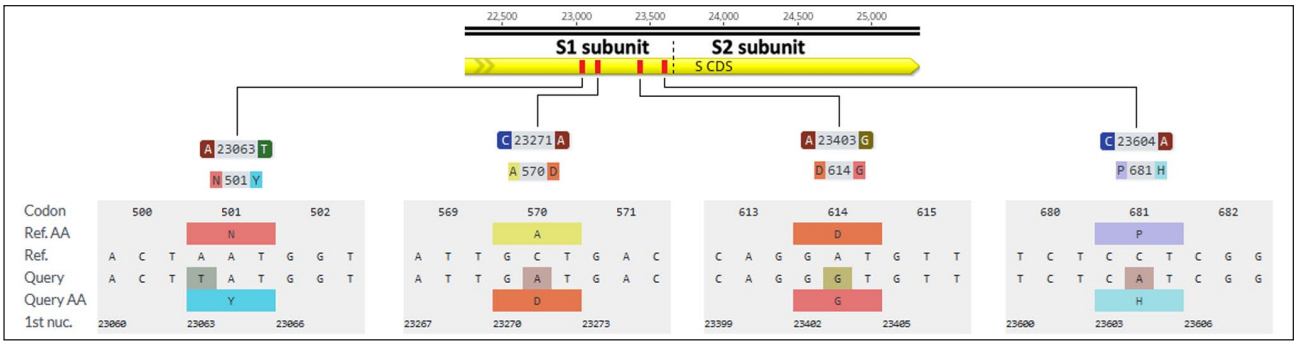

Figure 3 Amino acid substitutions at positions N501Y, A570D, D614G and P681H in the spike glycoprotein

genetically with 780 other sequences of Iranian strains, revealing that this particular virus has four mutations in the *S* gene. Amino acid substitutions in the spike glycoprotein at positions N501Y, A570D, D614G and P681H were recorded in the isolates (Figure 3). Comparing the sequences of Iranian SARS-CoV-2 viruses with the findings above revealed that this strain circulates in Tehran, Karaj, Gorgan, Rasht, Kashan, Semnan, Shiraz and Zahedan.

Discussion

The findings of this study indicate that a molecular test for SARS-CoV-2 was positive in cats exposed to positive owners, which is consistent with the results from a study in Spain in August 2020. In that study, cats, pigs, dogs and rabbits were evaluated, and the owners' positive molecular tests were considered, as was the case in the present investigation. The sole positive sample in their analysis was a cat with multiple underlying diseases.[10] In March 2021, in Chile, the infection of all three cats in a SARS-CoV-2-positive household was confirmed.[11] In a study in 2022, in northern Greece, molecular testing was positive for all three cats with positive owners.[12] In a 5-month longitudinal study in Rio, Brazil in April 2021, the gene sequences of eight dogs and four cats were positive for COVID-19 in molecular and serological studies of 39 domestic animals, which included dogs and cats that lived with positive owners who were not hospitalised.[13] However, molecular testing for cats was negative in a cross-sectional study in France.[8]

In a Thai study from January 2022, none of the animals tested were positive for COVID-19. The animals were tested in obviously high-risk areas. The lack of consideration given to the amount of contact these animals had with their deceased owners may explain why none of the cats in this study tested positive.[14]

The virus uses ACE2 receptors to enter the cell. This receptor is present in type 2 pneumocytes and tracheobronchial mucosal epithelial cells in ferrets. Two amino acids separate the SARS-CoV-2 spike binding sites in the ACE2 receptor of ferrets and cats, indicating that cats are extremely susceptible to COVID-19.[7]

Mutations in the spike gene are regarded as one of the most effective factors for viral infectivity and disease severity. Four mutations were detected in this research. Substitutions at positions N501Y and D614G make the protein structure more stable and allow the virus to bind to the cell receptor with high affinity and enhance viral replication and transmission in animals.[15–17]

By the third week of sampling, none of the positive cats in the present study were infected, and their molecular tests were negative. In a study in Japan, all cats were positive until the fifth day after SARS-CoV-2 inoculation.[18] In addition, according to the findings of a study in Spain, a positive cat's retest after 3 weeks was negative.[10] In a study in Chile, in 2021, the molecular tests in cats were positive on the 17th day.[11] A positive test in cats was no longer positive after 21 days in a study in northern Greece.[12]

According to the pet owners, only one of the cats in the current study displayed clinical signs, including vomiting, and other positive animals exhibited no additional clinical signs. The findings of the studies conducted in 2020 and 2022 are consistent with this finding;[7,12,18] however, in the studies from 2021, infected cats did exhibit clinical signs.[11,13,19]

The relationship between the pharyngeal and rectal samples was not statistically significant. The negligible amount of viral RNA released in faeces could also result in a negative test result from a rectal sample. However, according to the findings of a study published in 2022, in northern Greece, all rectal samples from all cats were positive, and there was no difference between pharyngeal and rectal samples in terms of the number of completed sequences.[12]

This virus was examined concerning the 44 lineages of animal SARS-CoV-2 viruses previously identified. A total of 3.4% of these viruses belonged to lineage B.1.1.7; they were isolated from a white-tailed deer and a tiger from the USA, a gorilla and a lion from the Czech Republic, a dog from Spain and a cat from Thailand a Spain.

Regarding disease susceptibility, the present study concurs with most previous studies on the infection of domestic cats with the SARS-CoV-2 virus. As evidenced by their positive molecular tests, it is believed that these

animals became infected owing to their proximity to diseased humans without clinical signs.

Conclusions

This study confirmed that the SARS-CoV-2 virus might be transmitted from humans to domestic cats; however, there is no evidence that the virus was transmitted from cats to humans. No positive rectal samples could be sequenced, which could be due to a low viral load in the rectal samples, sensitivity of RNA viruses and possible damage to the virus during storage and while being transported to the laboratory, or technical errors. All cats tested negative by the third week (21 days later). Surprisingly, the absence of clinical signs in COVID-19-positive cats suggests that, despite their susceptibility, cats have a very low mortality risk. Further studies may be needed to understand the molecular dynamics and evolution of SARS-CoV-2 in domestic cats.

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Conflict of interest The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval The work described in this manuscript involved the use of non-experimental (owned or unowned) animals and procedures that differed from established internationally recognised high standards ('best practice') of veterinary clinical care for the individual patient. The study therefore had prior ethical approval from an established (or ad hoc) committee as stated in the manuscript.

Informed consent Informed consent (verbal or written) was obtained from the owner or legal custodian of all animal(s) described in this work (experimental or non-experimental animals, including cadavers) for all procedure(s) undertaken (prospective or retrospective studies). No animals or people are identifiable within this publication, and therefore additional informed consent for publication was not required.

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