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First detection and molecular analysis of SARS-CoV-2 from a naturally infected cat from Argentina

Nadia Analía Fuentealba, Gastón Moré, María Emilia Bravi, Juan Manuel Unzaga, Lorena De Felice, Marcos Salina, Mariana Viegas, Mercedes Soledad Nabaes Jodar, Laura Elena Valinotto, Fernando David Rivero, David Di Lullo, Marcelo Pecoraro, Carlos Javier Panei

ABSTRACT

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the etiological agent of coronavirus disease 2019 (COVID-19), has rapidly spread worldwide. Studies of transmission of the virus carried out in animals have suggested that certain animals may be susceptible to infection with SARS-CoV-2. The aim of the present study was to investigate the infection of SARS-CoV-2 in pets (18 cats and 20 dogs) from owners previously confirmed as COVID-19-positive. Oropharyngeal and rectal swabs were taken and analyzed by real-time RT-PCR assays, while blood samples were taken for antibody detection. Of the total pets analyzed, one cat was found reactive to SARS-CoV-2 by real-time RT-PCR of an oropharyngeal and a rectal swab. This cat presented only sneezing as a clinical sign. Serological analysis confirmed the presence of antibodies in the serum sample from this cat, as well as in the serum from another cat non-reactive to real-time RT-PCR. Complete sequence and phylogenetic analysis allowed determining that the SARS-CoV-2 genome belonged to the B.1.499 lineage. This lineage has been reported in different provinces of Argentina, mainly in the Metropolitan Area of Buenos Aires. This study notifies the first detection of the natural infection and molecular analysis of SARS-CoV-2 in a cat from Argentina whose owner where COVID-19-positive. Although there is currently no evidence that cats can spread COVID-19, results suggest that health authorities should test pets with COVID-19-positive owners.

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1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the etiological agent of coronavirus disease 2019 (COVID-19), has rapidly spread worldwide. Although the available data suggest that SARS-CoV-2 emerged from an animal source, there is currently not enough evidence to corroborate either the source or route of transmission from the original animal reservoir to a putative intermediate host and then to humans. Thus, several studies are being carried out to gain insights into the susceptibility of different animal species, including domestic animals, to SARS-CoV-2. Coronaviruses (CoVs) belong to the order *Nidovirales* and the family *Coronaviridae*, which is further divided into the subfamilies *Letovirinae* and *Orthocoronavirinae*. The members of the subfamily *Orthocoronavirinae* are classified into four genera: *Alpha*, *Beta*, *Gamma* and *Delta* coronavirus according to their antigenic properties and phylogenetic relationship (https://talk.ictvonline.org/). The CoVs, have a single-stranded positive-sense RNA genome of 27–31-Kb. About two thirds of its genome code for the replicate gene, which consists of two overlapping open reading frames (ORFs), denominated ORF 1a and 1b, while other four ORFs code for the structural proteins of the virus. These
proteins are the spike (S), envelope (E), membrane (M) and nucleocapsid (N) proteins, necessary to complete the replication cycle of the virus. The S protein mediates viral binding to specific cell receptors and fusion between the envelope and the plasma membrane, and is the main inducer of neutralizing antibodies (Brian and Baric, 2005). The ability of CoVs to produce mutations in their genome facilitates the transmission from animals to humans and vice versa (Woo et al., 2009). These events could occur due to the accumulation of point mutations in different regions of the genome, and due to homologous recombination events between closely related genes of different circulating CoVs lineages in multiple host species, which have given rise to different viral strains (Graham and Baric, 2010; Ghosh and Malik, 2020; Terada et al., 2014; Vijgen et al., 2005, 2006). According to previous studies, SARS-CoV-2 is believed to have originated in bats (Zhou et al., 2020), similarly to that occurred with SARS-CoV and MERS-CoV, two other zoonotic coronaviruses reported in 2003 and 2012, respectively (Gautam et al., 2020). Since the species barrier jump from bats to humans is considered unlikely, the most probable hypothesis includes the existence of an intermediate host (Andersen et al., 2020). This highlights the importance of animals in the emergence of COVID-19 in China, which further emphasizes the need of a One-Health approach to tackling emerging diseases. Although there is currently no evidence that pets play a substantial role as anorexia, lethargy, diarrhea, cough and sneezing. Their COVID-19-positive owners. Most of the animals selected in this homes, all of which were located in the Metropolitan Area of Buenos Aires (AMBA), Argentina. All the animals were in close contact with...
quantifications were performed with the Qubit kit (Thermo Fisher Scientific, Waltham, MA, USA). For the sequencing process, a NextSeq500 platform with a NextSeq 500/550 Mid Output Kit v2.5 cartridges (300 cycles) was used.

2. Mapping-Alignment and complete genome sequencing obtaining.

The resulting reads were aligned with the BWA-MEM software to the SARS-CoV-2 reference genome with the ID EPI_ISL_402124 (GISAID), hCoV-19/Wuhan/WIV04/2019. Finally, the consensus sequence for each sample analyzed was generated in FASTA format with the help of the pileup command of the samtools software (Li et al., 2009) and the consensus command of bcftools (Li, 2011). To validate this consensus sequence, a second method, the de novo assembly with the MEGAHIT software (Li et al., 2015), was used to ensure that there was coincidence with the one previously obtained. Finally, a visual verification was performed using the IGV software.

2.4. Phylogenetic analysis

Regarding the viral data processing and analysis, the resulting reads of the sequencing process were aligned with the BWA-MEM software to the SARS-CoV-2 reference genome with the ID EPI_ISL_402124, and the consensus sequence for the sample was generated in FASTA format with a mean read depth of 4000X, covering around 94.01 % of the full-length genome (sequence data have been deposited in GISAID with accession number: EPI_ISL_1914577).

For the analysis of the cat sequence, a rapid lineage assignment was carried out using the Pangolin COVID-19 Lineage Assigner program (https://pangolin.coq-uk.io/). This tool allows determining the lineage through identification of complex patterns by using machine learning.

The lineage assigned was confirmed by phylogenetic analysis. The cat sequence was analyzed together with reference sequences from different lineages (https://github.com/hCoV-2019/pangolin) and sequences with the best alignment score by BLAST (10 hits per unknown sequence, against the GISAID database as of April 20th 2021, Supplementary data). The alignments were built with the MAFFT program (https://mafft.cbrc.jp/alignment/server/) with default parameters. The appropriate evolutionary model was selected with ModelFinder (Kalyaanamoorthy et al., 2017) according to Bayesian Information criteria, and the phylogenetic analysis was performed by Maximum Likelihood with the IQ program -TREE v. 2.1.2 COVID-edition (Minh et al., 2020). The reliability of the groups obtained was evaluated by the SH-like approximate likelihood ratio test (1000 replicates) (Guindon et al., 2010) and Ultrafast bootstrap Approximation (10,000 replicates) (Hoang et al., 2018). The SH-like / UFB values for the relevant groups are indicated in the tree branches.

2.5. Detail of non-synonymous substitutions (amino acid changes) in the proteins encoded in the cat SARS-CoV-2 genome

To determine the non-synonymous substitutions in comparison with the reference sequence hCoV-19/Wuhan/WIV04/2019, the CoV server tool was used and corroborated by visual inspection. The changes were compared with those reported in SARS-CoV-2 sequences worldwide using the GISAID COV-GLUE tool (Singer et al., 2020) and the outbreak info tool.

2.6. Serological analysis

The serum samples collected from the cats and dogs chosen for the present study were used to determine the presence of antibodies against SARS-CoV-2. Antibody detection were carried out at the Facultad de Farmacia y Bioquímica, Universidad Nacional de Buenos Aires, under the service modality using an ELISA 2.0 kit, developed by researchers from NANOBIOBEC and the LIE group (Chair of Immunology-IDEHU). The kit detects the specific antibodies against the S protein of SARS-CoV-2 in serum samples from different species.

3. Results

3.1. SARS-CoV-2 detection

The RNA extraction kit used includes an RNA carrier which allows the monitoring of the process by amplification of the internal control in the real-time RT-PCR in all samples. One out of the 18 cats analyzed in this study was detected as reactive by real-time RT-PCR from the OP and R swab samples. The ORF1ab and N genes were detected from the OP swab sample with a cycle threshold (Ct) of 35 and 30 respectively, while the N gene was detected from the R swab with a Ct 37.5, using the Cy5 DisCoVery SARS-CoV-2 RT-PCR detection kit. The results were then analyzed with a second kit of real-time RT-PCR, the GeneFinder™ COVID-19 Plus RealAmp kit. The results obtained showed the amplification for the three viral targets, RdRp, N and E genes, from OP samples with a Ct of 34.5, 31.5 and 36.5 respectively. Therefore, both kits amplified the OP sample, while the R sample was not reactive with the second kit.

The SARS-CoV-2-reactive cat was a 1.5-year-old female of mixed breed, living in a household in La Plata city, Buenos Aires, Argentina. The animal presented only sneezing for about 3 days after close contact with its COVID-19-positive owners. One of the owners developed symptoms on August 23rd 2020 and was then diagnosed COVID-19-positive by real-time RT-PCR two days later, on August 25th, whereas the other owner was confirmed as COVID-19-positive on August 28th 2020. Both owners were in close contact with the animal during the course of the disease. The samples were taken from the cat on September 5th, 2020.

To determine how long, the cat remaining reactive to SARS-CoV-2 by the molecular assay, additional OP and R swab samples were taken 6 days after the first samples. In this case, the presence of the genome of the virus was not detected by either real-time RT-PCR kit.

None of the dog samples analyzed in this study were found reactive to SARS-CoV-2 by real-time PCR with either kit. In addition, none of the dogs showed clinical signs, but some owners mentioned that their dogs were lethargic.

3.2. Next-generation sequencing of SARS-CoV-2 and phylogenetic analysis

The phylogenetic analysis determined that the cat SARS-CoV-2 genome identified belonged to the B.1.499 lineage. To study the genetic relationship of the cat sequence with sequences of the same lineage, a phylogenetic tree of the lineage B.1.499 was constructed (Fig. 1). In this phylogenetic tree, the cat sequence (blue) is included in a moderately supported cluster with sequences from Buenos Aires City (orange) and sequences from the Province of Buenos Aires (red) detected from June 2020 to December 2020. As compared with the Wuhan reference sequence, and based on 94.01 % of the genome covered, the SARS-CoV-2 cat sequence here found showed seventeen nucleotide mutations (Table 1).

3.3. Serological analysis

Serum samples from all the animals studied in the present study were taken at least 15 days after the first swab sampling, except the sample from the cat reactive to SARS-CoV-2 by molecular assay, which was taken 35 days after the first swab sample. The serological analysis by ELISA of the serum sample from this cat showed a strong positive signal against protein S of SARS-CoV-2. The serum sample from another cat also showed antibodies against SARS-CoV-2, indicating that the animal was also infected with the virus. The other two cats analyzed were negative by this technique.

The serum sample of the cat positive by real-time RT-PCR gave a positivity index (PI) value of 101.79. These values, clearly above the standard cut-off criteria (PI ≥ 10), revealed the presence of specific
antibodies against SARS-CoV-2. This result, combined with the positive real-time RT-PCR data obtained 35 days before serology, strongly suggests that SARS-CoV-2 actively replicated in this animal, inducing a robust anti-spike-protein antibody response. The other cat detected as positive by ELISA showed PI values of 21.07.

In all dog sera analyzed, no presence of specific antibodies against SARS-CoV-2 was detected by ELISA.

4. Discussion

At the beginning of September 2020, the Faculty of Veterinary Sciences of the National University of La Plata, Buenos Aires, Argentina, reported to the local health authorities the first case of a domestic cat infected with SARS-CoV-2, detected by real-time RT-PCR. This result was reported to the World Organization for Animal Health (OIE), being the first case of SARS-CoV-2 detected in an animal from Argentina. Different species, including domestic and wild animals, naturally infected with this novel coronavirus have been reported around the world (https://www.oie.int/en/scientific-expertise/specific-information-and-recommendations/questions-and-answers-on-2019novel-coronavirus/events-in-animals/). Moreover, experimental studies have demonstrated that cats are susceptible to SARS-CoV-2 infection and able to transmit the virus among them (Halfmann et al., 2020; Shi et al., 2020). The confirmed positive cat detected in this study was a 1.5-year-old female of mixed breed. The first samples (OP and R swabs) were taken on September 5th, 11 days after the first positive diagnosis of its owners. Although the exact moment of infection cannot be determined, the animal was detected as reactive to SARS-CoV-2 in these samples, with Ct values over 30, which could indicate a moderate viral load. Nevertheless, it also suggests that a potential replicative virus was present. Similar results have been previously found in two cats in Spain (Segales et al., 2020). In the present study, a second set of OP and R samples was taken from the cat on September 11th, and, in this case, the real-time RT-PCR results were not reactive in either swab. These results

Non-structural proteins (nsp); Open reading frame (ORF); Amino acid (aa); Single Nucleotide Polymorphism (SNP).
suggest a rapid clearance of the virus from the respiratory and intestinal tracts, which is consistent with experimental infection of cats, where the presence of viral RNA was detected until 5–8 days post-infection (Shi et al., 2020). The viral RNA expressed in the nasal turbinate, trachea, lung, and small intestine indicated that the virus replicated well in the upper respiratory tract of those cats. Viral RNA has also been detected in tonsils, lymph nodes, spleen, bone marrow, liver, kidney, heart, and olfactory bulb (Gaudreault et al., 2020; Shi et al., 2020). In natural infection of cats, it has been reported that the viral RNA may persist for about 10–11 days in nasal swabs (Barrs et al., 2020; Garigliany et al., 2020; Sailleau et al., 2020).

Although the SARS-CoV-2-positive cat studied in the present study did not show complications of its health during the course of the infection, it was clinically monitored until the real-time RT-PCR was not-reactive. Similarly, experimental studies have shown no clinical signs in any of the inoculated cats (Gaudreault et al., 2020; Shi et al., 2020). Moreover, other authors have described cases of natural infection that never showed clinical signs compatible with SARS-CoV-2 infection (Barrs et al., 2020; Segalés et al., 2020). Thus, infected cats with no clinical symptoms could act as a silent intermediate host of SARS-CoV-2. However, in a case of natural infection reported in Belgium, the cat presented clinical signs, such as lethargy, anorexia, loss of appetite, vomiting, and diarrhea, and later showed sneezing, productive cough, difficult breathing, and emaciation (Garigliany et al., 2020).

The phylogenetic analysis here performed allowed determining that the cat SARS-CoV-2 genome belonged to the B.1.499 lineage. This lineage, fully Argentine and initially detected in March 2020, has been reported in different provinces of the country, mainly in the region of the Metropolitan Area of Buenos Aires. In the analysis of genetic relationship with sequences of the same lineage, the cat sequence was included in the cluster with sequences from Buenos Aires City and sequences from the Province of Buenos Aires. This cluster included different cities of Buenos Aires province, all of which are located close to the city of La Plata, where the cat sample was obtained. Furthermore, within this cluster, the cat sequence formed a highly supported subcluster with a sequence that was detected in October 2020 in Lanús, a city within the Metropolitan Area of Buenos Aires. These two cities are connected by a large flow of people, a fact that supports this finding. When compared to the Wuhan reference sequence (Wu et al., 2020) and based on 94.01% of the genome covered, the SARS-CoV-2 cat sequence showed seventeen nucleotide mutations. Twelve out of these seventeen mutations have resulted in amino acid mutations, which have already been described in other sequences worldwide, except for the NSP2 K500R and NSP4 W6R combination, which was here observed for the first time and has not been previously reported. In particular, for the Spike region, the two changes observed (S,D614 G and S_E654Q) have already been reported in other sequences of the B.1.499 lineage from Argentina.

In this study, serological analysis by ELISA showed a strong positive signal against SARS-CoV-2 for the serum sample taken from the cat found reactive by real-time RT-PCR, 35 days (October 16th) after detection. The OD values, clearly above the standard cut-off criteria (PI ≥ 10), revealed the presence of specific antibodies against protein S of SARS-CoV-2. These serological results strongly suggest that SARS-CoV-2 actively replicated in this animal. The same has been observed by other authors (Sailleau et al., 2020), who confirmed the natural infection of a cat in France and detected the presence of specific antibodies against SARS-CoV-2 eight days after the beginning of signs. In addition, in other studies a study reactive by real-time RT-PCR showed specific antibody responses against SARS-CoV-2 by ELISA, indicating that the animal was infected by the virus. The cat began with clinical signs of lethargy and cough 4 days after the positive diagnosis of one of its owners, and the samples were taken 17 days after the SARS-CoV-2 reactive real-time RT-PCR of its owners. The negative results for the detection of viral RNA may have been due to the rapid clearance of the virus from the respiratory and intestinal tracts, as reported by other authors (Barrs et al., 2020; Garigliany et al., 2020; Sailleau et al., 2020; Shi et al., 2020).

5. Conclusion

Studies suggest that the SARS-CoV-2 pandemic outbreak involves a diversified host range. Although there is no evidence that domestic animals play a role in the onward transmission of the virus, the testing of pets with COVID-19-positive owners is suggested. The evidence suggests that human-to-animal transmission of SARS-CoV-2 can occur, and it is thus recommended that infected owners should avoid close contact with their pets. However, more studies will be needed to see whether cats and other domestic animals with co-morbidities may show a more severe clinical course of SARS-CoV-2 infection.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2021.109179.

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