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ORIGINAL ARTICLE

Detection of SARS-CoV-2 in two cats during the second wave of the COVID-19 pandemic in France

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Abstract

Although there are several reports in the literature of SARS-CoV-2 infection in cats, few SARS-CoV-2 sequences from infected cats have been published. In this study, SARS-CoV-2 infection was evaluated in two cats by clinical observation, molecular biology (qPCR and NGS), and serology (microsphere immunoassay and seroneutralization). Following the observation of symptomatic SARS-CoV-2 infection in two cats, infection status was confirmed by RT-qPCR and, in one cat, serological analysis for antibodies against N-protein and S-protein, as well as neutralizing antibodies. Comparative analysis of five SARS-CoV-2 sequence fragments obtained from one of the cats showed that this infection was not with one of the three recently emerged variants of SARS-CoV-2. This study provides additional information on the clinical, molecular, and serological aspects of SARS-CoV-2 infection in cats.

KEYWORDS
cats, mild-respiratory clinical signs, NGS, One Health, SARS-CoV-2, serology

1 | INTRODUCTION

SARS-CoV-2 has shown relatively generalist capacities by infecting many animal species, making it a good model in One Health research (MacLean et al., 2021). Indeed, SARS-CoV-2 infections have been detected in numerous animal species living in close contact with infected humans. Based on RNA detection, serological studies, and experimental infections, numerous animal species have proven susceptibility to SARS-CoV-2 (Shi et al., 2020), including Mustelidae (ferret and mink) (Oude Munnink et al., 2020), Canidae (dog) (Sit et al., 2020), *Felidae* (cat, tiger, and lion) (Sailleau et al., 2020), and *Cricetidae* (hamster, rat, and mouse) (Sia et al., 2020) as well as by the highly transmissible British variant (B.1.1.7) (Ferasin et al., 2021). On 24 March, the World Organisation for Animal Health reported cases of SARS-CoV-2 infections in cats in 17 countries (the United States, China, Belgium, Germany, Spain, France, Russia, the United Kingdom, Japan, Italy, Chile, Brazil, Greece, Canada, Argentina, Switzerland, and Latvia). In the medical and scientific literature, we found 23 papers examining natural SARS-CoV-2 infection in a total of 2242 cats (Barrs et al., 2020; Carlos et al., 2021; Chen et al., 2020; Deng et al., 2020; Ferasin et al., 2021; Fritz et al., 2021; Garigliany et al., 2020; Hamer et al., 2020; Hosie et al., 2020; Klaus et al., 2021; Michelitsch et al., 2020; Musso et al., 2020; Neira et al., 2020; Newman et al., 2020; Pagani et al., 2021; Patterson et al., 2020; Ruiz-Arrondo et al., 2020; Sailleau et al., 2020; Segalés et al., 2020; Stevanovic et al., 2020; Temmam et al., 2020; Villanueva-Saz et al., 2021; Zhang et al., 2020). Among these cats, only 94 were positive for ongoing or previous SARS-CoV-2 infection, as detected by qPCR or by serology. Additionally, the US Department of Agriculture has reported 67 cases of SARS-CoV-2 infection in cats identified by at least one of these assays. Viral RNA detected in 25 cats suggested that COVID-19 infections were transmitted from infected owners. These few sequences are available (31 on Global Initiative on Sharing All Influenza Data (Gisaid) and 18 on Genbank) (https://www.gisaid.org/ and https://www.ncbi.nlm.nih.gov/genbank/). While most cases of SARS-CoV-2 infection in cats were asymptomatic, some cats (14/94) experienced lethargy, mild respiratory, or digestive clinical signs (sneezing, coughing, ocular discharge, vomiting, and anorexia), and two studies reported severe respiratory problems in two cats (Garigliany et al., 2020; Musso et al., 2020). Recently, a study has shown an association between B.1.1.7 infection and clinical signs of myocarditis in cats (Ferasin et al., 2021). Moreover, several experimental studies have shown that SARS-CoV-2 can be transmitted between cats (Bosco-Lauth et al., 2020; Gaudreault et al., 2020; Halfmann et al., 2020). As yet, there is no evidence of cat-to-human transmission. Here, we present a clinical and biological investigation of SARS-CoV-2 infection of two cats that presented with mildly symptomatic disease, and that came from households with confirmed cases of COVID-19 sampled during the second wave (October–November 2020) of infections in France.

2 | MATERIALS AND METHODS

2.1 | First line diagnostic: Veterinary diagnostic laboratory

2.1.1 | RNA extraction

RNA extraction from nasopharyngeal and rectal swabs was done using QIAamp Viral RNA Mini Kit (QIAGEN). Swabs were resuspended in 200 μl of ATL + 20 μl of proteinase K then heated at 70°C for 10 min. Note that 200 μl of ATL + 20 μl of proteinase K was then added and again heated to 70°C. Finally, 200 μl of ethanol was added and the entire volume (640 μl) was transferred to a column. Subsequent steps proceeded according to the manufacturer's protocol.

2.1.2 | Real-time reverse-transcription PCR

Viral RNA was quantified using the Genesig Real Time PCR Coronavirus COVID-19 (CE IVD) kit (Primer Design) using the manufacturer's protocol and QuantStudio 5 Real-Time PCR System thermocycler (Thermo Fisher Scientific).

2.1.3 | Second-line diagnostic: Hospital virology laboratory

RNA extraction and removal of genomic DNA
Nasopharyngeal and rectal swabs were resuspended in 500 μl of phosphate-buffered saline (PBS), of which 140 μl was used for RNA extraction using the EZ1 RNA Tissue Mini kit (QIAGEN). The homogenate was then treated with the Turbo DNA-free kit (Thermo Fisher Scientific) according to the manufacturer's protocol in order to remove genomic DNA.

Ribosomal RNA depletion and real-time reverse-transcription PCR
To enable cost-effective sequencing of RNA samples, we depleted the eukaryotic rRNA using the NEBNext rRNA Depletion Kit (Human/Mouse/Rat). After rRNA depletion, double-stranded cDNA was synthesized by real time reverse-transcription PCR (RT-qPCR) using Superscript III platinum One-step Quantitative RT-PCR System (Invitrogen) as described previously with minor modifications (Corman et al., 2020). Briefly, a 25 μl reaction contained 5 μl of RNA, 12.5 μl of 2× reaction buffer, 1 μl of reverse transcriptase/Taq mixture, 0.4 μl of a 50 mM magnesium sulphate solution (Invitrogen), 1 μl of a 10 μM primer, and 0.5l μl of a 10 μM probe. Thermal cycling was performed at 50°C for 15 min for reverse transcription, followed by 95°C for 2 min and then 45 cycles of 95°C for 15 s and 60°C for 30 s using a Light Cycler 480 (Roche).

To increase the amount of genetic material available for sequencing, whole transcriptome amplification (WTA) was performed with QuantiTect Whole Transcriptome kit (QIAGEN) according to the manufacturer's instructions. Amplified DNA reactions was then purified using AMPure XP beads and quantified using Qbit dsDNA BR Assay Kit on a Qubit 3.0 fluorimeter (Invitrogen).

In complement to the de novo approach, we also used an adapted version of the published protocol from the ARTIC Network using ARTIC (Quick, 2020) primer scheme version 3, which produces ~400 bp overlapping amplicons over the SARS-CoV-2 genome.

Oxford Nanopore Technology library preparation and MinION sequencing

Libraries were prepared without shearing to maximize sequencing read length. The Oxford Nanopore Technology (ONT) protocol for native barcoding genomic DNA sequencing was followed using the barcoded ligation sequencing kit SQK-LSK108 and the EXP-NBD104 Native Barcoding kit. Sequencing libraries were constructed, and sequencing was performed according to the manufacturer's instructions, as briefly described below. First, the NEBNext Ultra II End Repair/dA-Tailing module (E7546S; NEB, USA) was used to prepare 1000 ng DNA samples. End-prepared DNA was ligated with native barcode adapters NBD04 using Blunt/TA Ligase Master Mix (M0367S; NEB). Following the barcode ligation reaction, the DNA was cleaned with AMPure XP beads. The two samples were then pooled to produce a 54 µl equimass pool used for adapter ligation with the Native Barcoding Adapter Mix (BAM). The final library was loaded onto an R9.4 flowcell (FLO-MIN106; Oxford Nanopore Technologies, UK), and the run was performed on a MinION Mk1B device (ONT).

Genome assembly

Following the MinION run, reads generated were basecalled and subsequently demultiplexed using Guppy GPU basecaller and barcoder (Oxford Nanopore Technologies). Reads were then mapped against a custom reference of SARS-CoV-2 genome comprising four Chinese and 70 early French sequences using Bowtie2 (Langmead & Salzberg, 2012) and minimap2 (Li, 2018). Finally, a consensus genome sequence based on mapped reads was generated with BCFtools consensus (Li, 2011). SARS-CoV-2 sequences were deposited on GISAID (EPI_ISL_1328819; EPI_ISL_1328821; EPI_ISL_1328824; EPI_ISL_1328826).

Microsphere immunoassay

Cat serum samples were tested using a multiplex microsphere immunoassay (MIA). Note that 10 µg of three recombinant SARS-CoV-2 antigens nucleoprotein (N), receptor-binding domain (RBD), and trimeric spike (tri-S) were used to capture specific serum antibodies, whereas a human protein (O6-methylguanine DNA methyltransferase) was used as a control antigen in the assay. Distinct MagPlex microsphere sets (Luminex Corporation) were respectively coupled to viral antigens using the amine coupling kit (Bio-Rad Laboratories) according to the manufacturer's instructions. The MIA procedure was performed as described previously (Fritz et al., 2021). Briefly, microsphere mixtures were successively incubated with serum samples (1:400) biotiny-lated protein A and biotinylated protein G (4 µg/ml each) (Thermo Fisher Scientific), and Streptavidin-R-Phycoerythrin (4 µg/ml) (Life Technologies) on an orbital shaker and protected from the light. Measurements were performed using a Magpix instrument (Luminex). To account for non-specific binding of antibodies to beads, relative fluorescence intensities (RFI) were calculated for each sample by dividing the median fluorescence intensity (MFI) signal measured for the antigen-coated microspheres by the MFI signal obtained for the control microspheres. Specific seropositivity cut-off values for each antigen were set at three standard deviations above the mean RFI of the 18 dogs and 14 cat serum samples sampled before 2019. Based on a pre-pandemic population, MIA specificity was set at 100% for dogs and cats.

Neutralization activity measurement

An MLV-based pseudoparticle carrying a GFP reporter pseudotyped with SARS-CoV-2 spike protein (SARS-CoV-2pp) was used to measure the neutralizing antibody activity in cats' sera. Each SARS-CoV-2 positive sample detected by MIA was processed according to a neutralization procedure as previously described (Legros et al., 2021). The level of infectivity is expressed as the percentage of GFP-positive cells and compared to cells infected with SARS-CoV-2pp incubated without serum. Pre-pandemic cats' sera from France was used as negative controls, and a commercial anti-SARS-CoV-2 RBD antibody (Sino Biological) was used as a positive control.

3 | RESULTS

Cat 1 was a solitary and sedentary 5-year-old female of European origin whose only contact was with her owner. Her last vaccination was 3 years prior and she was presented with no previous medical history. On 24 October 2020, 10 days after her owner developed symptoms and tested positive for SARS-CoV-2 infection, cat 1 developed sneezing with non-purulent nasal secretions but no digestive trouble or others notable clinical signs. Five days later, on 29 October, clinical examination of the cat showed pink mucous membranes, a heart rate of 175 bpm (110–200 bpm), a rectal temperature of 38.4°C (38–39°C), slight dehydration, a urea seric concentration of 0.49 g/l (0.4–0.6 g/l), and a creatinine seric concentration of 12.7 mg/l (10–20 mg/l). A 10-day treatment of 1 Doxybactin tablet in the evening and a daily dose of Meloxoral taken over 3 days with the morning meal was then initiated. An improvement of the clinical signs was observed in this cat 3 days after the onset of the treatment. Based on the owner's positive SARS-CoV-2 diagnosis and the cat's clinical signs, on the date of the clinical examination a blood sample and two oropharyngeal and rectal swabs were collected for further SARS-CoV-2 testing. Swabs were submitted to the veterinary diagnostic laboratory for RNA SARS-CoV-2 detection. The oropharyngeal swab tested positive by RT-qPCR targeting the ORF1ab with a cycle threshold (Ct) value of 20.41. No viral RNA was detected in the rectal swabs by both diagnostic laboratories: veterinary and hospital. The hospital virology laboratory confirmed the diagnosis following detection of viral RNA in the second ororpharyngeal swab

TABLE 1   Characteristics of cats sampled during the second wave of SARS-CoV2 infections in France

Cat ID	Age	Gender	Severity of cat's clinical signs	Nasopharyngeal swab	Rectal swab	Serology (MIA)	Serology (seroneutralization)
Cat 1	5	F	Mild	Positive (Ct: 20.41; 21.43)	Negative	Positive	Positive (<90%)
Cat 2	13	M	Mild	Positive (Ct: 20.55; 23.44)	Negative	N.D.	N.D.

Abbreviations: Ct, cycle threshold; N.D., not detected; MIA, microsphere immunoassay.

TABLE 2   Details of the five SARS-CoV2 genome fragments obtained from cat 2

Sequence fragments	Position (length)	Gene	Mutation[†]	AA change[†]	GISAID accession number
Fragment 1	3427-3623 (197)	ORF1a/NSP3	No	No	EPI_ISL_1328819
Fragment 2	11163-11484 (322)	ORF1a/NSP6	No	No	EPI_ISL_1328821
Fragment 3	18883-19002 (120)	ORF1ab	No	No	EPI_ISL_1328824
Fragment 4	25513-25667 (155)	ORF3a	G25563T	Q57H	EPI_ISL_1328826
Fragment 5	26769-26863 (95)	Membrane	No	No	Not available because <100pb

[†]as compared to the Wuhan_Hu-1 reference sequence.

by RT-qPCR targeting gene E with a Ct-value of 21.43 (Table 1). We did not obtain a SARS-CoV-2 sequence due to the sample's poor conservation condition prior to its arrival at the lab. To detect anti-SARS-CoV-2 IgG antibodies, the serum of cat 1 was analyzed using MIA and retrovirus-based pseudoparticle assay. Antibodies against N, RBD, and tri-S SARS-CoV-2 proteins were detected as well as a robust neutralization, allowing over 90% neutralization in the SARS-CoV-2pp assay confirming productive infection in cat 1 (Table 1).

Cat 2 was a 13-year-old male of European origin with chronic rhinitis and living with two other cats. The cat's owner, who had recently tested positive for SARS-CoV-2, reported an acute deterioration in his three cats' general condition without other details. A clinical investigation 20 November 2020 reported retro-mandibular adenopathy, and no other clinical signs was observed. As with cat 1, on the date of the clinical investigation oropharyngeal and rectal swabs were collected but with no blood sample. Similarly, veterinary diagnostic and hospital virology laboratories detected viral RNA in both oropharyngeal swabs with Ct-values of 20.55 and 23.44, respectively (Table 1). No viral RNA was detected in the rectal swabs. Again, due to poor conservation of the swabs prior to their arrival in our laboratory, only five partial fragments of the SARS-CoV-2 genome could be obtained by high-throughput sequencing on RNA derived from the oropharyngeal swabs. In fragment 2, we did not observe the 11288-11296 deletion characterizing three variants of concern (English B1.1.7, South African B.1.351, and Brazilian P.1) (Table 2), indicating that this cat was not infected by any of these three novel variants. Compared to the original Wuhan_Hu-1 reference sequence, we observed, in fragment 4, only one single nucleotide polymorphism (SNP) -G25563T- leading to the amino acid change Q57H. This H57 mutation is widely distributed around the world and was present in about 70% of the sequences reported in France between October and December 2020 according to Nextstrain (https://nextstrain.org/).

4 | DISCUSSION

Here, we report SARS-CoV-2 infection in two cats, diagnosed by molecular and serological assays and with mild clinical manifestations, including sneezing for one cat. Although we cannot definitively rule out infection of the cats by an individual outside the household, the information given by COVID-19-positive owners, including the exclusive and unique contact with its owner for cat 1 and the general deterioration in the condition of all cats of the cat 2 owner, strongly suggests a transmission from owners to cats.

Human-to-cat transmission is now reported worldwide, and while certainly not insignificant in COVID-19-positive households, precise estimates of the frequency of such transmission has not been established (Fritz et al., 2021; Hamer et al., 2020). To our knowledge, among the 23 papers investigating possible SARS-CoV-2 infection in cats, only 14 reported PCR results confirming infection in 27 cats (Barrs et al., 2020; Carlos et al., 2021; Ferasin et al., 2021; Garigliany et al., 2020; Hamer et al., 2020; Hosie et al., 2020; Klaus et al., 2021; Musso et al., 2020; Neira et al., 2020; Newman et al., 2020; Pagani et al., 2021; Ruiz-Arrondo et al., 2020; Sailleau et al., 2020; Segalés et al., 2020). The present study is only the second showing molecular results of cat infection in France, 8 months after the first case reported by Sailleau et al. (2020). It appears that the majority of infections in cats are asymptomatic, but can, at times, lead to lethargy, mild respiratory or digestive disease and, rarely, acute respiratory clinical signs (as observed in the two cats) (Garigliany et al., 2020; Musso et al., 2020). We report here one case of mild respiratory clinical signs (sneezing) in a cat, an observation consistent with symptomatic infections sporadically observed in other studies. We did not observe any digestive clinical signs, as previously reported in a French cat (Sailleau et al., 2020). In accordance with other recent studies, our results suggest that since the beginning of SARS-CoV-2 pandemic, almost 1 year after the first report of infection

in a cat, the pathology in cats has not changed globally, with only a relatively small proportion of cases reported as a result of clinical investigation by a veterinarian. This low pathogenicity can explain the paucity of studies reporting SARS-CoV-2 infection in cats in the absence of a global pet detection policy.

The two cats in the study were sampled during the second wave of infection in France and at the beginning of the ongoing emergence of multiple novel variants. Although we did not find evidence of infection by one of the three novel variants (B.1.1.7, B.1.351, and P.1) in cat 2, the emergence of these new variants raises the question of potential changes in pathogenicity or transmissibility in domestic animals. This question will become rapidly crucial in a very near future as the British variant, known to be much more infectious, is currently removing the ancestral variant of SARS-CoV-2 in France as well in other countries of Europe. Therefore, it is becoming more and more important to implement a One Health approach to face SARS-CoV-2 epidemic that takes into account infection and viral circulation in pets.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Formal analysis, investigation, methodology, validation, writing-original draft, and writing-review & editing: Matthieu Fritz. *Formal analysis, investigation, methodology, validation, and writing-review & editing*: Nicolas Nesi. *Formal analysis, investigation, methodology, validation, and writing-review & editing*: Solene Denolly. *Formal analysis, investigation, methodology, validation, and writing-review & editing*: Bertrand Boson. *Formal analysis, investigation, methodology, validation, and writing-review & editing*: Vincent Legros. *Conceptualization, resources, and writing-review & editing*: Serge Rosolen. *Conceptualization, resources, and writing-review & editing*: Alexandra Briend-Marchal. *Formal analysis, investigation, methodology, validation, and writing-review & editing*: Meriadeg Ar Gouilh. *Conceptualization, funding acquisition, project administration, validation, writing-original draft, and writing-review & editing*: Eric M Leroy.

PEER REVIEW

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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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