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INFECTIOUS DISEASE

Natural SARS-CoV-2 Infection in a Free-Ranging Black-Tailed Marmoset (*Mico melanurus*) from an Urban Area in Mid-West Brazil

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Summary

The emergence of spillover pathogens such as SARS-CoV-2 is a risk to vulnerable human populations. We report natural SARS-CoV-2 infection in a free-ranging adult female black-tailed marmoset (*Mico melanurus*) from an urban area of Cuiabá, Mato Grosso State, Brazil. The animal was found after a motor vehicle collision without previous clinical history. Necropsy confirmed polytrauma. Severe multifocal to coalescent haemorrhage and mild multifocal peribronchial lymphocytic hyperplasia were seen in lung sections. The alveolar septa were multifocally expanded by a few lymphocytes. Mild lymphocytic periportal hepatitis and interstitial nephritis were found. The lymphoid nodules of the large intestine showed marked lymphocytic hyperplasia. Infection by SARS-CoV-2 was established by viral RNA detection in a pool of nasopharyngeal and oropharyngeal swabs and liver samples. Immunohistochemistry detected the viral nucleocapsid protein in sections of lung, liver, spleen, lymph nodes and large intestine, and spike protein antigen in lung tissue. This is the first report of naturally occurring SARS-CoV-2 infection in a New World monkey. Platyrrhine species should be included as potential hosts of natural infection of SARS-CoV-2.

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Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent of coronavirus disease 2019 (COVID-19), an acute respiratory illness that is presumed to have emerged in late 2019 (Zhu *et al*, 2020). SARS-CoV-2 has led to a global pandemic associated with millions of infections, hundreds of thousands of human deaths and severe social and economic disruption (Griffin *et al*, 2021). Data about the susceptibility of animal hosts to SARS-CoV-2 are increasing rapidly.

Naturally acquired SARS-CoV-2 infections in wildlife have been described in Malayan tigers (*Panthera tigres jacksoni*), Amur tigers (*Panthera tigres altaica*) and African lions (*Panthera leo krugeri*) (McAloose *et al*, 2020). Some experimental studies have been conducted on Old World monkeys (Deng *et al*, 2020; Munster

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et al, 2020; Johnston *et al*, 2021) and New World monkeys (Clancy *et al*, 2021; Singh *et al*, 2021). Although non-human primates are considered to be a suitable model for SARS-CoV-2 infection, which reproduces several features of human COVID-19 (Lu *et al*, 2020), we have not found any reports of naturally occurring cases of SARS-CoV-2 in New World monkeys.

A free-ranging 0.4 kg adult female black-tailed marmoset (*Mico melanurus*) from an urban area of Cuiabá, Mato Grosso State, Brazil, was found on 19th January 2022 after a motor vehicle collision and without previous clinical history. The carcass was forwarded to the Veterinary Pathology Laboratory of the Federal University of Mato Grosso where a complete necropsy was performed and samples of multiple organs were fixed in 10% neutral buffered formalin. After fixation, these organs were processed conventionally, embedded in wax, cut at 3 μm, stained with haematoxylin and eosin (HE) and examined by optical microscopy. All procedures were conducted in full compliance with the approval of the Brazilian Ministry of the Environment (SISBIO 79317–1).

Swabs of the nasopharynx, oropharynx and rectum and samples of a range of other organs were placed in microtubes at necropsy and forwarded for SARS-CoV-2 molecular examination. The first protocol involved viral RNA extraction using a commercially available kit (MagMAX Viral/Pathogen Nucleic Acid Isolation Kit; Applied Biosystems, www.thermofisher.com) and detection by real-time polymerase chain reaction (RT-PCR) using a commercial kit (One/StepCOVID-19-IBMP Kit; Instituto de Biologia Molecular do Paraná, www.ibmp.org.br) that targets the N gene, ORF1ab and internal control (RNAse P). The second protocol comprised total RNA extraction carried out with a commercial kit (Maxwell RSC simplyRNA Tissue Kit; Promega Co, www.promega.com) and SARS-CoV-2 RT-qPCR via a commercial kit (Superscript III Platinum One-Step qRT-PCR System; Invitrogen, www.thermofisher.com) targeting the E gene of SARS-CoV-2. The viral load was indicated as the cycle threshold (Ct) value of the N gene, ORF1ab and E gene of SARS-CoV-2. According to the manufacturer's protocol, Ct values of <40 to the N gene and ORF1ab and <38 to the E gene were defined as positive for SARS-CoV-2 RNA.

Viral RNA was detected in the nasopharyngeal swabs, lung and liver. The Ct values of each SARS-CoV-2 target gene are summarized in Table 1.

Grossly, a body condition score of 3 (1–5 scale) was observed. A moderate amount of red serous fluid was found in the oral cavity. The sternocleidomastoid muscle was haemorrhagic and diffusely dark red to black in colour. A severe diaphragmatic rupture was present and the liver was dislocated to the thoracic cavity. All pulmonary lobes had multifocal to coalescent irregular dark brown to red areas. Multiple firm well-demarcated (0.2–0.4 cm), elevated, white nodules were observed on the mucosa of the caecum and colon.

Table 1
Cycle threshold (Ct) values of SARS-CoV-2 target genes in a naturally infected black-tailed marmoset (*Mico melanurus*)

	Target gene		
	E[1]	*N*[2]	*ORF1ab*[2]
Nasopharynx	34.2	35,1	ND
Lung	32.63	37.13	37.96
Liver	33.41	36.81	ND

[1], positive Ct cut-off value <38; [2], positive Ct cut-off value <40; ND, not detected.

On histological examination, about 70% of the airspace within lung sections was obliterated by numerous extravascular erythrocytes. Mild multifocal peribronchial lymphocytic hyperplasia and occasional areas of mild thickening of alveoli septa by lymphocytes and macrophages were seen (Fig. 1). Multifocally, the liver had a mild lymphocytic infiltration, proliferation of ductal epithelial cells and marked pericholangiolar thickening due to collagen deposition in portal spaces. There were occasional slightly swollen and hyaline muscle fibres in the myocardium. Mild multifocal interstitial lymphocytic nephritis and marked lymphoid hyperplasia of the large intestine were also seen. There were no evident histological changes in sections of spleen, stomach, small intestine, salivary gland, cerebrum or cerebellum.

Immunohistochemistry (IHC) was performed on lung, heart, liver, spleen, lymph node, stomach, large intestine, salivary gland, kidney, brain and cerebellum sections. Antigen retrieval was achieved with citrate buffer (10mM, pH 6.0) at 96°C for 30 min. The sections were then incubated in methanol:H_2O_2 solution (97%:3%) for 30 min to block non-specific binding. The sections were incubated at 37°C for 3 h with primary anti-SARS-CoV-2 nucleocapsid protein (NP) antibody (1:300; Novus Biologicals, www.novusbio.com) and an anti-SARS-CoV-2 spike protein antibody (clone mAb CR3022; Sigma-Aldrich, www.sigmaaldrich.com) diluted 1:100 in phosphate buffered saline (PBS). SARS-CoV-2 RT-PCR-positive human lung tissue with lesions typical of COVID-19 was used as a positive control. As a negative control, PBS was substituted for the primary antibody.

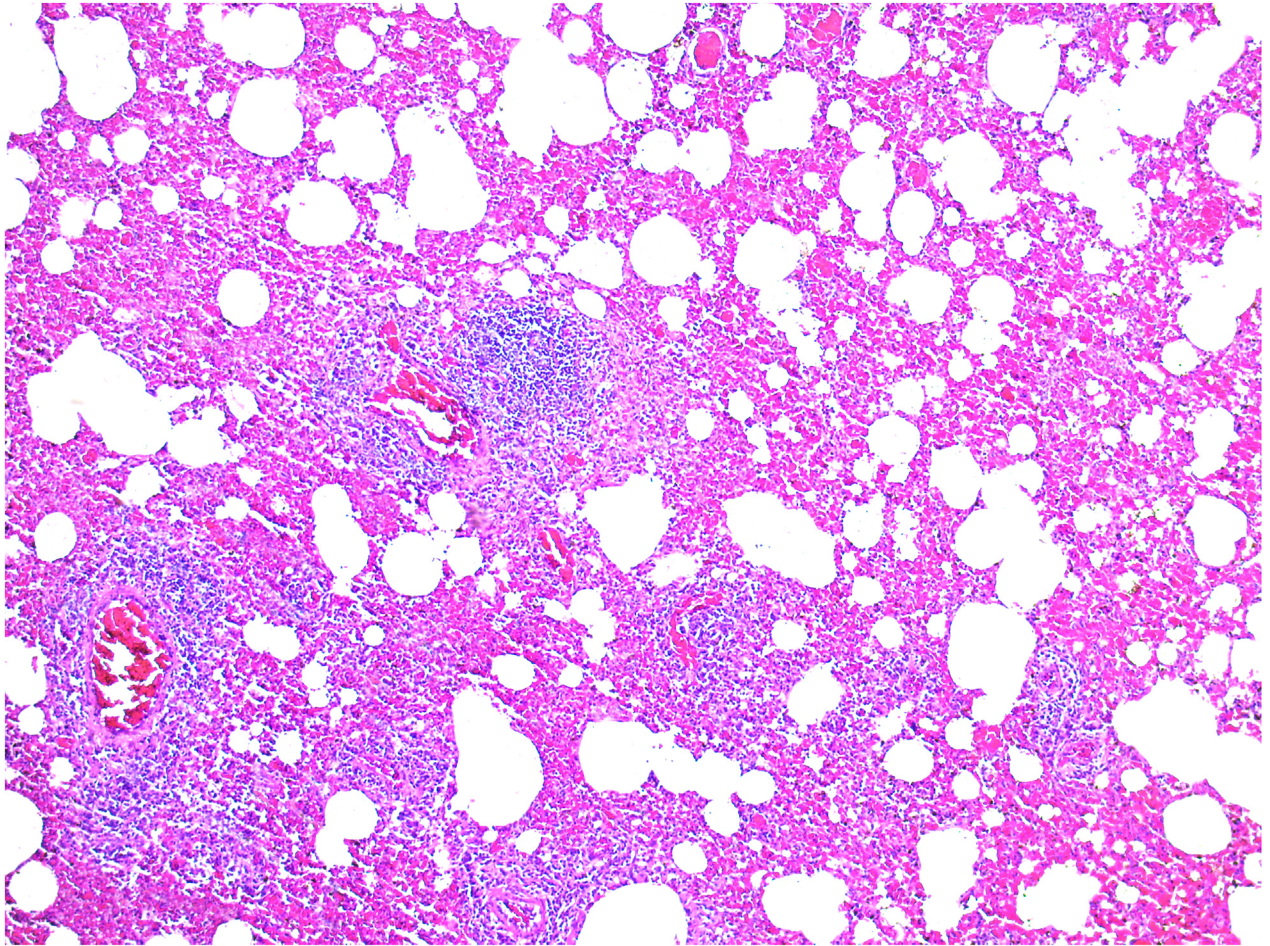

Fig. 1. SARS-CoV-2 infection, lung, black-tailed marmoset. Mild thickening of alveolar septa by lymphocytes and macrophages. HE. ×10.

A streptavidin–biotin–peroxidase commercial kit (LSAB + System HRP; Agilent Technologies, www.agilent.com) was used as the detection system. Peroxidase activity was visualized with EnVision FLEX HRP Magenta Substrate Chromogen System (Dako Cytomation, www.agilent.com/en/dako-products). The sections were counterstained with haematoxylin, coverslipped and viewed with an optical microscope.

IHC using the antibody against the NP of SARS-CoV-2 on positive control human lung tissue revealed intense cytoplasmic immunolabelling in pneumocytes (Fig. 2A). No immunolabelling was seen in the negative control (Fig. 2B). There was moderate, multifocal cytoplasmic immunolabelling of the nucleoprotein antigen in type 1 and 2 pneumocytes, macrophages in alveolar septa, and bronchiolar and bronchial lamina propria (Fig. 2C), lymph node, spleen and the lamina propria of the large intestine of the marmoset. Multiple hepatocytes had strong granular cytoplasmic immunolabelling. Rarely, the cytoplasm of renal convoluted tubule epithelial cells was moderately immunolabelled.

IHC using the antibody against the spike protein of SARS-CoV-2 on positive control human lung tissue revealed intense cytoplasmic immunolabelling in pneumocytes and macrophages in alveolar septa (Fig. 3A). No immunolabelling was seen in the negative control (Fig. 3B). There was mild, multifocal cytoplasmic immunolabelling in epithelioid macrophages in the bronchial submucosa and, rarely, in types 1 and 2 pneumocytes in lung tissue of the marmoset (Fig. 3C).

The diagnosis in this case was based on the detection of SARS-CoV-2 N gene, E gene and ORF1ab by RT-PCR and detection of viral nucleocapsid and spike protein antigens in tissues by IHC. SARS-CoV-2 diagnosis is most widely based on RT-PCR, which is considered to be the 'gold standard' method (WHO, 2020). Many RT-qPCR assays use a Ct cut-off of 40 as indicative of a positive result, and allows

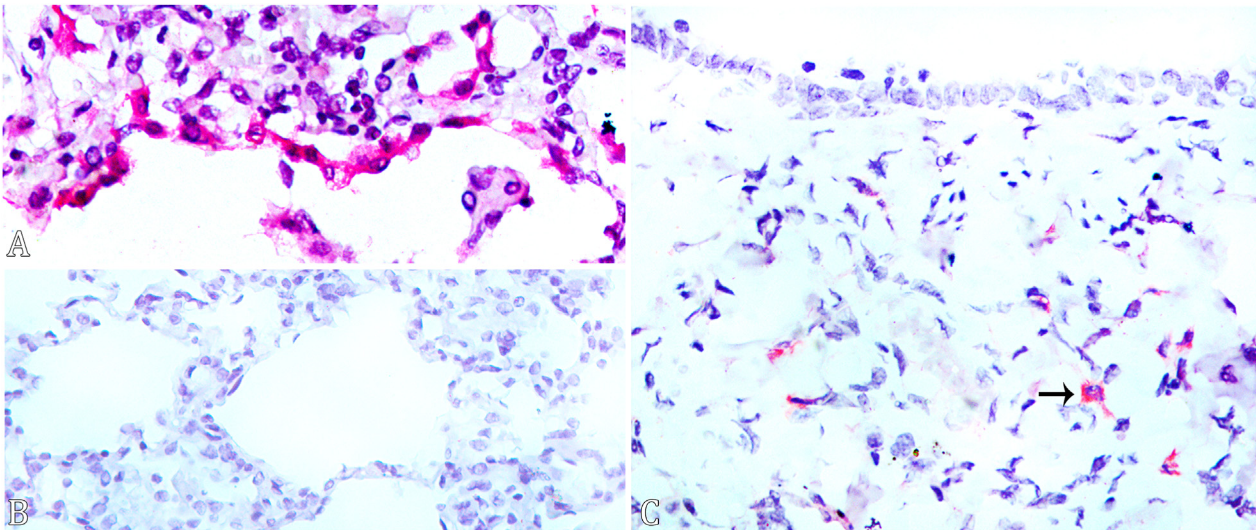

Fig. 2. SARS-CoV-2 infection, lung, black-tailed marmoset. Immunohistochemistry using antibody against nucleocapsid protein of SARS-CoV-2. (A) Positive control human lung tissue. Strong cytoplasmic immunolabelling in pneumocytes in alveolar septa. IHC. ×63. (B) Negative control. Absence of cytoplasmic immunolabelling in pneumocytes or macrophages in alveolar septa. IHC. ×40. (C) Moderate cytoplasmic immunolabelling in macrophages (black arrow) in bronchial lamina propria of SARS-CoV-2-infected marmoset. IHC. ×40.

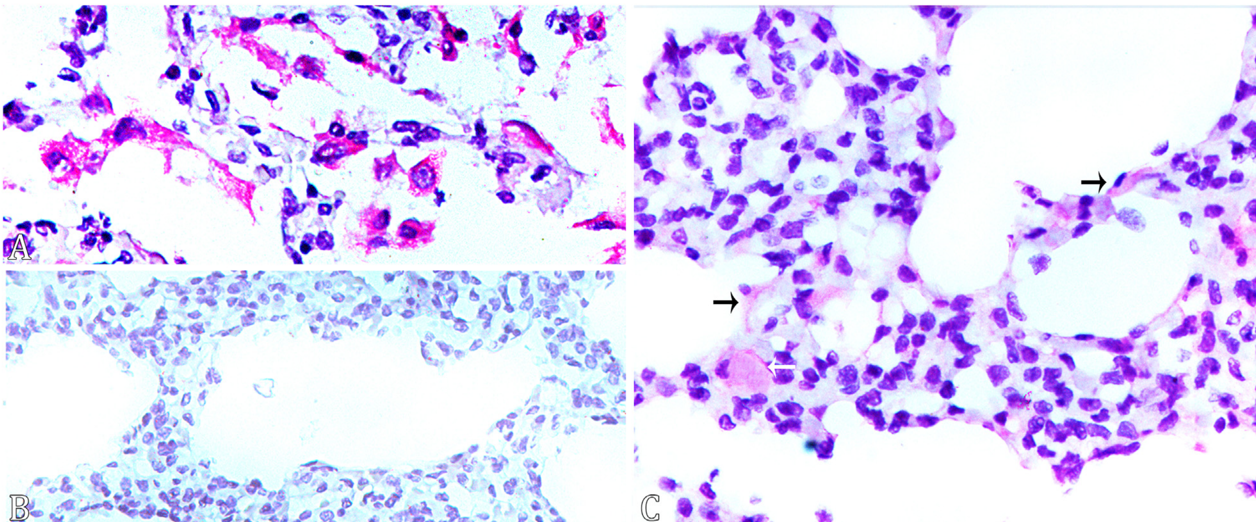

Fig. 3. SARS-CoV-2 infection, lung, black-tailed marmoset. Immunohistochemistry using antibody against spike protein of SARS-CoV-2. (A) Positive control human lung tissue. Strong cytoplasmic immunolabelling in pneumocytes and macrophages in alveolar septa. IHC. ×63. (B) Negative control. Absence of cytoplasmic immunolabelling in pneumocytes or macrophages in alveolar septa. IHC. ×40. (C) Lung sample. Mild cytoplasmic immunolabelling in pneumocytes (black arrows) and macrophages (white arrow) in alveolar septa in lung of SARS-CoV-2-infected marmoset. IHC. ×40.

detection of a very low number of RNA molecules (Tom and Mina, 2020), as found in this case. Our IHC findings are compatible with reports of experimental infections with SARS-CoV-2 virus in which low amounts of viral NP antigen was detected in types I and II pneumocytes and intra-alveolar macrophages, and in macrophages in the lymph nodes and lamina propria of the intestinal tract of rhesus monkeys (*Macaca mulatta*) (Munster *et al*, 2020).

Regardless of the severe lung damage in this case due to the polytrauma, the animal did not have diffuse alveolar damage (DAD) typical of COVID-19. No animal model has consistently reproduced DAD following inoculation with SARS-CoV-2 (Johnston *et al*, 2021). A mild interstitial lymphohistiocytic pneumonia was seen in this case, which is consistent with the results of a study in which pulmonary tissue did not usually develop lesions or foci of

severe pneumonia (Clancy *et al*, 2021). As in this marmoset, lymphoid hyperplasia was also found in African green monkeys (*Chlorocebus aethiops*) experimentally inoculated with SARS-CoV-2 (Johnston *et al*, 2021).

Non-human primates infected with SARS-CoV-2 can exhibit mild clinical signs (Gonçalves *et al*, 2021). Due to its free-living state and lack of previous clinical evaluation, it is not possible to say whether or not this marmoset had any clinical signs due to natural infection by SARS-CoV-2. The cause of death was attributed to polytrauma due to motor vehicle collision. We hypothesize that the accident may have occurred secondary to natural infection with SARS-CoV-2.

The source of SARS-COV-2 infection for this animal is unknown. Although descriptions of natural SARS-CoV-2 infection in wild animals have been linked to initial transmission from humans to captive animals (Delahay *et al*, 2021), this black-tailed marmoset was not under human care. Direct contact with infected humans may not be necessary for the transmission of SARS-CoV-2 (Delahay *et al*, 2021). It appears likely that this non-human primate contracted the pathogen from its urban environment, which could indicate the emerging potential of SARS-CoV-2 in vulnerable free-ranging non-human primates living in urban areas.

In the Americas, there is great concern about the risks of SARS-CoV-2 transmission from humans to wildlife, including neotropical non-human primates (Abreu *et al*, 2021). Previous molecular and serological studies have demonstrated no evidence of natural SARS-CoV-2 infection in non-human primates from urban, rural or sylvatic habitats (Abreu *et al*, 2021; Sacchetto *et al*, 2021). However, the explosive spread of SARS-CoV-2 in areas where contact between humans and native fauna is frequent increases the need for surveillance for early identification and response to the potentially harmful effects (Gryseels *et al*, 2020).

Our findings indicate that free-ranging non-human primates are potential hosts for natural SARS-CoV-2 infection. Control of the transmission of pathogens from humans to wildlife is extremely challenging, considering the potential indirect anthropogenic impacts. We suggest surveillance in free-ranging non-human primates at veterinary pathology laboratories, using RT-PCR and IHC for SARS-CoV-2 infection, even in cases without previous clinical history.

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CRediT Author Statement

M Souza, E Colodel: Necropsy, Histopathology. **A Pereira, D Ubiali:** Histopathology, Immunohistochemistry. **V Silva, B Nogueira, A Silva, R Pacheco, L Nakazato, V Dutra:** Molecular analysis. **A Pereira, A Vasconcelos, V Silva, B S Nogueira, A Silva, R Pacheco, M Souza, E Colodel, D Ubiali, A Biondo, L Nakazato, V Dutra:** Methodology, Data curation, Writing — reviewing, editing and formal analysis.

Data Availability Statement

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

Conflict of Interest Statement

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

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