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Investigation of Coronavirus (SARS-CoV-2) in Antillean manatees (*Trichechus Manatus Manatus*) in Northeast Brazil

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1. Abstract

1.1. Aims

Since 2019, the world has been experiencing a coronavirus (SARS-CoV-2) pandemic. Non-human animals are susceptible to the virus, including marine mammals. Here we aimed to test Antillean manatees, *Trichechus manatus manatus,* for SARS-CoV-2 contamination.

1.2. Methods and Results

We collected nasal swab samples from 19 individuals kept under the responsibility of the Brazilian centre for research and conservation of aquatic mammals (ICMBio/CMA) and we analysed the samples through RT-PCR and RT-LAMP-PCR. We found that two of the 19

manatees tested positive for SARS-CoV-2. SARS-CoV-2 contaminated animals did not experience any clear negative health consequences as a result of the virus's presence. This is the first case of potential SARS-CoV-2 detection in Sirenians.

1.3. Conclusion

Manatees may be susceptible to SARS-CoV-2 infection, and it was not possible to identify the species' ability to produce symptoms. As it is a zoonosis, this study demonstrated the importance of including the diagnosis of the virus in the animal health analysis protocols. Noting that all positive cases must be reported to the Ministry of Agriculture, Livestock and Supply (Brazil) and to the World Organization for Animal Health

1.4. Significance and Impact of the Study

This stud is first report of SARS-CoV-2 in sirenian of the world and possible the first in marine mammal. Such a result led to a new biosecurity protocol in the ICMBio/CMA to avoid potential human-manatee coronavirus transmission, showing how we can use simple genetic tools to improve the care of manatees. The health and conservation impacts of human-to-manatee SARS-CoV-2 transmission are still unclear, but given the conservation status of the species, we suggest that such transmission should be prevented until the risks to manatees are better understood.

2. Introduction

Since late 2019, the world has been experiencing a coronavirus (SARS-CoV-2) pandemic. The first reported case in Brazil was confirmed in February 2020 [1,2]. The transmission of the virus from humans to animals is possible, especially in mammals, which are already known to be susceptible to the disease (IUCN SSC 2020; [3]. Department of Agriculture - USDA 2021). Although other marine mammals' susceptibility has not been confirmed yet, some Asian Small-clawed Otters have already been infected with the COVID-19 virus (USDA 2021). Additionally, the possibility of other marine mammals' susceptibility to the virus contamination has already been postulated (IUCN SSC 2020; [4]. In addition to the direct inter-species (human – non-human animals) trajectory, wastewater, contaminated with SARS-CoV-2, discharged into natural water systems may pose a transmission risk to aquatic species [5-7]. West Indian manatees (*Trichechus manatus*) are marine mammals categorised worldwide by IUCN assessment as Vulnerable due to the sharp decline in their populations [8]. The subspecies *Trichechus manatus manatus* (Antillean manatee or Caribbean manatee) occurs in Brazil, being categorised as Endangered in the Brazilian list of threatened species (ICMBio 2022) and in the IUCN Red List of Threatened Species (Self-Sullivan and Mignucci-Giannoni, 2008). The species is targeted by a conservation action plan and a long-term reintroduction program in Brazil (Luna and Passavante 2010; Ordinance ICMBio N° 249/2018). The National Centre for Research and Conservation of Aquatic Mammals (ICMBio/CMA) is a Brazilian Government unit responsible for caring for Antillean manatees in captivity and rehabilitation and reintroduction processes (Ordinance No. 554 2020, May 25th, 2020). The centre carries out periodic health assessments of the manatees. Given the current coronavirus pandemic (SARS-CoV-2), the centre felt the need to verify whether the Antillean manatees were susceptible to the virus. Therefore, the present study aimed to test Antillean manatees for the coronavirus (SARS-CoV-2).

3. Materials and Methods

3.1. Study Animals

We collected samples from 19 Antillean manatees kept under the responsibility of the Centro de Pesquisa e Conservação de Mamíferos Aquáticos do Instituto Chico Mendes de Biodiversidade (ICMBio/CMA). Ten animals were kept in the rehabilitation captivity centre at Itamaracá Island, Pernambuco State, Northeast Brazil. Four animals were kept in acclimatization captivity and four animals in the wild (three of them previously released by the centre and one wild free-living) at the Costas dos Corais Environmental Protection Reserve (ICMBio/APA Costas dos Corais) in Alagoas State, Northeast Brazil. One animal was wild rescued in the Rio Grande do Norte State, Northeast Brazil. The animals in captivity at Itamaracá Island had no contact with the public, but they had close contact with the centre staff (i.e., biologists, veterinarians, and animal keepers). As general biosafety measures followed by the ICMBio/CMA, only the responsible team was allowed to be close to the animals and the area at the Itamaracá Island facility, always using Personal Protective Equipment (PPE – e.g., masks, gloves, 70% alcohol use). Additionally, some other measures, such as maintaining social distancing and reduced personal were also followed by the centre. The animals in the acclimatization captivity and wild had occasional contact with tourists and locals and regular close contact with the staff at APA Costas dos Corais.

3.2. Sample Collection

We collected samples from the nostrils of the 19 manatees with sterile cotton swabs (Figure 1) and preserved them in the field in Phosphate-buffered saline PBS (1ml of solution and 0,1X Phosphate-buffered saline PBS) for up to 24h before freezing the samples at -20C. We also collected biometric measurements and blood samples from all the animals to perform complete blood counts and serum biochemistry (urea, creatinine, GOT, GPT, glucose, triglycerides). Biometric and blood samples were collected on the same sampling day as the nasal swabs. This study complied with Brazilian law (Permit number: SISBIO/ICMBio N°77116-1).

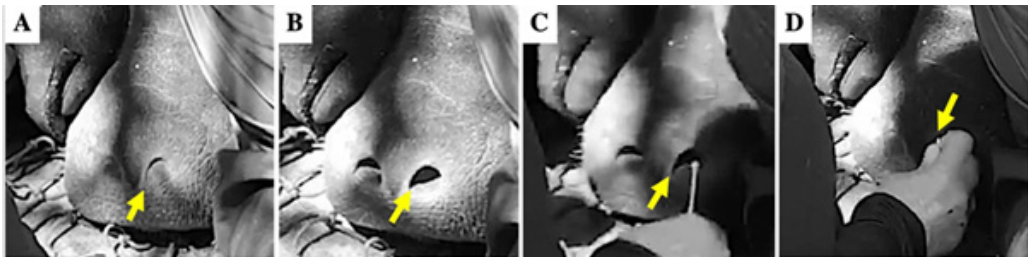

Figure 1: Method showing nasal swab sample collected from an Antillean manatee. The arrows point to the nasal opening. We introduced the swab into the nostril once it opened for breath. a-d: steps of the procedure.

3.3. Sample Analysis

We obtained the viral RNAs from the swab samples using the ReliaPrep™ Viral Total Nucleic Acid Purification Kit- Promega (extractions and purifications were done following the kit protocol). The RNAs were then analysed through RT-PCR and RT-LAMP and stored at -80°C. The RT-PCR technique isolates and purifies viral RNA from the samples, which is then reverse transcribed to cDNA and subsequently amplified in a real-time PCR thermocycler. RT-PCR is the test recommended by WHO (gold standard) to diagnose and monitor individuals with active infections. The RT-LAMP-PCR technique is a new experimental technique for fast and sensitive RNA detection. The RT-LAMP-PCR method is performed in simple isothermal conditions using four or six specific oligonucleotide primers for the target sequence of SARS-CoV-2.

3.4. RT-PCR assay

We used the MOLECULAR SARS-CoV2 (E) - Bio-Manguinhos kit for the diagnosis by quantitative reverse transcription PCR (RT-PCR). This kit uses the primer and probe sequences from the Berlin Protocol [9]. The viral target is located in the E gene, and for that, the Kit uses the fluorescence reporter FAM. The Kit provides a master mix with the enzyme, buffer and dNTPs Set, a probe for mixing and negative and positive controls for the reaction. For each reaction, 7.8 µl of the master mix and 2.2 µl of the probe mix are used, and 5 µl of the sample or controls is added to this mixture. The first step that precedes amplification is reverse transcription occurring at 45 °C for 15 min. After this step, the initial denaturation of the cycle occurs, and the material is subjected to a temperature of 95 °C for 2 min, and then, the 40 repetitions of the cycle are initiated. The cycling consists of two stages, the first at 95 °C for 15 seconds and the second at 58 °C for 30 seconds, where fluorescence acquisitions always take place at the end of the second stage. The reaction was performed using QuantStudio® 5 equipment from Thermo Fisher. The analysis parameters for the results are determined by the Kit, where the Threshold is set at 0.2, and the Baseline Start and End are set to AUTO. The detection routine is only valid when the analysis of the reaction controls is established as Ct undetectable for the negative control and Ct less than or equal to 37 for the positive control. The result for the samples is considered positive (detectable) if the Ct is less than 40. The PROBIT analysis (95% CI) indicated a sensitivity for target E: LOD of 0.97 copies/reaction (50% positivity) and 1.99 copies/reaction (95% positivity); In summary, for this kit, the detection limit for Coronavirus was established as 50 copies/reaction.

3.5. RT-LAMP-PCR assay

The LAMP reaction was performed with a final volume of 25 uL. 1.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP), 0.8 X of Isothermal Amplification Buffer (20 mM Tris-HCl (pH 8.8), 50 mM KCl, 10 mM (NH4)2SO4, 2 mM MgSO4, 0.1% Tween 20), 8 mM of MgSO4, 0.2 M of Betaine, 1.6 µM of inner primers (FIP, BIP), 0.2µM of outer primer (F3, B3) and 8 units of Bst 3.0 DNA Polymerase (NewEngland Biolabs) were used in the reaction. A total of 5 uL of RNA, previously extracted from the animals, was added to the LAMP reaction. It was incubated in the thermocycler (Veriti™ 96-Well Thermal Cycler) at 72°C for 60 minutes, followed by 5 min at 80 °C. Confirmation of a positive RT-LAMP reaction was achieved by observing the LAMP amplified products by the naked eye immediately after the addition of 2uL of Sybr Green ™I nucleic acid gel stain/Invitrogen Dilution 1:10) into the LAMP tube. LAMP products were exposed to UV light with a UV Lamp UVL-56, 6-watt, 365 nm Handheld (UVP, Upland, CA, USA) to observe the fluorescence. The results were confirmed by electrophoresis (Agarose Gel 2%). RT-LAMP primers: The primers used in this study were previously published by [10]. Four primers were used in the LAMP reaction, two inner primers (FIP-BIP) and two outer primers (F3- B3). The primer sequences are shown in Table 1. The target of the primers is a non-structural protein 3 (NSP3) of SARS-CoV-2.

Table 1: Primers used for SARS-COV-2 detection in *Trichechus manatus manatus*. Adapted by Lamb et al, 2020*.

Primer	Sequence (5′ to 3′)
F3	TCCAGATGAGGATGAAGAAGA
B3	AGTCTGAACAACTGGTGTAAG
FIP	AGAGCAGCAGAAGTGGCACAGGTGATTGTGAAGAAGAAGAG
BIP	TCAACCTGAAGAAGAGCAAGAACTGATTGTCCTCACTGCC

*Lamb LE, Bartolone SN, Ward E, Chancellor MB. Rapid detection of novel coronavirus/Severe Acute Respiratory Syndrome Coronavirus2 (SARS-CoV-2) by reverse transcription-loop-mediated isothermal amplification. PloS One. 2020; 15(6):e0234682.

4. Results

Despite the potential susceptibility for SARS-CoV-2 in marine mammals [11,12], to date, the infection of aquatic mammals had not been confirmed. Our results show evidence that Antillean manatees can be infected by SARS-CoV-2, and therefore, we confirmed the first potential case of SARS-CoV-2 detection in a Sirenia species. Our RT-PCR analysis showed that the viral target envelope protein gene (E gene) was reported in two of the 19 investigated samples: the Cycle of quantification (Ct) for sample 4 was 37.04, and the Ct of sample 14 was 38.58 (Figure 2). No other samples had amplification that was detectable by the Kit used in this study. The reaction controls were analyzed and were within the parameters established by the Kit manufacturer, where the negative control was undetermined, and the Ct of the positive control was less than or equal to 37, which in this case, had a Ct of 30.80. The E gene of SARS-CoV-2 detected in the Antillean manatees by Real-Time PCR is used as a first-line screening tool. This gene encodes the envelope (E) protein of SARS-CoV-2 playing a fundamental role in the viral assembly, envelope formation, pathogenesis, and viral replication. The E protein is mostly expressed during the virus replication cycle [13]. Our results of the RT-LAMP reaction confirmed that the same two animals were SARS-CoV-2 positive (Table 2; Figure 3). The RT-LAMP assays confirmed the presence of the NSP3 coding region of open reading frame (ORF) 1Ab. Previously published works suggest that the NSP3 protein is essential for SARS-CoV-2 replication, translation of the mRNA transcripts, and suppression of the immune response [14,15]. Blood counts were unaltered and biochemical serum tests results did not show any common abnormality between both contaminated individuals (Table 1). Furthermore, none of the detected SARS-CoV-2 animals presented clinical symptoms that could indicate COVID-19 development. Additionally, both animals were kept in different enclosures at the Itamaracá Island facility, one with other three animals that tested negative for SARS-CoV-2 and the other was kept alone in an isolated enclosure. Furthermore, after 15 days, new SARS-CoV-2 tests were conducted in the search for new cases of detection at the Itamaracá Island facility, and all the retested animals were negative for SARS-CoV-2 in both assay methods.

Table 2: Origin, living conditions, health and results of the Antillean manatees sampled.

Animal	Sex	Age	Living conditions	State	Overall health assessment	RT-PCR/RT-LAMP-PCR results
Xuxa	F	Adult	Rehabilitation captivity	PE	Blood count: Unaltered Biochemical: Low urea; high triglycerides.	Negative/Negative
Poque	M	Adult	Rehabilitation captivity	PE	Blood count: Absolute lymphopenia; thrombopenia. Biochemical: Low urea.	Negative/Negative
Carla	F	Adult	Rehabilitation captivity	PE	Blood count: Normocytic normochromic anaemia; lymphopenia leukopenia; absolute neutropenia Biochemical: High triglycerides	Negative/Negative

Sheila	F	Adult	Rehabilitation captivity	PE	Blood count: Absolute leukopenia Biochemical: High triglycerides	Negative/Negative
Canoa	F	Adult	Rehabilitation captivity	PE	Blood count: Absolute lymphopenia Biochemical: Unaltered	Negative/Negative
Daniel	M	Adult	Rehabilitation captivity	PE	Blood count: Unaltered Biochemical: High triglycerides	**Positive/Positive**
Bela	F	Adult	Rehabilitation captivity	PE	Blood count: Unaltered Biochemical: Low urea	Negative/Negative
Leno	M	Calf	Rehabilitation captivity	PE	Blood count: Unaltered Biochemical: Low urea	**Positive/Positive**
Mocinha	F	Juvenile	Rehabilitation captivity	PE	Blood count: Normocytic normochromic anaemia Biochemical: Unaltered	Negative/Negative
Zoe	M	Adult	Rehabilitation captivity	PE	Blood count: Normocytic normochromic anaemia Biochemical: Low urea; Low GPT	Negative/Negative
Paty	F	Adult	Acclimatization captivity	AL	Blood count: Absolute lymphopenia Biochemical: Low urea	Negative/Negative
Raimundo	M	Adult	Acclimatization captivity	AL	Blood count: Absolute lymphopenia Biochemical: Low urea	Negative/Negative
Netuno	M	Adult	Acclimatization captivity	AL	Blood count: Absolute lymphopenia Biochemical: High triglycerides	Negative/Negative
Assu	M	Adult	Acclimatization captivity	AL	Blood count: Absolute lymphopenia Biochemical: High triglycerides	Negative/Negative
Telinha	F	Adult	Released	AL	Blood count: Absolute lymphopenia Biochemical: Low urea	Negative/Negative
Joana	F	Adult	Released	AL	Blood count: Unaltered Biochemical: Low urea	Negative/Negative
Tinga	M	Adult	Released	AL	Blood count: Thrombocytopenia Biochemical: Low urea	Negative/Negative
Bacuri	M	Juvenile	Wild free-living	AL	Blood count: Intense thrombocytopenia Biochemical: Low urea	Negative/Negative
Natal	M	Adult	Wild free-living	RN	Blood count: Erythrocytosis; rubricytosis; heterophily; absolute lymphopenia; activated monocytes; phagocytosed coccos Biochemical: High urea	Negative/Negative

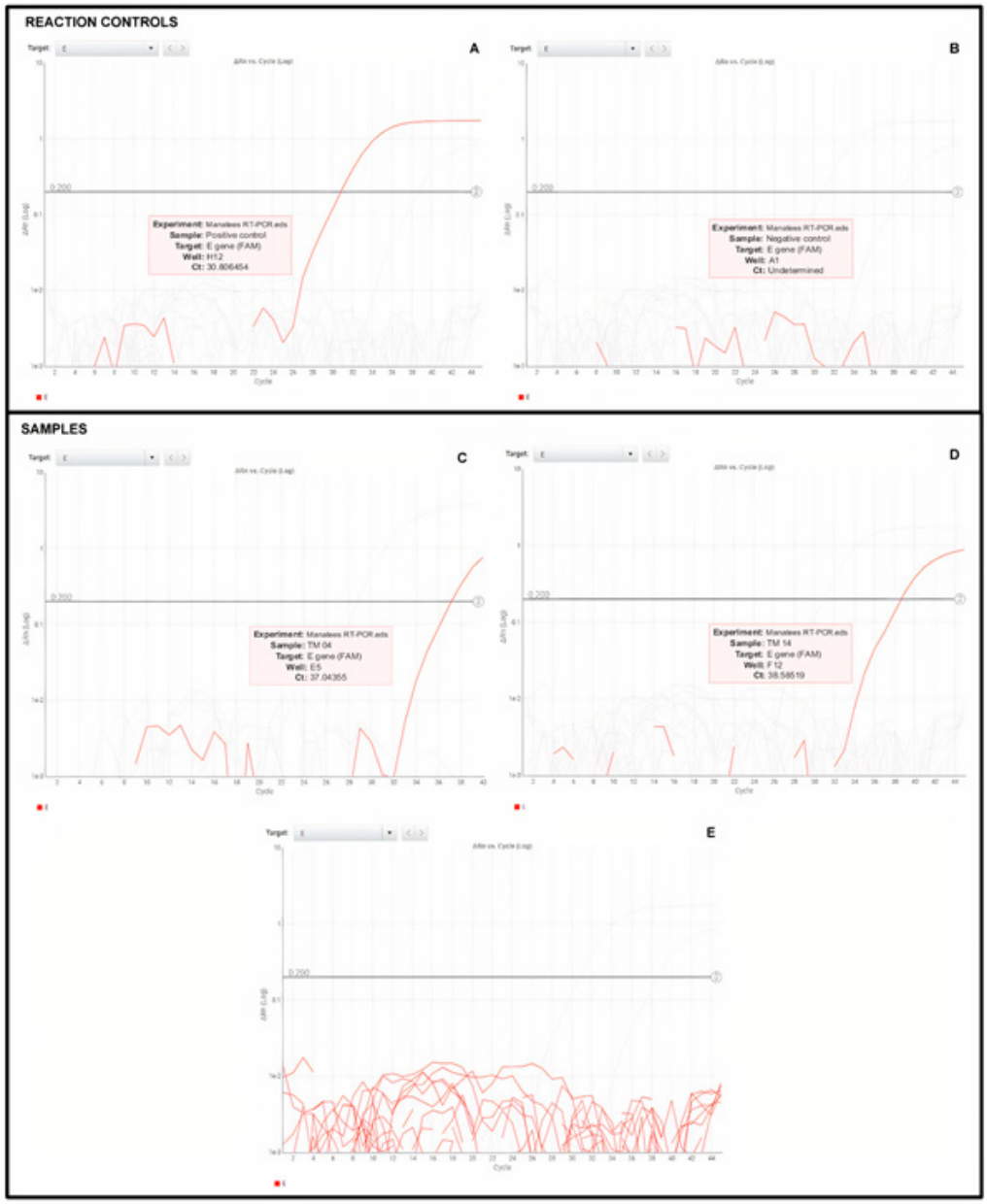

Figure 2: RT-PCR for SARS-CoV-2 in samples from *Trichechus manatus*: Section a and b shows the amplification graphs of the reaction controls with the positive control in 'a' and the negative control in 'b'. Sections c to e show the results of the samples processed in this experiment, where the two samples that had positive detection for SARS-CoV-2 are shown in 'c' and 'd', and in 'e', we see only the basal fluorescence reported by the equipment in all the other samples.

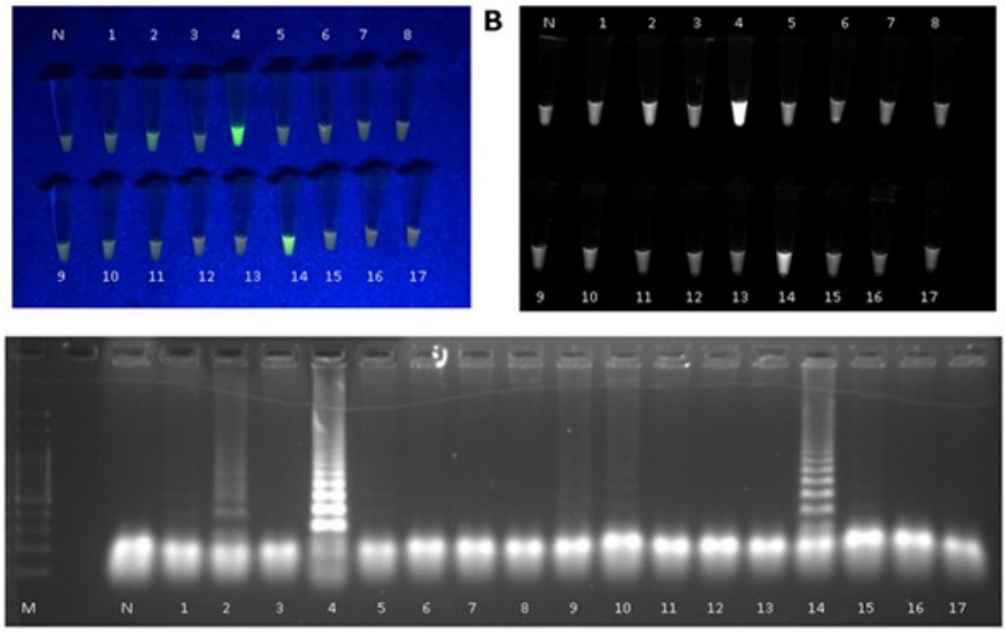

Figure. 3: LAMP for SARS-CoV-2 detection in *Trichechus manatus.* (N) Negative control, (1-17) Animal with positive tested.

5. Discussion

After SARS-CoV-2 detection in the manatees, all 24 staff at the Itamaracá captive centre were tested using an anti-SARS-CoV-2 antibodies search by an immunochromatographic method. Six staff members tested positive for the virus, of which two had direct contact with the animals. Although the keepers could have transmitted SARS-CoV-2 to the manatees, the actual source of infection is still uncertain. The water in the manatee pools comes directly from the sea, and the animals receive freshwater for hydration and bottle feeding [16]. Suggested that the lack of treatment of wastewater in common sewers could transfer SARS-CoV-2 to natural waters. Furthermore [17], found that fragments of the COVID-19 virus present in wastewater were capable of infecting tadpoles (*Physalaemus cuvieri*). Therefore, we highlight the potential risk of this transmission route to our study animals. Although detection of SARS-CoV-2 occurred, it is possible that this was due to contamination of the nasal passages with the virus, not a confirmed infection and replication within the Intensified animal observations regarding behavioural and breathing activities changes. Manatees are extremely docile animals and can usually interact with humans [18,19]. No evidence of transmission from manatees to humans was identified in the studies, however the results highlight the importance of not approaching manatees, except for animal care teams. In addition, it highlights the importance of using personal protective equipment such as masks and disposable gloves during procedures with manatees. While there are other available tests to detect SARS-CoV-2 in animals, RT-PCR and RT-LAMP assays are very precise regarding the detection of the virus within samples when conducted properly [20-22]. Thus, our results are conclusive and point to the susceptibility of the Sirenia group to SARS-CoV-2.

As the first potential case of SARS-CoV-2 in Antillean manatees, we highlight the importance of testing and reporting non-human species transmission cases. While the direct impact on the Antillean manatees' conservation remains unclear, detecting SARS-CoV-2, a potentially highly lethal virus, in a threatened species is of enormous and general concern for the Sirenia group, as well as to other marine mammal species. Asian Small-clawed Otters detected with SARS-CoV-2 showed clinical alterations including sneezing, lethargy, and coughing (USDA 2021). COVID 19 was a serious pandemic of social and economic impact in all countries, causing the whole world to seek rapid diagnostic solutions for the identification of the virus [23]. Our studies corroborate this need to search for a diagnosis, showing the effectiveness of the RT-LAMP reaction methodology adopted by the researchers of the Fundação Oswaldo Cruz to confirm the diagnoses in the manatees studied. This methodology can be applied to other manatees both in cases of clinical suspicion and in animal health assessment protocols.

The impact of COVID on aquatic ecosystems was not limited to the increase in the number of hospital waste such as masks and gloves in the environment that can be ingested by animals, wastewater from treatment plants that received viral loads or even from places where there were contaminated patients. , may have led to contamination of the environment, including drugs used on a large scale, such as ivermectin, azithromycin, analgesic, retroviral and other corticosteroids, affecting numerous species [24-26]. Manatees use coastal areas and may possibly be affected by this contamination if there is contact between the animal and the virus, and there should be an alert even for cases of drug residues, even if this is not the objective of the present study. Although our results only reported two specific cases of SARS-CoV-2 in Antillean mana-

tees individuals that have not developed any clinical complications due to the virus presence, the SARS-CoV-2 infection may result in several health complications in other individuals and annihilate an entire Antillean manatee's population. Our results also show how we can use genetic tools to improve the care and ultimately the conservation of the threatened Antillean manatees. We suggest prioritizing vaccination and regular SARS-CoV-2 testing of the staff and animals in all institutions that keep manatees and other animals in captivity. We also suggest further studies on how to avoid and protect threatened species from SARS-CoV-2 contamination and COVID-19 development risks.

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