

# Coronavirus (SARS-CoV-2) in Antillean Manatees (*Trichechus Manatusmanatus*)

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## Short Report

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# Abstract

For two years, 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 the coronavirus (SARS-CoV-2). We collected samples from 19 individuals kept under the responsibility of the Brazilian centre for research and conservation of aquatic mammals (ICMBio/CMA). We analysed the samples through RT-PCR and RT-LAMP-PCR and found that two of the 19 manatees tested positive for SARS-CoV-2. Such a result led to a new biosecurity protocol in the ICMBio/CMA to avoid potential human-manatee coronavirus contamination, showing how we can use simple genetic tools to improve the care and conservation of manatees.

## Introduction

Since late 2019, the world has been experiencing a coronavirus (SARS-CoV-2) pandemic, with the first case reported in Brazil in February 2020 (Werneck and Carvalho 2020). The transmission of the virus from humans to animals is possible, especially mammals (including marine ones), which are already known to be susceptible to the disease (IUCN SSC 2020; Gryseels et al. 2020). Infected wastewater discharged into natural water systems are supposed to contaminate aquatic species (Mathavarajah et al. 2020; Audino et al. 2021; Charlie-Silva et al. 2021). West Indian manatees (*Trichechus manatus*) are marine mammals categorised worldwide as Vulnerable due to the sharp decline in their populations (Deutsch et al. 2008). The subspecies *Trichechus manatus manatus* (Antillean manatee) occurs in Brazil, being categorised as Endangered in the Brazilian list of threatened species (ICMBio 2018). The species is targeted by a conservation action plan and a long-term reintroduction program in Brazil (Luna and Passavant 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).

## Materials & Methods

### Sample collection

We collected samples from 19 Antillean manatees (Supplementary Information – Table 1) kept under the responsibility of the Centro de Pesquisa e Conservação de Mamíferos Aquáticos do Instituto Chico Mendes de Biodiversidade (ICMBio/CMA). We collected samples from the nostrils of the manatees with 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 biometrics measures and blood samples from all the animals to perform blood and serum biochemicals

counts (urea, creatinine, GOT, GPT, glucose, triglycerides). This study complied with the Brazilian law (Permit number: SISBIO/ICMBio N°77116-1).

## 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 the COVID-19.

## 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 (Corman VM et al. 2020). 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 are 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.

## RT-LAMP-PCR assay

The LAMP reaction was performed with a final volume of 25 µL. 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 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 mM MgSO<sub>4</sub>, 0.1% Tween 20), 8 mM of MgSO<sub>4</sub>, 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 µL 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 2µL 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 Lamb et al. (2020). 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 Supplementary Information - Table 2. The target of the primers is a non-structural protein 3 (NSP3) of SARS-CoV-2.

## Results & Discussion

Despite the presumed susceptibility for SARS-CoV-2 in marine mammals (Nabi 2020; Mathavarajah et al. 2021), to date, the infection of aquatic mammals had not been confirmed. Our results show evidence that Antillean manatees can be contaminated by SARS-CoV-2, and therefore, we confirmed the first case of COVID-19 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 (Rahman et al. 2021).

Our results of the RT-LAMP reaction confirmed that the same two animals were COVID-19 positive (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 (Raj 2021; Emam et al. 2021).

Blood counts were unaltered and biochemical serum tests results did not show any common abnormality between both contaminated individuals (Supplementary information – table 1). Furthermore, none of the animals presented clinical symptoms that could indicate COVID-19 contamination.

After SARS-CoV-2 detection in the study manatees, all 24 staff at the Itamaracá captive centre were tested. Six staff members tested positive for COVID-19, from which two had direct contact with the

animals. Although the keepers could have transmitted COVID-19 to the manatees, the actual source of infection is still uncertain. The water in the manatee pools come directly from the sea, and they receive a freshwater source for hydration and bottle feeding. Mathavarajah et al. (2021) suggested that the lack of treatment of wastewater in common sewers could transfer SARS-CoV-2 to natural waters. Furthermore, Charlie-Silva et al. (2021) 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.

Many species, including manatees, requires human interventions to guarantee their populations' survival. Our results led to a new security protocol in the ICMBio/CMA captive centre to avoid potential human-manatee coronavirus contamination, showing 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 COVID-19 testing of the staff and animals in all institutions that keep manatees and other animals in captivity.

## Declarations

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Author Consent - All authors have read the final version of the manuscript and consent to its publication.

Author Contributions – FLNA and KL were responsible for the sample collections; FLM, NANB and RMLA were responsible for the genetic analyses; FL and BB were responsible for the elaboration of a first draft, and all authors reviewed, adjusted, and commented on the final draft. RU reviewed the grammar and language. All authors contributed to the study conception, design and execution.

Conflicts of Interest - The authors have no conflicts of interest to declare that are relevant to this article.

Ethics approval - The study complied with Brazilian law and was conducted under the SISBIO license 77116-1 held by Fernanda Attademo.

Data Availability - The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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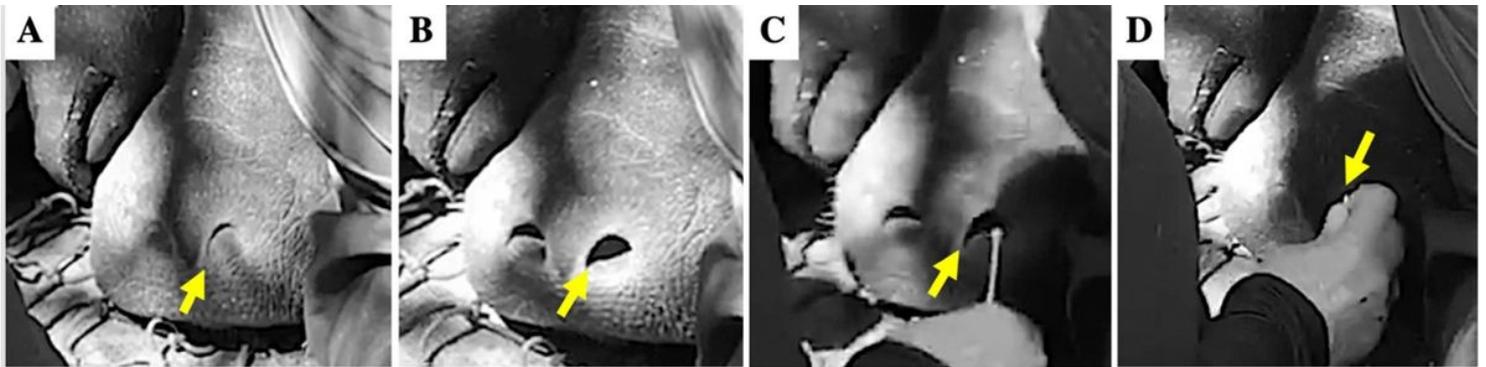
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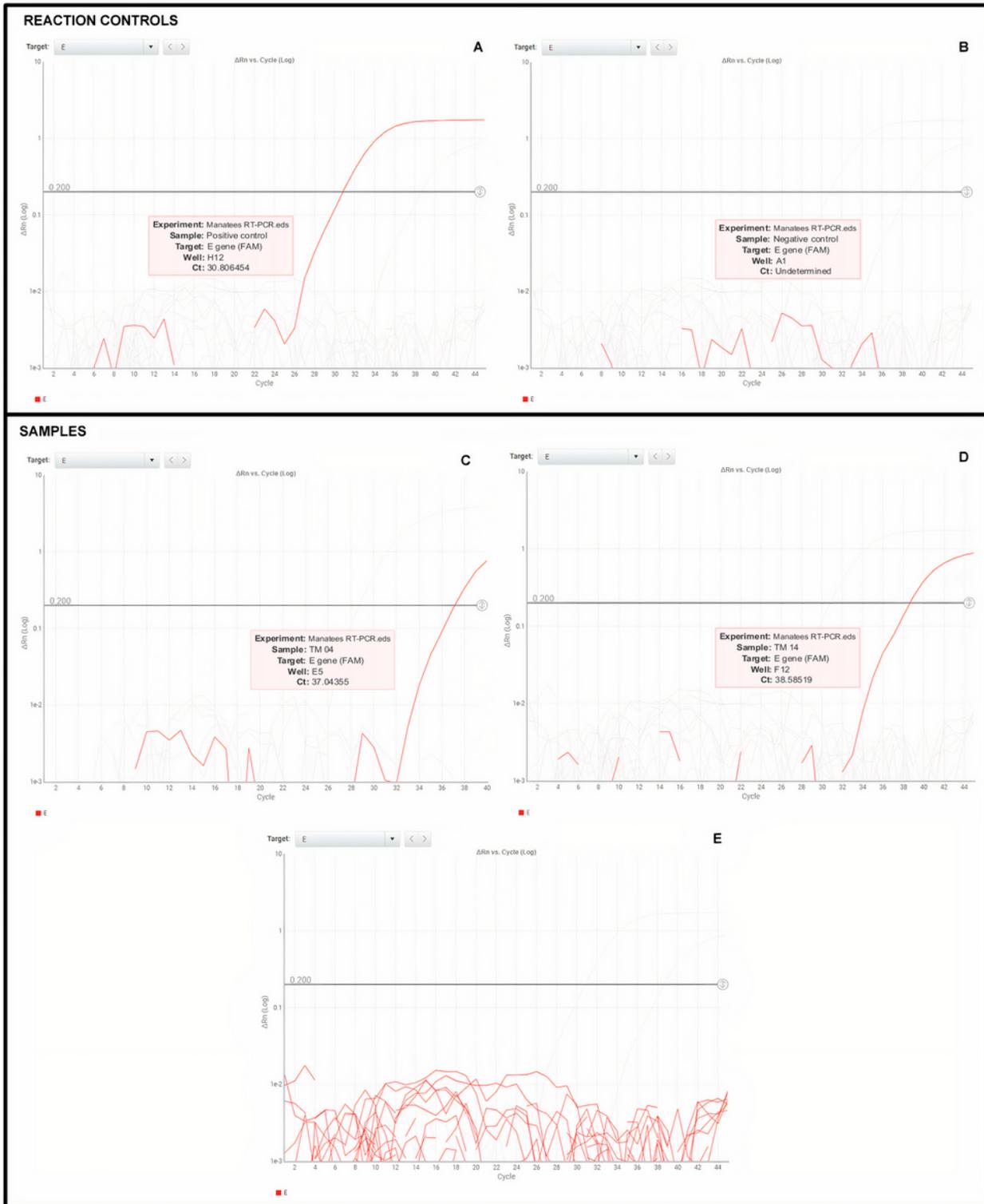
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## Figures



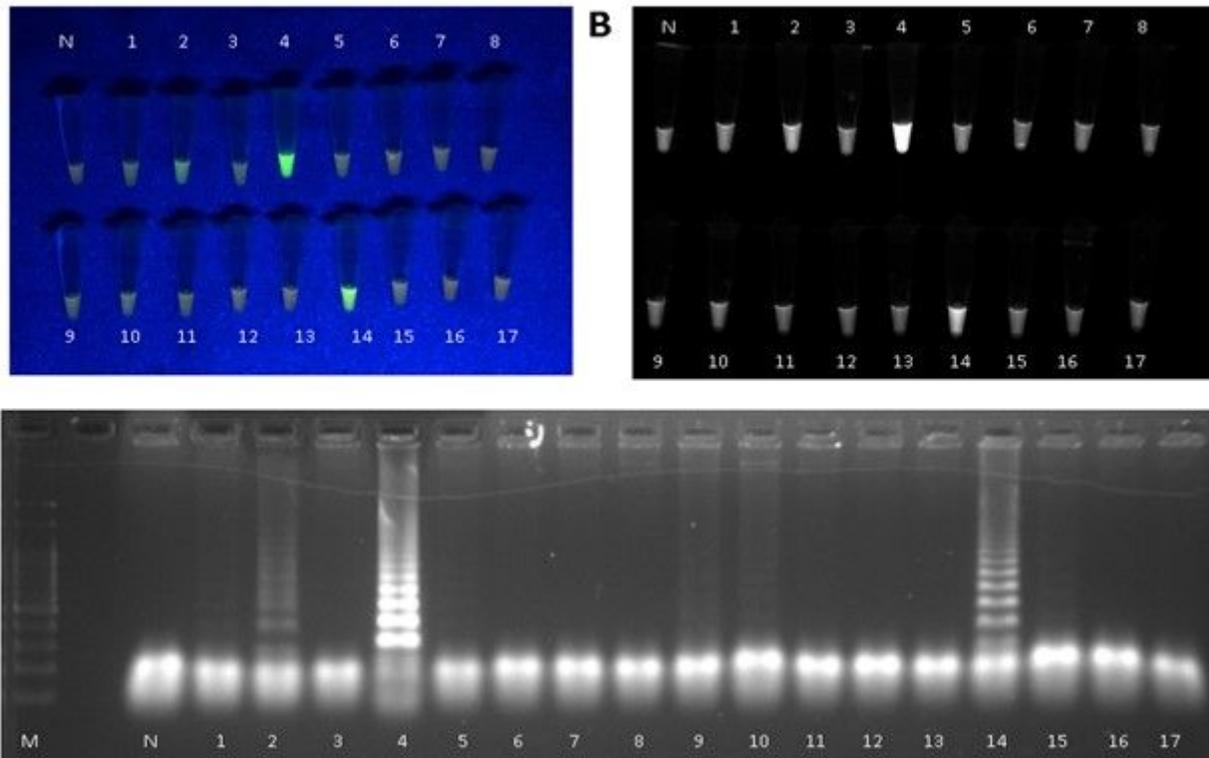
**Figure 1**

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



**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 'c', 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 tested.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementaryinformation14.12.docx](#)