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Short Communication

The presence of SARS-CoV-2 RNA in human sewage in Santa Catarina, Brazil, November 2019

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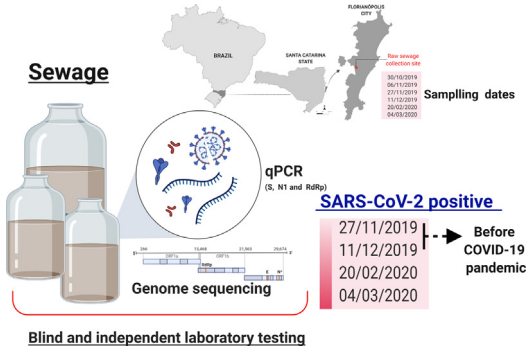
HIGHLIGHTS

- SARS-CoV2 was tested in human sewage from Florianopolis from Oct 2019 until Mar 2020.
- SARS-CoV-2 was detected on 27th Nov 2019 (5.49 ± 0.02 $\log_{10}$ genome copies L^{-1}).
- The samples in the subsequent three events were positive by all RT-qPCR assays.
- The overall load was 5.83 ± 0.12 $\log_{10}$ GC L^{-1}, ranging from 5.49 ± 0.02 (27th Nov 2019) to 6.68 ± 0.02 (4th Mar 2020).
- Our findings demonstrate that SARS-CoV-2 was circulating unnoticed in in Brazil since Nov 2019

GRAPHICAL ABSTRACT



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ABSTRACT

Human sewage from Florianopolis (Santa Catarina, Brazil) was analyzed for severe acute respiratory syndrome coronavirus-2 (SARS-CoV2) from October 2019 until March 2020. Twenty five ml of sewage samples were clarified and viruses concentrated using a glycine buffer method coupled with polyethylene glycol precipitation, and viral RNA extracted using a commercial kit. SARS-CoV-2 RNA was detected by RT-qPCR using oligonucleotides targeting N1, S and two RdRp regions. The results of all positive samples were further confirmed by a different RT-qPCR system in an independent laboratory. S and RdRp amplicons were sequenced to confirm identity with SARS-CoV-2. Genome sequencing was performed using two strategies; a sequence-independent single-primer amplification (SISPA) approach, and by direct metagenomics using Illumina's NGS. SARS-CoV-2 RNA was

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detected on 27th November 2019 (5.49 ± 0.02 log_{10} SARS-CoV-2 genome copies (GC) L^{-1}), detection being confirmed by an independent laboratory and genome sequencing analysis. The samples in the subsequent three events were positive by all RT-qPCR assays; these positive results were also confirmed by an independent laboratory. The average load was 5.83 ± 0.12 log_{10} SARS-CoV-2 GC L^{-1}, ranging from 5.49 ± 0.02 log_{10} GC L^{-1} (27th November 2019) to 6.68 ± 0.02 log_{10} GC L^{-1} (4th March 2020). Our findings demonstrate that SARS-CoV-2 was likely circulating undetected in the community in Brazil since November 2019, earlier than the first reported case in the Americas (21st January 2020).

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1. Introduction

The first reported cases of COVID-19, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) were described in Wuhan City, China in December 2019 (WHO, 2020). However, there is evidence to suggest that the virus had already been circulating a few months previously (Nsoesie et al., 2020). The first diagnosed COVID-19 case in the Americas was reported on 21st January 2020 in the USA (on 25th February in Brazil) (WHO, 2020). By November 2020, the World Health Organization had reported more than 1.3 million deaths worldwide from the disease, with over 160,000 occurring in Brazil (WHO, 2020).

SARS-CoV-2 is a respiratory virus and the transmission route is mainly through respiratory droplets or contact with contaminated surfaces (van Doremalen et al., 2020). It can also infect human gut enterocytes, and gastrointestinal manifestations have been reported (Lamers et al., 2020), with consequent prolonged faecal shedding of up to 10^8 SARS-CoV-2 genome copies per g of stool (Ding and Liang, 2020; Lescure et al., 2020).

The analysis of human and animal sewage has been successfully applied in monitoring the presence of biological risks (Choi et al., 2018), including enteric viruses (Prevost et al., 2015). A similar monitoring strategy has been applied for the detection of SARS-CoV-2 (Ahmed et al., 2020a; Chavarria-Miró et al., 2020; La Rosa et al., 2020; Medema et al., 2020; Nemudryi et al., 2020; Randazzo et al., 2020; Rimoldi et al., 2020; Wu et al., 2020; Wurtzer et al., 2020) demonstrating its value as a non-invasive early-warning tool for monitoring the trend of COVID-19 infection (Randazzo et al., 2020).

In this context, the present study aimed to evaluate the presence of SARS-CoV-2 in samples of human raw sewage in Santa Catarina, Brazil, collected in a period prior to the first clinical report (13th March 2020) of the disease in that region.

2. Methodology

Urban raw sewage samples were collected in the city of Florianopolis, Santa Catarina, Brazil (Supplementary Material Fig. 1) from a sewage system in central Florianopolis, serving a population of approximately 5000 inhabitants, in 6 independent sampling events from 30th October 2019 until 4th March 2020. The sampling was conducted using a submersible pump (Scheider, model BSC-94 3/4 hp) inside a raw sewage manhole to pump the raw sewage from the public collection system. The pump was automatically turned on for 5 min every 25 min for 24 h. The raw sewage was pumped to a closed fibre tank of 5 m^3. From this tank, 2 L of urban raw sewage samples were collected aseptically and 200 mL were used for further virus analysis. The samples had been collected by the Sanitary Engineering Laboratory for routine physical-chemical monitoring of urban sewage in the city, and were subsequently provided to the Laboratory of Applied Virology at the Federal University of Santa Catarina. The samples were stored frozen at −80 °C during six months. Florianópolis is an internationally known tourist and business destination in Brazil, which has been declared the South American Common Market capital of tourism. The raw sewage samples were collected from a sewage system in central Florianopolis, serving a population of approximately 5000 inhabitants. The main physical-chemical features of the urban sewage sampled are shown in Table 1 (Santos, 2019). 25 mL of each samples were thawed and clarified and viruses concentrated using a glycine buffer method coupled with polyethylene glycol precipitation (Viancelli et al., 2012; Schlindwein et al., 2009). Briefly, viral particles were eluted from the precipitated sample using a glycine buffer (pH 9.5) and concentrated by PEG 6000 precipitation. After centrifugation, the supernatant was discarded, and the resulting pellet containing the concentrated virus was suspended in 5.0 mL of 0.1 M phosphate buffer (pH 7.2). Murine Norovirus (MNV-1) was artificially inoculated into each raw sample as process control virus to estimate the efficiency of extraction (Diez-Valcarce et al., 2012). Viral RNA was extracted from 200 μL of concentrate using the QIAamp Viral RNA Mini kit (QIAGEN, CA, USA) according to manufacturer instructions, and eluted in 50 μL of RNAse free water. Two independent viral concentrations and RNA extractions were performed for each sewage sample. A negative control (H_2O) was included with each batch of samples. SARS-CoV-2 viral RNA was detected by reverse transcription followed by quantitative polymerase chain reaction (RT-qPCR) on a 7900 Real-Time PCR instrument (Applied Biosystems, USA). A 25 μL reaction contained 15 μL of RT-qPCR Master Mix containing 2 × reaction buffer provided with the OneStep qPCR Quantinova kit (QIAGEN, Germany), 1 U of reverse transcriptase/Taq mixture from the kit and 2 mM magnesium sulphate solution and 10 μL of RNA template (neat and 1:10 dilution). For all reactions, 400 nm of oligonucleotides and 200 nm of probe were used targeting SARS-CoV-2 specific genome sequences from regions N1 (CDC, 2020), S and two RdRp regions - from 14,965 to 15,302 bps (Chan et al., 2020) and from 15,361 to 15,460 bps (Corman et al., 2020). Thermal cycling for all RT-qPCRs was performed at 55 °C for 10 min for reverse transcription, followed by 95 °C for 3 min and then 45 cycles of 95 °C for 15 s, 58 °C for 30 s MNV-1 was detected according to Di Bartolo et al. (2012). The oligonucleotides and a commercial positive quantitative SARS-CoV-2 control (2019-nCoV_N_Positive Control, a purified plasmid DNA - 2 × 10^5 genome copies/μL) were obtained from IDT (Belgium). All RT-qPCRs were performed in duplicate in two independent experiments which incorporated RT-qPCR negative controls and sample process negative controls (field and blank controls).

Table 1
Sewage system and physical-chemical characterization.

	Sewer at collection point			Sewage physic-chemical characterization				
Samples collected	Average flow ($L.s^{-1}$)	Diameter (mm)	Population served (inhabitants)	COD[a] (mg L^{-1})	BOD[b] (mg L^{-1})	pH	Alkalinity (mg$CaCO_3$ L^{-1})	TSS[c] (mg L^{-1})
	1.5	100 mm	5.000	703	338	7.9	320	142

[a] COD: Chemical Oxygen Demand.
[b] BOD: 5 days Biochemical Oxygen Demand.
[c] Total Suspended Solids.

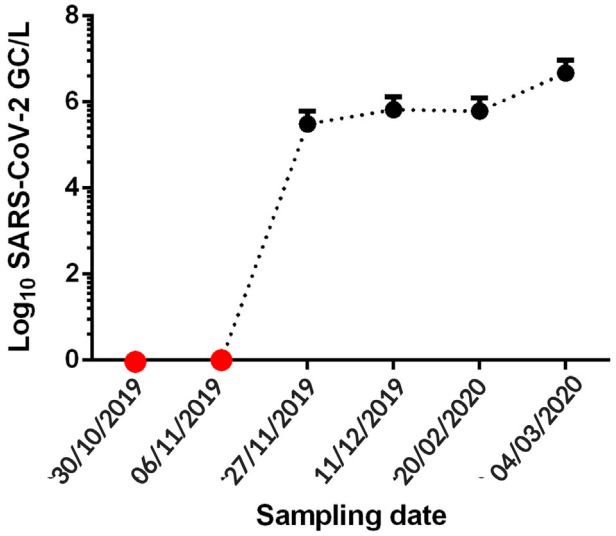

Fig. 1. Evolution of the presence of SARS-CoV-2 RNA in urban sewage, Florianopolis, Brazil. Red dots represent negative results. Black dots represent $\log_{10}$ SARS-CoV-2 genome copies per liter of urban sewage (M ± SE) from independent duplicate estimates. N1 RT-qPCR assay was used for quantification of SARS-CoV-2 genome copies per liter. All negative RT-qPCR and sample process controls in each sampling event were negative.

The results of all positive samples were further confirmed by an independent laboratory (Laboratório de Biologia Molecular, Microbiologia e Sorologia, UFSC University Hospital). These confirmatory tests were carried out using a blind, duplicate, independent concentration and RNA extraction of the original sample, and detection was performed using a RT-qPCR commercial kit (Seegene Allplex™ 2019-nCoV kit), that targets different SARS-CoV-2-specific RdRP, S and N regions. The S and RdRp amplicons were sequenced to confirm identity with SARS-CoV-2 in an independent genomic service (Neoprospecta Microbiome Technologies, Florianópolis, Brazil). Likewise, genome sequencing of the positive sample collected on 27th November 2019 was carried out using two different strategies. A sequence-independent single-primer amplification (SISPA) approach was performed as previously described (Lewandowski et al., 2019) with minor modifications. Briefly, reverse transcription was performed using primer A (5′-GTTTCCCACTGGAGGATA-N9–3′) and SuperScript IV (Thermo Fisher, USA). The second-strand synthesis was performed with Klenow enzyme (Promega, USA). cDNA amplification was performed by PCR and the reactions were pooled and purified using AMPureXP magnetic beads (Beckman Coulter) in 1:1 ratio. dsDNA samples concentrations were estimated using Picogreen (Invitrogen, USA) in a Qubit fluorometer (Thermo Fisher, USA) and used as input for an Illumina DNA Prep kit (Ilumina, USA) according to manufacturer instructions. In the second sequencing approach, the same cDNA prepared with primer A (in the RNA:DNA hybrid form) was used directly as input for the Illumina DNA Prep kit (Ilumina, USA) following the manufacturer protocol for dsDNA tagmentation. Samples from both approaches were sequenced on a 150 × 150 pb Miseq (Ilumina, USA) run with 1 M reads/sample. Sequence reads were mapped against a SARS-CoV-2 reference genome NC_045512.2 using Bowtie 2 software (Langmead and Salzberg, 2012). Reads were also mapped to other coronavirus reference genomes available in NCBI (Coronavirus 229E, HKU1, NL63, OC43, bat Hp-betacoronavirus, Pipistrellus bat coronavirus HKU5, Rousettus bat coronavirus HKU9, Tylonycteris bat coronavirus HKU4, Middle East Respiratory Syndrome Coronavirus -MERS-CoV-, and Pangolin coronavirus). In addition, a less stringent BLAST analysis was also performed against NCBI coronaviridae using default parameters. Mapping and BLAST identities and coverage were evaluated.

3. Results and discussion

The concentration method based on PEG used for SARS-CoV-2 concentration in human raw sewage in this study, had shown satisfactory recovery values (26%) for enteric viruses in a previous study performed by our research group (Schlindwein et al., 2009). Similarly, Ahmed et al. (2020b) compared different available virus concentration methods, and confirmed that a PEG-based concentration method obtained similar recovery values from untreated wastewater using murine hepatitis virus (SARS-MHV-3), a surrogate for SARS-CoV-2. In the present study, all sample analyses showed process efficiency values ranging from 16% to 26%, with an overall value of 21% ± 0.2% (M ± SE). Similarly, the average amplification efficiency and linearity of the N1-specific RT-qPCR were optimal; an average amplification efficiency of 99.8 (average slope of the standard curve, −3.333) and correlation coefficients (r^2) ranging from 0.991 to 0.997. The smallest number of copies detected was 10 copies / reaction.

All negative controls (RT-qPCR negative controls and sample process negative controls) were negative in each test. Notably, while samples were negative in the first two sampling events (30th October and 6th November 2019), all samples in subsequent three events were positive (since 27th November 2019 until 4th March 2020) by all RT-qPCR assays; these positive results were confirmed by an independent laboratory. Illumina sequencing of the RdRp and S amplicons revealed 100% identity with the respective available SARS-CoV2 target sequences (RdRp accession numbers MT925972, MT925973, MT925974, MT925975; and S accession numbers MT925976, MT925977, MT925978, MT925979). It is important to highlight that the SARS-CoV-2 positive sample from 27th November 2019 was subjected to deep Illumina sequencing: a total of 1,140,885 reads were obtained combining sequences from both library preparation strategies. From these reads, 25% of the SARS-CoV-2 genome was spanned (this sequence can be accessed at GeneBank number PRJNA679821; Supplementary Material Fig. 2). Other reads were mostly related to the human genome. Reads mapping against *Coronaviridae* genomes resulted in matches only with SARS-CoV-2, while BLAST analysis showed average similarities of 99.38% with SARS-CoV-2, and significantly lower similarities for other coronaviruses (79.5% and 91.30% for CoV-HKU and SARS-CoV-1).

The average SARS-CoV-2 load was 5.83 ± 0.12 $\log_{10}$ genome copies (GC) L^{-1}, ranging from 5.49 ± 0.02 $\log_{10}$ GC L^{-1} (Cq value of 36.1) (27th November 2019) to 6.68 ± 0.02 $\log_{10}$ GC L^{-1} (Cq value of 34.5) (4th March 2020) (Fig. 1). Those SARS-CoV-2 RNA loads are similar to those found in studies performed in France (Wurtzer et al., 2020), Spain (Chavarria-Miró et al., 2020; Randazzo et al., 2020), and the USA (Nemudryi et al., 2020; Wu et al., 2020). Prolonged faecal shedding of up to 10^8 SARS-CoV-2 genome copies per g of stool has been reported (Ding and Liang, 2020; Lescure et al., 2020), and SARS-CoV-2 RNA has been detected in faeces of not only symptomatic but also asymptomatic persons (Jiehao et al., 2020; Tang et al., 2020; Wölfel et al., 2020; Zhang et al., 2020).

SARS-CoV-2 RNA was detected as early as 27th November 2019, 56 days in advance of the first COVID-19 confirmed case in the Americas (in the USA), 91 days in advance of the first case in Brazil, and 97 days in advance of the first confirmed case in Santa Catarina Region. This demonstrates that SARS-CoV-2 was being shed within the community for several months prior to the first cases being reported by regional, national or Pan-American authorities. Unfortunately, we did not have access to human clinical samples of that period, nor there are any clinical data on SARS-CoV-2, as the investigation of COVID-19 cases in Brazil began after January 2020 and the first case reported in Santa Catarina was in March 2020. Few data are available on retrospective studies of SARS-CoV-2 RNA detection in sewage prior to onset of COVID-19 clinical cases. Randazzo et al. (2020) conducted a retrospective study of wastewater process plants in the Murcia Region, Spain, and compared the data to declared COVID-19 cases at

municipality level: the presence of SARS-CoV-2 RNA in sewage was also earlier than the first reported cases. Medema et al. (2020) observed the presence of SARS-Coronavirus-2 RNA in sewage in the early stage of the epidemic in the Netherlands. A similar situation was also observed in Barcelona, Spain, where SARS-CoV-2 was repeatedly detected in human sewage from January 2020 (Chavarria-Miró et al., 2020).

Interestingly, while the SARS-CoV-2 RNA loads were stable until late February (5.49 ± 0.02, 5.82 ± 0.01 and 5.65 ± 0.10 $\log_{10}$ SARS-CoV-2 genome copies L^{-1} in 27th November 2019, 11th December 2019 and 20th February 2020, respectively), an increase of approximately 1 $\log_{10}$ was observed on 4th March 2020 (6.68 ± 0.03 $\log_{10}$ SARS-CoV-2 genome copies L^{-1}) (Fig. 1) coinciding with the first COVID-19 case diagnosed in the region. Unfortunately, 10 days after the last sampling, the pandemic situation prevented the continuation of the sampling and therefore any confirmation of that increasing tendency. Likewise, there are no clinical records of human COVID-19 infection for the 2019 sample collection period.

4. Conclusions

We have confirmed the presence of SARS-CoV-2 RNA that strongly implies the presence of SARS-CoV-2 circulation in the Americas as early as 27th November, 56 days ahead of the reports of COVID-19 cases in the continent and more than 90 days in the case of Brazil. Therefore, our findings point that SARS-CoV-2 was circulating unnoticed in the community for some months before pandemic status was declared. Our results also show that the SARS-CoV-2 load remained constant until early March, then rose coinciding with the onset of COVID-19 cases in the Santa Catarina region.

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CRediT authorship contribution statement

GF, DRL coordinated the study, PR, MEM, DSMS performed sampling and detection, MAS, FHB, MLB performed virus detection, PHS, APC, LFVO, GW performed sequencing, PHS, APC, LFVO, GW, MH, DRL interpreted the sequencing analysis, GF, DRL, ECG, MH analyzed the results. DRL wrote the first version of the manuscript, and all authors contributed to the revision of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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