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SARS-CoV-2 in a stream running through an underprivileged, underserved, urban settlement in São Paulo, Brazil: A 7-month follow-up☆

Maria Tereza Pepe Razzolini a,b,*, Mikaela Renata Funada Barbosa b,c, Ronalda Silva de Araújo c, Ivo Freitas de Oliveira c, Maria Cássia Mendes-Correa d, Esté C. Sabino d, Suzi Cristina Garcia c, Anderson V. de Paula d, Lucy S. Villas-Boas d, Silvia Figueiredo Costa d, Milena Dropa a, Denise Brandão de Assis e, Beatriz S. Levin f, Antonio Carlos Pedroso de Lima g, Anna S. Levin d

a School of Public Health of Universidade de São Paulo, Brazil
b NARA - Center for Research in Environmental Risk Assessment, Brazil
c CETESB - Environmental Company of São Paulo State, Brazil
d Department of Infectious Diseases and Instituto de Medicina Tropical, Faculdade de Medicina, Universidade de São Paulo, Brazil
e São Paulo State Health Department, São Paulo, Brazil
f Guttman Community College, City University of New York, New York, USA
g Instituto de Matemática e Estatística, Universidade de São Paulo, Brazil

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ABSTRACT

COVID-19 pandemic has led to concerns on the circulation of SARS-CoV-2 in the environment, its infectivity from the environment and, the relevance of transmission via environmental compartments. During 31 weeks, water samples were collected from a heavily contaminated stream going through an urban, underprivileged community without sewage collection. Our results showed a statistically significant correlation between cases of COVID-19 and SARS in the community, and SARS-CoV-2 concentrations in the water. Based on the model, if the concentrations of SARS-CoV-RNA (N1 and N2 target regions) increase 10 times, there is an expected increase of 104% [95%CI: (62–157%)] and 92% [95%CI: (51–143%)], respectively, in the number of cases of COVID-19 and SARS.

We believe that differences in concentration of the virus in the environment reflect the epidemiological status in the community, which may be important information for surveillance and controlling dissemination in areas with vulnerable populations and poor sanitation. None of the samples were found infectious based cultures. Our results may be applicable globally as similar communities exist worldwide.

1. Introduction

During the COVID-19 pandemic, questions have arisen about circulation of SARS-CoV-2 in the environment and its relevance to the transmission of the disease. The presence of the virus in sewage raises the question of whether it is necessary to establish sanitary barriers in order to control the pandemic.

Monitoring of community wastewater is an effective tool to track diseases and to guide public health responses. Wastewater-based epidemiology (WBE) has been demonstrated as a complementary approach for spatial tracking of infectious diseases (Sims and Horden-Kasprzyk, 2020; Randazzo et al., 2020), including coronavirus disease 2019 cases (COVID-19), as well as early warning of the occurrence of infected populations (Medema et al., 2020; Prado et al., 2021). There has been extensive discussion on the role of aerosol generated by cough, talking and singing in the transmission of COVID-19. However, environmental-generated aerosols may also have a role. Giacobbo et al. (2021) state that aerosols generated from sewage and contaminated waters deserve a deeper discussion on the possible fecal-nasal transmissions. Likewise, Usman et al. (2020) brought the issue of the potential of the aerosols generated by contaminated waters could potentially be a route of transmission of the virus if present. The same was discussed by Al-Gheethi et al. (2020) whose literature review reveals that larger droplets can travel through aerosols at a short distance...
and, settle down very quickly on surfaces.

On January 31, 2020, the WHO declared the outbreak of COVID-19 to be a Public Health Emergency of International Concern (WHO, 2020). In Brazil the first COVID-19 case was confirmed in São Paulo on 26 February 2020 (Jesus et al., 2020).

SARS-CoV-2 was reported in sewage samples (Medema et al., 2020; Wu et al., 2020; Gonzalez et al., 2020) indicating that the virus circulates in the environment. However, these findings do not mean there is infectious virus in the environment. The finding of viable virus may pose a threat to populations who live in areas with poor sanitation, with a disproportionate exposure driven by inequality and social exclusion. Due to the importance to establish control strategies pandemic, SARS-CoV-2 viability has been investigating. Oliveira et al. (2021) demonstrated SARS-Cov-2 persistence in river water and wastewater with \( T_{90} \) values at 4 \( ^\circ \)C was 7.7 and 5.5 days, respectively, higher than observed for temperature at 24 \( ^\circ \)C.

Like other Latin American countries, Brazil presented a rapid and intense urbanization process, which outpaced the development of infrastructure, such as basic services and housing. Cities struggle to overcome deficiencies in sanitation, urban mobility, and adequate housing. The extent of informal settlements and the spatial segregation of low-income groups are demonstrated by the growth and proliferation of precarious and informal settlements, known as favelas (UN Habitat, 2013). In 2010, according to the Brazilian Census (IBGE, 2010) 11.4 million people lived in favelas, corresponding to 7% of the urban population.

Excretion of SARS-CoV-2 in the feces has been documented (Xiao et al., 2020; Fumian et al., 2021). Fumian et al. (2021) reported the presence of SARS-CoV-2 in stool samples from 10 patients, in which N1 was present in 100% of the samples with the viral load varying from \( 4.7 \times 10^3 \) to \( 4.7 \times 10^5 \) genomic copies/g of the stool, whilst N2 was present in 50% ranging from \( 2.6 \times 10^3 \) to \( 1.7 \times 10^4 \) genomic copies/g of stool. However, to date, it is not possible to assure that fecal-oral transmission of SARS-CoV-2 is a relevant route. On the other hand, as mentioned before SARS-CoV-2 can be detected in sewage of infected communities during outbreaks and sometimes even before the first clinical cases are confirmed (Prado et al., 2021; Gonzalez et al., 2020; Bowmick et al., 2020). Little research has been carried out on streams and rivers that run through areas in which sanitation is poor, and may provide estimations of the frequency of COVID-19 in a population. Guerrero-Latorre et al. (2020) conducted a study in Quito (Ecuador) that evaluated the levels of SARS-CoV-2 in three urban rivers and found significant concentrations of the virus in these waters.

This study aimed to evaluate the presence of SARS-CoV-2 in a stream that runs through a favela (slum) over a period of 7 months, and to investigate whether it correlates with the occurrence of clinical cases in this community.

2. Methods

2.1. Setting

The study was carried out in “São Remo Community ” located in western region of São Paulo city (Fig. 1). According to a census published in July 2019 the estimated population in the São Remo community is approximately 8000 inhabitants (https://jornal.usp.br/universidade/acoes-para-comunidade/cesso-coleta-dados-sobre-comunidades-proximas-a-usp/#:~:text=Segundo%20o%20Censo%20do%20IBGE,Vila%20Guaraciaba%2C%20cerca%20de%20500). The entire population of São Remo has free primary health care managed by the municipality.

This community is connected to a water supply network and all homes receive treated potable water. However, there is no sewer system. All wastewater generated in São Remo is released into a stream known as “Riacho Doce”, which runs through the community. Ultimately, the water from Riacho Doce ends up untreated in a main river of the São Paulo city, called Rio Pinheiros.

2.2. Water sampling

One liter water samples \((n = 31)\) were collected at 8 a.m. always on Mondays (from May to November 2020) at the same location of “Riacho Doce” in the community (Fig. 2). They were collected according to the Standard Methods for Examination of Water and Wastewater (APHA, Fig. 1. Location of São Remo Community, in the city of São Paulo, state of São Paulo, and Brazil (23°56’.29”S-46°74’.62”W). (Source: Google Earth; March 15, 2021).
Samples were transported to the Laboratory of the Division for Microbiology and Parasitology of CETESB, under refrigeration (20°C to 8°C) and analyzed within a maximum period of 24 h.

2.3. SARS CoV-2 detection and quantification by RT-qPCR

2.3.1. Ultracentrifugation

Briefly, 40 mL of sample was ultracentrifuged (110,000 g for 1 h at 4°C) to pellet all the viral particles together with any suspended material. The sediment was resuspended in 4 mL of 0.25 M glycine buffer, pH 9.5, on ice for 30 min. After the pellet elution, 4 mL of PBS 2x were added and the suspended solids were removed by centrifugation (3000 g for 15 min). The supernatant was transferred to pre-weight tubes and added and the suspended solids were removed by centrifugation (3000 g for 15 min). The supernatant was transferred to pre-weight tubes and ultracentrifuged at 110,000 g for 1 h at 4°C. Then the supernatant was carefully discarded, and the pellet was eluted with the remaining volume of the supernatant solution (approximately 300 μL) before the RNA extraction procedure. To estimate the final sample volume, the tubes were weighed again and the difference between the weights was calculated.

Method recovery was evaluated for each sample, with the addition of 10^3-10^6 gene copies equivalents of Bovine Coronavirus (BCoV) to the water sample. After 1 h of incubation at 4°C, the samples were ultracentrifuged according to the method described in the item 2.3.1.1.

2.3.2. Ultrafiltration - Centricon® Plus-70

Water samples submitted to the SARS-CoV-2 infectivity assay in cell culture were concentrated using the ultrafiltration method with Centricon® Plus-70 10 kDa (Millipore, USA). This procedure was carried out until August 31, while the concentrations of SARS-CoV-2 were in the order of 10^4 CG/L. The method consists of filtering approximately 100 mL of sample through a 0.22 μm PES filter (Millipore). Then, 60 mL of the filtered sample was centrifuged at 3000 g for 20 min. The procedure was repeated until the volume of the concentrated was reduced to approximately 1 mL. The filter was inverted, and the concentrate was recovered after centrifugation (1000 g for 2 min) and treated with 10 mL of 10 mg/mL gentamicin.

2.3.3. RNA extraction and RT-qPCR

RNA was extracted using QIAamp® Viral RNA Mini Kit (QIAGen, Germany) using 140 μL of sample. The final volume of extracted RNA was approximately 80 μL.

Standard quantification curves for N1 and N2 SARS-CoV-2 regions were constructed using serial dilutions of the 2019-nCoV N Positive Control kit (IDT, IA, USA), which consists in 2 × 10^4 to 2 copies/μL of a plasmid cloned with the N1 and N2 sequences of SARS-CoV-2. Primers and probes were also based on CDC protocol (https://www.cdc.gov/coronavirus/2019-ncov/lab/rt-qpcr-panel-primer-probes.html). RT-qPCR assays for SARS-CoV-2 were performed in 20 μL reaction volume containing 5 μL of TaqPath™ 1-Step RT-qPCR Master Mix, CG (ThermoFisher, MA, USA), 1.5 μL of 2019-nCoV_N1 (N1) primer–probe or 2019-nCoV_N2 (N2) primer–probe set (500 nM of primers and 125 nM of probe) (IDT, IA, USA) and 0.5 μL of the extracted RNA. To assess the presence of PCR inhibitors in the RNA samples, an internal positive control (0.8 μL of VetMAX Xeno Internal positive Control – VIC Assay ThermoFisher; 0.1 μL of 10,000 copies/μL of VetMAX Xeno Internal Positive Control RNA Thermo Fisher) was procedure in a duplex reaction with N1 target gene.

All reactions were performed in duplicate. A StepOne Plus (ABI, CA, USA) instrument was used for the RT-qPCR with the thermal cycling conditions as follows: initial incubation at 25°C for 10 min and initial denaturation at 95°C for 30 s, followed by 45 cycles of denaturation at 95°C for 5 s and primer annealing and extension reaction at 60°C for 30 s. A sample was considered positive if the cycle threshold (C_t) was below 40. The detection limit was determined to be around 1000 genomic copies/L. Results were expressed as copies of SARS-CoV-2 RNA (N1 or N2) per liter of water sample.

BCoV RT-qPCR reactions were performed using same conditions as described above with primers and probes described by Decaro et al. (2008). BCoV titers were estimated using standard curves prepared by five 10-fold dilutions (2.5 × 10^5 to 2.5 copies/μL) of 183 bp DNA fragment containing the targeted BCoV (GeneArt, Invitrogen, Thermo Fisher Scientific). To calculate the recovery efficiency of the total method, the number of RNA copies detected in 40 mL of water sample were divided by the number of RNA copies spiked and the results were expressed as a percentage. The mean recovery efficiency was 22.34% (±14.35%).

2.4. Virus identification: RNA extraction, PCR amplification and viral culture

All specimens were handled according to laboratory biosafety guidelines.

Samples were subjected to total nucleic acid extraction with the QIAamp viral RNA kit (QIAGEN, Hilden, Germany), according to the manufacturer instructions. Samples were then subjected to RT-PCR (RealStar® SARS-CoV-2 RT-PCR Kit 1.0, Altona Diagnostics) followed by DNA amplification (Roche LightCycler® 96 System).

Only samples confirmed to be positive by the two different RT-qPCR
assays, performed at Division for Microbiology and Parasitology of CETESB (N1 and N2 target regions) and at the Virology Laboratory at the Institute of Tropical Medicine of São Paulo (E and S genes) were sent for virus isolation.

Viral culture for SARS-CoV-2, conducted in a biosafety level-3 facility, utilized Vero CCL81 cells (ATCC® CCL-81™) in Dulbecco minimal essential medium supplemented with 10% heat inactivated fetal bovine serum and antibiotics/antimycotics. Samples were inoculated into a Vero cell culture in plastic bottles (Jet biofilm, 12,5 cm² area, 25 mL capacity) and incubated in a 37°C incubator in an atmosphere of 5% CO2. Cultures were maintained for at least 2 weeks and observed daily for evidence of cytopathic effects (CPEs). At least two subcultures were performed on each sample. The detection of CPEs was investigated using an inverted microscope (Nikon, Japan) and the presence of virus in supernatants from cultures showing CPEs was determined by specific RT-PCR, as described above. RT-PCR analysis was performed using RNA extracted from culture supernatants obtained two passages after the initial inoculation.

2.5. Data on respiratory infections in São Remo

During the same period, the cases of confirmed COVID-19, and the cases of Severe Acute Respiratory Syndrome (SARS) by any cause, that occurred in people residing in the zip codes referring to São Remo, were evaluated. The data was extracted from the SIVEP-Gripe database (available at: https://opendatasus.saude.gov.br/dataset/bd-srag-2021). The ZIP codes for the cases were obtained from the State of São Paulo notification system.

2.5.1. Data analysis

Based on the notification system from the State of São Paulo, weekly confirmed COVID-19 and Severe Acute Respiratory Syndrome (SARS) cases were combined - for each epidemiological week.

For statistical analysis, we adopted the logarithmic transformation for the concentration of N1 and N2 gene fragments. For cases in which the viral load was equal to the limit of detection, we assigned a value of 1000 genomic copies/L. In cases in which the viral load was below the limit of detection we could not quantify the load a value of 500 genomic copies/L was assigned.

In order to study the relationship between the number of cases and the RNA concentrations, we considered univariate Poisson regression models (Agresti, 1990) for N1 and N2 target regions. The number of SARS and COVID 19 cases was taken as the response variable and the logarithm of N1 and N2 concentrations as independent variables. Goodness-of-fit tests and residual analyses were performed showing that the model fits were adequate (not shown in this paper).

3. Results

From May to November 2020, 31 water samples were collected, on Mondays, and analyzed for the presence of SARS-CoV-2 RNA, of which 29 were positive. Fig. 3 presents the results of RT-qPCR of N1 and N2 concentrations in ultracentrifuged samples and the cases of SARS and COVID-19 in the São Remo community.

Among the 31 samples analyzed, only 1 showed a discordant result between the N1 and N2 assays (only N1 was detected at a concentration of 2.56 × 10^5 CG/L). Furthermore, the results with N1 showed higher concentrations in 64.5% of the samples, demonstrating that, for the US CDC assay, the 2019-nCoV_N1 (N1) primer–probe set was more sensitive than the 2019-nCoV_N2 (N2) primer–probe set in this study. The higher sensitivity of the N1 assay when compared to N2 has already been observed in other studies (Vogels et al., 2020; Pecson et al., 2021), but also with non-significant differences between the concentration values.

There is a visible linear relationship between the number of new disease cases and the logarithm of SARS-CoV-2 RNA concentrations (N1 and N2) found in the water samples (Fig. 4), motivating further analyses based on Poisson regression models.

For the logarithm of N1 and N2 gene fragments concentrations, separated Poisson regression models were fit and showed statistical significance on the relationship with the number of COVID-19 and Severe Acute Respiratory Syndrome (SARS) cases (p < 0.001 for both genes). In addition, for the first model fit, relating the number of cases with the log-concentration of N1, the estimated regression coefficient resulted 0.71 (standard error 0.12). This result can be interpreted in the following way: for an increase of one unit in the log-concentration of N1 the expected number of cases of COVID-19 will be multiplied by e^{0.71} = 2.04. In other words, if we multiply the concentration of N1 by 10 we will end up with an increase of 104% in the expected number of cases, with an associated confidence interval CI (95%) = [62%, 157%]. Similarly, from the model fit relating the number of cases with N2 log-concentrations, the estimated regression coefficient is 0.65 (0.12) meaning that an increase of one unit in the log-concentration of N2 implies the expected number of cases of COVID-19 will be multiplied by e^{0.65} = 1.92; alternatively, it is estimated an increase of 92% in the
expected number of cases of COVID-19 when the concentration of N2 is multiplied by 10 with CI (95%) = [51%, 143%]. Table 1 shows the descriptive statistics for the concentrations of the fragments genes N1 and N2 obtained from water samples. The missing data (Table 1) refers to samples from which the method applied detected these fragments, but the actual value was not registered.

Results of SARS-CoV-2 RT-qPCR and isolation in Vero cells assays are presented in Table 2.

Out of the 13 samples to evaluate viability and infectivity only seven confirmed to be positive by a different RT-qPCR assays (E and S genes). All seven samples were sent for virus isolation. In two samples we observed cytopathic effect (CPE), during the first passage in the Vero cells. However, we were not able to isolate culture-competent virus from any of the seven samples sent for virus isolation, after a second passage in Vero cells.

4. Discussion

During a 31-week serial evaluation of water from a stream running through an underprivileged, underserved, urban settlement (favela or slum), we observed a correlation between counts of SARS-CoV-2 in the community and the number of cases of COVID-19 and of Severe Acute Respiratory Syndrome (SARS) in the community. In this community wastewater runs directly into the stream as there is no sewage collection. Casanova et al. (2009) carried out a study based on surrogates for coronavirus (TGEV – transmissible gastroenteritis coronavirus and MHV – mouse hepatitis) and were able to demonstrate that the virus can remain in water and sewage from days to weeks (nine to 49 days at 25 °C in pasteurized settled sewage and seven to 70 days at 4 °C). According to Gundy et al. (2009) the human coronavirus is less stable remaining from 1.57 to 2.36 days. Studies using seeding of SARS-CoV-2 RNA in order to assess the persistence of this virus in water matrices were carried out by Bivins et al. (2020) and Oliveira et al. (2021), and both studies showed the presence of gene markers of SARS-CoV-2 and, some level of persistence in the environmental samples analyzed. Rimoldi et al. (2020) demonstrated the presence of SARS-CoV-2 RNA (orf)ab, N, E genes) in sewage and rivers but did not find cytopathic effect in none of the samples analyzed. Although virus had been detected in feces (Foladori et al., 2020; Chen et al., 2020), growth in culture is a rare event (Van Doorn et al., 2020; Amirian, 2020; Pumian et al., 2021). Viability of the virus in environmental matrices is still unknown. Studies carried out by several researchers in various countries (Medema et al., 2020; Ahmed et al., 2020; La Rosa et al., 2020; Randazzo et al., 2020; Guerrero-Latorre et al., 2020; Rimoldi et al., 2020) found SARS-CoV-2 in wastewater or river and streams samples but did not report if the virus was viable, which still remains undetermined.

Analyzing the water from a stream contaminated with human waste is probably a different scenario from evaluating sewage, because this natural environment probably receives none or very little of products such as chlorine and other disinfectants or other substances with potential inhibitory activity. Furthermore, it receives high levels of organic material. Both of these conditions may allow the virus to survive for longer periods of time in rivers and streams, which raises the question of whether SARS-CoV-2 in these waters can be a source of infections and thus contribute to the spread of COVID-19. The implications of this in communities such as Sao Remo would be tremendous as fecal-oral spread of COVID-19 would deepen the socioeconomic inequalities already observed for this disease (Costa et al., 2020; Wu et al., 2021). Because of this, we attempted viral culture. We found positive RT-qPCR only after the first passage in Vero cells as well as cytopathic effect. A positive PCR after the first passage in culture may represent viral fragments, other virus such as enterovirus, and not necessarily viable SARS-CoV-2. Thus, we considered a culture positive if PCR were positive after the second passage or later. This did not occur in our samples. As discussed by Jefferson et al. (2020) a positive culture would be the strongest evidence of potential for transmission. Cytopathic effect is considered to be a step below viral culture as evidence of transmission. In our study, 2 samples presented cytopathic effect during the first passage. We believe that this should not be taken into account, as we used extremely contaminated water. Cytopathic effect can be caused by a variety of enteric viruses (Sylvestre et al., 2021) and is not necessarily evidence of viable SARS-CoV-2.

However, the occurrence of this new virus in the environment is used as an early warning for surveillance systems, either national or

Table 1
Descriptive statistics for the measures of number of cases and for N1 and N2 fragments.

|          | Cases | Gene N1 | Gene N2 |
|----------|-------|---------|---------|
| Minimum  | 0     | 0       | 0       |
| Maximum  | 5.00  | 3,280,000 | 2,440,000 |
| Mean     | 1.16  | 223,434 | 197,302 |
| Standard Deviation | 1.24 | 695,280 | 611,874 |
| Median   | 1.00  | 26,900  | 17,450  |
| Missing Data | 0     | 0       | 1       |
Epidemiological status in the community, which may be important in conducting surveillance purposes and public health interventions. The usage of wastewater-based epidemiology can be a challenge for low- and middle-income countries due to most households are not connected to sewer networking (Pandey et al., 2021). Regardless the challenges, when SARS-CoV-2 RNA is detected in impacted water by sewage, may act as a signal that there are a significant number of cases of the disease, which urges immediate intervention measures. Monitoring the occurrence of SARS-CoV-2 RNA in the environment may be helpful as an epidemiological tool for evaluating COVID-19 prevalence in a community.

The main limitations of our study are that cultures were not done for all the water samples. Furthermore, due to the generalized lack of diagnostic resources we believe that COVID-19 cases in the database were not an accurate reflection of the cases in the community. Because of this, we decided to include cases of Severe Acute Respiratory Syndrome (SARS), to account for subnotification of COVID-19. Still, it is possible that mild cases of COVID-19 were missed.

Communities such as Sao Remo are common throughout the world. Although Sao Remo has slightly better conditions than many similar communities, with 2–3 persons per home, brick housing, electricity, and potable running water, our findings may be widely applicable.

5. Conclusion

Our results showed a correlation between cases of COVID-19 and SARS in an underprivileged community, and SARS-CoV-2 concentrations in the water of a stream running through this community. The differences in concentration of the virus in the environment reflect the epidemiological status in the community, which may be important information for tracking and preventing dissemination of the virus in areas with vulnerable population and poor sanitation. We did not find viable viral particles.

Contributors

MTPR and ASL: Conceptualization, writing, samples collection, literature search; MRFB: Analytical procedure for virus concentration (qPCR), review and editing; MCM-C, AVP and LSV-B: Analytical procedures for virus viability; MD, RSA, IFO, SCG: Analytical procedure for virus concentration (qPCR); SFC, DBA and ECS: review and editing; BSL and ACPL: Data analysis and writing.

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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