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Short communication

Metropolitan wastewater analysis for COVID-19 epidemiological surveillance

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ABSTRACT

The COVID-19 disease, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is a rapidly emerging pandemic which has enforced extreme containment measures worldwide. In the absence of a vaccine or efficient treatment, cost-effective epidemiological surveillance strategies are urgently needed. Here, we have used RT-qPCR for SARS-CoV-2 detection in a series of longitudinal metropolitan wastewaters samples collected from February to April 2020, during the earliest stages of the epidemic in the Region of Valencia, Spain. We were able to consistently detect SARS-CoV-2 RNA in samples taken in late February, when communicated cases in that region were only incipient. We also find that the wastewater viral RNA context increased rapidly and anticipated the subsequent ascent in the number of declared cases. Our results strongly suggest that the virus was undergoing community transmission earlier than previously believed, and suggest that wastewater analysis could be sensitive and cost-effective strategy for COVID-19 epidemiological surveillance. Routine implementation of this surveillance tool would significantly improve our preparedness against new or re-occurring viral outbreaks.

1. Introduction

The emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in Wuhan, China, has rapidly led to a pandemic scenario, with over 12 million COVID-19 confirmed cases globally as of July 9, 2020. COVID-19 symptoms are varied and often non-specific, including fever, cough, and diarrhea, among others. A non-negligible percentage of infected people develop pneumonia, which can subsequently lead to severe respiratory distress requiring mechanical ventilation, organ failure, viral sepsis, and death (Li et al., 2020). The widespread nature of the pandemics and the lack of easy symptom-based diagnosis, treatment, or vaccine has enforced drastic and extremely costly epidemiological control measures including worldwide lockdowns. Whereas massive RT-qPCR testing campaigns are being deployed in many countries to assess the actual prevalence of the virus, this is not a feasible surveillance strategy for the general population over the long term.

At the time this study was completed (May 13, 2020), Spain stood as the second most extensively affected country worldwide with over 228,000 confirmed COVID-19 infections and more than 27,000 deaths. The first three confirmed cases in the Iberian Peninsula were communicated on February 25, 2020 in Madrid, Barcelona, and Villareal, a small town nearby the city of Valencia. Furthermore, a retrospective analysis carried out in March showed that the first death in Spain from COVID-19 actually occurred on February 13 in Valencia. Therefore, the Valencian Region constituted the earliest known COVID-19 spot in Spain (Ministerio de Sanidad, Consumo y Bienestar Social - Professionals - Enfermedad por nuevo coronavirus, COVID-19). However, at the time, it was assumed that no community transmission was ongoing and, as a result, major containment measures were not enforced until March 15.

Setting up feasible and reliable methods for SARS-CoV-2 epidemiological surveillance is of utmost importance to successfully combat this pandemic virus and improve our preparedness in the event of viral re-emergences. Although SARS-CoV-2 is primarily a respiratory, airborne virus, previous studies with the related SARS-CoV-1 (the causative agent

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of the 2003 SARS outbreak) suggested the possibility of fecal-oral transmission based on detection of viral RNA by RT-qPCR in the stools of patients (Duan et al., 2003). Recent studies indicate that SARS-CoV-2 can be also excreted in feces and urine in asymptomatic carriers and in recently recovered patients (Cheung et al., 2020; Lescure et al., 2020; Lo et al., 2020; Sun et al., 2020; Wang et al., 2020; Wu et al., 2020; Xing et al., 2020; Zhang et al., 2020). Specifically, viral RNA was detected in feces up to 10 days after viral clearance from the respiratory tract, regardless of disease severity (Chen et al., 2020).

This implies that wastewaters may contain viral particles or viral RNA that could be used as an epidemiological surveillance tool. Wastewaters can also collect viruses present in the oral cavity and upper respiratory tract that are shed during personal hygiene. Compared to systematic testing of individuals, wastewater analysis is obviously less invasive, simpler and cheaper, but the sensitivity and reliability of this method remains to be shown. Previous work has established similar methods for the epidemiological surveillance of enteric viruses including norovirus, rotavirus (Santiso-Bellón et al., 2020), hepatitis E virus (Cuevas-Ferrando et al., 2020), influenza, and poliovirus (Heijnen and Medema, 2011; Hellmér et al., 2014; Hovi et al., 2012), and recent publications (Ahmed et al., 2020; Bivins et al., 2020; Haramoto et al., 2020; La Rosa et al., 2020; Lodder and de Roda Husman, 2020; Medema et al., 2020; Randazzo et al., 2020) suggest that COVID-19 detection in sewage is technically feasible, based on preliminary results obtained from a limited number of samples in China, Australia, the Netherlands, Italy, Japan, and USA. Here, we have analyzed sewage water collected in the Valencian region from February to April 2020 to assess our ability to detect the virus during the earliest stages of an outbreak.

2. Materials and methods

2.1. Wastewater sampling

Samples of metropolitan wastewater from Valencia (Spain) were taken at different time points in a two-month longitudinal study spanning from February 12 to April 14, 2020. Specifically, samples were taken from wastewater treatment plants Pinedo 1, Pinedo 2 and Quart-Benàger, all belonging to the Empresa Pública de Saneamiento de Aguas Residuales (Generalitat Valenciana). Grab samples of 200 mL of sewage water were collected in the morning, between 10 a.m. and 12 a.m. in all cases. Some samples were collected before and after wastewater treatment, and all samples were kept at 4 °C until analysis. Previous studies have shown that SARS-CoV-2 infectivity is stable at 4 °C for at least 14 days (Chin et al., 2020), although little is known about RNA stability. Since our earliest samples remained stored at 4 °C for almost two months, we cannot discard viral RNA degradation over time. However, this would lead to underestimation of the viral RNA present in these samples, which ultimately would strengthen our conclusion that RT-qPCR wastewater analysis allows early detection of disease outbreaks.

2.2. Sample processing and RNA extraction

Viral concentration was carried out by aluminum-driven flocculation. For this, 200 mL water samples were adjusted to pH 6.0 and an Al $(OH)_3$ precipitate was formed by adding 1:100 v:v of 0.9 N $AlCl_3$ solution. After pH readjustment to 6.0, samples were agitated slowly for 15 min at room temperature. Precipitates were collected by centrifugation at 1700×*g* for 20 min. Pellets were resuspended into 10 mL of 3% beef extract (pH 7.4), and samples were shaken for 10 min at 150 rpm (Randazzo et al., 2019). A concentrate was then formed by centrifugation at 1900×*g* for 30 min and the pellet resuspended in 1 mL of PBS ("9510 detection of enteric viruses (2017)," 2018). As a process control, samples were spiked with 10^5 PCR units of mengovirus vMC0 (CECT 100000) following a protocol similar to ISO 15216–2:2017 used in food products. This procedure has been previously validated for non-enveloped viruses (Cuevas-Ferrando et al., 2020; Randazzo et al., 2019) and more recently with a porcine epidemic diarrhea virus, an enveloped virus member of the *Coronaviridae* family used as a SARS-CoV-2 surrogate (Randazzo et al., 2020). RNA extraction was performed using the Nucleospin RNA virus Kit (Macherey-Nagel) following the recommended protocols and using a Plant RNA Isolation Aid (Ambion) pre-treatment (Cuevas-Ferrando et al., 2020).

2.3. SARS-CoV-2 RT-qPCR

The presence of SARS-CoV-2 was determined using the PrimeScript™ One Step RT-PCR Kit and the RT-qPCR diagnostic panel assays validated by the US Centers for Disease Control and Prevention (2019-nCoV RUO Kit) using the positive control (2019-nCoV_N_Positive Control) provided by IDT (Integrated DNA Technologies). The RT-qPCR was carried out following the manufacturer's instructions, recommended standards, and positive controls in a LightCycler 480 (Roche Diagnostics) instrument. Each RNA extract was analyzed in duplicate. Undiluted and tenfold diluted RNA extracts were analyzed for mengovirus to account for the presence of RT-qPCRs inhibitors. A calibration curve was performed using the 2019-nCoV_N_Positive Control provided by IDT. For each RT-qPCR run, a series of three positive and negative controls (extraction and PCR) were included. Cycle threshold (Ct) values were used to calculate gc/L in the original sample. Ct values lower than 40 were considered positive for SARS-CoV-2, as proposed previously (Wang et al., 2020). In all cases, the internal mengovirus control showed recovery rates >1%, an acceptable value according to previous work (Haramoto et al., 2018).

2.4. Epidemiological data

The sewage treatment plants under study collected wastewaters from approximately 1,200,000 inhabitants in 22 townships of the Valencian metropolitan area (Alaquas, Albal, Alcasser, Aldaia, Alfafar, Benetusser, Beniparell, Burjassot, Catarroja, Llocnou de la Corona, Manises, Massanassa, Mislata, Paiporta, Picanya, Picassent, Quart de Poblet, Sedavi, Silla, Torrent, Valencia, and Xirivella). The number of declared active cases per day for the Region of Valencia was obtained from Conselleria de Sanidad Universal y Salud Pública (Generalitat Valenciana).

3. Results and discussion

Here, we show that SARS-CoV-2 can be reproducibly detected by RT-qPCR in longitudinal samples from sewage treatment plants that receive wastewaters from over one million inhabitants in the metropolitan area of Valencia, Spain. We analyzed 15 samples taken between February 12 and April 14, 2020 at three wastewater treatment plants. Following concentration of viral content by flocculation, a standard RT-qPCR procedure allowed us to detect SARS-CoV-2 RNA in 12/12 samples collected from March 9 to April 14, 2020, with quantification cycles (Ct) values ranging between 34.00 and 37.84, correspondingly revealing between 5.22 and 5.99 $\log_{10}$ genomic copies (gc)/L (Table 1). Briefly, SARS-CoV-2 RNA was quantified as gc by plotting the Ct to an external standard curve built with 10-fold serial dilution of a quantified plasmid control (IDT). Calibration curves for N1 ($\log_{10}$ gc/L = −3.3774 Ct + 41.515, r^2 = 0.95), and N2 ($\log_{10}$ gc/L = −3.7752 Ct + 43.951, r^2 = 0.989), showed a linear dynamic range between 50 and 5 × 10^4. The limit of detection (LOD) was thus 50 gc per reaction, with Ct values of 37.05 ± 0.77 and 38.12 ± 0.24 for N1 and N2, respectively. The theoretical quantitation limits were 4.45 and 4.91 $\log_{10}$ gc/L for N1 and N2, respectively. SARS-CoV-2 RNA was not detected in a single sample from February 12, but was detected in one of the two samples collected in February 24 for RT-qPCR region N2, whereas region N1 remained negative for this time point. Comparison of RT-qPCR primers and probes for SARS-CoV-2 diagnostics suggests that N1 and N2 primers used here are efficient for SARS-CoV-2 RNA detection (Corman et al., 2020; Nalla

Table 1
Detection of SARS-CoV-2 RNA by RT-qPCR in untreated wastewater samples from three treatment plants in the metropolitan region of Valencia, Spain.

Sampling date (2020)	Water treatment plant	Recovery control (%)	Ct (N1)[a]	Log_{10} gc/L ± SD (N1)[a]	Ct (N2)[b]	Log_{10} gc/L ± SD (N2)[b]
February 12	Quart-Benàger	2.56	nd[c]	nd[c]	nd[c]	nd[c]
February 24	Pinedo 1	4.71	nd[c]	nd[c]	nd[c]	nd[c]
	Pinedo 2	7.34	nd[c]	nd[c]	37.84[d]	5.22[d]
March 9	Pinedo 1	5.40	35.16[d]	5.4[d]	36.88[d]	5.45[d]
	Pinedo 2	3.26	34.66, 35.89	5.37	35.55[d]	5.77[d]
March 11	Quart-Benàger	6.31	34.48, 34.80	5.56	36.95, 36.95	5.43
April 6	Pinedo 1	7.16	34.68, 34.81	5.53	34.71, 34.69	5.98
	Pinedo 2	4.51	34.81, 34.53	5.55	36.01, 36.94	5.55
	Quart-Benàger	2.60	35.19, 33.87	5.59	35.12, 34.17	5.99
April 9	Pinedo 1	12.07	36.02, 34.73	5.34	35.55, 36.66	5.64
	Pinedo 2	18.78	34.37, 35.48	5.47	35.91, 35.51	5.73
April 11	Quart-Benàger	11.17	34.58, 34.31	5.62	34.69, 35.52	5.88
April 13	Quart-Benàger	13.30	33.92, 34.08	5.75	35.12, 34.66	5.93
April 14	Pinedo 1	14.52	35.50, 34.79	5.41	35.82[d]	5.71[d]
	Pinedo 2	10.54	35.76, 35.2	5.31	35.12, 36.09	5.76

[a] PCR region encompassing 2019-nCoV_N1 Combined Primer/Probe Mix.
[b] PCR region encompassing 2019-nCoV_N2 Combined Primer/Probe Mix.
[c] Not detected (Ct > 40).
[d] Based on a single technical replicate.

et al., 2020). The recovery efficiency of the virus concentration method was quantitated including a mengovirus internal control and yielded values ranging between 2.56 and 18.78% in all samples (Table 1). The presence of inhibitors was checked in all the samples. No significant inhibition was detected for any of the samples based on mengovirus Ct values. This approach was not feasible for SARS-CoV-2 given the high Ct values for both molecular targets (N1 and N2). Hence, samples from February 24 provide the earliest piece of evidence that the virus was circulating in the community. Interestingly, we consistently detected SARS-CoV-2 RNA in samples collected on March 9 and March 11, when only 50 and 76 cumulative cases were declared in the entire Region of Valencia. This validates wastewater RT-qPCR analysis as a sensitive and reliable technique for early detection of SARS-CoV-2 outbreaks as earlier shown for the Murcia region (Spain) (Randazzo et al., 2020).

For each of the samples collected in April, we also analyzed treated wastewaters, which are routinely discharged to the sea or used for irrigation purposes. We found no evidence of viral RNA in 9/9 samples. These samples provide an additional negative control for our RT-qPCR analysis, reinforcing the conclusion that the signal obtained in untreated waters unlikely corresponds to background signal or non-specific DNA amplification. Importantly, these results confirm that current wastewater treatment procedures efficiently clear the virus.

In conclusion, our results show that the virus was probably undergoing local community transmission by the time the very first cases were declared in the Region of Valencia (Fig. 1). This contrasts with the previously accepted view that, in late February and early March, essentially all COVID-19 cases in Spain were imported or directly traceable through contacts, and that there was no ongoing community transmission at the time. We also find that the RT-qPCR signal in wastewaters increased and reached a plateau faster than declared cases. These results strongly suggest that analysis of wastewaters by RT-qPCR

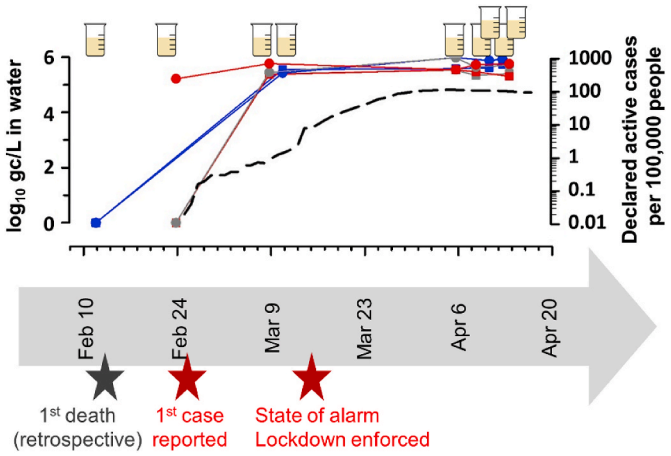

Fig. 1. Series of events and SARS-CoV-2 epidemiological follow-up by wastewater RT-qPCR analysis. Left axis: Mean viral load values as inferred by RT-qPCR. For each sampling time point (indicated with beakers), log viral load data shown on Table 1 are plotted. Treatment plants (colors: blue: Quart-Benàger, grey: Pinedo 1, red: Pinedo 2) and PCR regions (squares: N1, circles: N2) are indicated. Samples with undetectable RNA levels were arbitrarily assigned a log viral load of zero. Right axis: number of declared active cases in the Region of Valencia per 100,000 people (dashed black line). Bottom: series of events related to the COVID-19 pandemics in the Valencian Region. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

analysis is an efficient strategy for the epidemiological surveillance of COVID-19.

Extreme lockdown measures are currently allowing Spain and other countries to partially mitigate SARS-CoV-2 spread and may help us reduce disease prevalence within the next weeks or months. However, inevitable relaxation of current containment measures may lead to recurring local outbreaks or case imports from other regions. As such, it is of extreme importance to set up feasible and reliable epidemiological surveillance strategies that will improve our preparedness in the event of future viral re-emergencies.

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The funding sources had no rule in the study design, data collection, analysis of data, writing, or in the decision to publish.

Author contributions

W.R. performed the experiments and analyzed data; E.C–F. performed the experiments; R.S. analyzed data and co-wrote manuscript; P. D-C. conceived study, obtained the samples, analyzed data, and co-wrote manuscript; G.S. conceived study and supervised work. All authors have read and agreed to the published version of the manuscript.

Declaration of interests

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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