Detection of environmental SARS-CoV-2 RNA in a high prevalence setting in Spain

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Abstract

Since March 2020, Spain (along with many other countries) has been severely affected by the ongoing coronavirus disease 19 (COVID-19) pandemic caused by the rapid spread of a new virus (severe acute respiratory syndrome coronavirus 2; SARS-CoV-2). As part of global efforts to improve disease surveillance, we investigated how readily SARS-CoV-2 RNA could be detected in environmental samples collected from an isolated rural community in Spain with a high COVID-19 prevalence (6% of the population of 883 inhabitants). The first diagnosis of COVID-19-compatible symptoms in the village was recorded on March 3, 2020 and the last known active case resolved on June 5, 2020. By May 15, two months after strict movement constraints were imposed ("lockdown") the cumulative number of symptomatic cases had increased to 53. Of those cases, 22 (41%) had been tested and confirmed by RT-PCR. On May 13 and June 5, samples were collected from high-use surfaces and clothes in the homes of 13 confirmed cases, from surfaces in nine public service sites (e.g. supermarket and petrol station), and from the wastewater of the village sewage system. SARS-CoV-2 RNA was detected in 7 of 57 (12%) samples, including three households and three public sites. While there is not yet sufficient evidence to recommend environmental surveillance as a standard approach for COVID-19 surveillance, environmental surveillance research may contribute to advance knowledge about COVID-19 by further elucidating virus shedding dynamics and environmental contamination, including the potential identification of animal reservoirs.

Keywords: COVID-19; Environmental pathogen monitoring; Risk hotspot identification; Rural Spain; SARS-CoV-2

Introduction

Coronavirus disease 19 (COVID-19) has spread globally during early 2020. By July 28, over 16 million COVID-19 cases had been reported from 188 countries, causing more than 654,000 deaths worldwide, including 286,718 confirmed cases and 28,432 officially recorded fatalities in Spain (https://coronavirus.jhu.edu/map.html; last access 28/07/2020). The responses to this unprecedented challenge include travel bans, social distancing, and even stay-at-home ('lockdown') orders (Pung et al., 2020). These responses caused drastic changes in human behavior resulting in severe effects on the economy and many other areas (Gortazar & de la Fuente, 2020).

The causative agent of COVID-19 is a recently emergent zoonotic coronavirus officially named the Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) (Ward et al., 2020). This virus is transmitted not only by aerosols, but also indirectly through contaminated objects and surfaces, including human skin, on which it can survive for hours or days (Wakida et al., 2020; Eslami & Jalili, 2020; Ren et al., 2020; Kampf et al., 2020; Yan et al., 2020). The detection of RNA from SARS-CoV-2 in surfaces from supermarket trolleys, doorknobs, or garbage container handles, as well as the body surfaces or clothing of infected people, indicates the presence of potentially viable infective virus particles (Van Doremalen et al., 2020). Moreover, SARS-CoV-2 RNA has also been detected in wastewater (Rimoldi et al., 2020).

In Spain, a recent serosurvey showed a 5% average antibody prevalence in the national population, with up to 11% prevalence in the most affected provinces (Pollán et al., 2020). These results strongly suggest that the antibody prevalence in Spanish population is still well below the threshold believed to be required for herd immunity, which in turn suggests that the COVID-19 epidemic could potentially continue for many months or

even years if not controlled. As the number of confirmed cases in Spain is well below 5% of the population, the serosurvey results also imply that there have been many more cases of infection than those detected by PCR and officially recorded, indicating an urgent need for greater diagnostic effort.

Unfortunately, testing is often limited to severe symptomatic cases, and contact tracing was not yet in place in some Spanish regions. As of May 16, 2020, the regional health authority was still in the process of recruiting and training 400 healthcare workers for contact tracing of known COVID-19 cases. Efforts to control the spread of SARS-CoV-2 are therefore hampered by incomplete information on where and when the virus is present. Pathogen nucleic acids can be sampled in the environment for detection and monitoring purposes (Martínez-Guijosa et al., 2020). Environmental RNA surveillance could therefore contribute to improved spatial-temporal assessment of COVID-19 risk by monitoring suspected contaminated environments such as shopping malls, health centers, nursing homes, or households of people who have passed COVID-19. We hypothesized that nucleic acids of SARS-CoV-2 would be detectable in sites with known recent virus circulation. If true, environmental RNA sampling could contribute to monitoring of virus circulation, thereby identifying targets for a more efficient COVID-19 control.

Material and Methods

Study site

The village of Horcajo de los Montes (883 inhabitants in 2019; 4.6/km²) is part of the Ciudad Real province in Castilla – La Mancha (CLM), southern Spain. It is about 80 km away from the provincial capital, Ciudad Real, and the Hospital General Universitario Ciudad Real (HGUCR). As most villages in rural Spain, the population is steadily declining (10% loss in the last decade) and ageing (59% >65 years). Before lockdown,

Ciudad Real was one of the Spanish provinces with the highest human movement to and from Madrid (180 km from the study village), so had a high risk of SARS-CoV-2 introduction at the onset of the COVID-19 epidemic in Spain (Mazzoli et al., 2020). The first COVID-19 case recorded by the Horcajo de los Montes local medical services was diagnosed on March 3, 2020, 12 days before lockdown was imposed throughout Spain on March 15, 2020. By May 16, 2020, the cumulative number of symptomatic cases had reached 53 (6%; 3 cases remaining active), of which 22 (41%) had been confirmed by RT-PCR as SARS-CoV-2 infected. The last active case of the outbreak resolved on June 5, 2020.

Efforts to control COVID-19 spread in the village included not only the national lockdown and associated measures (home confinement or hospitalization of all known symptomatic cases, personal hygiene measures such as frequent handwashing, hand and household disinfection, and facemask use), but also, of particular relevance to this study, hypochlorite disinfections of public spaces. These disinfections by village municipality and a firm hired by the CLM authorities, started with disinfection of streets on March 14 and of public service sites on March 22. According to municipal records, disinfection with sprayed 2% hypochlorite took place 1 to 3 times weekly and included the exteriors of the medical center (12 times; occasionally including the inside), pharmacy (3 times, outside only), petrol station (7 times, outside only), and supermarket (8 times, outside only). The community spontaneously organized assistance for home-confined COVID-19 suspects, including food delivery, medicine delivery, cleaning service and medical assistance to avoid unnecessary movements, and requested police assistance to enforce home-confinement where needed.

Data sources and field sampling

We did not use individual patient data. From March 1, the local physician recorded all suspected COVID-19 cases along with the official testing results. On May 13, 2020, we sampled surfaces and clothing in 10 households (2 with PCR-confirmed active cases; 6 with PCR-confirmed recovered cases; 2 with not PCR-tested recovered cases). We also sampled 6 public service sites, and in addition the wastewater from the village sewage system. Environmental RNA sampling was repeated on June 5, 2020, re-visiting 9 of the previous sampling sites (4 households and 5 public service sites) and adding 6 new ones (3 households and 3 public service sites). The samples were tested for SARS-CoV-2 RNA.

Samples were collected using Dry sponges (3M™ Dry-Sponge; 3M-España, Madrid). These sponges were pre-hydrated with 15 ml of an isotonic surfactant and virus-inactivating liquid (patent pending) that allows to collect nucleic acids from surfaces and other substrates (Martínez-Guijosa et al., 2020). On each site visited, one to four sponges were gently rubbed over surfaces in contact with people's hands or gloves (Environment, E) and or over the gloves and clothing of the persons present (Clothing, C). Sample sites in households always included the toothpaste tube(s), fridge and oven handles, and the main door handle. Sampling sites in public service areas included surfaces such as keyboards, tables, chairs, refrigerators, and entry door handles. For wastewater sampling, 5 ml of liquid collected from the village's main sewage drain were mixed with an equivalent volume of the liquid used in the sponges. The collected samples were refrigerated until processed in the laboratory.

The data that support the findings of this study are available from the corresponding author upon request.

Laboratory procedures

Once in the laboratory, 2 ml of retained fluid was extracted from each sponge sample, collected in a screw cap tube and centrifuged at 12,000 x g for 10 min. Viral RNA was extracted from 200 µl of solution taken from the bottom of the tube using the NucleoSpin RNA Virus kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Detection of SARS-CoV-2 RNA was then performed by real-time RT-PCR targeting the envelop protein (E)-encoding gene and two targets (IP2 and IP4) of RNA-dependent RNA polymerase gene (RdRp), according to protocols included in the WHO guidelines (https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical-guidance/laboratory-guidance; last access June 1, 2020) (Corman et al., 2020; Grenga et al., 2020). Primer sets used are detailed in Table 1. The positive control for real-time RT-PCR is an *in vitro* transcribed RNA derived from the strain BetaCoV_Wuhan_WIV04_2019 (EPI_ISL_402124), loaned by the Pasteur Institute (Paris, France). Nuclease free water was used as negative control. Real-time RT-PCR was carried out using the SuperScript III Platinum One-Step qRT-PCR Kit (ThermoFisher, Massachusetts, USA), according to manufacturer's protocol. A CFX96 Touch Real-Time PCR Detection System Thermal Cycler (BioRad, Berkeley, USA) was used to carry out the reactions.

Results

In the first sampling event (May 13, 2020) we detected SARS-CoV-2 RNA on clothing in both of the households with known active cases and from a surface in one of the six households with recovered older PCR-confirmed cases (Table 2). SARS-CoV-2 RNA was also detected on surfaces in two of the six public service sites, the petrol station and the pharmacy, but virus RNA was not detected in the two wastewater samples. In the second sampling event (June 5, 2020), we detected SARS-CoV-2 RNA on clothing in one of seven households with recovered older PCR-confirmed cases (one of the positive

ones during the first sampling event) and in one public service site (City hall). There was no difference in SARS-CoV-2 RNA detection between sampling events (Fisher's test, p=1). Overall, 7 (12.28%) of the 57 samples and 6 (26%) of the sites surveyed were positive for SARS-CoV-2 RNA in at least two of the three RT-PCR reactions performed, and all samples were positive for the SARS-CoV-2-specific RdRP-IP4 and RdRP-IP2 PCRs targeting the coronavirus RNA-dependent RNA polymerase.

Although medical records indicate that there were few active cases present in the village at the time of sampling (possibly only three during the first sampling event and none in the second), our environmental RNA sampling indicated that SARS-CoV-2 RNA was not only present in the houses of active cases but in other households with confirmed older cases and in three of nine high-use public service sites.

Discussion

This small and relatively isolated village suffered a substantial COVID-19 outbreak between early March and mid-May 2020. Efforts to reduce the prevalence and spread of COVID-19 in the village succeeded in driving the number of known active cases to low levels by May 15, 2020. However, medical records indicated a very high likelihood that viable SARS-CoV-2 virus was still present and circulating 2.5 months after the first case was recorded.

Only three samples collected during the first sampling event in May tested positive for all three PCRs, and all Ct values were above 34 (Table 2). High Ct values could be indicative of a partially degraded RNA or a low viral load (Matson et al., 2020; Petrillo et al. 2020). Most research on environmental RNA does not include virus isolation attempts, since this requires biosafety level 3 facilities that are not widely available. Laboratory experiments with spiked samples have shown that SARS-CoV-2 can be stable in a favorable environment and can be isolated in appropriate cell cultures (Chin

et al. 2020). However, recent field studies reporting SARS-CoV-2 RNA detection from environmental samples and attempting virus isolation either failed to induce a cytopathic effect (Colaneri et al. 2020) or found only weak signals for the presence of replication competent virus (Santarpia et al. 2020).

While we do not know whether the RNA we detected at those sites was from live, potentially infectious viruses, the detection of SARS-CoV-2 RNA not only in the active- and recovered-case houses but at three of nine public service sites strongly suggested the possibility that live virus was still circulating outside the houses of active cases. This possibility suggests that disinfection activities should be continued and expanded to include the insides of all the public services as was already being done in the medical center, which tested negative despite of being a high-risk site. Households should receive additional information on good disinfection practices.

It is important to balance the societal controls required to minimize human-to-human transmission of COVID-19 against the need to minimize the resulting social disruption and adverse economic impact. While the WHO considers that there is not yet sufficient evidence to recommend environmental surveillance as a standard approach for COVID-19 surveillance, WHO also states that environmental surveillance research should be seen as an important public health objective to advance knowledge about COVID-19 since it could further elucidate virus shedding dynamics (Wu, et al. 2020) and has the potential to detect SARS-CoV-2 shedding from animal sources, such as animal production facilities and wet markets; potentially supporting identification of any animal reservoirs (https://apps.who.int/iris/bitstream/handle/10665/333670/WHO-2019-nCoV-Sci_Brief-EnvironmentalSampling-2020.1-eng.pdf; last access June 16, 2020).

Contributors

FR, IG and CG planned the study. Field data and samples were collected by FR, DH and CG. MD, LD, MP and IM performed laboratory procedures for environmental sampling. IG and JF performed and interpreted the RT-PCR testing. Data analysis was led by CG, IG and FR. All authors interpreted the study findings, contributed to the manuscript, and approved the final version for publication.

Declaration of interests

We declare no competing interests.

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Ethical approval

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as this study was based on environmental RNA sampling. We used no individual patient data and performed no animal sampling. The corresponding authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

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Data availability: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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329 **Table 1.-** Primer sequences and amplified fragment sizes in base pairs.

Primer target	Sequence 5'-3'	PCR fragment size
Gene RdRp / nCoV_IP2		
nCoV_IP2-12669Fw	ATGAGCTTAGTCCTGTTG	108 bp
nCoV_IP2-12759Rv	CTCCCTTTGTTGTGTTGT	
nCoV_IP2-12696b Probe(+)	AGATGTCTTGTGCTGCCGGTA [5']Hex [3']BHQ-1	
Gene RdRp / nCoV_IP4		
nCoV_IP4-14059Fw	GGTAACTGGTATGATTTCG	107 bp
nCoV_IP4-14146Rv	CTGGTCAAGGTTAATATAGG	
nCoV_IP4-14084 Probe(+)	TCATACAAACCACGCCAGG [5']Fam [3']BHQ-1	
Gene E / E_Sarbeco		
E_Sarbeco_F1	ACAGGTACGTTAATAGTTAATA GCGT	125 bp
E_Sarbeco_R2	ATATTGCAGCAGTACGCACACA	
E_Sarbeco_P1	ACACTAGCCATCCTTACTGCGCT TCG [5']Fam [3']BHQ-1	

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Table 2. Presence or absence of SARS-CoV-2 RNA in environmental samples.

Sampling site	Samples taken	RT-PCR results (Ct values for +)				Remarks
		RdRP-IP4	RdRP-IP2	Egene	Interpretation	
Medical center (May)	E, 2C	-	-	-	Negative	
Medical center (June)	E, 2C	-	-	-	Negative	
Pharmacy (May)	E	+36.55	+37.73	-	Positive	E positive
Pharmacy (June)	E	-	-	-	Negative	
Postal office (May)	E	-	-	-	Negative	
Postal office (June)	E	-	-	-	Negative	
Petrol station (May)	E	+38.37	+37.32	-	Positive	E positive
Petrol station (June)	E	-	-	-	Negative	
Supermarket (May)	E	-	-	-	Negative	
Supermarket (June)	E	-	-	-	Negative	
Police (May)	2C	-	-	-	Negative	
City hall (June)	E	+38.59	+39.1	-	Positive	E positive
Bar/restaurant (June)	E	-	-	-	Negative	
Vending shop (June)	E	-	-	-	Negative	
Household 1 (May*)	E, C	+37.71	+36.05	+38.27	Positive	C positive
Household 1 (June**)	E, C	-	-	-	Negative	
Household 2 (May**)	E, 2C	-	-	-	Negative	
Household 2 (June**)	E, C	-	-	-	Negative	
Household 3 (May**)	C	-	-	-	Negative	
Household 4 (May)	C	-	-	-	Negative	
Household 5 (May**)	E, C	-	-	-	Negative	
Household 6 (May**)	E, 3C	+38.26	+34.53	+39.15	Positive	E positive
Household 6 (June**)	E, 2C	+36.11	+41.06	-	Positive	C positive
Household 7 (May**)	E, 2C	-	-	+	Negative	
Household 8 (May**)	E, C	-	-	-	Negative	
Household 9 (May)	C	-	-	-	Negative	
Household 10 (May*)	E, C	+37.26	+37.22	+36.41	Positive	C positive
Household 10 (June**)	E, C	+	-	+	Negative	
Household 11 (June**)	E, C	-	-	+	Negative	
Household 12 (June**)	E, C	-	-	-	Negative	
Household 13 (June**)	E, C	-	-	-	Negative	
Wastewater (May)	2x5ml	-	-	-	Negative	
Total 23 sites	57 samples				7 positive samples; 6 sites	

Columns show the RT-PCR results for 23 sites or substrates where the environment (E) or gloves and clothing (C) were sampled for SARS-CoV-2 RNA in a rural village in Ciudad Real province, Spain, during the first COVID-19 outbreak. (*) indicates households with active cases on May 13, 2020; (**) indicates households with confirmed older cases.