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SARS-CoV-2 detection in sewage samples: Standardization of method & preliminary observations

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Background & objectives: Since its first recognition in Wuhan, China, in December 2019, the SARS-CoV-2 has spread rapidly across the world. Though SARS-CoV-2 spreads mainly *via* the droplets of respiratory secretions, it was also detected in stool samples of patients, indicating active infection of the gastrointestinal tract. Presence of SARS-CoV-2 RNA in sewage samples was reported in February 2020, raising the possibility of using environmental water surveillance to monitor SARS-CoV-2 activity in infected areas. The aim of this study was to standardize the methodology for detection of SARS-CoV-2 from sewage and explore the feasibility of establishing supplementary surveillance for COVID-19.

Methods: Sewage specimens were collected from six sites in Mumbai, India, using the grab sample method and processed using polyethylene glycol (PEG)-dextran phase separation method for virus concentration. Real-time reverse transcription-polymerase chain reaction (RT-PCR) assay was used to detect the presence of SARS-CoV-2 RNA.

Results: A total of 20 sewage samples collected from six different wards in Mumbai city, before the spread of SARS-CoV-2 infections and during May 11-22, 2020, were processed using the phase separation method. The WHO two-phase PEG-dextran method was modified during standardization. SARS-CoV-2 was found to concentrate in the middle phase only. All samples collected before March 16, 2020 were SARS-CoV-2 negative. Viral RNA was detected in sewage samples collected during the ongoing COVID-19 pandemic in all the six wards.

Interpretation & conclusions: PEG-dextran phase separation method was effectively used to concentrate SARS-CoV-2 from domestic waste waters to detection levels. It would be feasible to initiate sewage surveillance for SARS-CoV-2 to generate data about the viral transmission in various epidemiologic settings.

Key words COVID-19 - RT-PCR - SARS-CoV-2 - sewage - two-phase separation

The World Health Organization (WHO) declared COVID-19 a global pandemic on March 11, 2020[1].

The virus spread quickly across countries due to its respiratory route of spread and also the presence

of susceptible population globally[2]. The unique feature of SARS-CoV-2 infection is the prolonged shedding of virus in stools, with viral load being high in stool samples[3]. While the major focus remains on containment zones, it is critical to ensure adequate surveillance in apparently low-transmission zones to monitor the disease trends. Such surveillance includes serological assays to understand the seroprevalence of infection and in turn the disease transmission dynamics. However, because prolonged shedding of SARS-CoV-2 RNA has been reported in stool samples of both symptomatic and asymptomatic infected individuals[3], sewage surveillance seems to be a useful tool for monitoring the trend of COVID-19 disease in high- as well as low-transmission zones. Sewage surveillance has been used successfully as a tool to monitor the shedding of SARS-CoV-2 in stool samples in The Netherlands[4]. Presence of live virus in stool and the subsequent implications on faeco-oral transmission are still debated. Here, we describe the methodology standardized at the ICMR-National Institute of Virology, Mumbai Unit, Mumbai, India, for sewage surveillance for COVID-19. It was further planned to explore the feasibility of establishing sewage surveillance for COVID-19.

Material & Methods

Sewage samples were collected from a total of six sites in Mumbai, India. One of the sites was a sewage pumping station and the remaining sites were large open drains. Samples collected before the COVID-19 pandemic were available from each site. Fresh sewage samples were collected between May 11-22, 2020. One litre grab sample of sewage was collected by lowering a bucket in the flowing sewage water. The sample was transferred to a glass bottle containing 100 ml chloroform and a stirrer bar. The bottle contents were mixed well at the site. Samples were collected between 1000 and 1130 h. The samples were transported to the testing laboratory on wet ice within two hours. Personal protection equipment including face shield were used for protection. Materials used for sample collection were autoclave sterilized in the laboratory.

Sample processing for viral concentration: The sewage samples with chloroform were mixed thoroughly using a magnetic stirrer for 30 min at 4°C. The samples were centrifuged at $3000 \times g$ for 20 min at 4°C and supernatant was collected. To 500 ml clarified sewage sample, 39.5 ml of 22 per cent (w/w) dextran T40 (Sigma, USA), 287 ml of 29 per cent (w/w) PEG6000 (polyethylene glycol 6000) (Sigma, USA) and 35 ml of 5 M NaCl (Sigma, USA) were added sequentially with constant mixing. The mixture was stirred for one hour at 4°C before setting up phase separation. The sample was transferred to a one litre separating funnel and allowed to stand at 4°C for 16 h for partitioning of the virus into one of the phases[5-7]. After phase separation, the lower, middle/inter and upper phases were collected individually for SARS-CoV-2 detection by real-time reverse transcription-polymerase chain reaction (RT-PCR)[8].

RNA extraction and real-time RT-PCR: Viral RNA was extracted from the clarified sewage sample and the three phases using QIAamp Viral RNA Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. Presence of SARS-CoV-2 was inferred by detecting envelope (*E*) and RNA-dependent RNA polymerase (*RdRp*) genes amplification using LabGun COVID-19 real-time RT-PCR kit (LabGenomics, The Republic of Korea) as per the manufacturer's instructions. The kit manufacturer set a cut-off cycle threshold (Ct) for scoring a clinical sample positive for SARS-CoV-2 as Ct ≤40. The following reaction conditions were used: 50°C for 30 min, 45 cycles of 95°C for 15 min, 95°C for 15 sec and 60°C for one minute in a 7500 real-time PCR system (Applied Biosystems, USA). The RNA extracted from the clarified sewage sample and the lower, middle and upper phases was tested for SARS-CoV-2 RNA. Samples showing sigmoid curves of increasing fluorescence intensity in both the gene segments were considered positive for the virus.
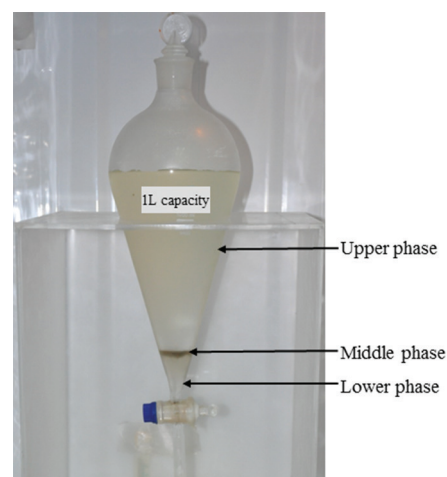
Results

The six sewage collection sites were chosen for detecting SARS-CoV-2 because of the presence of large slum-like areas housing low socio-economic groups and substantial migrant populations. These same sites were being used for poliovirus environmental surveillance (ES). The Wadala pumping station (Ward F/N) was the only site where piped sewage was collected. Table I presents the date of the first COVID-19 case and the increasing intensity of the pandemic in each ward. The first COVID-19 case in Mumbai was reported on March 11, 2020. The selected wards experienced COVID-19 cases in the mid-March, and the case numbers increased from <20 in the early April to more than 1000 by the mid-May 2020. At least one sewage sample collected before the COVID-19 outbreak and two samples during the COVID-19 pandemic were used in the study.

Table I. Confirmed COVID-19 cases in BMC wards where sewage samples were collected during May 11-22, 2020

Sewage site	BMC ward	Population	COVID-19 cases as of date		
			Date of the first case in March 2020	April 4	May 22
Wadala	F north	551079	20	5	1534
Dharavi	G north	627744	30	4	1969
Kurla	L	928813	22	6	1450
Shivaji Nagar	M east	840461	Not available	17	1140
Malad	P north	983421	22	18	534
Kanjur	S	773365	15	11	1373 (May 26)

As shown in Figure 1, PEG-dextran in water was separated in three phases, partitioning molecules as per their characteristics. If SARS-CoV-2 partitioned into lower or middle phase, it would get concentrated, enabling detection by PCR. The volume of middle phase varied from 2.2 to 4.5 ml (mean=2.7 ml) which was approximately 200-fold concentration if the virus partitioned into this phase and 7 to 10 ml (mean=8.3 ml) for the lower phase with a concentration factor of approximately 60-fold. In SARS-CoV-2 spiked sewage concentrates, no inhibitory effects of PEG/dextran T40 were observed (data not shown).

Real-time RT-PCR amplification curves of SARS-CoV-2 *E* and *RdRp* genes are presented in Figure 2. Figure 2A and B show completely flat curves for all the four RNA extracts (clarified sewage and that in lower, middle and upper phases) of a sewage sample collected in February 2020; figure 2C and D show the amplification curves of a sample collected in May 2020. RNA extracted from the middle phase gave the lowest Ct values of amplification for both the genes. The Ct values of middle phase RNA extracts were 3-9 points lower than the corresponding clarified sewage samples, confirming that SARS-CoV-2 partitioned only to the middle phase (Table II). Sewage samples with middle phase Ct values lower by 3 or more points were considered positive for SARS-CoV-2. Amplification curves (Ct >39) in the other phases could be due to a minor fraction of the viral particles or viral RNA that did not partition to the middle phase. Amplification curves with Ct >39 were observed in the RNA extracted directly from the sewage samples collected in May 2020 when the number of COVID-19 cases was increasing rapidly. Improved (lower) Ct values after concentration in the middle phase provided evidence of the presence of the virus.

Fig. 1. Separating funnel showing three phases of PEG6000-dextranT40-sewage sample mixture after 16 h at 4°C. The lower, middle and upper phases were collected individually for RNA extraction.

The final results of the 20 samples collected from the six sites before and during the COVID-19 pandemic period are shown in Table III. None of the sewage samples (from any site) collected before March 20, 2020 showed amplification of *E* and *RdRp* genes of SARS-CoV-2 RNA. In the absence of COVID-19 cases, the sewage concentrates (lower/middle phases) did not show any false-positive amplification in *E* and *RdRp* genes real-time RT-PCR. All sewage samples collected in May 2020 (except one sample from Kurla) were positive for SARS-CoV-2 (Ct values <36 for both *E* and *RdRp* genes for RNA extracted from the middle phase).

Although it may be tempting to argue that the late amplification (Ct >39) could be SARS-CoV-2 RNA specific, but we suggest that a viral concentration step be included and improvement of Ct value by about 3 points be used for the interpretation of positive results
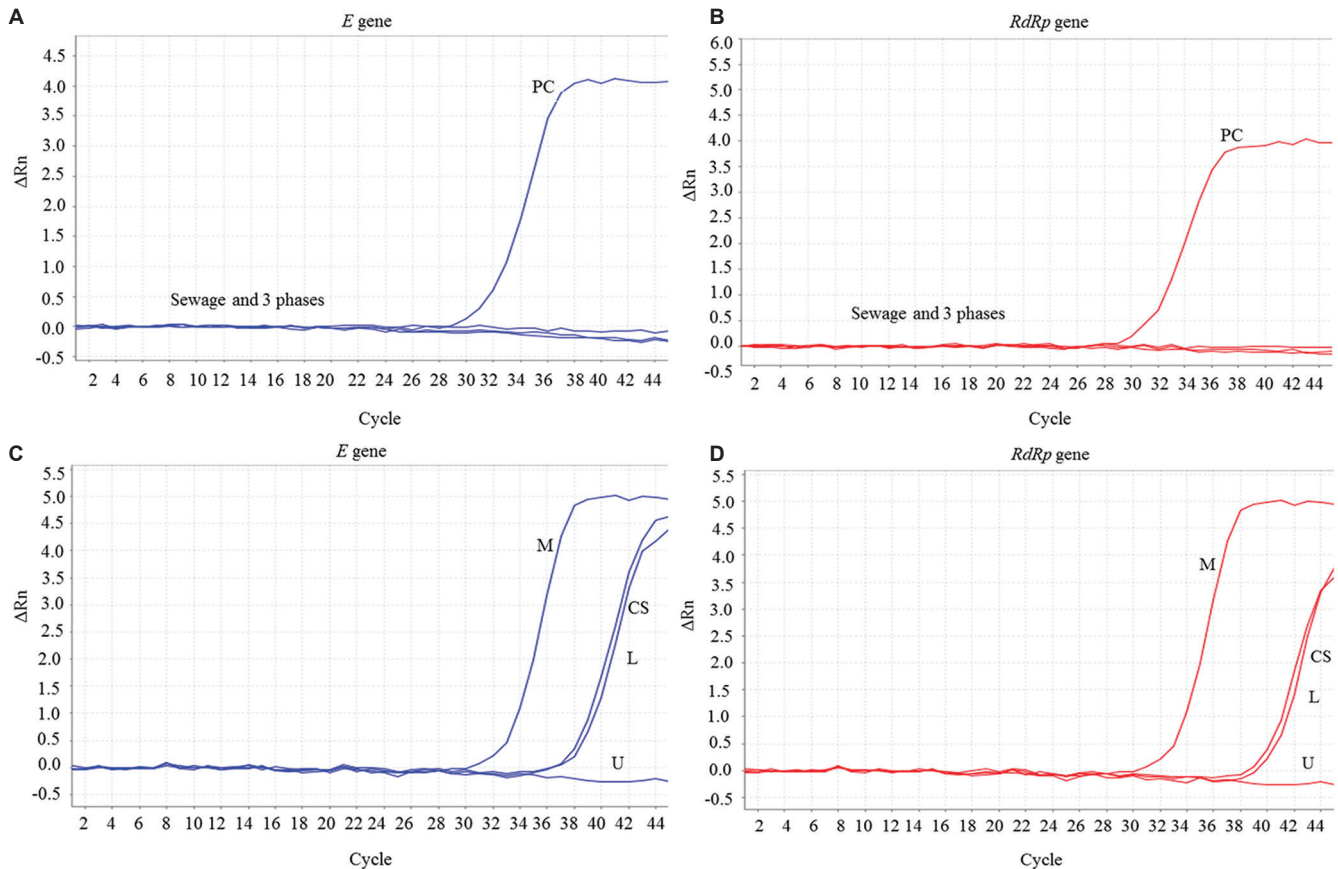
Fig. 2. Results of *E* and *RdRp* genes real-time RT-PCR of RNA extracted from clarified sewage and the three phases after phase separation. (**A** and **B**) SW-3713 (February 3, 2020); (**C** and **D**) SW-3737 (May 12, 2020). PC, positive control; CS, clarified sewage; L, lower phase; U, upper phase; M, middle phase. SARS-CoV-2 concentrated in to the middle phase. No amplification seen in SW-3713, the pre-COVID-19 sample.

until substantial additional data become available from several different SARS-CoV-2-infected and non-infected regions.

Discussion

A global emergency of pandemic was declared by the WHO on March 11, 2020 after the detection of SARS-CoV-2 in Wuhan, China, in December 2019[1]. The virus was found to be transmitting via respiratory route. However, SARS-CoV-2 RNA was also found to be excreted in human faeces[9]. This suggested that the virus could be detected from sewage water. ES for poliovirus detection has played a critical role in the eradication of wild poliovirus globally. The WHO-Global Polio Laboratory Network (GPLN) has established supplemental surveillance for polioviruses by testing sewage samples in several countries[10]. In India, ES was first initiated in Mumbai city in 2001 by ICMR-National Institute of Virology, Mumbai Unit (formerly known as ICMR-Enterovirus Research Centre).

The GPLN-validated PEG-dextran two-phase separation method to concentrate poliovirus from sewage recommends collection of the entire lower and interphase sample and treat it with chloroform to inactivate microbial contamination to render it compatible for virus isolation in cell cultures[7]. We, therefore, decided to employ the same two-phase separation methodology to concentrate and detect SARS-CoV-2 in sewage samples.

Risk analysis showed that SARS-CoV-2, an enveloped virus, is inactivated by household bleach, detergents/soaps and lipid solvents[11]. Sewage samples were collected in chloroform-containing bottles to inactivate the virus to further mitigate the risk and to allow sample handling in BSL-2 (Biosafety level 2) laboratories. This became the first modification of the WHO two-phase protocol. In our initial experiments with spiked sewage samples, SARS-CoV-2 could not be effectively concentrated in the lower phase (lower and middle layers collected together) collected as per

Table II. Results of real-time RT-PCR tests on RNA extracted from phase separation of sewage samples for SARS-CoV-2 detection

Sample_ID	Site	SARS-CoV-2 real time RT-PCR result							
		Ct values for gene *E*				Ct values for gene *RdRp*			
		Clarified sewage	Lower phase	Middle phase	Upper phase	Clarified sewage	Lower phase	Middle phase	Upper phase
SW-3713	Dharavi	UD	UD	UD	UD	UD	UD	UD	UD
SW-3731		UD	UD	UD	UD	UD	UD	UD	UD
SW-3737		38.64	39.4	33.02	UD	39.21	39.77	32.72	UD
SW-3741		39.4	37.72	33.15	UD	38.13	37.57	32.36	UD
SW-3714	Wadala	UD	UD	UD	UD	UD	UD	UD	UD
SW-3732		UD	UD	UD	UD	UD	UD	UD	UD
SW-3738		38.74	37.64	33.58	40.86	38.59	37.5	33.25	UD
SW-3742		36.35	37.61	31.92	40.95	35.67	37.19	30.96	UD
SW-3730	Shivaji Nagar	UD	UD	UD	UD	UD	UD	UD	UD
SW-3740		40.14	43.54	34.53	UD	40.18	41.38	38.76	UD
SW-3744		40.17	UD	36.27	UD	39.72	41.54	35.79	UD
SW-3734	Kurla	UD	UD	UD	UD	UD	UD	UD	UD
SW-3739		40.6	41.07	41.94	UD	40.56	40.66	39.3	UD
SW-3743		40.52	41.52	35.37	41.94	38.28	UD	35.98	UD
SW-3729	Kanjur	UD	UD	UD	UD	UD	UD	UD	UD
SW-3745		40.17	37.13	33.24	UD	39.32	38.1	33.5	UD
SW-3747		39.34	35.69	35.52	UD	35	34.1	32.85	37.42
SW-3733	Malad	UD	UD	UD	UD	UD	UD	UD	UD
SW-3746		37.33	39.28	33.11	41.84	38.95	41.53	33.64	42.19
SW-3748		37.13	35.65	33.56	40.07	39.83	34.11	30.46	35.25

UD, undetected, no amplification

Table III. Results of SARS-CoV-2 detection in sewage samples collected from six wards in Mumbai before and during the COVID-19 pandemic

Site	Pre-COVID-19 period				During COVID-19 period			
	Date of collection	Result	Date of collection in March 2020	Result	Date of collection in May 2020	Result	Date of collection in May 2020	Result
Dharavi	February 3, 2020	Negative	16	Negative	12	Positive	14	Positive
Wadala	February 3, 2020	Negative	16	Negative	12	Positive	14	Positive
Shivaji Nagar	X	X	11	Negative	13	Positive	15	Positive
Kurla	X	X	18	Negative	13	Positive	15	Positive
Kanjur	X	X	11	Negative	16	Positive	22	Positive
Malad	X	X	18	Negative	16	Positive	22	Positive

X, samples not available for testing

the WHO protocol[7]. The three layers of upper, middle and lower were collected separately and tested for SARS-CoV-2. The virus was found to be concentrated only in the middle phase. This was the second and major modification of the WHO two-phase separation method introduced during standardization.

PEG precipitation and PEG-dextran phase separation methods are widely used for concentrating viruses[5-7]. Pre-COVID-19 samples were collected for poliovirus ES in our laboratory. Sample collection was done during May 11-22, 2020 specifically for standardization of the method for detecting SARS-CoV-2 in sewage samples.

None of the pre-COVID-19 sewage samples showed amplification in any of the fractions (phase) tested, indicating that false amplification did not happen. All the samples collected in May 2020 were positive for SARS-CoV-2. Thus, the feasibility of using PEG-dextran phase separation method for ES of COVID-19 was established. The method may be further simplified by substituting gravity filtration for the centrifugation step and phase separation at ambient temperature. Other viral concentration methods may be compared to identify the most suitable one for long-term use.

Angiotensin-converting enzyme 2 (ACE2) was identified as the cellular receptor of SARS-CoV-2[12,13]. ACE2 is expressed on the cells of several different organs including lungs, kidneys and intestine. About 10^7 SARS-CoV-2 RNA copies/g of faeces may be excreted by symptomatic patients during the first week of the disease[9,14]. SARS-CoV-2 in sewage was first detected in The Netherlands within weeks after the reports of COVID-19 cases[4]. Viral concentration was achieved by ultra-filtration technique. Sewage samples were found to be negative for SARS-CoV-2 in the area where the prevalence of COVID-19 was 2.9 cases/100,000 population, with partial positivity at 3.5 cases/100,000 and high positivity when the prevalence reached 12.9 cases/100,000 population[4].

SARS-CoV-2 detection in waste waters could be used to understand the epidemiology of COVID-19. For example, (i) decreasing concentration or absence of virus at previously SARS-CoV-2-positive sewage sampling sites may indicate successful implementation of COVID-19 control strategies, and (ii) it may provide evidence of the presence or absence of SARS-CoV-2-infected populations and confirmation of COVID-19-free zones.

To conclude, SARS-CoV-2 was detected in sewage samples collected from six different sites in Mumbai during the ongoing pandemic of COVID-19. PEG6000-dextran T40 phase separation method was adopted to concentrate SARS-CoV-2 from sewage samples. The virus was found to partition and concentrate specifically at the middle phase. RNA extracted from the middle phase was suitable for SARS-CoV-2 detection by real-time RT-PCR.

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