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Detection of SARs-CoV-2 in wastewater using the existing environmental surveillance network: A potential supplementary system for monitoring COVID-19 transmission

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Abstract

The ongoing COVID-19 pandemic is caused by SARs-CoV-2. The virus is transmitted from person to person through droplet infections i.e. when infected person is in close contact with another person. In January 2020, first report of detection of SARS-CoV-2 in faeces, has made it clear that human wastewater might contain this virus. This may illustrate the probability of environmentally facilitated transmission, mainly the sewage, however, environmental conditions that could facilitate faecal oral transmission is not yet clear. We used existing Pakistan polio environment surveillance network to investigate presence of SARs-CoV-2 using three commercially available kits and E-Gene detection published assay for surety and confirmatory of positivity. A Two-phase separation method is used for sample clarification and concentration. An additional high-speed centrifugation (14000Xg for 30 min) step was introduced, prior RNA extraction, to increase viral RNA yield resulting a decrease in *Cq* value. A total of 78 wastewater samples collected from 38 districts across Pakistan, 74 wastewater samples from existing polio environment surveillance sites, 3 from drains of COVID-19 infected areas and 1 from COVID 19 quarantine center drainage, were tested for presence of SARs-CoV-2. 21 wastewater samples (27%) from 13 districts turned to be positive on RT-qPCR. SARs-COV-2 RNA positive samples from areas with COVID 19 patients and quarantine center strengthen the findings and use of wastewater surveillance in future. Furthermore, sequence data of partial ORF 1a generated from COVID 19 patient quarantine center drainage sample also reinforce our findings that SARs-CoV-2 can be detected in wastewater. This study finding indicates that SARs-CoV-2 detection through wastewater surveillance has an epidemiologic potential that can be used as supplementary system to monitor viral tracking and circulation in cities with lower COVID-19 testing capacity or heavily populated areas where door-to-door tracing may not be possible. However, attention is

needed on virus concentration and detection assay to increase the sensitivity. Development of highly sensitive assay will be an indicator for virus monitoring and to provide early warning signs.

Introduction

Novel coronavirus pneumonia (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-Cov-2) infection has become a global emergency through its widespread infection with 43,504,448 confirmed cases resulting 1,159,533 deaths in 213 countries as of 26th October, 2020 [1]. In December 2019, cluster of pneumonia like disease cases with symptoms including fever, difficulty in breathing, cough and invasive lesion on both lungs were reported from Wuhan, China [2]. The causative agent was identified as a Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) after ruling out SARS-CoV, MERS-CoV, influenza, avian influenza, adenovirus and other common respiratory pathogens [3]. Coronaviruses belonging to family *Cornaviridae* are enveloped, non-segmented positive sense RNA viruses distributed in human and mammals [4]. However, majority of human coronaviruses have mild infections but two betacornaviruses; severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) caused outbreaks in last two decades with 10% and 35.6% mortality rate respectively [5–7].

On March 11, 2020 WHO declared it as pandemic, when disease was reported in 114 countries [8]. The primary routes of viral transmission (SARS-CoV-2) was considered to be through droplet infections and person to person close contact, but later it is evident from various published studies that there is increasing possibility of fecal-oral transmission, however, environmental conditions that could facilitate faecal oral transmission is not yet clear [9, 10]. This shows the probability of environmentally mediated transmission. Since the early days of pandemic, we got interested in understanding and utilizing the role of environmental sampling mainly the sewage.

SARS-CoV-2 resembles 82% with SARS coronavirus (SARS-CoV) which caused an outbreak in 2003. Studies have shown the survival of SARS-CoV in stool for up to 4 days [7]. Another study described the presence of SARS-CoV and its infectious nature in water and sewage for days to weeks [11]. It was also described that faulty sewage system contaminated with SARs-CoV in a high-rise housing estate of Hong Kong during 2003 was linked to SARS outbreak involving large number of residents of surrounding buildings [12]. A recent study highlighted the shedding through stool of SARS-CoV-2 in cluster of 9 nCOVID-19 patients. It was reported that that the RNA concentration decreased from 10^7 RNA copies/g to 10^3 RNA copies/g after one week of symptom onset to third week [13]. Since the source of transmission of SARS-CoV-2 is still unknown therefore wastewater transmission pathway can become an important mode [14]. Hence, the presence of SARS-CoV-2 in contaminated sewage sample and its role in transmission needs to be investigated.

First COVID-19 case was reported in Pakistan on 26th February 2020 in a traveler returning from a religious congregation in Iran, now there have been 329,375 confirmed cases with 6,745 deaths as of 26th October 2020 [15]. Initially the cases were identified only in traveler returning from religious congregation, but soon afterwards local transmission was established with resident cases without any history of travel from mid-March. In this study, we used the existing polio environment surveillance network in Pakistan through which sewage samples were collected from designated sites on monthly basis in different districts of the country to

investigate poliovirus presence, its spread and molecular epidemiology. Same samples collected from mid-March till 30th April 2020 were processed and tested for detection of SARs-CoV-2 RNA.

Materials and methods

The study plan and use of environmental surveillance network was approved by National Institute of Health, Pakistan vide letter No NIH/ PHLD/21/VIR/RRL/001. Untreated wastewater samples (sewage samples) selected for testing in this study were collected using the grab sampling technique. Most of them were those collected for routine polio environment surveillance (ES). Polio ES sites are either open drains or pumping stations and are sampled routinely on monthly basis. Each sampling site represent 100,000–300,000 population [16]. Besides, wastewater from drains of some areas with recent history of SARS-CoV2 cases were also collected for detection and re-confirmation of SARs-CoV-2 detection. Sampling personnel strictly followed the standard safety guidelines for personnel protective equipment (PPE) required for wastewater sampling. One liter of sewage water was collected from the midstream into a sterile, leak proof container at a downstream sampling site during the peak morning flow. These samples were transported in properly sealed container with information form, indicating sampling site, district, sampling date and sampling time, to laboratory within 48 hours of collection maintaining reverse cold chain [16]. Samples were processed in laboratory for virus concentration using the two-phase separation method [17].

500 ml of each raw sewage specimens was concentrated. Firstly, clarification of the sample was done by pelleting of larger suspended solids by centrifugation (1500 X g for 20 min). The clarified sewage sample was mixed with defined amounts of polymers, 22% DextranT40 (Pharmacosmos A/S, Cat# 551000409006) and 29% polyethylene glycol (PEG-6000, Sigma Analytical, Cat# 81255) and 5N Sodium Chloride (Sigma-aldrich) [17]. The homogenous mixture obtained by vigorous shaking is left to stand overnight at 4˚C in a separation funnel. The polymer helped to form two distinct layers (phases) in the funnel which were collected and mixed with pellet formed in first step which was then treated with chloroform [17] (S1 Fig).

400 μl of processed sample was centrifuged at high speed (14000 X g) for 30 min to pellet the suspended solid particles that may increase the viral RNA yield. Virus may partly bound to these solids. Supernatant was discarded carefully without disturbing the pellet. Pallet was then mixed with 300 μl of RNase free water by vigorous vortexing and was processed for RNA extraction.

Spin star viral nucleic acid kit 1.0 (ADT Biotech, Phileo Damansara 1, Petaling Jaya Part No. 811803) was used to extract the viral RNA. Internal control provided with kits were added as an amplification control in rRT-PCR. Pellet was dissolved with 430 μl of lysis buffer supplied with kit, followed by 5 min vortexing to homogenously dissolve the pallet. Further processing was done as per the manufacturer's instructions. The final elution volume is 60μl. The extracted viral RNA was store at -20˚C till further testing.

Multiple qualitative reverse transcription real-time PCR kits for identification of SARS--CoV2 were used. These kits were already in use country wide for detection of SARS-CoV2 in human. These were (Kit 1) Real-Time Fluorescent RT-PCR Kit for detecting 2019-nCoV by BGI China (IVD &CE marked; Catalogue No. MFG030010), takes ORF 1ab gene as the target domain, (Kit 2) qRT-PCR for Novel Coronavirus (2019-nCoV) Nucleic Acid Diagnostic Kit (PCR-Flourescence Probing, IVD marked) by Sansure Biotech (Sansure Biotech Inc China, Ref No. S3102E). The kit utilizes novel coronavirus (2019-nCoV) ORF-1 gene and a conserved coding nucleocapsid protein N-gene as the target regions and finally (Kit 3) detection Kit for 2019 Novel Coronavirus RNA (PCR-Fluorescence Probing) targeting the ORF 1ab and N gene

of SARS-CoV-2/2019-nCoV by Da An Gene Co., China (IVD and CE marked; Catalogue No. DA-930). Thermal cycling and results interpretation were performed as per manufacturer's instruction.

For further confirmation these samples were also tested for envelop protein (E) gene detection using the primers / probe sets that was published by Corman V. M. et al. [18]. A 25 μl reaction contain 5 μl RNA, 12.5 μl 2x reaction buffer provided with the Superscript III one step RT-PCR with platinum Taq Polymerase (Invitrogen, Darmstadt, Germany; containing 0.4 mM of each deoxyribose triphosphates (dNTP) and 3.2 mM magnesium sulphate), 1 μl of reverse transcriptase / Taq mixture from Kit and different concentration of primers and probes (Table 1). Thermal cycling was carried out at 55˚C for 10 min, followed by 95˚C for 3min. Then 45 cycles of 95˚C for 15 sec, 58˚C for 30 sec on ABI 7500 real time system (Applied Bio Systems, US. Cat # 4351104).

Genetic sequencing was based on conventional amplification of genomic RNA by using Qiagen One step RT PCR kit as described by Shirato K et al [19]. The ORF 1a gene was amplified, using a pair of primers NIID_WH-1_F501 (TTCGGATGCTCGAACTGCACC) and NIID_WH-1_R913 (CTTTACCAGCACGTGCTAGAAGG). After 1st PCR, nested PCR was performed using 2nd PCR primers (Sense: NIID_WH-1_F509(CTCGAACTGCACCTCATGG), Antisense: NIID_WH-1_R854 CAGAAGTTGTTATCGACATAGC) and 1 μl of 1st PCR product under the same condition. PCR amplicons from 2nd round were purified by Qiaquick PCR purification Kit (Qiagen, Germany) and directly sequenced by using Sequencing primers (Sense: NIID_WH-1_Seq_F519 ACCTCATGGTCATGTTATGG, Antisense: NIID_WH-1_Seq_R840 GACATAGCGAGTGTATGCC) on ABI 3100 genetic analyzer using Big Dye Terminator kit V.3.0.cycle sequencing kit (ABI Foster City Canada, USA). The nucleotide sequences were assembled, edited and analyzed by Sequencher software v.4.9 (GeneCodes Incorporation, USA). The nucleotide sequence obtained was blasted against the available NCBI databank (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

A total of 76 wastewater samples were collected between 12 to 18 epidemiological weeks from 38 districts across country and were tested for SARs-CoV-2 RNA (Fig 1). 21 wastewater samples (27%) from 13 districts were positive on RT-qPCR (Table 2).

Primarily, 20 wastewater samples were collected during March 20, 2020 to April 09, 2020 from 17 districts of Pakistan; 18 samples from different polio environmental sites (ES) distributed across 16 districts and 02 samples in areas with recent history of SARS-CoV2 cases from capital city Islamabad. All these samples were tested against the three commercially available SARS-CoV-2 RNA detection diagnostic kits (mentioned under materials and methods). Negative control supplied with the diagnostic kits containing the internal control was added in each sample during extraction to ensure none of the wastewater extracted RNA samples had RT-qPCR inhibition. A total of 6 samples (30%) were positive for SARs-CoV-2 RNA, out of these all were detected on Kit 1, 2 (either one gene positive or both, results interpreted as per manufacturer's instruction) and E gene method whereas only 4 were positive on Kit 3. 4 positive samples had *Cq* values between 32 to 38, whereas remaining 2 samples collected in areas with history of SARs-CoV-2 cases from Islamabad were positive on all three diagnostic kits and E gene method having *Cq* between 36 to 38 (S1 Table).

A centrifugation step was also introduced before Viral RNA extraction to increase viral RNA yield. A decrease in *Cq* value has been observed, describing an increase in viral RNA concentration (S3 Table). Furthermore, 56 samples collected and received in laboratory from April 6, 2020 to April 28,2020 for polio diagnostics were also tested for detection of SARs-

Table 1. E Gene primers and probes for 2019 SARS-CoV2 real time- RT-PCR.

E gene	Oligonucleotide	Sequence [a]
	E_Sarbeco_F	ACAGGTACGTTAATAGTTAATAGCGT
	E_Sarbeco_P1	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ
	E_Sarbeco_R	ATATTGCAGCAGTACGCACACA

[a]FAM: 6-carboxyfluorescein; BBQ: blackberry quencher.

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CoV-2, out which 14 (24%) were positive. One sample was collected from the drain of a Rawalpindi Institute of Urology (RIU), a COVID-19 patient quarantine center, while other from a drain having catchment area with recent history of COVID-19 patients. Both collection sites are in district Rawalpindi. These samples were found positive for SARs-COV-2 RNA on RT-qPCR (S2 Table).

The sample collected from the drain of RIU, Rawalpindi was also subjected for partial sequencing of SARs-CoV-2 ORF-1a. Nucleotide sequence of partial SARs-CoV-2 ORF 1a is submitted in gene bank under accession number MT539157.

Discussion

The role of environmental surveillance in supporting Global Polio Eradication Initiative has already been acknowledged [20]. The environmental surveillance can be used as supplemental tool for detection pathogens circulating within the community. Wastewater provides a near-real-time data as it constantly collects feces, urine and traces of sputum that can contain SARs-CoV-2 shed by the infected individuals. Viral load estimation in COVID-19 positive patients

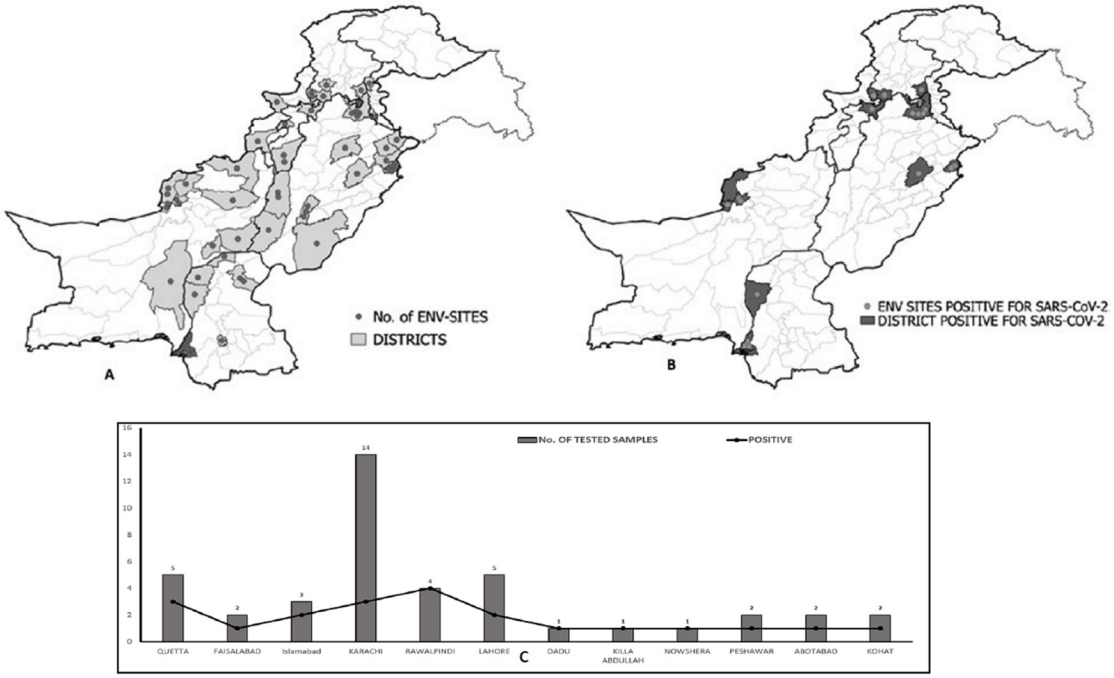

Fig 1. Geographical representation. A. Map indicating environmental sampling sites. Each dot represents a wastewater collection site B. Map representing districts with SARs-CoV-2 positive wastewater samples as dark grey color. Each dot represents a positive wastewater sample C. Graphical representation of positive samples among tested samples.

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Table 2. Wastewater sample tested for SAR-COV2 at virology department, National Institute of Health, Islamabad, Pakistan.

EPI Week	COVID-19 Detection		
	Samples Tested	Positive	Negative
Week12	2	1	1
Week14	13	2	11
Week15	16	4	12
Week16	32	10	22
Week17	9	1	8
Week18	6	3	3

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are still uncertain, however, recently N. Zhang *et al*. suggests levels as high as 600,000 viral genomes per ml of fecal material [21]. Similarly another study reported approximately 30,00,000 viral particles in a single fecal sample [21]. A previously published study on coronavirus reported that it remained infectious in water and sewage for days to weeks. Researchers reported time required for 99% reduction of virus infectivity was several days at room temperature in pure water or wastewater [22]. This adds another potential supplemental detection source of SARs-CoV-2 in communities.

In this study, we investigated the presence of SARs-CoV-2 RNA in wastewater using the existing poliovirus environment surveillance network that can be used in future as alternative system for the dependent area as SARS-CoV-2 supplementary surveillance. This definitely needs further evaluation and discussions; however, this seems to have a very interesting utilization in epidemiology. A total of 21 out of 78 positive wastewater samples for SARs-CoV-2 RNA clearly indicates viral RNA shedding in stool of infected individuals (Fig 1). Currently, there is no evidence of infection transmission of SARs-COV-2 or related SARs-Corona via wastewater [23].

We used three commercially available kits and Published E-Gene detection assay for surety and confirmatory of positivity. Samples collected from Quetta district at two different time intervals indicates COVID-19 prevalence and surge in infected individuals. This can be assumed from the decrease in *Cq* value in wastewater sample collected two weeks after. Detection of SARs-COV-2 RNA in two specific wastewater samples collected from areas with recent history of COVID-19 patients clearly explain that wastewater testing for COVID-19 can be used as a supplementary system. Likewise, SARs-COV-2 RNA positive samples from RIU, Rawalpindi and an area in Rawalpindi with COVID patients further strengthen the findings and use of this tool in future. This surveillance system can picks up vast majority of infected individuals with SARs-CoV-2 who do not present symptoms for the disease [23]. Furthermore, sequence data of partial ORF 1a generated from ICT-04 also reinforce our findings that SARs-CoV-2 can be detected in wastewater in Pakistan. Interestingly, the additional extra centrifugation step before viral RNA extraction seems to be encouraging in increasing the yield of viral RNA. This can be obvious from the data presented in S3 Table.

The surveillance through wastewater can be useful in remote or confined communities, however, further studies are needed on virus concentration and detection assay to increase the sensitivity. This has an epidemiologic potential for early detection of high burden area in advance; and heavily populated areas where door-to-door tracing may not be possible. This may also be more relevant to the developing countries with limited molecular testing. Development of highly sensitive assay will be an indicator for virus monitoring and to provide early warning signs. The developments on use of sewage samples for SARs CoV2 detection should

be further investigated and correlated with the quantitative data which has not been addressed in this study.

Conclusion

SARS-CoV-2 detection in wastewater using RT-qPCR assay, confirmed by sequencing, is a milestone in the field of epidemiology. The study finding indicates that environmental surveillance through wastewater could be used as a potential supplementary system (as used in case of poliovirus) to monitor viral tracking and circulation in settings where person to person testing capacity is limited. The virus concentration and detection method in wastewater needs attention to increase sensitivity of detection of SARs-CoV-2 in wastewater.

Supporting information

S1 Fig. Process flow chart: Sewage sample concentration of wastewater by two-phase separation method.
(TIF)

S2 Fig. Average COVID-19 positive case per epidemiological week in Pakistan.
(TIF)

S1 Table. Wastewater samples collected during March 20, 2020 to April 09, 2020. A comparative analysis.
(DOCX)

S2 Table. Details of wastewater samples tested for SARS-CoV2 at virology department, National Institute of Health, Islamabad, Pakistan.
(DOCX)

S3 Table. Sample preparation without and with centrifugation before viral RNA extraction. Comparison among the *Cq* values.
(DOCX)

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References

1. JHU. COVID-19 Dashboard by the Center for Systems Science and Engineering (CSSE) at Johns Hopkins University (JHU) 2020. https://coronavirus.jhu.edu/map.html.
2. WHO. Coronavirus disease (COVID-19) outbreak 2020. https://www.who.int/westernpacific/emergencies/covid-19.
3. WHO. WHO Statement regarding cluster of pneumonia cases in Wuhan, China 2020. https://www.who.int/china/news/detail/09-01-2020-who-statement-regarding-cluster-of-pneumonia-cases-in-wuhan-china.
4. Richman DD W R, Hayden FG, eds. Clinical virology. 4th ed2016.
5. Ramadan N, Shaib H. Middle East respiratory syndrome coronavirus (MERS-CoV): A review. Germs. 2019; 9(1):35–42. Epub 2019/05/24. https://doi.org/10.18683/germs.2019.1155 PMID: 31119115.
6. de Groot RJ, Baker SC, Baric RS, Brown CS, Drosten C, Enjuanes L, et al. Middle East respiratory syndrome coronavirus (MERS-CoV): announcement of the Coronavirus Study Group. J Virol. 2013; 87 (14):7790–2. Epub 2013/05/17. https://doi.org/10.1128/JVI.01244-13 PMID: 23678167.
7. Hui DS, Chan MC, Wu AK, Ng PC. Severe acute respiratory syndrome (SARS): epidemiology and clinical features. Postgrad Med J. 2004; 80(945):373–81. Epub 2004/07/16. https://doi.org/10.1136/pgmj.2004.020263 PMID: 15254300.
8. WHO. 2020. https://www.who.int/dg/speeches/details/who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19—11-march-2020.
9. Cheng PK, Wong DA, Tong LK, Ip SM, Lo AC, Lau CS, et al. Viral shedding patterns of coronavirus in patients with probable severe acute respiratory syndrome. Lancet. 2004; 363(9422):1699–700. Epub 2004/05/26. https://doi.org/10.1016/S0140-6736(04)16255-7 PMID: 15158632.
10. Adhikari SP, Meng S, Wu YJ, Mao YP, Ye RX, Wang QZ, et al. Epidemiology, causes, clinical manifestation and diagnosis, prevention and control of coronavirus disease (COVID-19) during the early outbreak period: a scoping review. Infect Dis Poverty. 2020; 9(1):29. Epub 2020/03/19. https://doi.org/10.1186/s40249-020-00646-x PMID: 32183901.
11. Casanova L, Rutala WA, Weber DJ, Sobsey MD. Survival of surrogate coronaviruses in water. Water Res. 2009; 43(7):1893–8. Epub 2009/02/28. https://doi.org/10.1016/j.watres.2009.02.002 PMID: 19246070.
12. Peiris JS, Chu CM, Cheng VC, Chan KS, Hung IF, Poon LL, et al. Clinical progression and viral load in a community outbreak of coronavirus-associated SARS pneumonia: a prospective study. Lancet. 2003; 361(9371):1767–72. Epub 2003/06/05. https://doi.org/10.1016/s0140-6736(03)13412-5 PMID: 12781535.
13. Woelfel Roman C VM, Guggemos Wolfgang, Seilmaier Michael, Zange Sabine, Mueller Marcel A, Niemeyer Daniela, et al. Clinical presentation and virological assessment of hospitalized cases of coronavirus disease 2019 in a travel-associated transmission cluster. medRxiv. 2020. https://doi.org/10.1101/2020.03.05.20030502
14. UNICEF W. Water, sanitation, hygiene, and waste management for the COVID-19 virus: interim guidance. 2020.
15. Government of Pakistan official portal: https://covid.gov.pk/stats/pakistan 2020.
16. WHO. Guidlines on Environmental Sureveillance for detection of poliovirus. 2015.
17. WHO. Guidelines for environmental surveillance of poliovirus circulation 2003. http://polioeradication.org/wp-content/uploads/2016/07/WHO_V-B_03.03_eng.pdf.
18. Corman VM, Landt O, Kaiser M, Molenkamp R, Meijer A, Chu DK, et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill. 2020; 25(3). Epub 2020/01/30. https://doi.org/10.2807/1560-7917.ES.2020.25.3.2000045 PMID: 31992387.
19. Shirato K, Nao N, Katano H, Takayama I, Saito S, Kato F, et al. Development of Genetic Diagnostic Methods for Novel Coronavirus 2019 (nCoV-2019) in Japan. Jpn J Infect Dis. 2020. Epub 2020/02/20. https://doi.org/10.7883/yoken.JJID.2020.061 PMID: 32074516.
20. Kroiss SJ, Ahmadzai M, Ahmed J, Alam MM, Chabot-Couture G, Famulare M, et al. Assessing the sensitivity of the polio environmental surveillance system. PLoS One. 2018; 13(12):e0208336. https://doi.org/10.1371/journal.pone.0208336 PMID: 30592720.
21. Wu FQ X A, Zhang JB, Gu XQ, Lee WL, Kauffman K, Hanage WP, et al. SARS-CoV-2 titers in wastewater are higher than expected from clinically confirmed cases. medRxiv. 2020. https://doi.org/10.1128/mSystems.00614-20 PMID: 32694130
22. Qu Guangbo L X, Hu Ligang, and Jiang Guibin. An Imperative Need for Research on the Role of EnvironmentalFactors in Transmission of Novel Coronavirus (COVID-19). Environmental Science & Technology 2020; 54(7):3730–2. https://doi.org/10.1021/acs.est.0c01102 PMID: 32202420

23. Ahmed W, Angel N, Edson J, Bibby K, Bivins A, O'Brien JW, et al. First confirmed detection of SARS-CoV-2 in untreated wastewater in Australia: A proof of concept for the wastewater surveillance of COVID-19 in the community. Sci Total Environ. 2020; 728:138764. Epub 2020/05/11. https://doi.org/10.1016/j.scitotenv.2020.138764 PMID: 32387778.