**Development of a surveillance system to detect SARS-CoV-2 genetic material in wastewater: a pilot study**

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**BACKGROUND**

Coronaviruses are zoonotic viruses that spread amongst animals and spill over to humans from time to time and have been causing illnesses ranging from mild symptoms to severe illness. They are positive-strand RNA viruses, around 30kb in genome size with 15 genes, enveloped, with a lipid membrane envelope around the surface of the virus. The lipid envelope makes coronaviruses more fragile than other viruses. Coronaviruses commonly infect animals, such as bats, birds, and mammals, which act as an intermediate host reservoir. Human coronaviruses were first identified in the mid-1960s and so far, a total of seven have been reported to be capable of infecting humans. Four of them, the 29E (alpha coronavirus); NL63 (alpha coronavirus); OC43 (beta coronavirus), and HKU1 (beta coronavirus), cause mild to moderate disease. Since the beginning of the 21st century, three more coronaviruses have crossed the animal-human species barrier to cause deadly pneumonia in humans, namely the Severe Acute Respiratory Syndrome (SARS-CoV-1), Middle East Respiratory Syndrome (MERS), and the current Severe Acute Respiratory Syndrome 2 (SARS-CoV-2). SARS-CoV-2is the newest of the family of coronaviruses associated with human infections that are grouped into the beta-CoV genus, with 79% genetic similarity to SARS-CoV-1. On 7 January 2020, Chinese authorities confirmed the outbreak of COVID-19, and on 30 January 2020, the Director-General of the World Health Organization (WHO) declared the COVID-19 outbreak a Public Health Emergency of International Concern. On 1st February 2020, 312 Bangladesh citizens were brought back from China’s Wuhan city and quarantined for 14 days. Eight of them were immediately isolated and three more were subsequently isolated upon showing symptoms. Their samples were tested on the 2nd of February 2020 in the laboratory of the Institute of Epidemiology, Disease Control and Research (IEDCR) and found negative for COVID-19. On 8 March 2020, Bangladesh confirmed its first COVID-19 case. On 11 March, WHO declared COVID-19 as a pandemic for its rapid global spreading. As of 20 January 2021, a total of **529,031**confirmed cases and **7,942** deaths were reported in Bangladesh.

Many COVID-19 infections are asymptomatic and unless tested, can remain undetected. Likewise, the current total picture of SARS-CoV-2 virus circulation in the population of Bangladesh is incomplete and the number of COVID-19patients most likely underestimated, mainly due to lack of testing. With limitations on COVID-19 testing making it hard to know how many people the disease has, turning to the sewer systems for a fast snapshot seems to be promising and a useful method to provide complementary and additional information to the clinical testing. The SARS-CoV-2’s fecal signature could turn out to be very useful, helping track how and where the disease is spreading among the population.

Once in the body, the virus can be shed through feces and urine, as well as through saliva and other respiratory discharges. The virus and/or its remnants are introduced into water resources and wastewater environment through the discharge of human waste and bodily fluids containing the virus, e.g. from brushing teeth, mouth washing, coughing and sneezing while bathing or showering, washing of hands or clothes, and discarding tissues and wipes into the toilet. As a complementary approach to monitoring the spread of COVID-19, many countries have since implemented wastewater-based epidemiology (WBE). WBE is a relatively new environmental concept for determining the exposure of populations to substances of concern and is based on the analysis of target biomarkers related to that substance of concern in raw wastewater to obtain qualitative and quantitative data on the health of communities within a given wastewater catchment. WBE has been used to help inform broader infectious disease epidemiological surveillance and mitigation efforts such as the Global Polio Eradication Initiative. Environmental water surveillance has also been used and recommended for monitoring the spread of other infectious disease-causing microorganisms such as typhoid, early warning of hepatitis A and Nor virus outbreaks, as well as for antimicrobial resistance.

Thus, the presence/absence of SARS-CoV-2 and/or remnants in wastewater treatment plant influent can determine the presence of infected individuals in a community and can be used as an epidemiological indicator, especially where community testing is not possible. The main aim of this study is to test the feasibility of applying the WBE and environmental water surveillance concept in Bangladesh as a tool that provides valuable additional information about the spread of the virus as a complement to health surveillance, and also as an early warning system for infection in a community providing a more sensitive and rapid indication of changes in infection rates before such effects become detectable by clinical health surveillance. Critically, this will provide decision support for officials determining the timing and severity of public health interventions to mitigate the overall spread of the disease. This study serves as a short-term, proof of concept study before the roll-out of national surveillance, and also involves preliminary testing, optimization, and validation of sampling and virus analysis methods, as well as results interpretation and reporting in the Bangladesh context.

**INTRODUCTION**

**The SARS-Cov-2 virus**

Coronaviruses (CoVs) belong to the family of Coronaviridae and they are a large and diverse family of viruses. The name ‘corona’ comes from their round appearance and the spikes on their surface that can be likened to a solar corona (Figure 1-1(a)). Coronaviruses are enveloped, which means that there is a lipid membrane envelope around the surface of the virus, while ‘naked’ viruses do not have this. The lipid envelope makes coronaviruses more fragile than other viruses (1) and is hence relevant to understanding their environmental persistence and transmission and their susceptibility to inactivation by disinfection. The lipid structure holds the membrane (M), envelope (E), and spike (S) proteins together, with the spike protein protruding from the envelope (Figure 1-1(a)). Since the spike protein is responsible for the connection with the host cells in humans, the virus loses its infectivity if the lipid envelope is destroyed (Figure 1-1(b)) (1).

Their genome is made up of single-stranded RNA (Figure 1-1(a)), which makes them highly susceptible to UV disinfection. When screening for the virus in wastewater, scientists detect the genetic information that codes for the key proteins in its structure. Euro surveillance and Centers for Disease Control and Prevention have provided references listing commonly used primers for the detection of the SARS-CoV-2 virus. The Euro surveillance E primers target regions of RNA that code for the envelope (E), while the CDC N1 and N2 primers detect fragments of RNA that code for the nucleocapsid (N) protein (Figure 1-1(a)).



**Fig 1****: SARS-CoV-2 key structure includes S, N, M, E and RNA (a); incapacitation process (b) and degradation (c). The subsequent analysis of SARS-CoV-2 RNA (typically after conversion to**

**DNA) may follow RNA extraction from intact, incapacitated or degraded virus and combinations**

**There of (Hill et al., 2020) (31).**

Coronaviruses mostly infect animals, such as, bats, birds, and mammals, which act as an intermediate host reservoir. Human coronaviruses (HCoVs) were first identified in the mid-1960s and so far, a total of seven have been reported to be capable of infecting humans. Four of them, the 29E (alpha coronavirus); NL63 (alpha coronavirus); OC43 (beta coronavirus), and HKU1 (beta coronavirus), cause mild to moderate disease, and may even go unnoticed. However, since the beginning of the 21st century, three more human coronaviruses have been identified and cause deadly pneumonia in humans (1). These include Severe Acute Respiratory Syndrome (SARS-CoV-1), Middle-East Respiratory Syndrome (MERS), and now Severe Acute Respiratory Syndrome 2 (SARS-CoV-2). This CoV is the newest of the family of coronaviruses associated with human infections that are grouped into the beta-CoV genus, with 79% genetic similarity to SARS-CoV-1 (21).

SARS-CoV-2 was revealed after testing of fluid from a patient’s lungs on 3 January 2020, following reports of several patients presenting with strange pneumonia in November and December 2019 in Wuhan Province, China. The first publications about this virus referred to it as the ‘novel coronavirus’, and the name 2019-nCoV was used to denote it. Since more has become known about the virus, it has been designated SARS-CoV-2 and is associated with the current pandemic of atypical pneumonia (the disease is designated as COVID-19). SARS-CoV-2 is transmitted from person-to-person via the respiratory system through sneezing, coughing and secretions, and by contact with contaminated surfaces (3).

Bangladesh scores very low in wastewater Management:

# Bangladesh is among the countries with the lowest level of wastewater treatment in the Asia Pacific region. Wastewater treatment and proper sanitation are the biggest problems for public health concern as well as for the environment. Moreover, treatment is rare with pipe connected areas with seldom practiced disinfection (4). Bangladesh is one of those developing countries, where wastewater and proper sanitation are currently challenging for a low economy and high population density. In the last few years, there has been considerable change in awareness for sanitation and hygiene. Several local and international organizations are trying to mitigate the waste water-related issues. There has also been a significant decrease in death due to water-borne diseases. Around 11% of deaths by diarrhea have been associated with the use of untreated groundwater those linked with wastewater (5). Contamination is more severe in areas with silt and clay layers. Besides, improper placement of latrines and discharge of untreated effluent in the surface water is causing more severe contamination. The condition of wastewater contamination is quite different in urban and rural areas. In urban areas, water scarcity is a major concern and mainly surface water is contaminated by the illegal effluent discharge into wastewater bodies. In rural areas, relatively more people have accessibility to wastewater sources.

## **Wastewater-Based Epidemiology**

Wastewater-Based Epidemiology (WBE), which can be answered through the investigation and surveillance of selected indicators of community health and behavior, reflected in the composition of urban wastewater (Ref.). Sampling and analyzing the wastewater can reveal the presence of the SARS-CoV-2 genetic fingerprint in wastewater for a specific community. Clinical diagnostic tests for COVID-19 are insufficient for rapid and cost-effective monitoring of the incidence and another problem concerns the worldwide high demand of consumables (e.g. swabs, reagents) needed for the collection and screening of samples for COVID-19(6). The fear of social stigma is also attached to the pandemic. For that COVID-19 diagnosis for how many people have the disease, turning to the sewer systems for a fast snapshot seems to be promising and a useful method to provide complementary and additional information to the clinical testing. Fecal signature for Covid-19 patients is very useful, helping track how and where the disease is spreading among the population. In many countries like Brazil and Israel, WBE has been used for decades to detect polio where the disease remains endemic (7). More recently, efforts have been made to set up a surveillance system for other viruses via wastewater, such as the Zika virus, when Brazil first confirmed a novel febrile illness outbreak to WHO and the virus emerged as a cause of serious birth defect microcephaly and the Guillain-Barre syndrome neurological disorder (8).

Sewage is a source of information that can be used for public health observation and used as an instrument for refining public health response in the pandemic. Public health authorities could use this information to evaluate when and how to start scaling up or back quarantine-style policies and recommendations. WBE provides significant advantages to face obstacles faced by other commonly applied techniques, such as the reliable provision of Spatio-temporal trends in human behavior and infection, near-real-time, and whole population data (Ref.). Sewer systems receive human excreta that contain viral particles shed by infected humans, regardless of their symptomatology status (symptomatic; asymptomatic [no symptoms]; paucisymptomatic or subclinical [mild symptoms]; and presymptomatic [no symptoms for the first few days before exhibiting COVID-19 symptoms]) (Ref.). Wastewater monitoring during air travel and cruises may provide information for the prevalence of SARS-CoV-2 infections among passengers, which can be spread in this way internationally (9). By using WBE, it is expected that the collected samples from both the sewer lines and the wastewater treatment facilities will enable the tracing of viral outbreaks.

WBE can also enable tracking the silent circulation of the virus due to the detection of low levels of the viral RNA before cases appear (10). Moreover, Boulos & Geraghty (11) have shown the incidence of SARS-CoV-2 in wastewater since November 2019, before the recording of the first COVID-19 patients in Santa Catalina, Brazil. This finding indicates the shedding of the virus from paucisymptomatic and asymptomatic persons in the community, months before the reporting of the first cases by the national authorities (12). One important feature of WBE relies on the fact that it can detect variations in the viral strains via phylogenetic analysis. According to Nemudryi et al. (13), the most prevalent strains of SARS-CoV-2 detected in the wastewater of Bozeman, Montana, were related to those previously observed in Europe (France and Iceland). Genome sequencing and phylogenetic analysis carried out by Chan, J.F, and coworkers (14), revealed similarities among the isolated viral strains with those found in Europe and the Lombardy region in northern Italy.

WBE can also track seasonal fluctuations in viral concentrations in wastewater which indicate the epidemiological patterns in a community.  Nordgren et al. (15) reported seasonal variations of Nor viruses (Nov. GGI and Nov. GGII), with the highest concentration of these viruses being recorded during winter and summer, respectively. Another study conducted by Li et al. (16) in China, has proved that the concentration levels of rotavirus were higher from November to March, corresponding to the clinical data of the virus reported in the country (17). Since a correlation between the COVID-19 spread and temperature exists (18), it is predicted that seasonal variations of the viral genetic material will also occur in the sewage.

WBE field is in early stages and the position of the scientific community is still unclear, especially methodology for the isolation, detection, and quantification of the virus genetic material in the wastewater. Hence, storage condition, RNA extraction, and concentration procedures for sewage samples have been reviewed from various research papers and standard methods have been optimized for the present study.

**Evidence for the presence of SARS-CoV-2 in wastewater**

Like other parts of the world in Bangladesh we detected Covid-19 genetic materials from Noakhali Isolation Center Wastewater from 10th July to 29th August.

First detections of CoVs in wastewater were achieved in 2013 (20). In the same year, a viral metagenomic investigation allowing for untargeted molecular analysis of the whole viral community identified the CoV HKU1 genome (a ‘common cold’ CoV) in sewage sludge (21), providing evidence for CoV presence in wastewater. A recent study reported the molecular detection of animal CoV belonging to the genus Alpha coronavirus in surface-water in Saudi Arabia (22). During the SARS eruption in 2004 in China, SARS-CoV RNA was detected in 100% (10/10) of untreated and 30% (3/10) of disinfected wastewater samples collected from a hospital in Beijing, China receiving SARS patients (23). There have been early reports of the molecular detection of SARS-CoV-2 in wastewater in the Netherlands, USA, France, and Australia (24). These studies reported the detection of SARS-CoV-2 RNA in untreated wastewater with maximum concentrations over 106 copies per liter. The study in France detected SARS-CoV-2 RNA in treated wastewater as well, with concentrations of up to nearly 105 copies per liter (35). Details of these reports on molecular detection of SARS-CoV-2 RNA in wastewater are summarized in Table 1.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Country** | **Period of Examination** | **Positive sample from total samples.** | **Ct value** | **Reference** |
| Italy | 14/04/2020 - 22/04/2020 | 4(12) | - | (25) |
| Spain | 06/04/2020 - 21/04/2020 | 7(7) | 33.61 – 39.60 | (27) |
| Turkey | 21/04/2020-25/04/2020 | 7(9) | 34.67 – 39.54 | (28) |
| Netherlands | 05/02/2020 - 16/03/2020 | 10(24) | - | (24) |
| France | 05/03/2020 - 23/04/2020 | 100 % of samples | - | (23) |
| Australia | 20/03/2020-01/04/2020 | 2(9) | 37.5 - 39 | (4) |
| The U.S.A. | 08/01/2020 - 25/03/2020 | 10(12) | 33.87 - 38.39 | (13) |
| India | 04/05/2020 - 12/06/2020 | 6(17) | 5.19 – 37.52 | (25) |
| Israel | 10/03/2020-21/04/2020 | 10(26) | 32.76 – 38.5 | (24) |

Table 1-**Comparison of different studies WBE**

**SARS-CoV-2 specific quantitative RT-qPCR**

Sewage RNA was analyzed by RT-qPCR using SansureCovid-19 RT-qPCR Kit usingCFX96 Real-Time System, with a C1000 Touch Thermal Cycler (Bio-Rad) where primer pairs for ORF1ab, N, Rnase-p primer pairs were used (25). The sequences of primers and probes are found in Table-2. For probe-based Luna Universal One-Step RT-qPCR kits, 2 μL of RNA were subjected to reverse transcription performed at 55 °C for 10 min. Initial denaturation was performed for 1 min at 95 °C followed by 45 cycles of denaturation for 10 s and combined annealing and extension for 30 s at 60 °C. For the LightCycler® Multiplex RNA Virus Master kit, 5 μL of template RNA were used. Reverse transcription was performed at 55 °C for 10 min. Initial denaturation was allowed for 30 s at 95 °C followed by 45 cycles of denaturation for 5 s, extension for 30 s at 60 °C and final cool-down to 40 °C for 30 s. The PCR runs were analyzed with Bio-Rad CFX Manager software version 3.1 (Bio-Rad Laboratories).

### Table -2 the sequences of primers and probes are found in various research papers

|  |  |  |
| --- | --- | --- |
| **Oligonucleotide sequence (5′-3′)** | **Gene** | **Reference** |
| ACAGGTACGTTAATAGTTAATAGCGT | S-gene | (38,26) |
| ATATTGCAGCAGTACGCACACA |
| GTGARATGGTCATGTGTGGCGG | ORF1ab | (38,26) |
| CARATGTTAAASACACTATTAGCATA |

### **Effect of wastewater sampling, storage conditions**

#### **Effect of wastewater sampling method**

Both grab (18, 19, and 20) and composite sampling methods have been reported for the collection of wastewater samples for the detection of SARS-CoV-2 were composite samples most reliable compared to grab samples. The viral titers in composite samples were found to be lower (∼1500 viral genomes/L) than those in the grab samples (∼2 × 104 viral genomes/L). There is limited literature regarding the impact of the type of sampling on the detection of viruses in wastewater. Gerba et al. (26) suggested that 24-h composite samples can enable catching the peak flows. The sampling time also plays significant role to the methodology applied for the detection of the virus. For example, in most cities, the flow rate of sewage is highest in the morning and evening hours. It is noted that only a few studies reported the time of grab sampling for the detection of SARS-CoV-2 in wastewater; 7-12 pm (26) and 1.00 pm (27).

#### **Effect of temperature of wastewater sample storage**

According to the virology and microbiology guidelines (28, 26), samples for viral analysis within less than 48 h are usually kept at 4 °C in the dark, whereas storage at lower temperatures (20 °C or−80 °C) for longer periods to maintain sample integrity. There is a scarcity of information available on the effect of the freezing process on the virus vitality (physiological capability of the live examined viruses (25). The study of Olson et al. (29) on the effect of storage temperature on the viability of the MS2 bacteriophage in wastewater, revealed that viral degradation does not seem to occur when samples are stored at 4 °C for one week before degradation of the virus equaled the initial virus loss due to freezing at −80 °C. It was also observed that the virus titers were substantially lower after sample storage for an approximately 40-day period at 4 °C compared to those observed upon sample storage at −80 °C. Interestingly, viral degradation was shown to increase at -20 °C compared to 4 °C and−80 °C, owing to the formation of large ice crystal, which provokes viral damage.

#### **Effect of thermal sample pre-treatment**

In some studies, wastewater samples were subjected to thermal treatment (56 °C for 30 min or 60 °C for 90 min), to increase the safety of the laboratory personnel during sample handling ([22](https://www.sciencedirect.com/science/article/pii/S2213343720306552#bib0110); [31](https://www.sciencedirect.com/science/article/pii/S2213343720306552#bib0155)). The thermal treatment of the sample was shown to reduce the infectivity of SARS-CoV-2([60](https://www.sciencedirect.com/science/article/pii/S2213343720306552#bib0300)). Also, raw wastewater samples were pasteurized at 60 °C for 90 min to inactivate SARS-CoV-2 ([31](https://www.sciencedirect.com/science/article/pii/S2213343720306552#bib0155)). The thermal treatment of samples is dependable with previous studies dealing with enveloped virus survival in pasteurized wastewater (32). The time required for 90% viral inactivation (T90) ranged between 7-13 h for the enveloped viruses ϕ6 and MHV in unpasteurized wastewater at 25 °C, whereas an increase in the T90 values to 28-36 h was observed at 10 °C.

**Sars-Cov-2 persistence and fate in the environment**

**SARS-CoV-2 shedding by infected individuals**

Wu et al. (33) demonstrated excretion of the SARS-CoV-2 virus after 3 weeks in phlegm and 4 weeks in stools. Other researchers have diagnosed clinical specimens from 73 hospitalized patients infected with SARS CoV-2 where thirty-nine patients tested positive for SARS-CoV-2 RNA in stool samples and 17 of those patients remained positive for SARS-CoV-2 in stools after becoming negative in respiratory samples (34, 35). This finding has now been repeated in various studies showing the extended duration of shedding of the virus in fecal samples. Sethuraman et al. (36) reported that in some cases, viral RNA can be detected in stool samples by RT-PCR 6 weeks after the first positive test.

**SARS-CoV-2 persistence in the environment**

Chin et al. (2020)(37) noted that the SARS-CoV-2 virus is vulnerable to standard disinfection methods and was undetected after 5-minute contact with household bleach (sodium hypochlorite) at various concentrations (1:49 and 1:99 dilution ratios), ethanol (70%), povidine-iodine (7.5%), chloroxylenol (0.05%) and chlorhexidine (0.05%). Chin et al. (2020)(38) also reviewed the stability of SARS-CoV-2 within the wastewater by incubating the virus in a virus transport medium at various temperatures for up to 14 days and then tested for infectivity. SARS-CoV-2 virus infectivity was also assayed following incubation on different surfaces, exposure to varying pH

values and different disinfectants. The researchers found that infectivity was still detectable on day 14 when the virus was incubated at 4°C, whereas at 70°C the virus was inactivated in 5 minutes. In the same article, the stability of the virus on various surfaces was tested by dropping the cultured virus onto surfaces left at room temperature (22°C) and a relative humidity of 65%. They found that treated smooth surfaces, particularly steel and plastic, support the persistence of infective virus more than rougher surfaces such as tissue paper, wood and cloth. The virus was stable at a range of pH values (at room temperature).Van Dormagen et al. (39) compared SARS-CoV-2 to the 2005 SARS-CoV-1 in terms of viability in aerosols, finding that, like SARS-CoV-1, SARS-CoV-2 also remains viable in aerosols (testing was for 3 h).

Although there is limited data on the survival of SARS-CoV-2 in water, because they behave similarly in aerosols, similar conduct is likely for SARS-CoV-1 and SARS-CoV-2 in water and wastewater. SARSCoV-1 was predicted to be very stable at 4°C in filtered tap water, and was found to remain live in stools for 6 days at room temperature, with fragments of SARS CoV-1 being detected in wastewater for up to 3 days, making it less stable in wastewater than poliovirus (40).

**Environmental surveillance of Sars-Cov-2**

Many COVID-19 infections are asymptomatic and unless tested, can remain undetected. As a complementary approach to monitoring the spread of COVID-19, many countries have since implemented wastewater-based surveillance of COVID-19 infections by monitoring the absence and presence, and concentration of SARS-CoV-2 viral particles in wastewater and contaminated environmental water sources (Ref.). Environmental surveillance has also been used and recommended for other infectious disease-causing microorganisms such as typhoid (WHO, 2018), early warning of hepatitis A and norovirus outbreaks (41), as well as for antimicrobial resistance (42), with modeling techniques used to assist both the design and interpretation of those efforts (44). A compartmental epidemiological model developed by (43) suggested that contaminated natural water bodies could become environmental reservoirs of SARS-CoVs, which would require the enforcement of strict post-epidemic measures to prevent re-infection. Currently, however, the minimal infectious dose (MID) of SARS-CoV-2, that is, the number of viral particles that causes an infection, for humans is unknown (45) and while SARS-CoV-2 has been detected in sewage and has been described to survive for 14 days in sewage at 4°C, and 2 days at 20°C, no fecal-oral transmission has yet been described for COVID-19. Due to its lipid envelope, it is expected that the new CoV will be less abundant as an infectious virus in wastewater when compared to other known enteric viruses, and less stable when exposed to water treatment processes in water and wastewater treatment plants (Ref.). In the context of surveillance, the presence or absence of the virus in wastewater is relevant, not due to the potential risk of infection spread, but because of the potential to determine the presence of infected individuals in a community (Ref.). More research is required to determine the potential for infection due to exposure to untreated wastewater or environmental sources contaminated with untreated wastewater. The following pilot study was planned and implemented to assess the justifiability of WBE in the context of Bangladesh.

**AIMS AND OBJECTIVES OF THE STUDY**

The aim of this study was to test the feasibility of applying the WBE and environmental water surveillance concept in Bangladesh as a tool to provide valuable information about the spread of Covid-19 virusand also as an early warning system for infection in a community providing a more sensitive and rapid indication of changes in infection rates before such effects become detectable by clinical health surveillance system. More specificay, this will provide decision support for officials determining the timing and severity of public health interventions to mitigate the overall spread of the disease. This experiment will also serve as a short-term, proof of concept study before the roll-out of national surveillance system, and also involves preliminary testing, optimization, and validation of sampling and virus analysis methods, as well as results interpretation and reporting in the Bangladesh context.

The specific objectives of the study were as follows:

* 1. To Detect SARS-CoV-2 from Wastewater samples from different geographical location of Bangladesh including Dhaka City;
  2. To develop waste water based surveillance system to detect the presence of COVID-19 in a community;
  3. To develop and establish the need for waste water based epidemiology in order to enable early identification and spatial-based monitoring of future outbreaks;
  4. To assist govt. and local authorities to upgrade the COVID-19 surveillance system.

**MATERIALS AND METHODS**

**ETHICS APPROVAL**

The study was reviewed and approved by the University of Pretoria (UP) Faculty of Health Sciences

Research Ethics Committee (Ethics Reference no.: 374/2020).

**SAMPLING SITES**

**Selection of sampling sites**

Sampling sites in this study were selected based on the COVID-19 hotspots (based on the number of Confirmed Covid-19 Positive Cases) .

According to the <http://dashboard.dghs.gov.bd/webportal/pages/covid19.php> confirmed cases are presented in Figure 2-1. For a more detailed case number of Bangladesh, please refer to Appendix A. From all places Dhaka had the highest number of confirmed cases at **109183**, followed by **Chattogram** at **19708**13 023 confirmed cases.

|  |  |
| --- | --- |
| **Distirct** | **Confirmed Cases** |
| **Barishal** | **3633** |
| **Brahmanbaria** | **2470** |
| **Chattogram** | **19708** |
| **Cox's Bazar** | **4855** |
| **Cumilla** | **7670** |
| **Dhaka** | **109183** |
| **Kishorgonj** | **2895** |
| **Khulna** | **6500** |
| **Rajshahi** | **5033** |
| **Gaibandha** | **1200** |
| **Rangpur** | **2909** |
| **Habiganj** | **1769** |
| **Sylhet** | **7126** |
| **Total** | **174951** |

**Sampling:**

The wastewater samples were collected from 16 locations of the seven divisions capturing major districts in Bangladesh. The selected districts for sampling were Dhaka, Sylhet, Chittagong, Mymensingh, Rajshahi, Khulna, Barisal, Rangpur Gaibandha, and Cox’s Bazar including Rohinga Camps at the Ukhia Upazila. This is to be mention-worthy that wastewater specimens were collected from various important locations of the capital Dhaka City as to be mentioned; Kuwait Moitri Hospital, Mugda Medical College and Hospital and Kurmitola General Hospital, Mohammadpur, Korail Slum, and Mirpur Slum (fig-2).

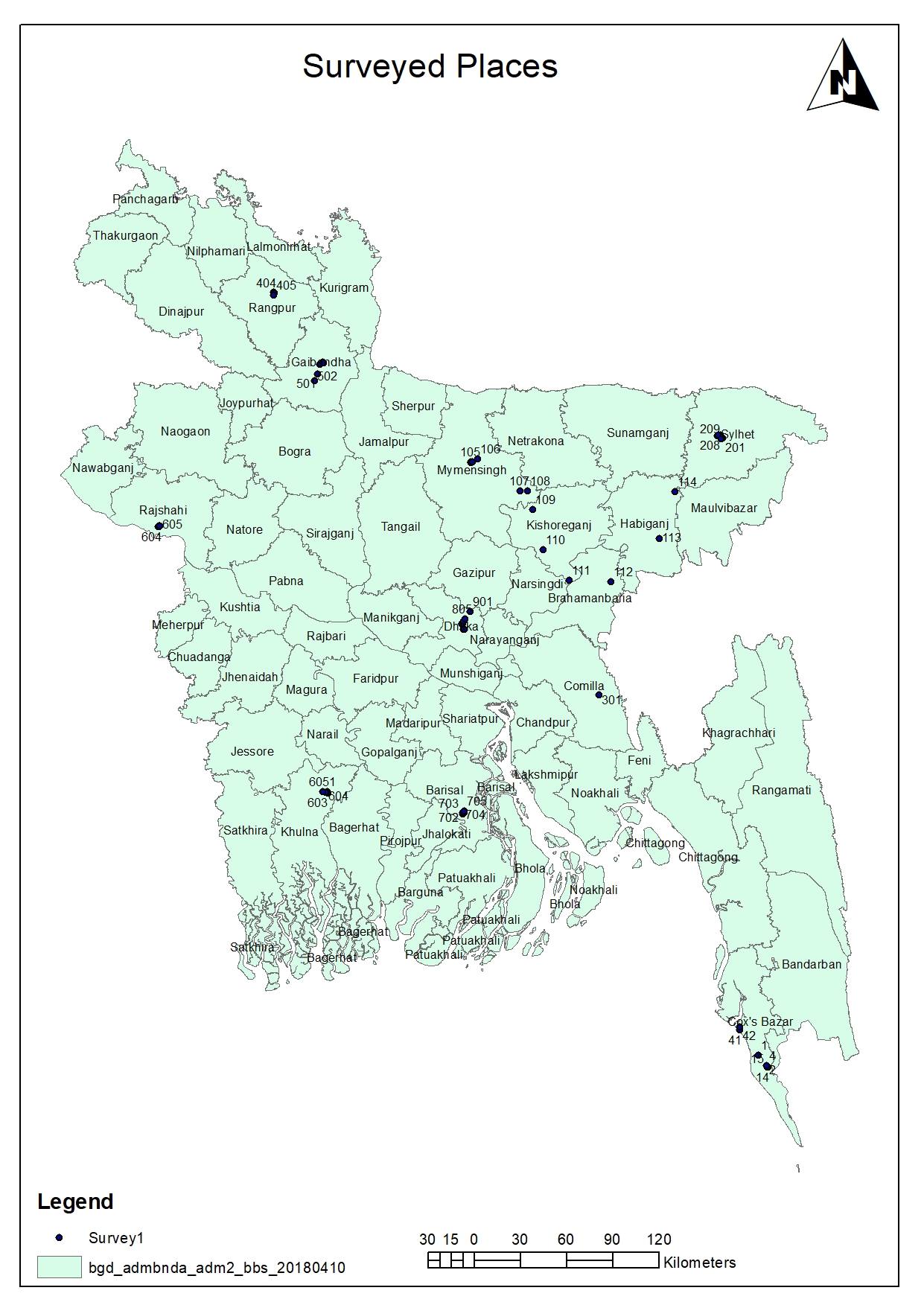


Fig-2 Sampling locations are indicated black dot

Specific locations (Table-3) were used for sample collection that continued two times during the tenure from November 2020 to January 2021. Relevant physicochemical and meteorological data were collected using sample collection form (Fig-3). Samples were collected in 50ml sterile falcon tubes and carried in the ice-box. All wastewater specimens were refrigerated at 4oC and brought to the laboratory for further analysis. Sterile falcon tubes used for sampling and blanks in the same group were analyzed to determine if there is any contamination during the transport. During sampling time various external factors are counted (Fig-3). All the experiments and analyses were carried out at the Covid-19 Diagnostic Lab, Dept. of Microbiology, Noakhali Science and Technology University.

Table-3 Sampling sites for each place

**Environmental factors**:

As environmental factors play significant role for Covid-19 temperature, PH of samples are measured. For that portable PH meter (Milwaukee) and thermometer (TP-300) are used. These parameters are measured immediately after sample collection within 5-10 minutes.

**Sample preparation, concentration, and RNA Extraction procedure**

We followed the same extraction procedure, as described by Kumar et al. (25). Briefly,

sewage samples (50 mL) were centrifuged (Thermo Scientific) at 4500×g for 30 min followed by

filtration of supernatant using 0.22-micron filters (Himedia). Further, each sewage filtrate was

concentrated using the polyethylene glycol (PEG) method. In this method, PEG 6000 (80 g/L) and NaCl (17.5 g/L) were mixed in 25 ml filtrate, which was then incubated at 17°C in 100 rpm shaking overnight. The next day, the mixture was centrifuged at 13000×g for 90 min. The supernatant was discarded after centrifugation, and the pellet was suspended in 300 μL RNase free water. This was further used as a sample for RNA isolation using a commercially available QIAamp Viral RNA Mini Kit Viral Nucleic Acid Extraction Kit. (Fig-4)

|  |
| --- |
| **QIAamp Viral RNA Mini Kit Protocol: Purification of Viral RNA (Spin Protocol)**  **Procedure:**  1. Pipet **560 μl** prepared Buffer AVL containing carrier RNA into a **1.5 ml** microcentrifuge tube.  2. Add **140 μl** plasma, serum, urine, cell-culture supernatant, or cell-free body fluid to the  Buffer AVL–carrier RNA in the microcentrifuge tube. Mix by pulse-overtaxing for **15 s.**  3. Incubate at room temperature for **10 min.**  4. Briefly centrifuge the tube to remove drops from the inside of the lid.  5. Add 560 μl ethanol (96–100%) to the sample, and mix by pulse-vortexing for **15 s**. After  mixing, briefly centrifuge the tube to remove drops from inside the lid.  6. Carefully apply **630 μl** of the solution from step 5 to the QIAamp Mini column (in a 2 ml  collection tube) without wetting the rim. Close the cap, and centrifuge at **6000 x g**  **(8000 rpm) for 1 min**. Place the QIAamp Mini column into a clean 2 ml collection tube,  and discard the tube containing the filtrate.  7. Carefully open the QIAamp Mini column, and repeat step 6. If the sample volume was  greater than 140 μl, repeat this step until all of the lysates have been loaded onto the spin column.  8. Carefully open the QIAamp Mini column, and add **500 μl Buffer AW1**. Close the cap,  and centrifuge at **6000 x g (8000 rpm) for 1 min**. Place the QIAamp Mini column in a  clean 2 ml collection tube (provided), and discard the tube containing the filtrate.  9. Carefully open the QIAamp Mini column, and add **500 μl Buffer AW2.** Close the cap  and centrifuge at full speed **(20,000 x g; 14,000 rpm) for 3 min.** Continue directly with  step 11; or to eliminate possible Buffer AW2 carryover, perform step 10 and then  continue with step 11.  10. Recommended: Place the QIAamp Mini column in a new 2 ml collection tube (not  provided) and discard the old collection tube with the filtrate. **Centrifuge at full speed for**  **1 min.**  11. Place the QIAamp Mini column in a clean 1.5 ml microcentrifuge tube (not provided).  Discard the old collection tube containing the filtrate. Carefully open the QIAamp Mini  column and add **60 μl Buffer AVE** equilibrated to room temperature. Close the cap, and  incubate at room temperature for **1 min.**  12. Centrifuge at **6000 x g (8000 rpm) for 1 min.**  A single elution with 60 μl Buffer AVE is sufficient to elute at least 90% of the viral RNA  from the QIAamp Mini column. Performing a double elution using 2 x 40 μl Buffer AVE  will increase yield by up to 10%. Elution with volumes of less than 30 μl will lead to  reduced yields and will not increase the final concentration of RNA in the eluate.  **Viral RNA is stable for up to 1 year when stored at −30 to −15°C or–90 to −65°C.** |

Fig-4 Protocol of Viral RNA extraction kit **(QIAamp Viral RNA Mini Kit)**

Besides, all the experiments were performed three times for confirmation of the results and

accepted where variations were less than 10%. Covid-19 positive patient samples were used as

an extraction control in each run. We employed qualitative measurement, and hence, increasing

and decreasing viral load is measured based on the Ct value. RNA concentrations were measured by NanoDrop (Thermo ScientificTMNanoDrop 2000 and 2000c, BioRad) and were stored at -70 °C until further use.

* 50ml sewage samples in Falcon Tube
* Centrifuge at 4,500×g for 30 min
* Transfer the Supernatant
* Filter through 0.22µl syringe filter
* Collect 25ml of filtered supernatant
* Add 2 g PEG 9000+0.437 g NaCl
* Incubate this mixture at17°C,100 rpm for overnight
* Centrifuge at 13,000×g for 90 min
* Discard the supernatant and resuspend the pellet in 10µl of Rnase free water
* RNA extraction by QIAamp Viral RNA Mini Kit Protocol
* RT-PCR for detection of Covid-19 gene

**2.3.** **RT-PCR Analysis**

RNAs were analyzed for the detection of SARS-CoV-2 by RT-PCR (CFX96, BioRad) using the

50ml sewage samples in Falcon Tube

Centrifuge at 4,500×g for 30 min

Transfer the Supernatant

Filter through 0.22µl syringe filter

Collect 25ml of filtered supernatant

Add 2 g PEG 9000+0.437 g NaCl

Incubate this mixture at17°C, 100 rpm for overnight

Centrifuge at 13,000×g for 90 min

Discard the supernatant and resuspend the pellet in 10µl of Rnase free water

RNA extraction by QIAamp Viral RNA Mini Kit Protocol

RT-PCR for detection of Covid-19 gene

Ensure RT-PCR kit (Sansure Biotech Inc., China). As described in the product manual, technical procedures carried out, and interpretations of results were made. In brief, we set the sample layout with RT-PCR protocol covering 45 cycles(Table-4)(Fig-4) containing FAM fluorescence select for ORF1ab, ROX for N gene as well as CY5 for Internal control.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| SL | Steps | Temperature(°C) | Time | Cycle No. |
| 1 | Reverse transcription | 50 | 30 min | 1 |
| 2 | cdna predenaturation | 95 | 1 min | 1 |
| 3 | Denaturation | 95 | 15 sec | 45 |
| 4 | Annealing, extension, and fluorescence collection | 60 | 30 sec |
| 5 | Device cooling | 25 | 10 sec | 1 |

Table-4 Steps and Cycle for Sansure RT-PCR Kit

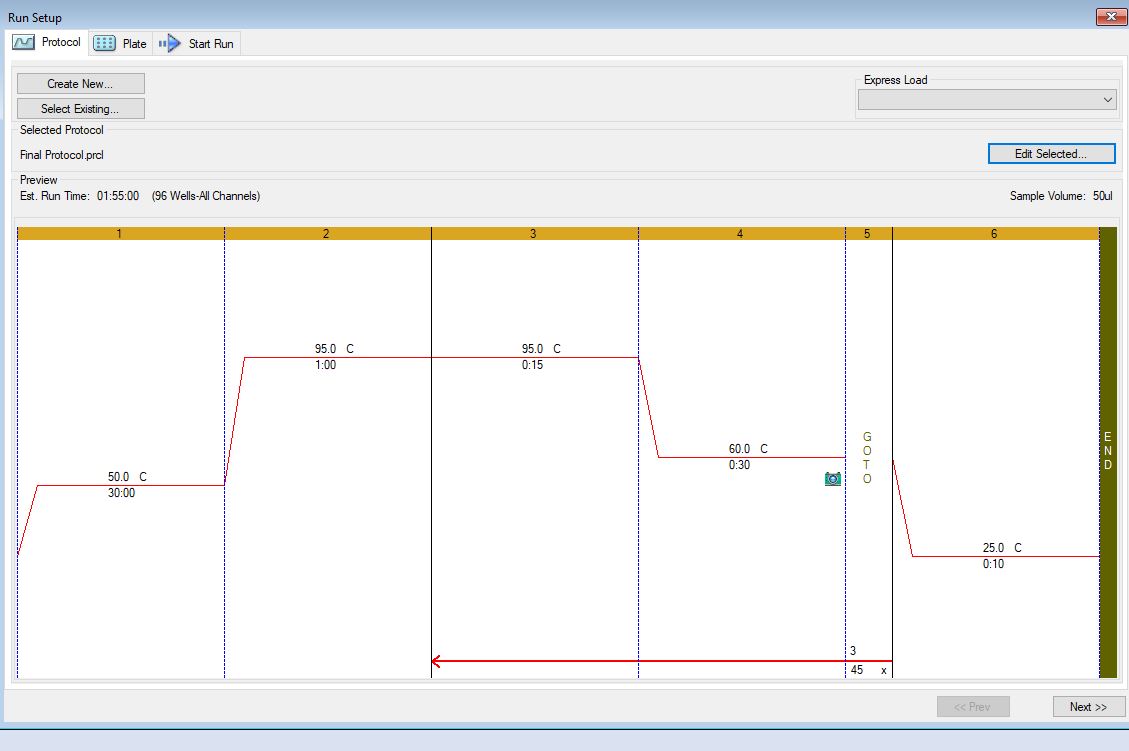


Fig-5 Protocol of Sansure RT-PCR Kit

As quality control measures, one positive control and one negative control were also run to validate the test procedure (Fig-5).

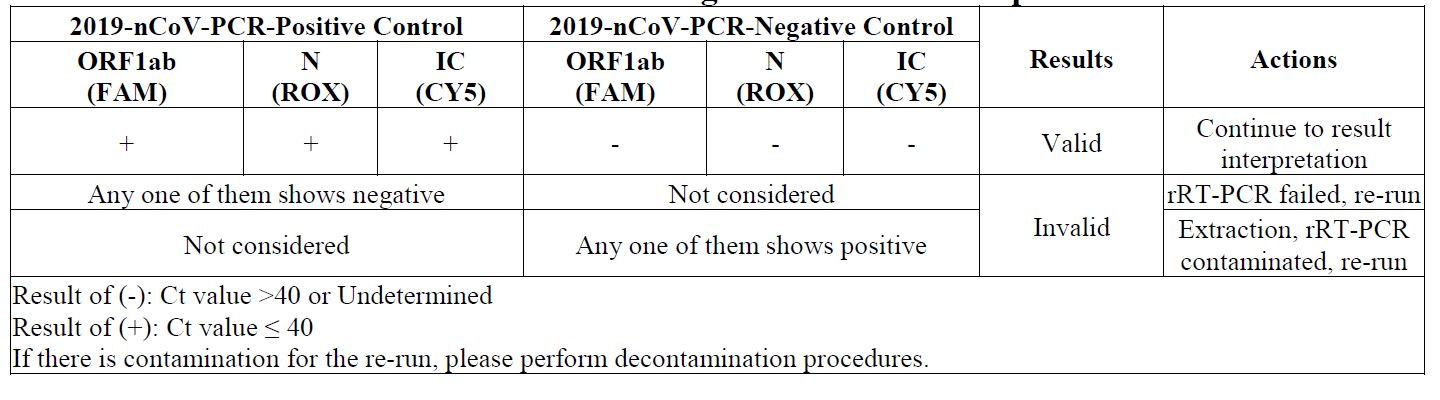


Fig-6 Positive and Negative control with interpretation

The Novel Coronavirus (2019-nCoV) Nucleic Acid Diagnostic Kit (PCR-Fluorescence Probing) is a real-time reverse transcription-polymerase chain reaction (qRT-PCR) test. The 2019-nCoV primer and probe set(s) is designed to detect RNA from SARS-CoV-2. Components Included within the RT-PCR kit given in table-5.

|  |  |  |  |
| --- | --- | --- | --- |
| **SL.** | **Reagent Name** | **Spec. & Qty.** | **Main Ingredients** |
| 1 | Sample Release Reagent | 1200 μL/tube x 2 | Lysis buffer(S03) |
| 2 | 2019-nCoV-PCR Mix | 1248 μL/ tube x 1 | Primers, Probes, dNTPs, MgCl2, Rnasin, PCR buffer |
| 3 | 2019-nCoV-PCR-Enzyme Mix | 192 μL/ tube x 1 | RT Enzyme, Taq Enzyme |
| 4 | 2019-nCoV-PCR-Positive Control | 500 μL/tube x 1 | In vitro transcriptional RNA for ORF1ab, N gene and internal control RNase P gene |
| 5 | 2019-nCoV-PCR-Negative Control | 500 μL/tube x 1 | Saline |
| 6 | Sample Storage Reagent | 2.0 mL/tube × 24 × 2 | 0.9% saline, Rnasin |

Table-5 **Components Included within treasure RT-PCR kit**

**RESULTS AND DISCUSSIONS**

**Sample Collection**

Text..District,num of sam ,date,…table lat,long,date

we take repeat sample from same place after 15 days where first round completed from 26th November to 11th December, 2020..Total sample number..district num..

**First round sample collection**

**Genes choose…for Ban perspective..DGSH….2 GENE BASED..(MS)**

**Human kits ..gene used for sewage sample ..DFA**

For the first sampling, we collected a total of 119 wastewater samples from 18 districts and found 114 positive samples (95%), for various genes while all three genes (ORF1ab, N, Internal Control-IC) found in 26 samples (21%), N gene, and IC in 15 samples (12%), ORF1ab gene and IC in 22 samples (18%) (Table-6).Rest of the samples…(MS)

Specific data from other counties of World ..MS

INDIA (Orophra…Patient sample..kit) DFA

Fig-Gene specific for Dye\Fluro..

|  |
| --- |
|  |
| **Fig 7:** Distribution of Ct values in seven divisions after first time(Decem..) and second time(Jan..) sampling in the wastewater specimens..CY5..IC,ROX-N gene,FAM-ORF1ab |

CT value defi…MS

**Second round sample collection**

After analysis of the second sample collection, which completed from 12th December to 27th December we found 117 specimens were positive(98%) while the three genes; ORF1ab, N, IC were found in 12 samples(10%), with N and IC genes in 23 samples(19%), and ORF1ab, IC genes in23 samples(19%) among the total 119 wastewater samples(Table-6).

Country wise comparision..MS

**Different gene comparison – Comparative analysis of gene expression**

Compared to the two sampling phases, the Cy5 gene in the first stage was more common, while the Cy5 gene was more common in some districts in the second process. The proportion of Cy5 genes in the first sampling in Dhaka City was nevertheless greater. The second large scenario is seen in Barishal.

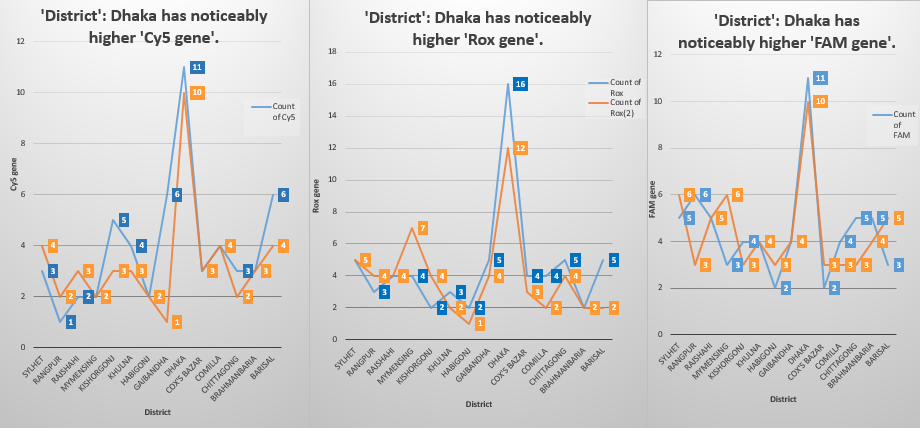


Fig-17: Dhaka has noticeable higher number of gene count…(ORF1ab,N,IC)

District vs Cy5,ROX, FAM gene confirmed the ratio

The situation for the FAM gene is a little like the Cy5 gene. In this situation, the share of FAM in the Dhaka section tends to be higher than in other districts. The FAM gene covered 11 positions out of 12 in Dhaka City as a positive ratio. Other districts have been averaged in the interim.

Here, in the Rox gene, the position of two additional genes is almost insensitive. However, in the second stage of Mymensingh, the proportion of Rox in the second component is relatively higher. Rox was confirmed in 16 places out of 18 samples, on the other hand.

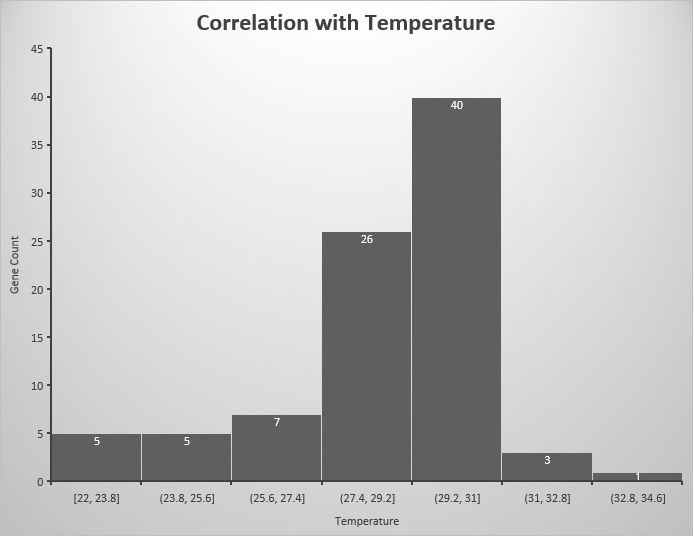


Fig-19: Gene correlation with temperature..(Scatter plot with line diagram.)

Here gene participation is between 27.4 and 31 degrees celsius in the warm temperature. With three genes combined between 27.4 and 29.2 grades Celsius, 26 samples are positive. Meanwhile, with 29,2 to 31 degrees Celsius 40 samples were confirmed.

Chart type: Histogram. Frequency of 'ph'

Description automatically generated

Fig-20: pH-gene association …..(Ph AND TEM in a same fig)

The neutral point requires the full commitment of confirmed ratios. The field of confirmed samples is better indicated by 6.7 to 7.4 areas.

**Comparison between First and Second round Sampling**

It was found genetic materials of Covid-19 in every places that indicates the confirmed cases of Covid-19 positive patients in all over the Bangladesh. In the both (1st time and 2nd time) sampling we found lowest number Cy5 gene and the highest number FAM gene .This particular findings indicate that genetic materials of Covid-19 are more resistant than that of the control gene. A total of 55 Cy5 gene was found from the first round sampling whereas it was 46 from second round sampling. The number of Rox and FAM gene were found 63 and 56 and 63 , 62 respectively(Fig-).

**Explanation of diff gene number….for tem and ph**

|  |  |
| --- | --- |
|  |  |
|  |  |

In the total 238 sewage samples we got 36%FAM,35%ROX and 29%CY5 gene.

**Heading..(Highest ct value found for ORF..)**

**Statistical Analysis of Data:**

From first time sampling mean of three genes are IC (35.97), O RF1ab (35.92), N (35.74) while SD of three genes is IC (2.78), ORF1ab (2.55), N (2.54) as well as second time mean of three genes are IC (36.83), ORF1ab (36.32), N (36.04) whereas SD of three genes is IC (1.66), ORF1ab (1.93), N(2.12) (Table-7,8).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Characteristics | Mean | SD | Min | Max |
| pH | 7.12 | 0.72 | 6.30 | 12.50 |
| Temperature | 28.68 | 2.15 | 22.10 | 32.60 |
| Cy5 (1) | 35.97 | 2.78 | 25.45 | 39.48 |
| FAM (1) | 35.92 | 2.55 | 26.14 | 39.37 |
| Rox (1) | 35.74 | 2.54 | 26.28 | 40.85 |
| Cy5 (2) | 36.83 | 1.66 | 31.53 | 39.70 |
| FAM (2) | 36.32 | 1.93 | 32.11 | 39.73 |
| Rox (2) | 36.04 | 2.12 | 31.00 | 39.65 |

Table 7: Descriptive analysis of result data

No significant differences were observed when the three genes Ct value of the first and second collection specimens was compared with each other.

**Correlation of ct values with sample’s PH and Temperature-(PH and Temp affect viral loads)**

**Heat map and PCO..(MS)**

Physicochemical parameters, such as PH (6.30-12.50) and Temperature (22.10-32.60) of all wastewater samples are measured. Samples mean PH observed as 7.12 with a standard deviation (SD) of 0.72 while the mean temperature was found 28.68ºC with 2.15ºC standard deviation.

Effect of different physicochemical parameters

Table rearrange..MS…Gene name..

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Parameters | pH | Temperature | Cy5 (1) | FAM (1) | Rox (1) |
| pH | 1 | **0.221\*** | 0.151 | **0.272\*** | 0.165 |
| Temperature | **0.221\*** | 1 | **0.367\*** | **0.746\*** | **0.379\*** |
| Cy5 (1) | 0.151 | **0.367\*** | 1 | 0.237 | 0.189 |
| FAM (1) | **0.272\*** | **0.746\*** | 0.237 | 1 | **0.489\*** |
| Rox (1) | 0.165 | **0.379\*** | 0.189 | **0.489\*** | 1 |

Since the p-value observed 0.021, which is less than 0.05 and this indicated that the overall model could significantly predict the dependent variable. So, we conclude that the effect of temperature has the effect or valid in predicting pH. Here, R square is 0.22 or 22% of the variation in Temperature can be predicted from the variables pH. The regression equation was: 5+0.076x

The equation showed that the coefficient for Temperature is +0.076. The coefficient indicated that for every additional unit in Temperature, pH increased by an average of 0.076 units.

Tempara and PH correlation with ct value…MS

Correlation of **patients data** with factors…tem or others..

|  |
| --- |
|  |
| **Fig-12** Strong Correlation of pH &Temperature, Cy5 (1) & Temp, FAM (1) & Temp, FAM (1) & Temp with Ct value |

The result was statistically significant…(p=..)

Since the p-value found0.0014, which is less than 0.05 and this indicates that the overall model can significantly predict the dependent variable. So, we conclude that the effect of temperature has the effect or valid in predicting Cy5(1). Here R square is 0.37 or 37% of the variation in Temperature can be predicted from the variables Cy5(1). The regression equation was: 22+0.49x

The equation showed that the coefficient for Temperature is +0.49. The coefficient indicated that for every additional unit in Temperature, Cy5(1) increased by an average of 0.49 units.



Since the p-value is <0.001 which is less than 0.05 and this indicates that the overall model can significantly predict the dependent variable. So, we conclude that the effect of Temperature has the effect or valid in predicting FAM (1). Here R square is 0.88 or 88% of the variation in Temperature can be predicted from the variables FAM (1). The regression equation was: 11+0.88x

The equation shows that the coefficient for Temperature is +0.88. The coefficient indicates that for every additional unit in Temperature, FAM (1) increases by an average of 0.88 units.



Since the p-value is 0.0012 which is less than 0.05 and this indicates that the overall model can significantly predict the dependent variable. So, we conclude that the effect of Temperature has an effect or valid in predicting Rox (1). Here R square is 0.38 or 38% of the variation in Temperature can be predicted from the variablesRox (1). The regression equation was: 23+0.43x

The equation shows that the coefficient for Temperature is +0.43. The coefficient indicates that for every additional unit in Temperature, Rox (1) increases by an average of 0.43 units.







**SARS-COV-2 ANALYSIS IN Different WASTEWATER SAMPLES**

**Rohinga Camp-**

**About Rohinga Camp…Why ….Patient sample .stigma….Wastewater monitoring.**

We collected 23 sewage samples(Treated and raw ) from Rohinga Camp ,Kutupalong RC Camp-2E,2W,4 and 4 Ext where we found Covid-19 genetic materials all types of samples. Interestingly we found positive genes from waste water treatment plant in various stages of treatment.

By comparing with all other places the presence of Covid-19 genetic material is very low although the number of population in Rohinga camp more than others area. That denotes food habit, life style strategy, immunity, hygiene, education also responsible for affecting Covid-19.

But the sewage treatment in Rohinga camp can’t able to degrade viral genetic materials as we found positive genes in the sewage collected from outlet of Upflow filter ,Trickling filter, Maturation pond,Laggon-1, Lagoon-2,Digest bed even Lime treated discharge. We found highest ct value from Rohinga camp 39.85 and maximum ct value.



**1st round and 2nd round should be …graph**

**This findings indicates that the strain of Covid-19 from Rohinga Camp are less virulent as we found less N gene and higher SD value…..strategy ….this reults also proved by talking by loacal people and alao authority…death and positive less..need more research and seq of covid-19**

**Possible explanation..**

**Rox high**

**1.virulant…spreading**

**2….**



**Dhaka City**

****

**Dhaka is the capital of Bangladesh, largest city and center of all kinds of business where people come from different parts of the world, living huge number of population including various slum area which indicates the genetic materials of Covid-19 high and patients are affecting highest ratio with both confirmed cases and death cases.**

**In this study we observed several important places, slum areas as well as hospital’s sewage sample where we found highest number of all Covid-19 genetic materials from whole over the Bangladesh. After analysis it was demonstrated that all samples collected from Dhaka city was positive for Covid-19 various genes either first time or second time.**

**We detected the Ct value range in the first and second round sampling respectively CY5 (30.72-38.44), FAM (32.12-37.61), ROX (33.11-37.48) where CY5 (31.8-39.7), FAM (33.3-38.75), ROX (31.0-39.32), where second round Ct value was higher than first round sampling. It specified that in the second time moth of January Covid-19 patient number was lower than first time sampling month of December.**

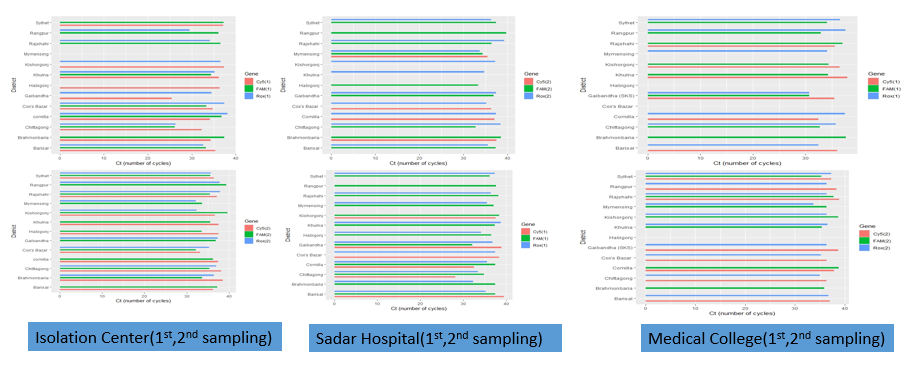
**Another findings of our study proved that in the Dhaka city the number of bacteria was highest per ml from all other samples. It opens another door of research that we might be get any indicator bacteria in the community which able to help easily detect Covid-19 gene without expensive molecular laboratory.**

**It might be claimed that number of bacterial population has strongly association with Covid-19 genetic materials as we also found higher number of ct value with lower number of bacterial population in Rohiga camp sewage sample from both treated and row samples.**

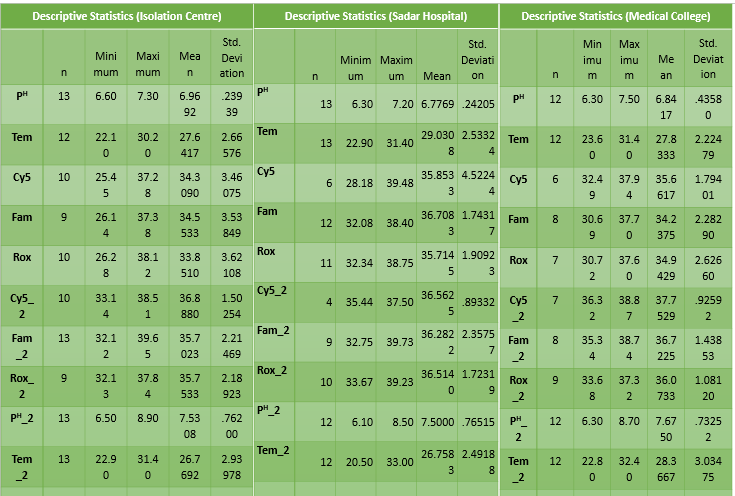
**Another reason for highest number of bacterial population might be for PH of Dhaka city that was first and second round sequentially (6.60-8.70) and (6.60-8.90).From literature review it found that in the neutral conditions are favorable for bacterial population that was not found from other places.**

**Others place**

|  |  |  |
| --- | --- | --- |
|  |  |  |
|  |  |  |



**The most outstanding result in our experiment was the Ct value of isolation centers sewage sample that indicates the main source of Covid-19 genetic materials as we got lowest ct value from all results. We found the lowest Ct CY5(25.45),FAM and ROX(26.14 AND 26.28) from Gaibandha and Chittagong isolation center respectively.**

****

**This result also denotes we need to take highest precautions for treatment of isolation centers sewage and research needs to continue to find out the proper treatment system for these types sewage as all of Cocid-19 positive patients stay here before recovery. Another way the genetic materials might be mixed with aquatic system and that will turn dangerous thing by incorporating and genetic materials transformation using horizontally or vertically gene transformation method in others pathogens.**

**To stop the unwanted scenario, it is high time to change the waste water system of whole Bangladesh and monitoring the status of waste water regularly for all the individual areas.**

**From various places it could be strongly agree that the viral genetic material and spreading are not same in all over the country. For that individual area should be monitored that cann’t match with one another for various factors like strategy of life style, availability of antibiotics, age of populations as well as environmental factors. Again we found the different ct values during two time sampling that indicates continuous monitoring system should be developed. Seasonal functions play important role for pathogenic virus or bacteria like in the summer diarrheal patient could be found more than winter season again in the low temperature flu like disease spraed more the in the summer season.**

**SUMMARY OF THE RESULTS**

The monitoring of COVID-19 genes by wastewater opened the door to further investigation employing an updated research method. However, we have done the same with some new approaches to guarantee the existence and interaction of selective genes with the unwelcome outbreak of additional viral flue. We also concurrently focused on monitoring the continuing spread of COVID and proposed wastewater treatment solutions. What we know will be useful for the time being.

Samples were obtained in 11 districts of Bangladesh along with the Rohingya Camp. In two stages of sampling, we stressed the interaction of the COVID-19 gene with pH and temperature. Here, the combination of pH and temperature with these genes-ROX, FAM, and Cy5-has shown a comparatively large ratio. Between the two stages of the data study, the first-time data revealed a more correlated role for both environmental and non-environmental variables.

Both drainage samples are measured by their physiochemical parameters, including PH (6.30 - 12.50) and temperature (22.10 - 32.60) ºC. Samples mean PH observed as 7.12 with a standard deviation (SD) of 0.72 while the mean temperature was found to be 28.68ºC with a standard deviation of 2.15ºC.

We assume that the temperature effect has an effect or is true when forecasting pH as the p-value measured was 0.021, which was below 0.05. The overall model could forecast the variable significantly. Here, R square is 0.22 or 22% of the difference in temperature from pH variables can be expected. The equation suggested that the temperature coefficient is +0.076. The coefficient revealed that pH improved by an average of 0.076 for each additional unit at temperature.

We also infer that the influence of temperature in predicting Cy5 is successful or true. Since p is 0.0014, which is less than 0.05, the overall model will forecast the dependent variable significantly. In this case, the R square is 0.37 or 37% of the temperature differences from the Cy5 variables. The equation for regression was: 22+0.49x. The equation suggested that the temperature coefficient is +0.49. The coefficient revealed that Cy5 improved by an average of 0.49 for each additional unit within the Temperature.

We found that the effect of Temperature has an effect or valid in predicting FAM since the p-value is <0.001 which is less than 0.05. Here R square is 0.88 or 88% of the variation in Temperature can be predicted from the variables; FAM. The regression equation was: 11+0.88x. The equation shows that the coefficient for Temperature is +0.88. The coefficient indicates that for every additional unit in Temperature, FAM increases by an average of 0.88 units.

We conclude that the effect of Temperature has an effect or valid in predicting Rox since the p-value is 0.0012 which is less than 0.05. Here R square is 0.38 or 38% of the variation in Temperature can be predicted from the variablesRox. The regression equation was: 23+0.43x. The equation shows that the coefficient for Temperature is +0.43. The coefficient indicates that for every additional unit in Temperature, Roxincreases by an average of 0.43 units.

From our analysis of the study, at least three genes- FAM, Rox, and Cy5 can be taken as part of the sewage sample. Moreover, the association between ph, temperature, and the Covid gene is important. The percentage of FAM gene takes the lion’s share 36% (125 samples remains positive for this category). Sample collection is performed in two stages and the first stage delivers more active genes. More results (66 confirmed) at 27-31 degrees Celsius were involved. In the 6.5 to 7.4 zone, ph addiction was observed.

**RECOMMENDATIONS**

This study proves clear proof of concept for the use of wastewater-based epidemiology as a complementary surveillance tool for the management of the Covid-19 pandemic. It is essential for human, animal, and the environment. So, continued sampling of whole country wastewater will allow for the expansion of trend monitoring, and it is recommended that more sampling from all districts as this study was a pilot phase study.

Bangladesh is passing the second stage of the pandemic, but the experience of other countries teaches us that second and even third waves of infection will be more dangerous. For that, it will be best for monitoring the whole county by WBE.

Translating the viral titers from wastewater into the actual number of cases within a community is highly challenging, if not impossible. This type of calculation relies on many assumptions, which remain poorly quantified, for example, the amount and dynamics of viral shedding in feces, viral persistence in the sewer network, and variation in wastewater flow and temperature due to climatic conditions.

But, it will be possible to calculate the total number of positive patients either symptomatic or asymptomatic usingWBE.

**Developing a Wastewater Based Surveillance**

**Proactive strategy for developing WBS-**

**Based on the Our Experience and Sample Plan Design**

General considerations need to consider several factors. First and foremost, the goals of the monitoring program must be established. In addition to study goals, key considerations include:

1. Worker safety, Laboratory Safety
2. Environmental factors for wastewater sample
3. Wastewater system characteristics
4. Sample type, timing, and location
5. Sample frequency and duration
6. Sample collection, transport, preservation, and storage
7. Consistency in sampling methods
8. Availability and collection of metadata
   1. physicochemical parameters of samples
   2. Clinical data of specific sample location
   3. Discussion with respective correspondents of wastewater treatment plants.

8. Proper disposal and discard system (Including autoclave and Incineration Point)

**Safety First**

Standard practices associated with water resource recovery facility operations should be sufficient to protect worker safety. Such practices include the use of the Personal Protective Equipment (PPE) normally required when handling untreated wastewater, such as safety gloves, glasses, masks, or face shields. Safety recommendations may vary between handling wastewater and processing samples.

It must be maintained BSL-2(Biosafety Label-2) lab for sample handing with proper disposal system. All laboratory experiments should be continue in Biosafety Cabinets.

**Environmental factors for wastewater sample**

Various environmental factors influence the results like PH, temperature, salinity etc. Environmental factors for wastewater samples should be recorded for correlation of viral CT value.

**Wastewater system characteristics**

The complexity of wastewater infrastructures varies widely around the world: from latrines to single-family septic systems, from community systems to conventional wastewater treatment systems with hundreds of miles of pipes, lift stations, and multiple facilities serving millions of

people. When determining sites for sample collection for more complex analyses, it is important

to coordinate with wastewater utility personnel and local public health officials who can provide

context on preferred sampling points. This is particularly true when attempting to determine community prevalence or to identify specific community locations with changing trends in infection.

In most situations, sampling of the effect of the water-recovery system will provide the population served by the facility an overall consolidated signal intensity measurement. In urban areas where complex sewage network is used by large communities, the study and detection of sites in which a large proportion of cases of COVID-19 are suspected can be carried out using separate sewer trunk pipes.

**Sample Type, Timing, and Location**

A representative sample is critically important, otherwise, artifacts of sample design could skew

results and conclusions. Therefore, in terms of sample type, composite samples are generally preferred over grab samples, though grab samples may be acceptable if composite sampling is not practical. For example, the use of grab samples may make it easier to monitor multiple locations within a service area, including pump stations or hospitals that could act as possible sentinels for infection. Grab sampling may also be the only practical approach to sample smaller and more rural communities or communities that are not served by central water resource recovery facilities. Additional research will help to inform the degree to which the SARS-CoV-2 genetic signal varies with a grab sample and the sensitivity of the detection methods.

It is also presently unclear how significantly the SARS-CoV-2 signal in sewage varies throughout the day. Viruses exhibit different behaviors in response to chemicals and can demonstrate significant retardation in the sewer shed, enabling them to be detected over several

days. Longer composite durations (e.g., 24-hour) will presumably give the most representative signal, though alternative durations (e.g., 2-, 4-, or 8-hour composites) may also be adequate. If collecting grab samples, consideration may need to be given to the time of day sampled. It is also important to understand travel time in the sewer and hydraulic retention times within the water resource recovery facility (e.g., primary clarifier) to understand what time of day is reflected by a particular sample. It may be beneficial to try to capture the morning flush, though there is some debate as to whether this would capture the strongest signal or result in a more diluted signal.

When attempting to match the SARS-CoV-2 signal to local public health results, the effect of the regular commuting and tourism operations must be acknowledged in passing services regions. No noticeable signal variation can be found over one day or a week in more complex networks covering broader areas with low base line flows.

The composite form (i.e., time-proportional versus flow-proportional) of samples does not have a direct effect on the SARS-CoV-2 signal surveillance performance. Thus in most centralized reclaiming water resources facilities flow proportional composite samples could be readily available.

The array of composite samples can also cool where possible. The most suitable sampling position can be determined by each case of usage. For instance, upstream sampling in the sewer shed is most likely important to track the hot spot, while the sampling at the water supply recovery facility may be best suited to pattern analysis or to determine population prevalence overall.

The most important representation of the population served by the water resource recovery facility are influential wastewater (post head work), but the main wastewater may also be appropriate if influential wastes are not usable.

If the target matrix is a representative water source, samples from constantly fluid, well mixed areas should be obtained to prevent aggregation of solids. Most laboratories are still in the absence of an optimized protocol for a certain position and case to evaluate 1 L samples mainly for practical purposes.

The optimal sample volume for analysis will also rely on the analysis approach. A 1-L sample can be divided into four 250 mL aliquots for immediate testing, archiving and quality monitoring for prospective or retrospective review (e.g., replication, method development, recovery spikes). The occurrence and length of the sample depend on the calendar of events in a local community, particularly in the case of COVID-19 and other outbreaks of diseases.

The survey should preferably catch key points in the epidemiological curve for pattern analysis (e.g., emergence, peak, waning). Sample frequency and period changes may be appropriate over time.

The genetic signal ofSARS-CoV-2 is unlikely to differ dramatically from day to day if a representative composite sample is presumed, particularly because fecal removal of SARS-CoV-2 by an infected person can persist for weeks. Therefore, if adequate resources are available, it will not be appropriate to test every day.

In the early stages of an epidemic, a potential exception may be made where the caseload may increase in a short period by magnitude orders (i.e. from 1 cases to 10 to 100 cases). The hot spot surveillance using samples is another potential exception. Without raising the pace of collecting samples, essential 'pulses' for this use case can become more difficult to track

As the disease subsides within the population, a long-term surveillance network may be useful to rapidly detect and respond to secondary waves. By reducing sampling frequency, such a program can be applied for longer times (e.g., every two weeks or even monthly).

Where appropriate, sample bottles should be fresh, gathered, carried, processed and deposited. Autoclaved bottles or at the least bottles that have been washed with chlorine will be used as a substitute and thoroughly rinsed in order to keep the SARS-CoV-2 genetic sign from being compromised by leftover bleach. When autoclaving, autoclave compatibility materials should be checked.

Samples should be cooled during transport after processing or kept cold with ice if cooling is not feasible. The samples should be held at 4°C (up to two weeks) and evaluated as soon as possible. Samples should be frozen at -80°C, -40°C or -20°C automatically, where this is unlikely (in decreasing order of preference). But the storage temperature may be dictated by practical conditions, since this is given as instructions rather than as absolute specifications.

It could be easier to store filtered and/or condensed samples rather than row waste samples to minimize storage space demand and to theoretically boost SARS-CoV-2 signal retention.

The pasteurization of the wastewater could be another step taken by certain establishments to ensure the safety of the worker. It is important to remember that pasteurization may have detrimental effects in the genetic signal of SARS-CoV-2. This is also important during the final review of the results.

**Consistency in Sampling Methods**

Users should ensure that the procedure can be implemented in all cases before beginning the

study, and staff taking samples should be properly trained. Quality assurance elements such as

audits and data traceability reviews should be included in a monitoring program. Consistent and

thorough documentation of the sampling protocol and metadata will enable more universal use of

the data. Also, when deviating from the sampling plan, changes should be documented.

**Critical Metadata**

For starters, the ambient air temperature, the water temperature of the sample, and the sample temperature as obtained in the laboratory and in subsequent storage represent the possibility for an effect of the sample outcome.

It would be difficult to establish an optimal method for gathering transport, handling, and analysis samples for the genetic signal of SARS-CoV-2 before the unique survival characteristics of both SARS-CoV-2 and their genetic signal are understood.

Further information on the state of the activity of the water resource recovery system may be available on other water quality metrics including drainage flow, pH, gross suspended solids and ammonia.

This may be beneficial in recognizing events which can influence the frequency of the genetic signal (e.g., dilution due to ingress of stormwater runoff). Depending on the particular usage case of the wastewater monitoring effort, more complete characterization of flows (e.g. diurnal variability) or structure may be warred.

**Recommendations:**

**Sampling methodology**

Based on the findings of this study it is recommended that1 sewage samples should be taken during the morning peak flow period between 8 and 10 am. These samples should be kept cool and transported to the relevant laboratory on the day of sampling, stored at 4°C, and viral recovery performed within 24 h of sampling.

**SARS-CoV-2 detection**

It is recommended that the evaluation and validation of methods includes a minimally acceptable QA/QC including:

1. Positive control;

2. Negative control;

3. Extraction control;

**SARS-CoV-2 gene assays**

It is recommended that 3 gene-based detections will be more effective than two genes detection for Covid-19. The N target was detected most frequently, then ORF1ab then E gene.

**Upstream sampling and monitoring of a smaller area**

The potential to use this methodology for testing the wastewater of smaller area, defined communities, such as prisons, mines, and community. It demonstrated, with positive results found at wastewater treatment works serving mines and industries, as well as sewer sampling downstream of a prison and hospital. A sampling of combined sewage for a defined population can be useful for surveillance of increased viral load to give early warning of a possible surge in infections. It is important however that regular samples should be taken over time to establish

trends and baselines. This could provide a cost-effective and less invasive means of continuous screening. Where increasing trends in viral load are noted then additional clinical test methods could be rolled out based on an early warning system.

**Data visualization and trend monitoring**

Based on the limited dataset, plotting of weekly sample results appeared to be sufficient to indicate trends, as such weekly sampling of identified sites for national surveillance is recommended. It is recommended that viral load be quantified making use of the Ct number with a proposed categorical data analysis recommended based on the Global Polio Surveillance scheme.

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