

# Science of the Total Environment

## First detection of SARS-CoV-2 genetic material in the vicinity of COVID-19 isolation centre in Bangladesh: Implications for wastewater surveillance of sewer network --Manuscript Draft--

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|-------------------------------|---|
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| <b>Abstract:</b>              | We made the first and successful attempt to detect SARS-CoV-2 genetic material in the vicinity wastewaters of an isolation centre i.e. Shaheed Bhulu Stadium, situated at Noakhali. Owing to the fact that isolation center, in general, always contained a constant number of 200 COVID-19 patients, the prime objective of the study was to check if several drains carrying RNA of coronavirus are actually getting diluted or accumulated along with the sewage network. Our finding suggested that while the temporal variation of the genetic load decreased in small drains over the span of 50 days, the main sewer exhibited accumulation of SARS-CoV-2 RNA. Other interesting finding displays that probably distance of sampling location in meters is not likely to have a significant impact on gene detection concentration, although the quantity of the RNA extracted in the downstream of the drain was higher. These findings are of immense value from the perspective of wastewater surveillance of COVID-19, as they largely imply that we do not need to monitor every wastewater system, and probably major drains monitoring may illustrate the city health. Perhaps, we are reporting the accumulation of SARS-CoV-2 genetic material along with the sewer network i.e. from primary to tertiary drains. The study sought further data collection in this line to simulate conditions prevailed in the most of developing countries and to shed further light on decay/accumulation processes of the genetic load of the SARS-CoV-2. |
| <b>Response to Reviewers:</b> | Response to the Editor and Reviewers<br><br>Ms. Ref. No.: STOTEN-D-20-24176R2<br>Title: First detection of SARS-CoV-2 genetic material in the vicinity of COVID-19  |

isolation centre in Bangladesh: Variation along the sewer network  
Journal: Science of the Total Environment

Dear Editor

Our rebuttal to each comments of the referee is appended for your final consideration to check the suitability of our work for possible publication in your journal "Science of the Total Environment".

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The revised version still presents some important weaknesses in the experimental design, which make conclusions reached by the authors cannot be fully supported by the results shown in the manuscript.

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---X---



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Assistant Professor, Earth Sciences, 336A, Block-5

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Dated: 3<sup>rd</sup> January, 2021

Dear Prof Barcelo,

It is unfortunate to see a short communication that is worth disseminating quickly (being novel in terms of distance investigation along the sewer network) is being dragged and facing complete vague comments. Last revision, we were confident that you would make an informed decision at your desk, but it came for revision again. Our rebuttal can be found in the response sheet. Mostly we do not find any clear cut pin-pointing comments and mostly all of them lack the merit.

Comments are such that we all authors suspect if referee has the enough expertise to review this work (as it seems referee has no idea how to convert Ct value to gene copies, so he says it is difficult to understand) . Has he/she is of microbiology background? We feel that referee never ever done any such analyses by him/herself. Lack of expertise and experience is highly evident in several comments.

We are sure that we have made at least two referees satisfied with our work/revision. Now we leave it upto you that how do you judge the referee comment and how do you judge three revisions that are carried out. Now it is upto you to understand if the referee has got any serious worth objection or its conflict of interest or competing spirit or the work really lack the merit.

If you remember in the second revision we wrote to you that "Referee 1 has been given more clarity to understand our work." Even that time we felt that referee is unable to grasp our work, and explained it in very detail. But this time comments are too vague to even make any point or revision.

In addition we have changed the title a little and re-drawn the graphical abstract to make it look nice and stand alone. I believe in the best interest to COVID-19 research, you will make a decision rapidly at this short communication. Considering the importance of our work, we request you to make the informed decision at your desk without making us wait for another 15 days. We do not mind if paper is rejected in that process but we do believe paper will not undergo for further review. We are keeping fingers crossed that the eminent editor will be satisfied with the revisions and recommend the paper for publication.

Best Regards

Manish Kumar

# First detection of SARS-CoV-2 genetic material in the vicinity of COVID-19 isolation centre in Bangladesh: Variation along the sewer network

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## Response to the Editor and Reviewers

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36

37     **Abstract**

38

39 We made the first and successful attempt to detect SARS-CoV-2 genetic material in the vicinity  
40 wastewaters of an isolation centre i.e. Shaheed Bhulu Stadium, situated at Noakhali. Owing to the  
41 fact that isolation center, in general, always contained a constant number of 200 COVID-19  
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54 on decay/accumulation processes of the genetic load of the SARS-COV-2.

55

56 **Keywords:** SARS-COV-2, Environmental surveillance, Sewage waste, Isolation centre, COVID-19.

57

58

59

60 **1. Introduction**

61 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the strain of coronavirus that  
62 causes coronavirus disease 2019 (COVID-19), that are now being frequently reported in  
63 specimens collected from the wastewater treatment plants around the world (*Ahmed et al.,*  
64 *2020a; Haramoto et al., 2020; Kumar et al., 2020a,b,c,d,e; La Rosa et al., 2020; Lodder et al.,*  
65 *2020; Medema et al., 2020; Nemudryi et al., 2020; Or et al., 2020; RBarcelo et al., 2020a,b, Bivins*  
66 *et al., 2020*). However, the wastewater surveillance of COVID-19 (WWSOC-19) has been mostly  
67 been reported from the wastewater treatment plants, and there is a dearth of SARS-CoV-2 RNA  
68 data in the ambient waters, and in the sewer system (Orive et al. 2020, *andazzo et al., 2020;*  
69 *Rimoldi et al., 2020; Sherchan et al., 2020; Wu et al., 2020b; Wurtzer et al., 2020a,b,c; Tang et*  
70 *al., 2020; Zhang et al., 2020*). On the other hand, several developing countries like India,  
71 Bangladesh, Pakistan, and others do not have plenty of water treatment plants and thus there  
72 requires a need of WBE validity and effectiveness to monitor a sewer system. The results may  
73 help the policymakers of these countries to make a decision pertaining to the national scale  
74 implementation of WWSOC-19.

75

76 Further, while the infectivity issues of SARS-CoV-2 RNA are not yet neglected or proved in the  
77 scholarly world, the public around the globe is sceptical about the wastewaters generating from  
78 the isolation centres. There have been some reports on decay of genetic loading of SARS-CoV-2  
79 (*Ahmed et al., 2020b, Kumar et al., 2020b, Kumar et al., 2021*) in the wastewater systems, yet  
80 accumulation/decay needs to be still investigated in sewer networks. Overall, there is a lack of

81 explicit understanding of process of SARS-CoV-2 gene enrichment in the sewer systems along the  
82 distance (downstream of the source/COVID-19 hotspot); and following the sewer network i.e.  
83 from small to larger drains; larger drains to the canal, and canals to main sewer system.

84

85 Further, a broad observation is that the most of WWSOC-19 studies either correlated Ct-value or  
86 gene copies with the total infected person in the corresponding city or community. Uncertainties  
87 are high pertaining to the average amount of SARS-CoV-2 genes being shredded by an infected  
88 person, and its relationship with the number of genes detected during WWSOC-19. While we  
89 already know about the variations that exist in the length of viral shedding (Wu et al., 2020b; Xu  
90 et al., 2020), the magnitude of the shedding keep varying that ranges between  $10^2$  and  $10^8$  copies  
91 of RNA per gram of human waste (Lescure et al., 2020; Pan et al., 2020; Wölfel et al., 2020). The  
92 general trend has been to see the fluctuation in the Ct value and then estimate the corresponding  
93 increase or decrease of the COVID-19 patient in a given vicinity of the treatment plants. However,  
94 there has been a complete lack of studies on WWSOC-19, with known variation in infected  
95 symptomatic and asymptomatic individuals.

96

97 Accordingly, we conducted a preliminary detection survey of SARS-CoV-2 RNA in wastewater  
98 samples collected from the sewage network in the vicinity of isolation centre at Noakhali,  
99 Bangladesh. The primary objective of the study was to understand the genetic load variation  
100 along the sewer network in the vicinity of the isolation centre, under the preview of tracing the

101 decay/accumulation processes of the SARS-CoV-2 RNA. We intended to contribute in policy  
102 decision regarding the WBE inclusion in developing countries by tracing the change among the  
103 primary, secondary and tertiary drains. The results are likely to appeal to the policy makers  
104 worldwide especially to the developing/low sanitation countries to adopt for wastewater  
105 surveillance for COVID-19 pandemic.

106

107 **2. Material and Methods**

108 ***2.1. Sampling***

109 Wastewater samples were collected from the three different drains, i.e., coming out of Shaheed  
110 Bhulu Stadium at Noakhali, Bangladesh ( $22.8763^{\circ}$  N,  $91.0973^{\circ}$ E), which connect to a canal  
111 (secondary drainage system) and eventually meets the main sewer system (tertiary drains)  
112 (**Figure 1**). For this study, the sampling location was selected based on the fact that Shaheed  
113 Bhulu Stadium is the largest detention Centre for COVID-19 patients in the Noakhali district,  
114 Bangladesh. This facility has been established to accommodate more than two hundred COVID-  
115 19 positive patients for isolation purposes but kept around 200 patients all the time during the  
116 monitoring period. This preliminary study has been carried out with samples collected from the  
117 three different drains i.e., coming out of the stadium (primary drains), which connects to a canal  
118 (secondary drainage system) and eventually meets the main sewer system (tertiary drains). The  
119 terminal end of all three primary drains opens in the canal which is interlinked with the main  
120 sewer. Hence, the prime source of SARS-CoV-2 RNA remains the isolation center which we  
121 targeted to understand accumulation/dilution of it along the drainage system. It is noteworthy  
122 that the main sewer is constructed as a drainage system for municipal sewage that connects the

123 Maijdee city with ~0.1 million inhabitants in the upstream to the Bay of Bengal. In order to  
124 understand the weather during sampling, physico-chemical characteristics of sampled drains,  
125 canal and main sewer, and number of patients being treated on the date of samples collected,  
126 information is given in supplementary file 1a,b,&C.

127

128 In order to understand the distance impact on genetic loading along the drains, we collected  
129 samples at various distances i.e. 100m, 200m, 300m, and 400m as presented in **Table 1**.  
130 Specimens were aseptically collected in a 50 ml sterile falcon tube, transported in the laboratory  
131 keeping inside the ice-box, refrigerated at 4°C during preparatory activities, and were analyzed  
132 on the same day. Sterile falcon tubes for sampling with identical blanks were analyzed to  
133 determine any possible contamination during the transport. All analyses were done at the  
134 Microbiology Laboratory of the Department of Microbiology, Noakhali Science and Technology  
135 University (NSTU), Bangladesh. One argument is quite obvious here that main drain and canal are  
136 likely to bring SARS-CoV-2

137

138 **2.2. Sample preparation, and procedure for the RNA extraction and concentration**

139 We followed the same extraction procedure, as described by Kumar et al. (2020a). Briefly,  
140 sewage samples (50 mL) were centrifuged (Thermo Scientific) at 4500×g for 30 min followed by  
141 filtration of supernatant using 0.22-micron filters (Himedia). Further, each sewage filtrate was  
142 concentrated using the polyethylene glycol (PEG) method. In this method, PEG 6000 (80 g/L) and  
143 NaCl (17.5 g/L) were mixed in 25 ml filtrate, which was then incubated at 17°C in 100 rpm shaking

144 for overnight. The next day, the mixture was centrifuged at 13000×g for 90 min. The supernatant  
145 was discarded after centrifugation, and the pellet was resuspended in 300 µL RNase free water.  
146 This was further used as a sample for RNA isolation using a commercially available Favor Prep™  
147 Viral Nucleic Acid Extraction Kit. In brief, PEG concentrated samples were transferred in a  
148 collection tube with a VNE-carrier RNA buffer. After the appropriate mixing of samples with  
149 proper incubation, a conventional column-based ethanol extraction procedure was followed  
150 using the VNE column. The RNase P (RP) primer and probe set was included with the commercial  
151 Sansure RT-PCR kit.

152

153 In addition, all the experiments were performed three times for confirmation of the results, **and**  
154 **accepted where variations were less than 10%**. Covid-19 positive patient samples were used as  
155 an extraction control in each run. We employed qualitative measurement, and hence, increasing  
156 and decreasing viral load is measured based on the Ct value. RNA concentrations were measured  
157 by NanoDrop (Thermo Scientific™ NanoDrop 2000 and 2000c, BioRad) and were stored at -70 °C  
158 until further use.

159

160 **2.3. RT-PCR Analysis**

161 RNAs were analyzed for the detection of SARS-CoV-2 by RT-PCR (CFX96, BioRad) using the  
162 Sansure RT-PCR kit (Sansure Biotech Inc., China). As described in the product manual, technical  
163 procedures carried out, and interpretations of results were made. In brief, we set the samples

164 layout with RT-PCR protocol covering 45 cycles containing FAM fluorescence select for ORF1ab,  
165 ROX for N gene as well as CY5 for Internal control. As quality control measures, one positive  
166 control and one negative control were also run to validate the test procedure. The Novel  
167 Coronavirus (2019-nCoV) Nucleic Acid Diagnostic Kit (PCR-Fluorescence Probing) is a real-time  
168 reverse transcription polymerase chain reaction (rRT-PCR) test. The 2019-nCoV primer and probe  
169 set(s) is designed to detect RNA from SARS-CoV-2.

170

171 **2.4 Methods for gene copies calculations:**

172 We used qualitative estimation of gene copies per unit of sample volume based on the  
173 correlation of the  $C_T$  value to copy numbers provided by the Sansure kit that was used for the  
174 gene detection. Correlation diagram was prepared using the principle of 3.3  $C_T$  change  
175 equivalence to the 10-fold change in gene copies as described by Kumar et al, 2020a,b. For  
176 relative quantification, we quantified extracted RNA of the SARS-Cov-2 positive samples and  
177 calculated the enrichment factor based on the least Ct value that has been described to contain  
178 200 gene copies as per the Sansure kit protocol. A gradient with various dilutions was prepared  
179 to get linear regression lines between Ct values and gene copies, and then each obtained Ct  
180 values from RT-PCR were converted into gene copies per ml of the sample.

181

182 **3. Results and discussion**

183 We collected 16 specimens between 10<sup>th</sup> July and 29<sup>th</sup> August 2020 from the drain, sewage, and  
184 toilets near Shaheed Bhulu Stadium Detention Centre (DC) for COVID-19 patient at Noakhali,  
185 Bangladesh. A summary of the results of amplification cycles (Ct) of various sampled water along

186 with the distance of sampling in the vicinity of isolation centre with 50 days temporal resolution  
187 i.e. in between 10<sup>th</sup> July and 29<sup>th</sup> August 2020 has been presented in **Table 1** and amplification  
188 plots obtained through RT-PCR illustrating temporal variation through Ct value in various  
189 sampling drains, (a) Drain 1 (b) Drain 2 (c) Drain 3, and (d) Main sewer is presented in **Figure 2**.

190

191 **Table 1** summarizes the Ct values obtained during the monitoring which ranged between 20.42  
192 to 39.38 for N genes which corresponds to 10,000 gc/ml to not detected, and 22.35 (8620 gc/ml)  
193 to 40.72 (ND) for ORF1ab genes, implying a huge variation in gene copies of SARS-CoV-2.  
194 Interestingly as their lowest and highest values belong to corresponding samples of the same  
195 date i.e. 17<sup>th</sup> and 26<sup>th</sup> August, it seems sewer systems played a critical factor WWSoC-19. Other  
196 than this anomaly 17<sup>th</sup> August samples exhibited higher loading of genetic material than 29<sup>th</sup>  
197 August 2020, while the number of patients in the containment remained the same during the  
198 monitoring period. This emphasizes the fact that just becoming COVID-19 positive is not a  
199 measure of the viruses shedding by the infected person, but perhaps the state of infection  
200 matters. It is easy to speculate that with each day passing owing to aggressive testing and  
201 capacity building of carrying out the tests, early detections of COVID-19 positive people were  
202 happening, and thus probably it is reflecting on the genetic load. Moreover, temporal  
203 environmental variations due to huge rainfall with temperature and humidity fluctuations along  
204 with inadequate sewage treatment might have significant impact on the quantitative variations  
205 of SARS-CoV-2 viral genetic material.

206

207 As far as different characteristics of sampled drains are concerned, as depicted in **Figure 2**, drain  
208 3 seems to carry the greater RNA load of SARS-CoV-2 followed by drain 2 and drain 1. Although  
209 dissimilarities observed between the primary drainage line (drain samples) and secondary  
210 drainage system (canal) and tertiary drainage system (main sewer), however, a trend of higher  
211 genetic material loading in the secondary and tertiary system was also found on twilight tenure.  
212 This is a unique finding where gene accumulation has been observed instead of the general  
213 expectation of dilution in the larger sewer system. The probable reasoning, other than the  
214 accumulation of loading from various drains of the isolation centre, in support of this observation  
215 can be the additional contribution of RNA excreted from the asymptomatic patient as well as yet  
216 to be diagnosed people into the sewer system.

217

218 The Ct values of different genes are presented as Box and Whisker plots in **Figure 3**. The average  
219 calculated for different genes viz. ORF1ab, N genes, and RNase P were found to be 34.62, 31.69  
220 and 36.93, respectively. N genes showed the lowest Ct values, followed by ORF1 ab and RNase P.  
221 Also, 50% Ct values (Q1 and Q2) were ranged between 29.03 to 31.36 for N genes and 31.03 to  
222 34.78 for ORF1ab. We employed the distance factor in our sampling strategies and the results  
223 are presented in **Figure 4**. While drain 3 in general showed the increase in the ORF1ab and N  
224 genes, drain 1 was not showing a consistent trend in genetic material loading of SARS-CoV-2  
225 along with the distance. We tend to propose that probably distance in meters is not likely of a  
226 critical factor capable of producing a trend.

227

228 **Table 2** shows a comparative analysis of the monitoring period, percentage of positive samples  
229 detected, and range of Ct-value along with their reference (Ahmed et al., 2020a; Balboa et al.,  
230 2020; Kocamemi et al., 2020; Kumar et al., 2020; Medema et al., 2020; Or et al., 2020; Rimoldi et  
231 al., 2020; Wu et al., 2020b; Wurtzer et al., 2020a). One finding stands out on this comparison is  
232 the Ct value of 20.42 which corresponds to much higher genetic material loading of SARS-CoV-2  
233 than any other study reported. This may be because we sampled in the vicinity of the isolation  
234 centre, while other studies compared in **Table 2** have reported the values from the wastewater  
235 treatment plant. Overall, we successfully detected ORF1ab and N protein genes from the  
236 wastewater samples of Bangladesh, which is for the first time, reported the data from the  
237 containment centre. Our study provides an opportunity to produce a realistic coefficient in the  
238 future for the conversion of genetic material loading with the number of infected people in the  
239 community.

240

241 Further, referring to the limitations of the present study, surrogate samples were sent to other  
242 laboratories to check how precisely the Sansure kit data matches with others. We also tested the  
243 filtration methods to check the efficiency of the PEG method used in this study. Although, Hata  
244 and Honda (2020) reported a high efficiency of the PEG method in Japanese wastewater, and the  
245 same has been found true by Kumar et al. (2020g) while testing the sample concentration  
246 efficiency between PEG and filtration. While lack of supply always poses critical challenges in any  
247 research, during the lockdown we could not find a supply of MS2, as used by Kumar et al.  
248 (2020a,g) or could perform the whole process control (WPC) together with MPC as recommended  
249 by Haramoto et al., (2020). We had to use a swab sample of a symptomatic person as our control

250 as indicated by CY-5 as quality control of our analyses, which makes sense and provides a low-  
251 cost control for such analyses, yet it may be controversial, less precise and at times comes  
252 negative as the case in a few samples in our case (drain 1 samples at 300 and 400m and main  
253 sewer sample on 07<sup>th</sup> and 26<sup>th</sup> August). By applying fluorescence quantitative RT-PCR technology,  
254 this test utilized the novel Covid-19 ORF1ab and the specific conserved sequence of coding  
255 nucleocapsid protein N gene as the target regions, which were designed for conserved sequences  
256 of the double target genes to achieve detection of wastewater samples RNA through fluorescent  
257 signal changes. The PCR detection system used the positive internal control, which monitors the  
258 presence of PCR inhibitors in test specimens by detecting whether the internal control signal is  
259 normal, to avoid a false negative result when used for human RT-PCR experiments. In our  
260 experiments with wastewater, few specimens were negative for CY5, indicating that the human  
261 gene RNase P gene was missing in the samples. Hence it has been noted that the human RNase  
262 P gene is more vulnerable to degradation than Covid-19 viral genes.

263

264 In the present study, we also have abstained from putting any semi-quantitative calculation of  
265 gene copies due to lack of enough replicates, kit intended for the human sample, as well as  
266 uncertainties involving RT-PCR (Stuart et al. 2014). Nevertheless, the bottom line is that the  
267 patterns of obtained Ct values suggest successful detection of SARS-CoV-2 RNA from the  
268 wastewater samples in Bangladesh. Also, their increasing abundance in tertiary drain  
269 demonstrated that it is not difficult to employ the COVID-19 surveillance through wastewater in  
270 the sewer systems to know the community health as we probably do not need extensive and

271 rigorous sampling. However, the major drains may be enough to use the capability of  
272 wastewater-based epidemiology in south-Asian settings.

273

274 Pertaining to the limitation of this study, the sampling design strategies, influences of  
275 environmental factors, contribution of viral loads other than the symptomatic/asymptomatic  
276 patients at the isolation centre, as well as the condition of sewer (canals/drains) system are not  
277 explicitly characterised. It may also be argued that the assumption that the discharge from the  
278 isolation centre is constant due to the constant number of COVID-19 patients, can introduce a  
279 bias in the interpretation. This biasness discussion is far-fetching as WBE capabilities are too  
280 limited to determine the number of viruses being shredded from the patients (before, during and  
281 after COVID-10 infection). Thus the observation still holds fairly well with or without that  
282 assumption.

283

284 Further, since sampling of the different parts of the network were not sampled on the same day,  
285 another argument is quite possible about the validity of RNA loading comparisons among  
286 different dates. However, the focus of the present study was to understand the distance impact  
287 on SARS-CoV-2 gene loading along the sewage network under the preview of possible  
288 decay/accumulation of the same in a given sewer network. We put effort to generate information  
289 on the pertinent question related to the minimum number of samples required in a given sewer  
290 network of the community. Such information will be vital for the promotion of WBE study for  
291 COVID-19. To the best of our knowledge, this is the first ever study aimed at understanding the  
292 accumulation and decay of SARS-CoV-2 in a sewer/ drain system, that can be helpful in triggering

293 further research, and comparisons on decay/accumulation along the drainage system, which is  
294 vital for ascertaining monitoring locations for COVID-19.

295

296 **4. Conclusions**

297 While the wastewater surveillance of COVID-19 has been focused on wastewater treatment  
298 plants worldwide, we have opted for drain waters monitoring in the vicinity of the isolation  
299 center, which is first of its kind. Apart from this being the first detection report of SARS-CoV-2  
300 RNA in the wastewaters of Bangladesh. Further, the uniqueness of the study has been the tracing  
301 of the genetic load in the vicinity of the isolation center that contains almost the constant number  
302 with 200 COVID-19 patients, which takes the variable of the number of infected persons out of  
303 the equation. This has been the key feature of this study as most of the study reported worldwide  
304 has either reported total infected person in the city or country. There has been a complete lack  
305 of infected person information contributing to the total genetic load to the sampled wastewater.  
306 We have found about 75% of our sampled water positive based on the absence or presence of  
307 ORF1ab gene. However, the critical observation has been the temporal variation where small  
308 drains showed an easing of the loading of genetic load, the bigger canal, and main sewer city  
309 exhibited temporal accumulation of SARS-CoV-2 RNA genetic materials. On the other hand, the  
310 distance of sampling location appears to be insignificant from the perspective of wastewater  
311 surveillance of COVID-19. We intend to analyze further samples taken in June, July, and August  
312 and preserved to shed further light on the temporal variation and decay/accumulation processes  
313 of the genetic load of the SARS-COV-2.

314

315 **Ethics Statement**

316 The work did not involve any human subject and animal experiments.

317

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322 Technology University, Bangladesh.  
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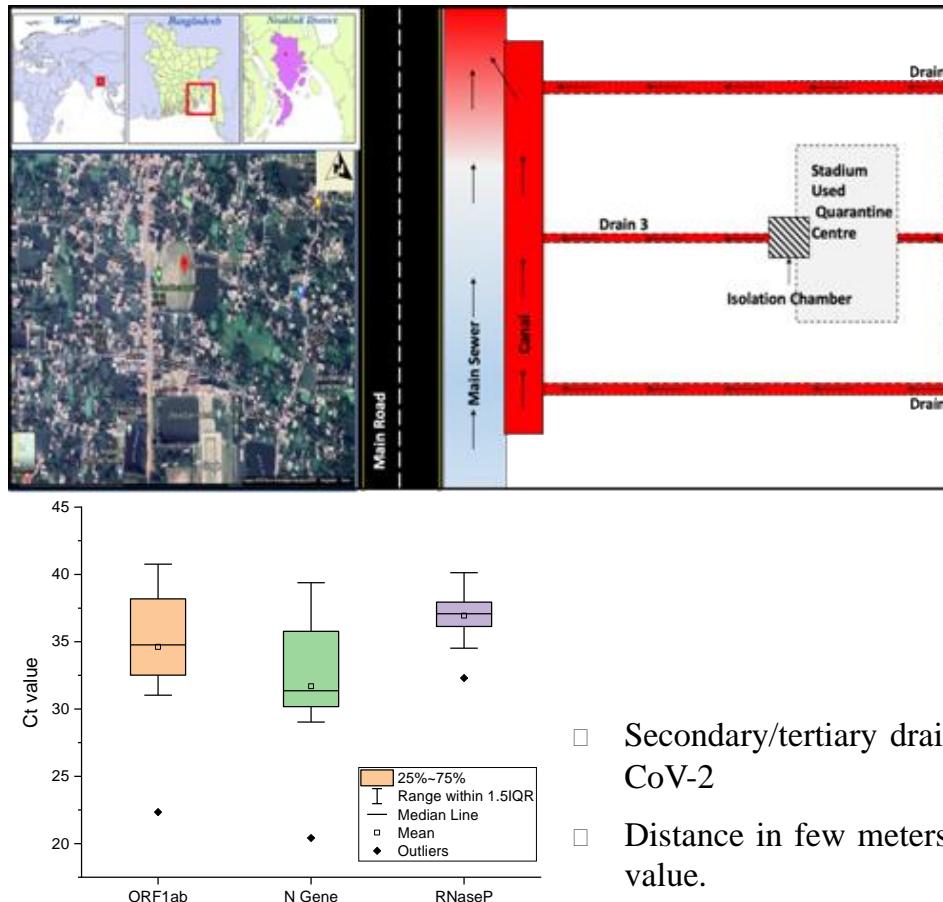
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## Graphical abstract

## Do the genes accumulate or dilutes in the sewer network?: First detection in the vicinity of detention centre in Bangladesh\*



| Source     | Distance from Isolation Center (m) | Date of Sample collection | Ct Value        |                 |                 |
|------------|------------------------------------|---------------------------|-----------------|-----------------|-----------------|
|            |                                    |                           | (Sansure Kit)   |                 |                 |
|            |                                    |                           | FAM<br>(ORF1ab) | ROX<br>(N gene) | Cy5<br>(RNaseP) |
| Drain 1    | 100                                | 18-08-2020                | 34.69           | 32.25           | 37.91           |
|            |                                    | 29-08-2020                | ND              | ND              | 36.42           |
|            | 200                                | 17-08-2020                | 40.72           | ND              | 36.95           |
|            | 300                                | 07-08-2020                | ND              | ND              | ND              |
| Drain 2    | 400                                | 26-08-2020                | 39.43           | ND              | ND              |
|            | 200                                | 18-08-2020                | 36.94           | 35.78           | 37.57           |
| Drain 3    | 400                                | 26-08-2020                | ND              | ND              | 36.14           |
|            | 200                                | 17-08-2020                | 36.78           | 35.82           | 39.08           |
|            |                                    | 18-08-2020                | 22.35           | 20.42           | 32.3            |
|            | 300                                | 17-08-2020                | 31.03           | 29.03           | 37.08           |
| Canal      |                                    | 26-08-2020                | 32.85           | 30.47           | 40.13           |
|            | NTS                                | 10-07-2020                | 40.77           | 39.38           | 38.79           |
|            |                                    | 26-08-2020                | 32.18           | 30.18           | 35.32           |
| Main Sewer | NTS                                | 07-08-2020                | ND              | ND              | ND              |
|            |                                    | 18-08-2020                | 34.85           | 33.24           | 37.94           |

- Secondary/tertiary drains of sewer network exhibited RNA accumulation of SARS-CoV-2
- Distance in few meters from the excretion point has no significant influence on Ct-value.

Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)

## Highlights

- First detection report of SARS-CoV-2 RNA in the wastewaters of Bangladesh.
- Probably first report on wastewater surveillance in the vicinity of COVID-19 isolation center.
- Secondary/tertiary drains of sewer network exhibited RNA accumulation of SARS-CoV-2
- Distance in few meters from the excretion point has no significant influence on Ct-value.

1      **First detection of SARS-CoV-2 genetic material in the vicinity of COVID-19**  
2                   **isolation centre in Bangladesh: Variation along the sewer network**

3  
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35

36

37     **Abstract**

38

39 We made the first and successful attempt to detect SARS-CoV-2 genetic material in the vicinity  
40 wastewaters of an isolation centre i.e. Shaheed Bhulu Stadium, situated at Noakhali. Owing to the  
41 fact that isolation center, in general, always contained a constant number of 200 COVID-19  
42 patients, the prime objective of the study was to check if several drains carrying RNA of  
43 coronavirus are actually getting diluted or accumulated along with the sewage network. Our  
44 finding suggested that while the temporal variation of the genetic load decreased in small drains  
45 over the span of 50 days, the main sewer exhibited accumulation of SARS-CoV-2 RNA. Other  
46 interesting finding displays that probably distance of sampling location in meters is not likely to  
47 have a significant impact on gene detection concentration, although the quantity of the RNA  
48 extracted in the downstream of the drain was higher. These findings are of immense value from  
49 the perspective of wastewater surveillance of COVID-19, as they largely imply that we do not need  
50 to monitor every wastewater system, and probably major drains monitoring may illustrate the city  
51 health. Perhaps, we are reporting the accumulation of SARS-CoV-2 genetic material along with the  
52 sewer network i.e. from primary to tertiary drains. The study sought further data collection in this  
53 line to simulate conditions prevailed in the most of developing countries and to shed further light  
54 on decay/accumulation processes of the genetic load of the SARS-COV-2.

55

56 **Keywords:** SARS-COV-2, Environmental surveillance, Sewage waste, Isolation centre, COVID-19.

57

58

59

60 **1. Introduction**

61 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the strain of coronavirus that  
62 causes coronavirus disease 2019 (COVID-19), that are now being frequently reported in  
63 specimens collected from the wastewater treatment plants around the world (*Ahmed et al.,*  
64 *2020a; Haramoto et al., 2020; Kumar et al., 2020a,b,c,d,e; La Rosa et al., 2020; Lodder et al.,*  
65 *2020; Medema et al., 2020; Nemudryi et al., 2020; Or et al., 2020; RBarcelo et al., 2020a,b, Bivins*  
66 *et al., 2020*). However, the wastewater surveillance of COVID-19 (WWSOC-19) has been mostly  
67 been reported from the wastewater treatment plants, and there is a dearth of SARS-CoV-2 RNA  
68 data in the ambient waters, and in the sewer system (Orive et al. 2020, *andazzo et al., 2020;*  
69 *Rimoldi et al., 2020; Sherchan et al., 2020; Wu et al., 2020b; Wurtzer et al., 2020a,b,c; Tang et*  
70 *al., 2020; Zhang et al., 2020*). On the other hand, several developing countries like India,  
71 Bangladesh, Pakistan, and others do not have plenty of water treatment plants and thus there  
72 requires a need of WBE validity and effectiveness to monitor a sewer system. The results may  
73 help the policymakers of these countries to make a decision pertaining to the national scale  
74 implementation of WWSOC-19.

75

76 Further, while the infectivity issues of SARS-CoV-2 RNA are not yet neglected or proved in the  
77 scholarly world, the public around the globe is sceptical about the wastewaters generating from  
78 the isolation centres. There have been some reports on decay of genetic loading of SARS-CoV-2  
79 (*Ahmed et al., 2020b, Kumar et al., 2020b, Kumar et al., 2021*) in the wastewater systems, yet  
80 accumulation/decay needs to be still investigated in sewer networks. Overall, there is a lack of

81 explicit understanding of process of SARS-CoV-2 gene enrichment in the sewer systems along the  
82 distance (downstream of the source/COVID-19 hotspot); and following the sewer network i.e.  
83 from small to larger drains; larger drains to the canal, and canals to main sewer system.

84

85 Further, a broad observation is that the most of WWSOC-19 studies either correlated Ct-value or  
86 gene copies with the total infected person in the corresponding city or community. Uncertainties  
87 are high pertaining to the average amount of SARS-CoV-2 genes being shredded by an infected  
88 person, and its relationship with the number of genes detected during WWSOC-19. While we  
89 already know about the variations that exist in the length of viral shedding (Wu et al., 2020b; Xu  
90 et al., 2020), the magnitude of the shedding keep varying that ranges between  $10^2$  and  $10^8$  copies  
91 of RNA per gram of human waste (Lescure et al., 2020; Pan et al., 2020; Wölfel et al., 2020). The  
92 general trend has been to see the fluctuation in the Ct value and then estimate the corresponding  
93 increase or decrease of the COVID-19 patient in a given vicinity of the treatment plants. However,  
94 there has been a complete lack of studies on WWSOC-19, with known variation in infected  
95 symptomatic and asymptomatic individuals.

96

97 Accordingly, we conducted a preliminary detection survey of SARS-CoV-2 RNA in wastewater  
98 samples collected from the sewage network in the vicinity of isolation centre at Noakhali,  
99 Bangladesh. The primary objective of the study was to understand the genetic load variation  
100 along the sewer network in the vicinity of the isolation centre, under the preview of tracing the

101 decay/accumulation processes of the SARS-CoV-2 RNA. We intended to contribute in policy  
102 decision regarding the WBE inclusion in developing countries by tracing the change among the  
103 primary, secondary and tertiary drains. The results are likely to appeal to the policy makers  
104 worldwide especially to the developing/low sanitation countries to adopt for wastewater  
105 surveillance for COVID-19 pandemic.

106

107 **2. Material and Methods**

108 ***2.1. Sampling***

109 Wastewater samples were collected from the three different drains, i.e., coming out of Shaheed  
110 Bhulu Stadium at Noakhali, Bangladesh ( $22.8763^{\circ}$  N,  $91.0973^{\circ}$ E), which connect to a canal  
111 (secondary drainage system) and eventually meets the main sewer system (tertiary drains)  
112 (**Figure 1**). For this study, the sampling location was selected based on the fact that Shaheed  
113 Bhulu Stadium is the largest detention Centre for COVID-19 patients in the Noakhali district,  
114 Bangladesh. This facility has been established to accommodate more than two hundred COVID-  
115 19 positive patients for isolation purposes but kept around 200 patients all the time during the  
116 monitoring period. This preliminary study has been carried out with samples collected from the  
117 three different drains i.e., coming out of the stadium (primary drains), which connects to a canal  
118 (secondary drainage system) and eventually meets the main sewer system (tertiary drains). The  
119 terminal end of all three primary drains opens in the canal which is interlinked with the main  
120 sewer. Hence, the prime source of SARS-CoV-2 RNA remains the isolation center which we  
121 targeted to understand accumulation/dilution of it along the drainage system. It is noteworthy  
122 that the main sewer is constructed as a drainage system for municipal sewage that connects the

123 Maijdee city with ~0.1 million inhabitants in the upstream to the Bay of Bengal. In order to  
124 understand the weather during sampling, physico-chemical characteristics of sampled drains,  
125 canal and main sewer, and number of patients being treated on the date of samples collected,  
126 information is given in supplementary file 1a,b,&C.

127

128 In order to understand the distance impact on genetic loading along the drains, we collected  
129 samples at various distances i.e. 100m, 200m, 300m, and 400m as presented in **Table 1**.  
130 Specimens were aseptically collected in a 50 ml sterile falcon tube, transported in the laboratory  
131 keeping inside the ice-box, refrigerated at 4°C during preparatory activities, and were analyzed  
132 on the same day. Sterile falcon tubes for sampling with identical blanks were analyzed to  
133 determine any possible contamination during the transport. All analyses were done at the  
134 Microbiology Laboratory of the Department of Microbiology, Noakhali Science and Technology  
135 University (NSTU), Bangladesh. One argument is quite obvious here that main drain and canal are  
136 likely to bring SARS-CoV-2

137

138 **2.2. Sample preparation, and procedure for the RNA extraction and concentration**

139 We followed the same extraction procedure, as described by Kumar et al. (2020a). Briefly,  
140 sewage samples (50 mL) were centrifuged (Thermo Scientific) at 4500×g for 30 min followed by  
141 filtration of supernatant using 0.22-micron filters (Himedia). Further, each sewage filtrate was  
142 concentrated using the polyethylene glycol (PEG) method. In this method, PEG 6000 (80 g/L) and  
143 NaCl (17.5 g/L) were mixed in 25 ml filtrate, which was then incubated at 17°C in 100 rpm shaking

144 for overnight. The next day, the mixture was centrifuged at 13000×g for 90 min. The supernatant  
145 was discarded after centrifugation, and the pellet was resuspended in 300 µL RNase free water.  
146 This was further used as a sample for RNA isolation using a commercially available Favor Prep™  
147 Viral Nucleic Acid Extraction Kit. In brief, PEG concentrated samples were transferred in a  
148 collection tube with a VNE-carrier RNA buffer. After the appropriate mixing of samples with  
149 proper incubation, a conventional column-based ethanol extraction procedure was followed  
150 using the VNE column. The RNase P (RP) primer and probe set was included with the commercial  
151 Sansure RT-PCR kit.

152

153 In addition, all the experiments were performed three times for confirmation of the results, and  
154 accepted where variations were less than 10%. Covid-19 positive patient samples were used as  
155 an extraction control in each run. We employed qualitative measurement, and hence, increasing  
156 and decreasing viral load is measured based on the Ct value. RNA concentrations were measured  
157 by NanoDrop (Thermo Scientific™ NanoDrop 2000 and 2000c, BioRad) and were stored at -70 °C  
158 until further use.

159

160 **2.3. RT-PCR Analysis**

161 RNAs were analyzed for the detection of SARS-CoV-2 by RT-PCR (CFX96, BioRad) using the  
162 Sansure RT-PCR kit (Sansure Biotech Inc., China). As described in the product manual, technical  
163 procedures carried out, and interpretations of results were made. In brief, we set the samples

164 layout with RT-PCR protocol covering 45 cycles containing FAM fluorescence select for ORF1ab,  
165 ROX for N gene as well as CY5 for Internal control. As quality control measures, one positive  
166 control and one negative control were also run to validate the test procedure. The Novel  
167 Coronavirus (2019-nCoV) Nucleic Acid Diagnostic Kit (PCR-Fluorescence Probing) is a real-time  
168 reverse transcription polymerase chain reaction (rRT-PCR) test. The 2019-nCoV primer and probe  
169 set(s) is designed to detect RNA from SARS-CoV-2.

170

171 **2.4 Methods for gene copies calculations:**

172 We used qualitative estimation of gene copies per unit of sample volume based on the  
173 correlation of the  $C_T$  value to copy numbers provided by the Sansure kit that was used for the  
174 gene detection. Correlation diagram was prepared using the principle of 3.3  $C_T$  change  
175 equivalence to the 10-fold change in gene copies as described by Kumar et al, 2020a,b. For  
176 relative quantification, we quantified extracted RNA of the SARS-Cov-2 positive samples and  
177 calculated the enrichment factor based on the least Ct value that has been described to contain  
178 200 gene copies as per the Sansure kit protocol. A gradient with various dilutions was prepared  
179 to get linear regression lines between Ct values and gene copies, and then each obtained Ct  
180 values from RT-PCR were converted into gene copies per ml of the sample.

181

182 **3. Results and discussion**

183 We collected 16 specimens between 10<sup>th</sup> July and 29<sup>th</sup> August 2020 from the drain, sewage, and  
184 toilets near Shaheed Bhulu Stadium Detention Centre (DC) for COVID-19 patient at Noakhali,  
185 Bangladesh. A summary of the results of amplification cycles (Ct) of various sampled water along

186 with the distance of sampling in the vicinity of isolation centre with 50 days temporal resolution  
187 i.e. in between 10<sup>th</sup> July and 29<sup>th</sup> August 2020 has been presented in **Table 1** and amplification  
188 plots obtained through RT-PCR illustrating temporal variation through Ct value in various  
189 sampling drains, (a) Drain 1 (b) Drain 2 (c) Drain 3, and (d) Main sewer is presented in **Figure 2**.

190

191 **Table 1** summarizes the Ct values obtained during the monitoring which ranged between 20.42  
192 to 39.38 for N genes which corresponds to 10,000 gc/ml to not detected, and 22.35 (8620 gc/ml)  
193 to 40.72 (ND) for ORF1ab genes, implying a huge variation in gene copies of SARS-CoV-2.  
194 Interestingly as their lowest and highest values belong to corresponding samples of the same  
195 date i.e. 17<sup>th</sup> and 26<sup>th</sup> August, it seems sewer systems played a critical factor WWSoC-19. Other  
196 than this anomaly 17<sup>th</sup> August samples exhibited higher loading of genetic material than 29<sup>th</sup>  
197 August 2020, while the number of patients in the containment remained the same during the  
198 monitoring period. This emphasizes the fact that just becoming COVID-19 positive is not a  
199 measure of the viruses shedding by the infected person, but perhaps the state of infection  
200 matters. It is easy to speculate that with each day passing owing to aggressive testing and  
201 capacity building of carrying out the tests, early detections of COVID-19 positive people were  
202 happening, and thus probably it is reflecting on the genetic load. Moreover, temporal  
203 environmental variations due to huge rainfall with temperature and humidity fluctuations along  
204 with inadequate sewage treatment might have significant impact on the quantitative variations  
205 of SARS-CoV-2 viral genetic material.

206

207 As far as different characteristics of sampled drains are concerned, as depicted in **Figure 2**, drain  
208 3 seems to carry the greater RNA load of SARS-CoV-2 followed by drain 2 and drain 1. Although  
209 dissimilarities observed between the primary drainage line (drain samples) and secondary  
210 drainage system (canal) and tertiary drainage system (main sewer), however, a trend of higher  
211 genetic material loading in the secondary and tertiary system was also found on twilight tenure.  
212 This is a unique finding where gene accumulation has been observed instead of the general  
213 expectation of dilution in the larger sewer system. The probable reasoning, other than the  
214 accumulation of loading from various drains of the isolation centre, in support of this observation  
215 can be the additional contribution of RNA excreted from the asymptomatic patient as well as yet  
216 to be diagnosed people into the sewer system.

217

218 The Ct values of different genes are presented as Box and Whisker plots in **Figure 3**. The average  
219 calculated for different genes viz. ORF1ab, N genes, and RNase P were found to be 34.62, 31.69  
220 and 36.93, respectively. N genes showed the lowest Ct values, followed by ORF1 ab and RNase P.  
221 Also, 50% Ct values (Q1 and Q2) were ranged between 29.03 to 31.36 for N genes and 31.03 to  
222 34.78 for ORF1ab. We employed the distance factor in our sampling strategies and the results  
223 are presented in **Figure 4**. While drain 3 in general showed the increase in the ORF1ab and N  
224 genes, drain 1 was not showing a consistent trend in genetic material loading of SARS-CoV-2  
225 along with the distance. We tend to propose that probably distance in meters is not likely of a  
226 critical factor capable of producing a trend.

227

228 **Table 2** shows a comparative analysis of the monitoring period, percentage of positive samples  
229 detected, and range of Ct-value along with their reference (Ahmed et al., 2020a; Balboa et al.,  
230 2020; Kocamemi et al., 2020; Kumar et al., 2020; Medema et al., 2020; Or et al., 2020; Rimoldi et  
231 al., 2020; Wu et al., 2020b; Wurtzer et al., 2020a). One finding stands out on this comparison is  
232 the Ct value of 20.42 which corresponds to much higher genetic material loading of SARS-CoV-2  
233 than any other study reported. This may be because we sampled in the vicinity of the isolation  
234 centre, while other studies compared in **Table 2** have reported the values from the wastewater  
235 treatment plant. Overall, we successfully detected ORF1ab and N protein genes from the  
236 wastewater samples of Bangladesh, which is for the first time, reported the data from the  
237 containment centre. Our study provides an opportunity to produce a realistic coefficient in the  
238 future for the conversion of genetic material loading with the number of infected people in the  
239 community.

240

241 Further, referring to the limitations of the present study, surrogate samples were sent to other  
242 laboratories to check how precisely the Sansure kit data matches with others. We also tested the  
243 filtration methods to check the efficiency of the PEG method used in this study. Although, Hata  
244 and Honda (2020) reported a high efficiency of the PEG method in Japanese wastewater, and the  
245 same has been found true by Kumar et al. (2020g) while testing the sample concentration  
246 efficiency between PEG and filtration. While lack of supply always poses critical challenges in any  
247 research, during the lockdown we could not find a supply of MS2, as used by Kumar et al.  
248 (2020a,g) or could perform the whole process control (WPC) together with MPC as recommended  
249 by Haramoto et al., (2020). We had to use a swab sample of a symptomatic person as our control

250 as indicated by CY-5 as quality control of our analyses, which makes sense and provides a low-  
251 cost control for such analyses, yet it may be controversial, less precise and at times comes  
252 negative as the case in a few samples in our case (drain 1 samples at 300 and 400m and main  
253 sewer sample on 07<sup>th</sup> and 26<sup>th</sup> August). By applying fluorescence quantitative RT-PCR technology,  
254 this test utilized the novel Covid-19 ORF1ab and the specific conserved sequence of coding  
255 nucleocapsid protein N gene as the target regions, which were designed for conserved sequences  
256 of the double target genes to achieve detection of wastewater samples RNA through fluorescent  
257 signal changes. The PCR detection system used the positive internal control, which monitors the  
258 presence of PCR inhibitors in test specimens by detecting whether the internal control signal is  
259 normal, to avoid a false negative result when used for human RT-PCR experiments. In our  
260 experiments with wastewater, few specimens were negative for CY5, indicating that the human  
261 gene RNase P gene was missing in the samples. Hence it has been noted that the human RNase  
262 P gene is more vulnerable to degradation than Covid-19 viral genes.

263

264 In the present study, we also have abstained from putting any semi-quantitative calculation of  
265 gene copies due to lack of enough replicates, kit intended for the human sample, as well as  
266 uncertainties involving RT-PCR (Stuart et al. 2014). Nevertheless, the bottom line is that the  
267 patterns of obtained Ct values suggest successful detection of SARS-CoV-2 RNA from the  
268 wastewater samples in Bangladesh. Also, their increasing abundance in tertiary drain  
269 demonstrated that it is not difficult to employ the COVID-19 surveillance through wastewater in  
270 the sewer systems to know the community health as we probably do not need extensive and

271 rigorous sampling. However, the major drains may be enough to use the capability of  
272 wastewater-based epidemiology in south-Asian settings.

273

274 Pertaining to the limitation of this study, the sampling design strategies, influences of  
275 environmental factors, contribution of viral loads other than the symptomatic/asymptomatic  
276 patients at the isolation centre, as well as the condition of sewer (canals/drains) system are not  
277 explicitly characterised. It may also be argued that the assumption that the discharge from the  
278 isolation centre is constant due to the constant number of COVID-19 patients, can introduce a  
279 bias in the interpretation. This biasness discussion is far-fetching as WBE capabilities are too  
280 limited to determine the number of viruses being shredded from the patients (before, during and  
281 after COVID-10 infection). Thus the observation still holds fairly well with or without that  
282 assumption.

283

284 Further, since sampling of the different parts of the network were not sampled on the same day,  
285 another argument is quite possible about the validity of RNA loading comparisons among  
286 different dates. However, the focus of the present study was to understand the distance impact  
287 on SARS-CoV-2 gene loading along the sewage network under the preview of possible  
288 decay/accumulation of the same in a given sewer network. We put effort to generate information  
289 on the pertinent question related to the minimum number of samples required in a given sewer  
290 network of the community. Such information will be vital for the promotion of WBE study for  
291 COVID-19. To the best of our knowledge, this is the first ever study aimed at understanding the  
292 accumulation and decay of SARS-CoV-2 in a sewer/ drain system, that can be helpful in triggering

293 further research, and comparisons on decay/accumulation along the drainage system, which is  
294 vital for ascertaining monitoring locations for COVID-19.

295

296 **4. Conclusions**

297 While the wastewater surveillance of COVID-19 has been focused on wastewater treatment  
298 plants worldwide, we have opted for drain waters monitoring in the vicinity of the isolation  
299 center, which is first of its kind. Apart from this being the first detection report of SARS-CoV-2  
300 RNA in the wastewaters of Bangladesh. Further, the uniqueness of the study has been the tracing  
301 of the genetic load in the vicinity of the isolation center that contains almost the constant number  
302 with 200 COVID-19 patients, which takes the variable of the number of infected persons out of  
303 the equation. This has been the key feature of this study as most of the study reported worldwide  
304 has either reported total infected person in the city or country. There has been a complete lack  
305 of infected person information contributing to the total genetic load to the sampled wastewater.  
306 We have found about 75% of our sampled water positive based on the absence or presence of  
307 ORF1ab gene. However, the critical observation has been the temporal variation where small  
308 drains showed an easing of the loading of genetic load, the bigger canal, and main sewer city  
309 exhibited temporal accumulation of SARS-CoV-2 RNA genetic materials. On the other hand, the  
310 distance of sampling location appears to be insignificant from the perspective of wastewater  
311 surveillance of COVID-19. We intend to analyze further samples taken in June, July, and August  
312 and preserved to shed further light on the temporal variation and decay/accumulation processes  
313 of the genetic load of the SARS-COV-2.

314

315 **Ethics Statement**

316 The work did not involve any human subject and animal experiments.

317

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1 **Table 1.** Summary of the results of amplification cycles (Ct) of various sampled wastewater along with the distance of sampling in the  
2 vicinity of isolation center with 50 days temporal resolution i.e. in between 10<sup>th</sup> July and 29<sup>th</sup> August 2020.

| Source        | Distance<br>(m) and<br>Sample<br>collection | Date<br>of<br>Sample<br>collection | RNA Quantity  |                  | Ct Value           |               |               |               | Gene Copies (gc/ml) |               |               |  |
|---------------|---|------------------------------------|---------------|------------------|--------------------|---------------|---------------|---------------|---------------------|---------------|---------------|--|
|               |   |                                    | (Sansure Kit) |                  |                    |               |               |               |                     |               |               |  |
|               |   |                                    | ID            | Conc.<br>(ng/μl) | Ratio<br>(260/280) | FAM<br>ORF1ab | ROX<br>N gene | Cy5<br>RnaseP | FAM<br>ORF1ab       | ROX<br>N gene | Cy5<br>RnaseP |  |
| Drain 1       | 100 (D1-1)                                  | 18-08-2020                         | 12.42         | 1.97             | 34.69              | 32.25         | 37.91         | 524           | 1,385               | 725           |               |  |
|               |   | 29-08-2020                         | 25.5          | 1.95             | ND                 | ND            | 36.42         | ND            | ND                  | ND            | 1747          |  |
|               | 200(D1-2)                                   | 17-08-2020                         | 12.37         | 1.99             | 40.72              | ND            | 36.95         | 14            | ND                  | ND            | 1277          |  |
|               | 300(D1-3)                                   | 07-08-2020                         | 24.68         | 1.92             | ND                 | ND            | ND            | ND            | ND                  | ND            | ND            |  |
|               | 400(D1-4)                                   | 26-08-2020                         | 12.23         | 2                | 39.43              | ND            | ND            | 31            | ND                  | ND            | ND            |  |
| Drain 2       | 200 (D2-2)                                  | 18-08-2020                         | 13.18         | 1.92             | 36.94              | 35.78         | 37.57         | 137           | 153                 | 886           |               |  |
|               | 400 (D2-4)                                  | 26-08-2020                         | 14.37         | 2.01             | ND                 | ND            | 36.14         | ND            | ND                  | ND            | 2060          |  |
| Drain 3       | 200 (D3-2)                                  | 17-08-2020                         | 17.47         | 1.97             | 36.78              | 35.82         | 39.08         | 151           | 149                 | 363           |               |  |
|               |   | 18-08-2020                         | 22.61         | 1.98             | 22.35              | 20.42         | 32.3          | 827,624       | 2,225,489           | 19863         |               |  |
|               | 300 (D3-3)                                  | 17-08-2020                         | 15.97         | 1.92             | 31.03              | 29.03         | 37.08         | 4,655         | 10,330              | 11863         |               |  |
|               |   | 26-08-2020                         | 22.16         | 1.99             | 32.85              | 30.47         | 40.13         | 1,571         | 4,206               | 196           |               |  |
|               | 400 (D3-4)                                  | 26-07-2020                         | 15.31         | 1.95             | 32.84              | 30.35         | 34.52         | 1,581         | 4,533               | 5359          |               |  |
| Canal         | NTS   | 10-07-2020                         | 27.31         | 1.97             | 40.77              | 39.38         | 38.79         | 14            | 16                  | 431           |               |  |
|               |   | 26-08-2020                         | 18.14         | 2                | 32.18              | 30.18         | 35.32         | 2,344         | 5,040               | 3343          |               |  |
| Main<br>Sewer | NTS   | 07-08-2020                         | 24.63         | 1.96             | ND                 | ND            | ND            | ND            | ND                  | ND            |               |  |
|               |   | 18-08-2020                         | 16            | 1.94             | 34.85              | 33.24         | 37.94         | 476           | 747                 | 712           |               |  |

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4 NTS= Distance not to scale; ND: Not Detected

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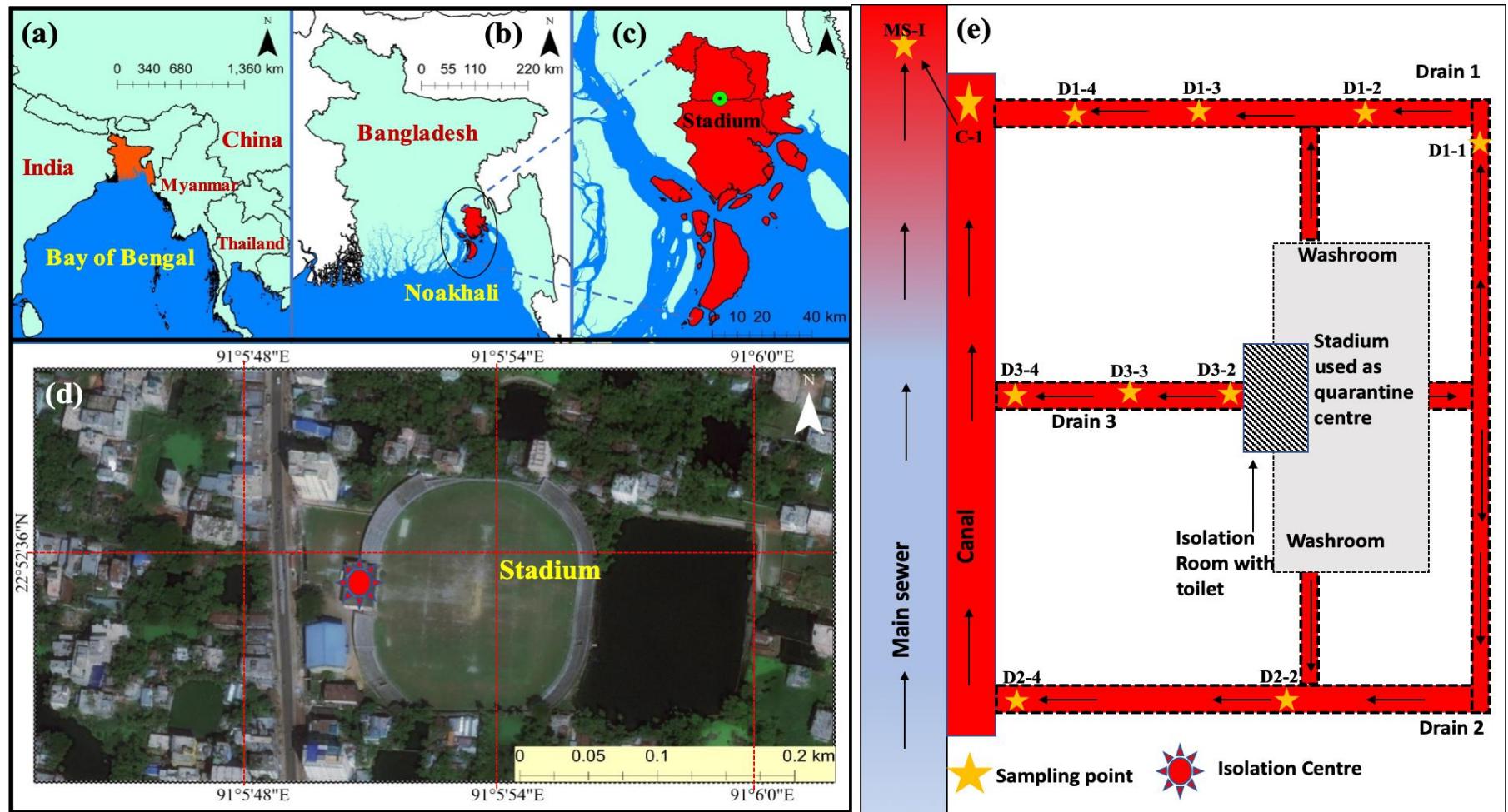
6 **Table 2.** Comparative positive sample and the range of Ct value reported of ORF-lab genes of  
7 SARS-CoV-2 in the wastewater of various countries.

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| Country     | Period of Examination            | Positive sample from total samples | Ct                   | Reference                    |
|-------------|----------------------------------|------------------------------------|----------------------|------------------------------|
| Italy       | 14/04/2020<br>22/04/2020         | - 4(12)                            | -                    | (Rimoldi et al., 2020)       |
| Spain       | 06/04/2020<br>21/04/2020         | - 7(7)                             | 33.61 – 39.60        | (Balboa et al., 2020)        |
| Turkey      | 21/04/2020<br>25/04/2020         | - 7(9)                             | 34.67 – 39.54        | (Kocamemi et al., 2020)      |
| Netherlands | 05/02/2020<br>16/03/2020         | - 10(24)                           | -                    | (Medema et al., 2020)        |
| France      | 05/03/2020<br>23/04/2020         | - 100 % of samples                 | -                    | (Wurtzer et al., 2020a)      |
| Australia   | 20/03/2020<br>01/04/2020         | - 2(9)                             | 37.5 - 39            | (Ahmed et al., 2020a)        |
| U.S.A.      | 08/01/2020<br>25/03/2020         | - 10(12)                           | 33.87 - 38.39        | (Wu et al., 2020a)           |
| India       | 04/05/2020<br>12/06/2020         | - 100 % of samples                 | 32.65 – 34.18        | (Kumar et al., 2020)         |
| Israel      | 10/03/2020<br>21/04/2020         | - 10(26)                           | 32.76 – 38.5         | (Or et al., 2020)            |
| Bangladesh  | <b>10/07/2020<br/>29/08/2020</b> | <b>- 12 (16)</b>                   | <b>20.42 – 40.77</b> | <b>(Present study, 2020)</b> |

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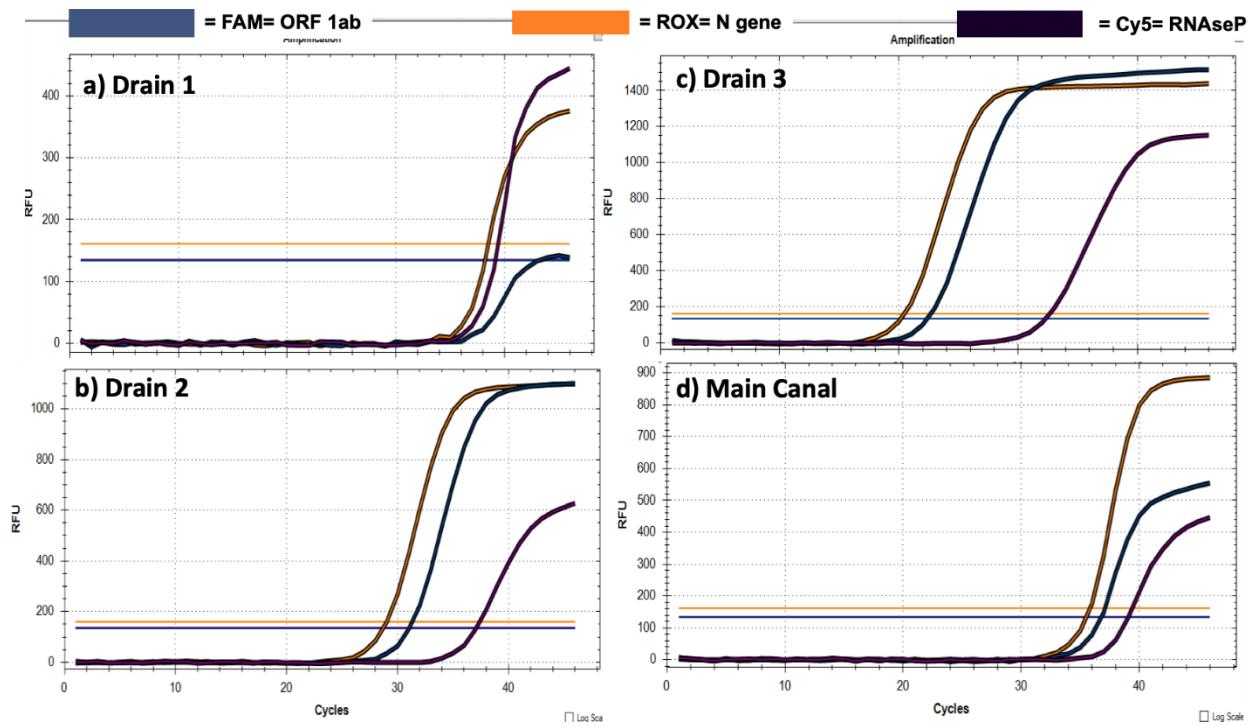
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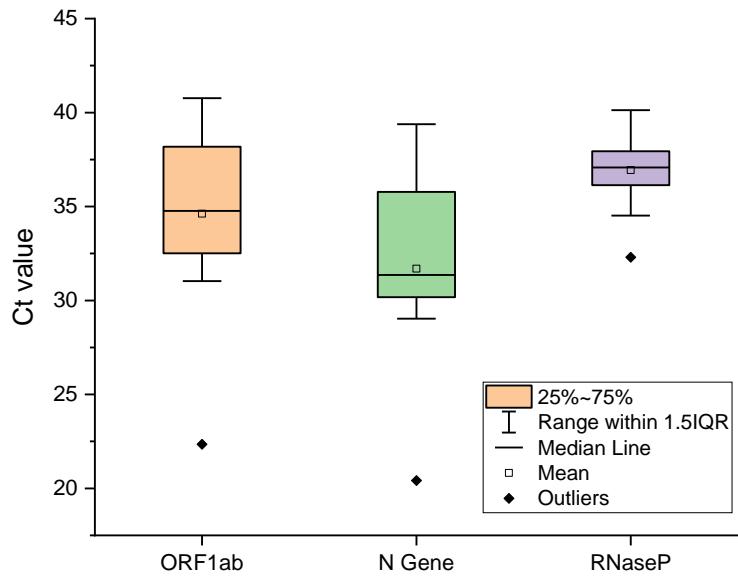
3 **Figure 1.** Study area location depicting, geographical locations of a)Bangladesh; b)Noakhali District in the Southern Bangladesh; c)  
 4 Stadium location in the district; d) Stadium and isolation Centre established at the Shaheed Bhulu Stadium; as well as e) Schematic  
 5 detail of the sampling location at various drains outlets, canal and main sewer line.

6



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8 **Figure 2.** Amplification plots obtained through RT-PCR in the samples from the drains and the  
9 main canal.



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11 **Figure 3.** Box-whisker plot illustrating the Ct-value variations of three genes for the entire  
12 monitoring period along with the outliers.

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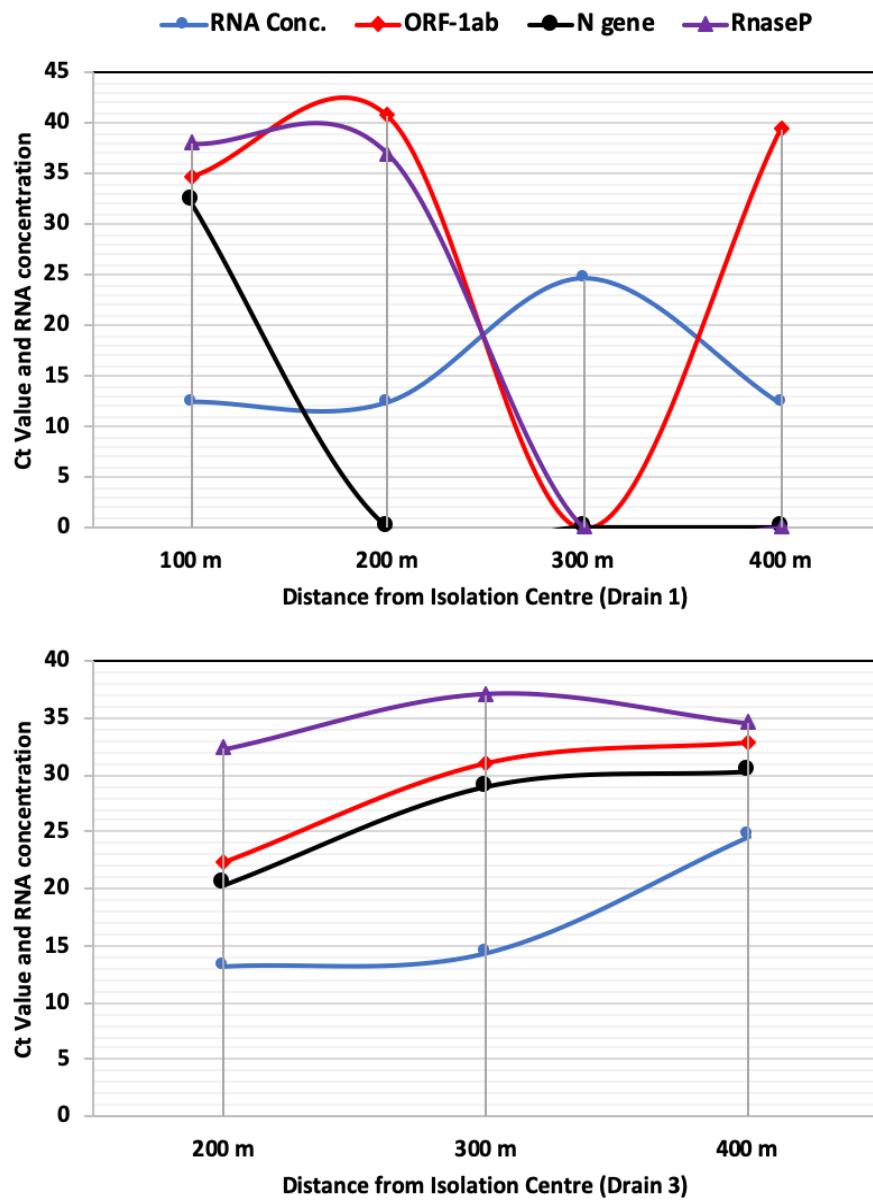


Figure 4. Trend in genetic material loading of SARS-CoV-2 along with the distance.

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**Supplementary Bangladesh 2021.docx**

## Author Contributions

**Firoz Ahmed:** Conceptualization, Formal analysis, Funding, Investigation, Methodology, Project administration, Supervision, Writing - review & editing

**Md. Aminul Islam:** Conceptualization, Data curation, Formal sample analysis, Investigation, Methodology, Validation, Roles - original draft, Writing - review & editing

**Manish Kumar:** Conceptualization, Visualization, Methodology, Data curation, Data analysis and interpretation, Writing - original draft,

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**Md. Main Uddin:** Investigation, Validation, Roles/Writing - original draft

**Md. Nur Islam:** Data curation, Investigation, Software

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All authors critically scrutinised and approved the final version of the manuscript.

**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: