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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
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Abstract:	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.
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October, 2, 2020

Professor Damia Barcelo

Editor-in-Chief

Science of the Total Environment

Dear Professor Barcelo:

As Editorial Members of *npj Clean Water*, we are pleased to contribute and submit a Review article to *npj Clean Water*, entitled **"First detection of SARS-CoV-2 genetic material in the vicinity of COVID-19 isolation centre through wastewater surveillance in Bangladesh".** Please find the enclosed proposal and the current version of the manuscript.

A pre-print of this work has been published by medrxiv **doi:** https://doi.org/10.1101/2020.09.14.20194696 https://www.medrxiv.org/content/10.1101/2020.09.14.20194696v2.full.pdf for helping the research community to start implementing the recommendations of this study. The work has been drawing significant media attention in Bangladesh and elsewhere, as evident by media reports. Considering the recent rapid progress in COVID-19 related studies, we will be grateful if you may handle it with accelerated reviewing and production process for prompt dissemination of the key findings of our work with monitoring management implications.

A number of studies have been reported detection SARS-CoV-2 in wastewater; some reported even in treated wastewater and river water. However no study, to the best of our knowledge has monitored the SARS-CoV-2 RNA fluctuation in the vicinity of isolation centre for COVID-19 patients. The major challenges in WBE study of COVID-19 has been the correlation between COVID-19 patients and the number of gene copies indicated through Ct value, owing to the difficulties in ascertaining the number of symptomatic/ asymptomatic patients a STPs is actually receiving. In our study that variable is supposed to be constant and thus provides a scenario to understand the dilution, and accumulation of genes along the sewer system; as well as the temporal variations. Thus apart from the study being the proof of first breakthrough analytical capacity development in Bangladesh, it also provides significant addition to the existing knowledge on wastewater surveillances of COVID-19. One of such major observations that we are reporting here is accumulation of gene copies in secondary/tertiary drains with respect to primary ones. This imply that we can analyse major drains of a sewer network in a given city to understand the COVID-19 situation, or relate earl warning, per se.

We firmly believe that this collaborative work has a great impact and interest on readers of your journal, including researchers, wastewater engineers, policy makers, etc. All authors have agreed with this submission and accept responsibility for this manuscript. We confirmed that this manuscript has only been published as pre-print and it is not under consideration by another scholarly journal. The authors have no

conflicts of interest to declare. All images were generated and compiled by authors following Guide for Authors. We have multiple co-corresponding authors to signify their key roles in data generation (Firoz Ahmed), analysis supervision and interpretation (Manish Kumar) and co-ordination of the collaboration among different stakeholders and institutes (Md. Jakariya).

We would like the following competing researchers to be excluded from peer reviewers of this manuscript.
- ✧ Dr. Warish Ahmed, CSIRO, Australia
- ✧ Dr. Masaaki Kitajima, Hokkaido University
- ✧ Dr. Eiji Haramoto, Yamanashi University
- ✧ Dr. Sudipti Arora, Dr. B. lal Institute of Biotechnology, Jaipur (India)

We look forward to hearing from you at your earliest convenience.

Yours sincerely,

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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

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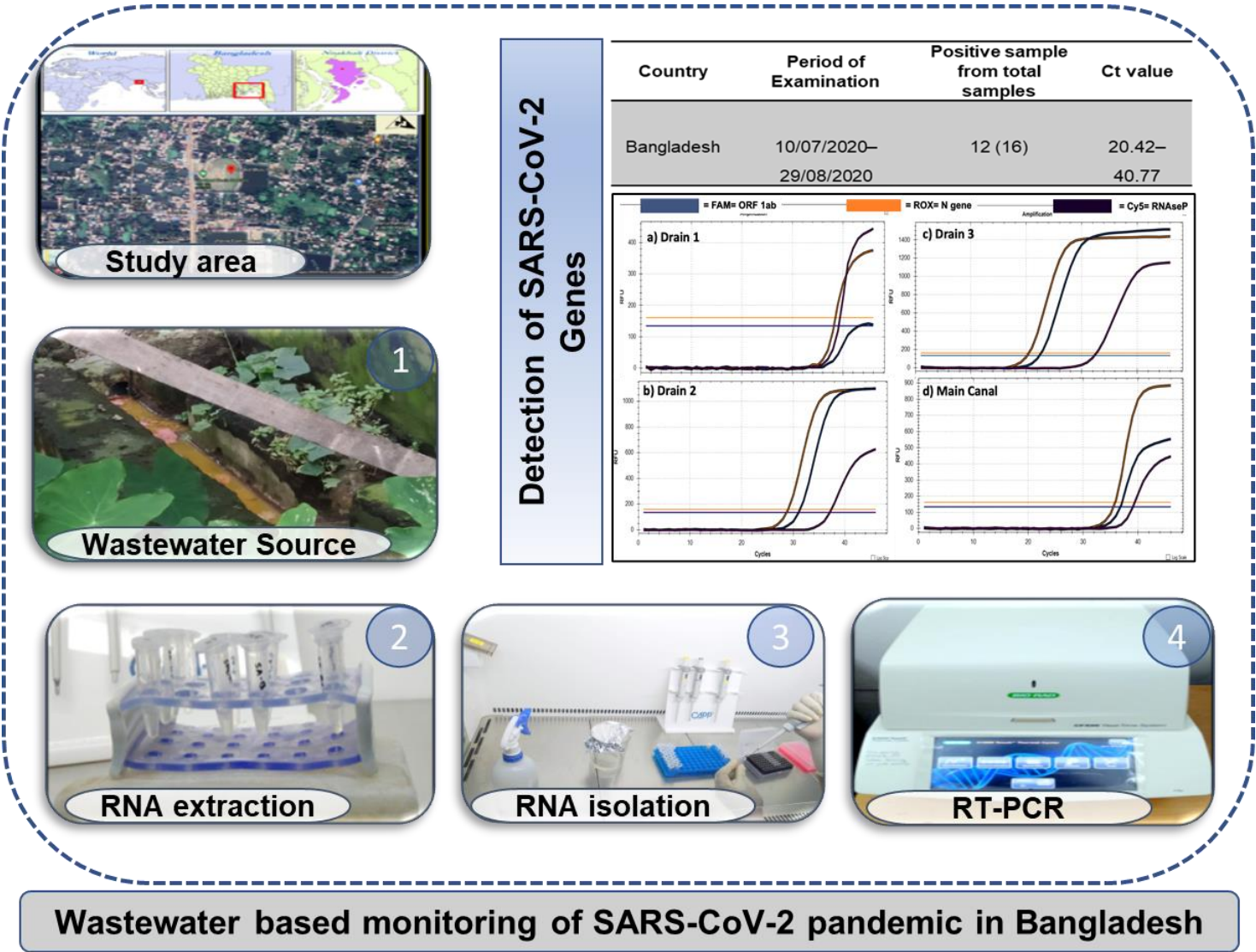Email: manish.kumar@iitgn.ac.in manish.env@gmail.com

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Abstract

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.

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

1

Graphical Abstract

Graphical abstract

Wastewater based monitoring of SARS-CoV-2 pandemic in Bangladesh

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 centre.

- Secondary/tertiary drains of sewer network exhibited RNA accumulation of SARS-CoV-2

- Distance in few 100 meters from the excretion point has no significant influence on Ct-value.

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the strain of coronavirus that causes coronavirus disease 2019 (COVID-19), that are now being frequently reported in specimens collected from the wastewater treatment plants around the world owing to the shredding of the symptomatic/asymptomatic COVID-19 infection (Ahmed et al., 2020; Haramoto et al., 2020; Kumar et al., 2020; La Rosa et al., 2020; Medema et al., 2020; Nemudryi et al., 2020; Or et al., 2020; Randazzo et al., 2020; Rimoldi et al., 2020; Wu et al., 2020b; Wurtzer et al., 2020). In Milano, Italy, ORF1ab assay and N protein assay was used to detect SARS-CoV-2 RNA from untreated wastewater and river water samples (Rimoldi et al., 2020). However, the wastewater surveillance of COVID-19 (WWSoC-19) has been mostly focused on wastewater treatment plants around the globe, and there is slow progress on ambient waters and especially in the sewer system. This is critical because several developing countries like India, Bangladesh, Pakistan, and others do not have proper wastewater systems even in their class-I cities. As a result, the policymakers in these countries are in confusion pertaining to the national scale implementation of WWSoC-19.

Further, while the infectivity issues of SARS-CoV-2 RNA present in the wastewater as well as ambient environmental waters are not yet neglected or proved in the scholarly world, the public around the world are very skeptical about the wastewaters coming from the isolation centers mainly that are not equipped with wastewater treatment systems. There have been some decay reports of genetic loading of SARS-CoV-2 (Ahmed et al., 2020b, Kumar et al., 2020b) in the wastewater system, but sewer systems are not yet investigated. In addition, there is a lack of

22   explicit understanding of either decay or accumulation of Covid-19 genetic material along with the

23   sewer systems with distance and the networking from small drain to larger drains followed by the

24   canal and main sewer system.

25

26   Furthermore, it is a general observation that most of the WWSoC-19 studies reported worldwide

27   have either correlated their C_t-value or detected gene copies with the total infected person in the

28   corresponding monitoring city or country. Uncertainty is very much evident on the average

29   amount of gene copies shredded by an infected person, and its relationship with the number of

30   genes detected during WWSoC-19. While we already know about the variations that exist in the

31   length of viral shedding in wastewater from the various studies (Wu et al., 2020b; Xu et al., 2020),

32   the magnitude of the shedding ranges between 10^2 and 10^8 copies of RNA per gram of human

33   waste (Lescure et al., 2020; Pan et al., 2020; Wölfel et al., 2020). The general trend has been to

34   see the fluctuation in the Ct value and then estimate the corresponding increase or decrease of

35   the COVID-19 patient in a given vicinity of treatment plants. However, there has been a complete

36   lack of WWSoC-19 study where infected individuals' information is known and then a variation in

37   genetic loading has been studied.

38

39   Accordingly, we conducted a preliminary detection of SARS-CoV-2 RNA in wastewater samples

40   from the sewage network in the isolation centre at Noakhali, Bangladesh. The aim of the study

41   was to: i) understand the genetic load in the vicinity of the isolation centre with an almost constant

42 number of COVID-19 patients. ii) analyse the distance impact on genetic loading and tracing the

43 decay/accumulation of the same along with the sewage network, iii) and temporal variation in

44 genetic material shedding from the infected patient.

45

46 **2. Material and Methods**

47 *2.1. Sampling*

48 Wastewater samples were collected from sewage waste tank, passage drain, and toilets near

49 Shaheed Bhulu Stadium at Noakhali, Bangladesh (**Figure 1**). The sampling location for this study

50 was selected based on the fact that Shaheed Bhulu Stadium is the largest detention Centre for

51 COVID-19 patients at Noakhali district, Bangladesh. This facility has been established to

52 accommodate more than two hundred COVID-19 positive patients for isolation purposes but kept

53 around 200 patients all the time during the monitoring period. This preliminary study has been

54 carried out with samples collected from the three different drains that are coming out of the

55 stadium and can be considered as primary drains, which connects to a canal (secondary drainage

56 system) and eventually meets the main sewer system (tertiary drains).

57

58 In order to understand the distance impact on genetic loading along the drains, we collected

59 samples at various distances i.e. 100m, 200m, 300m, and 400m as presented in **Table 1**. Specimens

60 were aseptically collected in a 50 ml sterile falcon tube, transported in the laboratory keeping

61 inside the ice-box, refrigerated at 4°C during preparatory activities, and were analyzed on the

62 same day. Sterile falcon tubes for sampling with identical blanks were analyzed to determine any

3

63   possible contamination during the transport. All analyses were done at the Microbiology

64   Laboratory of the Department of Microbiology, Noakhali Science and Technology University

65   (NSTU), Bangladesh.

66

67   *2.2. Sample preparation, and procedure for the RNA extraction and concentration*

68   We almost followed the same procedure of extraction described in Kumar et al. (2020). Briefly,

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

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

71   concentrated using the polyethylene glycol (PEG) method. In this method, PEG 6000 (80 g/L) and

72   NaCl (17.5 g/L) were mixed in 25 ml filtrate, which was then incubated at 17°C in 100 rpm shaking

73   for overnight. The next day, the mixture was centrifuged at 13000×g for 90 min. After

74   centrifugation, the supernatant was discarded and the pellet was resuspended in 300 μL RNase

75   free water. This was further used as a sample for RNA isolation. RNA isolation was carried out using

76   a commercially available kit according to the manufacturer's instructions. RNA concentrations

77   were measured by NanoDrop (Thermo Scientific™ NanoDrop 2000 and 2000c, BioRad) and were

78   stored at -70 °C until further use.

79

80   *2.3. RT-PCR Analysis*

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

82   RT-PCR kit (Sansure Biotech Inc., China). Technical procedures carried out as described in the

83   product manual and interpretations of results were analyzed as instructed in the manual. In brief,

84   we set the samples layout with RT-PCR protocol covering 45 cycles containing FAM fluorescence

85   select for ORF1ab, ROX for N gene as well as CY5 for Internal control. As quality control measures,

86   one positive control and one negative control were also run for validation of the test procedure.

87

88   **3. Results and discussion**

89   We collected 16 specimens between 10th July and 29th August 2020 from the drain, sewage, and

90   toilets near Shaheed Bhulu Stadium Detention Centre (DC) for COVID-19 patient at Noakhali,

91   Bangladesh. A summary of the results of amplification cycles (Ct) of various sampled water along

92   with the distance of sampling in the vicinity of isolation center with 50 days temporal resolution

93   i.e. in between 10th July and 29th August 2020 has been presented in **Table 1** and amplification

94   plots obtained through RT-PCR illustrating temporal variation through Ct value in various sampling

95   drains, (a) Drain 1 (b) Drain 2 (c) Drain 3, and (d) Main sewer is presented in **Figure 2**.

96

97   **Table 1** summarizes the Ct values obtained during the monitoring which ranged between 20.42 to

98   39.38 for N genes, and 22.35 to 40.72 for ORF1ab genes, implying a huge variation in gene copies

99   of SARS-CoV-2. Interestingly as their lowest and highest values belong to corresponding samples

100   of the same date i.e. 17th and 26th August, it seems sewer systems played a critical factor WWSoC-

101   19. Other than this anomaly 17th August samples exhibited higher loading of genetic material than

102   29th August 2020, while the number of patients in the containment remained the same during the

103   monitoring period. This emphasizes on the fact that just becoming COVID-19 positive is not a

104   measure of the viruses shedding by the infected person, but perhaps the state of infection

105   matters. It is easy to speculate that with each day passing owing to aggressive testing and capacity

106 building of carrying out the tests, early detections of COVID-19 positive people were happening,

107 and thus probably it is reflecting on the genetic load. Moreover, temporal environmental

108 variations due to huge rainfall with temperature and humidity fluctuations along with inadequate

109 sewage treatment might have significant impact on the quantitative variations of SARS-CoV-2 viral

110 genetic material.

111

112 As far as different characteristics of sampled drains are concerned, as depicted in **Figure 2**, drain 3

113 seems carrying the heavier RNA load of SARS-CoV-2 followed by drain 2 and drain 1. Although

114 dissimilarities observed between the primary drainage line i.e. drain samples and secondary

115 drainage system i.e. canal and tertiary drainage system i.e. the main sewer, however, trend of

116 higher genetic material loading in the secondary and tertiary system was also found on twilight

117 tenure. This is a unique finding where gene accumulation has been observed instead of the general

118 expectation of dilution in the larger sewer system. The probable reasoning, other than the

119 accumulation of loading from various drains of the isolation centre, in support of this observation

120 can be the additional contribution of RNA excreted from the asymptomatic patient as well as yet

121 to be diagnosed people into the sewer system.

122

123 The Ct values of different genes are presented as Box and Whisker plots in **Figure 3.** The average

124 calculated for different genes viz. ORF1ab, N genes, and RNase P were found to be 34.62, 31.69

125 and 36.93, respectively. N genes showed the lowest Ct values, followed by ORF1 ab and RNase P.

126 Also, 50% Ct values (Q1 and Q2) were ranged between 29.03 to 31.36 for N genes and 31.03 to

127   34.78 for ORF1ab. We employed the distane factor in our sampling strategies and the results are

128   presented in **Figure 4**. While drain 3 in general showed the increase in the ORF1ab and N genes,

129   drain 1 was not showing a consistent trend in genetic material loading of SARS-CoV-2 along with

130   the distance. We tend to propose that probably distance in meters is not likely of a critical factor

131   capable of producing a trend.

132

133   **Table 2** shows a comparative analysis of monitoring period, percentage of positive samples

134   detected, and range of Ct-value along with their reference (Ahmed et al., 2020; Balboa et al., 2020;

135   Kocamemi et al., 2020; Kumar et al., 2020; Medema et al., 2020; Or et al., 2020; Rimoldi et al.,

136   2020; Wu et al., 2020b; Wurtzer et al., 2020). One finding stands out on this comparison is the Ct

137   value of 20.42 which corresponds to much higher genetic material loading of SARS-CoV-2 than any

138   other study reported. This may be because we sampled in the vicinity of the isolation centre, while

139   other studies compared in **Table 2** have reported the values from the wastewater treatment plant.

140   Overall, we successfully detected ORF1ab and N protein genes from the wastewater samples of

141   Bangladesh, which is for the first time, reported the data from the containment centre. Our study

142   provides an opportunity to produce a realistic coefficient in the future for the conversion of

143   genetic material loading with the number of infected people in the community.

144

145   Further, referring to the limitations of the present study, surrogate samples were sent to other

146   laboratories to check how precisely the Sansure kit data matches with others. We also tested the

147   filtration methods to check with the efficiency of the PEG method used in this study. Although,

148   Hata and Honda (2020) reported a high efficiency of the PEG method in Japanese wastewater, and

149   the same has been found true by Kumar et al. (2020g) while testing the sample concentration

150   efficiency between PEG and filtration. While lack of supply always poses critical challenges in any

151   research, during lockdown we could not find a supply of MS2, as used by Kumar et al. (2020a,g) or

152   could perform the whole process control (WPC) together with MPC as recommended by Haramoto

153   et al. (2020). We had to use a swab sample of a symptomatic person as our control as indicated

154   by CY-5 as quality control of our analyses, which makes sense and provides a low-cost control for

155   such analyses, yet it may be controversial, less precise and at times comes negative as the case in

156   a few samples in our case (drain 1 samples at 300 and 400m and main sewer sample on 07th and

157   26th August). By applying fluorescence quantitative RT-PCR technology, this test utilized the novel

158   Covid-19 ORF1ab and the specific conserved sequence of coding nucleocapsid protein N gene as

159   the target regions, which were designed for conserved sequences of the double target genes to

160   achieve detection of wastewater samples RNA through fluorescent signal changes. The PCR

161   detection system used the positive internal control, which monitors the presence of PCR inhibitors

162   in test specimens by detecting whether the internal control signal is normal, to avoid a false

163   negative result when used for human RT-PCR experiments. In our experiments with wastewater,

164   few specimens were negative for CY5 indicated that the human gene RNase P gene was missing in

165   the samples. Hence it has been noted that the human RNase P gene is more vulnerable to

166   degradation than Covid-19 viral genes.

167

168   We also refrained to put here any semi-quantitative calculation of gene copies owing to lack of

169   enough replicates, kit designed for the human sample as well as uncertainties involving RT-PCR

170   (Stuart et al 2014) and will induct such calculation in our future study when there will be a method

171   at hand for precise copy calculations using suitable methods. Nevertheless, the bottom line is that

172   the patterns of obtained Ct values suggest successful detection of SARS-CoV-2 RNA from the

173   wastewater samples in Bangladesh, and their increasing abundance in tertiary drain demonstrated

174   that it is not difficult to employ the COVID-19 surveillance through wastewater in the sewer

175   systems to know the community health as we probably do not need dense sampling but the major

176   drains may be enough to use the capability of wastewater-based epidemiology in south-Asian

177   settings.

178

179   ## 4. Conclusions

180   While the wastewater surveillance of COVID-19 has been focused on wastewater treatment plants

181   around the world, we have opted for drain waters monitoring in the vicinity of the isolation center,

182   which is first of its kind. Apart from this being the first detection report of SARS-CoV-2 RNA in the

183   wastewaters of Bangladesh, the uniqueness of the study has been the tracing the genetic load in

184   the vicinity of the isolation center that contains almost the constant number with 200 COVID-19

185   patients, which takes the variable of the number of infected persons out of the equation. This has

186   been the key feature of this study as most of the study reported worldwide has either reported

187   total infected person in the city or country. There has been a complete lack of infected person

188   information that is contributing to the total genetic load to the sampled wastewater. We have

189   found about 75% of our sampled water positive based on ORF1ab gene absence or presence.

190   However, the critical observation has been the temporal variation where small drains showed an

191   easing of the loading of genetic load, the bigger canal, and main sewer city exhibited temporal

192 accumulation of SARS-CoV-2 RNA. On the other hand, the distance of sampling location in meters

193 appears to be insignificant from the perspective of wastewater surveillance of COVID-19. We

194 intend to analyze further samples taken in June, July, and August and preserved to shed further

195 light on the temporal variation and decay/accumulation processes of the genetic load of the SARS-

196 COV-2.

197

198 **Ethics Statement**

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

200 **Acknowledgement**

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343 **Table 1.** Summary of the results of amplification cycles (Ct) of various sampled wastewater along with the distance of sampling in the

344 vicinity of isolation center with 50 days temporal resolution i.e. in between 10th July and 29th August 2020.

| Source	Distance from Isolation Center (m)	Date of Sample collection	RNA Quantity		Ct Value		
					(Sansure Kit)		
			Concentration (ng/µl)	Ratio (260/280)	FAM (ORF1ab)	ROX (Ngene)	Cy5 (RnaseP)
Drain 1	100	18-08-2020	12.42	1.97	34.69	32.25	37.91
		29-08-2020	25.5	1.95	ND	ND	36.42
	200	17-08-2020	12.37	1.99	40.72	ND	36.95
	300	07-08-2020	24.68	1.92	ND	ND	ND
	400	26-08-2020	12.23	2	39.43	ND	ND
Drain 2	200	18-08-2020	13.18	1.92	36.94	35.78	37.57
	400	26-08-2020	14.37	2.01	ND	ND	36.14
Drain 3	200	17-08-2020	17.47	1.97	36.78	35.82	39.08
		18-08-2020	22.61	1.98	22.35	20.42	32.3
	300	17-08-2020	15.97	1.92	31.03	29.03	37.08
		26-08-2020	22.16	1.99	32.85	30.47	40.13
	400	26-07-2020	15.31	1.95	32.84	30.35	34.52
Canal	NTS	10-07-2020	27.31	1.97	40.77	39.38	38.79
		26-08-2020	18.14	2	32.18	30.18	35.32
Main Sewer	NTS	07-08-2020	24.63	1.96	ND	ND	ND
		18-08-2020	16	1.94	34.85	33.24	37.94

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346 *NTS= Distance not to scale; ND: Not Detected*

347 **Table 2.** Comparative positive sample and the range of Ct value reported of ORF-lab genes of SARS-

348 CoV-2 in the wastewater of various countries.

349

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

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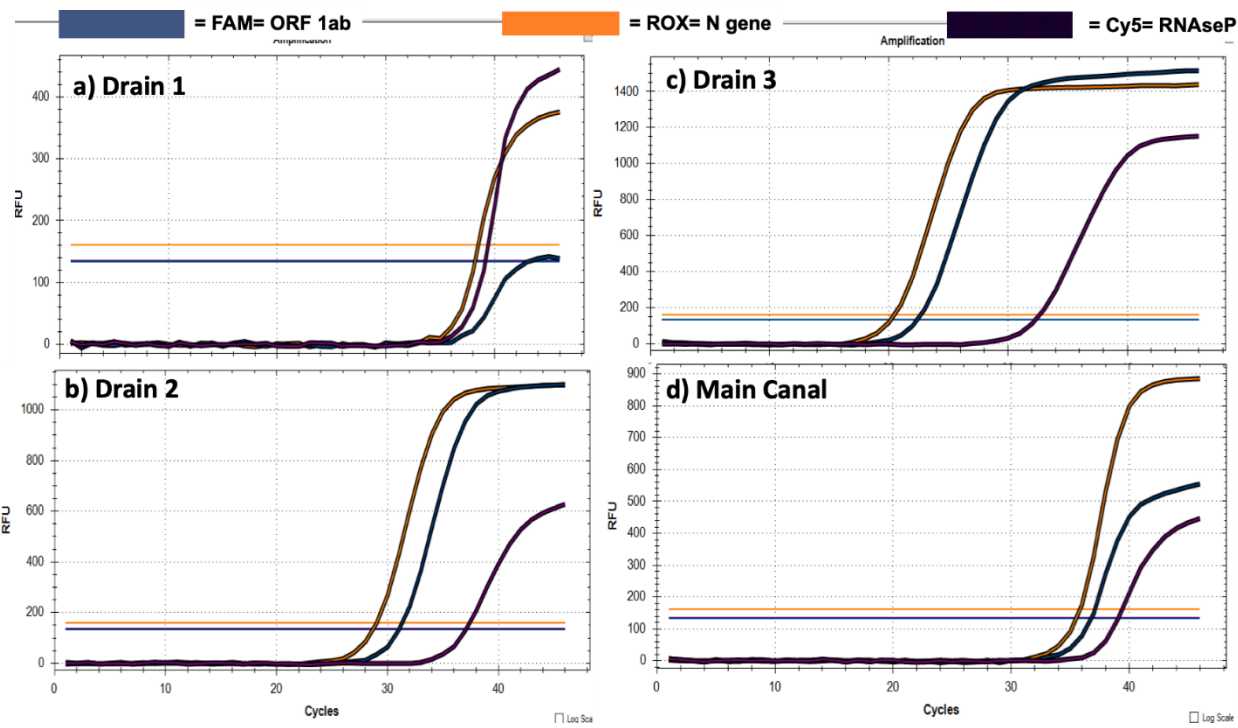
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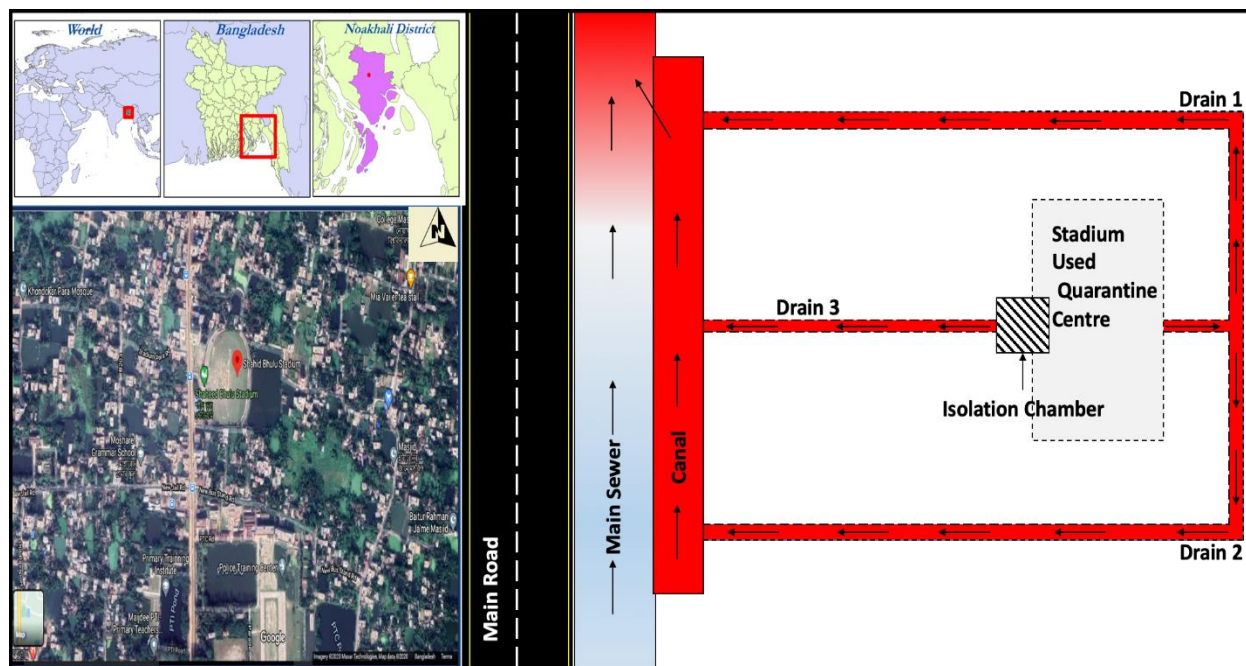
Figure 1. Study area location depicting the isolation camp established at the Shaheed Bhulu Stadium at Noakhali along with the schematic diagram of sampling drains.

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357 **Figure 2.** Amplification plots obtained through RT-PCR illustrating temporal variation through C_t
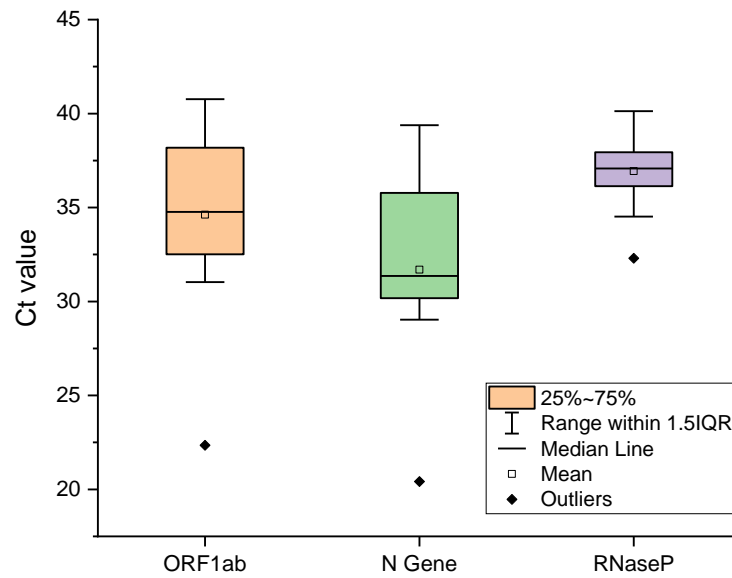
358 value in various sampling drains.

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Figure 3. Box-whisker plot illustrating the Ct-value variations of three genes for the entire

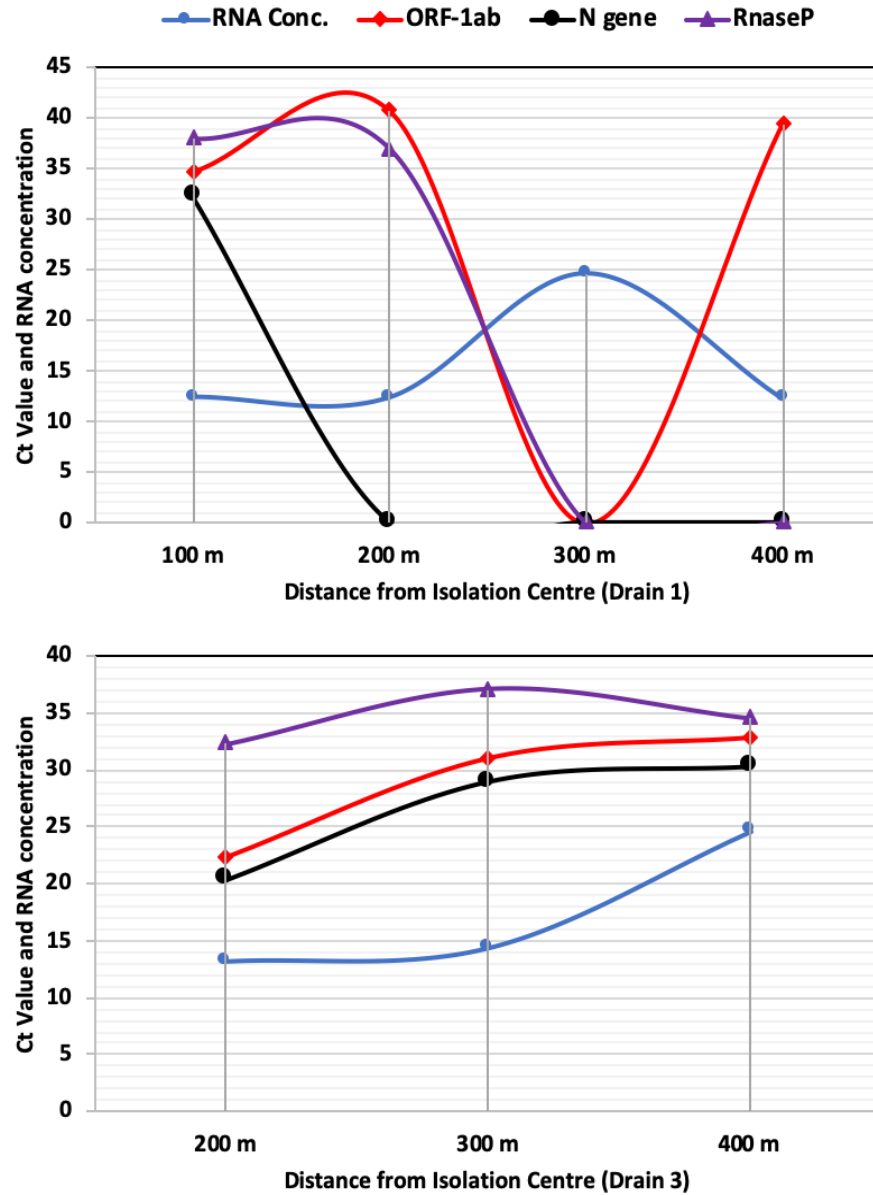361   monitoring period along with the outliers.

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

Author Contributions

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

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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: