Evaluating Recovery Control Concentrations of Bovine Coronavirus (EVAg 015V-02282) Used for SARS-CoV-2 Wastewater Surveillance

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Abstract. SARS-CoV-2 is a pathogenic strain of coronavirus which causes acute respiratory disease in humans. Community spread of COVID-19 was difficult to assess in the beginning of the pandemic, so new methods of detecting the virus had to be discovered. For this reason, national wastewater surveillance systems were implemented in order to detect the spread of SARS-CoV-2 virus. However, to establish such systems, there's a need to standardize protocols for coronavirus concentration, as well as finding the optimal titre of BCoV, generally used as a recovery control. In our study, we used a strain of Betacoronavirus 1 (EVAg 015V-02282) provided by EVAg as our process control. In order to set up which concentration of the Bovine coronavirus (BCoV) will give the most conclusive results, we diluted the virus three times by the decimal method before running the samples in the QIAcuity Digital PCR (dPCR). The evaluation was made so that the BCoV concentration could be used for future studies.

1 Introduction

Wastewater surveillance has been implemented for detecting and quantifying SARS-CoV-2. This method has been applied in countries like Italy, Netherlands, Germany, France and other non-EU countries like Japan, Australia and USA [1-4]. Wastewater surveillance was adopted because it's a cost-effective way of monitoring SARS-CoV-2 and it has the advantage of being independent of the availability of clinical testing resources [5]. At this moment, several studies were already conducted for wastewater surveillance of SARS-CoV-2 RNA to see the genomic variations of the virus [6]. However, as it is a new area of studies, and because wastewater is a complex system, at the moment there's no standardized protocol regarding the choice of recovery control, concentration and detection methods, given that different results have been reported [7].

The use of a recovery control for viral concentration from water is needed to measure the viral losses during the process [8]. Therefore, for SARS-CoV-2, viruses such as bovine

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coronavirus (BCoV), bovine respiratory syncytial virus (BRSV) and human coronavirus OC43 have been used for this purpose. These types of viruses, also called "proxies" are added before or after sample storaging, although the second option is preferred [9]. The proxy virus can also be incubated in the wastewater sample at a known concentration before RNA extraction. Depending on points of inoculation three types of process control exist: whole process controls, molecular process controls and RT-qPCR controls. Another important aspect for the proxy is the genetic similarity with the SARS-CoV-2 [10]. Therefore, in our study, we used bovine coronavirus (BCoV) as a process control which was added before ultrafiltration of our wastewater sample.

The bovine coronaviruses (BCoVs) are responsible for respiratory and enteric infections in cattle and other wild ruminants. Like SARS-CoV-2, BCoVs are part of the family Coronaviridae, order Nidovirales [11]. Similar to COVID-19, BCoV virus has the structural proteins spike (S), envelope (E), the membrane (M), nucleocapsid (N) and additionally, the hemagglutinin-esterase (HE) protein [12]. BCoV uses 5-N acetyl-9-O-acetylneuraminic acid as a receptor to cellular binding, while SARS-CoV-2 uses angiotensin-converting enzyme receptor for the same purpose [13, 14].

The bovine coronavirus resemblance with SARS-CoV-2 makes it a good candidate as a process control, because it may behave the same way as our target virus when undergoing the concentrating and extraction process. Therefore, BCoV has been previously used so the viral losses during the process could be monitored [15, 16]. According to LaTurner et al. [17] study, BCoV represents a viable option as a process control, because it had an overall low coefficient of variance among different concentration methods and a good recovery rate. However, further studies are needed in order to establish parameters such as recovery control titre, etc.

The present study aims to assess the recovery rate of BCoV and the presence of SARS-CoV-in wastewater samples. Additionally, we evaluated which concentration is better suited for the recovery control. For our analysis we chose to apply RT dPCR (QIAcuity Digital PCR) as it was considered the most efficient method for this type of samples [18].

2 Characteristics of SARS-CoV-2

SARS-CoV-2 is a monocatenary virus comprised of positive-sense RNA [19]. The genome has 30 kilobases of which two-thirds code for the replicase complex (ORF1a and ORF1b) and 16 non-structural (Nsps 1-16) proteins have a role in virus replication. The other one-third consists of structural and accesory genes [20]. The nucleocapsid protein (N) forms the capsid outside the genome which is also packed by an envelope which consists of: the membrane protein (M), spike protein (S), and envelope protein (E) (Fig. 1.) [21].

The spike's glycoprotein (S) main role is pathogeny as it has the ability to bind to the host cell through its RBD (receptor-binding domain). The infection is facilitated by the protein subunits (S1, S2 and S2') which adhere to the host cell. By doing this, the RNA genome is further released in the host cell which starts the replication cycle trough the ORF genes [22]. Envelope proteins (E) are smaller proteins found in the virion which are important for viral morphogenesis [23]. Just like the N protein, it also contains conserved regions among the BAT-CoV, SARS-CoV, and SARS-CoV-2 versions of coronavirus. The membrane's protein (M) function is RNA packaging and is the most abundant protein of coronaviruses, as it gives the distinct shape of the virus [21].

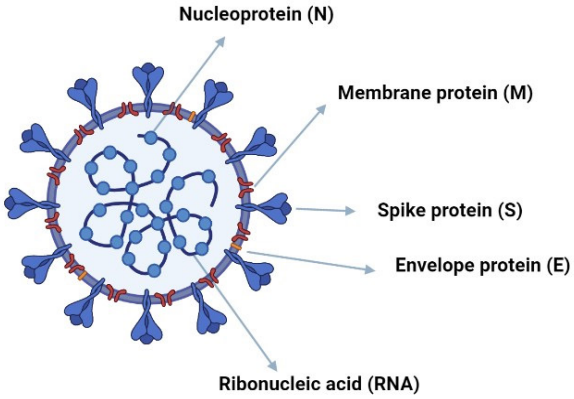

Fig. 1. Schematic representation of the SARS-CoV-2 structure [24]

The nucleocapsid (N) protein is a multivalent RNA-binding protein as it has more domains like disordered N-terminal domain (NTD), an RNA-binding domain (RBD), a predicted disordered central linker (LINK), a dimerization domain, and a disordered C-terminal domain (CTD). These domains were predicted to interact with both RNA structures and other proteins [25]. The role of nucleocapsid proteins (N) is packaging viral RNA into ribonucleocapsid as well as mediating the assembly of the viral particle by interacting with the genome and M protein. This mediation also helps RNA transcription and replication [26]. According to Naqvi et al. [19], aligning N sequences from BAT-CoV, SARS-CoV and SARS-CoV-2 showed highly conserved regions.

While studying the SARS-CoV-2 virus, PCR-based assays have used different regions of the coronavirus, such as: ORF1a and ORF1b regions, the nucleocapsid region (N), the envelope (E), the spike (S) protein and the RNA-dependent RNA polymerase (RdRP) genes [27]. However, the most frequent used regions in research are the nucleocapsid (N) and envelope (E) genes. Less often, some studies used the ORF1a, ORF1b and S genes [20].

When choosing which target genes had the most sensitivity in detecting SARS-CoV-2, studies had to asses Ct (cycle threshold) values for different coronavirus genes. Therefore, by targeting the E, N1, N2, N3 genes using qPCR it was noted that the E gene was the least sensitive, having low Ct values, and that the N gene had high Ct values, even if the level of RNA was low. One explanation for the sensitivity difference between genes was ascribed to nucleotide mutations [20, 28]. The World Health Organisation and the United States Center of Disease Control recommended as well the use of the nucleocapsid gene targets (N1 and N2) [29]. According to a comparative study [30] more primers were assessed for their efficiency towards SARS-CoV-2 detection. As they targeted E, N and RdRp genes, they observed that the use of N1 primers had the most robust results. Therefore, it can be considered that the N1 primer/probe set is a sensitive option when it comes to detecting SARS-CoV-2.

3 Materials and Methods

3.1 Wastewater Samples

The wastewater sample was collected from one location in Bucharest (N 44.408031, E 26.141911) in a 1 liter container and immediately stored at 4 °C. After collection it was transported to the National Institute for Research and Development in Environmental Protection for processing. In our present study, we used the sample of wastewater received on 18th May 2023 so we could test the best concentration that needs to be used for our recovery control (Bovine coronavirus). For this analysis we used the QIAcuity Digital PCR.

3.2 Bovine coronavirus internal recovery control

Bovine coronavirus strain S379 Riems was prepared from a stock solution provided by EVAg (015V-02282). 1 mL of ultrapure water was added at first in order to restore the lyophilizate virus. After this step, 100 µL BCoV stock was aliquoted in 1,5 mL tubes. The aliquots were stored immediately at -80 °C in the ultrafreezer.

To define the best concentration for BCoV process control, it was necessary to obtain an intermediate phase dilution. Hence, 540 µL water were mixed with 60 µL BCoV stock to acquire the intermediary BCoV solution. To continue the dilution, we added 100 µL of intermediary BCoV in 30 mL of ultrapure water and obtained the working solution for BCoV. At last, the working solution of BCoV was used for decimal dilution method from a solution of 30 mL ultrapure water and 100 µL BCoV intermediate phase. At the end of this stage we had three different concentrations of our process control (1:10, 1:100, 1:1000).

3.3 Sample preparation and ultrafiltration

For the pre-concentration stage, we transferred 40 mL from our wastewater sample into six conical tubes and mixed them with 100 µL BCoV working solution. Each tube was inoculated with a different concentration of the process control as shown in Table 1. Before the ultrafiltration step, the tubes had to be centrifuged at 5500xg for 10 minutes to obtain and collect the supernatant. The ultrafiltration stage was done through centrifugal concentrators provided by Sartorius and Millipore. Therefore, 14 mL of the supernatant were transferred with a pipette in three Amicon® Ultra-15 tubes (#VS2032) and three Vivaspin 20 tubes (#UFC903024) as indicated in Table 1. Two types of concentrators were chosen in order to establish which will get the best results. All tubes were centrifuged at 5500xg for 20 minutes until the whole volume was filtered through the tube membranes. After completion of this step, our concentrated samples were recovered from the filter tubes with a pipette and transferred in a 2 mL Eppendorf tube [31].

Table 1. Distribution of diluted BCoV in concentrators tubes

SAMPLE	CONCENTRATOR TUBE	DILUTION
SAMPLE 1	Amicon® Ultra-15 tubes (Sartorius)	10^{-1}
SAMPLE 2	Amicon® Ultra-15 tubes (Sartorius)	10^{-2}
SAMPLE 3	Amicon® Ultra-15 tubes (Sartorius)	10^{-3}
SAMPLE 4	Vivaspin 20 tubes (Millipore)	10^{-1}
SAMPLE 5	Vivaspin 20 tubes (Millipore)	10^{-2}
SAMPLE 6	Vivaspin 20 tubes (Millipore)	10^{-3}

3.4 Nucleic acid extraction

The RNA from our wastewater samples was extracted using the AllPrep® PowerViral® kit for RT-dPCR, following the manufacturer instructions. From our previous concentrated viral samples, we added 200 µl in each 2 mL tube. 600 µl PM1/β-ME mix was further added to the sample tubes and then vortexed. 150 µl IRS solution was mixed and vortexed with our samples followed by incubation at 2-8°C for 5 minutes. Subsequently, samples were centrifuged at 13000xg for 1 minute following supernatant collection into 2 mL tubes. In the end, 600 µl of each supernatant was used for viral RNA extraction using the automated nucleic acid extraction instrument QIAcube Connect (QIAGEN, USA). The obtained RNA has been stored at -80 °C for future applications [31].

3.5 Detection and quantification of N1, N2 genes by RT-Digital PCR

For the quantification step of N1 and N2 regions from SARS-CoV-2 and BCoV we used the QIAcuity Digital PCR from Qiagen [32]. The technology was chosen as wastewater samples need a more sensitive approach [18]. Before running the analysis, we had to prepare the extracted RNA. Therefore, we added 20 µL master mix and 20 µL of our RNA samples and controls (negative and positive) in eight 200 µL Eppendorf tubes. After mixing the components, we loaded 39 µl of our obtained samples in the QIAcuity 26K Nanoplate (Fig. 2) and sealed it. The set up for the RT-Digital PCR was made according to the manufacturer instruction using QIAcuity® Software Suite. The targeted regions were read on three different channels at different intensities. The green and yellow channel corresponding to N2 and BCoV respectively had an exposure duration of 300 ms. For N1 gene, the red channel had an exposure duration of 700 ms. In the end of the analysis, the amplification data was obtained through the QIAcuity® Software Suite.

The samples were loaded in the QIAcuity 26K Nanoplate as indicated in Fig. 2 in the following order: the first three wells (A1, B1, C1) contained the samples concentrated with Sartorius tubes that were inoculated with 3 different concentrations of BCoV (1:10, 1:100, 1:1000) (#VS2032) following another three wells (D1, E1, F1) containing samples concentrated with Millipore tubes (#UFC903024). The last two wells (G1, H1) were loaded with one positive control and one NTC (no template control).

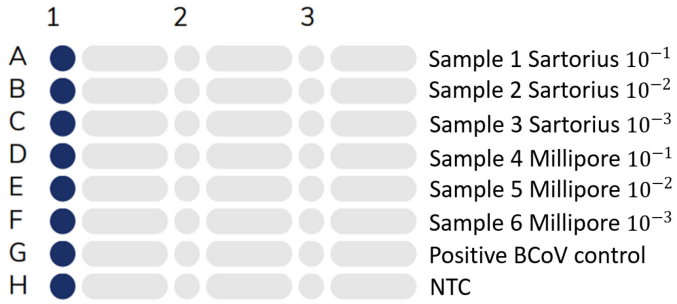

Fig. 2. Sample arrangement in the QIAcuity 26K Nanoplate, where A1– Sample 1 Sartorius 10^{-1}; B1– Sample 2 Sartorius 10^{-2}; C1– Sample 3 Sartorius 10^{-3}; D – Sample 4 Millipore 10^{-1}; E1– Sample 5 Millipore 10^{-2}; F1– Sample 6 Millipore 10^{-3}; G1– Positive BCoV control; H1– NTC.

4 Results and discussion

In this study, we focused on revealing which of the two concentrators we used for the ultrafiltration obtained the best results and which process control (BCoV) dilution will be the most suited for future studies on SARS-CoV-2 Wastewater Surveillance.

According to the table below (Table 2), which was obtained through QIAcuity® Software Suite, it can be observed that the samples that were spiked with 1:10 diluted BCoV (A1, D3) had the most conclusive results. The sample corresponding with A1 had 18 positive partitions for N2, 23 for BCoV gene and 27 for N1 and a concentration of 0.928, 1.186, 1.393 copies/ µl. The sample from D3 plate well had 22, 96, 27 partitions for N2, BCoV, N1 and a concentration of 1.180, 5.156, 1.448 copies/ µl respectively. These results had also revealed that the most suited concentrators were the ones from Millipore, as the corresponding values were higher in comparison with the Sartorius concentrated samples. Additionally, positive partitions were observed in almost every well except for the negative control. All of our samples presented the required number of valid partitions so the Digital PCR analysis could be considered viable.

4.1 Fluorescent intensity of sample concentrations

Beside the numbers we obtained from our analysis, the QIAcuity® Software Suite permitted us to go further with our research and represent our results more clearly. Therefore, a scatter plot point graphic was made to represent the fluorescent intensity of our targeted genes N1, N2 and BCoV. As it can be seen in Fig. 3, the most abundant fluorescence was present in the G1 well which corresponded with the positive control. The fluorescence could be observed for all targeted regions. Additionally, in accordance with the previous table, it is noticeable that the D1 well, corresponding to the Millipore concentrated sample, is more abundant in fluorescence than the other samples. Another aspect that can be observed in Fig. 3 is that both N1 and N2 targeted regions presented fluorescence which indicates the presence of SARS-CoV-2 in the wastewater samples.

Testing SARS-CoV-2 from wastewater samples can be a difficult assessment because of the organic matter that can interact with the viral particles. Depending on the concentration and extraction method results can vary. For example, other studies chose to use electronegative membranes [3] because of their high adsorption of enveloped viruses or methods based on polyethylene glycol (PEG 8000) [33] when concentrating their samples. However, the data on recovery rates of the process control is limited and none of the studies reported the percent of the virus that was recovered.

Table 2. RNA quantification of SARS-CoV-2 from wastewater samples and bovine coronavirus

	SAMPLE	TARGET	CONCENTRATION (COPIES/ µL)	PARTITIONS		
				valid	positive	negative
A1	T4 – Sartorius (1:10 BCoV)	N2	0.928	25329	18	25311
		BCoV	1.186	25329	23	25306
		N1	1.393	25329	27	25302
B1	T4 – Sartorius (1:100 BCoV)	N2	0.527	25419	10	25409
		BCoV	0.000	25419	0	25419
		N1	0.844	25419	16	25403
C1	T4 – Sartorius (1:1000 BCoV)	N2	1.230	25311	23	25288
		BCoV	0.160	25303	3	25300
		N1	1.284	25311	24	25287
D1	T4 – Millipore (1:10 BCoV)	N2	1.180	25403	22	25381
		BCoV	5.156	25403	96	25307
		N1	1.448	25403	27	25376

E1	T4 – Millipore (1:100 BCoV)	N2	1.281	25457	24	25433
		BCoV	0.053	25457	1	25456
		N1	0.747	25457	14	25443
F1	T4 – Millipore (1:1000 BCoV)	N2	1.278	25481	24	25457
		BCoV	0.373	25481	7	25474
		N1	1.545	25481	29	25452
G1	Positive Control	N2	66.08	25433	1217	24216
		BCoV	80.11	25435	1468	23967
		N1	66.30	25433	1221	24212
H1	Negative control	N2	0.000	25443	0	25443
		BCoV	0.000	25443	0	25443
		N1	0.000	25443	0	25443

According to Feng et al. study [5], that used droplet digital PCR (ddPCR), the N1 gene proved to be more sensitive to this detection method. Similarly, the same observation could be made in our case, where the targeted N1 region had a higher fluorescent intensity. Moreover, we could also see in our case that the BCoV gene was not correlated with the N1, N2 regions meaning that the SARS-CoV-2 and the process control may recover differently after ultracentrifugation and extraction. The reason for this may be that the SARS-CoV-2 virus and the recovery control had different concentrations, but also that the coronavirus from wastewater had more contact time with the samples [9].

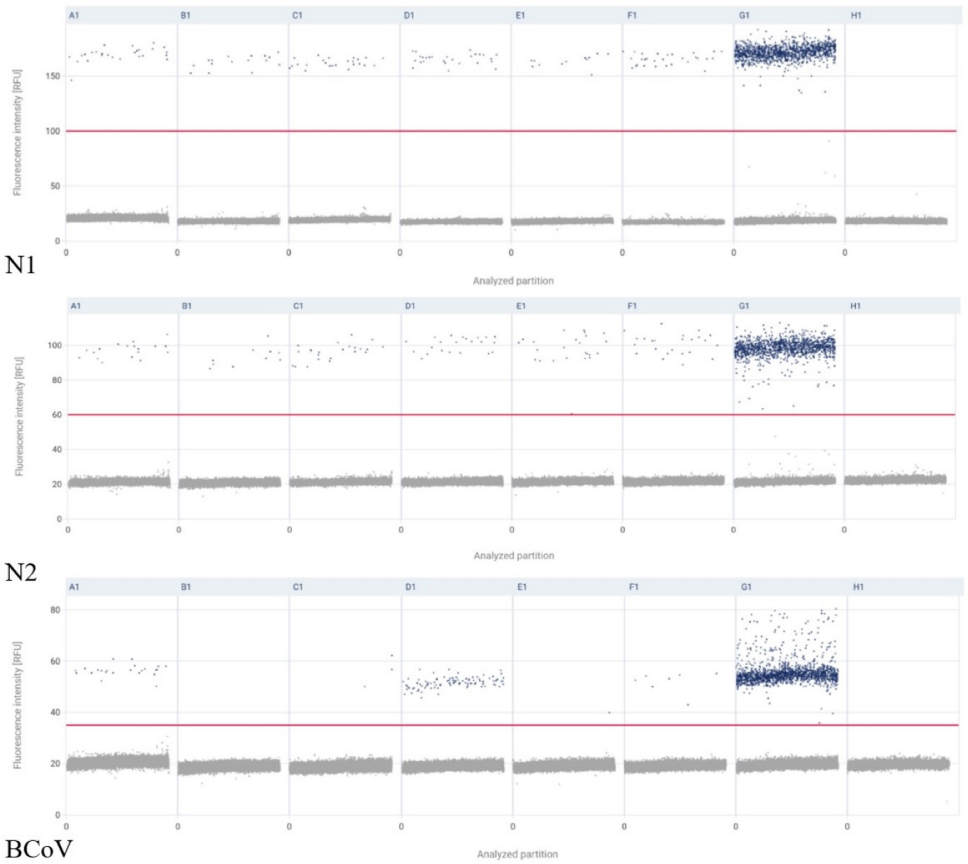

Fig. 3. Scatter plot of fluorescent intensity for N1, N2, BCoV

5 Conclusions

By using different concentrations of BCoV and two types of concentrator tubes (Sartorius and Millipore), we assessed which ones will obtain the most optimal results. Our findings showed us that both samples spiked with 10^{-1} BCoV dilution obtained higher values than the other samples when using RT-dPCR. This means that the bovine coronavirus with the highest concentration will be a more suitable option for future studies. Additionally, it could be noted that the samples that went through ultrafiltration with Millipore concentrators were better according to the number of positive partitions and the fluorescence intensity from our analysis. Overall, our study demonstrates that the BCoV virus is a viable option as a process control and that the used concentration is an important parameter that must be taken into account for the wastewater surveillance of SARS-CoV-2.

Acknowledgments

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