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Journal of Virological Methods 297 (2021) 114249

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Short communication

Evaluation of low-cost viral concentration methods in wastewaters: Implications for SARS-CoV-2 pandemic surveillances

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ARTICLE INFO

Keywords:
BCoV
PP7
Polyethylene glycol precipitation
Skimmed milk flocculation
Aluminum polychloride flocculation

ABSTRACT

In the pandemic of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) many strategies have been performed in order to control viral spread in the population and known the real-time situation about the number of infected persons. In this sense, Wastewater Based Epidemiology (WBE) has been applied as an excellent tool to evaluate the virus circulation in a population. In order to obtain reliable results, three low-cost viral concentration methods were evaluated in this study, polyethylene glycol (PEG) precipitation, skimmed milk flocculation (SM) and Aluminum polychloride flocculation, for *Pseudomonas aeruginosa* bacteriophage PP7 as a surrogate for non-enveloped viruses and Bovine Coronavirus (BCoV) as a surrogate for enveloped virus, with emphasis for SARS- CoV-2. Our results suggest that PEG precipitation for viral concentration, for both enveloped and non-enveloped virus from wastewater is an appropriate approach since it was more sensitive compared to SM flocculation and Aluminum polychloride flocculation. This methodology can be used for WBE studies in order to follow the epidemiology of the SARS-CoV-2 pandemic, mainly in developing countries where the economic resources are frequently limited.

Wastewater Based Epidemiology (WBE) as an indicator of human viruses circulating in a population is a useful approach already used in different studies around the world (Pina et al., 2001; Villena et al., 2003; La Rosa et al., 2014; Prevost et al., 2015; Victoria et al., 2016). One of the most remarkable WBE approaches is the surveillance of wild and vaccine strains of poliovirus, given support to the worldwide program for the eradication of poliomyelitis (WHO, 2015).

Since the initial outbreak of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) in December 2019, and the posterior declaration of pandemic by the World Health Organization (WHO) in March 2020, many strategies have been performed in order to control viral spread in the population and known the real state of situation about the number of infected persons in a determined region. Conventional strategies to assess SARS-CoV-2 circulation are based on the direct detection of the virus (nucleic acids by molecular methods or viral antigens by immunoassays) (La Marca et al., 2020) or by indirect tests, such as serological testing (Koopmans et al. 2020), both analyzing directly each suspected person with the infection. This approach is time consuming and expensive since thousands of molecular tests are performed daily in each country in order to track both symptomatic and asymptomatic patients. WBE appears as an excellent strategy, since it can evaluate indirectly, the trend of the viral circulation in the entire population, with the analysis of just a few wastewater samples. This WBE approach is also cheaper than the evaluation of each suspected infection in the population, mainly in developing countries, where the resources are scarce; moreover, indirectly allows the detection of asymptomatic, paucisymptomatic and presymptomatic individuals (Hart and Halden, 2020; Polo et al., 2020). In this way, the detection of SARS-CoV-2 in samples of the gastrointestinal tract and feces (Holshue et al., 2020; Xiao et al., 2020; Wu et al., 2020a) from infected individuals support the use of WBE as an indicator of virus dispersion in the population. Many studies have been detected SARS-CoV-2 in wastewater samples around the world even before the identification of the virus in the population of the studied regions (Ahmed et al., 2020; La Rosa et al., 2020a; Medema et al., 2020; Wu et al., 2020b; Wurtzer et al., 2020).

Concentration method of viral particles present in wastewater samples is a very important step in order to obtain reliable results. Since current methods used for concentration of viral particles were developed

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https://doi.org/10.1016/j.jviromet.2021.114249
Received 19 May 2021; Received in revised form 28 July 2021; Accepted 28 July 2021
Available online 31 July 2021

and mainly used for non-enveloped virus, comparison of methods could shed light concerning a consistent method to concentrate SARS-CoV-2 (an enveloped virus) in wastewater samples. In this way, some authors have analyzed distinct methods such as ultrafiltration, absortion-elution based methods, PEG precipitation, skimmed milk (SM) flocculation, aluminum polychloride, bag mediated filtration system, ultracentrifugation, using different viruses as surrogates of SARS-CoV-2 like *Bovine coronavirus*, *Feline Calicivirus*, *Human coronavirus* OC43, *Porcine epidemic diarrhea virus* and *Mengovirus* (Gonzalez et al., 2020; Jafferali et al., 2021; Barril et al., 2021; Philo et al., 2021; Pérez-Cataluña et al., 2021). Because wastewater profile can vary due to different conditions as geography or population, more research is needed in order to evaluate reliable wastewater surveillances (La Rosa et al., 2020b).

The aim of this study was to evaluate three viral concentration methods, polyethylene glycol precipitation, skimmed milk flocculation and Aluminum polychloride flocculation, for *Pseudomonas aeruginosa* bacteriophage PP7 as a surrogate for non-enveloped viruses and Bovine Coronavirus (BCoV) as a representative of enveloped viruses, with emphasis for SARS-CoV-2. PP7 as surrogates of non-enveloped virus have been already used in several studies of environmental water matrices (Ahmed et al., 2020; Assis et al., 2018; Balboa et al., 2020; Barril et al., 2021; Blanco Fernández et al., 2017; Calgua et al., 2013; Castells et al., 2019; Blanco Fernández et al., 2017; Rajal et al., 2007), and BCoV was chosen as a specific representative of SARS-CoV-2 since their high similarity and phylogenetic proximity, both belonging to the *Betacoronavirus* genus in the *Coronaviridae* family (LaTurner et al., 2021).

The concentration methods compared in this study were chosen since they are used in developing countries mainly due to their inexpensive reagents and devices. PEG, SM and aluminum polychloride flocculation are more accessible methods compared with ultrafiltration or ultracentrifugation methods, which are generally used in high income countries (Medema et al., 2020; Wurtzer et al., 2020; Nemudryi et al., 2020; Balboa et al., 2020).

Three independent experiments were performed with three independent samples which were collected between November and December of 2020 from the influent of a wastewater treatment plant in the city of Salto, Uruguay. Samples containing three liters of raw wastewater were collected each time, refrigerated and immediately transported to the laboratory for further analysis. In a biosafety cabinet (BSL2), the wastewater samples were partitioned to concentrate the same sample in duplicated by the three viral concentration methods. Two volumes of 500 mL were concentrated by PEG precipitation method, two volumes of 200 mL were concentrated by Aluminum polychloride flocculation and two volumes of 50 mL were concentrated by skimmed milk flocculation.

Each volume of wastewater sample was seeded with 50 µL of BCoV (10^5 genomic copies/µL), kindly provided by Dr. Mirazo and Dr. Berois from Virology Section from School of Sciences, UdelaR, previously cultured in HRT-18 cells and 50 µL of PP7 (10^6 genomic copies/µL), kindly provided by Dr. Rajal from Salta University, Argentina, previously cultured in *Pseudomonas aeruginosa* cell suspension. After the inoculation of the viruses in the wastewater, samples were subjected to viral concentration. PEG precipitation method was performed according to the protocol described by Lewis and Metcalf (1988) with modifications (Lewis and Metcalf, 1988). First, weights of centrifugation pots

Table 1
Percentage of recovery success and recovery efficiency of PP7 in three independent experiments. Each experiment was performed with a different sample in duplicate. 6.2×10^7, 3.0×10^7 and 6.1×10^7 genomic copies were inoculated in experiment 1, 2 and 3 respectively.

PP7 Recovery efficiency									
	PEG precipitation			SM flocculation			Aluminum polychloride flocculation		
	Sample	Concentrated sample[#]	% of recovery	Sample	Concentrated sample[#]	% of recovery	Sample	Concentrated sample[#]	% of recovery
	1a	256,667	0.4	1a	3,833,333	6.2	1a	333	0.0005
	1b	456,667	0.7	1b	510,000	0.8	1b	333	0.0005
Experiment 1	1a 1/10	2,675,000	4.3	1a 1/10	16,400,000	26.5	1a 1/10	6567	0.0106
	1b 1/10	6,975,000	11.3	1b 1/10	17,666,666	28.5	1b 1/10	13,883	0.0224
	2a	9,666,667	16.4	2a	2,150,000	7.3	2a	667	0.0023
	2b	2,916,667	4.9	2b	2,883,333	9.8	2b	1000	0.0034
Experiment 2	2a 1/10	19,833,333	33.6	2a 1/10	1,065,000	3.6	2a 1/10	ND	ND
	2b 1/10	13,416,667	22.7	2b 1/10	813,333	2.8	2b 1/10	5000	0.0169
	3a	3,396,667	5.6	3a	1,901,833	3.1	3a	667	0.0011
	3b	3,874,167	6.4	3b	2,338,333	3.8	3b	1167	0.0019
Experiment 3	3a 1/10	4,083,333	6.7	3a 1/10	13,385,000	22.0	3a 1/10	ND	ND
	3b 1/10	9,600,000	15.8	3b 1/10	10,905,000	17.9	3b 1/10	167	0.0003
Mean (±SD)	PEG	3,427,917 ± 3,413,756	5.7 ± 5.8	SM	2,269,472 ± 1,102,035	5.2 ± 3.2	Al	695 ± 340	0.0016 ± 0.0011
	PEG 1/10	9,430,556 ± 6,393,679	15.7 ± 10.9	SM 1/10	10,039,167 ± 7,432,831	16.9 ± 11.2	Al 1/10	6404 ± 5681	0.0125 ± 0.0095
PP7 Recovery success[*]	Undiluted sample		6/6(100 %)			6/6(100 %)			6/6(100 %)
	Diluted sample		6/6(100 %)			6/6(100 %)			4/6(66 %)
	Total		12/12(100 %)			12/12(100 %)			10/12(83 %)

ND: Not detected. SD: Standard deviation.
[*] Positive qPCR reactions/total qPCR reactions (% positive samples).
[#] genomic copies.

were registered. 500 mL of sample was distributed in two bottles (250 mL each) and centrifuged at 4750 xg for 20 min at 4 °C. Supernatant (S1) was maintained at 4 °C to be used later. Pots were weighted again and weight of each pellet was calculated. Sediment was mixed with 3 volumes respect to pellet volume with 3% Beef extract (Oxoid, Hampshire, England), 2 M $NaNO_3$ eluant (pH 5.5). Volumes of the same sample were mixed in one pot, pH was adjusted to 5.5 and sample was stirred for 1 h at 4 °C. Solids were then removed by centrifugation at 10, 000 xg for 20 min and the eluted was mixed with the first supernatant obtained (S1) and adjusted to pH 6.5−7.2. PEG 6000 (Sasol. Hamburg, Germany) was added to a final concentration of 10 % (w/v) and NaCl to 2% (w/v). The resulting suspension was stirred for 6 h at 4 °C and centrifuged at 10,000 xg for 25 min. The supernatant was discarded and the pellet was resuspended in 5 mL of PBS buffer (Dako Inc., California, USA) (pH 7.2), adjusted to pH 8.0, incubated for 1 h with occasional vortex, and centrifuged at 10,000 xg for 20 min. Supernatant was stored for nucleic acid extraction.

Skimmed milk flocculation method was performed according to the protocol described by Calgua et al. (2013). 50 mL of each sample were mixed with glycine buffer 0.25 N, pH 9.5 (1:2 v/v) to elute viruses from organic matter, centrifuged at 8000 xg after shaking 30 min on ice. The resulting supernatant was acidified to pH 3.5 by the addition of HCl 1 N. Pre-flocculated skimmed milk (Difco™, France) was added to supernatant and the mixture stirred for 8 h to allow the viral adsorption to the flocs. Samples were directly centrifuged at 8000 xg for 30 min at 4 °C and the pellet was resuspended in 1 mL of phosphate buffer at pH 7.5 (1:2, v/v of Na_2HPO_4 0.2 M and NaH_2PO_4 0.2 M).

Flocculation with aluminum polychloride was performed according to method described by Randazzo et al. (2020). Briefly, 200 mL of the sample was transferred to 250 mL centrifugal pots, pH was adjusted to 6.0 with HCl 1 N and 2 mL of a 4% $AlCl_3$ (Acros organics, Geel, Belgium) solution was added. pH was readjusted to 6.0 and the sample was shaked for 15 min at 150 rpm at room temperature. Then, the sample was centrifuged at 1700 xg for 20 min and the supernatant was discarded. The pellet was resuspended with 10 mL of 3% beef extract solution (Oxoid, Hampshire, England), shaked for 10 min at 200 rpm, and centrifuged for 30 min at 1900 xg. Finally the pellet was resuspended in 1 mL of PBS (pH 7.2).

Nucleic acid was extracted from 300 µL of each concentrated sample, and 300 µL of a dilution 1/10 of each sample (to evaluate inhibition of the enzymatic reaction by fulvic and humic acid, proteins, metals and salts), using a Liferiver viral DNA/RNA isolation kit (Shanghai ZJ Biotech Co., Ltd.) following the manufacturer's instructions.

Reverse transcription was performed with random primers pd(N)6 (Macrogen, Republic of Korea) and RevertAid reverse transcriptase (RevertAid RT Thermo Scientific™, Carlsbad, CA, USA) according to manufacturer's instructions. Quantitative PCR reactions were performed in duplicate using Rotor-gene Q thermocycler (Qiagen, Hilden, Germany). RNAse/DNAse-free water was used as negative control to validate nucleic acid extraction and enzymatic reactions, PP7 and BCoV cultures were used as positive control of these reactions.

Detection and quantification of PP7 was performed by qPCR in a final volume of 25 µL, containing a final concentration of 0.4 µM of primers 247 F and 320 R, 1X Sensifast Probe No-Rox Kit (Bioline, UK), 274 probe in a final concentration of 0.4 µM, 5.2 µL of DNAse/RNAse free water and 5 µL of cDNA. The amplification was performed with one step of denaturation at 95 °C for 5 min and 45 cycles at 95 °C for 15 s and 60 °C for 1 min (Rajal et al., 2007).

Detection and quantification of BCoV was carried out by qPCR in a final volume of 25 µL, containing a final concentration of 0.4 µM of primers BoCV Minn F, BoCV Minn R, 1X Sensifast Probe No-Rox kit (Bioline, UK), BCoV Minn S probe in a final concentration of 0.2 µM, 5 µL of DNAse/RNAse free water and 5 µL of cDNA. The amplification was performed with one step of denaturation at 95 °C for 10 min and 45 cycles at 95 °C for 15 s and 60 °C for 1 min (Castells et al., 2019).

We used previously established standard curves available in our laboratory in order to quantify the spiked and recovered viruses and all the samples were previously checked for natural contamination of BoCoV.

The recovery of PP7 as a surrogate of non-enveloped viruses is shown in Table 1. As we can see, PEG precipitation and SM flocculation methods detected PP7 in all the analyzed samples (percentage of recovery success of 100 %) presenting similar virus efficiency recovery (≈5%). Considering the volume of the analyzed sample, PEG methodology has an advantage over SM since the same quantity of viral particles where added to 50 mL and 500 mL of samples concentrated by SM and PEG, respectively. These results suggest that PEG methodology is more sensitive than SM since the original sample is 10 times larger for PEG (500 mL) than for SM (50 mL) concentration. The results of the 1:10 dilution evidences the presence of inhibitors in the samples. These inhibitors did not affect the recovery success since the virus was detected in all the analyzed samples without dilution, nevertheless, partial inhibition affects the virus concentration recovery efficiency with an increase of one fold observed in diluted samples. Viral concentration with aluminum polychloride flocculation recovered the virus in all the undiluted analyzed samples (percentage of recovery success of 100 %), however, the percentage of recovery efficiency is far less sensitive than the other evaluated methods (three folds less than PEG and SM). Dilution 1:10 of samples concentrated by aluminum polychloride flocculation showed a recovery success of 66 %, two out of six analyzed samples could not detect the virus, however, percentages of recovery efficiency are better than samples without dilution, nevertheless, values are lower than the percentages obtain with PEG and SM method. It is worth mentioning that the recovery efficiency of all the analyzed samples with Aluminum polychloride flocculation were under 0.05 %.

BCoV recovery values, as a surrogate of SARS-CoV-2 demonstrated that PEG methododology perform better than SM flocculation. Mean percentage of recovery efficiency were similar for both methods but for one sample (with and without dilution) concentrated by SM, the virus was not detected (percentage of recovery success of 66 %). Dilution of the samples (1:10) showed better values of recovery efficiency than samples without dilutions but in the same order of magnitude. Similar to the results obtained for PP7, considering the same virus inoculum and the different samples volumes for PEG (500 mL) and SM (50 mL) methodologies, it is likely that PEG viral concentration is a methodology with higher sensitivity than SM for BCoV detection and quantification in wastewater.

Our results of BCoV concentration recovery are in concordance with data published by Gonzalez et al. (2020) and Jafferali et al. (2021) since they obtained percentage of recovery around 5%, although they used different concentration methodologies (InnovaPrep, electronegative filtration and adsorption-elution).

Results obtained with aluminum polychloride flocculation showed the lowest recovery efficiency and recovery success of the viral concentration methodologies analyzed in this study for both undiluted and diluted samples.

Methods evaluated in this works shows that PEG precipitation and SM flocculation have similar percentage of recovery for enveloped and non-enveloped viruses, using PP7 and BCoV as surrogates of each one. Nevertheless, aluminum polychloride flocculation demonstrated that this method recovered PP7 (with a low percentage of recovery), but not BCoV (only one replica of three samples was detected).

Considering these results obtained for the three methodologies, they are partially in accordance with data published by other studies (Pérez-Cataluña et al., 2021, Barril et al., 2021). In these works they recovered a Porcine Epidemic Diarrhea Virus (PEDV) and Feline Calicivirus (FCV) as surrogates of enveloped and non-enveloped viruses using viral concentration methods based on PEG and aluminum-based adsorption-precipitation. Percentage of recovery using PEG is similar to that described in our study, but by using aluminum polychloride flocculation we could not recover BCoV. These differences could be explained since we used a different virus as surrogate of SARS-CoV-2 or

Table 2
Percentage of recovery success and recovery efficiency of BCoV in three independent experiments. Each experiment was performed with a different sample in duplicate. 3.2×10^7, 1.3×10^7 and 5.7×10^6 genomic copies were inoculated in experiment 1, 2 and 3, respectively.

BCoV Recovery efficiency									
	PEG precipitation			SM flocculation			Aluminum polychloride flocculation		
	Sample	Concentrated sample[#]	% of recovery	Sample	Concentrated sample[#]	% of recovery	Sample	Concentrated sample[#]	% of recovery
Experiment 1	1a	48,333	0.2	1a	1,159,500	3.6	1a	ND	ND
	1b	67,500	0.2	1b	150,167	0.5	1b	500	0.002
	1a 1/10	858,333	2.6	1a 1/10	5,055,000	15.6	1a 1/10	ND	ND
	1b 1/10	1,641,667	5.1	1b 1/10	3,541,667	10.9	1b 1/10	ND	ND
Experiment 2	2a	525,833	4.0	2a	ND	ND	2a	ND	ND
	2b	157,500	1.2	2b	ND	ND	2b	ND	ND
	2a 1/10	891,667	6.7	2a 1/10	ND	ND	2a 1/10	ND	ND
	2b 1/10	558,333	4.2	2b 1/10	ND	ND	2b 1/10	ND	ND
Experiment 3	3a	250,833	4.4	3a	2833	0.05	3a	ND	ND
	3b	235,833	4.1	3b	197,000	3.4	3b	ND	ND
	3a 1/10	400,000	7.0	3a 1/10	3333	0.06	3a 1/10	ND	ND
	3b 1/10	291,667	5.1	3b 1/10	1,051,666	18.4	3b 1/10	ND	ND
Mean (±SD)	PEG	214,305 ± 173,880	2.4 ± 2.0	SM	377,375 ± 527,939	1.9 ± 1.9	Al	NA	NA
	PEG 1/10	773,611 ± 488,433	5.1 ± 1.6	SM 1/10	2,412,917 ± 2,303,170	8.9 ± 8.0	Al 1/10	NA	NA
BCoV Recovery success[*]	Undiluted sample		6/6(100 %)			4/6(66 %)			1/6(17 %)
	Diluted sample		6/6(100 %)			4/6(66 %)			0/6
	Total		12/12 (100 %)			8/12(66 %)			1/12(8 %)

NA: Not applicable, ND: Not detected. SD: Standard deviation.
[*] Positive qPCR reactions/total qPCR reactions (% positive samples).
[#] genomic copies.

due to the composition of the wastewater samples which could affects considerable the performance of the concentration methodologies since the presence of inhibitors in the samples was evidenced by the results of the efficiency recovery for 1:10 dilutions (Table 2).

Several studies that used different concentration methods using different viruses as surrogates of SARS-CoV-2 described the highest recovery efficiency for different concentration methods: Pérez-Cataluña et al. (2021) described that the best percentage of recovery was obtained with PEG, Philo et al. (2021) described the best results using Skimed milk flocculation, Barril et al. (2021) using aluminum polychloride flocculation and Jafferali et al. (2021) using ultrafiltration. There is an enormous difficult in order to determine which is the best viral concentration method for SARS-CoV-2 since each study compare different methodologies with different viruses as surrogates. Differences in recoveries were already reported for non-enveloped viruses (Haramoto et al., 2018), and it is likely that consistent differences are observed when comparing different viral concentration methods for enveloped virus. Difference in chemical composition of wastewater between different countries and type of viruses (Pons et al., 2004; Lu et al., 2020) could be affecting results obtained by different concentration methodology, especially when viral concentration methods are based on chemical modification of the matrix.

Based in our results we suggest that PEG precipitation for viral concentration, for both enveloped and non-enveloped virus from wastewater is an appropriate approach since it was more sensitive when compared with SM flocculation and aluminum polychloride flocculation. This methodology can be used for WBE studies in order to follow the epidemiology of the SARS-CoV-2 pandemic, mainly in developing countries where the economic resources are frequently limited for an exhaustive surveillance of clinical cases of SARS-CoV-2 infections.

Author statement

Conceived the study: MV and RC. Wrote the manuscript: MS. Performed the laboratory experiments: MS and AM. Supervised data collection and laboratory work: MV, RC, EA and PG. Critical reviewed the manuscript: MV, RC, AM, EA and PG. All authors read and approved the final manuscript.

Financial support

We want to thank the financial support by the program 'Polo de Desarrollo Universitario' (PDU), Universidad de la República (UdelaR), Uruguay and the staff of the wastewater treatment plant.

Declaration of Competing Interest

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.

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