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Detection of SARS-CoV-2 in wastewater is influenced by sampling time, concentration method, and target analyzed

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ABSTRACT

The detection of SARS-CoV in wastewater has been proposed as a tool for monitoring COVID-19 at the community level. Although many reports have been published about detecting viral RNA in wastewater and its presence has been linked to infected people, appropriate analytical methodologies to use this approach have not yet been established. In this study, we compared ultrafiltration, polyethylene glycol precipitation, flocculation using $AlCl_3$, and flocculation with skim milk for the recovery of SARS-CoV-2, using RNA from patients with positive diagnoses for COVID-19 and *Pseudomonas* phage φ6 as the control. We also evaluated the primers for detecting the E, RdRp, and N genes of the virus, as well as different storage times. Differences in the recovery efficiencies were evident with the different concentration methods, the best being ultrafiltration and precipitation with aluminum, which had recovery rates of 42.0% and 30.0%, respectively, when virus was present at high levels. Significant differences were found between the recoveries using wastewater and deionized water and between different storage times, with better recoveries for 6 and 12 h samplings. The E gene was the only one detected in all the samples analyzed. The results show that although this approach can provide important data for studying the pandemic, clear protocols are necessary for investigations to be comparable.

Key words: COVID-19, epidemiology, method validation, RT-PCR, SARS-Cov-2, wastewater

HIGHLIGHTS

- RNA of SARS-CoV-2 was used to evaluate the recovery of different methods in wastewater.
- The utility of phage φ6 of *Pseudomonas* as a control for evaluation of recovery methods of SARS-CoV-2 from wastewater was demonstrated.
- Differences due to the target selected for the detection of SARS were demonstrated.
- The results show that it is possible to detect SARS RNA in wastewater, however its epidemiological usefulness will depend on the correct validation of the technique used for detection.

INTRODUCTION

The pandemic caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has caused many social, economic, and public health concerns (Kaye *et al.* 2020; Tisdell 2020). To control its effects, different actions have been proposed, including the mass testing of people, social distancing, contact tracing, isolating infected individuals, and in many countries, the complete restriction of people's mobility (Block *et al.* 2020). However, given the impossibilities of analyzing an entire population and maintaining long-term confinements, other tools such as wastewater-based epidemiology (WBE) provide information about the virus's behavior in a community and thus allow for making correct public health decisions have gained attention (Sims & Kasprzyk-Hordern 2020).

Wastewater-based epidemiology is a new approach being used to provide comprehensive health information in communities. The concept is based mainly on the extraction, detection, and subsequent analysis and interpretation of chemical or biological compounds present in wastewater, the receptor of waste from all human activities. Thus, WBE has been proposed

as a tool for monitoring the spread of infectious diseases at the community level (Sims & Kasprzyk-Hordern 2020). SARS-CoV-2 is mainly a respiratory virus; however, its RNA has also been detected and quantified in high concentrations in the fecal matter of both symptomatic and asymptomatic patients (Chen *et al.* 2020; Wölfel *et al.* 2020). This information has served as the basis for several investigations in different countries that used the WBE concept by measuring SARS-CoV-2 RNA in wastewater to understand and study the virus's circulation within communities (Ahmed *et al.* 2020a; Hasan *et al.* 2020; Hata *et al.* 2020; Sherchan *et al.* 2020).

Studies have reported that the virus can be detected in the wastewater of a given place even before the first patient reports (Medema *et al.* 2020). Others have reported that wastewater detection can provide more reliable information about the number of infected in a population (Ahmed *et al.* 2020a), as most asymptomatic patients are not detected by the classical epidemiological approach; this would serve to detect spikes of contagion and prepare health systems in advance. This is why the application of the WBE methodology has attracted attention for rapid community-level monitoring for COVID-19, which can then be verified by many emerging scientific protocols. The development of a rapid and inexpensive WBE-based methodology could facilitate real-time surveillance and predict future COVID-19 pandemic outbreaks for public health authorities.

Although the information that WBE provide can be of great value for monitoring the pandemic, most of the reported investigations have used different virus concentration and detection methods. Thus far, there are no standardized and validated protocols that allow information to be compared between different countries. Critical steps in testing for SARS-CoV-2 RNA in wastewater include the sampling and storage of the wastewater, the method of virus concentration, the selected genes to test for and their appropriate reverse transcription polymerase chain reaction (RT-PCR) primers, and data analysis. Studies have reported using different controls and viruses such as the murine hepatitis virus, bovine coronavirus, and bacteriophage φ6 as internal controls (Ahmed *et al.* 2020b; Torii *et al.* 2020), but there is no consensus on which virus should be used as a control for the concentration process. Most papers published on this topic lack complete information on how they validated the analytical technique used for the detection and quantification of the SARS-CoV-2 particles, which increases the uncertainty of the measurements and therefore the possible usefulness of the data to make inferences about the population. For example, some studies did not provide any information on the method's recovery efficiency, control used, and limits of detection (Ahmed *et al.* 2020b). For this reason, our aim in this study was to compare the most reported concentration methods to determine the efficiency of the recovery methods using real RNA from patients with positive COVID-19 diagnoses. We used bacteriophage φ6 as an internal control because this virus is easy to grow and can be easily stored in most laboratories, and therefore available for use in routine testing for SARS-CoV-2 in wastewater. We also evaluated different primers to determine their effect on the detection and quantification of SARS-CoV-2 virus RNA in wastewater and calculated validation parameters such as the efficiency, detection limit, quantification limit, repeatability, and precision.

MATERIALS AND METHODS

Preparation of *Pseudomonas* phage φ6

Pseudomonas phage φ6 (DSM 21,518) was propagated by the top agar layer method using *Pseudomonas syringae* (DSM-21,482) as the host strain. To obtain the inoculum, cultures were prepared with approximately 10^4 PFU/mL in 5 mL phosphate buffer and slowly agitated at room temperature for 4 h. The phage suspension was collected, and bacteria removed by centrifugation at 5,000 $\times g$. The supernatant was then filtered (0.45 μm) to remove any remaining bacteria.

Preparation of wastewater samples

Wastewater samples were obtained from the entrance of a treatment plant located in the metropolitan area of Valle de Aburrá, Medellín, Colombia. A 24-hour composite sampling was performed using an autosampler, and the samples were refrigerated until use. Physicochemical parameters are shown in the supplementary material. To evaluate the extraction efficiency, 200 mL each of wastewater or deionized water were inoculated with 1 mL of the phage suspension and 500 μL of SARS-CoV-2 RNA, which was obtained from samples taken from patients diagnosed as positive for COVID-19 at the teaching laboratory of the Microbiology School at the University of Antioquia. The samples were concentrated as described below, and RNA extractions were performed using the E.Z.N.A.® Total RNA Kit I according to the manufacturer instructions. To determine the stability of the RNA in wastewater, recovery tests were conducted at 6, 12, and 24 h.

Sample concentration

Four previously published concentration methods were evaluated for obtaining SARS-CoV-2 RNA from wastewater: flocculation with aluminum chloride (Randazzo *et al.* 2020), precipitation with polyethylene glycol (PEG) (Wu *et al.* 2020), flocculation with skim milk (Assis *et al.* 2017), and ultrafiltration (Barril *et al.* 2020). The recovery efficiency for each concentration method was calculated based on the number of copies quantified by RT-qPCR as follows:

$$\text{Recovery efficiency}(\%) = \left(\frac{\text{virus copies recovered}}{\text{virus copies added}}\right) \times 100.$$

All assays were made by triplicate. The mean and standard deviation were also calculated for each concentration method.

RT-qPCR analysis

Recently published RT-qPCR (quantitative PCR) primers and assays were used for detecting SARS-CoV-2 RNA in the wastewater samples. The primer and probe sequences are shown in Table 1 along with the PCR parameters. For all RT-qPCR assays, gBlocks® gene fragments were purchased from Integrated DNA Technologies (Coralville, IA, USA) and used as positive standards or controls and to construct standard curves from serial dilutions. For the RT-qPCR assays, the High-Capacity cDNA RT Kit (Thermo Fisher Scientific) was used to obtain the cDNA; qPCR was then performed using TaqMan™ Gene Expression Master Mix per the manufacturer protocol with a Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratories). All RT-qPCRs were performed in triplicate. An extraction blank, sample blank, and positive and negative controls were used in each analysis, and *Pseudomonas* phage φ6 cDNA was used as an internal amplification control. All samples were analyzed undiluted and diluted 10-fold to evaluate the effect of RT-PCR inhibitors.

Validation of RT-PCR for detecting SARS-CoV-2 in wastewater

RT-PCR dynamic range, efficiency, and linearity

For each RT-qPCR assay, 3 standard curves were generated by a linear regression analysis of the threshold cycle (Ct) versus the $\log_{10}$ copy number of the fragments of each gene. The amplification efficiency (E) was calculated with the slope (a) of the linear regression for each standard curve using the formula $E = 10^{\cdot|1/a|} - 1$. An efficiency of 90–110%. was accepted. To assess the linearity, the concentration of each gene was determined retrospectively using the formula $\log_{10}(x) = [Ct - b]/a$. The values obtained were compared with the theoretical quantities of each gene and provided the mean bias. The maximum allowed bias for the measured quantities was established at $\pm 0.25 \log_{10}$ per PCR.

Table 1 | Primers and probes used in this study

Organisms	Target	Primer type and probe	Sequence	Cycling parameters	References
Phage ϕ6	phi-6S_1 gene	Forward	5′-TGGCGGCGGTCAAGAGC-3′	35 cycles of 94 °C for 15 s and 60 °C for 1 min	Gendron *et al.* (2010)
		Reverse	5′-GGATGATTCTCCAGAAGCTGCTG-3′		
		Probe	FAM-CGGTCGTCGCAGGTCTGACACTCGC-TAMRA		
SARS-CoV-2	N protein	Primer type and probe	F-CACATTGGCACCCGCAATC	45 cycles of 95 °C for 15 s, 58 °C for 30 s.	Corman *et al.* (2020)
		Forward	R-GAGGAACGAGAAGAGGCTTG		
		Reverse	P-FAM-ACTTCCTCAAGGAACAACA		
		Probe	TTGCCA-BHQ1		
	E protein	Forward primer	ACAGGTACGTTAATAGTTAATAGCGT	45 cycles of 95 °C for 10 and 60 °C for 30 seconds	Medema *et al.* (2020)
		Reverse primer	ATATTGCAGCAGTACGCACACA		
		Probe	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ		
	RdRP gene	Forward primer	GTGARATGGTCATGTGTGGCGG	45 cycles of 95 °C for 15 s, 58 °C for 30 s	Corman *et al.* (2020)
		Reverse primer	CARATGTTAAASACACTATTAGCATA		
		Probe	FAM-CAGGTGGAACCTCATCAGGAGATGCBHQ1		

Limits of detection and quantification

The limit of detection (LOD) was defined as the target concentration at which the amplification product is detected with a probability of at least 0.95 ($LOD_{95\%}$). The LOD was determined by serial dilutions of the target. Twelve PCR replicate measurements were performed for each dilution level, and the level with the lowest number of copies for which the 12 replicates were positive was considered the approximate $LOD_{95\%}$ value.

The limit of quantification (LOQ) was defined as the lowest amount of target in a sample that can be reliably quantified. The LOQ was determined as the minimum concentration of the target for which 12 replicates give a positive result with the coefficient of variability for Ct being no more than 0.5 Ct. This quantification was performed by adding a known concentration of the target before sample preparation and RNA extraction.

Repeatability and reproducibility

For this test, several RT-qPCRs were performed with a sample in the same (intra-assay) and different (inter-assay) series, and the standard deviation of the quantitation cycle (Cq) and coefficients of variation were calculated.

Statistical analysis

A one-way analysis of variance (ANOVA) was used to determine whether there was a difference in the recoveries between the analyzed methods. A two-factor analysis of variance was also performed to determine differences between storage times, concentration methods, and virus recovery efficiencies. The association between the RNA recovery rates for SARS-CoV-2 and *Pseudomonas* phage φ6 was evaluated by a Spearman correlation.

RESULTS AND DISCUSSION

RNA recovery assays

Table 2 shows the extraction efficiency for the 4 evaluated methods. The quantification was conducted using gene E because in the initial evaluation of the samples, it was the one that showed the highest reproducibility and concentration. The tests were conducted using 3 different concentrations defined as high, medium, and low levels of presence. The results show that the best concentration methods were ultrafiltration and aluminum precipitation, with recovery efficiencies of 42.0% and 30.0%, respectively, at high levels of presence. Comparing the recoveries of the different methods, there were significant differences between them ($p < 0.05$). While it was possible to detect and quantify SARS-CoV-2 RNA at different concentrations with the methods, the best recovery efficiencies were obtained at the high and medium concentrations; this suggests the uncertainty measurement is higher at low concentrations, making it difficult to relate the viral RNA concentration with the real number infected in a community. However, measurements at low levels could still be useful for the early detection of outbreaks or infected people, which would be helpful for the authorities in making public health decisions, especially in developing countries, where it is not possible to do many tests on the population.

Significant differences were found when comparing the recovery efficiencies using wastewater and deionized water, which suggests that water quality affects the recovery process. Therefore, it is necessary to analyze the matrix effect each time a SARS-CoV-2 analysis is initiated. As the effect on recovery could be due to the physicochemical characteristics of the water, it is necessary to make determinations such as the biological oxygen demand, total organic carbon, and pH, which

Table 2 | Extraction efficiency for the evaluated methods

	Wastewater Recovery Rate % ± SD			Deionized Water Recovery Rate % ± SD		
Concentration method	High level 2×10^5 copies	Medium level 2×10^3 copies	Low level 2×10^1 copies	High level 2×10^5 copies	Medium level 2×10^3 copies	Low level 2×10^1 copies
PEG Precipitation	30.0 ± 11.3	22.8 ± 4.0	9.6 ± 4.0	85.8 ± 6.0	82,0 ± 6.7	79.0 ± 5.3
Aluminum flocculation	41.6 ± 9.1	37.6 ± 7.7	17.5 ± 8.0	84.1 ± 7.4	80.4 ± 9.4	78.7 ± 10.2
Skim milk flocculation	16.2 ± 8.8	15.0 ± 9.7	8.4 ± 2.4	79.6 ± 5.2	74.6 ± 8.1	72.1 ± 9.6
Ultrafiltration	42.0 ± 10.4	39.1 ± 8.3	22.3 ± 12.6	86.5 ± 5.5	87.2 ± 9.8	82.6 ± 8.3

affects the recovery of viral genetic material (Hamouda *et al.* 2020). These results demonstrate the importance of including a control that, in addition to the traditional positive and negative amplification controls, evaluates the overall correct functioning of the method with the concentration present.

Recovery at different times

The SARS-CoV-2 RNA recovery results at different times are shown in Figure 1. Due to its chemical nature, the RNA molecule is less stable under environmental conditions; therefore, it is necessary to establish how long wastewater sampling should last in order for the natural destruction of the molecule not to affect the stability of RNA. Most studies have reported composite sampling times of 6, 12, and 24 h. However, there is still not enough evidence to indicate which is the optimal sampling time and considers the fragility of the RNA and representativeness of the sample for the community. The results of this study suggest that sampling should last between 6 and 12 h because the recovery rate decreased significantly for the 24 h storage. However other studies focused on studying RNA decay in wastewater reported the average T_{90} (time required for 1-$\log_{10}$ reduction) of SARS-CoV-2 RNA ranged from 3.3 to 27.8 days in untreated wastewater (Ahmed *et al.* 2020c; Bivins *et al.* 2020). The rapid decay of RNA in this study suggest that additional studies are needed to explore how physicochemical characteristics of wastewater affect the SARS-CoV-2 RNA integrity in wastewater systems.

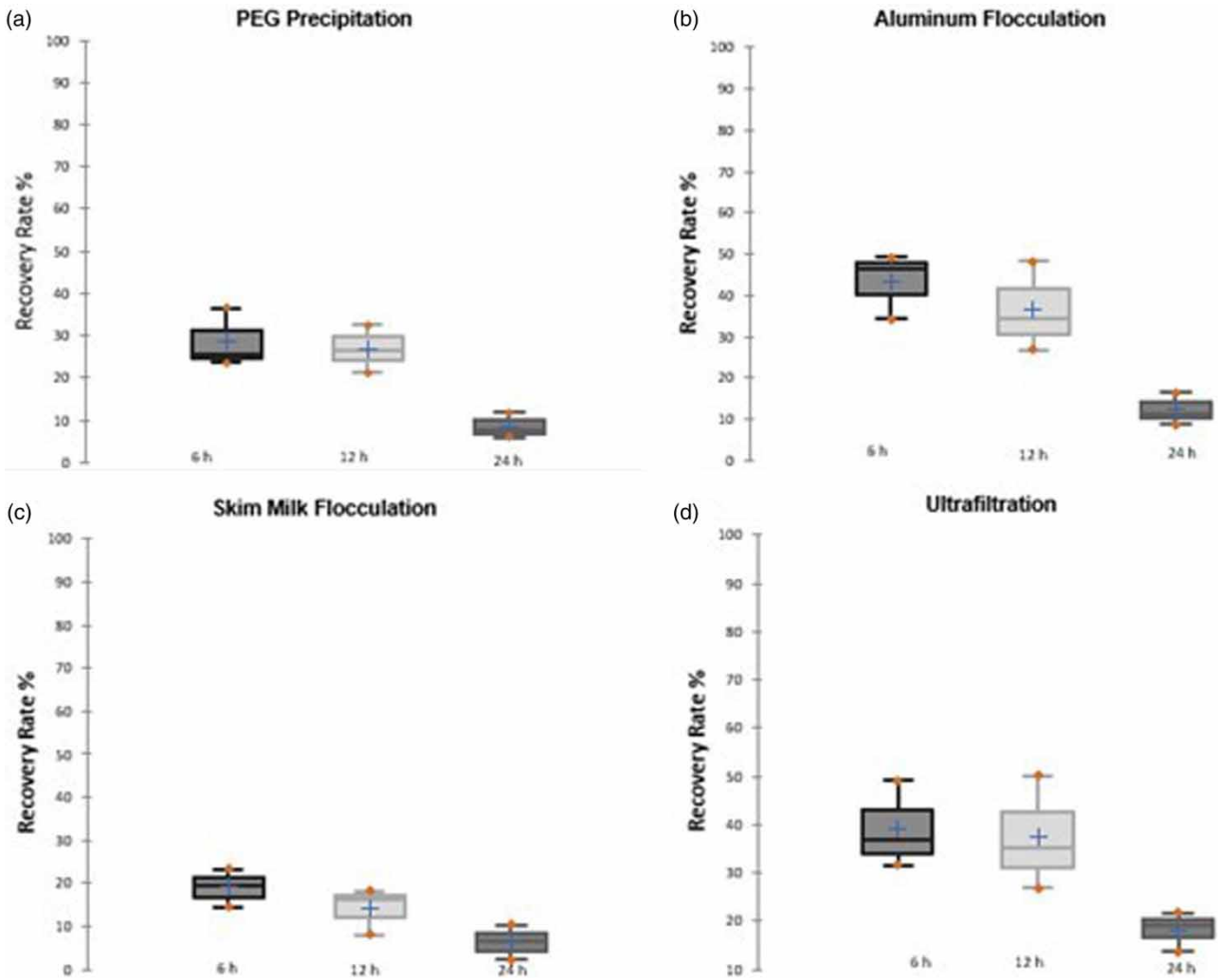

Figure 1 | SARS-CoV-2 RNA recovery rate at different times. (a) PEG precipitation, (b) aluminum flocculation, (c) skim milk flocculation, (d) ultrafiltration.

This aspect is important for the design of epidemiological surveillance plans based on monitoring RNA in wastewater, since it involves selecting sampling points that ensure the RNA is sampled at appropriate times and is representative of the behavior of the population. It is also necessary to consider whether sampling at the entrances of treatment plants will be representative, as the water may spend a long time in the system before reaching the plant, allowing much of the RNA to be lost, especially when ambient temperatures are high as in tropical countries (Ahmed *et al.* 2020c). The selection of the sampling point and time will also depend on the objective of the surveillance, which could be to evaluate the presence of COVID-19 in the community or to correlate its presence with the actual number of infected, as has been proposed by other authors (Medema *et al.* 2020; Randazzo *et al.* 2020).

Recovery of *Pseudomonas* phage φ6

Several viruses have been proposed for use as an internal control during the analysis of SARS-CoV-2, and some have shown good results. In this study, we used *Pseudomonas* phage φ6 to relate its recovery rate to that of SARS-CoV-2 RNA and thus establish its usefulness as a recovery control. The recovery results are shown in Figure 2. The Spearman correlation analysis showed a positive association between the recovery efficiencies for SARS-CoV-2 RNA and the phage. The results suggest that this phage can be used as an internal control and that its recovery efficiency is positively related to that of SARS-CoV-2 RNA. Although other viruses have been used for this control, phages are easier and cheaper to grow and thus their availability in laboratories is more common than animal viruses. Although *Pseudomonas* phage φ6 is not phylogenetically related to SARS-CoV-2, the results indicate that it may behave similarly to SARS-CoV-2 in wastewater due to its envelope and because it is an RNA virus, which is key to its usefulness as an internal control in these types of assays. For both viruses, recovery decreased when found at low levels, which shows the influence that residual water has on the recovery efficiency. It has been reported that particulate matter can influence the recovery of different types of viruses, including SARS-CoV-2 (Barril *et al.* 2020). Given that concentration is one of the critical reasons for monitoring COVID-19 in wastewater, more research will be required to understand how the physicochemical parameters of wastewater influence virus recovery and thus be able to select the most appropriate method (Lu *et al.* 2020).

Validation results

In this study, we validated the methodology using three primer pairs with different gene targets to determine the effect of the target used on the detection of SARS-CoV-2 RNA in wastewater. Although differences in the detection of RNA in wastewater using different primers has been previously reported, in this study, the validated methods were compared similarly to rule out that any differences found were due to the method's performance rather than the target. The results for the dynamic range, efficiency, and R^2 are shown in Table 3.

The results show that the methods had similar and acceptable characteristics for the dynamic range, linearity, and efficiency criteria, and therefore they could be compared during a wastewater sample analysis. The LOQ (copies/mL) for the method used for the N1 gene was 1.5×10^1, while those for the E and RdRp genes were 2.0×10^1 and 2.5×10^1, respectively. The repeatability results are shown in Table 4.

The evaluated validation parameters showed that the methods performed well at laboratory with the different types of primers used for detecting SARS-CoV-2. However, previous studies have reported differences in virus detection associated with the analyzed target (Barril *et al.* 2020). In this study, the concentration and detection processes were previously validated, and the quality control recommendations (EPA 2004) were followed, indicating that the differences found in SARS-CoV-2 detection were due to changes in the target sequences. Thus, the sequences of the previously designed primers could have prevented detection and quantification. It has been demonstrated that the N gene is one of the most non-conservative genes in the SARS-CoV-2 genome and has the most mutations on the targets of various nucleocapsid (N) gene primers and probes (Wang *et al.* 2020), which suggests that this gene is not the most recommended for WBE studies.

One of the major drawbacks of this type of analysis is the inhibitors present in wastewater, which can lead to the reporting of false negatives. The reliability of the detection and quantification data is related to the quality assurance processes for the tests, which include a series of controls that allow for demonstrating that the method works correctly. Although the methods used in this study were previously validated, when analyzing residual samples, the detection and quantification capacity of the individual primer sets differed in each sampling. To evaluate the behavior of the methods with residual water samples, samples were obtained as described above for 5 days and analyzed using the aluminum chloride method for RNA concentration and the three pairs of primers for detection. The results of these tests are shown in Table 5.

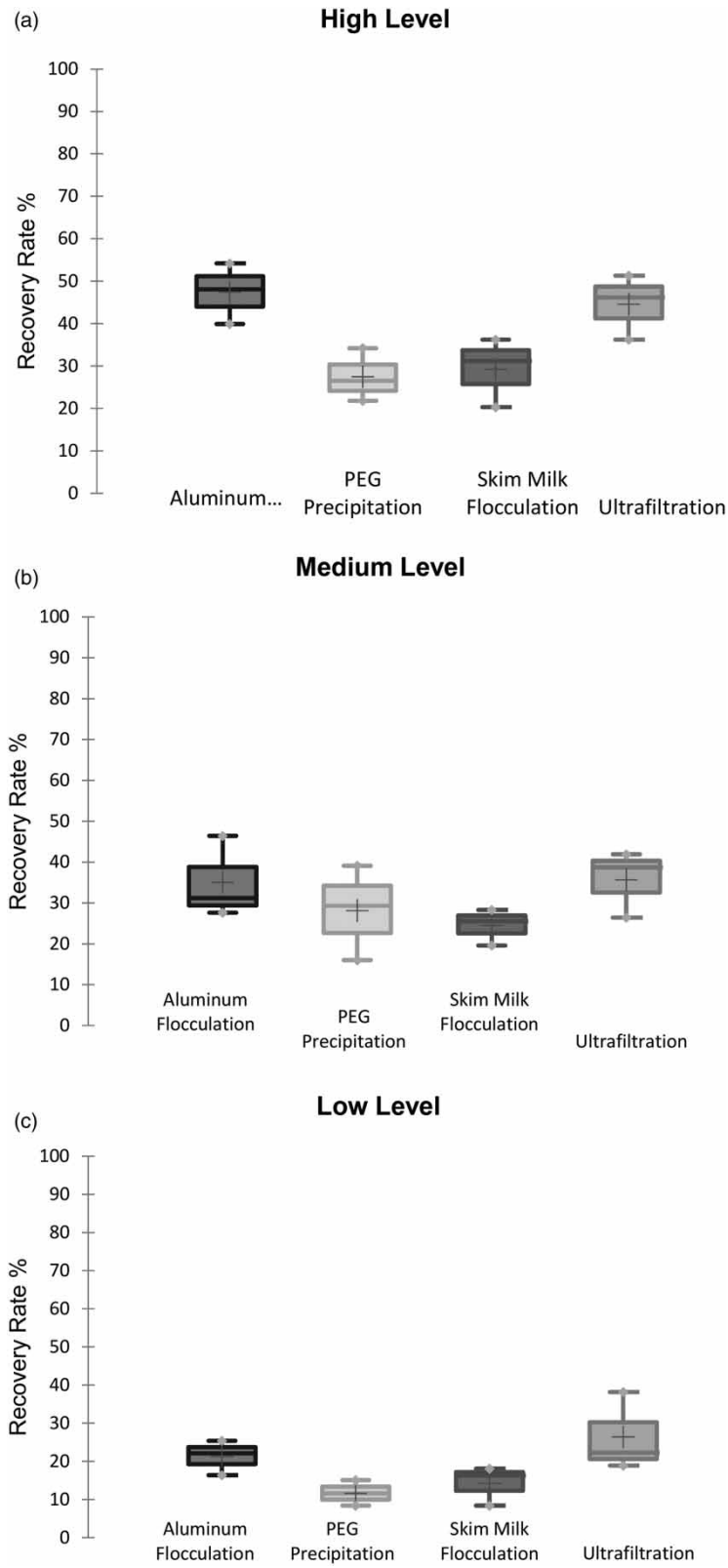

Figure 2 | Recovery of *Pseudomonas* phage $\varphi 6$ with different concentration methods. (a) High level 1.5×10^5 copies, (b) medium level 1.5×10^3 copies, (c) low level 2×10^1 copies.

Table 3 | Results for the dynamic range, efficiency, and R^2

Gen	Dynamic range (copies/mL)	Efficiency %	R^2
N1	1.5×10^1–1.5×10^7	103	0.998
E	1.0×10^1–1.0×10^7	97	0.999
RdRp	2.0×10^1–2.0×10^6	90	0.996

Table 4 | Repeatability results using different primers

	Gen E		Gen N1		Gen RdRp	
Copies/mL	Cq	%CV	Cq	%CV	Cq	%CV
1×10^3	26.9	0.5	20.6	2.0	24.9	2.7
1×10^2	30.2	10.8	24.31	1.1	27.4	1.8
1×10^1	32.8	2.2	27.76	0.9	30.5	3.0

Table 5 | SARS-CoV-2 RNA quantification from wastewater samples

	SARS-CoV-2 recovered copies/mL		
Sample	Gen E	Gen RdRp	Gen N1
1	3.3×10^3	1.4×10^3	1.1×10^2
2	1.6×10^2	ND	ND
3	4.3×10^2	ND	ND
4	4.8×10^4	3.1×10^2	ND
5	2.4×10^3	2.6×10^2	1.2×10^2

ND: no detected.

The primers used for the E gene detected it in all the analyzed samples, while the N and RdRp genes were detected in 2 and 3 samples, respectively. Because the amplifications obtained using *Pseudomonas* phage φ6 showed no evidence of PCR inhibition, the quantification differences are related to the target. The survival of RNA of SARS-CoV-2 in wastewater is mediated by different factors such as the presence of other microorganisms and by physicochemical factors such as pH, solids, and disinfectant compounds, which destroy the genetic material of the virus, making it more difficult to detect (Hamouda *et al.* 2020). Studies in humans have shown that as infection progresses, there are differences in detection depending on the type of gene used; this has been found to be related to the sensitivity of the primers and the time of infection (Nalla *et al.* 2020; van Kasteren *et al.* 2020). The differences found in this study could be related to the damage of the virus in the wastewater. It is possible that when RNA of SARSCoV-2 reaches the wastewater, a progressive disappearance of the different genes occurs due to the destruction of the molecule. The non-detection of a gene (e.g., RdRp) in these cases could be interpreted as a loss of the replicative capacity of the virus and therefore of its contagiousness or infectivity (Reina & Suarez 2020). However, more investigations are necessary to establish with certainty the behavior of the viral particle in wastewater, and in this way, to establish which are the most appropriate genes for monitoring the virus.

CONCLUSIONS

In this study, we showed that although experimentally the methods worked adequately when detecting the virus in wastewater, the genes used produced different results, making it necessary to conduct further studies to establish which gene is the most suitable target. The E gene was detected in all wastewater samples, suggesting its usefulness as a target in WBE analysis. Ultrafiltration and precipitation with $AlCl_3$ obtained the best RNA recovery percentages. The differences found in

the concentration methods also showed that the type of matrix influences the recovery of the virus, meaning that a method cannot be selected without first evaluating its recovery efficiency in the laboratory. The recovery of RNA from *Pseudomonas* phage φ6 was influenced in a similar way to that of RNA SARS-CoV-2, making it a good candidate to be used as a control in this type of assay. Although a large amount of information has been published regarding sampling times, concentration methods, controls used, and primers evaluated, the variability in our results shows that more studies are still necessary to establish clear protocols for sampling, concentration, and detection.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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