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Evaluation of Pre-Analytical and Analytical Methods for Detecting SARS-CoV-2 in Municipal Wastewater Samples in Northern Italy

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Abstract: (1) Background: The surveillance of SARS-CoV-2 RNA in urban wastewaters allows one to monitor the presence of the virus in a population, including asymptomatic and symptomatic individuals, capturing the real circulation of this pathogen. The aim of this study was to evaluate the performance of different pre-analytical and analytical methods for identifying the presence of SARS-CoV-2 in untreated municipal wastewaters samples by conducting an inter-laboratory proficiency test. (2) Methods: three methods of concentration, namely, (A) Dextran and PEG-6000 two-phase separation, (B) PEG-8000 precipitation without a chloroform purification step and (C) PEG-8000 precipitation with a chloroform purification step were combined with three different protocols of RNA extraction by using commercial kits and were tested by using two primers/probe sets in three different master mixes. (3) Results: PEG-8000 precipitation without chloroform treatment showed the best performance in the SARS-CoV-2 recovery; no major differences were observed among the protocol of RNA extraction and the one-step real-time RT-PCR master mix kits. The highest analytic sensitivity was observed by using primers/probe sets targeting the N1/N3 fragments of SARS-CoV-2. (4) Conclusions: PEG-8000 precipitation in combination with real-time RT-PCR targeting the N gene (two fragments) was the best performing workflow for the detection of SARS-CoV-2 RNA in municipal wastewaters.

Keywords: environmental surveillance; urban wastewater; PEG-8000 precipitation; SARS-CoV-2

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1. Introduction

Amid the pandemic of SARS-CoV-2 [1], communities have faced the rapid spread of the virus and its related disease—called COVID-19—affecting the testing capacity of public health systems and microbiological laboratories [2,3]. Strong evidence has shown the utility of viral RNA monitoring in municipal wastewater samples (sewage) for SARS-CoV-2 infection surveillance at a population-wide level—according to the wastewater-based epidemiology (WBE) approach [4–6]. Since SARS-CoV-2 is shed by feces in the early stage of infection and can cause asymptomatic infection in a large proportion of individuals, it is an ideal target for WBE. This strategy may allow to: (i) estimate the real prevalence of SARS-CoV-2 infection at a population level, (ii) monitor SARS-CoV-2 spread after the implementation of containment measures and restrictions and (iii) provide an early warning of virus re-introduction [4–6]. Moreover, surveillance of SARS-CoV-2 in sewage may provide timely indications on SARS-CoV-2 infection dynamics, overcoming the lag in monitoring exclusively COVID-19 symptoms and tests, since the onset of symptoms might be 2 weeks apart from viral infection [7–9]. Moreover, this approach of surveillance can overcome test availability and indications that can result under pressure during the surge of new outbreaks.

Several proof-of-principle studies on SARS-CoV-2 monitoring in municipal wastewater samples were designed and conducted through a number of different pre-analytical and analytical protocols, encompassing sewage concentration, RNA extraction and SARS-CoV-2 molecular detection, making it difficult to compare inter-laboratory results [5,9–11]. However, methods optimization and quality control are crucial for generating reliable public health information among countries and over time [4–6], as demonstrated in the surveillance of poliovirus in environmental wastewater samples in the framework of the global polio eradication initiative [12].

In Italy, a WBE network in the Lombardy Region (a region in Northern Italy accounting for nearly 10 million inhabitants) was recently established [13] in order to provide local support in SARS-CoV-2 infection surveillance in one of the Italian epidemic hot-spots. Different research institutions (co-authoring this work) in the Lombardy Region have collaborated to develop a common protocol of analysis by optimizing and standardizing the methods for the pre-analytical and analytical workflow in order to make results of inter-laboratory analysis comparable and applicable on a wider scale.

Initially, in order to evaluate the sensitivity and turn-around time of the different pre-analytical and analytical methods for detection of SARS-CoV-2 in municipal wastewater samples, an inter-laboratory proficiency test (PT) was carried out by the laboratories that participated in the WBE network, allowing researchers to identify the best-performing laboratory protocol to be included in the WBE network pipelines. The optimized protocol will be adopted for future regional and national surveillance studies in order to improve the quality and reproducibility of the results.

2. Materials and Methods

2.1. Generation of Wastewater Samples Stock

Two composite 24 h raw, untreated urban wastewater samples were collected at the inlet of two wastewater treatment plants in the Lombardy Region. The wastewater treatment plants are in a high-density urban setting in Milan, serving a population of nearly 1 million inhabitants each and receiving mainly municipal waste. Sampling was done in volume- or time-proportional mode, depending on the automatic sampler available. After the collection, samples were immediately processed for viral concentration or were stored at −80 °C until analysis.

The first sample was collected in March 2019, in Milan municipality, almost one year in advance of the COVID-19 pandemic onset and was considered as the blank negative control (NC); this sample was tested for the presence of SARS-CoV-2 RNA by carrying out real-time RT-PCR assays targeting the ORF-1ab and the N gene in triplicate in four different laboratories. This sample was analyzed following a preservation step at −80 °C.

The second sample, collected in December 2020, in Monza-Brianza municipality, was analyzed following a preservation step at −80 °C, and was split into two separate untreated wastewater aliquots: one was spiked with SARS-CoV-2 culture supernatant (SARS-CoV-2 viral load; 4.7×10^7 copies/mL; cycle threshold [Ct] 20) and was considered a positive control (PC); one was directly processed as an "unknown sample" in terms of the presence of SARS-CoV-2, but was expected to be weak positive. These sewage samples were then split into identical aliquots to be tested in parallel by the WBE Lombardy Network collaborating laboratories.

2.2. Pre-Analytical Process: Concentration of Sewage Samples

Untreated urban wastewater samples were processed using three different protocols for sample concentration:

(1) Dextran and polyethylene glycol-6000 (PEG) two-phase separation according to the 2003 WHO Guidelines for Environmental Surveillance of Poliovirus protocol [14], omitting the chloroform treatment to preserve the integrity of the SARS-CoV-2 envelope, as described, firstly, by La Rosa, G. et al. [15]. Briefly, 250 mL of wastewater sample was centrifuged for 30 min at 4500× *g* to pellet the wastewater solids, retaining the pellet for further processing. The clarified wastewater was mixed with dextran and PEG-6000 (19.8 mL of 22% dextran, 143.5 mL 29% PEG 6000, 17.5 mL 5 N NaCl); after a constant agitation for 30 min using a horizontal shaker, the mixture was left to stand overnight at 4 °C in a separation funnel. The bottom layer and the interphase were then collected drop-wise; this concentrate was added to the wastewater solids [14].

(2) PEG-8000 precipitation of 90-mL sewage, modified from Wu, F. et al. [16] and described, firstly, by Castiglioni, S. et al. [17], as follows:

A total of 80 mL of wastewater sample was centrifuged for 30 min at 4500× *g* and 4 °C without break to pellet the wastewater solids. Two aliquots of 40 mL of the clarified wastewater was mixed with 4 g PEG-8000 and 0.9 g sodium chloride (Carlo Erba, Milan, Italy) and were left in a shaker for 15 min at room temperature to dissolve the PEG-8000. Samples were centrifuged for 2 h at 12,000× *g* and 4 °C without break. After centrifugation, the supernatant was discarded and the tubes were returned to the centrifuge at 4 °C for a second centrifugation step at 12,000× *g* for 5 min. The pellet in each tube was suspended in 750 µL of Tryzol (Life Technologies, Monza and Brianza, Italy) and stored at −20 °C until RNA extraction.

(3) PEG-8000 precipitation of 250 mL of sewage, modified from Wu, F. et al. [16], as follows:

A 250 mL wastewater sample was centrifuged for 30 min at 1200× *g* and 4 °C with break to pellet the wastewater solids. Four aliquots of 50 mL of the clarified wastewater were mixed with 4 g PEG-8000 and 0.9 g sodium chloride (Carlo Erba, Milan, Italy) and were left in a shaker for 60 min at room temperature to dissolve the PEG-8000. Samples were centrifuged for 30 min at 10,000× *g* and 4 °C with break. After centrifugation, the supernatant was discarded and the tubes were returned to the centrifuge at 4 °C for a second centrifuge step at 10,000× *g* for 5 min. The pellet in each tube was suspended in 5 mL of PBS (Life Technologies, Monza and Brianza, Italy), treated with chloroform (1:4 *v*/*v*) and centrifuged for 10 min at 1000× *g* and 4 °C. The supernatant was stored at −20 °C until RNA extraction.

A UV treatment of samples (30 min) or a heat treatment (56 °C, 30 min) of the wastewater sample was included before all concentration processes to increase the safety for the laboratory personnel during sample manipulation.

2.3. Pre-Analytical Process: RNA Extraction from Concentrated Sewage Samples

RNA was extracted by means of two commercial kits according to manufacturer's instructions and by combining three different protocols, as follows:

(A) QIAamp MinElute Virus Spin Kit (QIAGEN, Hilden, Germany) with an input of 400 µL of sample and an elution volume of 60 µL, as previously described [17]. (B) NucliSens EasyMag (bioMerieux, Marcy-l'Étoile, France), with an input of 400 uL and 500 µL of sample and an elution volume of 100 µL, (C) NucliSens EasyMag (bioMerieux, Marcy-l'Étoile, France), with an input of 4 mL of sample and an elution volume of 100 µL.

2.4. Analytical Process: Real-Time RT-PCR Assays

The primer/probe sets used in this study targeted two different regions of the nucleocapsid (N) gene, namely, N1 and N3, as listed by the CDC (USA) (2020), and ORF-1b-nsp14, according to the methods described by La Rosa, G. et al. [15]. Three different one-step RT-PCR assays for SARS-CoV-2 were performed using: (1) AgPath-ID One-Step RT-PCR™ kit (Thermofisher Scientific, Waltham, MA, USA), (2) TaqMan™ Fast Virus 1-Step Master Mix (Thermofisher Scientific, Waltham, MA, USA), and (3) QScript XLT 1-Step RT-PCR ToughMix® (QuantaBio, Beverly, MA, USA). Primers and probes were obtained from Eurofins genomics (Eurofins Genomics Germany GmbH, Ebersberg, Germany).

To determine any potential contamination and/or inhibition, specific positive (EURM-019) and negative (DNAse/RNAse-free distilled water) controls were included in each real-time RT-PCR run. A sample was considered positive for SARS-CoV-2 when N1 or N3 or both viral targets showed a cycle threshold (Ct) $\leq$ 39. Real-time RT-PCR runs were performed by using the QuantStudio 5 Real-time RT-PCR system (thermofisher Scientific, Waltham, MA, USA), the ABI PRISM 7500 Sequence Detection System (Thermofisher Scientific, Henogen, Saudi Arabia) and the CFX96 BIo-Rad Detection System (Bio-Rad, Milan, Italy). All samples were tested in triplicate and in three different runs. Since all semi-quantitative assays were performed in triplicate, the reported Ct corresponded to the mean value of the three triplicates.

To minimize contamination risk, RNA extraction, molecular assays set-up and real-time RT-PCR runs were performed in separate rooms, according to good laboratory practice for molecular assays.

2.5. Pre-Analytical and Analytical Workflows

In the preliminary PT workflow, 8 different combinations of methods were run, as shown in Figure 1. Briefly, the three different methods of concentration, namely, (A) Dextran and PEG-6000 two-phase separation, (B) PEG-8000 precipitation without a chloroform purification step and (C) PEG-8000 precipitation with a chloroform purification step were combined with the three different protocols of RNA extraction by using commercial kits.

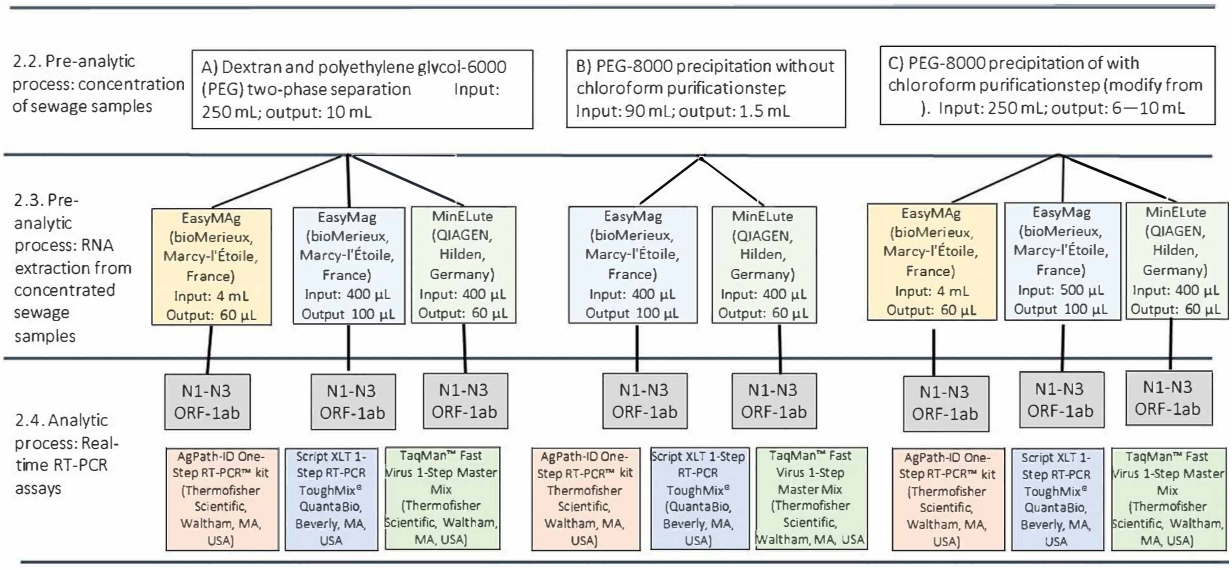

Figure 1. Flowchart of the pre-analytical and analytical workflow in this proficiency test [14,16,17].

2.6. Evaluation of SARS-CoV-2 Recovery Efficiency

The SARS-CoV-2 recovery efficiency of each replicate for each concentration method was calculated based upon the copies of RNA quantified by RT-PCR as follows:

$$\text{Recovery Efficienty (\%) = SARS-CoV-2 copies/}\mu\text{L recovered/ SARS-CoV-2 copies/}\mu\text{L seeded} \quad (1)$$

$$\text{Recovery Efficiency}(\%) = \frac{\text{SARS-CoV-2 copies recovered}}{\text{SARS-CoV-2 copies seeded}} \quad (2)$$

For each concentration method, the mean and standard deviation were calculated.

2.7. Evaluation of SARS-CoV-2 RT-PCR Assays Efficiency

Once the best pre-analytical protocol in the tested workflows was assessed, it was implemented in all research laboratories involved in the WBE network in Lombardy. In order to evaluate the analytical processes, to explore SARS-CoV-2 RT-PCR assays performance and to calibrate RT-PCR methods, the standard curves were constructed using the SARS-CoV-2 Research Grade Test Reference Material (RGTM 10169) from the National Institute of Standards and Technology (NIST). It consists of a synthetic RNA fragment from the SARS-CoV-2 genome (Fragment 1—Total length: 3985 nt, SARS-CoV-2 sequence: 25,949–29,698,) with a concentration of approximately 5×10^6 copies/µL.

Evaluation of the analytical processes was conducted by comparing AgPath-ID One-Step RT-PCR™ kit (Thermofisher Scientific, Waltham, MA, USA), TaqMan™ Fast Virus 1-Step Master Mix (Thermofisher Scientific, Waltham, MA, USA) and QScript XLT 1-Step RT-PCR ToughMix® (QuantaBio, Beverly, MA, USA) efficiencies based on SARS-CoV-2 standard curves generated for both the N1 and N3 target sequences, using the following amplification efficiency formula (Wong and Medrano, 2005):

$$\text{Efficiency} = [10(-1/\text{slope})] - 1 \quad (3)$$

2.8. Data Analysis

The QuantStudio 5 Real-time RT-PCR system (Thermofisher Scientific, Waltham, MA, USA), the ABI PRISM 7500 Sequence Detection System (Thermo Fisher Scientific, Henogen, Saudi Arabia) and the CFX96 Bio-Rad Detection System (Bio-Rad, Milan, Italy). were used to analyze all RT-PCR tests; data were collected and managed using Microsoft Excel (Microsoft Corp., Redmond, WA, USA). Samples with reduced fluorescence, as evident in the RT-PCR curves, were considered inhibited; samples with non-exponential multiplication were considered false positives.

The delta ct value (ΔCt) was calculated by comparing the mean value of the Ct of N1 recovered vs. seeded.

All samples with a Ct $\leq$ 39 cycles were considered positive.

The one-way analysis of variance (ANOVA) was used to determine whether there was a difference in SARS-CoV-2 recovery among the concentration methods tested.

3. Results

3.1. Performance of the Concentration Methods

Method A of concentration allowed researchers to recover nearly 10 mL of samples' concentrates from 250 mL of untreated sewage, with a turnaround time (TAT) of 14–16 h; method B allowed researchers to recover 1.5 mL of concentrated sample from 80 mL of untreated sewage with a TAT of 3–4 h; method C allowed researchers to recover from 6 to 10 mL of concentrated sample from 250 mL of untreated sewage with a TAT of 3–4 h. Overall, N1, N3 and ORF-1b-nsp14 were identified in sewage samples spiked with 2.5×10^9 copies/mL of SARS-CoV-2 by using all the evaluated workflows, with 100% of positive replicates (Table 1). Method B for wastewater concentration (the PEG-8000 precipitation without chloroform purification step) showed the best ΔCt values, which

resulted in −1.9 by using the QIAamp MinElute Virus Spin Kit (QIAGEN, Hilden, Germany) and −0.6 by using the NucliSens EasyMag (bioMerieux, Marcy-l'Étoile, France) ($p > 0.05$) (Table 1). In all considered wastewater concentration methods, the Ct values of ORF-1b-nsp14 were shown to be statistically lower ($p < 0.005$) than those of N1 and N3, with a mean ΔCt between ORF-1b-nsp14 and N1–N3 of 4.71 (SD: ±1.56).

Considering the sewage "unknown sample" for the presence of SARS-CoV-2, the detection of N1, N3 and ORF-1b-nsp14 with 100% of positive replicates was identified only by using method B of concentration, with mean Ct values of N1 ranging from 33 (SD: ±0.4) by using the QIAamp MinElute Virus Spin Kit, QIAGEN to 34 (SD: ±0.2) by using NucliSens EasyMag, bioMerieux. The mean Ct values of ORF-1b-nsp14 of 37 (SD: ±0.3) were obtained by using the QIAamp MinElute Virus Spin Kit (QIAGEN, Hilden, Germany) and of 37 (SD: ±0.6) by using NucliSens EasyMag (bioMerieux, Marcy-l'Étoile, France). When the sewage sample was concentrated by using method A and C, ORF-1b-nsp14 always tested negative; the N1 positive replicates ranged from 17% (1/6) to 83% (5/6), with the Ct values ranging from 37.3 (SD: undeterminable) and 38.7 (SD: ±0.3) (Table 1); N3 positive replicates resulted in 67%, with the Ct values ranging from 36.8 (SD: ±0.5) and 37.9 (SD: ±0.1) (Table 1).

3.2. SARS-CoV-2 Recovery Efficiency

For the 500 mL untreated wastewater sample seeded with SARS-CoV-2, method B (i.e., PEG-8000 precipitation without chloroform purification step) provided the highest ($p < 0.001$) SARS-CoV-2 recovery of 76% by using the QIAamp MinElute Virus Spin Kit (QIAGEN, Hilden, Germany) and 31.4% by using NucliSens EasyMag (bioMerieux, Marcy-l'Étoile, France) (Table 1). The other concentration methods showed a SARS-CoV-2 recovery efficiency < 18% (Table 1) and were, thus, excluded from the workflow.

3.3. Real-Time RT-PCR Efficiency

The calculated efficiencies were significantly lower for the TaqMan™ Fast Virus 1-Step Master Mix (N1 = 85.2%, N3 = 90.8%) compared to the AgPath-ID One-Step RT-PCR™ kit (N1 = 98.4%, N3 = 98.2%) and to QScript XLT 1-Step RT-PCR ToughMix® (N1 = 98.8%, N3 = 99.5%) (Table 2, Figure 2).

Table 1. Results from the different workflows implemented in this inter-laboratory proficiency test (PT).

								Spiked Sample by SARS-CoV-2 Viral Load: 4.7×10^7 Copies/mL					Unknown Sample		
Volume of Sewage to be Concentrated	**Methods of Concentration**	**Volume of Concentrating Sewage**	**RNA Extraction Kit**	**Extraction Input Elution Volume**	**RT-qPCR Kit**	**RT-PCR Instrument**	**Tournaround Time**	**Target**	**Positive Replicates**	**Mean Ct Value**	**SD Ct Value**	**ΔCt (Recoverded vs. Seeded)**	**Mean Recovery Efficiency (%)**	**Positive Replicates**	**Mean Ct Value**	**SD Ct Value**
250 mL	(A) Dextran and PEG-6000 two-phase separation	nearly 10 mL	QIAamp MinElute Virus Spin Kit (QIAGEN)	400 µL 60 µL	AGPATH-ID ONE-STEP RT-PCR (Thermo Fisher)	Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher)	16-h	N1	100%	29.2	0.62	7.2		17%	37.27	/
								N3	100%	30.4	0.85			und	und	/
								ORF	100%	36.5	0.79			und	und	/
			NucliSens EasyMag (bioMerieux)	400 µL 100 µL	AGPATH-ID ONE-STEP RT-PCR (Thermo Fisher)		16-h	N1	100%	26.2	0.38	4.2	12%	67%	37.9	0.45
								N3	100%	25.6	0.41			und	und	/
								ORF	100%	28.9	0.23			und	und	0.02
			NucliSens EasyMag (bioMerieux)	4 mL 60 µL	AGPATH-ID ONE-STEP RT-PCR (Thermo Fisher)			N1	100%	27.6	0.25	5.6		33%	38.2	0.02
								N3	100%	27	0.22			67%	37.6	0.2
								ORF	100%	30.4	0.17			und	und	/
80 mL	(B) PEG-8000 precipitation without chloroform purification step	1.5 mL	QIAamp MinElute Virus Spin Kit (QIAGEN)	400 µL 60 µL	AGPATH-ID ONE-STEP RT-PCR (Thermo Fisher)	Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher)	8-h	N1	100%	20.1	0.13	−1.9	76%	100%	33.1	0.4
								N3	100%	20.5	0.20			100%	33.4	0.34
								ORF	100%	24.6	0.30			100%	37.5	0.75
			NucliSens EasyMag (bioMerieux)	500 µL 100 µL	AGPATH-ID ONE-STEP RT-PCR (Thermo Fisher)		8-h	N1	100%	21.4	0.38	0.6		100%	34.1	0.22
								N3	100%	19.7	0.31			100%	32.6	0.19
								ORF	100%	24.6	0.43			100%	37.2	0.34
250 mL	(C) PEG-8000 precipitation with chloroform purification step	6–10 mL	QIAamp MinElute Virus Spin Kit (QIAGEN)	400 µL 60 µL	QScript XLT 1-Step RT-PCR ToughMix (QuantaBio)	CFX96 BioRad real-time PCR System (Biorad)	8-h	N1	100%	25.6	1.27	3.6	18%	83%	37.7	1.69
								N3	100%	26.9	1.05			67%	36.8	0.46
								ORF	100%	32.8	1.08			und	und	/
			NucliSens EasyMag (bioMerieux)	500 µL 100 µL	QScript XLT 1-Step RT-PCR ToughMix (QuantaBio)		8-h	N1	100%	29.2	0.33	7.2		50%	38.7	0.3
								N3	100%	29.5	0.43			67%	37.4	0.01
								ORF	100%	32.5	0.57			und	und	/
			NucliSens EasyMag (bioMerieux)	4 mL 60 µL	QScript XLT 1-Step RT-PCR ToughMix (QuantaBio)		8-h	N1	100%	29.3	0.5	7.3	1%	17%	38.03	/
								N3	100%	26.1	0.38			67%	37.9	0.46
								ORF	100%	33.1	1.41			und	und	/

Table 2. Comparison of AgPath-ID One-Step RT-PCR, QScript XLT 1-Step RT-PCR ToughMix® and TaqMan™ Fast Virus 1-Step master mix efficiencies.

	Target	Standard Curve	R^2	Efficiency
AgPath-ID One-Step RT-PCR™ kit	N1	y = −3.3863x + 37.009	0.9984	98.4%
	N3	y = −3.3677x + 38.426	0.9982	98.2%
QScript XLT 1-Step RT-PCR ToughMix®	N1	y = −3.279x + 39.076	0.9988	98.8%
	N3	y = −3.3073x + 40.08	0.9995	99.5%
TaqMan™ Fast Virus 1-Step Master Mix	N1	y = −3.7356x + 41.786	0.9985	85.2%
	N3	y = −3.5652x + 38.426	0.9971	90.8%

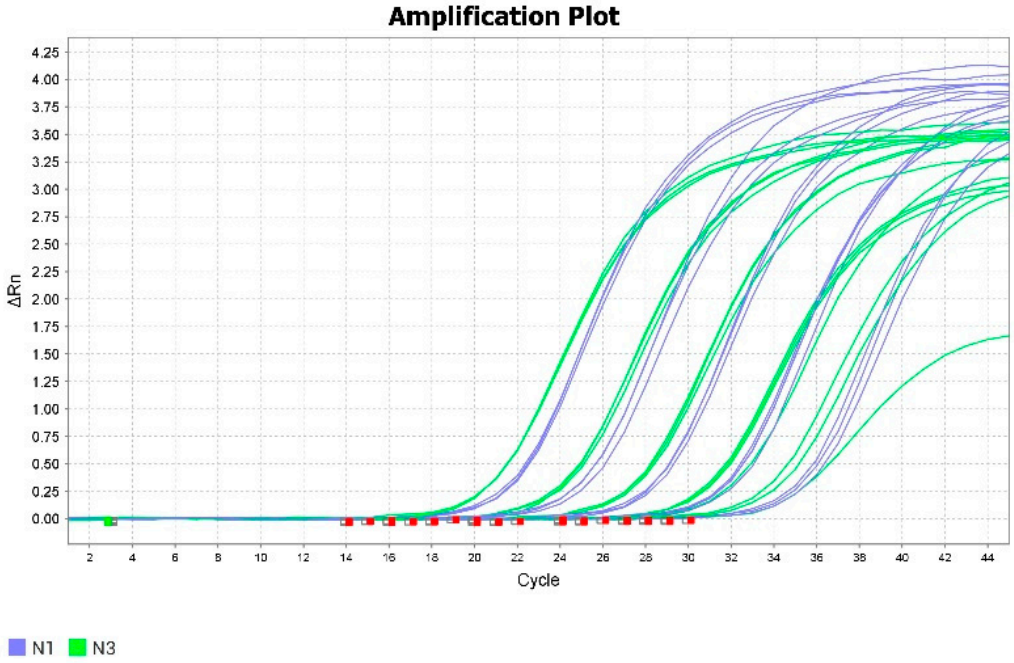

Figure 2. Real-Time RT-PCR amplification plot of the assay targeting N1 and N3 of the 10-fold dilutions of the SARS-CoV-2 Research Grade Test Reference Material (RGTM 10169) by using AgPath-ID One-Step RT-PCR™ kit.

4. Discussion

The development of a surveillance system through the implementation of the WBE approach may serve to monitor viral transmission in the community and to act as an early-warning system, allowing timely interventions to face new pathogens that may threaten human health [18]. The WBE approach has been used for decades to detect poliovirus and to track other viruses—able to persist long enough in untreated wastewater to allow reliable detection—in consideration that the sewage system can blend viral shedding variation among single individuals and over the course of their infection, into an average amount that represents the entire community under investigation [19–23]. Recently, several studies have reported the detection of SARS-CoV-2 RNA in wastewater samples worldwide [16,24–28] and have also shown a good correlation between the number of active COVID-19 cases and the SARS-CoV-2 RNA concentration in wastewater samples from different cities in Europe, Asia, the USA and Australia [16,24,25].

The sensitive detection of SARS-CoV-2 RNA in wastewater and, thus, the identification of SARS-CoV-2 infections within a community, depends on both the wastewater concentration pre-analytical phase and the molecular methods employed for the analysis, which are often different and lack standardization. Considering the pre-analytical process, SARS-CoV-2 concentration methods are particularly important because the concentration of this virus in wastewater samples is expected to be low at the onset or at the offset of the COVID-19 epidemic curve [16,17,24,25]; thus, the concentration methods must be sensitive enough to detect a very low concentration of SARS-CoV-2 in an environmental

matrix to provide an effective early warning system and to track in a real-time manner the introduction of SARS-CoV-2 in a community.

Nowadays, a number of virus concentration methods have been developed for the detection of enteric viruses in water and wastewater matrices [4,18]. In this study, nine different workflows, including pre-analytical and analytical processes, were evaluated to then be implemented in the WBE of the Lombardy Region; these included three different methods of concentration, three different protocols of RNA extraction and three different one step real-time RT-PCR reagents. In this study, the method showing the best performance in the recovery of SARS-CoV-2, from both mock and unseeded samples, was that carried out by using PEG-8000 precipitation without chloroform treatment. In particular, this method allowed for a better recovery efficiency of SARS-CoV-2 when compared to Dextran and polyethylene glycol-6000 (PEG) two-phase separation, in contrast to other Italian preliminary results [15]. PEG-8000 precipitation without chloroform has also shown a good performance in concentrating SARS-CoV-2 from wastewater matrices in other published studies [11,29,30]. In the study from Ahmed, W. et al., the mean ± SD of the recovery of murine hepatitis virus (as a proxy of SARS-CoV-2) was shown to be 44.0% ± 27.7, similar to that observed in our study, where the recovery of spiked SARS-CoV-2 ranged from 31.4% to 76% by using PEG-8000 precipitation [30]. The only equipment needed to carry out PEG-8000 precipitation is a centrifuge that reaches up to 12,000× g, thus, resulting in a relatively simple and inexpensive protocol; moreover, it allows one to process larger volumes (e.g., 1 L) of wastewater and to concentrate SARS-CoV-2 from both the solid and the liquid phases, as well as being non-time consuming (3–4 h). On the other hand, this method requires handling of hazardous chemicals (such as Tryzol) that, however, could be replaced by elution in phosphate-buffered saline (PBS).

In respect to the evaluation of the three different protocols of RNA extraction by commercially available kits, no major differences were identified, as observed elsewhere [4,31]. Regarding SARS-CoV-2 real-time RT-PCR assays efficiency, in combination with all evaluated concentration methods, we observed that the Ct values for ORF-1b-nsp14 were statistically higher than those for N1 and N3, with a mean ΔCt between ORF-1b-nsp14 and N1-N3 of nearly 5, meaning a loss of sensitivity of nearly 2 Log when using ORF-1b-nsp14 instead of N viral targets, as also described in other studies that investigated the analytical sensitivity and efficiency of different SARS-CoV-2 real-time RT–PCR primer–probe sets [31,32].

Recently, the Water Research Foundation (WRF) released a question survey via social media to collect information on the development of methods for the detection of genes that indicate the presence of SARS-CoV-2 in wastewater samples [33]; feedback was obtained by 35 countries, with results showing that the concentration methods most frequently used were the PEG-8000 precipitation, followed by nucleic acid extraction and assay for primarily nucleocapsid gene targets (N1, N2, and/or N3) [33]. These results from WRF are in line with the output of our study.

A limitation of this study is that there was a limited number of replicates of the tested methods due to the time required for processing and restrictions on people and laboratory spaces during the pandemic, which is when this experimental work was carried out.

5. Conclusions

In conclusion, a new pre-analytical and analytical workflow to detect SARS-CoV-2 from wastewater samples was implemented in the framework of the WBE laboratories' network in the Lombardy Region.

The main innovation of this surveillance approach relies on the fact that it can overcome the testing availability, rates and indications and that it can capture the viral spread from symptomatic and asymptomatic individuals, offering a comprehensive and cost-effective solution for SARS-CoV-2 surveillance and providing a strong and independent signal of how much the virus is circulating in a given community. All these aspects make the WBE an innovative real-time cost-effective tool for community-based surveillance

that can also be used for other emerging pathogens of concern for human health, to track outbreaks and guide public health interventions of prevention and control.

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