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Evaluation of different molecular systems for detection and quantification of SARS-CoV-2 RNA from wastewater samples

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ABSTRACT

Wastewater-based epidemiology has proved to be a suitable approach for tracking the spread of epidemic agents including SARS-CoV-2 RNA. Different protocols have been developed for quantitative detection of SARS-CoV-2 RNA from wastewater samples, but little is known on their performance. In this study we compared three protocols based on Reverse Transcription Real Time-PCR (RT-PCR) and one based on Droplet Digital PCR (ddPCR) for SARS-CoV-2 RNA detection from 35 wastewater samples. Overall, SARS-CoV-2 RNA was detected by at least one method in 85.7 % of samples, while 51.4 %, 22.8 % and 8.6 % resulted positive with two, three or all four methods, respectively. Protocols based on commercial RT-PCR assays and on Droplet Digital PCR showed an overall higher sensitivity *vs.* an in-house assay. The use of more than one system, targeting different genes, could be helpful to increase detection sensitivity.

1. Introduction

Detection of infectious agents in wastewater samples has allowed the development of wastewater-based epidemiology (WBE), a powerful approach for monitoring the presence and dissemination trends of infectious agents in large communities (Sims and Kasprzyk-Hordern, 2020). Indeed, WBE has proved feasible for monitoring the dissemination of several infectious agents, including SARS-CoV-2, the viral agent responsible for the recent COVID-19 pandemic (Hamza & Hamza, 2018; La Rosa et al., 2013; Sinclair et al., 2008; Sims and Kasprzyk-Hordern, 2020), on a population scale that cannot be easily addressed by individual testing.

Detection of SARS-CoV-2 in wastewaters can be carried out by reverse-transcription real-time PCR (RT-qPCR) and Droplet Digital PCR (ddPCR) assays (Alygizakis et al., 2021), or even by whole-genome sequencing (WGS) approaches (Barbé et al., 2022; Crits-Christoph et al., 2021; Rios et al., 2021). The latter can be useful for an earlier recognition of viral variants but are associated with higher costs and are more labour-intensive.

Previous studies evaluated the performance of different protocols for concentration and extraction of SARS-CoV-2 RNA from wastewater specimen (Barril et al., 2021; Pino et al., 2021; Dimitrakopoulos et al., 2022; Peinado et al., 2022), while little is known on the performance of currently available amplification systems. In this study we evaluated different quantitative amplification methods, including three RT-qPCR and one ddPCR methods, for the detection of SARS-CoV-2 from wastewater samples.

2. Materials and methods

2.1. Wastewater samples

A total of 35 wastewater samples were collected from "Ponte a Niccheri" wastewater treatment plant (PNi-WWTP) (managed by

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Publiacqua S.p.A.), located in the southern area of Florence (Tuscany, Italy) during the period March-October 2021. The combined sewer system basin served by PNi-WWTP is constituted by approximately 52 kilometres of both pressure and conventional gravity sewers covering an area of about 5.8 square kilometres, with a catchment population of nearly 14,000 inhabitants, also including a secondary care hospital with around 400 beds and COVID-19 dedicated wards during the pandemic emergency. Physicochemical analyses on PNi-WWTP samples were performed following standard methods (APHA et al., 2005). Each wastewater sample (200 mL) was a 24-hours composite sample (time proportional), collected with a refrigerated (4 °C) AS950 automatic sampler (Hach Company, Loveland, CO, U.S.A.) at the inlet of the PNi-WWTP. After collection, each sample was rapidly transported in a cool box with ice packs and stored in the lab at 4 °C before being processed. Laboratory analysis started within 48 hours from samples collection. Appropriate biosafety practices were followed for the handling and transport of the samples.

2.2. Samples concentration and extraction

Twenty-three samples were processed using an in-house protocol provided by the Italian National Institute of Health (Istituto Superiore di Sanità, ISS). Each sample underwent a pre-treatment of 30 minutes at 56 °C for inactivation of infectious viral particles. After cooling (10–15 minutes at room temperature), 45 mL of each sample were transferred in Clearline 50 mL tubes (Biosigma, Cona, VE, Italy) and centrifuged at 4500 x *g* for 30 minutes at 4 °C. The supernatant (40 mL) was collected in a new tube containing 4 g of polyethylene glycol (PEG 8000, Fisher Scientific, Geel, Belgium) and 0.9 g of NaCl (Merck KGaA, Darmstadt, Germany). After the components were completely dissolved, tubes were centrifuged at 12000 x *g* for two hours at 4 °C. The supernatant was discarded, and tubes were left upside down to favour the complete removal of PEG/NaCl. The pellet was eventually resuspended in 200 μL of ASL stool lysis buffer (Qiagen GmbH, Hilden, Germany). The extraction of nucleic acids was performed using the STARMag 96 ×4 Universal Cartridge Kit (Seegene Inc., Seul, Republic of Korea) in Microlab NIMBUS (Hamilton Company, Reno, NV, U.S.A.) and the eluate (100 μL) was stored at -80 °C pending further analysis. Twelve additional samples were concentrated using the Zymo Environ™ Water RNA Kit (Zymo Research, Irvine, CA, U.S.A.) following manufacturer's instructions. In details, 5 mL of sample were directly subjected to RNA enrichment and purification to get a final amount of 25 μL of eluate, which is ready for downstream molecular analysis.

2.3. Quantitative detection of SARS-CoV-2 RNA

Quantitative detection of SARS-CoV-2 viral RNA was performed using three different RT-qPCR methods, using a CFX96 thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.), and a ddPCR method using a C1000 Touch thermal cycler (Bio-Rad), as detailed below.

2.3.1. Manual method developed by ISS [method A]

This RT-qPCR protocol was developed by ISS for the detection of SARS-CoV-2 in urban wastewater samples, targeting a region of ORF1b (nsp14) specific for the SARS-CoV-2 genome (La Rosa et al., 2021). A standard dsDNA obtained from cultured wild-type SARS-CoV-2 (provided by ISS) was used, starting from a concentration of $1{\cdot}10^5$ copies/μL serially diluted (1:10) in TE buffer pH 8.0 (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.) up to $1{\cdot}10^1$ copies/μL to generate a calibration line to be included in the PCR run. A volume of 5 μL of RNA template (both samples and quantification controls) was used in each reaction. AgPath-ID™ One-Step RT-PCR Reagents kit (Thermo Fisher Scientific) was used for PCR mix. Thermal protocol consisted of 30 minutes at 50 °C, 10 minutes at 95 °C, then 15 seconds at 95 °C and 45 seconds at 60 °C for 45 cycles. Samples were considered positive when a signal was detected at cycle threshold (Cq) <40. A limit of detection (LoD) of $5.6{\cdot}10^4$ genome copies per liter of processed wastewater was previously reported for this method (La Rosa et al., 2021).

2.3.2. Wastewater SARS-CoV-2 RT-qPCR system (Promega Corporation, Madison, WI, U.S.A.) [method B]

This multiplex RT-qPCR method, specifically designed for the quantification of SARS-CoV-2 RNA from wastewater samples, targets a region of the nucleocapsid (N) and envelope (E) genes of SARS-CoV-2 genome. Quantitative evaluation of SARS-CoV-2 RNA is achievable by constructing a four-point calibration curve starting from a standard DNA (at the concentration of $2{\cdot}10^5$ copies/μL) provided by the manufacturer (Mondal et al., 2021). A volume of 5 μL of RNA template (both samples and quantification controls) was used in each reaction. As process control, the kit allows to detect Pepper Mild Mottle Virus (PMMoV), an abundant and common RNA virus in wastewater samples. Thermal protocol consisted of 15 minutes at 45 °C, 2 minutes at 95 °C, 3 seconds at 95 °C and 30 seconds at 62 °C for 40 cycles. Samples were considered positive when a signal was detected at Cq <40. A LoD of 5 copies per reaction was previously reported for this method (Mondal et al., 2021).

2.3.3. Quanty COVID-19v2 (Clonit Srl, Milan, Italy) [method C]

This *in vitro* diagnostic (IVD) multiplex RT-qPCR method was developed and CE-IVD marked for the detection and quantitative evaluation of SARS-CoV-2 RNA in biological samples (e.g., nasopharyngeal swabs) but has also been tested with different matrices (Mancusi et al., 2022). Two different targets on the N gene (N1 and N2) of SARS-CoV-2 genome are detected and the evaluation of viral load is performed on the N1 target using a four-point RNA calibration curve (concentrations ranged from $1{\cdot}10^5$ to $1{\cdot}10^2$ copies/μL) provided by the manufacturer. A volume of 5 μL of RNA template (both samples and quantification controls) was used in each reaction. Thermal protocol consisted of 2 minutes at 25 °C, 15 minutes at 50 °C, 2 minutes at 95 °C, then 3 seconds at 95 °C and 30 seconds at 60 °C for 45 cycles. Samples were considered positive when a signal was detected at Cq <40. A LoD of 8.15 and 5.45 copies/μL of RNA extract for the N1 and N2 target, respectively, were reported by the manufacturer (www.clonit.it).

2.3.4. Droplet digital PCR (ddPCR) technology [method D]

ddPCR is a method based on water-oil emulsion droplet technology providing an absolute count of target nucleic acid copies for each sample without the need of running calibration curves (Hindson et al., 2013). One-Step RT-ddPCR Advanced Kit for Probes reagent kit (Bio-Rad) was used with the QX200 Droplet Digital PCR System (Bio-Rad). Primers and probe used were the same as for the [A] methodology (volumes and concentrations of reagents were adjusted according to the manufacturer's indications). A volume of 5.5 μL of RNA template was used for samples and for a positive control represented by an RNA extract from a SARS-CoV-2-positive nasopharyngeal swab. Thermal protocol consisted of 50 minutes at 60 °C, 10 minutes at 95 °C then 45 cycles of 30 seconds at 95 °C and 60 seconds at 58 °C, followed by 10 minutes at 98 °C and 30 minutes at 4 °C. Data analysis was performed with QX manager 1.2 (Bio-Rad).

The RT-qPCR using the A, B and C methods were performed simultaneously, while ddPCR was performed after an additional freeze-thawing step of the RNA extract. Each quantitative result was subsequently normalized in copies of viral genomic RNA per litre of wastewater (c.g./L). 95 % confidence intervals (CI) were calculated using the "Jeffreys" method available at EPITOOLS (https://epitools.ausvet.com.au/). Data were plotted and analysed using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, U.S.A.).

3. Results

During the study period, a total of 35 wastewater samples were processed with the four different qRT-PCR methods. Detection of PMMoV using the B method (Cq mean 27.1, Cq range 22.5–30.2)

(Table S1) with all the 35 samples tested, confirmed the efficacy of the concentration and extraction protocols used, and the lack of invalid results with this molecular method. No internal amplification controls were included with the other methods.

A total of 8/35 (22.9 %), 14/35 (40.0 %), 19/35 (54.3 %) and 19/35 (54.3 %) samples were reported positive for SARS-CoV-2 with the A, B, C and D methods, respectively (Table 1 and **S1,** Fig. 1). Overall, SARS-CoV-2 RNA was detected by at least one method from 30/35 samples (85.7 %), while 18 samples (51.4 %) resulted positive with at least two methods, 8 samples (22.8 %) with at least three methods, and 3 samples (8.6 %) with all four methods.

All methods reported a concordant result with 8 of 35 samples (22.9 %, 95 % CI 10.4–40.1 %) (Table S1). Of these samples, five (14.3 %) were negative and three (8.6 %) were positive for SARS-CoV-2 by all methods. In the latter samples, the B and C methods reported slightly higher SARS-CoV-2 RNA c.g./L compared to A and D methods, which reported comparable results in terms of nucleic acid concentration (Fig. 1). Considering as true negatives the five samples which tested negative with all methods, the NPV of the four methods ranged from 18.5 % to 31.3 % (Table 1).

Considering as true positives specimens testing positive by at least one molecular method (N=30), sensitivity was 26.7 % and 46.7 % for A and B methods, respectively, and 63.3 % for both C and D methods, (Table 1). Considering as true positives specimens testing positive by more than one method (N=18), sensitivity was 27.8 %, 66.6 %, 94.4 %, and 77.8 % for A, B, C, and D methods, respectively, while specificity was 88.2 % for both B and C methods, and 82.3 % and 70.6 % for A and D methods, respectively (Table 1). Concerning viral load, B and C methods yielded the highest mean viral load, resulting as $1.3{\cdot}10^4$ and $9.2{\cdot}10^3$ c.g./L, respectively (Table 1). However, it should be noted that the D method was run with RNA extracts that were subjected to an additional cycle of freezing and thawing.

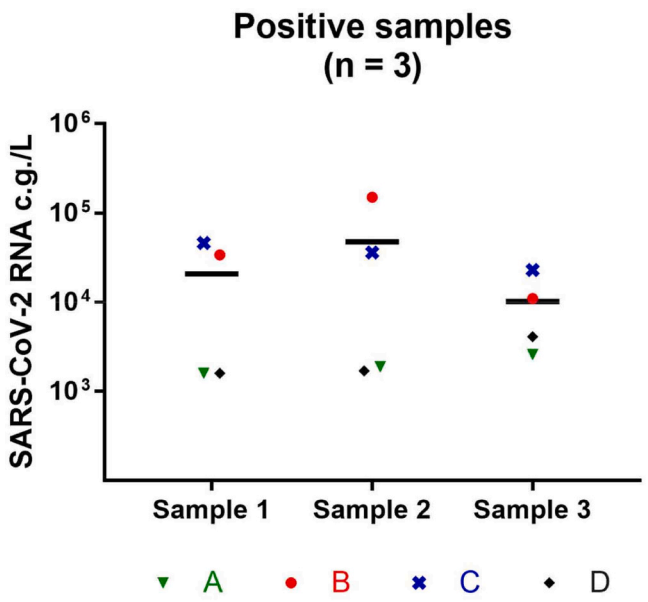

Fig. 1. Comparison between the three samples detected as positive by each method tested. Bold horizontal lines represent SARS-CoV-2 RNA c.g./L mean for each sample. A) manual method developed by ISS; B) Wastewater SARS-CoV-2 RT-qPCR System (Promega Corporation); C) Quanty COVID-19v2 (Clonit Srl); D) ddPCR method.

4. Discussion

The COVID-19 pandemic renewed the attention on the need for large-scale surveillance for tracking positive cases, implementing infection control measures in the population, and monitoring possible shedding of new SARS-CoV-2 variants (Carducci et al., 2020; Medema et al., 2020; Barbé et al., 2022; Marchini et al., 2023). However, tracking SARS-CoV-2-positive cases with viral RNA detection in respiratory samples (e.g., nasopharyngeal swabs) may not be suitable for large-scale screening due to logistic and/or economic reasons, and may lead to underestimation of the viral diffusion in the population. As previously reported in several studies (Ahmed et al., 2020; Bivins et al., 2020; Bonanno Ferraro et al., 2022; La Rosa et al., 2020; La Rosa et al., 2021; Randazzo et al., 2020; Sherchan et al., 2020; Westhaus et al., 2021), WBE could represent a suitable alternative for large-scale and low-cost screening of SARS-CoV-2, providing an early warning system for new pandemic waves (Alygizakis et al., 2021).

The optimization of wastewater samples concentration has been addressed by several authors (Barril et al., 2021; Pino et al., 2021; Dimitrakopoulos et al., 2022; Peinado et al., 2022). Flood et al. (2021) used spiked wastewater samples to evaluate the efficiency of concentration of PEG precipitation and ultrafiltration by using two RT-qPCR assay and ddPCR showing a higher sensitivity of the latter in SARS-CoV-2 RNA detection. Besides SARS-CoV-2, other surrogates such as Mengovirus (Salvador et al., 2021) could be helpful as process control and for methodologies comparison. RT-qPCR is widely used as efficient and sensitive method for SARS-CoV-2 RNA detection in wastewater allowing also high throughput analysis, as well as WGS approaches used also for SARS-CoV-2 variants detection. On the other hand, ddPCR were less investigated (Alygizakis et al., 2021), and there seems to be no consensus regarding the optimal molecular method for WBE of SARS-CoV-2.

In this study, three RT-qPCR and one ddPCR methods were evaluated with a collection of 35 wastewater samples collected from a wastewater treatment plant serving the southern area of Florence (Italy) during an eight-months surveillance in 2021. The commercial methods (B and C) exhibited an overall higher sensitivity compared with the in-house method (A), while the ddPCR method (D) exhibited a similar sensitivity to the most sensitive commercial method (Table 1), confirming the

Table 1
Summary of molecular methods result. NPV: negative predictive value; CI: confidence interval. A) manual method developed by ISS; B) Wastewater SARS-CoV-2 RT-qPCR System (Promega Corporation); C) Quanty COVID-19v2 (Clonit Srl); D) ddPCR method.

	RT-qPCR			ddPCR
	A	B	C	D
c.g./L mean (range)	$3.0{\cdot}10^2$ ($3.8{\cdot}10^2$ - $2.6{\cdot}10^3$)	$1.3{\cdot}10^4$ ($6.8{\cdot}10^2$ - $2.2{\cdot}10^5$)	$9.2{\cdot}10^3$ ($3.2{\cdot}10^3$ - $4.7{\cdot}10^4$)	$2.5{\cdot}10^3$ ($7.9{\cdot}10^2$ - $4.4{\cdot}10^4$)
Positive samples (%)	8 (22.9 %)	14 (40.0 %)	19 (54.3 %)	19 (54.3 %)
95 % CI	11.4–38.5 %	25.1–56.5 %	37.9–69.9 %	37.9–69.9 %
Sensitivity* (95 % CI)	26.7 % (13.5–44.1 %)	46.7 % (29.8–64.1 %)	63.3 % (45.5–78.7 %)	63.3 % (45.5–78.7 %)
NPV* (95 % CI)	18.5 % (7.4–35.9 %)	23.8 % (9.7–44.6 %)	31.3 % (13.0–55.6 %)	31.3 % (13.0–55.6 %)
Sensitivity (95 % CI)**	27.8 % (11.5–50.6 %)	66.6; % (43.7–84.7 %)	94.4 % (76.8–99.4 %)	77.8 % (55.4–92.0;%)
Specificity (95 % CI)**	82.3 % (59.9–94.8 %)	88.2 % (67.6–97.5 %)	88.2 % (67.6–97.5 %)	70.6 % (47.0–87.8 %)
NPV (95 % CI)**	51.8 % (33.6–69.7 %)	71.4 % (50.3–87.1 %)	93.7 % (74.3–99.3 %)	75.0 % (50.9–90.9 %)

* calculated considering as true positive samples where SARS-CoV-2 RNA was detected by at least one molecular method (N=30)
** calculated considering as true positive samples where SARS-CoV-2 RNA was detected by at least two molecular methods (N=18)

good sensitivity of the ddPCR-based method, as previously reported (Flood et al., 2021). The slightly lower mean SARS-CoV-2 RNA c.g./L observed with the latter method could have been related with the fact that RNA extracts had been subjected to an additional freezing-thawing cycle before performing the ddPCR run. No further investigations were performed in this study to evaluate this effect. Nevertheless, previous findings suggested that a single freeze-thawing step (from -80 °C) did not significantly affect RNA stability in concentrated wastewater samples (Huge et al., 2022; Thapar et al., 2023).

However, the sensitivity of each method was relatively low, as well as their concordance. Discordant results could in part be related to the different viral targets detected by each method. A possible approach to increase sensitivity in SARS-CoV-2 RNA detection could be the combination of at least two methods. In this perspective, combination of the in-house A method with the two commercials methods (B and C) achieved a sensitivity of 60.0 % (18/30) and 73.3 % (22/30), respectively, while combination of the two commercial B and C methods achieved a sensitivity of 73.3 % (22/30) (Figure S1). ddPCR method combined with the B and C methods, on the other hand, achieved a sensitivity of 80.0 % (24/30) and 96.7 % (29/30) (Figure S1), respectively, suggesting that the latter combination could be the best option.

The wastewater characteristics of PNi-WWTP are those typical of a municipal area, albeit often diluted due to the collection of rainwater during the rainy periods and due to the unintended infiltration of parasitic waters (Weiss et al., 2002). Such additional inflows and unintended infiltrations cause wastewater dilution estimated by the local water service provider in a yearly average and dry period (i.e. July-August) dilution factor of approximatively 2.0 and 1.4, respectively. Furthermore, the combined sewer system is characterized by the presence of watertight concrete static septic tanks placed in buildings basements with the function of faeces/large debris retention to avoid settling within the gravity sewer pipes where the outflow is collected. In summary, the municipal wastewater used in this study is particularly complex due to the time variable combined effect of rainwater inflow, parasitic waters infiltration and septic tank ubiquitous presence. The physicochemical characterization of the wastewater used, relative to the period covered by the present study is reported in the supplementary material (Table S2).

Limitations of this study were represented by the overall low number of samples tested, which may lead to statistical biases, and by the use of two different extraction methods, which may have contributed to the variability of results. Moreover, extraction/amplification internal control were not available for all evaluated molecular methods.

5. Conclusions

WBE used for monitoring the spread of SARS-CoV-2 opens novel horizons to address an important healthcare challenge. However, the optimization of viral nucleic acids concentration/extraction steps and detection methods from wastewater samples remain a key point to obtain sensitive results. In this study we observed significant variability among three different RT-qPCR detection methods, and also showed that ddPCR could represent a reliable tool for SARS-CoV-2 detection from environmental samples, while combination of this technology with conventional qRT-PCR methods could improve the sensitivity of viral nucleic acids quantification.

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CRediT authorship contribution statement

Gian Maria Rossolini: Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Fabio Morecchiato:** Writing – original draft, Investigation, Formal analysis, Data curation. **Tommaso Lotti:** Writing – review & editing, Conceptualization. **Claudio Lubello:** Writing – review & editing, Supervision, Resources. **Alberto Antonelli:** Writing – review & editing, Methodology, Data curation, Conceptualization. **Leandro Di Gloria:** Investigation, Formal analysis. **Marco Coppi:** Writing – original draft, Methodology, Formal analysis, Data curation. **Claudia Niccolai:** Writing – original draft, Investigation, Formal analysis. **Matteo Ramazzotti:** Writing – original draft, Methodology, Conceptualization. **Piergiuseppe Calà:** Supervision, Resources. **Fabrizio Mancuso:** Supervision, Resources, Conceptualization.

Declaration of Competing Interest

G.M.R. reports grants, consulting fees, and payment or honoraria for lectures, presentations, speakers’ bureaus, manuscript writing or educational events from bioMérieux, MSD, Shionogi, Zambon, Menarini, Angelini; grants from Accelerate, Cepheid, Nordic Pharma, Seegene, Arrow, Symcel, DID, Hain Lifescience, Meridian, Setlance, Qvella, Qlinea, Biomedical Service, Quidel, QuantaMatrix, SD Biosensor; consulting fees from Pfizer and Qiagen; payment or honoraria for lectures, presentations, speakers’ bureaus, manuscript writing or educational events from Becton Dickinson, Cepheid and Pfizer, outside the submitted work. All other authors declare no competing interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jviromet.2024.114956.

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