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Adapted methods for monitoring influenza virus and respiratory syncytial virus in sludge and wastewater

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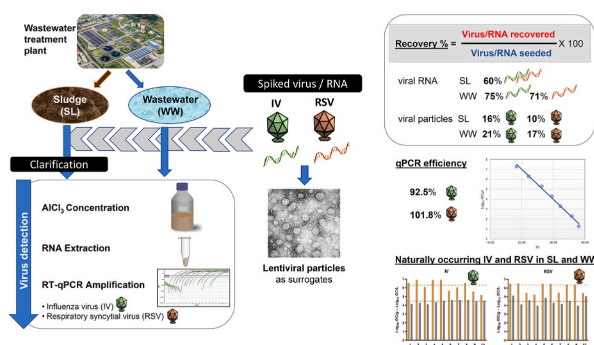
HIGHLIGHTS

- An efficient method targeting enveloped viruses in sludge is presented.
- Lentiviral particles are useful surrogates for IV and RSV monitoring.
- The protocol allows quantification of naturally occurring IV and RSV in sludge.
- Sludge is a significant reservoir of IV and RSV.
- Winter wastewater and sludge samples were positive for IV and RSV.

GRAPHICAL ABSTRACT



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ABSTRACT

Wastewater-based surveillance constitutes a valuable methodology for the continuous monitoring of viral circulation, with the capacity to function as an early warning system. It holds particular significance in scenarios where respiratory viruses exhibit overlapping clinical presentations, as occurs with SARS-CoV-2, influenza virus (IV), and respiratory syncytial virus (RSV), and allows seasonal virus outbreaks to be distinguished from COVID-19 peaks. Furthermore, sewage sludge, given it harbors concentrated human waste from a large population, serves as a substantial reservoir for pathogen detection.

To effectively integrate wastewater-based epidemiology into infectious disease surveillance, the detection methods employed in wastewater samples must be adapted to the distinct characteristics of sludge matrices. In this study, we adapted and applied protocols for the detection of IV and RSV in sewage sludge, comparing their performance with the results obtained in wastewater. To assess the efficiency of these protocols, sludge and wastewater samples were spiked with IV and RSV RNA, either free or incorporated in lentiviral particles. Samples were concentrated using the aluminum hydroxide adsorption-precipitation method before viral RNA extraction. Absolute virus quantification was carried out by RT-qPCR, including an internal control to monitor potential inhibitory factors. Recovery efficiencies for both free IV and RSV RNA were 60 % in sludge, and 75 % and 71 % respectively in wastewater, whereas the values for IV and RSV RNA in lentiviral particles were 16 % and 10 % in sludge and 21 % and 17 % in wastewater respectively. Additionally, the protocol enabled the quantification of

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naturally occurring IV and RSV in wastewater and sludge samples collected from two wastewater treatment plants during the winter months, thus affirming the efficacy of the employed methodologies.

1. Introduction

Acute respiratory illnesses represent a substantial public health challenge, imposing a considerable burden in terms of mortality, morbidity, and socioeconomic ramifications, a scenario that has increased in complexity since the advent of SARS-CoV-2 (Safiri et al., 2023; Wang et al., 2020). The resurgence of seasonal respiratory viruses such as influenza viruses (IV) and respiratory syncytial viruses (RSV) since the suppression of COVID-19 control measures, and their frequent coexistence and co-infection with SARS-CoV-2, is a major concern for health authorities (García-Arroyo et al., 2022; Lansbury et al., 2020). There is therefore an urgent need for diligent monitoring of respiratory viruses using improved models and estimations able to unravel the intricate dynamics governing their circulation (World Health Organization, 2023).

In the realm of disease surveillance, wastewater-based epidemiology (WBE) has emerged globally as a valuable methodology to analyze the dynamic evolution of excreted viruses, with the potential to enhance conventional infectious disease monitoring systems and serve as an early warning mechanism for outbreaks (Chau et al., 2022; Jiménez-Rodríguez et al., 2022; Monteiro et al., 2022; Schmitz et al., 2021; Sims and Kasprzyk-Hordern, 2020). However, for an effective integration of WBE into infectious disease surveillance, pivotal challenges need to be addressed, such as the complex nature of wastewater matrices, the recovery and concentration of targets expected to occur in low amounts, the selection of representative samples, and the critical step of population normalization (Azubuike et al., 2022; Polo et al., 2020). WBE research has already established the correlation between respiratory viruses, RNA levels in wastewater, and incidence rates obtained from clinical epidemiological data, demonstrating the credibility of this approach for disease tracking (Hirose et al., 2016; Minodier et al., 2019; Peccia et al., 2020; Perlas et al., 2023; Wolfe et al., 2021). Notably, the RNA of respiratory viruses remains detectable in wastewater even after the virus is no longer infectious, providing insights into persistent viral genetic material (Hughes et al., 2022; Wolfe et al., 2022).

The successful adoption of WBE for SARS-CoV-2 monitoring has naturally spurred interest in its broader application in public health surveillance, encompassing other relevant respiratory viruses such as IV and RSV (Ando et al., 2023; Heijnen and Medema, 2006; Koureas et al., 2023; Markt et al., 2023). This expansion aligns with the European Commission recommendation to increase the range of viral pathogens in urban wastewater surveillance systems (European Commission, 2022).

Numerous national wastewater surveillance programs are currently monitoring the spread of respiratory diseases by measuring the concentration of genetic markers, not only in wastewater but also in primary settled solids. As sewage sludge contains concentrated human waste from a vast population, it potentially holds substantial pathogen diversity (Bibby and Peccia, 2013; Gholipour et al., 2022). Moreover, previous research indicates that viruses and their genetic markers exhibit a greater affinity for the solid rather than liquid fraction of wastewater matrices (Graham et al., 2021; Kim et al., 2022; Kitamura et al., 2021; Li et al., 2021; Mercier et al., 2022; Roldan-Hernandez and Boehm, 2023; Ye et al., 2016). These findings consistently highlight the propensity of viruses, particularly enveloped ones, to partition to wastewater solids, in several instances resulting in higher concentrations of viral genetic markers in primary sludge compared to paired wastewater influent samples (Graham et al., 2021; Kim et al., 2022; Li et al., 2021; Wolfe et al., 2021).

Given the potential for influenza and other respiratory viruses to persist in the environment, particularly within solid matrices (Boehm et al., 2023; Li et al., 2021; Mercier et al., 2022; Perlas et al., 2023), the aim of this work was to assess the feasibility of detecting IV and RSV in sewage sludge. The study leveraged knowledge acquired by established methods for virus detection in sludge (Martín-Díaz and Lucena, 2018) and the efficiency of an extraction method previously validated by our group for the detection of SARS-CoV-2, IV, and RSV (Martín-Díaz and Lucena, 2018; Toribio-Avedillo et al., 2023).

2. Material and methods

2.1. Sample collection

Twenty wastewater samples were collected during 2022 from two prominent wastewater treatment facilities (Baix Llobregat and Besos). These facilities collectively cater to the sanitation needs of the city of Barcelona, which boasts a population of approximately 3.3 million residents. Specifically, Baix Llobregat serves approximately 65 % of this population, while Besos serves the remaining 35 %. Both wastewater treatment plants extend their services to other municipalities within the province of Barcelona, as corroborated by official data accessible through the Barcelona metropolitan area public administration webpage (AMB, 2021a; AMB, 2021b).

Twenty sludge samples from the same wastewater treatment plants were also collected at the same dates as the wastewater samples. These sludge samples were a composite mixture of raw primary sludge (approximately 60 %) and secondary sludge (40 %) which had undergone a sequence of processes including mechanical thickening. For specific case of Baix Llobregat WWTP the process continues with an anaerobic-mesophilic (35 °C) digestion for 20–25 days, dewatering, and thermal drying.

Ten samples of each matrix selected for being negative for IV and RSV (Toribio-Avedillo et al., 2023) were used to analyze the recovery of the spiked RNA and viruses respectively. Negative samples were processed in duplicate using the protocol in this study and were considered those being negative in both replicates. In these negative samples, the internal control was always positive, indicative of no inhibition. These negative samples were collected between May and early October, where the presence of IV and RSV, as seasonal respiratory viruses, was not expected.

Other ten samples of wastewater and ten samples of sludge, which were collected during winter months (November–February), were used to analyze naturally occurring IV and RSV.

2.2. Protocol for sludge clarification and elution

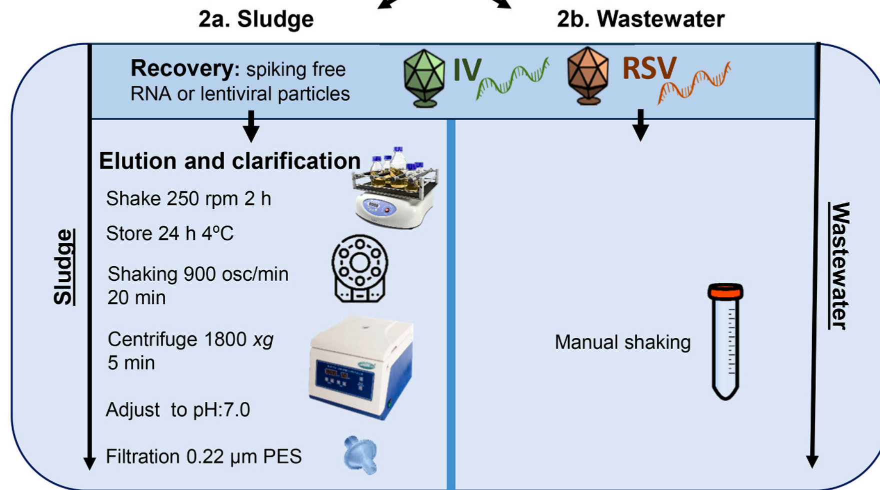
Before the analysis, the sludge samples underwent elution and clarification following a previously published methodology (Martín-Díaz and Lucena, 2018) (Fig. 1). In brief, 1/10 dilution of 20 g of the sample was performed with 180 ml of glycine buffer solution at pH 9.5. Viruses attached to solid particles in this solution were subsequently eluted by agitation with a wrist-action shaker for 20 min at 900 osc/min. The sample was then clarified by centrifugation at 1800 $\times g$ for 15 min. The resulting pellet was discarded, and the pH of the supernatant was adjusted to 7.0 by gradually adding 1 M HCl. The supernatant was then filtered using 0.22 μm pore-size polyethersulfone non-protein-binding membrane filters (Millipore, MA, USA) to obtain the viral extract.

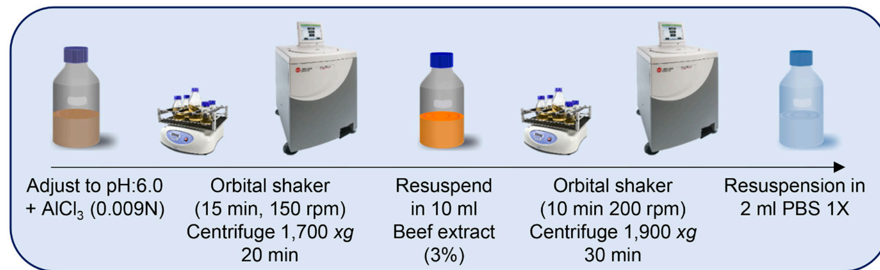
2.3. Sample concentration

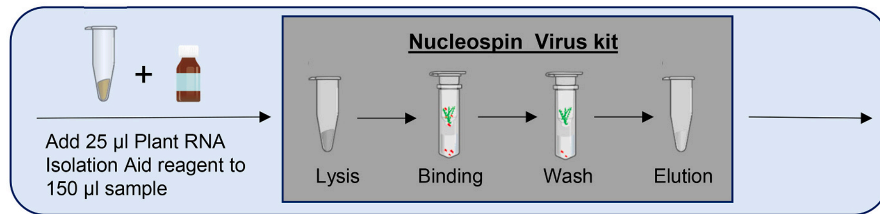
The concentration of both wastewater and eluted sludge was carried out using the adsorption-precipitation method with aluminum hydroxide (Fig. 1). This procedure utilized by multiple research groups in Spain

for SARS-CoV-2 analysis (Carcereny et al., 2021; Randazzo et al., 2020), was recently shown to be useful for IV and RSV monitoring in wastewater (Toribio-Avedillo et al., 2023). The method was chosen for its efficiency in detection, speed, simplicity, and cost-effectiveness (Pérez-Cataluña et al., 2021).

Accordingly, samples of wastewater (200 ml) and eluted sludge were collected, and their pH was adjusted to 6.0. An $AlCl_3$ solution was added to achieve a final concentration of 0.009 N, and the mixture was thoroughly agitated by hand. The pH was readjusted to 6.0, and the mixture was agitated further using an orbital shaker for 15 min at 150 rpm,







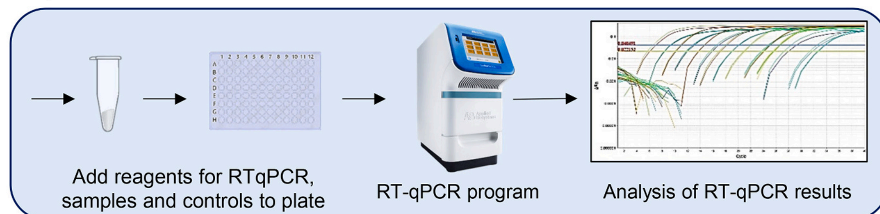


Fig. 1. Methodology used in this study. Schematic presentation of the method used to determine influenza virus (IV) and respiratory syncytial virus (RSV) in sludges and wastewater.

followed by centrifugation at 1700 ×g for 20 min. The supernatant was discarded, and the resulting pellet was resuspended in a 10 ml solution of 3 % beef extract (Becton, Dickinson and Company, Sparks, MD, US). After 10 min of agitation at 200 rpm, another centrifugation step at 1900 ×g for 30 min was performed. The supernatant was once again discarded, and the pellet was resuspended in a 2 ml 1× phosphate-buffered saline solution.

2.4. Nucleic acid extraction

Nucleic acid extraction was carried out according to established procedures in a Class II biosafety cabinet, employing the Nucleospin RNA Virus kit (Macherey-Nagel GmbH & Co., Düren, Germany) according to the manufacturer's instructions, with some modifications favoring RNA stability and recovery as previously described (Randazzo et al., 2020). Modifications included the addition of 25 μl of Plant RNA Isolation Aid (Thermo Fisher Scientific, Vilnius, Lithuania) and 600 μl of lysis buffer from the NucleoSpin virus kit to 150 μl of the concentrated sample. The mixture was subjected to pulse-vortexing for 1 min. Then, the homogenate was centrifuged for 5 min at 10,000 ×g to remove the debris. The supernatant was subsequently processed according to the manufacturer's instructions and eluted in 100 μl of RNAse free dH_2O. One extraction was performed for each sample and quantification was performed within 24 h after extraction.

2.5. RT-qPCR quantification

The genes targeted for quantification through real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) included the matrix protein M1 genes from influenza A viruses (World Health Organization, 2015) and the nucleoprotein N genes from RSV-A viruses (Hu et al., 2003). RT-qPCR was conducted using Viasure Real-Time PCR Detection kits (CerTest Biotec, Barcelona, Spain), which provided all the necessary components for real-time PCR in a single reaction mix, including specific primers, probes, dNTPs, buffer, polymerase, and retrotranscriptase. To rule out polymerase activity inhibition, the kit contains an internal control that targets an exogenous gene absent in humans, animals and plants (specific gene information is not available as it is under patent protection). Real-time qPCR employing TaqMan hydrolysis probes was carried out in Class II biosafety cabinets, using the StepOne Real-Time PCR System (Applied Biosystems, Foster City, USA). A 20 μl reaction mixture was prepared, and all samples, including standards and negative controls, were subjected to duplicate analysis following the program outlined in Table 1. After the amplification reaction, virus genes were detected in the FAM channel and the internal control in the VIC/HEX/JOE channel. Samples were considered positive (and quantifiable) if they reached a cycle threshold (Ct) value within the values of the standard curve and if the internal control included in the reaction was amplified correctly.

Table 1
Quantitative RT-PCR conditions for IV and RSV.

Virus	Step	Temperature	Time	Number of cycles	Limit of detection (GC/l)
Influenza	Reverse transcription	45 °C	15 min	1	6.01×10^3
	Initial denaturalization	95 °C	2 min	1	
	Denaturalization	95 °C	10 s	45	
	Annealing/ Extension	60 °C	50 s		
RSV	Reverse transcription	45 °C	15 min	1	7.31×10^3
	Initial denaturalization	95 °C	2 min	1	
	Denaturalization	95 °C	10 s	45	
	Annealing/ Extension	60 °C	50 s		

These Viasure RT-qPCR assays were initially designed for qualitative assays. We adapted these assays for absolute quantification, that required the preparation of standard curves. Standard curves were prepared using serial decimal dilutions with double-distilled water of commercial synthetic RNA (CerTest Biotec) for IV and RSV containing 10^8 gene copies (GC)/μl, as indicated by the manufacturer. Each dilution was amplified in triplicate in at least five independent runs and the average threshold cycle (Ct) values and GC for each dilution were used to calculate the abundance of each virus in the volume tested (GC)/l. A duplicate of each dilution of the standard curve and of each sample and of the negative control (nuclease-free water) were run in each plate. If the two replicates showed consistent values, the GC number represented the mean value from duplicate analyses. In case of no consistent results between the two replicates, the samples were amplified again in duplicate. Samples were deemed negative in the absence of an amplification signal and if the internal control exhibited a positive signal ($Ct \leq 30$). If the negative control produced a signal, the positive control lacked a signal, or the internal control was not as expected ($Ct < 30$), the results were considered invalid.

2.6. Assessing protocol efficiency for the recovery of viral RNA and viral particles in wastewater and sludge

To evaluate the recovery of viral RNA by the protocol used, viral RNA sequences homologous to IV and RSV (CerTest Biotec, Barcelona, Spain) were used. The sequences consisted of RNA homologous to the isolate A/Arkansas/08/2020 belonging to the influenza A(H1N1) pdm09 subtype; RNA homologous to the isolate B/Arizona/12/202 belonging to the influenza B/Yamagata lineage, and RNA homologous to the strain S2 ts1C belonging to RSV subtype A. GenBank accession numbers were MW130270-77, MT499475-82 and NC_001803.1, respectively.

Sludge and wastewater samples negative for IV and RSV were spiked with free RNA of each virus (Fig. 1) to reach a final concentration of 200 GC/ml. Recovery efficiency was assessed by comparing the expected concentrations in the original samples to the actual concentrations determined by RT-qPCR analysis after viral RNA purification. Recovery efficiency for each viral RNA was calculated based on the GC quantified by RT-qPCR as follows: Recovery efficiency (%) = (N° GC viral RNA recovered / N° GC viral RNA seeded) × 100.

To assess the efficiency of the protocol for virus recovery, non-replicative and non-infectious recombinant viral particles with genetic material from each target virus (Viasure Viral Influenza A, Influenza B & RSV. CerTest Biotec, Barcelona, Spain), incorporated with a lentiviral vector system (Sakuma et al., 2012), were used. IV surrogate particles contained the whole genome of IV and RSV surrogate particles contained the M, N and F genes and two fragments of the L gene (12440-12610 and 13810-13920) of RSV.

Ten sludge and ten wastewater samples confirmed as negative for IV and RSV as described above, were spiked with a final concentration of 200 viral particles per ml or g of sample. Viral particles in wastewater samples were distributed by gentle agitation. In the case of sludge, a uniform distribution within the sample before the elution and clarification step was achieved by their addition to 100 g of sludge and agitation with an orbital shaker at 250 rpm for 2 h. After 24 h of decantation at 4 °C to allow viral attachment to solid particles, the aqueous fraction was removed, and the solid fraction was collected for analysis.

Recovery efficiency was assessed by comparing the expected viral concentrations in the original samples to the actual concentrations determined by RT-qPCR analysis after virus concentration, purification, and quantification. Calculations for each virus were based on the copies quantified by RT-qPCR as follows: Recovery efficiency (%) = (Viral particles recovered / Viral particles seeded) × 100.

2.7. Transmission electron microscopy studies of the viral particles

The lentiviral particles containing IV and RSV RNA used for the recovery assessment were analyzed by transmission electron microscope (TEM). For this, 10 μl of the stock containing ca. 10^8 particles/ml was dropped onto copper grids with carbon-coated Formvar films, negatively stained with 2 % ammonium molybdate (pH 6.8) and examined under a Jeol 1010 transmission electron microscope (JEOL Inc. Peabody, MA, US) operating at 80 kV.

2.8. Statistical analysis

Computation of data and statistical tests were carried out using GraphPad Prism 10 software (GraphPad Software, San Diego, CA, US). Viral concentrations were 10-log transformed and the Mann-Whitney test was used to evaluate the differences between the counts; evaluations were based on significance levels of p value = 0.05. Unpaired t-test was used to evaluate differences between recovery efficiencies of IV and RSV in sludge and wastewater.

3. Results

3.1. Validation of RT-qPCR assays

The RT-qPCR standard curves (Fig. 2) prepared for the absolute quantification of target viruses indicated efficiencies of 92.5 % for IV and 101.8 % for RSV. Additionally, the limits of quantification (LOQ) for these assays were determined using serial decimal dilutions of RNA stocks containing 200 GC/ml of IV and RSV extending the series to a dilution as low as 1 GC/ml. LOQ was determined to be 6.01 GC/ml for IV and 7.31 GC/ml for RSV.

3.2. Recovery efficiency of free RNA in sludge and wastewater

To evaluate the efficiency of free RNA recovery, ten sludge and ten wastewater samples were spiked with free RNA and the expected concentrations in the original samples were compared with the actual concentrations determined by RT-qPCR analysis after virus purification. In sludge samples, recovery rates for viral RNA were 60 % for IV (IV-A) and 60 % for RSV (RSV-A). Higher recovery rates were obtained in wastewater, with 75 % for IV and 71 % for RSV (Table 2).

3.3. Comparison of viral particle recovery in sludge and wastewater

The effectiveness of the viral concentration and extraction methods was assessed through an experiment in which ten wastewater and ten sludge samples were spiked with a stock of known concentration of lentiviral particles containing RNA of IV-A or RSV-A.

The integrity of the particles in these lentiviral stocks was validated by TEM microscopy, which revealed heterogeneous spherical particles within the size range of 40 ± 14 nm (Fig. 3).

After the samples were spiked with viral particles, they were processed as described above for sludge and wastewater samples from the same water treatment facilities. Recovery efficiency was determined by comparing the theoretically expected concentrations in the original samples and the real concentrations determined by RT-qPCR analysis. Accordingly, recovery rates of lentiviral particles in sludge were 16 % for IV and 10 % for RSV, being higher in wastewater: 21 % and 17 %, respectively (Table 2), although no statistically significant differences were obtained between sludge and wastewater for IV (unpaired t-test, $p = 0.3607$) or for RSV (unpaired t-test, $p = 0.2082$).

3.4. Analysis of environmental samples

When the protocol was applied to analyze naturally occurring viruses in ten sludge and wastewater samples collected during winter months, all the winter samples were positive for both viruses (Fig. 4). The median concentrations for IV and RSV in sludge were 2.41×10^6 and 2.51×10^6 GC/g respectively, the values per g of sludge being significantly higher than in wastewater (Mann-Whitney test, $p < 0.0001$), which showed median counts of 3.06×10^4 GC/l for IV and 2.77×10^4 GC/l for RSV (Fig. 4). Comparing the viruses per sample, the median IV values in sludge and wastewater did not differ significantly from RSV values (Mann-Whitney test, $p = 0.247$ for sludge and $p > 0.999$ for wastewater), even though RSV showed more variability between samples. The internal control, analyzed using the VIC channel, showed Ct values between 26.5 and 27.2 in all the wastewater and sludge samples analyzed, confirming the absence of inhibitors.

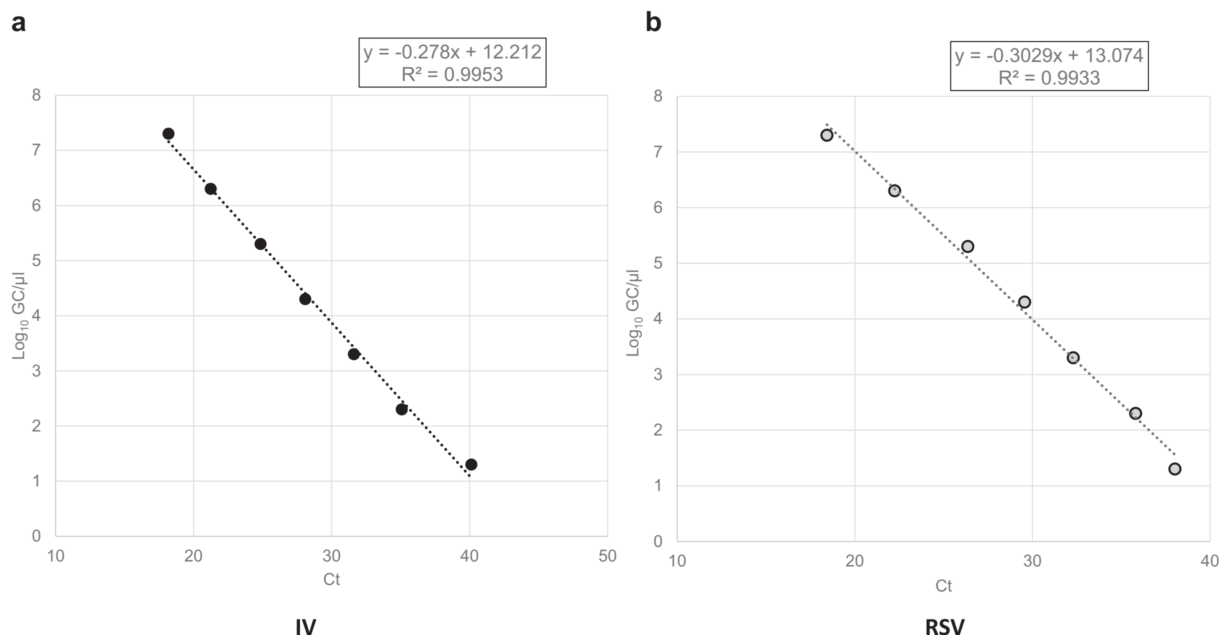


Fig. 2. RT-qPCR standard curve obtained with influenza virus (IV) and respiratory syncytial virus (RSV). Data are the average of three replicates in five independent experiments. Upper right, standard curve and R^2 value of each virus.

Table 2
Efficiency of recovery of IV and RSV free RNA and IV and RSV lentiviral particles in wastewater and sludge.

Sample	Material	Virus	Theoretical concentration ml/g of sample	Averaged amount recovered[a]	Averaged % recovery efficiency	Limit of detection
Wastewater	Viral RNA	IV	200 GC	150.75 GC	75.38 ± 35.24	7.46 GC
		RSV	200 GC	141.64 GC	70.82 ± 38.30	8.73 GC
	Viral particles	IV	200 particles	41.80 particles	20.93 ± 14.80	13.39 particles
		RSV	200 particles	34.21 particles	17.10 ± 15.50	9.01 particles
Sludge	Viral RNA	IV	200 GC	119.18 GC	59.59 ± 29.58	10.75 GC
		RSV	200 GC	120.71 GC	60.36 ± 44.04	15.59 GC
	Viral particles	IV	200 particles	31.91 particles	15.95 ± 7.93	12.30 particles
		RSV	200 particles	20.50 particles	10.25 ± 5.92	17.56 particles

[a] Average results of ten experiments. Limit of Detection: The minimal amount of RNA gene copies or lentiviral particles added to the samples that could be detected.

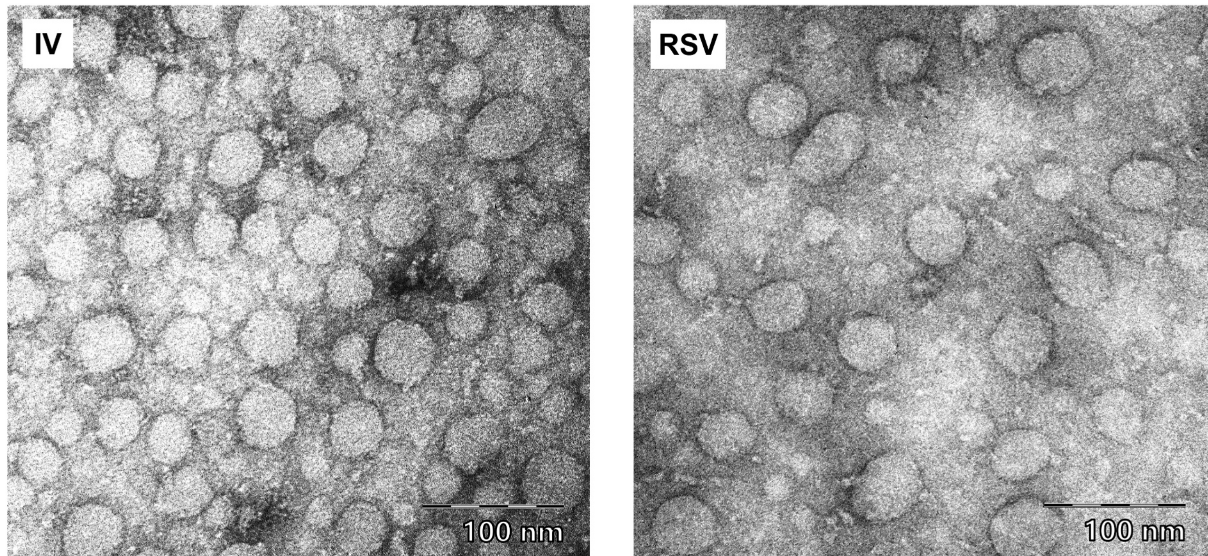


Fig. 3. Electron microscopy analysis of viral particles. Transmission electron microscopy images of lentiviral particles containing influenza virus (IV) and respiratory viruses (RSV) used to assess recovery efficiency.

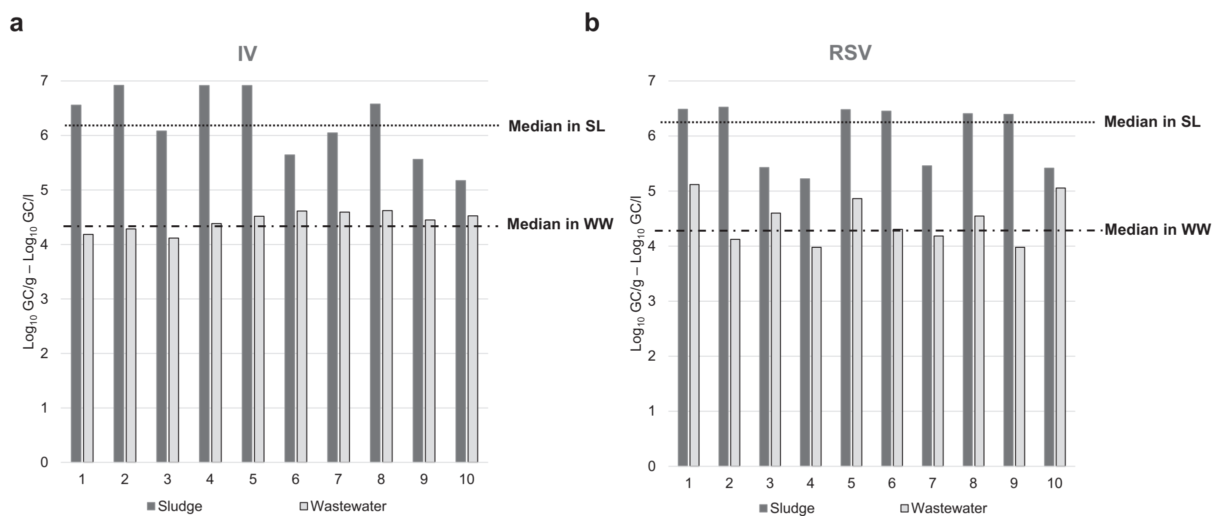


Fig. 4. Naturally occurring IV and RSV. Values of naturally occurring influenza virus (IV) and respiratory syncytial virus (RSV) in samples of sludge (SL) ($\log_{10}$ GC/g) and wastewater (WW) ($\log_{10}$ GC/l). Values are the mean of two replicates. Dotted lines show the median of values obtained with both viruses for all wastewater and sludge samples.

4. Discussion

Since the recent pandemic, when WBE proved to be a valuable tool for monitoring the prevalence of SARS-CoV-2, there has been growing interest in studying a range of respiratory viruses in different environmental matrices to control the possible spread of virulent strains (Ando et al., 2023; Boehm et al., 2023), and it has been proposed that pathogens other than SARS-CoV-2 should be included in existing surveillance

programs (Markt et al., 2023). In addition, as the symptomatology of SARS-CoV-2 resembles that of other respiratory viruses, a differential detection system could shed light on their spread and provide useful epidemiological data. Among the most prevalent respiratory viruses in the population are IV and RSV, particularly in winter months (García-Arroyo et al., 2022), and a significant correlation between their concentrations in wastewater and the incidence of clinical cases has been reported in several studies (Hughes et al., 2022; Mercier et al., 2022). Therefore, with advancements in analytical methodology, it has become evident that WBE can be usefully applied to monitor the presence and stability of a broad range of viruses and thus enhance global health protection.

Due to the challenges of handling infectious viruses, surrogates were employed to evaluate the efficiency of the methods for monitoring respiratory viruses in water and sludge matrices. Although phage Φ6 has commonly been used as a surrogate for respiratory viruses (French et al., 2023), lentiviral particles were chosen as a suitable alternative for our study, not only because of the enveloped nature of lentiviruses, but also because they contain the viral RNA of interest. These attributes rendered the particles particularly useful for assessing the different steps of the protocol: elution, concentration, recovery, and detection by RT-qPCR. Although the lentiviral particles were of the expected size for lentiviral particle vectors (Parr-Brownlie et al., 2015; Rahman et al., 2013), confirming their correct formation, they were slightly smaller than real IV and RSV virions (Berthiaume et al., 1974; Tiwari et al., 2014; Vajda et al., 2016). Because of their larger size, the retention of IV and RSV in solids can be expected to be higher than the retention of lentiviral particles in particulate material with small pore sizes. Nevertheless, lentiviral particles behave like enveloped RNA viruses, showing a similar low level of stability, showing sensitivity to low pH, high salt levels, temperature, or shear forces, among other conditions (Moreira et al., 2021). Therefore, these surrogates offer a safer approach for the evaluation of viral recovery efficiency, avoiding the risks associated with pathogenic viruses.

The RT-qPCR assays and standard curves used for the absolute quantification of both viruses revealed a high level of recovery efficiency, the use of an internal control ruling out interference by inhibitors. The results of the assays carried out with particles and free RNA strongly suggest that the methodology designed for wastewater analysis, after some modifications, can be applied to sludge matrices with comparable efficiency. However, the recovery rate for particles or free RNA was somewhat lower in sludge than in wastewater, which could be primarily attributed to the adsorption of viral material to particulate matter in sludge or its entrapment in sludge flocs (Bhattarai et al., 2021; Otawa et al., 2007; Yang et al., 2020). The efficiency of recovery of seeded RNA, both in sludge and wastewater, was equal to or higher than previously reported recoveries of enveloped viruses (Ahmed et al., 2021; Rusiñol et al., 2020).

The recovery of free RNA of both viruses surpassed that of particles, possibly due to the smaller size of RNA molecules, and the absence of capsids that need to be broken, resulting in a lower loss of genetic material compared to the particles (Ikner et al., 2012; Kumblathan et al., 2023). A greater persistence of viral particles compared to free RNA would be expected. However, unlike what occurs in natural environments, in our study, the time between free RNA inoculation and analysis was short, reducing the degradation of free RNA. Nevertheless, the recovery efficiencies in sludge in the present work (16 % for IV and 10 % for RSV) were comparable to those of previous studies analyzing enveloped virus RNA in sludges, which reported recoveries of 12–16 % for phage PP7 (Assis et al., 2017) and below 10 % for enveloped phage Φ6 RNA, the latter being attributed to a strong phage adsorption to the sludge (Yang et al., 2022). In wastewater, the recovery rate of enveloped virus RNA depends on the spiked viruses with recoveries of 8 to 24 % for SARS-CoV-2 (Barril et al., 2021), 1 to 25 % for different coronaviruses (La Rosa et al., 2020). In addition to the viruses analyzed, variable efficiencies for enveloped viruses are reported depending on the concentration method used, some showing improved recovery efficiency (Rusiñol et al., 2020). For example, for SARS CoV-2 in wastewater, the concentrated pipette method showed recoveries of 25.1 %, the adsorption-extraction method of 26 % while ultracentrifugation accounted for 12 to 25.4 % (Ahmed et al., 2021). Other studies analyzing the recovery of SARS-CoV2 in wastewater, ultracentrifugation showed a viral recovery efficiency of 25 % (Rusiñol et al., 2020; Zheng et al., 2022), $AlCl_3$ showed a recovery efficiency of 11 %, PEG of 11.8 %, $MgCl_2$ of 12.4 %, ultrafiltration (Amicons) of 7.9–9.6 % and membrane adsorption of 11 % (Zheng et al., 2022).

Regarding potential differences in recovery between viruses, we obtained similar counts for IV and RSV in all wastewater and sludge samples. However, it is worth noting that RSV shows more variability throughout the year (Toribio-Avedillo et al., 2023). The positivity results were probably affected by the use of samples collected in the winter months, as both IV and RSV are known to be seasonal, with the highest peaks of incidence occurring in January–February (García-Arroyo et al., 2022), particularly for IV. Over a year, the incidence of IV might be lower compared to RSV, as was shown in wastewater (Toribio-Avedillo et al., 2023).

Solid matrices, including sludge and settled solids, are well-established as significant reservoirs of both enveloped and non-enveloped viral particles (Bibby and Peccia, 2013; Cantalupo et al., 2011). In addition, the viruses removed from raw wastewater by treatment plants are transferred to the sludge, where they accumulate and become concentrated, especially hydrophobic enveloped viruses (Yang et al., 2020), implying a potential risk of exposure and transmission during sludge treatment and disposal. Accordingly, despite the lower recovery efficiency observed in sludge, our method detected a higher GC in sludge compared to wastewater. This result was expected, as previous studies have reported a higher viral presence in the solid fraction of wastewater (Roldan-Hernandez and Boehm, 2023; Serra-Compte et al., 2021). It should be considered, however, that the viral loads of the two matrices are difficult to compare because of the lack of correspondence of a unit of sludge (g) to one ml of wastewater.

The vast quantity of sludge generated every day by wastewater treatment plants, which renders it suitable for the epidemiological monitoring of respiratory viruses, calls for different reuse strategies. One of the most practicable environmental options available for the application of treated municipal sludge is in agriculture (Levantesi et al., 2015). To guarantee the safety of these practices, it would be useful to evaluate different potential hazards, including that of respiratory viruses. The monitoring of wastewater can detect the outbreak of respiratory viruses faster than clinical surveys (Ando et al., 2023; Carcereny et al., 2021; Heijnen and Medema, 2011; Kitajima et al., 2022; Koureas et al., 2023; Randazzo et al., 2020; Schmitz et al., 2021; Wolfe et al., 2022). Its potential advantage for public health is the possibility to conduct screening of a large population using a few samples without depending on the availability of clinical testing (Thompson et al., 2020), with transmission collections in large geographic regions that can assist in rapid efforts by public health authorities. In addition, the monitoring of sludges could provide additional tools for respiratory virus tracking in wastewater treatment plants, especially in communities with low caseloads or in early detection periods (D'Aoust et al., 2021). However, for introducing sludges in wastewater-based epidemiology, the development of suitable, cost-effective and reliable methodologies for virus determination in sludge as the one presented in this study is crucial for a comprehensive and in-depth examination of viral transmission in the environment. The method presented in this study shows a recovery efficacy comparable with other concentration methods. It has been adapted from previous validated protocols for viral determination in sludge (Martín-Díaz and Lucena, 2018), and allows the viral analysis in sludges and in wastewater simultaneously, with the addition of only few additional steps when analyzing sludges. It presents the advantage of being based on the adsorption-precipitation method with aluminum hydroxide, that has already been shown effective for monitoring

respiratory viruses such as SARS-CoV-2 (Carcereny et al., 2021; Randazzo et al., 2020) and IV and RSV in wastewater (Toribio-Avedillo et al., 2023).

5. Conclusion

The proposed method proved effective for detecting IV and RSV in sewage sludge, the recovery efficiencies being in line with available data on viral recovery in this matrix. By taking advantage of methods originally designed for SARS-CoV-2 detection, thus simplifying usage for laboratories with experience only in wastewater testing, the developed protocol facilitates sludge analysis. Despite the recognized utility of wastewater-based surveillance, more research is needed to understand the behavior of viral genetic markers within wastewater solids, particularly those with similar symptomatology and seasonality. Existing methods are mainly aimed at the detection of non-enveloped viruses and are not suitable for enveloped viruses. Also, the presence of natural inhibitors (humic acids, heavy metals, etc.) in sludge may have adverse effects on the amplification reaction of viral genes, leading to false-negative results. Therefore, there is a need to implement efficient extraction and detection methods targeting enveloped viruses in sludge.

CRediT authorship contribution statement

Daniel Toribio-Avedillo: Writing – review & editing, Writing – original draft, Investigation, Data curation. **Clara Gómez-Gómez:** Writing – review & editing, Validation, Investigation. **Laura Sala-Comorera:** Writing – review & editing, Visualization, Formal analysis. **Belén Galofré:** Writing – review & editing, Resources. **Maite Muniesa:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

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.

Data availability

Data will be made available on request.

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