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Using Molecular Tools to Compare Various Concentration Protocols Prior to Detecting SARS-Cov-2 RNA in Wastewater

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Abstract

Background: Reports showed presence of SARS-CoV-2 genetic material in wastewater. Wastewater concentration methods are optimized for detection of non-enveloped viruses so need to be adopted for enveloped viruses and their genetic material. Methods: Conventional (cRT-PCR) and quantitative real time RT-PCR (qRT-PCR) were used as readouts to compare 4 water concentration methods namely, A) filtration on negatively charged membrane followed by extracting RNA from it, B) adsorbtion-elution method, C) flocculation with skimmed milk and D) polyethylene glycol precipitation, to detect SARS-CoV-2 RNA and 229E human coronavirus (229E-HCoV) as a model for spike-containing enveloped virus from fresh and wastewater. Results: On using cRT-PCR: recovery rate of SARS-CoV-2 RNA was better using method A then B for fresh water and method B then D for wastewater. 229E-HCoV recovery from fresh water was better using method C then A and methods B then D for wastewater. On using qRT-PCR, both methods A and B were better for SARS-CoV-2 RNA recovery from both fresh and wastewater. For the 229E-HCoV methods A was the most efficient for fresh water and method B for wastewater. Conclusion: Method B is recommended for SARS-CoV-2 RNA or whole 229E-HCoV recovery from wastewater.
Keywords: SARS-CoV-2, Human corona virus 229E, molecular tools, wastewater, Concentration methods

1. Introduction

In March 2020, Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was classified by WHO as the cause of global pandemic [1]. It is an enveloped virus belonging to family Coronaviridae, its envelope is associated by three structural proteins: spike, envelop and membrane protein [2]. The virus can be detected in nasal, pharyngeal swabs, sputum and stool samples [3]. High viral RNA concentration was detected in stool samples of infected persons [4] which makes it main source of virus shed in wastewater beside nasal discharge, sputum and other virus containing human body fluids/secretions that can also reach wastewater [5].

This stimulated scientists to think that detecting the virus load in wastewater of a given area can enable approximate estimation of the number of infected persons among the total population living in it [6] and number of asymptomatic carriers as well [7] and based on this many studies were conducted to detect the virus in wastewater [8,9].

Several wastewater concentration methods are known including ultrafiltration, adsorbtion-elution, and precipitation by polyethyleneglycol (PEG). Those methods were optimized for non-enveloped viruses which are known to be prevailing in wastewater. Many reports compared those concentration methods to know which of them can lead to the best recovery of SARS-CoV-2 to be used in its detection in wastewater.

Pérez-Cataluña et al [10] inoculated wastewater samples with a number of enveloped and non-enveloped viruses and compared percentage of viral recovery by precipitation using the aluminum-based adsorption or polyethylene glycol (PEG), results suggest usage of the aluminum-based adsorption method.

Jafferali et al [11] also compared ultrafiltration and adsorption on negatively charged membrane in recovering enveloped and non-enveloped viruses including SARS-CoV-2 and results showed that ultracentrifugation was better.

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Evaluating wastewater concentration methods before conducting a study was also shown by Ahmed et al [12] to be of high importance as he recorded that the optimum concentration method depended on the type of virus that you target in your study, and that it differs not only among enveloped and non enveloped viruses but also among structurally similar viruses. Beside wastewater concentration methods comes the RNA extraction methods that also affects viral recovery rates [13].

Although all those comparative studies were really of benefit but they were focusing on finding best method to recover enveloped viruses from wastewater and did not pay attention that in reports that proved detecting SARS-CoV-2 in wastewater only the genetic material of the virus was found but not the whole viral particle [14,15]. Few scientists compared wastewater concentration methods that enabled higher RNA recovery [16].

From here our research aimed to compare four concentration methods, A) filtration on negatively charged membrane followed by extracting RNA from it, B) adsorbtion-elution method, C) flocculation with skimmed milk and D) polyethylene glycol precipitation) to detect genetic material of SARS-CoV2 and the 229E human coronavirus (229E-HCoV) as spike-containing enveloped virus both from fresh and wastewater by both conventional and real-time PCR.

2.Experimental:

2.1.Viruses:

a)SARS-CoV-2: Nasal sample was collected in March 2021 from SARS-CoV-2 infected female Egyptian patient with mild symptoms (Rhinorrhea, chest pain, coughing fever and headache). Patient was positive for SARS-CoV-2 IgM/IgG by rapid test (AMEDA, labordiagnostik GmbH) then further confirmed for active infection by RT-PCR and sequencing of the whole Spike and polymerase genes. Sequencing results showed that the causative strain for infection was related to the C.36.5 and C.37 known as lambda variant. Sample was used as source for SARS-CoV-2 RNA.

b) 229E-HCoV (ATCC-VR-740): Prepared stock with final viral count 1.6×10^4 PFU/ml

2.2.Water samples preparation:

Two kinds of water samples were included: a) distilled (fresh) water, b) wastewater collected from the inlet of Zeinin wastewater treatment plant in October 2022 and confirmed to be negative for both SARS-CoV-2 and 229 E-HCoV virus by conventional PCR.

Tested water samples, each of volume 1liter, were inoculated with a) 100µl of SARS-CoV-2 RNA of with concentration of 306 ng/µl. b) 300µl of 229E-HCoV.

2.3.Water concentration methods:

2.3.1. Filtration on negatively charged membrane (Method A) [17].

A volume of 1liter Fresh and wastewater samples inoculated with SARS-CoV-2 RNA and 229E-HCoV were used. Initially, pH of both were adjusted to 3.5 using 1N HCl then both water types were filtered through 0.45 µm nitrocellulose membrane (Hi-GenoMB, Mumbai, India). Membrane was removed from holder, cut into small pieces and put in 5 ml lysis buffer (QIAGEN, Hilden, Germany) for direct RNA extraction. Extracted nucleic acids were stored at -80ºC till use.

2.3.2. Adsorption-elution (Method B) [18]:

A volume of 50 ml of 1M aluminum chloride was added to 1 liter of seeded fresh and waste water samples with both the SARS-C0V-2 RNA and 229E-HCoV to give a final concentration 0.05M. pH was adjusted to be 3.5 by 1N HCl. Water samples were filtered through 0.45 µm nitrocellulose membrane followed by passing 200 ml 0.5 mM H_2SO_4, pH 3.0 to ensure viral particle retention and eliminate all bio-solids. Membrane was removed from holder and soaked in a Petri dish in11 ml of 1 mM NaOH; pH 10.5 for 10 minutes. Later, the upper surface of the membrane was scraped to elute the virus/virus RNA. The elute was neutralized by adding 50 µl 50 mM H_2SO_4 and 50 µl 100x Tris–EDTA pH 8.0 and stored at -80ºC till use.

2.3.3. Skimmed milk flocculation (Method C) [19]:

A total of 10 grams skimmed milk powder (Miro, Egypt) were dissolved in 1liter distilled water, pH was adjusted to 3.5 with 1N HCl. pH of seeded fresh and wastewater with the SARS-CoV-2 RNA and 229E-HCoV was adjusted to 3.5 by 1N HCl. A volume of 10 ml of the acidified milk was added to each liter of the water samples to reach a final concentration of 0.01% milk followed by 8 hours stirring at room temperature then the mixture was left overnight at 4 °C in the refrigerator to allow flocks to settle down. The supernatants were discarded and pellets were centrifuged at 8000×*g* for 30 min at 4 °C. The obtained pellets were dissolved in phosphate buffered saline (PBS) to reach final volume of 10 ml and stored at -80ºC till use.

2.3.4. Polyethylene glycol precipitation (PEG 800) (Method D) [20]:

Volumes of 100 ml of both of seeded fresh and wastewater with the SARS-CoV-2 RNA and 229E-HCoVwere divided into two 50 ml sterile tubes and centrifuged at 3000×*g*; 4ºC for 30 min. Supernatants were removed without discarding the pellet and their pH was adjusted to 7-7.5 by 1M NaOH. PEG 800 (40%) was added to NaOH (8%) to a final ratio 1:3 ratio to make their final concentrations 10 % and 2%, respectively. The mixture was inverted several times then incubated overnight at 4^0C followed

centrifugation at 10000xg at 4^{0}C for 30 min. Supernatants were discarded and pellets were resuspended in 1 ml PBS and store at -80°C till use.

2.4.Detection methods:

2.4.1.Semi-quantitative conventional reverse transcriptase-polymerase chain reaction (cRT-PCR): A volume of 140 µl of each concentrated water samlpe was subjected to viral RNA extraction (QIAGEN, Hilden, Germany), reverse transcription (Omniscript RT, Hilden, Germany) then PCR (Taq polymerase QIAGEN, Hilden, Germany) using inhouse designed primers (Table 1). Reaction mixture was subjected to the following temperature conditions, 94 °C for 3 min., denaturation at 94 °C for 30 seconds, annealing at 57 °C for 1 min., extention at 72 °C for 1 min. for 40 cycles followed by extension at 72 °C for 10 min. The amplification products were visualized after electrophoresis on agarose gel (1.1%) containing ethidium bromide. Band intensities were calulated using the UVP VisionWorks LS image analysis software, version:8.16.16089.9066.

2.4.2.Quantitative RT-PCR (qRT-PCR)

Concentrated water samples were subjected to viral RNA extraction, reverse transcription then qPCR using the unrevealed florescent dye read on SYBR/FAM channel (Luna, New England) and in-house designed primers (Table 1). Reaction mixture was subjected to the following temperature conditions, Initial denaturation: 95°C for 60 sec., denaturation at 95°C for 15 sec., annealing: 57°C for 10 sec, extension at 60°C for 15 sec. for 40 cycles [17].

3.Results

3.1.SARS-CoV-2 cRT-PCR

Visualization of the SARS-CoV-2 RNA cRT-PCR amplification products corresponding to concentrated samples by the 4 different used protocols post electrophoresis on agarose gel (Figure 1a) and % band intensity corresponding to each amplification product as deduced by the UVP VisionWorks LS image analysis software (Figure 1b) compared to positive control. Results revealed that, for distilled water, method A gave the sharpest band and highest % band intensity compared to positive control indicating its the highest recovery rate. This was followed by method B then C and method D came at the end with the lowest recovery rate. Regarding viral RNA recovery from wastewater, both methods B and A gave the best nearly equal recovery followed by method D, whereas, method C gave zero recovery as demonstrated by absence of any amplification products.

3.2.229E-HCoV cRT-PCR

Visualization of the 229E-HCoV cRT-PCR amplification products corresponding to concentrated samples by the 4 different used protocols post electrophoresis on agarose gel (Figure 2a) and % band intensity corresponding to each amplification product as deduced by the UVP VisionWorks LS image analysis software (Figure 2b) for compared to positive control. Results showed that, For distilled water, Method C gave sharpest band and highest % of band intensity compared to positive control which indicates highest recovery rate, this was followed by method A then B and comes at the end method D with lowest recovery rate. For viral recovery from waste water Method B then method D showed nearly equal results but method A and C showed no band.

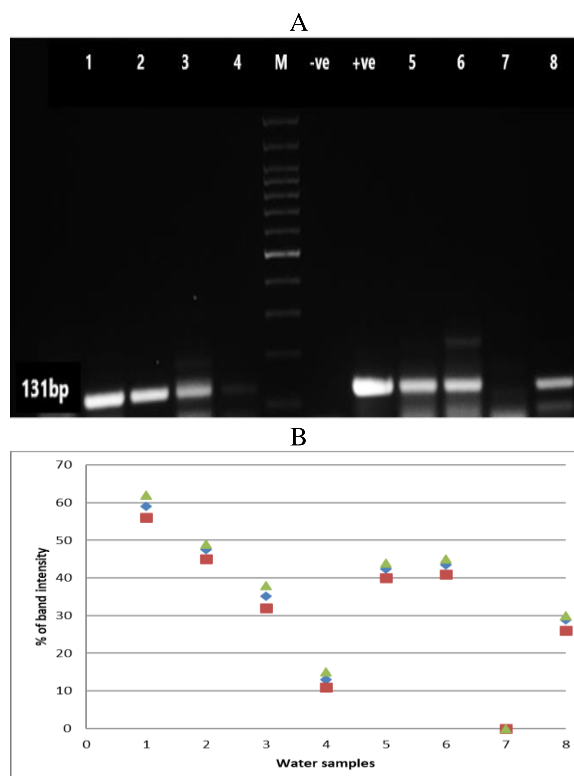

Figure 1: Semi-quantitative transformation of the visualized SARS-Co-V2 cRT-PCR products on agarose gel corresponding to recovered viral RNA by each of the used four water concentration protocols into % band intensity by the UVP VisionWorks LS image analysis version:8.16.16089.9066 software. Results of Polymerase chain reaction to detect RNA of SARS-Co-V2, A: electrophoresis agarose gel, where 100 DNA ladder is used and the one with higher intensity is equivalent to 500bP, B: % of band intensity and dots represent three readings from three independent experiments, where M: DNA ladder, -Ve: negative control, +ve: positive control, 1:Dist.Water/method A, 2: Dist.Water/B, 3: Dist.Water/C, 4: Dist.Water/D, 5: Waste water/A, 6: Waste water/B, 7: Waste water/C, 8: Waste water/D.

Table 1: Sequence of primers used for detection of SARS-CoV-2 and 229E-HCoV.

Virus	Forward	Product seize	Test used
SARS-CoV-2.	F:AATTACCCCCTGCATACACTAA R:ATGGAACCAAGTAACATTGGAAA	131bp	cRT-PCR and qRT-PCR
229E-HCoV –short.	F:TACCCATCAACAAGAAAGACAAA R:TGGGTGACAAATCCACCCGT	98bp	qRT-PCR
229E-HCoV.	F:TCTGAACCACAACGTGGTCG R:TGTGGTATCTCTGGTTCTGAAT	356bp	cRT-PCR

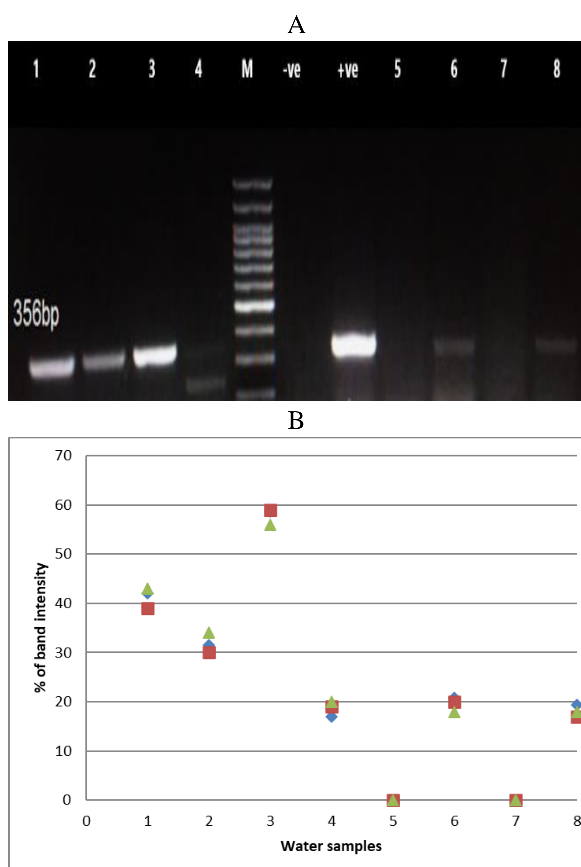

Figure 2: Semi-quantitative transformation of the visualized 229E-HCoV cRT-PCR products on agarose gel corresponding to recovered viral RNA by each of the used four water concentration protocols into % band intensity by the UVP VisionWorks LS image analysis version:8.16.16089.9066 software. Results of Polymerase chain reaction to detect RNA of 229E-HCoV, A: electrophoresis agarose gel, where 100 DNA ladder is used and the one with higher intensity is equivalent to 500bP,B: % of band intensity and dots represent three readings from three independent experiments, where M: DNA ladder, -Ve: negative control, +ve: positive control, 1:Dist.Water/method A, 2: Dist.Water/B, 3: Dist.Water/C, 4: Dist.Water/D, 5: Waste water/A, 6: Waste water/B, 7: Waste water/C, 8: Waste water/D.

3.3.SARS-CoV-2 real time qRT-PCR

Cycle thresholds (Ct), the number of cycles at which the linear amplification occurs, corresponding to SYBR green quantitative detection of SARS-CoV-2 amplicons recovered from fresh and wastewater using the four used water concentration protocols compared to positive control are presented in Figure 3A&B. In case of distilled (fresh) water results showed that method A gave the best recovery of the viral RNA as demonstrated by the least Ct value compared to the positive followed by method B then C and D which nearly gave the same Ct values.
As for the waste water, method B gave the highest recovery rate followed by method A with little difference in recovery rate then came method D then method C, respectively.

According to these results method A is the best for recovering SARS-CoV-2 RNA from distilled water and Method B was the best in case of waste water .
Melting curve shown in Figure 3C confirmed Single target amplification.

3.4.229E-HCoV real time qRT-PCR

Cycle thresholds (Ct), the number of cycles at which the linear amplification occurs, corresponding to SYBR green quantitative detection of 229E-HCoV amplicons recovered from fresh and wastewater using the four used water concentration protocols compared to positive control methods suggested that method A then C then B gave very close recovery rate then comes method D.

But for waste water results were different as method B showed highest recovery rate followed by method D with slight difference then comes method A then method C.

So for 229 E-HCoV in distilled water method A showed best results and method B was the best for viral isolation from wastewater (Figure 4A&B).

Single target amplification was shown in melting curve in Figure 4C.

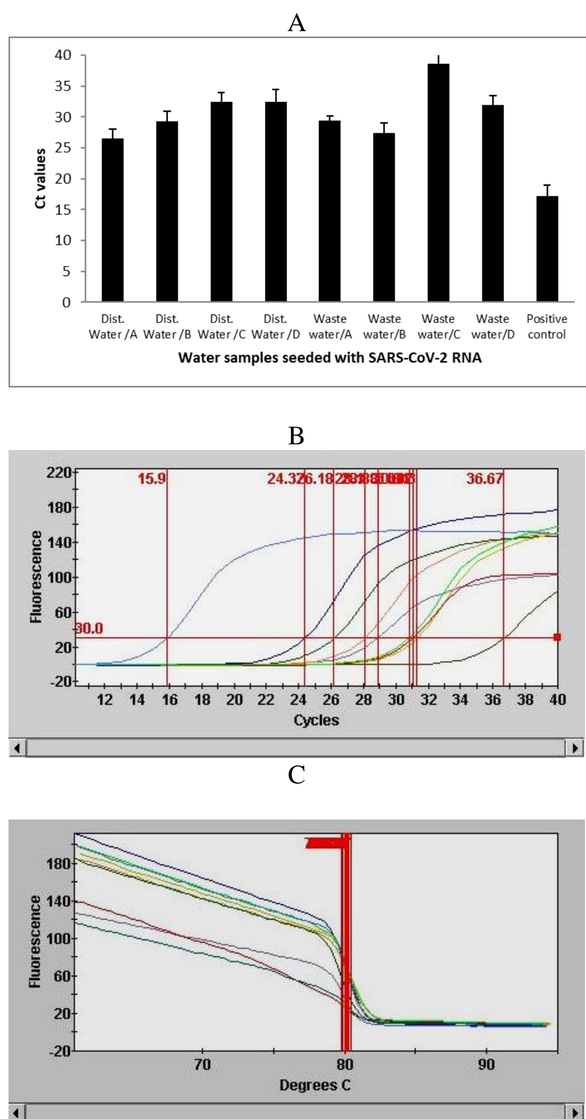

Figure 3: A:Cycle thresholds (Ct), the number of cycles at which the linear amplification occurs, B:Amplification curve and C: melting curve corresponding to quantitative detection of SARS-CoV-2 amplicons recovered from fresh and wastewater using the four used water concentration protocols compared to positive control.

4.Discussion

Since the beginning of the novel coronavirus pandemic reports speculated about possible virus shed in various human excretory/secretory products including stool, vomiting, nasal discharge and sputum [3] which more likely end in the drain. Thus scientist started to think of using the level of the detected SARS-CoV-2 (or its RNA) in wastewater to guess (approximately estimate) the number of infected humans in a given district [6].

However, scientists need to test all available wastewater concentration methods to enhance the sensitivity of detecting SARS-CoV-2 in wastewater, specially that reports showed that only the viral RNA and not the whole virus is found in wastewater [14-15].

Here we used both conventional and quantitative real time RT-PCR (qRT-PCR) as readouts to compare efficiencies of 4 water concentration methods namely, A) filtration on negatively charged membrane followed by extracting RNA from it, B) adsorption-elution method, C) flocculation with skimmed milk and D) polyethylene glycol precipitation to enhance sensitivity of detecting genetic material of both the SARS-CoV-2 and 229E-HCoV as a model for spike-containing enveloped viruses both from fresh and waste water.

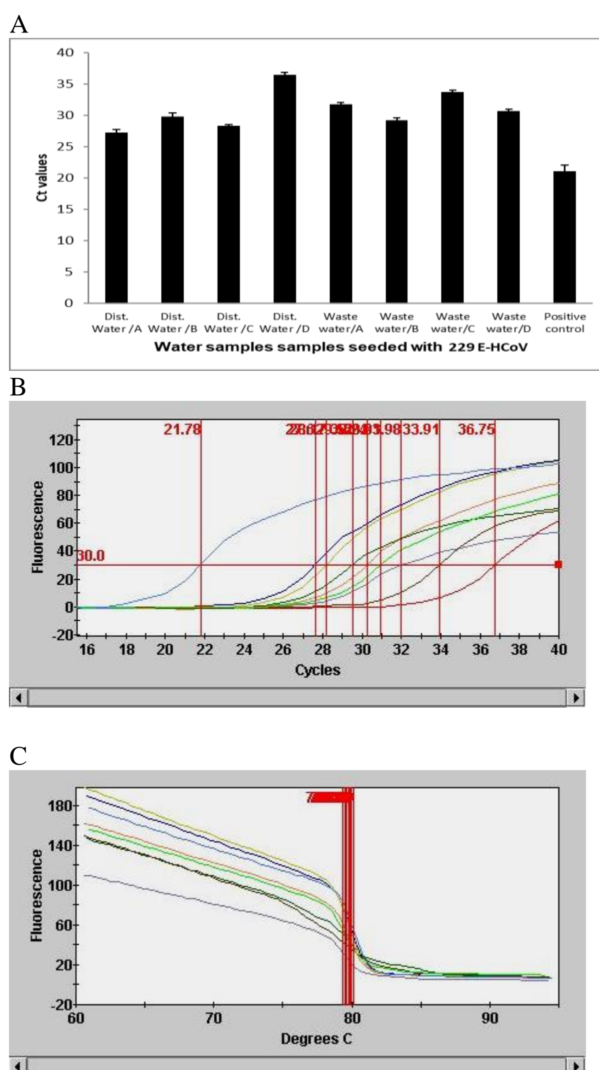

Figure 4: A:Cycle thresholds (Ct), the number of cycles at which the linear amplification occurs, B:Amplification curve and C: melting curve corresponding to SYBR green quantitative detection of 229E-HCoV amplicons recovered from fresh and wastewater using the four used water concentration protocols compared to positive control.

Results showed ability of real time qRT-PCR to detect both the genetic material of SARS-CoV-2 and 229E-HCoV in fresh and wastewater with different Ct values but recovered RNA from the four used concentration protocols were all within the detection limits. Unlikely, cRT-PCR was not sensitive enough to detect recovered SARS-CoV-2 RNA for wastewater samples by the concentration method C were no amplification products were visualized on the agarose gel and similar recovery and detection limitations were recorded upon using cRT-PCR to detect recovered RNA of both viruses by methods A and C.

Generally, we can say that methods A and B can be used with high % recovery of both SARS-CoV-2 RNA and whole 229E-HCoV viral particle, however method B gave better results as on cutting nitrocellulose into pieces and put it in lysis buffer as done with method A, this might cause RNA or viral particle to be entangled (lost) between membrane pieces. So on using conventional PCR or real time PCR method B is recommended as it gave the best recovery of both SARS-CoV-2 RNA and whole 229E-HCoV when compared to other methods and this agreed with Pino et al [16].

Method C can be used for RNA and viral isolation from fresh but not wastewater which might be due to competition (interference) between milk particles and present particles in wastewater to adsorb RNA or viral particles carrying negative charge in acidic pH.

Although it gave different band intensities or Ct value in each test, method D comes second in the recommendation as both cRT-PCR and qRt-PCR results were within the detection limit. Also this method gave better results on isolating RNA or viral particle from wastewater than from fresh water. This might be due to the higher molecular weight protein content in wastewater which are not or rarely present in fresh water which will be precipitated by PEG. As the virus/RNA are more likely adsorbed on such high molecular weight proteins they will be co-precipitated by PEG [22] making the PEG preferable approach to recover corona virus/RNA from wastewater by many researchers [23] regardless of the limited sample volume used.

5.Conclusions

Since Viral particles or viral RNA are found diluted in wastewater so usage of wastewater concentration methods of higher recovery rates is very important in order not to miss any positive samples. on comparing four different wastewater concentration methods we found that for SARS-CoV-2 RNA or whole 229E-HCoV one can recommend adsorbtion-elution method for recovery from fresh water and adsorbtion-elution method then polyethylene glycol precipitation method for recovery from wastewater, also we can recommend qRT-PCR for detection.

List of abbreviations:

Dist.water/A: Distilled water spiked with RNA of SARS-Co-V2 or Full virus of human corona virus 229E then concentrated with method A.
Dist.water/B: Distilled water spiked with RNA of SARS-Co-V2 or Full virus of human corona virus 229E then concentrated with method B.
Dist.water/C: Distilled water spiked with RNA of SARS-Co-V2 or Full virus of human corona virus 229E then concentrated with method C.
Dist.water/D: Distilled water spiked with RNA of SARS-Co-V2 or Full virus of human corona virus 229E then concentrated with method D.
Wastewater/A: Wastewater spiked with RNA of SARS-Co-V2 or Full virus of human corona virus 229E then concentrated with method A.
Wastewater/B: Wastewater spiked with RNA of SARS-Co-V2 or Full virus of human corona virus 229E then concentrated with method B.
Wastewater/C: Wastewater spiked with RNA of SARS-Co-V2 or Full virus of human corona virus 229E then concentrated with method C.
Wastewater/D: Wastewater spiked with RNA of SARS-Co-V2 or Full virus of human corona virus 229E then concentrated with method D.

6. Conflict of interest:

All authors declare that they have no conflict of interest.

7.Funding:

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8.References:

1.Ahmed, W., Bivins, A., Simpson, S.L., Smith, W.J.M., Metcalfe, S., McMinn, B., Symonds, E.M., Korajkic, A.; Comparative analysis of rapid concentration methods for the recovery ofSARS-CoV-2 and quantification of human enteric viruses and a sewage associated marker gene in untreated wastewater. Sci Total Environ. 799:149386.2021. doi:10.1016/j.scitotenv.2021.149386.

2.Brian, D.A., Baric, R.S.; Coronavirus genome structure and replication. Curr.Topics Microbiol. Immunol. 287, 1–30. 2005.doi: 10.1007/3-540-26765-4_1.

3.Wang, W.,Xu, Y., Gao, R., Lu, R., Han, K., Wu, G., Wenjie, Tan, W.; Detection of SARS-CoV-2 in Different Types of Clinical Specimens.

JAMA. 323(18), 1843-1844. 2020.doi:10.1001/jama.2020.3786

4.Wölfel, R., Corman, V.M., Guggemos, W., Seilmaier, M., Zange, S., Mueller, M.A., Niemeyer, D., Vollmar, P., Rothe, C., Hoelscher, M., Bleicker, T., Bruenink, S., Schneider, J., Ehmann, R., Zwirglmaier, K., Drosten, C., Wendtner, C.; Virological assessment of hospitalized cases of coronavirus disease 2019. Nature. 581, 465–469.2020.doi: 10.1038/s41586-020-2196-x.

5. Lahrich, S., Laghrib, F., Farahi, A., Bakasse, M., Saqrane, S., El Mhammedi, M.A.; Review on the contamination of wastewater by COVID-19 virus: impact and treatment. Sci. Total Environ. 751, 142325.2021. https://doi.org/10.1016/j.scitotenv.2020.142325.

6.Medema, G., Heijnen, L., Elsinga, G., Italiaander, R., Brouwer, A.; Presence of SARSCoronavirus-2 RNA in sewage and correlation with reported COVID-19 prevalence in the early stage of the epidemic in the Netherlands. Environ. Sci. Technol. Lett.A-F.2020. doi: 10.1021/acs.estlett.0c00357.

7. Islam, A., Hossen, F., Rahman ,A., Sultana, K.F., Hasan, M.N., Haque, A., Sosa-Hernández, J.E., Oyervides-Muñoz, M.A., Parra-Saldívar, R., Ahmed, T., Islam, T., Dhama, K., Sangkham, S., Bahadur, N.M., Reza, H.M., Jakariya Al Marzan, A., Bhattacharya, P., Sonne, C., Ahmed, F.; An opinion on Wastewater-Based Epidemiological Monitoring (WBEM) with Clinical Diagnostic Test (CDT) for detecting high-prevalence areas of community COVID-19 Infections. Current Opinion in Environmental Science & Health,2022.https://doi.org/10.1016/j.coesh.2022.100396.

8.Sharif, S., Ikram, A., Khurshid, A., Salman, M., Mehmood, N., Arshad, Y., Ahmed, J., Safdar, R.M., Rehman, L., Mujtaba, G., Hussain, J., Ali, J., Angez, M., Alam, M.M., Akthar, R., Malik, M.W., Baig, M.Z.I., Rana, M.S., Usman, M., Ali, M.Q., Ahad, A., Badar, N., Umair, M., Tamim, S., Ashraf, A., Tahir, F., Ali, N.; Detection of SARs-CoV-2 in wastewater using the existing environmental surveillance network: A potential supplementary system for monitoring COVID-19 transmission. PLOS ONE 16(6): e0249568. 2021.https://doi.org/10.1371/journal.pone.0249568.

9.Nag, A., Arora, S., Sinha, V., Meena, E., Sutaria, D., Gupta, A.B., Medicherl, K.M.; Monitoring of SARS-CoV-2 Variants by Wastewater-BasedSurveillance as a Sustainable and Pragmatic Approach—A Case Study of Jaipur (India). Water. 14, 297. 2022.https://doi.org/10.3390/w14030297.

10.Pérez-Cataluña, A., Cuevas-Ferrando,E.,Randazzo,W., Irene Falcó, I., Allende, A., Sánchez, G.; Comparing analytical methods to detect SARS-CoV-2 in wastewater. Science of the Total Environment. 758, 143870. 2021.doi: 10.1016/j.scitotenv.2020.143870.

11. Jafferali, M.H., Khatami, K., Atasoy, M., Birgersson, M.,Williams, C., Cetecioglu, Z.; Benchmarking virus concentration methods for quantification ofSARS-CoV-2 in raw wastewater. Science of the Total Environment 755 142939. 2021.doi.org/10.1016/j.scitotenv.2020.142939.

12. Ahmed, W., Bivins, A., Simpson, S.L., Metcalfe, S., McMinn, B., Symonds, E.M., Korajkie, A.; Comparative analysis of rapid concentration methods for the recovery of SARS-CoV-2 and quantification of human enteric viruses and a sewage-associated marker gene in untreated wastewater. Science of The Total Environment. 799: 149386. 2021. doi.org/10.1016/j.scitotenv.2021.149386.

13. Zheng, X., Deng, Y., Xu, X., Li, S., Zhang, Y., Ding, J., On, H.Y., Lai, J.C.C., Yau, C., Chin, A.W.H., Poon, L.L.M., Tun, H.M., Zhang, T.; Comparison of virus concentration methods and RNA extraction methods for SARS-CoV-2 wastewater surveillance. Science of The Total Environment. 824: 153687. 2022. doi.org/10.1016/j.scitotenv.2022.153687

14. Rimoldi, S.G., Stefani, F., Gigantiello, A., Polesello, S., Comandatore, F., Mileto, D.,Maresca, M., Longobardi, C., Mancon, A., Romeri, F., Pagani, C., Cappelli, F., Rosci-oli, C., Moja, L., Gismondo, M.R., Salerno, F.; Presence and infectivity of SARS-CoV-2 virus in wastewaters and rivers. Sci. Total Environ. 744, 140911.2020. doi:10.1016/j.scitotenv.2020.140911.

15.Bivins, A., Greaves, J., Fischer, R., Yinda, K.C., Ahmed, W., Kitajima, M., Munster, V.J., Bibby,K.; Persistence of SARS-CoV-2 in Water and Wastewater. Environ. Sci. Technol. Lett. 7(12): 937–942. 2020.https://doi.org/10.1021/acs.estlett.0c00730.

16. Pino, N.J., Rodriguez, D.C., Laura Castrillón Cano, L.C., Rodriguez, A.; Detection of SARS-CoV-2 in wastewater is influenced by sampling time, concentration method, and target analyzed. Journal of Water and Health. 19 (5): 775. 2021.doi: 10.2166/wh.2021.133.

17. McQuaig, S.M., Scott, T.M., Lukasik, J.O., Paul, J.H., Harwood, V.J.; Quantification of human polyomaviruses JC virus and BK virus byTaqMan quantitative PCR and comparison to other water quality indicatorsin water and fecal samples. Appl Environ Microbiol. 75:3379–

3388.2009.http://dx.doi.org/10.1128/AEM.02302-08.

18. Katayama, H., Shimasaki, A., Ohgaki, S.; Development of a virus concentration method and its application to detection of enterovirus and norwalk virus from coastal seawater. Applied and Environmental Microbiology, 68, 1033–1039. 2002.doi: 10.1128/AEM.68.3.1033-1039.2002.
19. Bofill-Mas, S., Hundesa, A., Calgua, B., Rusinol,M., Maluquer de Motes, C., Girones, R.;Cost-effective method for microbial source tracking using specific human and animal viruses. J. Vis.Exp. 3(58), 2820. 2011. http://dx.doi.org/10. 3791/2820.
20. Farkas, K. , Hillary, L.S., Thorpe, J., Walker, D.I., Lowther,J.A. ,McDonald, J.E., Malham,S.k., Jones, D.L.;Concentration and Quantification of SARS-CoV-2 RNA in Wastewater Using Polyethylene Glycol-Based Concentrationand qRT-PCR. Methods Protoc.4, 17.2021. https://doi.org/10.3390/mps4010017.
21. Buston, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T.; The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem., 55 (4): 611-622.2009. doi: 10.1373/clinchem.2008.112797.
22. Sim, S., He, T., Tscheliessnig, A., Mueller, M., Tan, R.B.H., Jungbauer, A.;Protein precipitation by polyethylene glycol: a generalized model based on hydrodynamic radius. J Biotechnol. 20;157(2):315-9. 2012.doi:10.1016/j.jbiotec.2011.09.028.
23. Farkas, K. , Hillary, L.S., Thorpe, J., Walker, D.I., Lowther,J.A. ,McDonald, J.E., Malham,S.k., Jones, D.L.; Concentration and Quantification of SARS-CoV-2 RNA in Wastewater Using Polyethylene Glycol-Based ConcentrationandqRT-PCR.2021. MethodsProtoc.4: 17.https://doi.org/10.3390/mps4010017.