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ORIGINAL ARTICLE - PUBLIC HEALTH

UTILITY OF SKIMMED MILK FLOCCULATION TECHNIQUE FOR ROUTINE SURVEILLANCE OF SARS COV-2 IN WASTEWATER SAMPLES -PRELIMINARY FINDINGS FROM A LONGITUDINAL STUDY

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Abstract

INTRODUCTION : Ongoing pandemic of coronavirus disease (COVID-19) caused by a new beta coronavirus named Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has warranted a global responsibility for its prevention and control. The use of an environmental approach called wastewater-based epidemiology (WBE) for virus detection has proved to be an additional tool to support COVID-19 prevention activities. Among the several methods available, skimmed milk flocculation method has proven to be simple and effective in low-resource settings.

METHODS : In the study period between 11 July – 12 September 2022, 227 wastewater samples from 40 different samplings sites from 4 zones of Trichy City were analyzed using the Skimmed milk flocculation technique. The isolation and detection of SARS COV-2 in wastewater were done by the COSARA kit using Open System real-time PCR.

RESULTS : Out of the 227 samples,92 (40.5%) tested positive for SARS COV-2 virus RNA. Mean Ct values for the RdRp gene were 35.5 and 34.2 for the E gene. Samples collected from all 4 zones and 37 (92.5%) wards showed positivity. There was no significant difference observed between the SARS Cov-2 waste water positivity (p=0.6) and mean Ct values of RdRp (p=0.9) and E Gene(p=0.1) between different zones of the city.

CONCLUSION : Skimmed milk flocculation technique used in the present study had advantages like yielding faster results, ease of use, and non-requirement of robust laboratory infrastructure which matched well with its use in other settings further validating its utility in low resource settings and as a routine surveillance measure.

KEY WORDS : Surveillance, wastewater surveillance, SARS-CoV-2, COVID-19, Wastewater-Based Epidemiological Monitoring, Skimmed milk flocculation.

INTRODUCTION

A global pandemic of coronavirus disease (COVID-19) caused by a new beta coronavirus named Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is currently ongoing. The outbreak, first detected in Wuhan (China) in December 2019 spread rapidly to 213 countries/ territories causing massive loss of life, economies, and livelihoods.[1] Globally, till January 11 2023, there have been 660,378,145 confirmed cases of COVID-19, including 6,691,495 deaths, reported to WHO.[2]

Though the respiratory route has been the primary mode transmission of SARS-CoV-2 infections, multiple studies have demonstrated high levels of viral RNA in stools of infected patients, more so in children giving rise to possibilities of even a faeco–oral mode of transmission.[3,4,5]

This faecal shedding of the virus was quantified since the start of the COVID-19 pandemic through quantitative Polymerase Chain Reaction (RT qPCR) as a routine public health surveillance measure in various countries.[6,7] Detection of SARS-CoV-2 in faeces and early shedding demonstrated by virus detection in faeces has resulted in the use of an environmental approach called wastewater-based epidemiology (WBE), an additional tool to support COVID-19 prevention and control actions in several regions.[7]

In the context of COVID-19, an effective public health surveillance can be defined as a process of, systematic, continuous, collection, analysis, and interpretation of relevant epidemiological data which could predict rise of COVID-19 cases. An effective disease surveillance system is thus imperative to detect impending disease outbreaks before they spread and cause significant morbidity and mortality lives.[8,9,10]

Though an integral part of surveillance systems, wastewater surveillance (WWS) is less explored and tracked by authorities.[11,12] In a study conducted in the US, the wastewater SARS COV-2 viral titres were consistent and appeared to

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precede, clinical COVID-19 surveillance indicators and daily new cases.[13] Several studies have shown that SARS-COV 2 is detectable in wastewater 3 weeks before an impending outbreak.[13-17] This presence of SARS-COV-2 in wastewater could be exploited for routine environmental surveillance by public health authorities. This routine surveillance could not only help predict an upcoming COVID wave but also prove a useful tool for monitoring SARS CoV-2 virus elimination in the future.

The strength of the wastewater surveillance technique could depend upon various factors predominantly, the sensitivity and specificity of the concentration method, the sample type, the sample volume, sample processing time, and the availability of cost-effective laboratory equipment.[18]

The Centres for Disease Control (CDC) has evaluated that various sample concentrations methods like ultrafiltration, filtration through an electronegative membrane with sample pre-treatment by addition of $MgCl_2$ or acidification, polyethylene glycol (PEG) precipitation, and Skim milk flocculation technique can yield the detection SARS COV-2 in wastewater.[18]

In a comparative study of various wastewater sample concentration methods like the bag-mediated filtration system (BMFS), polyethylene glycol (PEG) precipitation, ultrafiltration, and sludge extraction, the Skimmed milk flocculation extraction method showed consistent results over time and between various treatment places.[19]

Here in this communication, we attempt to elucidate the process of skimmed milk flocculation method for wastewater SARS-COV-2 (WW SARS-CoV-2) extraction that can be used in public health laboratories for routine environmental surveillance.

SUBJECTS AND METHODS :

The present communication is part of an ongoing study on the development of an early warning system for COVID-19. Below we describe the methods of only the wastewater sample collection method and analysis using Skimmed milk flocculation technique.

1. PROCEDURE FOR SAMPLE COLLECTION :

In the study period between 11 July 2022 – 12 September 2022, a total of 227 waste samples water samples were collected. Every week 42 samples were collected from a total of 41 different sites in the city, 40 from the ward outlets and 2 from the sewage treatment plant (STP). The 40 different sites for wastewater sample collection were selected from sewage outlets of the specific ward after consultation with the sanitary inspector of the ward.

Samples were collected 4 days a week ,1 sample each from every ward =10 samples /day .So from 4 days ,data collection 40 samples were collected from the wards outlets and 2 samples were collected in the week from Sewage treatment plant

The sampling points were selected such that they would be at the confluence of major sewer outlets representing the outflow of the particular ward (Insert Figure 1) The sample collection for every zone /ward was done on a particular day to confer uniformity in the collection of the sample and reporting of results. Wastewater samples were collected during the early morning hours between 7 am – 10 am, as this time represents the maximum sewer outflow.

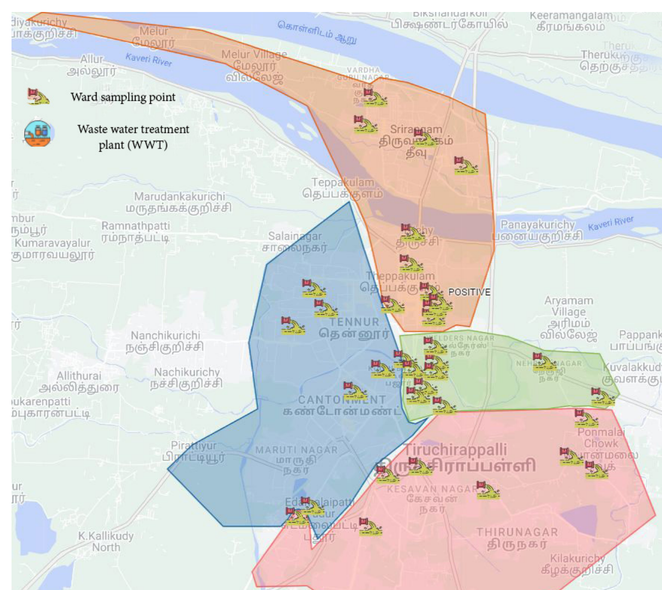

Figure 1: Sampling points over the city

A health worker was trained in the method of wastewater sample collection under the guidelines laid down by the Central pollution control board of India.[20] In the present study, the investigator used the manual sampling method for wastewater sample collection. One litre grab sample of sewage was collected by lowering a bucket in the flowing sewage water. The investigator used multiple grabs (3 times) to increase the yield of sample.[21] Of the 1 litre grab, the health worker collected 200 ml of wastewater sample under universal safety precautions in a wide-mouth polypropylene bottle and transported it to the laboratory at a temperature of <4oC, to ensure virus viability.[21] The investigator ensured that the samples reached the laboratory within 2 hours of the first collected sample.18 Materials used for sample collection were sterilized using an autoclave in the laboratory.

2. SAMPLE PROCESSING FOR VIRAL CONCENTRATION :

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As the concentration of SARS-CoV-2 from wastewater is a bioaerosol-generating process, the procedure was carried out in the Biosafety Level 2 (BSL2) facility with unidirectional airflow. All BSL-2 precautions, including respiratory protection and a designated area to don on and don off personal protective equipment, were followed. The laboratory waste from wastewater samples that may contain SARS-CoV-2 was autoclaved and managed by BSL2 biosafety guidelines.[18]

In the present study, the following steps were followed for estimating the presence of SARS COV-2 in wastewater samples using the skimmed milk flocculation method (Insert Figure 2 here). [22]

Step 1. Pretreatment of wastewater sample and reagent preparation.

a. Reagent Preparation of 5% pre-flocculated Skim Milk:

i. To make a 5% pre-flocculated skim milk solution (w/v), 5 g skimmed milk powder was dissolved in 100 ml MilliQ water.

ii. The solution was autoclaved for 15 minutes at 115°C, 18 psi, and then allowed to cool at room temperature.

iii. The prepared skim milk solution was stored at 4°C for up to three days.

b. Pre-treatment:

a. For the virus concentration assay, a total of 200 mL of wastewater was collected, and divided into two aliquots (100 mL).

b. To each 100 mL sample, 1 mL 5% pre-flocculated skim milk was added and the pH was adjusted to 3.5-4.0 by the addition of 1 M HCl.

c. The samples were placed on a horizontal shaker and incubated at 200 rpm for 2 hours at room temperature to allow the floccules to form.

d. After incubation, the floccules were allowed to sediment by centrifugation at 3500 x g for 30 min at 4°C using a swinging bucket rotor.

e. The supernatant was removed carefully from the tube without disturbing the bottom and the pelleted virus suspend with buffer.

f. 1 set of pellets was used for RNA extraction using the QIAamp viral RNA mini kit by suspending it with the lysis buffer provided in the kit.

g. Another aliquot was dissolved in 1.5 mL of sterile 1x PBS (pH 7.4) and vortex both suspensions for 5-10 minutes at maximum speed to completely dissolve the pellet.

h. The final viral concentrates were stored at -80 °C until further processing.

Step 2. RNA Extraction

a. Isolation and Detection of SARS-COV-2.

The isolation and detection of SARS COV-2 in wastewater samples were done by the COSARA kit using Open System Real-time PCR. The detection is based on the amplification of the RdRp and E gene (RNA-dependent RNA polymerase enzyme, and Envelope gene) sequence and the measurement of fluorescence increase. The mechanism of duplex targeting ensures maximum sensitivity and specificity and enables the detection of the virus in a sample before seroconversion. The presence of SARS-COV-2 is indicated by the increased FAM fluorophore fluorescence. An internal Control (IC), which is a part of the PCR kit, is used as a control for the whole diagnostic process, i.e. RNA extraction efficiency, reverse-transcription step efficiency (transcription of RNA into cDNA), and PCR amplification efficiency (PCR inhibition). The IC positive amplification is detected in the Cal Red fluorophore fluorescence channel. The PCR kit is designed for in-vitro diagnostics for both qualitative and quantitative detection and it utilizes the "hot start" technology minimizing non-specific reactions and ensuring maximum sensitivity. Ready to Use Master Mix contains uracil-DNA-glycosylase (UDG) which eliminates possible contamination of the PCR with amplification products.

b. Viral RNA Extraction.

140µl of the sample was collected in a 2ml Collection tube and 560µl of Carrier RNA – Lysis Solution (HRL) was added to the cell-free sample. It was incubated for 10 minutes at room temperature (15-25°C). These samples were Centrifuged for 10 seconds to remove any droplets formed inside the cap of the collection tubes. 560µl of ethanol (96-100%) was added to the sample and mixed well by gentle pipetting. The samples were then centrifuged for 10 seconds to remove any droplets formed inside the cap of the collection tubes. The lysate thus obtained was transferred onto the Hi Elute Miniprep spin column. It was then Centrifuged at 8000 rpm for 1 minute. The flow-through was discarded after the spin. The previous step was repeated with the remaining sample. First, wash -Add 500 µl of wash (WS) solution, and centrifuge at 8000rpm for 1 minute. Discard the flow-through. Reuse the collection tube

Second Wash – Add another 500µl of wash solution (WS) onto the column. Close the tube gently and centrifuge for 3 minutes at 14000 rpm to wash the column. Discard the flow-through. Reuse the collection tube, and centrifuge for 1 minute at 14000 rpm to dry the membrane. Discard the flow-through. Reuse the collection tube, and centrifuge for 1 minute at 14000 rpm to dry the membrane. Close the tube gently and centrifuge for 1 min at 8000rpm. Transfer the elute to a new capped 1.5ml collection tube for long-term storage.

Storage of the elute with purified RNA:

The elute contains purified RNA, and it is recommended to be stored at a lower temperature (-80° C). Utmost care was taken to avoid repeated freezing and thawing of the sample which may cause denaturing of RNA.22

3. Step 3. RNA amplification SOP – PCR (COSARA).

a. Master Mix:

Master Mix was thawed on a frozen tray/ Ice Vortex and centrifuged for 3 seconds; it was then placed on a frozen tray/ Ice. PCR tubes were placed on the frozen tray for carrying out a reaction. Aliquot 5µL of Master Mix into desired wells. Tighten the strip tube caps and transfer them to the Template addition passage.

b. Template addition:

Vortex and centrifuge purified RNA for a few seconds. Add 5µL of Purified RNA sample to each well using a new tip between each sample. Thaw Positive control on ice. Vortex and centrifuge Positive control for a few seconds. Add 5µL of Nuclease-free water (NTC) in the appropriate well. Add 5 µL of Positive control to the appropriate well. Place caps on the PCR tubes and ensure proper fitting.

The Reaction tubes were loaded in a real-time PCR machine and the run was started.

Table 1 : Thermal Cycling set up.

Temperature	Time	Cycles	Capture
45°C	15 min	Hold	N/A
95°C	2 min	Hold	N/A
95°C	03 sec	45	N/A
55°C	32 sec		Green (FAM), Yellow (Cal Fluor 560), Orange (Cal Fluor Red 610), and Red.

Ensure that the Run file is saved and select Run. Label the good position according to wells loaded in Real-time PCR. If controls pass, then interpret the sample results. If controls fail, then the run is invalid, and all should be repeated.

Table 2 : Interpretation of Results.

RdRp Gene	E Gene	Internal Control	Result
No Amplification	No Amplification	Amplification	Negative
No Amplification	Amplification	Amplification	Positive
Amplification	Amplification	Amplification	Positive
Amplification	No Amplification	Amplification	Positive
Any result	Any result	No Amplification*	Invalid

*Repeat extraction, the absence of IC amplification indicates that there is no nucleic acid material in the reaction. The results will be interpreted as the presence or absence of SARS COV-2 RNA with Ct values.

In our surveillance system, we found skimmed milk flocculation useful as it can be easily used in low-resource settings, and yields faster results.

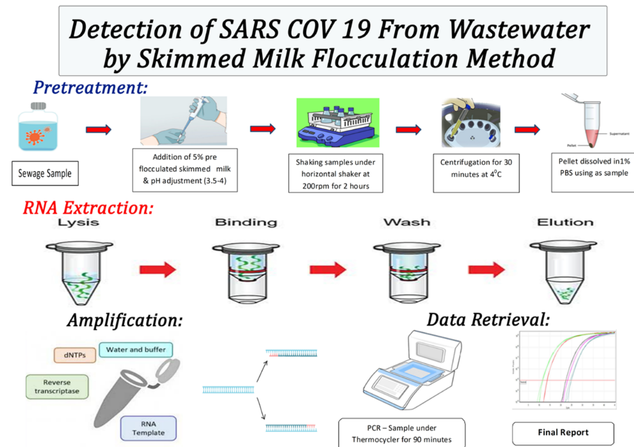

Figure 2 : Procedure of Skimmed Milk Flocculation technique

Data management and statistical analysis.

The data regarding SARS-COV-2 positivity was shared by the Laboratory technician in an application (app) developed by the investigator for the surveillance named WAP. The app generated a unique ID. Following the testing of samples, the sample data with Unique ID regarding sample positivity and Ct values for the RdRp gene and E gene were entered in the app. The data entered in the app was accessed by the investigator on a dashboard constructed for surveillance purposes. A line list of all the sample data was accessed from this dashboard and summarized. The data obtained were analyzed using SPSS v21. According to the manufactures instruction (COSARA -Saragene), a Ct value < 40 was considered positive. Means and standard deviation were calculated for Ct values of positive samples. Correlation statistics (r) were used to find out the relationship between daily wastewater SARS-COV-2 sample positivity and daily COVID-19 reported cases. The chi-square test was used to find the significant difference between the wastewater SARS CoV-2 positivity between the four zones. ANOVA statistics were used to find out the significant difference between CT values of RDRP and E gene between all 4 zones.

RESULTS

A total of 227 wastewater samples were collected and analyzed from the 40 wards during the study period. Out of the 227 samples,94 samples (40.5%) tested positive for SARS COV-2 virus RNA. (Insert Table 3 here). (Insert Fig 3 here). Mean Ct values for the RdRp gene were 35.5 with an (sd=2.0, maximum = 40 and minimum =28) and for the E gene 34.2 (sd=1.5, maximum 37 and minimum =31.)

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Samples collected from all 4 zones and 37 (92.5%) out of the 40 wards showed SARS-COV2 positivity. There was no significant difference observed between the wastewater SARS Cov-2 positivity among different zones (X2, df=3, p=0.60.

Table 3 : SARS COV-2 wastewater sample positivity among different zones.

Zone	Negative	Positivity	Total samples analysed	Positivity (%)	P value
Zone 4	29	25	54	46	0.6
Zone 3	30	15	45	33	
Zone 2	31	23	54	43	
Zone 1	45	29	74	39	
Total	135	92	227	41	

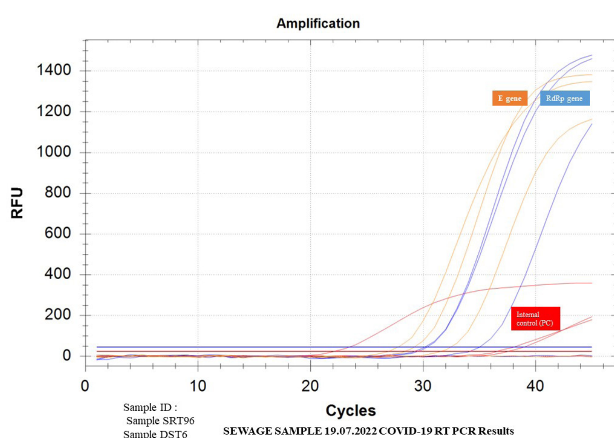

Figure 3 : Gene Amplification Results

ANOVA suggests that there were no significant differences observed in the mean Ct values of the RdRp gene (F3, 90 = 9.24, p =0.9) and E gene (F3, 30 = 2.06, p =0.1) between the various zones of the city.

Table 4 : Comparison of CT values of RdRP gene and E gene between different zones.

Ct Values	Zone	N	Mean	Std. Deviation	Tests of homogeneity of Variances		ANOVA	
					Levenes statistics	Sig.	F	Sig.
Ct-RdRP Gene	Zone 1	29	35.5	2.5	1.945	0.123	9.24	0.9
	Zone 2	23	35.7	1.8				
	Zone 3	16	35.3	2.7				
	Zone 4	26	35.7	1.3				
	Total	94	35.5	2.1				
Ct -E gene	Zone 1	14	34.1	1.9	5.036	0.006	2.06	0.13
	Zone 2	8	33.4	0.9				
	Zone 3	6	35.0	0.9				
	Zone 4	6	35.0	1.3				
	Total	34	34.2	1.5				

The COVID-19 cases and the wastewater SARS COV-2 showed a significant positive correlation (r= 0.86, p<0.01). (Insert Figure 4 and Figure 5 here).

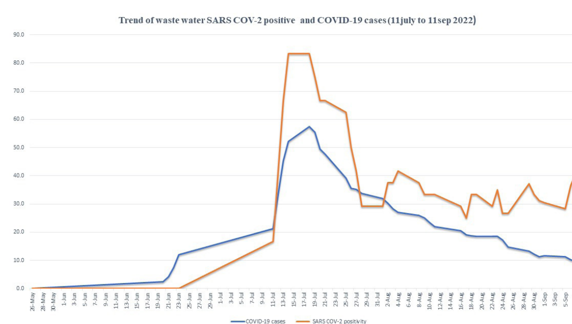

Figure 4 : Trend of Waste water SARS COV-2 and COVID-19 cases.

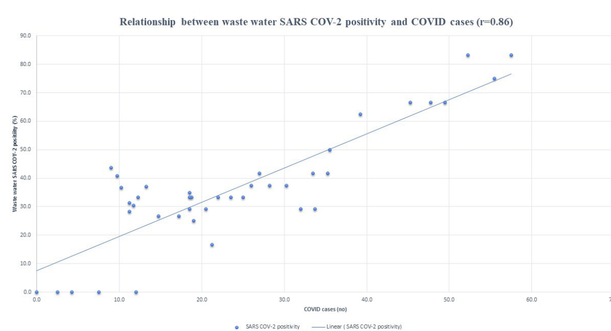

Figure 5 : Relationship of waste water SARS COV-2 and COVID-19 cases.

DISCUSSION

The present discussion regarding the standardization of skimmed milk flocculation (SMF) technique used in the present study has to be viewed in the context of the limited availability of comparable evidence in global and Indian settings as a routine surveillance method. An epidemiological approach to monitoring wastewater systems may be relevant for the early detection, which allows public health administrators to respond on time and eliminate a potential catastrophe.[23] Monitoring of centralized wastewater thus allows the detection of intentional, natural, or accidental contamination events.

The direct examination of wastewater for SARS COV-2 is difficult due to the low and fluctuating nature of concentrations of the organisms. Concentration procedures are usually organism and/or matrix specific and most techniques employed have high or unknown variability parameters.18 Alternative methods of SARS-COV-2 detection have been tried out globally.

Javier Martin et al in a study in South-East England isolated SARS-CoV-2 RNA in wastewater samples using a novel nested RT-PCR.[1] Diana M. Toledo et al her study

in Northern New England using reverse transcriptase-quantitative PCR (RT-qPCR) and reverse transcriptase-droplet digital PCR (RTddPCR) detection methods, detected SARS-CoV-2 RNA in samples from all 9 municipalities tested, including cities and small towns within this region, and showed wastewater positivity as an early indicator of active case count increases.[17] Both the above methods require robust laboratory infrastructure and seem unsuitable for routine environmental surveillance.

In a study conducted by Sharma DK et al in Mumbai, a two-phase PEG-dextran method was effectively used to concentrate SARS-CoV-2 from domestic wastewater. Viral RNA was detected in sewage samples collected during the ongoing COVID-19 pandemic in all six locations.[21] In this method, the phase separation takes around 16 hrs making it more time-consuming and laborious and thus less suitable for day-to-day surveillance in low resource settings.[21]

Of all the methods described by the Centre for Disease Control, studies had concluded that the skimmed milk flocculation (SMF) technique is an efficient method to concentrate viruses in all types of environmental water matrices such as river water, seawater, groundwater, and wastewater.[19,22,24,25]

Sarah E. Philo et al in her study compared multiple methods for concentration and recovery of SARS-CoV-2, concluding that skimmed milk flocculation without vertrel extraction performed consistently over time and between different treatment places.[19] Further, it was observed that the skimmed milk flocculation technique is easy, less time-consuming; and can be carried out in low-resource settings with minimal laboratory infrastructure.[19]

A study conducted by the same author Sarah E Philo for validation of the skimmed milk pellet extraction protocol for SARS-CoV-2 wastewater Surveillance concluded that the method reduced time spent per sample and more samples could be run at a single time making it a viable method for quick turnaround of wastewater data for public health interventions.[25]

ADVANTAGES OF SKIMMED MILK FLOCCULATION TECHNIQUE OVER OTHER METHODS

In the present study, the investigator emulated the method developed by Sarah E Philo for wastewater SARS COv-2 surveillance.[25] There was consistency in the positive reporting of samples from various zones and wards of the city. There was no significant difference observed in the wastewater positivity of samples in different zones, which proves that the results were valid and consistent. As the role of any early warning system is to be timely, in our study we observed that the SARS COV-2 extraction by skimmed milked protocol is simple, less time-consuming, and doesn't need any costly laboratory equipment.

In the present study, we analyzed a total of 227 samples for 2 months duration,6 samples per day with a mean extraction time of approximately 8 hrs. (sample processing started at 8 am and results were out by 4 pm). This fast processing of samples helped us in the daily reporting of results which contributed to the dynamic early warning COVID-19 surveillance system that further assisted health administrators for efficient decision-making.

CORRELATION OF WASTEWATER SARS COV-2 AND CLINICAL COVID-19 CASES

In the present study, we observed that the rise of waste water SARS CoV-2 positivity shows a positive linear relationship with the daily rise in COVID-19 cases daily. This finding is similar to the studies from various parts of the world which showed a strong positive correlation when comparing wastewater data to daily new clinical cases.[15-17,26]

CONCLUSION

The present study concludes that the skimmed milk flocculation technique for waste water SARS CoV-2 is easy, feasible, less resource intensive can be used for daily environmental COVID-19 surveillance by public health department.

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CONFLICT OF INTRESTS

All authors disclose that there are no conflicts of interest.

REFERENCES

1. Martin J, Klapsa D, Wilton T, Zambon M, Bentley E, Bujaki E, et al. Tracking SARS-CoV-2 in sewage: Evidence

of changes in virus variant predominance during COVID-19 pandemic. Viruses. 2020 Oct 1;12(10).

2. WHO WHO. https://covid19.who.int/ [Internet]. 2023. Available from: https://covid19.who.int/

3. Xu Y, Li X, Zhu B, Liang H, Fang C, Gong Y, et al. Characteristics of pediatric SARS-CoV-2 infection and potential evidence for persistent fecal viral shedding. Nat Med. 2020 Apr 1;26(4):502–5.

4. Zhang JC, Wang S Bin, Xue YD. Fecal specimen diagnosis 2019 novel coronavirus–infected pneumonia. J Med Virol. 2020;92(6):680–2.

5. Xing YH, Ni W, Wu Q, Li WJ, Li GJ, Wang W Di, et al. Prolonged viral shedding in feces of pediatric patients with coronavirus disease 2019. J Microbiol Immunol Infect [Internet]. 2020;53(3):473–80. Available from: https://doi.org/10.1016/j.jmii.2020.03.021

6. Bar-Or I, Weil M, Indenbaum V, Bucris E, Bar-Ilan D, Elul M, et al. Detection of SARS-CoV-2 variants by genomic analysis of wastewater samples in Israel. Sci Total Environ. 2021;789(January):1408.

7. Prado T, Fumian TM, Mannarino CF, Resende PC, Motta FC, Eppinghaus ALF, et al. Wastewater-based epidemiology is a useful tool to track SARS-CoV-2 and support public health policies at the municipal level in Brazil. Water Res. 2021;191(January).

8. CIHR - Institute of Population and Public Health. The Future of Public Health in Canada: Developing a Public Health System for the 21st Century The Ad Hoc Committee on the Future of Public Health in Canada. 2003;(June).

9. Choi BCK. The Past, Present, and Future of Public Health Surveillance. Hindawi Publ Corp. 2012;2012(Table 1):26.

10. World Health Organization. Surveillance in emergencies [Internet]. Available from: https://www.who.int/emergencies/surveillance#:~:text=An effective disease surveillance system, or following a natural disaster.

11. WHO WHO. Status of environmental surveillance for SARS-CoV-2 virus. Sci Br [Internet]. 2020;(August):1–4. Available from: https://www.who.int/publications/i/item/WHO-2019-nCoV-sci-brief-environmentalSampling-2020-1

12. Centers for Disease Control and Prevention. National Wastewater Surveillance System (NWSS) [Internet]. Available from: https://www.cdc.gov/nwss/

13. Wu F, Xiao A, Zhang J, Moniz K, Endo N, Armas F, et al. Wastewater surveillance of SARS-CoV-2 across 40 U.S. states from February to June 2020. Water Res. 2021;202(January).

14. Prado T, Fumian TM, Mannarino CF, Maranhão AG, Siqueira MM, Miagostovich MP. Preliminary results of SARS-CoV-2 detection in sewerage system in niterói municipality, Rio de Janeiro, Brazil. Mem Inst Oswaldo Cruz. 2020;115(7):1–3.

15. Saguti F, Magnil E, Enache L, Churqui MP, Johansson A, Lumley D, et al. Surveillance of wastewater revealed peaks of SARS-CoV-2 preceding those of hospitalized patients with COVID-19. Water Res [Internet]. 2021;189(January):116620. Available from: https://www.sciencedirect.com/science/article/pii/S0043135420311556?via%3Dihub

16. Castiglioni S, Schiarea S, Pellegrinelli L, Primache V, Galli C, Bubba L, et al. SARS-CoV-2 RNA in urban wastewater samples to monitor the COVID-19 pandemic in Lombardy, Italy (March–June 2020). Sci Total Environ. 2022;806(January).

17. Toledo DM, Robbins AA, Gallagher TL, Hershberger KC, Barney RE, Salmela SM, et al. Wastewater-Based SARS-CoV-2 Surveillance in Northern New England. Microbiol Spectr. 2022;10(2):1–14.

18. Centers for Disease Control and Prevention. Wastewater Surveillance Testing Methods [Internet]. Available from: https://www.cdc.gov/nwss/testing/index.html?CDC_AA_refVal=https%3A%2F%2Fwww.cdc.gov%2Fhealthywater%2Fsurveillance%2Fwastewater-surveillance%2Ftesting-methods.html.

19. Philo SE, Keim EK, Swanstrom R, Ong AQW, Burnor EA, Kossik AL, et al. A comparison of SARS-CoV-2 wastewater concentration methods for environmental surveillance. Sci Total Environ. 2021;760(December):144215.

20. India Central Pollution Control Board. P R Division. Guide Manual: Water and Wastewater Analysis. 2011;1–195.

21. Deepa Kailash Sharma, Uma Prajwal Nalavade, Kamlesh Kalgutkar NG&, Deshpande JM. SARS-CoV-2 detection in sewage samples: Standardization of method & preliminary observations. Indian J Med Res. 2021;153(January & February 2021):159–65.

22. Dilip Abraham, Venkata Raghava Mohan G. Skimmed Milk Flocculation Technique for Waste Water. 2021;(January). Available from: https://www.protocols.io/view/skimmed-milk-flocculation-technique-for-waste-wate-81wgb79wqvpk/v1

23. Sinclair RG, Choi CY, Riley MR, Gerba CP. Pathogen Surveillance Through Monitoring of Sewer Systems. Adv Appl Microbiol. 2008;65(January):249–69.

24. Yanaç K, Adegoke A, Wang L, Uyaguari M, Yuan Q. Detection of SARS-CoV-2 RNA throughout wastewater treatment plants and a modeling approach to understand COVID-19 infection dynamics in Winnipeg, Canada. Sci Total Environ. 2022;825(January).

25. Philo SE, Ong AQW, Keim EK, Swanstrom R, Kossik AL, Zhou NA, et al. Development and Validation of the Skimmed Milk Pellet Extraction Protocol for SARS-CoV-2 Wastewater Surveillance. Food Environ Virol [Internet]. 2022;14(4):355–63. Available from: https://doi.org/10.1007/s12560-022-09512-5

26. Wu F, Xiao A, Zhang J, Moniz K, Endo N, Armas F, et al. SARS-CoV-2 RNA concentrations in wastewater foreshadow dynamics and clinical presentation of new COVID-19 cases. Sci Total Environ. 2022;805(January).