A Novel Wastewater-Based Serological Approach for Detecting Anti-SARS-CoV-2 IgM Antibodies

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Abstract:

The emergence of Severe Acute Respiratory Syndrome - Coronavirus-2 (SARS-CoV-2) necessitate innovative surveillance strategies beyond conventional diagnostics. This study investigates the feasibility of wastewater-based epidemiology (WBE) for serological surveillance by detecting anti-SARS-CoV-2 membrane IgM antibodies using an optimized indirect enzyme-linked immunosorbent assay (ELISA) protocol. Wastewater samples (n = 10) from diverse localities across Nagpur city were processed using polyethylene glycol (PEG) precipitation to concentrate immunoglobulins. A checkerboard titration approach optimized assay conditions, identifying 5 ng of membrane peptide (LSYFLASFR), undiluted sample input, and a 1:5000 dilution of HRP-conjugated anti-human IgM as ideal. The assay demonstrated strong diagnostic performance with 80% sensitivity and 100% specificity, as validated by ROC curve analysis (AUC > 0.9, p = 0.0013). Real time PCR confirmed positive samples showed significantly elevated absorbance compared to negative controls. These findings suggest that antibody detection in wastewater is a robust, semi-quantitative, cost-effective and non-invasive tool for early community-level surveillance. This pilot study highlights the potential of serological WBE to complement traditional diagnostics, particularly in resource-limited settings and paves the way for scalable epidemiological monitoring of viral immunity at the population level.

Keywords: anti SARS-CoV-2 IgM detection; Indirect ELISA; Serological surveillance; Wastewater-based Epidemiology (WBE)

I. INTRODUCTION

The outbreak of the novel coronavirus, Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), triggered an unprecedented global health crisis. In January 2020, the World Health Organization (WHO) characterized this outbreak as a public health emergency of international concern, resulting in the WHO's highest level of alarm (WHO, 2021). On March 11, 2020, the WHO declared COVID-19 a pandemic (WHO, 2020). Characterized by its rapid transmission and diverse clinical manifestations, COVID-19 has underscored the urgency for innovative approaches to its surveillance, diagnosis, and containment. Central to this paradigm shift is the recognition of wastewater as a reservoir for invaluable insights into the epidemiology and dynamics of SARS-CoV-2 transmission.

SARS-CoV-2 is classified as a betacoronavirus, characterized by its enveloped structure and helical capsid, and it contains a positive-sense single-stranded RNA genome. Among RNA viruses, coronaviruses are notable for their large size and are classified within the realm Riboviria, order Nidovirales, suborder Cornidovirineae, family Coronaviridae, and subfamily Orthocoronavirinae (Cavanagh, 1997). These viruses are further categorized into Alphacoronavirus, Betacorona virus, Gammacorona virus, and Deltacoronavirus genera, which are capable of infecting mammals and birds (Kirtipal et al., 2020). SARS-CoV-2 possesses various structural proteins, including the small envelope protein (involved in viral assembly), hemagglutinin esterase protein (responsible for binding to the cell membrane), membrane protein (required for viral assembly), nucleocapsid protein (facilitating RNA packaging and genome protection), and spike protein (a glycoprotein on the viral envelope responsible for binding to and fusing with the host cell membrane). The virus is believed to have originated in animals and exhibits zoonotic transmission, underscoring the complex dynamics of viral spillover and interspecies transmission. Predominantly targeting the respiratory epithelium, SARS-CoV-2 utilizes the angiotensin- converting enzyme 2 (ACE2) receptor for cellular entry, a mechanism similar to that of SARS-CoV. Among the human coronaviruses (HCoV), OC43 and NL63 are among the most commonly encountered strains.

A. Epidemiology:

Since the initial report from China, the disease has spread rapidly and the number of incident cases has increased exponentially. Globally an estimated 774 million individual have been reported to date. An estimated 45 million cases of SARS-CoV-2 infection

have been reported in India. The infection rate (per million) by age was highest among those aged 50-70

years and lowest among those aged < 10 years. Males (41.6) were more likely to be susceptible to infection than females (24.3). The global distribution of SARS- CoV-2 reflects the interplay of environmental, socio- economic, and demographic factors, with urban centers and densely populated regions serving as epicenters of transmission. Variability in viral lineage dynamics and the emergence of variants of concern underscore the evolutionary plasticity of SARS-CoV-2 and its potential to evade immune surveillance mechanisms.

B. Wastewater surveillance:

Wastewater surveillance has emerged as a robust method for tracking the spread of infectious diseases within urban communities and offers predictive insights into future spikes in infections and hospital admissions. The conventional approach for disease surveillance involves quantifying the presence of viral genetic material in wastewater samples collected from areas with well-defined populations. This strategy has been widely adopted globally, particularly amidst the SARS-CoV-2 pandemic, l eading to the establishment of comprehensive wastewater monitoring systems [4].

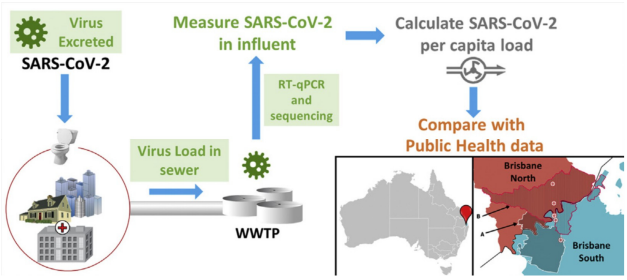


Fig. 1: Schematic representation of monitoring SARS-CoV-2 prevalence through wastewater surveillance.

Wastewater surveillance has demonstrated effectiveness in tracking SARS-CoV-2 infection trends at both the regional and local levels, facilitating rapid responses to emerging outbreaks. Surveillance during the COVID-19 pandemic has predominantly focused on quantifying viral RNA and sequencing variants in wastewater samples. However, the potential of monitoring other immune-relevant molecules in wastewater remains underexplored, with antibodies against SARS-CoV-2 emerging as a promising targets. Assessment of the neutralization activity against the virus directly correlates with the levels of anti-SARS-CoV-2 IgG or IgA antibodies. A decrease in community-wide antibody levels in wastewater could indicate a decline in durable immunity following widespread infection or seasonal vaccination campaigns. Short-term longitudinal studies have revealed that antibody protection against future infections diminishes over the course of months among naturally infected, vaccinated, or boosted individuals.

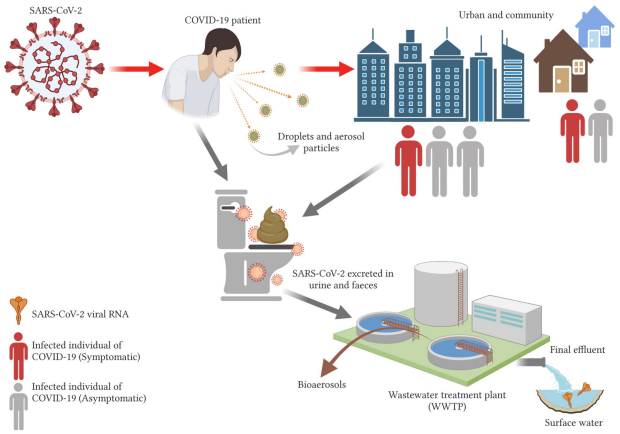


Fig. 2: The possible transmission routes for SARS-CoV-2 RNA through wastewater.

Individuals previously infected with SARS-CoV-2 excrete spike IgG and IgM antibodies in their feces, which can subsequently be identified in wastewater. This capability enables wastewater surveillance to detect the presence of SARS-CoV-2 antibodies in both symptomatic and asymptomatic individuals, thereby serving as an early indicator of potential COVID-19 transmission within a community.

Comprehending the vulnerability of populations to potential future waves of infection is paramount for averting an endemic state associated with undesirable levels of illness and death. Broadening surveillance efforts to monitor antibody levels is a proactive strategy that can pinpoint at-risk populations susceptible to future infections.

Integrating wastewater surveillance for antibody abundance would serve as a supplementary, not substitute, method for traditional serology. Linking ELISA measurements to serum concentrations is challenged by individual immune response variations and fecal shedding heterogeneity of detectable antibodies, similar to viral particle shedding rates. Wastewater surveillance can address gaps in settings where population-wide serological studies are impractical, identify neighborhoods or populations with low or undetectable antibody levels, and potentially mitigate geographic health disparities. This approach is valuable for assessing vaccine-mediated immunity, especially given the low and uneven rates of booster uptake, particularly in countries like the United States.

Predicting the local impact of future SARS-CoV-2 waves requires estimating the immune response durability from prior vaccination or infection, making wastewater-based assessments crucial for rapid data collection to identify at-risk populations [4].

This study presents a robust protocol for conducting enzyme-linked immunosorbent assay (ELISA) to detect anti-SARS-CoV-2 membrane IgM antibody in wastewater. We investigated the detection efficiency across various dilutions and validated our method by using both freshly collected and archived frozen samples. This protocol not only opens avenues for future research in monitoring efforts but also allows

retrospective analysis of samples collected during the SARS-CoV-2 pandemic. By integrating antibody level assessment with viral detection and sequencing, our approach enhances the utility of wastewater surveillance, providing indispensable data for informed public health policy formulation and decision-making.

C. Polyethylene Glycol Precipitation method:

Wastewater serves as a reservoir for a diverse array of pathogens, including viruses, bacteria, and parasites, which are pivotal for the dissemination of diseases within communities. Consequently, wastewater-based surveillance has emerged as a vital strategy for the early detection and monitoring of enteric viruses such as poliovirus, norovirus, and hepatitis. This surveillance methodology has been increasingly used in recent years to establish an early warning system for identifying and tracking the transmission patterns of these pathogens (Hovi et al., 2012; Smith et al., 2016; Tiwari and Dhole, 2018).

Environmental surveillance via wastewater analysis has a longstanding history in public health, particularly in the surveillance of poliovirus and, more recently, antimicrobial resistance (AMR). Amidst the ongoing COVID-19 pandemic, wastewater surveillance has garnered additional attention as a means of detecting SARS-CoV-2 shed into wastewater from the upper gastrointestinal and respiratory tracts, as well as through fecal matter. This approach provides valuable insights into the prevalence and circulation dynamics of viruses within communities, thereby facilitating early detection and response initiatives.

Filtration, Centrifugation and Precipitation Purpose

PEG precipitation, combined with NaCl as a co-precipitant, is an effective method for purifying viruses by altering their solubility, inducing precipitation, and eliminating large wastewater solids, prokaryotic and eukaryotic cells, and other debris.

Definition/Acronyms

PEG:- Polyethylene glycol (PEG) is a synthetic, hydrophilic, biocompatible polymer that is widely used in biomedical and other applications. PEG functions to provide a hydrophobic environment, whereas salt cations are used to neutralize the negative charge of the phosphate backbone.

NaCl:- Sodium chloride is the salt responsible for the salinity of seawater and the extracellular fluid of many multicellular organisms. An initial extraction step using saturated NaCl solution facilitates the separation of nucleic acids from contaminants, followed by further extraction with organic solvents and precipitation with 2-propanol to obtain total RNA of high purity, which is suitable for applications such as cDNA synthesis, RT-PCR and Northern blot hybridization.

Supernatant:- A supernatant liquid occurs during the chemical process of precipitation, and it is typically a clear liquid free of precipitate located above the solid part during settling.

Pellet:- A pellet, in ornithology, is the mass of undigested parts of a bird's food that some bird species occasionally regurgitate.

Enzyme-linked immunosorbent assay (ELISA):
In the context of serological detection of SARS-CoV-2 antibodies in wastewater samples, the availability of an alternative assay method that is sensitive, specific, cost-effective, and widely applicable is invaluable. Enzyme-linked immunosorbent assays (ELISA) have emerged as promising candidates to fulfill these requirements.
In the proposed ELISA method, antibodies extracted from concentrated wastewater samples were immobilized by binding to anti-human IgM antibodies (enzyme-linked secondary antibodies). Horseradish peroxidase was then conjugated to specifically purified anti-human IgM antibodies to enable the detection of acute infections within the community. The quantity of immobilized horseradish peroxidase was assessed throughanenzymatic reaction involving tetramethylbenzidine, a yellow compound known for its strong absorbance at 450 nm.

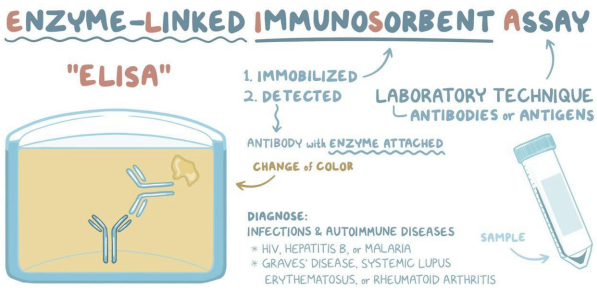


Fig. 3: Principle of Enzyme-linked immunosorbent assay.

In indirect ELISA, both antibodies and antigens can be assayed. The molecules were immobilized in the following sequence: polystyrene surface, antigen, primary antibody, and antiglobulin-enzyme conjugate. When assaying antibodies, the antigen concentration remains constant, whereas for antigen assays, the antibody concentration remains constant. In both scenarios, the color developed during the substrate reaction step correlated with the quantity of assayed material present, enabling quantitative analysis.

Checkerboard method of titration:-

The checkerboard titration method facilitates the simultaneous assessment of two variables: antibody concentration and sample concentration. By varying the sample-to-antibody ratio in each well, the optimal concentrations of both components, as well as the ideal ratio between them, can be determined. This information obtained from the checkerboard assay can then be applied to optimize the conditions for subsequent ELISA assays, resulting in improved results. For instance, in a typical setup, columns 1-12 may represent different antibody dilution factors, whereas rows A-H correspond

to the sample dilution factors. Starting with a known concentration of antigen, such as 5 ng/mL, doubling

dilutions across the plate can be performed, resulting in concentrations of 10 ng/mL, 20 ng/mL, and 30 ng/mL, allowing for a comprehensive evaluation of the assay conditions.

Wastewater surveillance is a powerful tool for monitoring the prevalence of infectious diseases in urban populations, with a predictive value for upcoming increases in cases and hospitalizations. The approach primarily used in disease surveillance has been to measure the number of viral copies detected in wastewater for a study area of a known population, and systems for wastewater monitoring have been established worldwide during the SARS-CoV-2 pandemic. However, the potential of measuring other biomarkers, such as proteins and metabolites in wastewater has not been fully explored. Here, we developed an approach to determine the optical density of antibodies in wastewater. We measured the abundance of anti-SARS-CoV-2 membrane IgM in typical fresh samples of community wastewater, and from Sewage Treatment Plants (STPs) using an indirect ELISA assay.

II. MATERIALS AND METHODS

Day	Monday
Date	5/2/2024
Time	4:10 PM
Latitude	21.128339
Longitude	79.061392
Area	Bajaj nagar
Source	Sewage treatment plant (STP)
Temperature	27°C
pH	5.7
Color	Dark yellow
Weather temperature	33°C
Humidity	45%
Collected by	Ashish Tule

Table 1 : Performa for wastewater sample collection.

Study area:-

This study was performed on wastewater samples from different localities in the Nagpur district of Maharashtra State, India.

Study subjects and Wastewater samples:

Wastewater studies typically focus on monitoring and analyzing the composition of wastewater to gain insights into public health, environmental impact, and community health trends. To obtain samples from different areas, we require permission from the Municipal Corporation. Samples were collected from the Sewage Treatment Plant (STP) of CIIMS Hospital, Nagpur, and the rest of the samples were provided by the CIIMS-ARC team from their ongoing DBT-INSACOG project on wastewater surveillance. Samples were taken while wearing the PPE kit into the sample bottles. 70% ethanol was used for sanitization whenever required. Sites are selected by considering the population size, development of diseases, history of cases, etc. The study was a prospective study that included a total of n=10 sewage wastewater samples collected from various areas of Nagpur City (Itabhatti-01, Itabhati-02, Hiwari Nagar- 02 , Sonegaon, Pardi- 01 , Hazaripahad, Mokshdham, Somalwada, Pohra, Dabha and RO (CIIMS-ARC)). 500 ml of sewage samples were collected from the areas mentioned in the morning.

Sample collection:-

Wastewater samples were collected from Sewage Treatment Plants (STPs) in different localities in Nagpur. The date, time, location, and physical characteristics of the water and the weather conditions were recorded. The wastewater samples were collected in high density polyethylene (HDPE) bottles, washed with 70% ethanol and stored in a refrigerator at 7°C.

A. Materials required:-

- Microtiter plate
- Micropipettes [variable volume 10µl, 100µl, 1000µl]
- Microtips [10µl, 100µl, 1000µl]
- Eppendorf vials
- Tissue papers
- Spatula
- Beakers
- Test tubes
- Glass rods
- Volumetric flask

Equipments:-

- Incubator
- Cyclo mixer for vortexing
- ELISA plates reader
- pH meter
- Laboratory centrifuge
- Weighing balance
- Autoclave
- Refrigerator

SOLUTIONS:

Phosphate buffer saline (1x-PBS), Bovine Serum Albumin (BSA), primary antibody [PEG-concentrated wastewater samples], secondary antibody (anti human IgM HRP conjugate), substrate (TMB), and Stop Solution (2.5N H_2SO_4).

PREPARATION OF SOLUTIONS:

1) **Sample dilution preparation:** For antibody detection (1:5, 1:50, 1:100 and 100) take 20µL wastewater sample in 80µL 1x PBS then vortex it for 15-20secs.

2) **Phosphate Buffer Saline (PBS):**

Composition for 10x PBS:
- Sodium chloride (NaCl) - 80g
- Potassium chloride (KCl) - 2g
- Disodium hydrogen phosphate (Na2HPO4) - 14.4g
- Sodium dihydrogen phosphate (NaH2PO4) - 2.4g
- Distilled water - 1000mL

pH - 7.4

Preparation of 1X Phosphate Buffer Saline:

Take 100mL of 10x PBS solution raise volume up to a level 900mL. Adjust the pH to 7.4 and finally make up to 1000mL. Each dilution was prepared in 1x PBS.

3) Blocking Buffer:

Bovine Serum Albumin (0.5%) - For 100mL, 500mg BSA in 100mL of 1x PBS.

4) Peptide dilution: Dilution is 1:5 (400µL sample:1600µL PBS), 1:50 (40µL sample:1960µL PBS), 1:100 (20µL sample:1980µL PBS) and undiluted (100µL sample)

- **Secondary antibody dilution:**

The secondary antibody dilutions used for antibody detection were 1:5000, 1:10000, 1:15000 and 1:20000 for anti human IgM HRP conjugate. To prepare it take 1µL IgM antibody in 5mL of 1x PBS (1:5000), 2µL IgM antibody in 5mL of 1x PBS (1:10000), 3µL IgM antibody in 5mL of 1x PBS (1:15000) and 4µL IgM antibody in 5mL of 1x PBS (1:20000).

5) Substrate:

TMB commercially available in ready to use form.

6) Stop solution:

2.5N H_2SO_4. For 500mL, take 35mL of H_2SO_4 in 450mL of distilled water.

B. Standardization of the peptide using checkerboard titration method

Peptide use:

The SARS-CoV-2 membrane protein (LSYFLASFR) was selected as the antigenic peptide for this study. Subsequently, this peptide was selected for IgM antibody detection in wastewater samples using an anti-human IgM antibody by indirect ELISA.

Checkerboard titration method:

Standardization of the peptides was performed using the checkerboard titration method in which various concentrations of peptides, sample dilutions, and secondary antibody dilutions were evaluated. Furthermore, the concentration that gave the best optimal readings was selected to evaluate the suspected and confirmed samples for COVID-19. The different concentrations used to standardize the assay are described below.

Peptide concentration:

The peptide concentration used for the assay was 5ng, 10ng, 20ng, and 30ng

Secondary IgM dilution:

The dilutions for secondary antibody IgM used in the assay were 1:5000, 1:10000, 1:15000, and 1:20000.

Sample dilution:

The samples were diluted to per different dilutions such as 1:5, 1:50, 1:100, and undiluted. Wastewater samples were diluted within 1x PBS.

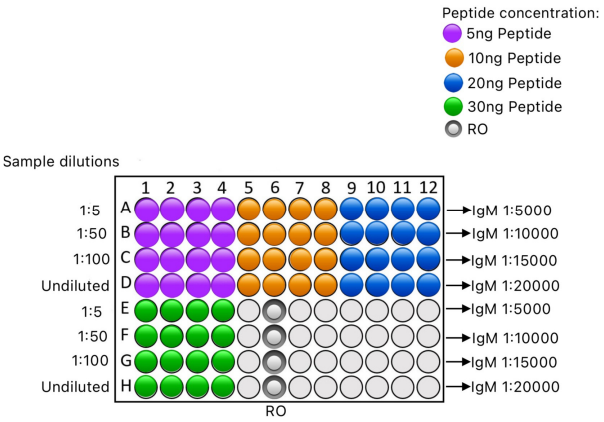


Fig. 4: The image represents the checkerboard titration method with various peptide concentration, sample dilutions and secondary IgM dilutions.

C. Evaluation of wastewater samples:

A total (n=10) wastewater samples were evaluated. As per the data found, tables and figures were prepared as per the standard protocol 5ng/100µl of the antigen concentration, undiluted (PEG Concentrated) sample concentrations, and 1:5000 of the secondary antibody dilution (IgM). ROC curve data was also prepared for the developed peptide assay to define sensitivity and specificity along with the cut-off range of the samples.

D. Statistical analysis:

ROC curve analysis comparison between the developed and existing technologies was performed using the MedCalc statistical software. Figures and graphs were prepared using the MedCalc statistical software. Concordance between individual tests was calculated using MedCalc statistical software.

METHODS:

A. *PEG Precipitation method:*

The PEG precipitation method completes within three steps as follows:

1. Filtration
2. Centrifugation, and
3. Precipitation

Day-2:

Transfer the collected 35mL of wastewater samples to 50mL of centrifuge tube.

⬇️

Centrifuge the tubes at 4000rpm for 40 min at 10-12°C temperature.

⬇️

After centrifugation, take 30mL of supernatant without disturbing the bottom pellet.

⬇️

Filter the supernatant with 0.22µm syringe filter in fresh centrifuge tube.

⬇️

Add 2gm Polyethylene glycol (PEG-8000) (8%PEG) (Himedia) and 0.437gm sodium chloride (NaCl) (0.3M NaCl) to the collected supernatant.

⬇️

Keep it overnight at 7°C refrigerator.

Day-3:

The centrifuge tube was incubated according to the PEG protocol and centrifuged at 14,000rpm for 70 min at 4°C.

⬇️

After centrifugation, the supernatant was discarded and resuspended the pellet in 250µl of nuclease-free water.

⬇️

Vortex and centrifuge at 4000rpm for 30 sec at 4°C.

⬇️

Transfer the entire 250µl of the resuspended sample to an Eppendorf tube (2.0 mL).

⬇️

Store it at -80°C until further use.

B. *Indirect ELISA:*

Indirect enzyme linked immunosorbent assay (ELISA) is a biochemical technique used mainly in immunology to detect the presence of antibodies in wastewater samples. This study was based on a semiquantitative indirect IgM ELISA for the detection of anti-SARS-CoV-2 membrane IgM antibody from wastewater samples.

Antibody detection by Indirect ELISA:-

Protocol:

PEG (Polyethylene glycol) Protocol

⬇️

Membrane SARS-CoV-2 (LSYFLASFR) peptide Coating

⬇️

anti-SARS-CoV-2 IgM detection

Add 100µl of the peptide antigen (Membrane SARS-CoV-2) at different concentrations (5ng, 10ng, 20ng, and 30ng) to the microtiter plates. Incubate the same at 4°C overnight.

⬇️

Next day wash the plates one time with PBS-T.

⬇️

Add 0.5% BSA 100µl in each well. Incubate the plates for two hours at 37°C.

⬇️

Wash the plate three times with PBS-T

⬇️

Add 100µl of concentrated wastewater sample as per to different sample dilutions (1:5, 1:50, 1:100, undiluted concentrations) and RO sample in a previously coated well.

⬇️

Incubate the plate for 60 min at 37°C.

⬇️

After incubation, wash the wells thrice with PBS-T.

⬇️

Now add the Goat anti-human IgM-HRP Conjugated (secondary antibody) 100µl to each well at different concentrations (1:5000, 1:10000, 1:15000, 1:20000).

⬇️

After the addition of the secondary antibody incubate it further for 45 mins at 37°C.

⬇️

After incubation, wash the plate four times with PBS-T. Then add 100µl of substrate solution (TMB/H202) in each well and keep it for 15 min.

⬇️

Then to stop the reactions, add the stop solution (H_2SO_4) 100µl in each well. The absorbance in each well was measured at 450nm wavelength using an ELISA reader.

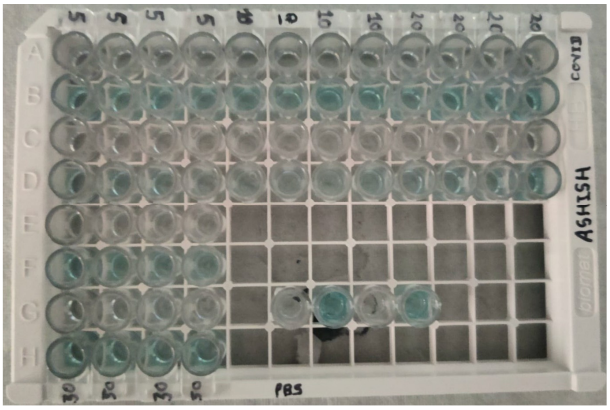
III. RESULTS AND DISCUSSION

Wastewater surveillance is a potent tool for monitoring infectious diseases in urban populations, and offers predictive insights into cases. While primarily focused on viral load detection, the potential to measure other biomarkers, such as proteins and metabolites, remains underexplored. This study introduces a novel approach to determine antibody optical density in wastewater, specifically anti-SARS-CoV-2 membrane IgM levels, using ELISA techniques on samples from community wastewater and Sewage Treatment Plants (STPs).

The antigenic peptide of Membrane SARS-CoV-2 was standardized using the checkerboard titration method to detect the anti-SARS-CoV-2 IgM antibody in wastewater samples. Furthermore, antigenic peptides were used against different wastewater samples (n=10), which comprises confirmed positive samples (n=5) of COVID RT-PCR by the CIIMS Research team and healthy control samples (n=5). After evaluating (n=10) samples, the concentration of antigenic peptide with 5ng along with undiluted (PEG Concentrated) samples and 1:5000 secondary IgM dilution was selected with optimal results. The selected concentration was used to evaluate suspected and confirmed wastewater samples of COVID-19.

Sample code	Area	Site
1	Pohara	STP
2	Hazaripahad	STP
3	Dabha	STP
4	Mokshdham	STP
5	Itabhatti - 02	STP

Table 2 : STPs sites (1)



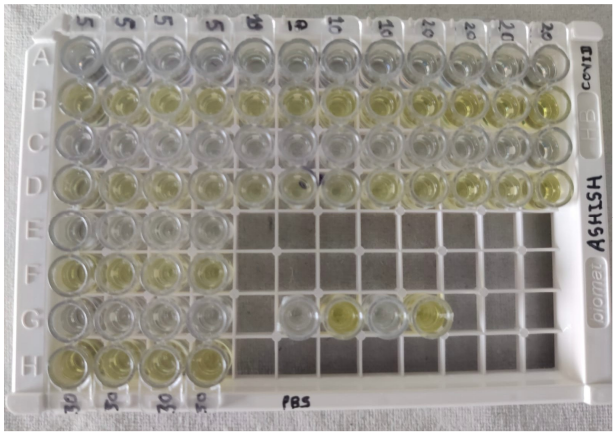


Fig. 5: Concentrated wastewater evaluated for anti-SARS-CoV-2 membrane protein IgM by Indirect ELISA (1).

Results

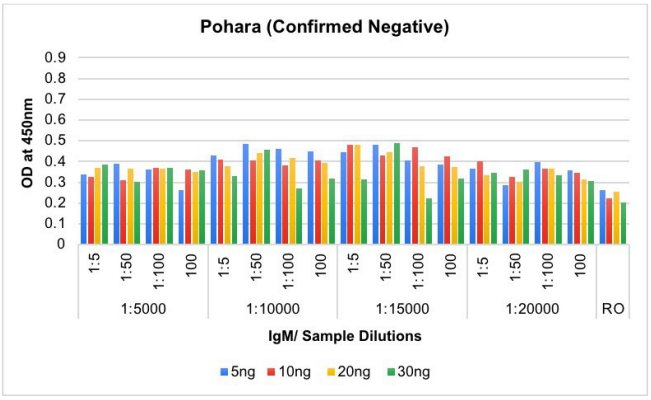


Fig. 6: Bar graph showing the standardization of IgM antibody detection based ELISA assay in COVID Negative wastewater sample using checkerboard titration method in Pohara STP.

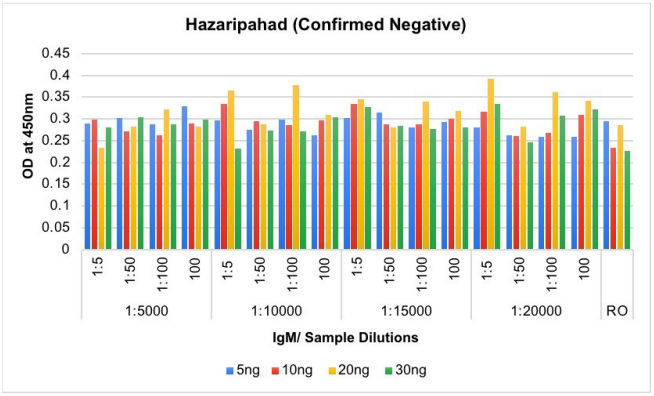


Fig. 7: Bar graph showing the standardization of IgM antibody detection based ELISA assay in COVID Negative wastewater sample using checkerboard titration method in Hazaripahad STP.

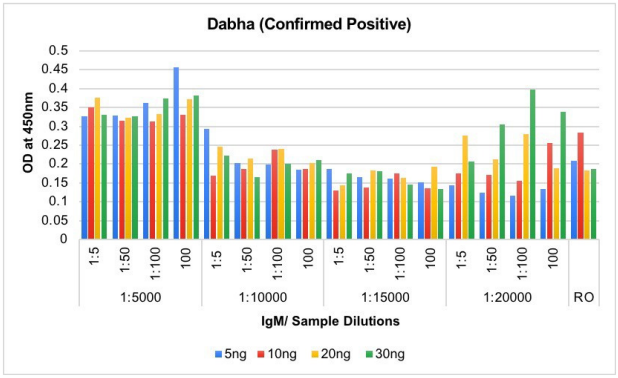


Fig. 8: Bar graph showing the standardization of IgM antibody detection based ELISA assay in COVID Positive wastewater sample using checkerboard titration method in Dabha STP.

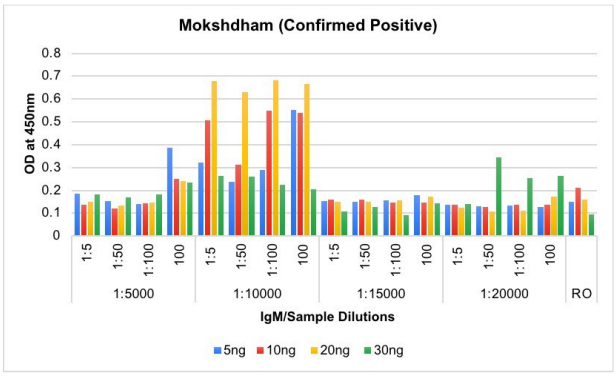


Fig. 9: Bar graph showing the standardization of IgM antibody detection based ELISA assay in COVID Positive wastewater sample using checkerboard titration method in Mokshdham STP.

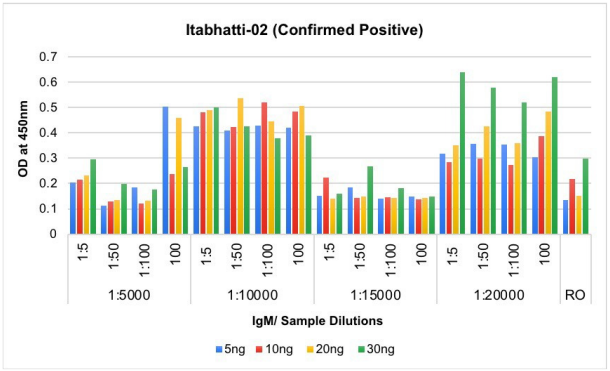


Fig. 10: Bar graph showing the standardization of IgM antibody detection based ELISA assay in COVID Positive wastewater sample using checkerboard titration method in Itabhatti-02 STP.

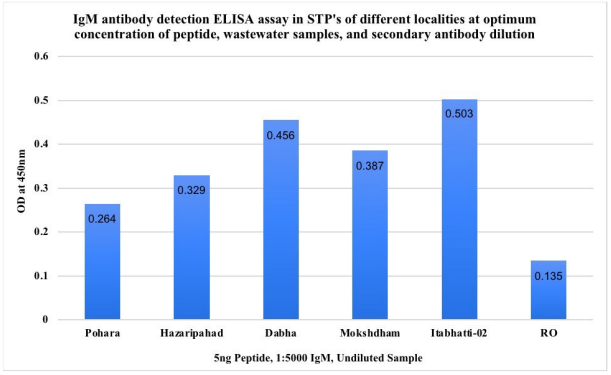


Fig. 11: Bar graph showing absorbance values of IgM antibody detection based ELISA assay at 450nm in COVID positive and negative wastewater samples in different STP's at optimal concentrations of 5ng peptide dilution, 1:5000 IgM antibody dilution and 100µL of wastewater samples.

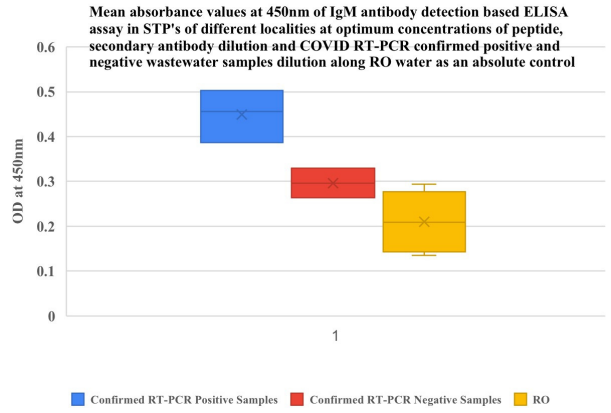


Fig. 12: Box whisker plot showing mean absorbance values of IgM antibody detection based ELISA assay at 450nm in COVID confirmed RT-PCR positive and COVID confirmed RT-PCR negative wastewater samples of different STP's along with RO water as an absolute control using optimal concentrations (5ng peptide dilution, 1:5000 IgM dilution and 100µL undiluted sample addition).

Confirmed positive: Those wastewater samples which are positive by COVID Real time PCR.

Confirmed negative: Those wastewater samples which are negative by COVID Real time PCR.

Sample code	Area	Site
1	Hazaripahad	STP
2	Mokshdham	STP
3	Somalwada	STP
4	Hiwari nagar-02	STP
5	Itabhatti-01	STP
6	Itabhatti-02	STP
7	Pohara	STP
8	Pardi-01	STP
9	Dabha	STP
10	CIIMS-ARC	RO

Table 3 : STPs sites (2)

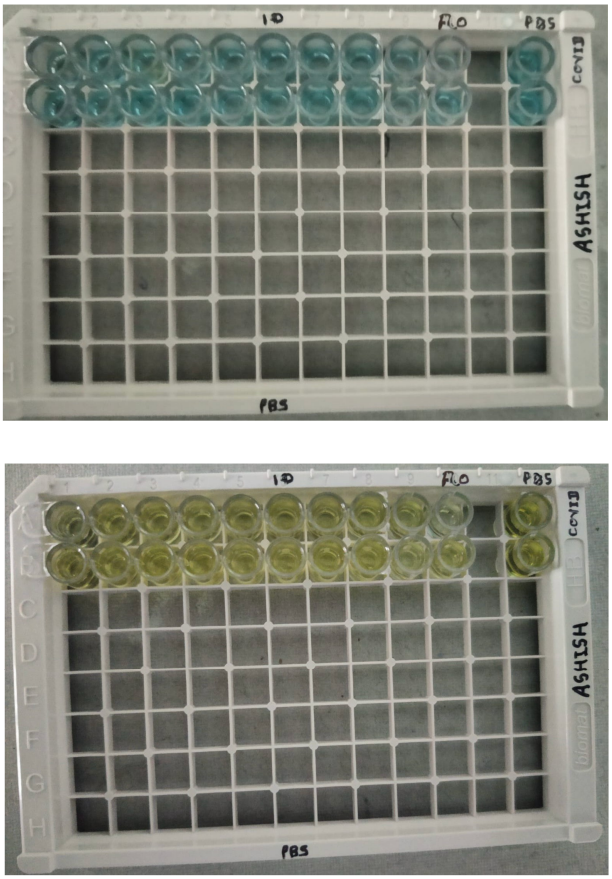


Fig. 13: Concentrated wastewater evaluated for anti-SARS-CoV-2 membrane protein IgM by Indirect ELISA (2).

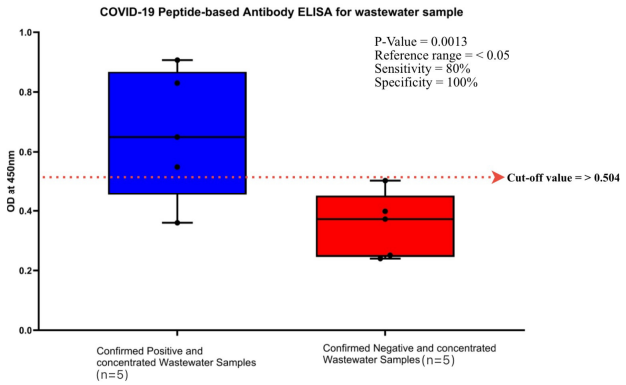


Fig. 14: Box whisker plot showing mean absorbance values of COVID-19 confirmed (Positive and Negative) concentrated wastewater samples using COVID-19 peptide based IgM Antibody detection ELISA assay.

Confirmed positive: Those wastewater samples which are positive by COVID Real time PCR.

Confirmed negative: Those wastewater samples which are negative by COVID Real time PCR.

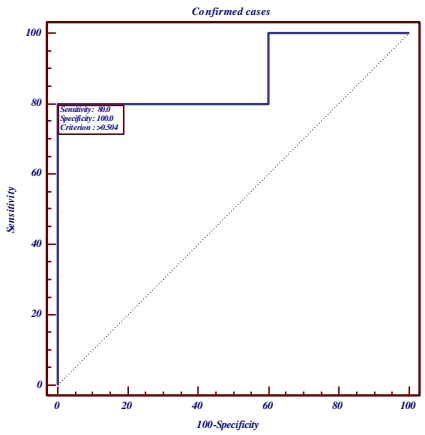


Fig. 15: Mean absorbance values of Membrane SARS-CoV-2 confirmed COVID positive wastewater samples and confirmed COVID negative wastewater samples with ROC (receiver operating characteristic) curve.

Virus	Peptide	Significance level P (Area=0.5)	Reference P Level	Cut-off value	(AUC) 95% confidence interval	% Sensitivity	% Specificity
SARS-CoV-2	Membrane SARS-CoV-2	0.0013	< 0.05	> 0.504	> 0.9	80	100

Table 4 : P-value along with cut-off value and Area Under Curve (AUC) determined by ROC analysis of Membrane SARS-CoV-2 Peptide for detection of COVID-19 infection using ELISA in wastewater samples.

With respect to the objective of the study, for the Membrane SARS-CoV-2 specific antigenic peptide, four different concentrations of Peptide sequence (LSYFLASFR) were coated on an ELISA well plate, as 5ng, 10ng, 20ng, and 30ng. The wastewater sample was also taken at different dilutions of 1:5, 1:50, 1:100 and undiluted (PEG Concentrated). Four dilutions of the secondary antibodies were used i.e. 1:5000, 1:10000, 1:15000, and 1:20000. From **Figure 13 to 17**, the bar graph shows the standardization of IgM antibody detection-based ELISA assay in COVID Positive and Negative wastewater samples using the checkerboard titration method in different STP's. We can clearly observe that at the different antigen concentrations, the undiluted sample yielded the highest titre value among the three. Therefore, we selected this sample dilution for proceeding ahead with the further analysis. In addition, because the coating concentration of 5ng peptide concentration yielded good results, we decided to proceed with the same. For the concentrations of secondary antibody, we used 1:5000 dilution to proceed with the further protocol. In **Figure 18**, the combined graph of checkerboard titration is showing an absorbance values of IgM antibody detection-based ELISA assay at 450nm in COVID positive and negative wastewater samples in different STP's at optimal concentrations of 5ng peptide dilution, 1:5000 IgM antibody dilution and 100µL of wastewater samples. The box whisker plot in **Figure 19** shows the mean absorbance values of IgM antibody detection based ELISA assay at 450nm in COVID confirmed RT-PCR positive and COVID confirmed RT-PCR negative wastewater samples of different STP's along with RO water as an absolute control (using optimal concentrations of 5ng peptide dilution, 1:5000 IgM dilution and 100µL undiluted sample addition.

Based on these observations, we selected 5ng as the peptide concentration, sample dilution of undiluted 100µL, and the secondary antibody dilution of 1:5000, total of 10 samples were taken out of which five samples were confirmed positive and four samples were confirmed negative, and one RO water sample (By COVID RT-PCR).

As shown in **Figure 20,** the PEG-concentrated wastewater samples were evaluated for anti-SARS-CoV-2 membrane protein IgM using indirect ELISA. In **Figure 21**, the box whisker plot shows the mean absorbance values of COVID-19 confirmed (Positive and Negative) concentrated wastewater samples using COVID-19 peptide based IgM Antibody detection ELISA assay. The ROC Curve was constructed for the Membrane SARS-CoV-2 peptide-based indirect ELISA assay as shown in **Figure 22**, and the P-Value along with cut-off for optimal sensitivity and specificity for the ELISA test was determined by ROC curve analysis as shown in **Table 4**.

The Area under the curve (AUC) was calculated to evaluate the accuracy of each test. The AUC of the Membrane SARS-CoV-2 peptide was > 0.9 with a cut-off value of > 0.504, indicating the high discriminatory ability of the peptide for confirmed and control cases. From the ROC curve analysis, it was found that for the detection of the Membrane SARS-CoV-2 antibody from wastewater samples, the designated peptide showed a sensitivity of 80% and specificity of 100% (p= 0.0013).

The mean absorbance values of COVID-19 confirmed (positive and negative) concentrated wastewater samples using the COVID-19 peptide-based IgM Antibody detection ELISA assay are shown in **Figure 21**. IgM antibody detection using the synthetic peptide of Membrane SARS-CoV-2 (active COVID-19 biomarker) was associated with relatively high mean absorbance values in confirmed COVID-19 wastewater samples as compared to non-COVID wastewater samples.

This was a pilot study, and the sample size was small. Further studies on large number of wastewater samples are required. Another limitation is that, the analysis requires raw wastewater to be concentrated.

Agan et al., (2022): Wastewater samples naturally accumulate antibodies from varying numbers of individuals, ranging from a few to potentially thousands, posing challenges for directly interpreting the detected antibody levels and immune status. Nonetheless, methods can be devised to infer the immune status of the population.

COVID-19 wastewater surveillance initiatives contributing to the CDC's National Wastewater Surveillance System gather site metadata, including water flow rates and the populations within the sewershed contributing to the collection point signal. Using these data, ELISA optical density or titer measurements can potentially be extrapolated to estimate bulk copies per person, similar to the approach used for

viral copy number data. By detecting antibodies in archived frozen wastewater samples, it is feasible to retrospectively analyze the SARS-CoV-2 pandemic and develop predictive models while validating them against historical incidence levels. Utilizing existing samples collected throughout the COVID-19 pandemic enables longitudinal studies to quantify antibody concentrations and integrate this information with viral load and case data for the corresponding sewershed population. This approach represents an exciting advancement in understanding the potential of antibody detection in wastewater.

A wastewater surveillance strategy focusing on antibody abundance would serve as a complement rather than a replacement for traditional serology methods. The task of correlating ELISA optical density or titer measurements with equivalent concentrations in serum is hindered by individual variations in immune responses and diversity in the rate of fecal shedding of detectable antibodies, which parallels the variability observed in the shedding of viral particles through feces. Wastewater surveillance holds promise for addressing gaps in geographic regions where population-wide serological studies are impractical. It can also help identify vulnerable neighborhoods or populations with low or undetectable antibody levels, thereby aiding the identification and mitigation of geographic health disparities. Notably, vaccine-mediated immunity relies on timely boosting with updated vaccines targeting circulating variants. Currently, the rates of boosting in countries such as the United States are low and exhibit significant disparities among populations. Given that predicting the local impact of the next SARS-CoV-2 infection wave necessitates estimating the durability of immune responses from previous vaccinations or natural infections, wastewater-based assessments could expedite data collection and facilitate the identification of at-risk populations.

IV. CONCLUSION

In the present study, we aimed to optimize a wastewater-based checkerboard titration mediated indirect ELISA protocol for the detection of anti-SARS-CoV-2 membrane IgM antibody. The peptide was evaluated using the checkerboard titration method to determine the suitable concentrations and dilutions for antibody detection. The sensitivity and specificity of the developed assay were 80% and 100% respectively, with a cut-off value > 0.504; the developed test had inherent rapidity and sensitivity in detecting acute COVID infection.

In conclusion, we have reported the utility of a simple and cost effective anti-SARS-CoV-2 membrane IgM antibody detection ELISA protocol using wastewater samples against the Membrane SARS-CoV-2 peptide (LSYFLASFR). The main advantage of using wastewater for indirect ELISA is that it can provide semiquantitative analysis and is useful in the early stages of infection. This study indicated that the peptide-based ELISA assay can be used as a reliable tool for wastewater surveillance and in studies of wastewater-based epidemiology of infection, especially in developing countries. Since, various molecular techniques are also available for detection of the assay, this could be expensive and require high infrastructure, serology-based assays could be used as a reliable tool to assess the prevalence of acute COVID-19 infection from wastewater sources within the community and in highly suspected cases of the infection.

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