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Target gene influences detection of SARS-CoV-2 virus in wastewater concentrated samples: an Egyptian study

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Abstract
Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) detection in wastewater could help in predicting number of infected persons in a given area. We aimed to detect SARS-CoV-2 in wastewater by qRT-PCR targeting different virus genes using in house designed primers. A total of 21 wastewater samples (2 liters each) were collected from influent of Zenin wastewater treatment plant (WWTP) in Egypt between August 2021 and March 2022 and primers targeting SARS-CoV-2 S, N and E genes were used for detection. Results showed that detection sensitivity was highly affected by the target gene which was 95, 90, and 76% upon targeting the virus N, S, and E genes, respectively. Testing sensitivity of our designed N-specific primers on serial template dilutions revealed its ability to quantify SARS-CoV-2 until virus RNAconcentration 2.5pg /reaction mixture. Multiple alignments of the used N-specific primer pair to all SARS-CoV-2 variants reported by the CDCincluding all omicron variants showed 100 % alignment except the zeta P2.In conclusion, we recommend targeting the SARS-CoV-2 N gene using the herein designed N-specific primers to detect the virus in wastewater samples.

Key words: *qRT-PCR, SARS-CoV2, Target gene, Wastewater.*

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Highlights:

- SARS-CoV-2 was detected in 20/21 concentrated wastewater samples collected from Zenin wastewater treatment plant.
- Number of positive samples was affected by the target gene and experiment conditions.
- Usage of primers targeting the N-gene isrecommended in detection compared to primers targeting the S and E genes.
- The N-gene-specific primer pair showed high sensitivity even at very low template concentrations.
- The N-gene-specific primer pair showed 100 % annealing when aligned with all SARS-CoV-2 variants except Zeta P2.

I. Introduction

WHO classified severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) as the cause of global pandemic in March 2020 (Ahmed et al., 2021). Viral RNA was detected with high concentration in stool samples of infected persons (Wolfel et al., 2020) making it the main source for viral presence in wastewater. Besides, viral particle present in nasal, pharyngeal and sputum samples (Wang et al., 2020) were an additional source as they could reach wastewater as well.

From here scientists thought that quantitative detection of SARS-CoV-2 viral particles in wastewater could help in predicting number of infected persons living in a given area (Medema et al., 2020),and based on that manystudies were conductedto detect the virus in wastewater (Sharif et al., 2021; Nag et al., 2022).

Target genes used in detection differ among studies.When the N, ORF1ab and S genes of the SARS-CoV-2 were targeted for PCR detection results showed that some samples were positive for the three genes and some were only positive for two target genes but others were only positive for one gene (Thongpradit et al., 2022).

When other study targeted theN1, N2, E, IP2 and IP4 genomic fragments of SARS-CoV-2 results showed detection percentages of 91.2, 85.3, 70.6, 79.4 and 73.5% respectively with the N1 givingboth the highest detection percentage and the highest percentage (77%) of positive replicates (Perez-Cataluna et al., 2021).

Others focused only on N1 and N2 genes for detection as recommended by CDC (Li et al., 2022)

We aimed to detect SARS-CoV-2 in wastewater by qRT-PCR targeting different virus genes (S, N and E) using in house designed primers in wastewater samples collected from influent of a wastewater treatment plant (WWTP) in Egypt.

II. Material And Methods

Positive control:

Nasal sample was collected in March 2021 from SARS-CoV-2infected female Egyptian patient with mild symptoms (Rhinorrhea, chest pain, coughing fever and headache). Patient was positive for SARS-CoV-2 IgM/IgGby 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.

Wastewater samples

Two liters of wastewater were collected weekly through the period between August 2021 and March 2022 (21 samples in total) from influent ofZenin wastewater treatment plant (WWTP). It represents one of the largest WWTPs in Giza government, Egypt as it receives 275,000 to 550,000 m^3/day with an average of 385,000 m^3/day of wastewater flow (Latif 2022).

Waste water sample concentration by Adsorption-elution method (Katayama wt al., 2002):

A volume of 50 ml 1MAluminium chloride were added to 1 liter of waste water samples to give final concentration of 0.05M then pH was adjusted to be 3.5 by 1N HCl. Water samples were filtered through 0.45 µm nitrocellulose membrane (Hi-GenoMB, Mumbai, India) follwed by passing 200 mL of 0.5 mM H_2SO_4, pH 3.0 over the membrane to ensure viral particle retention and eliminate all biosolids. Membrane was removed from holder and put in a petridishfollowed by adding 11 mL of 1 mMNaOH, pH 10.5 over its surface and left for 10 minutes, then its upper surface was scrapedfor eluting viruses. Then elute was neutralized by adding 50 µL of 50 mM $H_2SO_4$and 50 µL of 1nM Tris–EDTA, pH 8.0 and stored at -20^{o}C till use. Of note, each sample was filtered through two nitrocellulose membranes, 1 liter each to avoid its blocking and the received elutes were finally mixed.

Quantitative RT-PCR (qRT-PCR)

Concentrated water samples as well as positive control were subjected to viral RNA extraction (QIAGEN; Hilden, Germany),reverse transcription using the reverse primer of each gene (super script III Reverse transcriptase, Invitrogen, USA). cDNA from individual samples was subjected for quantitative PCR using theunrevealed florescent dye read on SYBR/FAM channel(Luna, New England) and in-house primers (Table 1). Reaction mixture was subjected to the following cyclingconditions, initial denaturation: 95^{o}C for 60 sec., denaturation at 95^{o}Cfor 15 sec., annealing: 57oC for 10 sec and extension at 60^{o}C for 15 sec. for 40 cycles (Buston et al., 2009).

Table 1: Sets of in house primers used inviral detection.

SARS-CoV-2 target gene	Primer sequence
S-gene	F:AATTACCCCCTGCATACACTAA
	R:ATGGAACCAAGTAACATTGGAAA
N-gene	F:ATTCAACTGGCAGTAACCAGAA
	R:TTAAGGTCTTCCTTGCCATGTT
E-gene	F:TGTACTCATTCGTTTCGGAAGA
	R:ACGCACACAATCGAAGCGCA

The sensitivity of the N-gene-specific primers in the SARS-CoV-2 qRT-PCR detection was carried out as previously described. RNA concentrations 150, 100, 50, 25, 12.5, 10, 7.5, 5, 2.5, 1.25 ng, 750, 500, 250, 125, 75, 50, 25, 12.5, 10, 7.5, 5 and 2.5pg were used to prepare cDNAwhich was used in the qRT-PCR reaction mixture.

Multiple alignment of primer pair on all SARS-CoV-2 variants

The Clustal Omega version 1.2.4 online tool was used to perform multiple alignments of the used N-specific primer pair to one example of each SARS-CoV-2 variant referred to by CDC (CDC, 2020) in addition to the omicron recent Egyptian isolate.

III. Results and discussion

Since the beginning of the novel coronavirus pandemic, various reports have been concerned with the detection of SARS-CoV-2 in wastewater. This was due to virus shed in different human excretory/secretory products including stool (main source of viral RNA in wastewater), vomiting, nasal discharge and sputum (Wang et al., 2020) which all reach wastewater. Thus, scientist detectedSARS-CoV-2 (or its RNA) in wastewater and use it to guess (approximately estimate) the number of infected humans in a given area (Medema et al., 2020).

Here, we collected wastewater samplesthrough period between August 2021 and March 2022 thus including summer vacation months and school months where gathering of people specially children increase rate of spreading of respiratory viruses.

Although reports concerned with wastewater sample concentration used polyethylene glycol (PEG) precipitation technique (Farkas et al 2021) which utilizes a maximum sample volume of 200-250 ml, here we used the adsorption elution method as it gives the chance to filter larger volume of the sample (1-2 liters) thus increases the possibility for viral detection.This was also supported by others (Perez-Cataluna et al., 2021) who inoculated wastewater samples with a number of enveloped and non-enveloped viruses,compared both concentration methodsandresults showed higher recovery rate on using the aluminum-based adsorption method.

Weused in house designed primers targetingthe S, N and E genes for SARS-CoV-2 detection in wastewater. On using oligo-dTin reaction mixture of reverse transcriptionof RNA all wastewater samples were negativein conventional RT-PCR (cRT-PCR), although it worked on detecting virus from clinical samples as nasal or throat swabs (Park et al., 2020).This might be due to1) presence of RNA of different organisms in wastewater samples that masked our target genes, 2) the limited sensitivity of cRT-PCR.

On using our homemade designed primers targeting the S-gene in quantitative RT-PCR (qRT-PCR), CT values showed presence of SARS-CoV-2 in 90% (19/21)of the wastewater samples (Figure 1).Althoughmany samples showed high CT values (33-38), yet, they are still in the accepted range.

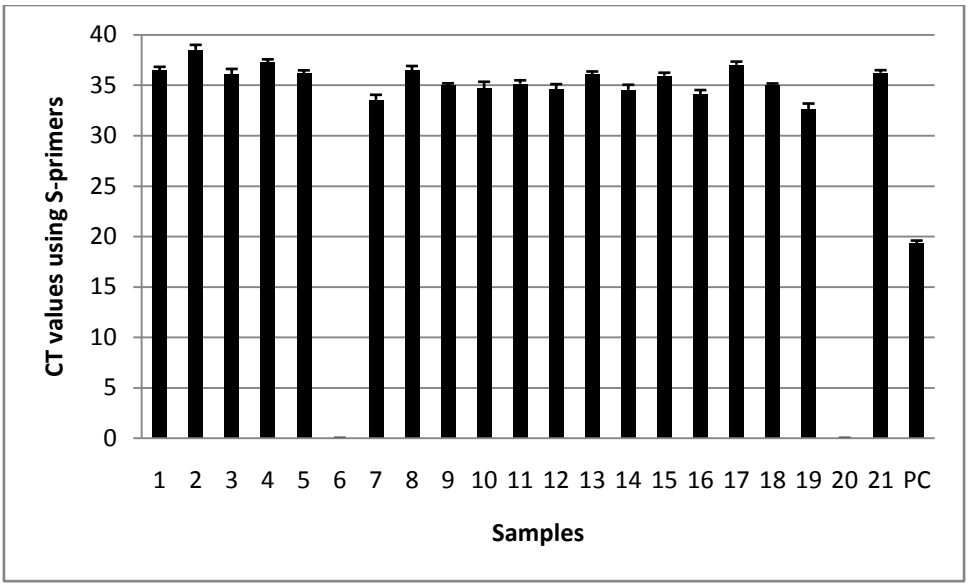


Figure 1:CT values for qRT-PCR detection of the SARS-CoV-2 in concentrated wastewater samples compared to positive control (PC) using homemade S-gene-specific primers of the virus.

Using of N-gene-specific primers showed better sensitivity as 95.2 % (20/21) of the samples were SARS-CoV-2-positive withCT values rangingbetween 31-34 except for two samples which showed amplification aftergave 36 and 38 cycles and one sample which was positive for the N-gene and negative for the S-gene (Figure 2). This might be due to mutation in S-gene to corresponding sequence of our used primer on the viral genome leading to decrease in annealing capability.

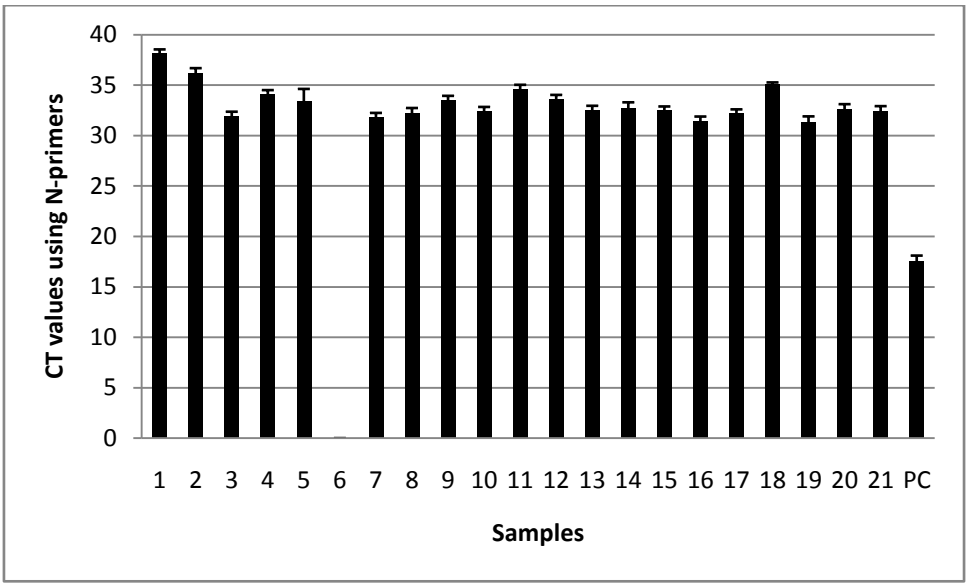


Figure 2:CT values for qRT-PCR detection of the SARS-CoV-2 in concentrated wastewater samples compared to positive control (PC) using homemade N-gene-specific primers of the virus.

On using E-gene as target for SARS-CoV-2 detection results showed that only 76% (16/21) samples were positive (Figure 3). Actually that agreed with number of reports wheredifferent targets were used for SARS-CoV-2 detection and results confirmed that the E-gene showed the poorest detection sensitivity compared toothertarget gene (Khoshchehreh et al., 2020; Perez-Cataluna et al., 2021) which was attributed to the very low expression level of the E-gene in SARS-Cov-2 (Kim et al., 2020).

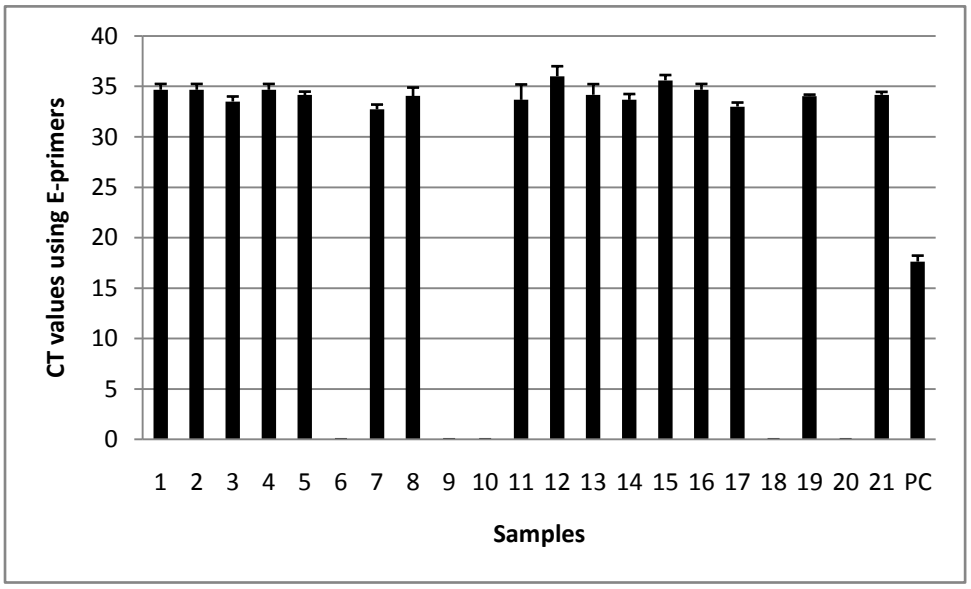


Figure 3:CT values for qRT-PCR detection of the SARS-CoV-2 in concentrated wastewater samples compared to positive control (PC) using homemade E-gene-specific primers of the virus.

In agreement, previous report recommended usage of N-gene primers for SARS-CoV-2 detection in concentrated wastewater samples as the low expression level of the E-gene caused missing of some infected samples as well as the continuous recorded mutations in S-gene (Li et al., 2020)made it not the perfect target for detection.

Since presence of viral genome in wastewater samples is highly diluted beside that the wastewater concentration techniques had recovery limits, altogether, highlighted the need for highly sensitive and specific assays to avoid missing infected samples during detection. Therefore,we tested the sensitivity of our in house N-gene primers to check its concentration limits for qRT-PCR detection of SARS-CoV-2.

Serial concentrations of positive control SARS-CoV-2 RNA ranging 150, 100, 50, 25, 12.5, 10, 7.5, 5, 2.5 and 1.25 ng RNA/reaction mixture and 750, 500, 250, 125, 75, 50, 25, 12.5, 10, 7.5, 5, 2.5pg RNA/reaction

mixture were used in qRT-PCRand results showed that at RNA concentration 150ng/reaction CT value was 17.97 and it increased gradually with decreasing RNA concentrations to reach 37.44 at RNA concentration 2.5 pg/reaction mixture confirming the high sensitivity of the N-specific primers even at very low template concentrations(Figures 4 & 5).

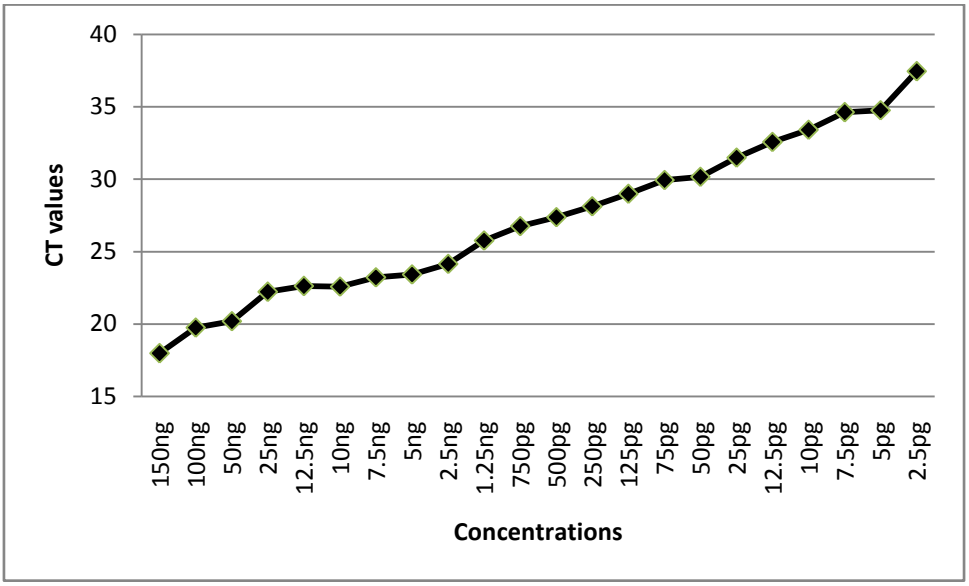
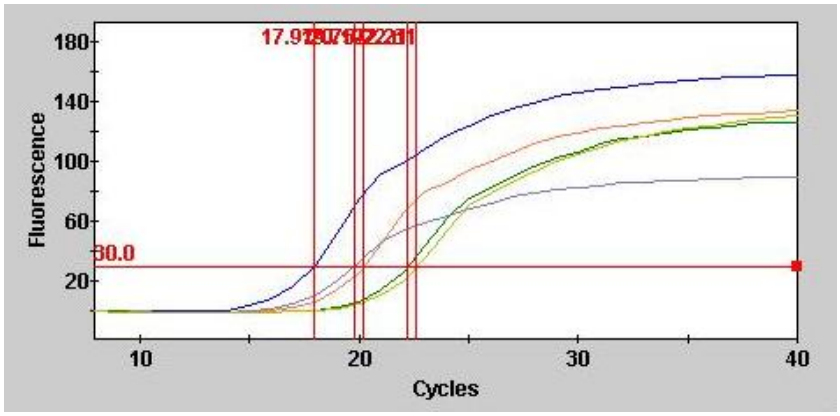
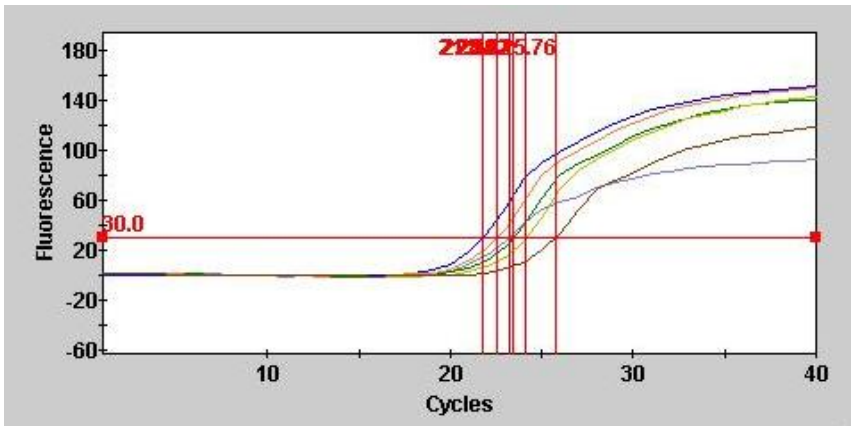


Figure 4: CT values of SARS-CoV-2 detection N-gene-specific primers at serial concentrations of the virus RNA. Results revealed extremely high detection sensitivity even at very low viral RNA concentrations.

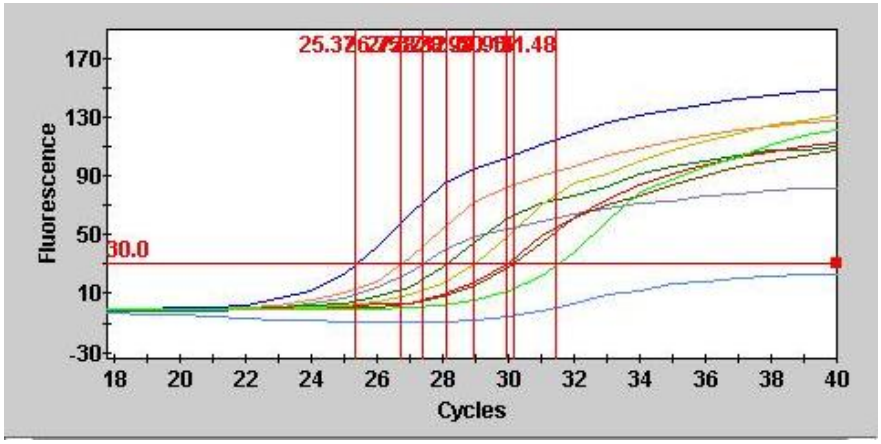
A



B



C



D

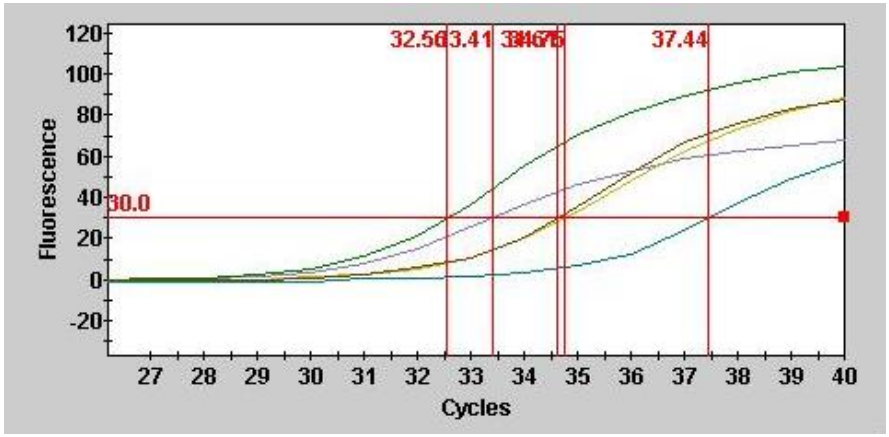


Figure 5:CT values of qRT-PCR of different cDNA volumes equivalent to A: 150, 100, 50, 25, 12.5 ng RNA/ reaction mixture, B: 12.5, 10, 7.5, 5, 2.5, 1.25 ng RNA/ reaction mixture, C: 1250, 750, 500, 250, 125, 75, 50, 25 pg RNA/ reaction mixture and negative control and D: 12.5, 10, 7.5, 5 and 2.5 pg RNA/ reaction mixture.

To confirm that our N-gene targeting primers were suitable for all SARS-CoV-2 variants, multiple alignmentof the primer pair was carried out on various SARS-CoV-2 variants using the Clustal Omega online tool.Results showed that our N-specific primer pair showed 100 % identity to all checked SARS-CoV-2 variants (Summarized in Table 2) except for zeta P2 variant (Figure 6).

Table 2: Variants included in the multiple alignments of our homemade SARS-CoV-2 N-specific primers.

Variant name	Accession number on NCBI	Variant name	Accession number on NCBI
Wuhan isolate, reference isolate	NC_045512.2	**Kappa B.1.617.1**	MZ571142.1
Beta B.1.351	MZ433432.1	**Zeta P2**	FJ882963.1
Beta	OM366051.1	**Omicron BA.1**	OP090659.1
gamma	OM367886.1	**Omicron BA.4**	OP093374.1
Delta B.1.617.2	OK091006.1	**Omicron BA.5**	OP062268.1
Eta B.1.525	MZ362439.1	**Omicron (Egyptian isolate)**	OP183454.1
Iota B.1.526	MZ702413.1		

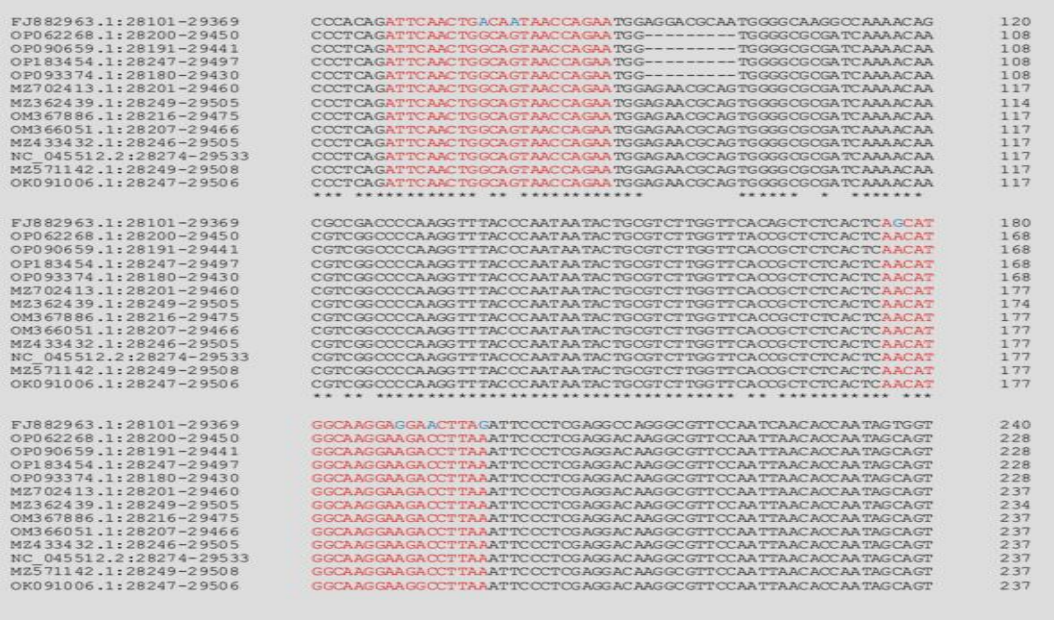


Figure 6: Multiple alignment of our homemade N-specific primer pair to SARS-CoV-2 variants by using the Clustal Omega online tool. Forward primer was marked in red in first raw, reverse and complement of reverse primer was marked in red between second and third columnsand mismatching nucleotides of the zeta P2 variant were marked in blue.

IV. Conclusions:

Detection of SARS-CoV-2 in wastewater samples collected from influent of Zenin WWTP revealed presence of the virus in 95% of the collected samples. Detection of SARS-CoV-2 in wastewater is highly affected by target gene and primers used for detection as well as the experimental conditions. Based on our results, we recommend usage of our homemade primer targeting the SARS-CoV-2 N-gene that showed very high sensitivity even at extremely diluted template virus RNA concentrations upon detection and 100 % annealing with all SARS-CoV-2 variants referred to by CDC as well as all omicron variants.

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Conflict of interest
All authors declare no conflict of interest.

Referances

[1]. Ahmed, W., Bivins, A., Simpson, S.L., Smith, W.J.M., Metcalfe, S., McMinn, B., Symonds, E.M., Korajkic, A. 2021 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.Sci Total Environ, 10, 799:149386.

[2]. 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 .2009 The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem., 55 (4): 611-622.

[3]. CDC: https://www.cdc.gov/coronavirus/2019-ncov/variants/variant-classifications.html.

[4]. Farkas, K. , Hillary, L.S., Thorpe, J., Walker, D.I., Lowther,J.A. ,McDonald, J.E., Malham,S.k., Jones, D.L., 2021. Concentration and Quantification of SARS-CoV-2 RNA in Wastewater Using Polyethylene Glycol-Based ConcentrationandqRT-PCR.MethodsProtoc,4: 17.https://doi.org/10.3390/mps4010017.

[5]. Katayama, H., Shimasaki, A., Ohgaki, S. 2002 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.

[6]. Kim,D., Lee,J., Yang, J., Kim, J.W., Kim, V.N., Chang, H. 2020 The Architecture of SARS-CoV-2 Transcriptome. Cell, 181: 914-921

[7]. Khoshchehreh, M., Wald-Dickler, N., PaulHoltom, P., Butler-Wu, S.M. 2020 A needle in the haystack? Assessing the significance of envelope (E) gene-negative, nucleocapsid (N2) gene-positive SARS-CoV-2 detection by the Cepheid Xpert Xpress SARS-COV-2 assay.JClinVirol, 133:104683.doi: 10.1016/j.jcv.2020.104683.

[8]. Latif, E.F. 2022 Applying novel methods in conventional activated sludge plants to treat low-strength wastewater. Environ. Monit.Assess: 194:323.

[9]. Li, Q., Wu, J., Nie, J., Zhang, L., Hao, H., Liu, S., Zhao, C., Zhang, Q., Liu, H., Nie, L., Qin, H., 2020. The impact of mutations in SARS-CoV-2 spike on viral infectivity and antigenicity. Cell, 182 (5), 1284–1294.

[10]. Li, L., Mazurowski, L., Dewan, A., Carine, M., Haak, L., Guarin, T.C., Dastjerdi, N.G., Gerrity, D., Mentzer, C., Pagilla, K.R. 2022 Longitudinal monitoring of SARS-CoV-2 in wastewater using viral genetic markers and the estimation of unconfirmed COVID-19 cases. Science of the Total Environment, 817: 152958.

[11]. Medema, G., Heijnen, L., Elsinga, G., Italiaander, R., Brouwer, A. 2020 Presence of SARS Coronavirus-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.doi: 10.1021/acs.estlett.0c00357.

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

[13]. ParkM.,WonJ., ChoiB. Y., LeeC. J.2020 Optimization of primer sets and detection protocols for SARS-CoV-2 of coronavirus disease 2019 (COVID-19) using PCR and real-time PCR. Experimental and Molecular Medicine, 52(6), 963–977.

[14]. Pérez-Cataluña , Cuevas-Ferrando, E., Walter Randazzo, W., Irene Falcó, I., Ana Allende, A., Gloria Sánchez, G. 2021Comparing analytical methods to detect SARS-CoV-2 in wastewater. Science of the Total Environment, 758: 143870.

[15]. 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. 2021 Detection of SARs-CoV-2 in wastewater using the existing environmental surveillance network: A potential supplementary system for monitoring COVID-19 transmission. PLOSONE, 16(6): e0249568. https://doi.org/10.1371/journal.pone.0249568.

[16]. Thongpradit, S., Prasongtanakij, S., SupanartSrisala, S., Kumsang, Y., Chanprasertyothin, S., Boonkongchuen, P., Pitidhammabhorn, D., Manomaipiboon, P., Somchaiyanon, P., Chandanachulaka, S., Hirunrueng, T., Ongphiphadhanakul, B. 2022 A Simple Method to Detect SARS-CoV-2 in Wastewater at Low Virus Concentration. Journal of Environmental and Public Health, 2022.

[17]. Wang, W.,Xu, Y., Gao, R., Lu, R., Han, K., Wu, G., Wenjie Tan, W. 2020 Detection of SARS-CoV-2 in Different Types of Clinical Specimens. JAMA, 323(18):1843-1844. doi:10.1001/jama.2020.3786.

[18]. 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. 2020 Virological assessment of hospitalized cases of coronavirus disease 2019. Nature, 581: 465–469.

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