Supporting Information

Detection, Quantification, and Simplified Wastewater Surveillance Model of SARS-CoV-2 RNA in the Tijuana River

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1 Supplemental Materials and Methods

Samples were analyzed for SARS-CoV-2 (accession number: MN908947.3) nucleocapsid (N) gene via N1 region with starting position 28287 and N2 region with starting position 29164, as well as PMMoV (accession number: NC_003630.1) with starting position 1878. The US CDC has stated that these two primers for SARS-CoV-2 provide useful information for epidemiology and surveillance, and they have been widely used for wastewater surveillance. In addition, an *in silico* BLAST (performed on March 28, 2022) using the primer set from N1 on the nr database in NCBI did not yield hits on any genes or genomes except for those associated with SARS-CoV-2, even when allowing up to two total mismatches.

The Taqman® Fast Virus 1-step master mix includes AmpliTaq® Fast DNA Polymerase, Thermostable MMLV enzyme, dNTPs (dATP, dGTP, dCTP, and dTTP), RNaseOUT™ Recombinant Ribonuclease Inhibitor, ROX™ dye (passive reference), and buffer components to control several common RT-PCR inhibitors. Results were analyzed via the ThermoFisher Design and Analysis software (v1.5.2). For RT-qPCR, the threshold was set at 0.09 for both nCoV-N1 and nCoV-N2 and 0.06 for PMMoV.

The LOD for RT-qPCR was determined for each assay using the pooled data from standards analyzed on all plates via Microsoft Excel, Version 2202[2].For nCoV-N1 and nCoV-N2, the LOD was 8 and 8.5 copies/reaction at 95% probability, respectively. For PMMoV the LOD was 5 copies/reaction at 95% probability. For SARS-CoV-2 plasmids, the DNA concentration range measured was 4 - 400,000 gc/rxn where the lowest dilution 4gc/rxn resulted in a standard deviation of 0.67 for all detected Cq values determined with N1 assay and 1.02 for all detected Cq values determined with N2 assay. For PMMoV gBlocks, the range measured was 1.56 – 1,560,000 gc/rxn where the lowest dilution 1.56gc/rxn resulted in a standard deviation of 1.3 for all detected Cq values.

Intra-assay repeatability for samples was checked by calculating the standard deviation of Cq values for technical replicates. The standard deviations were below 0.1 Cq units for most samples and were below 0.5 for all samples for the N1 and PMMoV assays. Only for the N2 assay, the standard deviation of the Cq values for technique replicates was greater than 0.5 for samples from Radio Club collected on 7/31/2020, 8/6/2020, and 3/14/2021. However, the Cq values of N2 for these three samples were close to and below the threshold associated with a 95% probability of detection.

Sample concentrations were determined via Microsoft Excel, Version 2202 using the Cq values obtained, based on the values of the standard curves analyzed on the same plate as the samples (to factor out any variability between plates). Visual outliers which corresponded to likely errors in pipetting or in the preparation of dilutions, such as wells that were unreliable due to human error were not included in the compiling of the standard curve for each assay. The plasmids and gBlocks are double stranded but the sample target RNA is single stranded, so the concentrations of the standards were divided by two prior to the completion of the regression to adjust for the offset of amplification of RNA in our samples compared to our standards.

A conversion quantified the number of copies per liter of sample using subsequent volumes that were filtered, extracted, reverse transcribed, and analyzed.

$$N = \frac{1}{C\%} \times \frac{1}{P\%} \times \frac{1}{D\%} \times \frac{1}{R\%} \times \frac{G}{V} \times \frac{1000mL}{L}$$

Where C% is the conversion factor of cDNA to RNA which was assumed to be 100% for one-step RT-qPCR kits, P% is the percent of extracted DNA used in qPCR in which 2 µL of sample was used in each well/50 µL extracted, D% is dilution of RNA sample which was either never diluted (100%) or diluted at 10%, R% is the nucleic acid recovery during the extraction process and assumed to be 100%, G is the concentration in copies/reaction unit as determined by Cq Values, and V is the volume of sample (mL) filtered through the 0.45 µm membrane. It should be noted that recovery was not measured using matrix spikes in this study, but it has previously been reported for murine hepatitis virus (MHV, a surrogate of SARS-CoV-2) to be 65% using the adsorption-extraction method with $MgCl_2$ addition, and 27% using the adsorption-extraction method with acidification but no $MgCl_2$ addition[3]. As such, the concentrations reported here may be underestimates of the true concentrations.

The Qubit 4 Fluorometer was used with the Qubit RNA XR Assay - Extended Range kit for quantification of RNA in extracted samples, and the Qubit 1X dsDNA HS Assay Kit was used for quantification of DNA in gBlock standards. The purity and yield of gBlocks and plasmids were quantified and verified by the manufacturer (IDT).

To reduce the possibility of cross-contamination, master mixes were prepared separately from samples and standards, in an AirClean 600 PCR Workstation. A biosafety cabinet was used to add samples and standards to the plate after the master mix was already aliquoted. Different sets of pipettes were also used in each of these stations to further reduce the possibility of nucleic acid contamination in the PCR workstations. Gloves were replaced after handling standards as an additional precaution. Spaces used for processing samples were disinfected before and after with 10% beach, 70% ethanol, and UV light. All equipment used to concentrate samples, such as beakers, graduated cylinders, and filtration devices were autoclaved using the Hirayama Hiclave HVA-100.

2 Supplemental Discussion

To assess the impact of differences in the fecal strength of Tijuana River samples[1], concentrations of SARS-CoV-2 were normalized by PMMoV (Figure S1). However, normalizing to PMMoV in this setting was not consistent from sample to sample, potentially suggesting that there is less impact of non-fecal sources of pollution and no need to normalize especially since it is a smaller sewer-shed.

3 Supplemental Figures

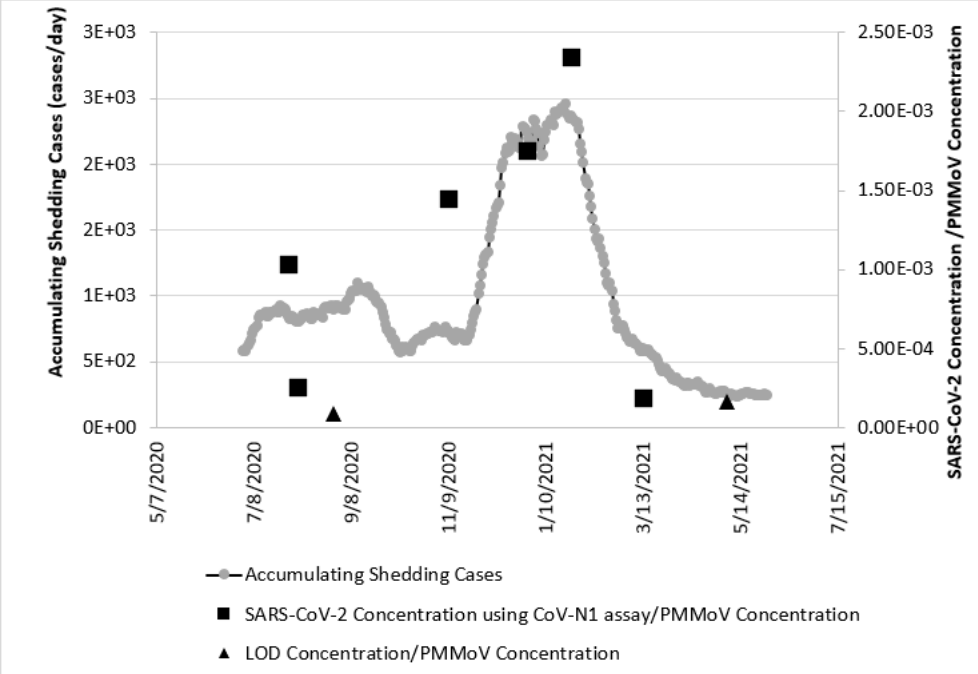

Figure S1. Changes in accumulating shedding COVID-19 cases with time and SARS-CoV-2 concentrations normalized to respective PMMoV concentrations at Tijuana River Radio Club site.

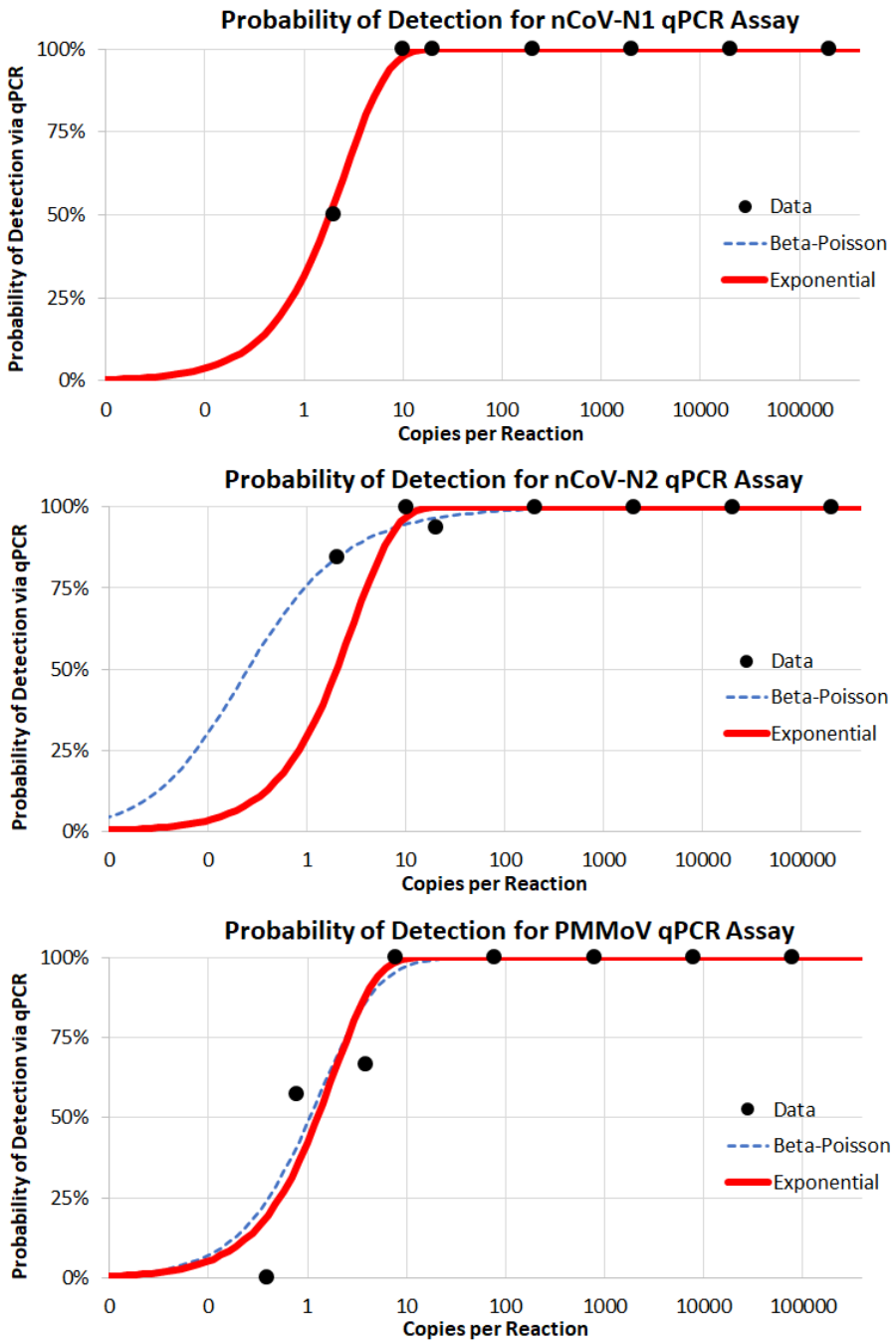

Figure S2. RT-qPCR Probability of Detection for nCoV-N1, nCoV-N2, and PMMoV.

4 Supplemental Tables

Table S1. Physical and chemical water quality parameters measured at the Boca Rio and Radio Club sampling sites

Date	DO (mg/L)	pH	Cond. (mS/cm)	TDS (g/L)	Turb. (NTU)	COD (mg/L)	DOC (mg/L)	TN (mg/L)
			Boca Rio					
31 Jul 2020	19.63	8.71	36.18	18.45	3.48	NM[a]	2.30	0.26
6 Aug 2020	NA[b]	7.76	38.05	19.05	7.22	NM[a]	6.88	1.59
28 Aug 2020	10.51	8.01	98.87	29.00	4.26	NM[a]	3.45	0.43
10 Nov 2020	NA[b]	7.16	44.97	22.53	11.62	NM[a]	7.03	3.94
30 Dec 2020	10.35	7.90	51.67	25.83	4.92	NM[a]	1.50	0.42
27 Jan 2021	9.72	7.99	55.10	26.53	7.28	NM[a]	2.53	1.10
14 Mar 2021	10.35	8.13	57.30	29.60	2.58	NM[a]	1.71	0.50
5 May 2021	5.54	7.98	56.80	29.60	7.65	NM[a]	2.98	0.84
Arithmetic Average	11.02	7.96	54.87	25.08	6.13	NM[a]	3.55	1.13
Standard Deviation	4.63	0.43	19.59	4.56	2.92	NM[a]	2.20	1.22
			Radio Club					
31 Jul 2020	0.49	7.92	2.44	1.22	59.05	256	16.56	32.20
6 Aug 2020	NA[b]	7.59	2.65	1.32	45.55	201	18.00	31.43
28 Aug 2020	3.75	7.96	4.97	1.49	90.00	234	35.53	9.33
10 Nov 2020	NA[b]	7.45	2.87	1.44	29.57	180	39.35	61.14
30 Dec 2020	6.01	7.91	2.97	1.49	59.47	144	38.80	55.99
27 Jan 2021	8.22	7.92	3.00	1.58	45.47	159	23.28	23.71
14 Mar 2021	6.01	7.71	2.79	1.46	23.87	113	18.47	56.26
5 May 2021	8.08	8.69	2.75	1.46	47.63	243	55.38	17.41
Arithmetic Average	5.43	7.89	3.05	1.43	50.08	191	30.67	35.93
Standard Deviation	2.92	0.37	0.79	0.11	20.42	51	13.83	19.59

Note: Dissolved oxygen (DO); electrical conductivity (Cond.); turbidity (Turb.); chemical oxygen demand (COD); dissolved organic carbon (DOC); total nitrogen (TN).

[a] Not measurable (NM) due to high chlorine interference.

[b] Not available (NA) due to inavailability of DO meter.

Table S2. Collection times and field conditions during sampling dates of Tijuana River

Sample Collection Date	Collection Time at Radio Club	Collection Time at Boca Rio	24-h Average Flow Rate at International Boundary (m^3/h)	Instantaneous Flow Rate at International Boundary[a] (m3/h)	72-h Precipitation Accumulated (mm)	Antecedent Dry Days[b] (d)	Mean Lower Low Water Tide Level[c] (m)	Field Notes
31 Jul 2020	9:20	10:14	1.15E+03	6.1E+02	0.0	32	1.1	-
6 Aug 2020	10:05	11:05	3.72E+02	1.1E+01	0.0	38	1.2	-
28 Aug 2020	12:20	13:20	2.90E+02	0.00E+00	0.0	60	0.8	Stagnant flow
10 Nov 2020	9:40	10:40	8.68E+02	3.6E+02	7.6	2	0.6	-
30 Dec 2020	10:16	11:12	4.94E+03	5.8E+03	14.0	3	1.3	-
27 Jan 2021	10:05	11:10	1.10E+04	9.4E+03	19.6	2	0.9	-
14 Mar 2021	10:20	11:28	8.32E+03	6.2E+03	48.2	2	1.5	-
5 May 2021	10:01	10:33	0.00E+00	0.00E+00	0.0	51	0.3	Stagnant flow; Abnormal green color

[a] Instantaneous flow rate measurements were obtained from the International Boundary Water Commission at the time of collection at Radio Club to the nearest 15-minute interval.

[b] Dry days were observed as 2.5 mm or less of precipitation, if any, following San Diego Water Board guidelines.

[c] Mean lower low water tide levels were obtained from the National Oceanic and Atmospheric Administration Imperial Beach, California station located 2.5 km northwest of Boca Rio.

Table S3. Effective Volume Filtered and Equivalent Volume for samples

Sample Collection Date	Effective Volume Filtered (mL)	Fraction of RNA Analyzed	Equivalent Volume Analyzed (mL)[b]
Boca Rio			
31 Jul 2020	198	2µL/50µL = 0.04	7.9
6 Aug 2020	198	2µL/50µL = 0.04	7.9
28 Aug 2020	495[a]	2µL/50µL = 0.04	19.8
10 Nov 2020	297	2µL/50µL = 0.04	11.9
30 Dec 2020	198	2µL/50µL = 0.04	7.9
27 Jan 2021	396	2µL/50µL = 0.04	15.8
14 Mar 2021	495	2µL/50µL = 0.04	19.8
5 May 2021	495	2µL/50µL = 0.04	19.8
Radio Club			
31 Jul 2020	48.6	2µL/50µL = 0.04	1.9
6 Aug 2020	49.5	2µL/50µL = 0.04	2.0
28 Aug 2020	99.0 [a]	2µL/50µL = 0.04	4.0
10 Nov 2020	49.5	2µL/50µL = 0.04	2.0
30 Dec 2020	39.6	2µL/50µL = 0.04	1.6
27 Jan 2021	69.3	2µL/50µL = 0.04	2.8
14 Mar 2021	69.3	2µL/50µL = 0.04	2.8
5 May 2021	99.0	2µL/50µL = 0.04	4.0

[a] Samples were pre-filtered using 8µm filter

[b] Equivalent Volume Analyzed = Equivalent Volume Filtered * Fraction of RNA Analyzed

Table S4. Primer/probe[a] sequences for each virus assay

Assay	Sequence (5'-3')	Amplicon Length	Accession Number (Start Position)	Reference(s)
nCoV-N1	F: GACCCCAAAATCAGCGAAAT R: TCTGGTTACTGCCAGTTGAATCTG [FAM]-ACCCCGCATTACGTTTGGTGGACC-[BHQ1]	72 bp	MN908947.3 (28287)	4
nCoV-N2	F: TTACAAACATTGGCCGCAAA R: GCGCGACATTCCGAAGAA [FAM]-ACAATTTGCCCCCAGCGCTTCAG-[BHQ1]	67 bp	MN908947.3 (29164)	4
PMMoV	F: GAGTGGTTTGACCTTAACGTTTGA R: TTGTCGGTTGCAATGCAAGT [FAM]-CCTACCGAAGCAAATG-[MGB]	68 bp	NC_003630.1 (1878)	5,6

[a] Primers were purified by standard desalting; probes were HPLC purified

Table S5. Slope, y-intercept, and efficiency of nCoV-N1, nCoV-N2, and PMMoV assays using TaqMan Fast Virus One Step RT-qPCR

Parameter	**nCoV-N1**	**nCov-N2**	**PMMoV**
slope	-3.20	-3.28	-3.41
intercept	37.27	39.15	37.28
efficiency	105%	102%	96%
R^2	0.9557	0.9559	0.9779

Table S6. MIQE checklist: Essential and Desirable Information[7]

ITEM TO CHECK	IMPORTANCE	CHECKLIST
EXPERIMENTAL DESIGN		
Definition of experimental and control groups	E	Included in Materials and Methods
Number within each group	E	Included in Materials and Methods
Assay carried out by core lab or investigator's lab?	D	Included in Materials and Methods
Acknowledgement of authors' contributions	D	Included in Acknowledgements
SAMPLE		
Description	E	Included in Materials and Methods/SI
Volume/mass of sample processed	D	Included in Materials and Methods/SI
Microdissection or macrodissection	E	Not Applicable
Processing procedure	E	Included in Materials and Methods
If frozen - how and how quickly?	E	Included in Materials and Methods
If fixed - with what, how quickly?	E	Not Applicable
Sample storage conditions and duration (especially for FFPE samples)	E	Included in Materials and Methods
NUCLEIC ACID EXTRACTION		
Procedure and/or instrumentation	E	Included in Materials and Methods
Name of kit and details of any modifications	E	Included in Materials and Methods
Source of additional reagents used	D	Not Applicable
Details of DNase or RNAse treatment	E	Not Applicable
Contamination assessment (DNA or RNA)	E	Included in Materials and Methods
Nucleic acid quantification	E	Included in SI
Instrument and method	E	Included in SI
Purity (A260/A280)	D	Included in SI
Yield	D	Included in SI
RNA integrity method/instrument	E	Not Determined
RIN/RQI or Cq of 3' and 5' transcripts	E	Not Determined
Electrophoresis traces	D	Not Determined
Inhibition testing (Cq dilutions, spike or other)	E	Included in Materials and Methods
REVERSE TRANSCRIPTION		
Complete reaction conditions	E	Included in Materials and Methods
Amount of RNA and reaction volume	E	Included in Materials and Methods
Priming oligonucleotide (if using GSP) and concentration	E	Not Applicable
Reverse transcriptase and concentration	E	Included in Materials and Methods
Temperature and time	E	Included in Materials and Methods

Manufacturer of reagents and catalogue numbers	D	Included in Materials and Methods
Cqs with and without RT	D*	Not Applicable
Storage conditions of cDNA	D	Not Applicable
qPCR TARGET INFORMATION		
Gene symbol	E	Included in SI
Sequence accession number	E	Included in SI
Location of amplicon	D	Included in SI
Amplicon length	E	Included in SI
In silico specificity screen (BLAST, etc)	E	Not Applicable
Pseudogenes, retropseudogenes or other homologs?	D	Not Applicable
Sequence alignment	D	Not Applicable
Secondary structure analysis of amplicon	D	Not Applicable
Location of each primer by exon or intron (if applicable)	E	Not Applicable
What splice variants are targeted?	E	Not Applicable
qPCR OLIGONUCLEOTIDES		
Primer sequences	E	Table S4 of SI document
RTPrimerDB Identification Number	D	Not Applicable
Probe sequences	D**	Table S4 of SI document
Location and identity of any modifications	E	Table S4 of SI document
Manufacturer of oligonucleotides	D	Included in Materials and Methods
Purification method	D	Included in SI
qPCR PROTOCOL		
Complete reaction conditions	E	Included in Materials and Methods
Reaction volume and amount of cDNA/DNA	E	Included in Materials and Methods
Primer, (probe), Mg++ and dNTP concentrations	E	Included in Materials and Methods
Polymerase identity and concentration	E	Included in Materials and Methods
Buffer/kit identity and manufacturer	E	Included in Materials and Methods
Exact chemical constitution of the buffer	D	Not Provided
Additives (SYBR Green I, DMSO, etc.)	E	Included in Materials and Methods
Manufacturer of plates/tubes and catalog number	D	Included in Materials and Methods
Complete thermocycling parameters	E	Included in Materials and Methods
Reaction setup (manual/robotic)	D	Included in Materials and Methods
Manufacturer of qPCR instrument	E	Included in Materials and Methods
qPCR VALIDATION		
Evidence of optimisation (from gradients)	D	Not Determined
Specificity (gel, sequence, melt, or digest)	E	Not Determined

For SYBR Green I, Cq of the NTC	E	Not Applicable
Standard curves with slope and y-intercept	E	Included in SI
PCR efficiency calculated from slope	E	Included in SI
Confidence interval for PCR efficiency or standard error	D	Not Determined
R^2 of standard curve	E	Included in SI
Linear dynamic range	E	Included in SI
Cq variation at lower limit	E	Included in SI
Confidence intervals throughout range	D	Not Determined
Evidence for limit of detection	E	Included in SI
If multiplex, efficiency and LOD of each assay.	E	Not Applicable
DATA ANALYSIS		
qPCR analysis program (source, version)	E	Included in SI
Cq method determination	E	Included in SI
Outlier identification and disposition	E	Included in SI
Results of NTCs	E	Included in Materials and Methods
Justification of number and choice of reference genes	E	Not Applicable
Description of normalisation method	E	Included in SI
Number and concordance of biological replicates	D	Not Determined
Number and stage (RT or qPCR) of technical replicates	E	Included in Materials and Methods
Repeatability (intra-assay variation)	E	Included in SI
Reproducibility (inter-assay variation, %CV)	D	Not Determined
Power analysis	D	Not Determined
Statistical methods for result significance	E	Not Applicable
Software (source, version)	E	Included in SI
Cq or raw data submission using RDML	D	Not Provided

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