Supporting information for

More than a tripledemic: Influenza A Virus, Respiratory Syncytial Virus, SARS-CoV-2, and Human Metapneumovirus in wastewater during winter 2022-2023

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Additional details of sample collection. At seven of the eight POTWs, settled solids were collected from the primary clarifier. Settled solids samples were grab samples except for at SJ where staff collected a 24-h composite sample; samples were collected every 6 hours and composited[1]. At Gil, solids were settled from a 24-h composite influent sample using standard method 160.5[2].

Additional details of sample processing and nucleic-acid extraction. Briefly, solids were dewatered by centrifugation, and an aliquot of dewatered solids was dried to determine its dry weight, and another aliquot was resuspended in the bovine coronavirus (BCoV)-spiked DNA/RNA shield (Zymo Research) at a concentration of 75 mg/ml. Bovine coronavirus (BCoV) was used as a positive recovery control. This concentration of solids was chosen as previous work titrated solutions with various concentrations of solids to identify a concentration that minimized inhibition while maintaining sensitivity of the assays[3,4]. The suspension was homogenized, and then centrifuged. The supernatant was subjected to nucleic acid extraction. RNA was extracted from 10 replicate supernatant aliquots per sample. For each replicate, RNA was extracted from 300 µl of homogenized sample using the Chemagic Viral DNA/RNA 300 kit H96 for the Perkin Elmer Chemagic 360 followed by PCR inhibitor removal with the Zymo OneStep-96 PCR Inhibitor Removal kit.

Additional details of digital droplet RT-PCR. Digital droplet PCR was performed on 20-µl samples from a 22-µl reaction volume, prepared using 5.5-µl template, mixed with 5.5 µl of One-Step RT-ddPCR Advanced kit for Probes (catalog no. 1863021; Bio-Rad), 2.2 µl reverse transcriptase, 1.1 µl dithiothreitol (DTT), and primers and probes at a final concentration of 900 nM and 250 nM, respectively. Droplets were generated using the AutoDG Automated Droplet Generator (Bio-Rad). PCR was performed using Mastercycler Pro with the following protocol: reverse transcription at 50°C for 60 min, enzyme activation at 95°C for 5 min, 40 cycles with 1 cycle consisting of denaturation at 95°C for 30 s and annealing and extension at either 59°C or 61°C (for human viruses) or 56°C (for PMMoV/BCoV duplex assay) for 30 s, enzyme deactivation at 98°C for 10 min, and then an indefinite hold at 4°C. The ramp rate for temperature changes was set at 2°C/s, and the final hold at 4°C was performed for a minimum of 30 min to allow the droplets to stabilize.

All samples were processed for HMPV in multiplex as described in the Data Descriptor by Boehm et al.[4]. For the remaining human viral assay, samples collected between 7/1/22 and 3/11/23, were processed in multiplex as described in the Data Descriptor by Boehm et al.[4] Thereafter, for all samples collected between 3/12/23 and 5/7/23 (month/day/year format), assays for SARS-CoV-2 N gene (FAM), RSV (Cy5), and IAV (Cy5.5) were multiplexed using the probe mixing method; the multiplex assay also contained assays for influenza B and norovirus GII for which results are not presented herein. This assay was run at an annealing temperature of 61°C. BCoV and PMMoV were run in a duplex assay.

Each sample was run in 10 replicate wells. On each 96 well plate, extraction-negative controls were run in 3 wells, and extraction-positive controls in 1 well. PCR-positive controls of SARS-CoV-2, IAV, RSV, HMPV, BCoV, and PMMoV were run in 1 well, and no-template controls (NTC) were run in 3 wells. Positive controls consisted of BCoV and PMMoV gene block controls and the same human virus controls described in the main text as positive extraction controls. Results from replicate wells were merged for analysis.

Droplets were analyzed using the QX200 or QX600 Droplet Reader (Bio-Rad). Each sample was run in 10 replicate wells on 96 well plates that also contained positive and negative extraction and PCR controls. Thresholding was done manually using QuantaSoft Analysis Pro software (Bio-Rad, version 1.0.596). We have found that the automatic thresholding is not accurate. We thresholded the entire plate by referencing the positive and negative controls, and then examined the output of every individual well to ensure the thresholding appropriately separated negative and positive populations. In order for a sample to be recorded as positive, it had to have at least three positive droplets. Three positive droplets corresponds to a concentration between ~500 and 1000 copies (cp)/g dry weight; the range in values is a result of the range in the equivalent mass of dry solids added to the wells. Any plates for which negative controls were positive or positive controls were negative were discarded and the samples re-processed and rerun.

IAV subtype assay design and testing. To design the primers and probes, influenza A genome sequences were downloaded from NCBI in January 2023 and aligned to identify conserved regions in the specified regions of the genome. Then primers and probes targeting those conserved regions were developed in silico using Primer3Plus (https://primer3plus.com/) (Table S2).

Primers and probes were then screened for specificity in silico, and in vitro against other respiratory and enteric viruses. including adenoviruses, coronaviruses, metapneumovirus, parainfluenza, RSV, coxsackievirus, echovirus, parechovirus (NATtrol Respiratory Verification Panel NATRVP2.1-BIO and NATtrol EV Panel NATEVP-C viral panels, Zeptometrix, Buffalo, NY), as well as synthetic genomic RNA from influenza A H1N1 and influenza A H3N2 (Twist Bioscience, South San Francisco, CA).

Measuring IAV subtype markers in wastewater solids. RNA extracts from the two POTWs (SJ and Ocean) were stored at -80°C for 1-5 months. RNA was subjected to a single freeze-thaw and used as template undiluted in digital droplet PCR using the same methods outlined above. Assays were run in multiplex using the probe mixing approach. H1 and N1 were multiplexed in the HEX and Cy5.5 channels, respectively. H3, N2, and IAV were multiplexed in the HEX, FAM/HEX, and ROX channels. Ten replicates were run for each sample, for each sample with the same number of positive and negative extraction and PCR controls per plate as described for the prospective measurements described in the main text. Positive controls consisted of synthetic genomic RNA from influenza H1N1 and H3N2 (Twist). Results are reported as copies per gram dry weight.

Additional description of clinical testing data. CDPH does not publish data on the number of tests provided across the state in sentinel laboratories for influenza, RSV, and HMPV. However, CDC does provide some indication of these numbers through their FluView website for influenza

(https://www.cdc.gov/flu/weekly/fluactivitysurv.htm) and their National Respiratory and Enteric Virus Surveillance System (NREVSS) website for RSV (https://www.cdc.gov/surveillance/nrevss/rsv/state.html#CA). However, it should be noted that the exact data used to generate the CDC positivity rates for influenza and RSV are not the exact same as those used to generate the data provided at the CDPH website, so the number of tests described next should be viewed as approximate. In the time period represented by the current study, the state of California clinical laboratories reporting to NREVSS tested a median of 8767 (interquartile range (IQR) = 5330 - 13482) clinical samples per week for influenza and a median 7500 (IQR = 4300 - 9900) clinical samples per week for RSV. Note that CDC only reports three-week aggregated means for the number of RSV tests. The number of HMPV tests is not publicly available, but according to CDPH, during the time period of this study a median of 1993 (IQR = 1574 - 2115) HMPV tests per week were administered by state sentinel laboratories.

The number of COVID-19 tests administered across the state of California per day is publicly available at the weblink provided in the main text of the paper. The median daily number of COVID-19 tests administered in the state during the time period of this study was 65666 (IQR = 42084 - 102078) per day.

According to Figure S3, the peak in positivity rates for influenza, RSV, and COVID-19 occurred in November 2022, December 2022, and December 2022, respectively. The peak in positivity rates for HMPV occurred in March 2023. It should be noted that positivity rates for influenza, RSV, and HMPV are biased because those tests are usually only administered by clinicians on individuals suffering from severe illnesses or with comorbidities. Also, the data for influenza, RSV, and HMPV represent results from sentinel laboratories across the state, and do not include results from outside the sentinel laboratories. COVID-19 test positivity rates are also biased because with the increased availability of at-home antigen tests, the results of which are not reportable to public health agencies in California, the number of COVID-19 testing rates have decreased and may be limited to severely ill individuals[5].

Correlations between wastewater and clinical positivity rates. IAV in wastewater solids was significantly associated with state-aggregated weekly clinical specimen influenza positivity rates at all POTWs (tau between 0.50 and 0.72, all $p<10^{-5}$) except for SVCW and Sun where the association was not statistically significant. Weekly median normalized RSV in wastewater solids was significantly associated with state-aggregated weekly clinical specimen RSV positivity rates at all plants (tau between 0.61 and 0.69, all $p<10^{-8}$). Weekly median normalized HMPV in wastewater solids was significantly associated with state-aggregated weekly clinical specimen HMPV positivity rates at all plants (tau between 0.53 and 0.73, $p<10^{-6}$). Daily normalized SARS-CoV-2 was positively associated with daily state-aggregated COVID-19 positivity rates (tau between 0.26 and 0.46, all $p<10^{-11}$), as has been shown extensively in previous work.[1,6]

References

(1) Wolfe, M. K.; Topol, A.; Knudson, A.; Simpson, A.; White, B.; Duc, V.; Yu, A.; Li, L.; Balliet,

M.; Stoddard, P.; Han, G.; Wigginton, K. R.; Boehm, A. High-Frequency, High-Throughput Quantification of SARS-CoV-2 RNA in Wastewater Settled Solids at Eight Publicly Owned Treatment Works in Northern California Shows Strong Association with COVID-19 Incidence. *mSystems* **2021**, *6* (5), e00829-21. https://doi.org/10.1128/mSystems.00829-21.

(2) AWWA. *Standard Methods for the Examination of Water and Wastewater*, 21st ed.; Eaton, A. D., Clesceri, L. S., Rice, E. W., Greenberg, A. E., Series Eds.; American Public Health Association, American Water Works Association, Water Environment Federation: Baltimore, 2005.

(3) Huisman, J. S.; Scire, J.; Caduff, L.; Fernandez-Cassi, X.; Ganesanandamoorthy, P.; Kull, A.; Scheidegger, A.; Stachler, E.; Boehm, A. B.; Hughes, B.; Knudson, A.; Topol, A.; Wigginton, K. R.; Wolfe, M. K.; Kohn, T.; Ort, C.; Stadler, T.; Julian, T. R. Wastewater-Based Estimation of the Effective Reproductive Number of SARS-CoV-2. *Environmental Health Perspectives* **2022**, *130* (5), 057011–1.

(4) Boehm, A. B.; Wolfe, M. K.; Wigginton, K. R.; Bidwell, A.; White, B. J.; Hughes, B.; Duong; Chan-Herur, V.; Bischel, H. N.; Naughton, C. C. Human Viral Nucleic Acids Concentrations in Wastewater Solids from Central and Coastal California USA. *Scientific Data* **2023**, *10*, 396.

(5) Radar, B.; Gertz, A.; Iuliano, A. D.; Gilmer, M.; Wronski, L.; Astley, C. M.; Sewalk, K.; Varrelman, T.; Cohen, J.; Parikh, R.; Reese, H. E.; Reed, C.; Brownstein, J. S. Use of At-Home COVID-19 Tests — United States, August 23, 2021–March 12, 2022. *MMWR Morb Mortal Wkly Rep* **2022**, *71*, 489–494. http://dx.doi.org/10.15585/mmwr.mm7113e1.

(6) Duvallet, C.; Wu, F.; McElroy, K. A.; Imakaev, M.; Endo, N.; Xiao, A.; Zhang, J.; Floyd-O'Sullivan, R.; Powell, M. M.; Mendola, S.; Wilson, S. T.; Cruz, F.; Melman, T.; Sathyanarayana, C. L.; Olesen, S. W.; Erickson, T. B.; Ghaeli, N.; Chai, P.; Alm, E. J.; Matus, M. Nationwide Trends in COVID-19 Cases and SARS-CoV-2 RNA Wastewater Concentrations in the United States. *ACS EST Water* **2022**, *2* (11), 1899–1909. https://doi.org/10.1021/acsestwater.1c00434.

(7) Borchardt, M. A.; Boehm, A. B.; Salit, M.; Spencer, S. K.; Wigginton, K. R.; Noble, R. T. The Environmental Microbiology Minimum Information (EMMI) Guidelines: QPCR and DPCR Quality and Reporting for Environmental Microbiology. *Environ. Sci. Technol.* **2021**, *55* (15), 10210–10223. https://doi.org/10.1021/acs.est.1c01767.

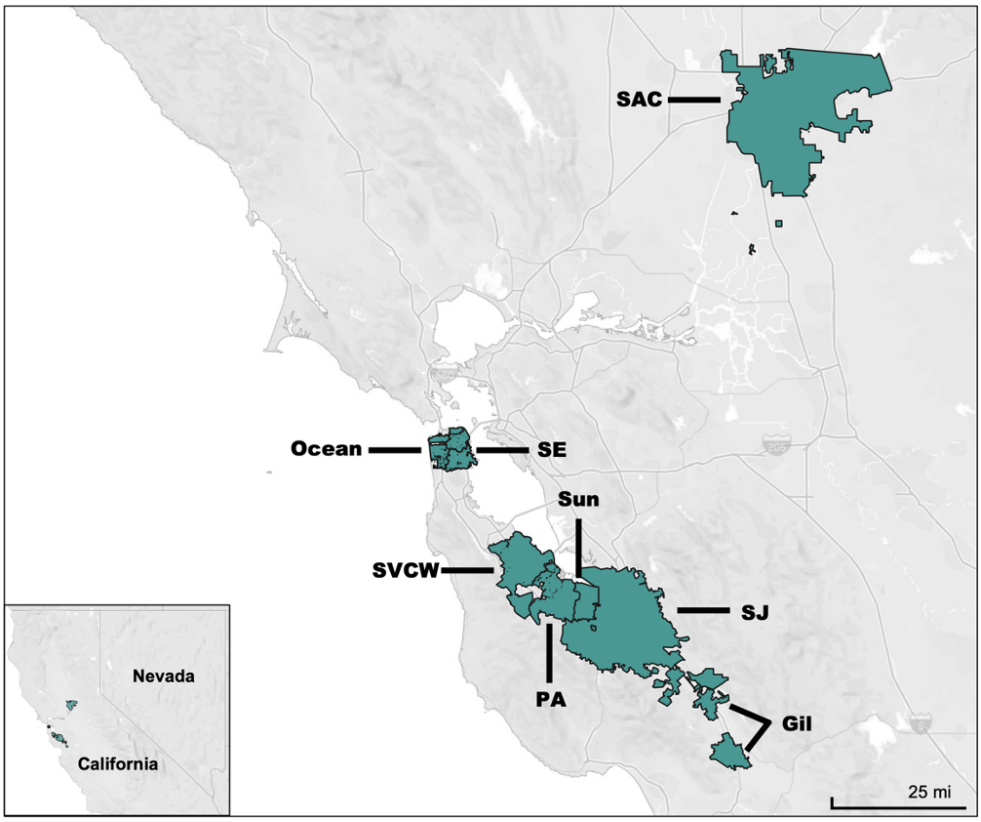

Figure S1. Map of sewersheds participating in the study. The most distant sites are 175 km apart.

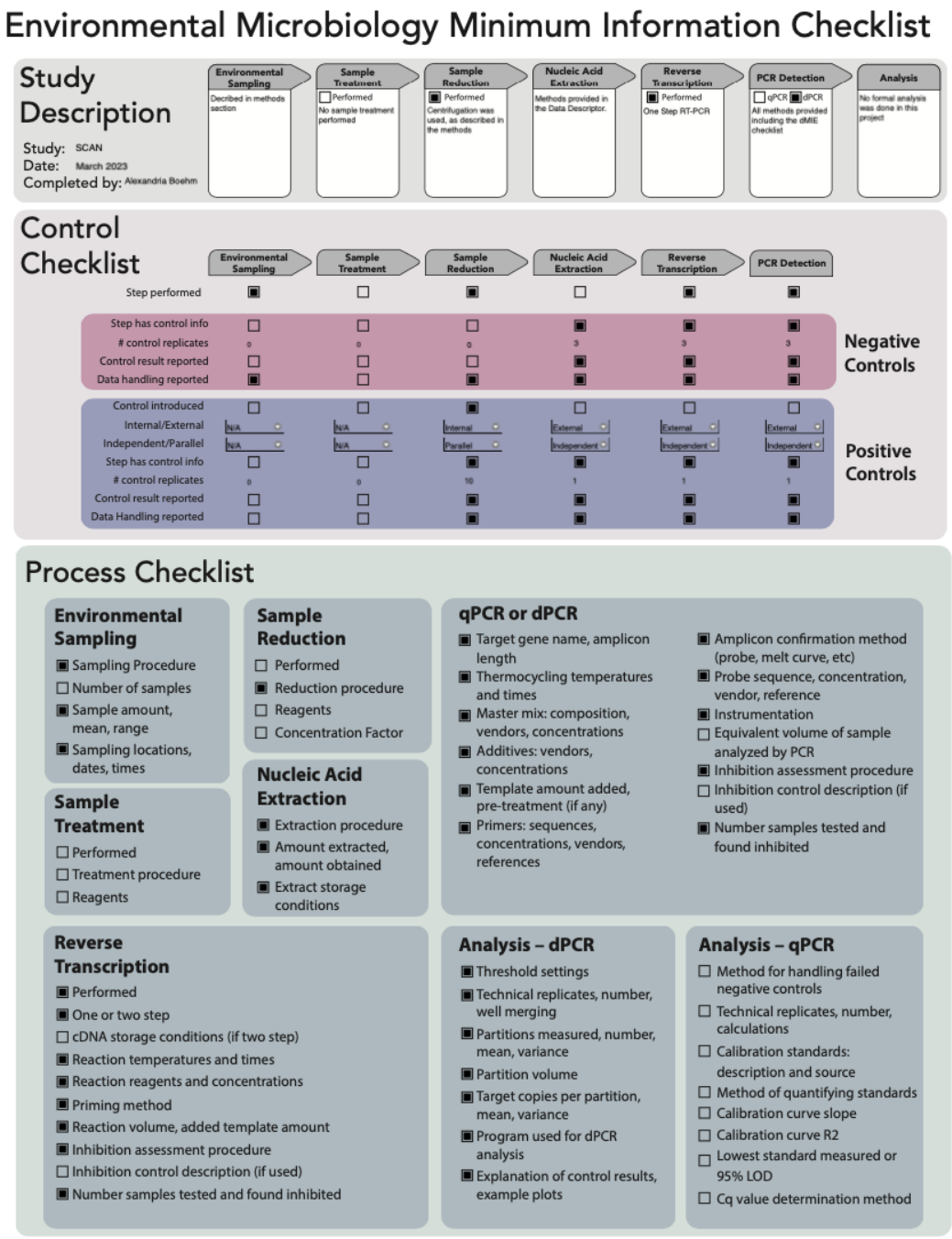

Figure S2. EMMI checklist[7]. Details of the partition numbers and volume, and copy numbers per partition are reported in a Data Descriptor[4].

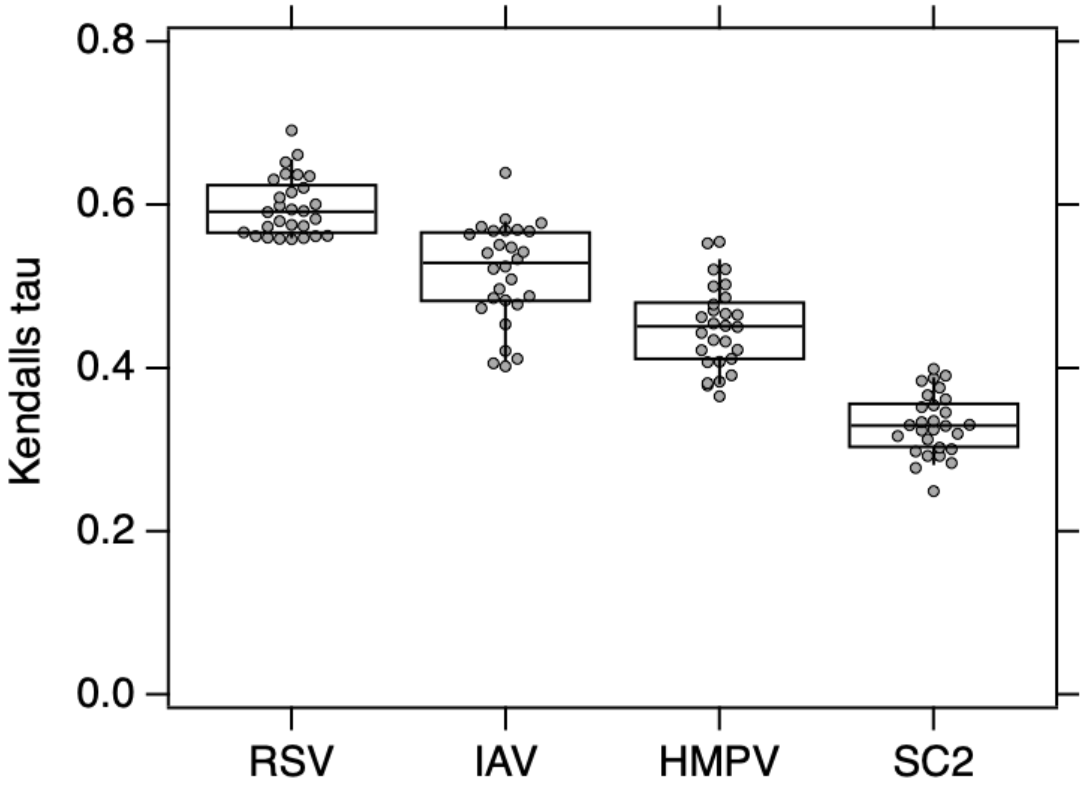

Figure S3. The distribution of between POTW Kendalls tau values for each human viral target. There are 28 tau values for each virus (each combination of 2 POTW among the 8), and those are shown as gray circles. The box and whisker plot provides a visualization of the distribution. The whiskers extend from the 9th to 91st percentiles. The bottom and top of the box represent the 25th and 75th percentiles, the line through the middle of the box represents the median.

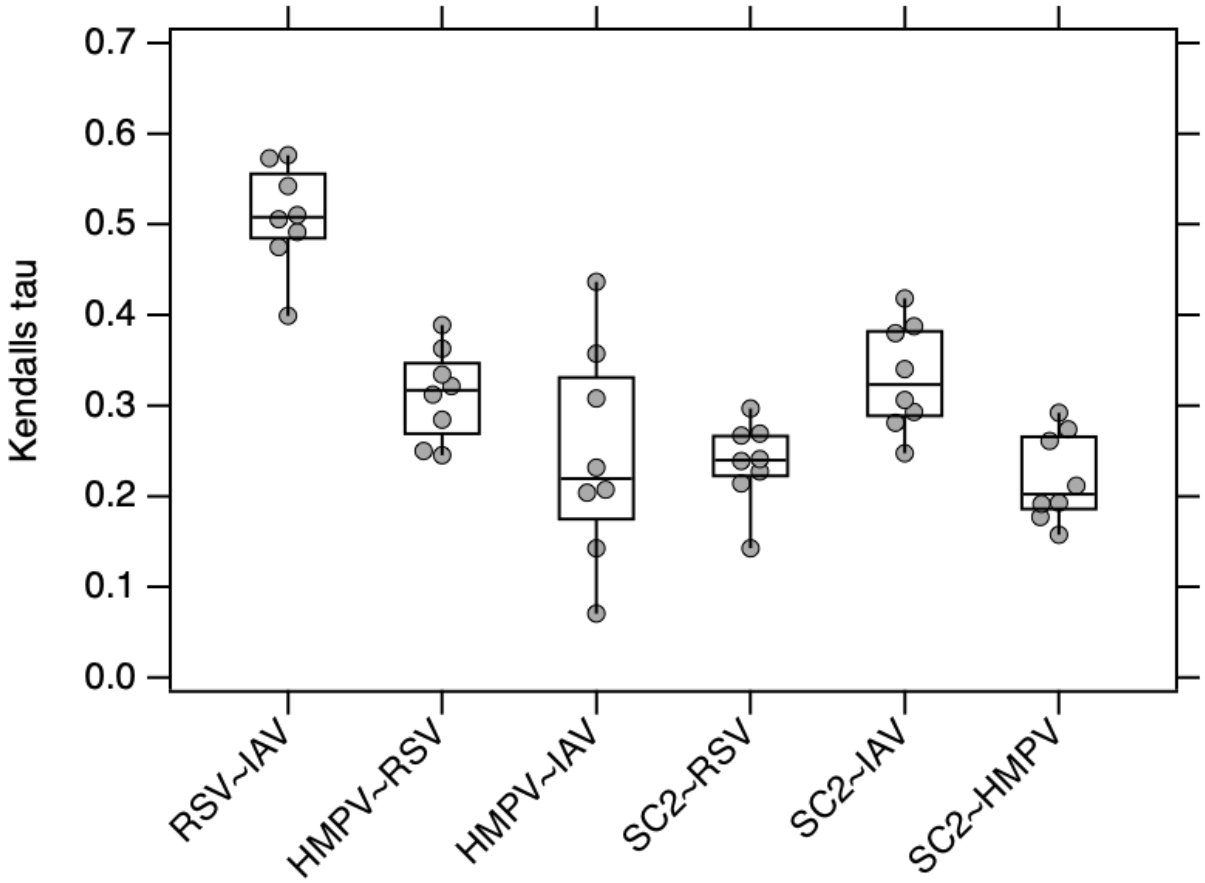

Figure S4. The distribution of within POTW Kendalls tau values for different human viral targets comparisons (bottom axis). There are 8 tau values for each virus combination (1 for each POTW), and those are shown as gray circles. The box and whisker plot provides a visualization of the distribution. The whiskers extend from the 9th to 91st percentiles. The bottom and top of the box represent the 25th and 75th percentiles, the line through the middle of the box represents the median. SC2 is SARS-CoV-2. All tau values are shown, even those that are not significantly different from 0.

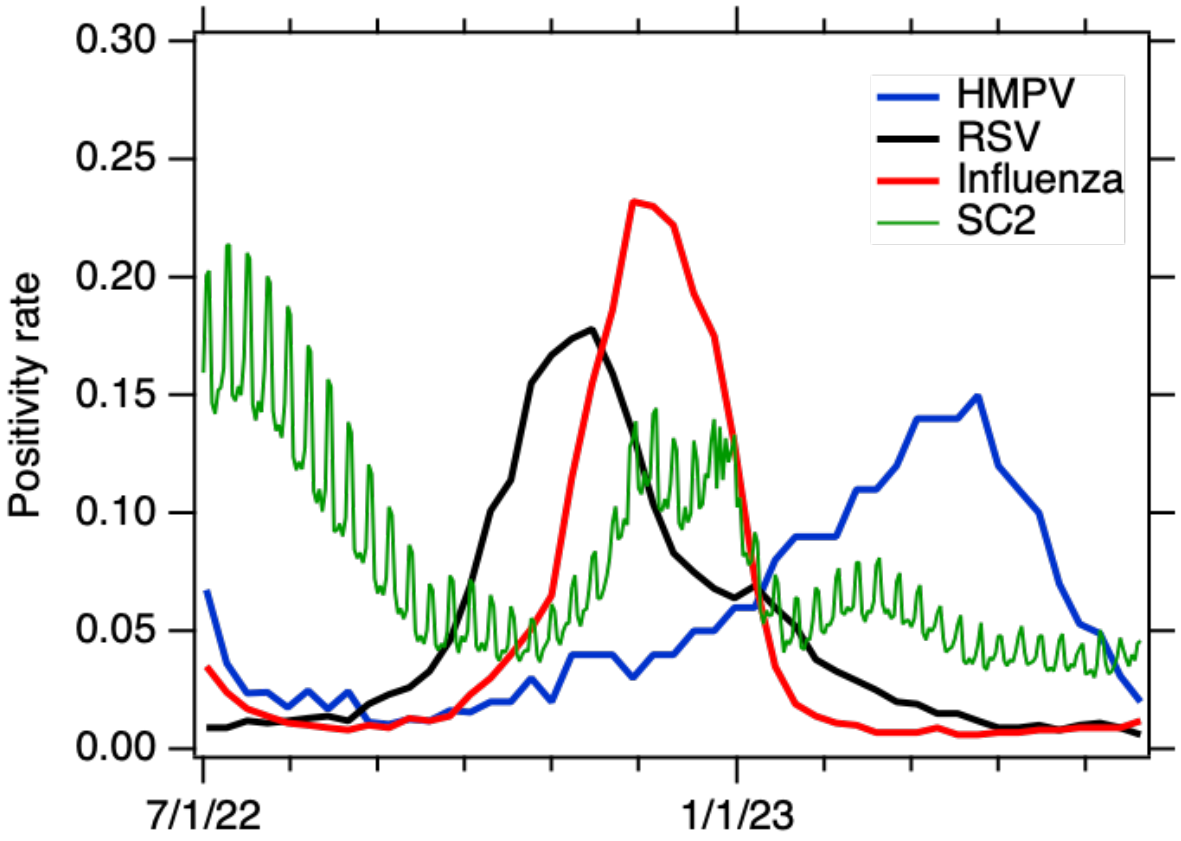

Figure S5. Positivity rates for influenza, RSV, human metapneumovirus (HMPV) and SARS-CoV-2 (SC2) in clinical specimens aggregated across the state of California. Data are reported weekly for influenza, RSV, and HMPV, and daily for SARS-CoV-2.

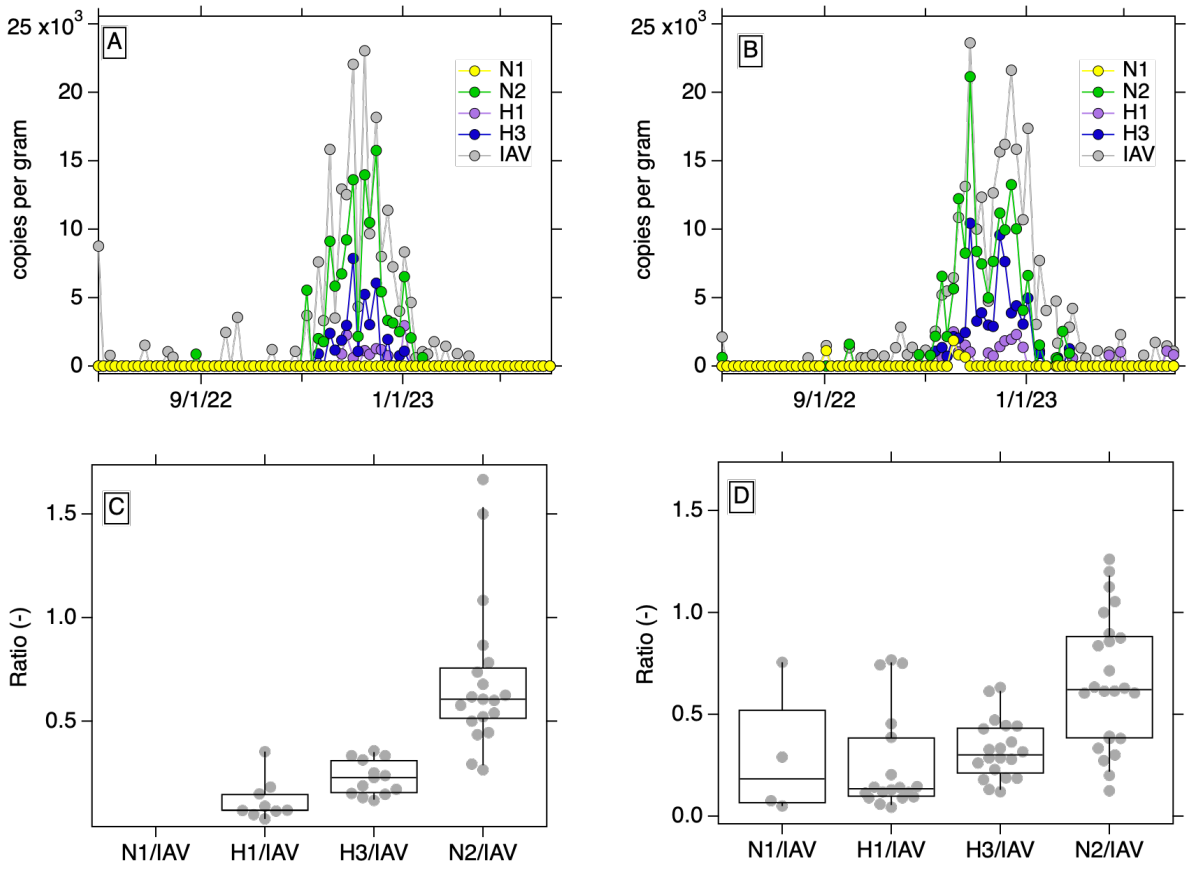

Figure S6. Panel A. Concentrations of IAV, N1, N2, H1, and H3 in copies per gram dry weight in archived samples from SJ POTW. Panel B. Concentrations of IAV, N1, N2, H1, and H3 in copies per gram dry weight in archived samples from Ocean POTW. Panels C and D. Distributions of the ratio of each IAV subtype marker and IAV measured in the archived samples; panel C shows results for SJ, and panel D for Ocean. Only results for samples in which the subtype marker was detected are shown. The whiskers extend from the 9th to 91st percentiles. The bottom and top of the box represent the 25th and 75th percentiles, the line through the middle of the box represents the median.

Table S1. Primers and probes of previously published assays.

Target	Primer/Probe	Sequence
SARS-CoV-2 N Gene	Forward	CATTACGTTTGGTGGACCCT
	Reverse	CCTTGCCATGTTGAGTGAGA
	Probe	CGCGATCAAAACAACGTCGG
BCoV	Forward	CTGGAAGTTGGTGGAGTT
	Reverse	ATTATCGGCCTAACATACATC
	Probe	CCTTCATATCTATACACATCAAGTTGTT
PMMoV	Forward	GAGTGGTTTGACCTTAACGTTTGA
	Reverse	TTGTCGGTTGCAATGCAAGT
	Probe	CCTACCGAAGCAAATG
Influenza A	Forward	CAAGACCAATCYTGTCACCTCTGAC
	Reverse	GCATTYTGGACAAAVCGTCTACG
	Probe	TGCAGTCCTCGCTCACTGGGCACG
RSV	Forward	CTCCAGAATAYAGGCATGAYTCTCC
	Reverse	GCYCTYCTAATYACWGCTGTAAGAC
	Probe	TAACCAAATTAGCAGCAGGAGATAGATCAG
HMPV	Forward	ACTTTATTGGAGAAGGAGCAGG
	Reverse	GGGTAATGRTGATCAAGRTCA
	Probe	AYTGGATGGCMAGAACAGCA

Table S2. Parameters used with primer design software to design the influenza subtype marker assays..

- Product size ranges: 60-275
- Primer size: min 15, opt 20, max 36
- Primer melting temperature: min 50°C, optimal 60°C, max 65°C - GC% content: min 40%, optimal 50%, high 60%
- concentration of divalent cations = 3.8 mM
- concentration of dNTPs needs to be 0.8 mM
- Internal Oligo: size min 15, optimal 20, max 30
- Internal Oligo: Melting temp min 62°C, optimal 63°C, max 70°C
- Internal Oligo: GC% min 30%, optimal 50%, max 80%

Table S3. Novel influenza A (IAV) H1, H3, N1, and N2 assays developed in this study. Forward and. reverse primers and probes are provided. Assays were run at 59°C annealing temperatures.

H1 (IAV)	Forward	GTGAATCACTCTCCACAGCA
	Reverse	TGATTRGGCCATGAACTTGT
	Probe	TGGAACGTGTTACCCAGGAGA
H3 (IAV)	Forward	GAGRTCAGATGCACCCATTG
	Reverse	TCTGGTACATTYCGCATCCC
	Probe	TGCATCACTCCAAATGGAAGCA
N1 (IAV)	Forward	TCYCCCTTGGAATGCAGAAC
	Reverse	ACCAAGCGACTGACTCAAAT
	Probe	AGGAGYCCATATCGAACCCT
N2 (IAV)	Forward	GTGTTATCAATTTGCCCTTGG
	Reverse	GGTCCGATAAGGGGTCCTAT
	Probe	CAGGGAACAACACTAAACAACGTG