Environmental Science & Technology Letters

pubs.acs.org/journal/estlcu

Letter

Wastewater-Based Detection of Two Influenza Outbreaks

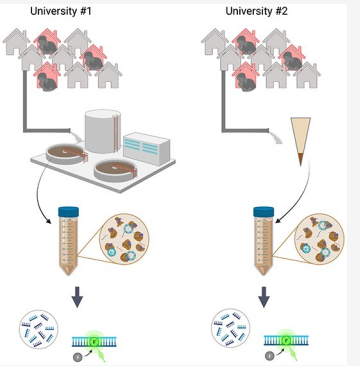
Marlene K. Wolfe, Dorothea Duong, Kevin M. Bakker, Michelle Ammerman, Lindsey Mortenson, Bridgette Hughes, Peter Arts, Adam S. Lauring, William J. Fitzsimmons, Emily Bendall, Calvin E. Hwang, Emily T. Martin, Bradley J. White, Alexandria B. Boehm,* and Krista R. Wigginton*

Cite This: *Environ. Sci. Technol. Lett.* 2022, 9, 687−692

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Traditional influenza surveillance informs control strategies but can lag behind outbreak onset and undercount cases. Wastewater surveillance is effective for monitoring near real-time dynamics of outbreaks but has not been attempted for influenza. We quantified influenza A virus (IAV) RNA in wastewater during two active outbreaks on university campuses in different parts of the United States and during different times of year using case data from an outbreak investigation and high-quality surveillance data from student athletes. In both cases, the IAV RNA concentrations were strongly associated with reported IAV incidence rates (Kendall's τ values of 0.58 and 0.67 for the University of Michigan and Stanford University, respectively). Furthermore, the RNA concentrations reflected outbreak patterns and magnitudes. For the University of Michigan outbreak, evidence from sequencing IAV RNA from wastewater indicated the same circulating strain identified in cases during the outbreak. The results demonstrate that wastewater surveillance can effectively detect influenza outbreaks and will therefore be a valuable supplement to traditional forms of influenza surveillance.



KEYWORDS: *wastewater, influenza, settled solids, surveillance, outbreak*

INTRODUCTION

Influenza surveillance is critical for determining the timing, location, and magnitude of outbreaks. Public health agencies use several combined data sources for influenza surveillance, including outpatient visits, hospitalizations, and clinical laboratory results. These methods can take 1−2 weeks or longer to detect increases in influenza activity.[1] Moreover, these surveillance systems generally characterize the beginning and peak of influenza season rather than their magnitude[2] and capture only a small fraction of influenza illnesses.[3] People with milder illnesses, illnesses outside "typical" influenza season, or with limited access to medical care are generally unaccounted for. The COVID-19 pandemic has highlighted the need for more robust estimates and models of respiratory virus circulation, especially methods that can differentiate causative agents and identify outbreaks occurring outside typical seasonal patterns.

Wastewater-based epidemiology (WBE) is a surveillance technique that has recently been adopted widely. Other alternative surveillance approaches have been proposed to augment conventional influenza surveillance, including the use of online activity[4] and symptom data;[3] however, these tend to be nonspecific to influenza and biased.[3,5] WBE targets genetic material from pathogens and is therefore highly specific; a number of teams have demonstrated that SARS-CoV-2 RNA levels in wastewater correlate with community incidence rates[6] and identify the presence and trends of variants of concern.[7,8] Because fecal shedding of viruses that primarily infect the respiratory tract has been poorly characterized, WBE has not historically been used for these viruses. However, many respiratory viruses, such as RSV and influenza A, are shed and detectable in stool, and stool is not the only human contribution to wastewater.[9−12] We have built on our successful deployment of WBE for COVID-19 surveillance to expand the use of these tools for RSV[13] and in this study for the influenza A virus (IAV).

Measures to mitigate the transmission of COVID-19 dramatically limited the transmission of influenza over the first two years of the pandemic,[14] hindering efforts to investigate whether wastewater surveillance can be used for IAV. In the fall of 2021, the University of Michigan (UM) in Ann Arbor, MI, experienced a rapid increase in influenza A (H3N2) cases, representing some of the first detected influenza activity in the United States since the start of the COVID-19 pandemic.[15] Given the outbreak's novelty, several agencies, including members of our team, collaborated to collect high-resolution clinical data beyond the usual surveillance.[15] In the spring of 2022, Stanford University in Stanford, CA, saw an increase in influenza A activity, as

Received: May 25, 2022
Revised: June 27, 2022
Accepted: June 28, 2022
Published: July 5, 2022

detected by comprehensive surveillance by the athletic department. These two outbreaks in different university communities, along with complementary wastewater solid samples collected throughout the outbreaks, provided an opportunity to assess whether IAV RNA is detectable in wastewater solids and determine the relationship between wastewater concentrations and clinical data.

MATERIALS AND METHODS

Assay Choice for Influenza A. Droplet digital polymerase chain reaction (PCR) was used to quantify a target specific to the influenza A M1 gene using an assay developed by the U.S. Centers for Disease Control and Prevention (CDC).[16] Multiple primer sets are available for this target from the CDC. On the basis of alignment with influenza A sequences over the past 4 years in the United States, primers INF A forward 1 and reverse 1 were chosen (see the Supporting Information).

Wastewater Samples. Samples were collected from the City of Ann Arbor Wastewater Treatment Plant (AA WWTP). AA WWTP is a publicly owned treatment works that serves Ann Arbor, MI, including the University of Michigan Ann Arbor campus. AA WWTP receives an inflow of 17 million gallons per day (MGD) from a sewershed with approximately 130 000 people, including students on campus and in surrounding areas who utilize University Health Services. Further details about AA WWTP are provided by Kim et al.[17] and in the Supporting Information. Samples were collected as part of a routine wastewater monitoring program, and samples between October 4, 2021, and December 4, 2021, were chosen for IAV analysis to match the time period of a known influenza A outbreak. During the study period, AA WWTP staff collected daily settled solids from the primary clarifier and 24-hour composite influent samples in sterile 50 mL conical tubes. These samples were stored immediately at 4 °C and sent by courier to a laboratory at the University of Michigan where they were maintained at 4 °C unless otherwise described; more details about sample storage are available in the Supporting Information.

Samples from Stanford University were collected from a manhole that accesses a large sewer main that conveys wastewater exclusively from the campus, from nearly 200 buildings on campus, including student and faculty housing for 10 000 people. During the study period, 24-hour composite samples were collected and analyzed for IAV RNA 6 days per week as part of a routine monitoring program. A temperature-controlled autosampler was set at 4 °C and collected a raw wastewater sample every 30 min. The sample was then placed in an Imhoff cone to settle the solids; the solids were collected and stored at 4 °C before processing on the same day. Samples between January 1, 2022, and April 28, 2022, during the period prior to and during an outbreak are included here.

Sample Preparation. Solids samples from both sites were dewatered by centrifugation followed by decanting and then resuspended in a buffer prior to direct extraction as described by Wolfe et al.[5] Whole samples from the AA WWTP were pasteurized (60 °C for 1 h) prior to analysis to adhere to UM biosafety protocols. Influent samples from AA WWTP were concentrated using PEG to precipitate viruses as described by Flood et al.[18] Additional details are available in the Supporting Information.

RNA Extraction. RNA extraction was performed on 10 replicate aliquots of dewatered settled solids or PEG pellets generated from the influent as described by Wolfe et al.[6] To remove inhibitors, samples were passed through a Zymo OneStep-96 PCR Inhibitor Removal Kit (Zymo Research). Negative controls (water) and positive controls (BCoV spiked in DNA/RNA Shield; Zymo Research) were included for each extraction plate, and 4 μL of poly-A carrier RNA was added to the extraction positive controls prior to extraction.

Droplet Digital RT-PCR. Extracted RNA samples from both sites were immediately analyzed using two multiplex droplet digital RT-PCR (ddPCR) assays. One multiplex assay targeted the IAV M1 gene and SARS-CoV-2 nucleocapsid (N) and spike (S) genes; the IAV M1 gene alone is presented here. A second multiplex assay targeted pepper mild mottle virus (PMMoV) and bovine coronavirus (BCoV) and is described elsewhere.[6] PMMoV is a plant virus that is shed in human stool and abundant in wastewater globally.[19,20] It is used here to control for fecal strength and recovery of viral RNA.[21] BCoV was used as an exogenous recovery control; samples were required to have >10% BCoV recovery. PCR negative and positive and extraction controls were included to ensure no contamination as described by Wolfe et al.[6] Additional details of assays, controls, and thermocycling conditions are available in the Supporting Information.

Each of the 10 replicate RNA-extracted aliquots was run in its own well, and results from 10 wells were merged for analysis. Thresholding was done using QuantaSoft Analysis Pro Software (Bio-Rad, version 1.0.596). Concentrations for each RNA target were converted using dimensional analysis to concentrations of target copies per gram of dry weight of wastewater solids unless otherwise stated.[22] The dry weight of dewatered solids was determined by drying. The total error includes both the error associated with the Poisson distribution and the variability among merged replicates and is reported as the standard deviation.

Sequencing Methods. IAV RNA from a settled solid sample collected at AA WWTP during the peak of the outbreak (November 11, 2021) was sequenced to determine if the IAV genotype in wastewater matched that identified in clinical specimens. RNA was converted into cDNA and then amplified with a SuperScript IV One-step RT-PCR kit with Platinum Taq High Fidelity DNA Polymerase using primers targeting the IAV HA sequence. The product was resolved with a 1% agarose gel, and the putative HA amplicon was excised and amplified with primers targeting the HA1 subunit sequence. The product was purified using magnetic beads (AmpureXP, Beckman Coulter) and sequenced using the Sanger method with the HA1 subunit primers. Details of sequence generation, including PCR primers and thermal cycler parameters, are provided in the Supporting Information.

Clinical Data. For the UM outbreak, the daily total number of positive clinical specimens (hereafter "daily cases") was provided by University Health Service at the University of Michigan (UHS). Data were collected by UHS as part of an outbreak investigation, and positive cases are presented as a function of specimen collection date.[15] A majority of cases reported to the county were captured by the UHS outbreak data (97%); we therefore calculated IAV incidence rates using the number of daily cases from UHS and the total sewershed population of 130 000. A 5-day centered, smoothed average was used in analysis to limit the impact of testing bias. These activities were conducted consistent with applicable federal law and determined not to be regulated by the IRB (HUM00209156).

https://doi.org/10.1021/acs.estlett.2c00350
Environ. Sci. Technol. Lett. 2022, 9, 687−692

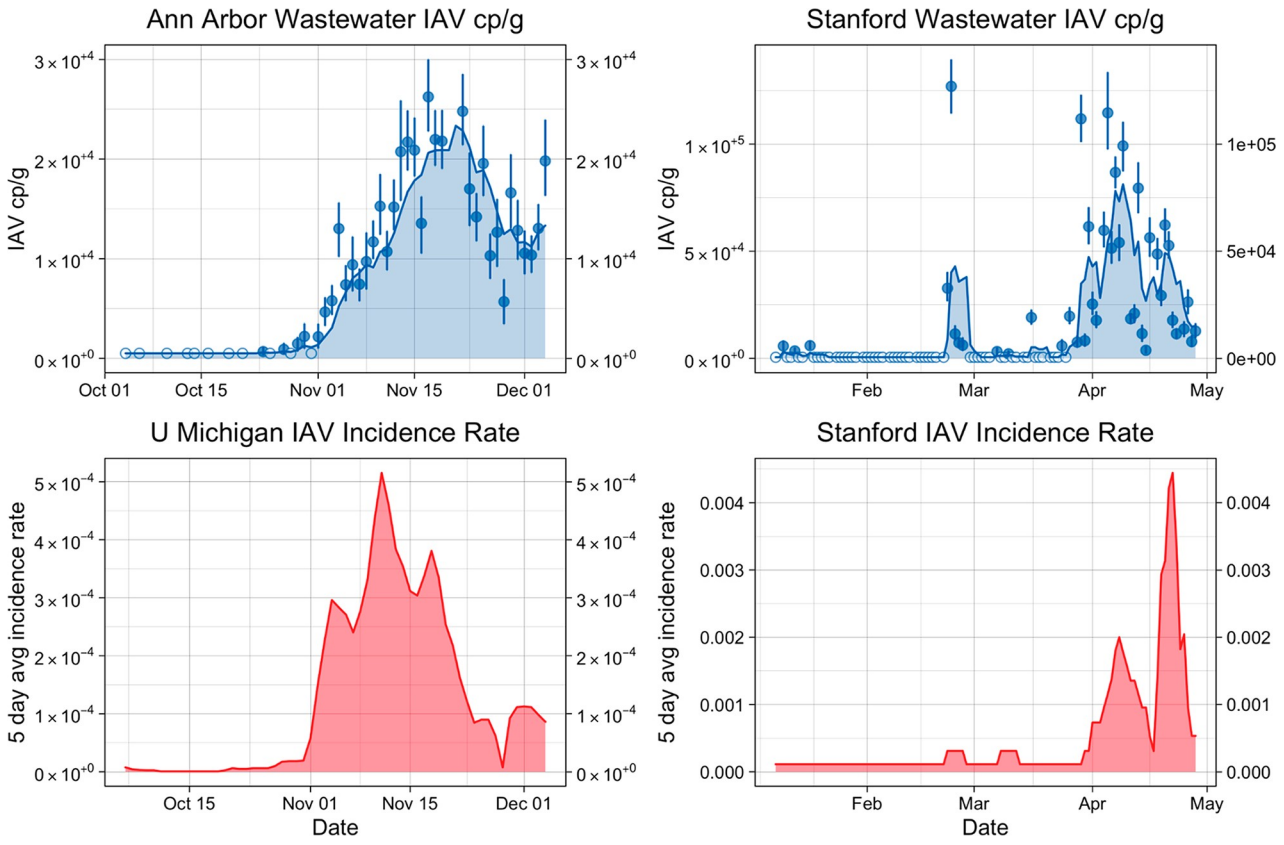


Figure 1. Time series of the UM and Stanford University outbreaks, as shown in wastewater IAV concentrations and confirmed influenza A incidence rates. In the top row, points show daily IAV M1 RNA concentrations in copies per gram of dry weight of wastewater solids with error bars representing standard deviations of replicates as total errors from the ddPCR instrument software. Empty circles indicate nondetect measurements. The area under the line represents the 5-day smoothed average copies per gram of IAV M1 RNA in wastewater. In the bottom row, the red area represents the 5-day smoothed number of reported cases.

At Stanford University, case data were provided from surveillance of student athletes. During the study period, any student athlete (approximately 900 total) presenting with flu-like symptoms was screened for COVID-19, and if negative, specimens were screened at the Stanford Virology Laboratory for IAV. Positive cases are presented as a function of specimen collection date. Student athletes live throughout campus and do not have a housing preference; therefore, it is assumed that the fraction of athletes living in the sewershed is approximately equivalent to the fraction of campus served by the sewershed and that incident IAV cases among students on campus are proportional to the number of incident cases in the sewershed. Thus, the IAV incidence rate was calculated using the number of daily cases from student athlete surveillance and the total student athlete population of 900. This study was approved by the Stanford University IRB (57358) with a waiver of informed consent.

Statistical Analysis. For the outbreak periods at both UM and Stanford University, primary analysis was performed using the daily IAV M1 gene concentration along with the 5-day smoothed incidence rate. This analysis was repeated with data normalized by PMMoV and is included in the Supporting Information. We tested the null hypothesis that daily wastewater concentrations were not associated with 5-day smoothed incidence rates using Kendall's τ as data were not normally distributed at either UM or Stanford University (Shapiro–Wilk normality test, both $p < 0.05$). The rate of change of the smoothed IAV incidence rate with IAV wastewater concentration was estimated using linear regression. Measurements below the detection limit were replaced with 500 copies/g (the approximate detection limit), and days with zero clinical cases reported were replaced with 0.1 for analysis. All statistical analyses were implemented in Rstudio (version 1.3.1073).

RESULTS AND DISCUSSION

We found that IAV RNA in wastewater was correlated with the IAV incidence rate at both UM and Stanford University. The first clinical case was reported at UM on October 6, 2021. No more cases were reported until October 20; the outbreak then increased to a peak of 84 daily cases on November 10, 2021 (Figure 1 and Figure S1). Measurements of the IAV M1 gene in archived wastewater were below the detection limit (approximately 500 copies/g) from October 4 until the first positive measurement on Oct 21, 2021, and then increased and peaked on November 17, 2021, before the end of the study in early December (Figure 1). IAV M1 gene concentrations ranged from nondetect to 2.63×10^4 copies/g (median of 1.03×10^4 copies/g). Overall, 857 cases were reported by UHS during the study, accounting for 97% of cases reported at the county level and thus approximating the cases in the Ann Arbor sewershed (Figure S2). Positive and negative controls were positive and negative, respectively.

The first clinical case of IAV infection in a Stanford athlete was reported on February 22, 2022, with only one other case reported until March 30, after which cases increased to a

https://doi.org/10.1021/acs.estlett.2c00350
Environ. Sci. Technol. Lett. 2022, 9, 687−692

maximum of 7 per day on April 19, 2022 (Figure 1). Measurements of the IAV M1 gene from a real-time, daily monitoring program were below the detection limit when monitoring began with only a few positive measurements in January 2022 during a period without recorded cases after which there were increases in concentration seen starting on February 22 and again around March 26, 2022 (Figure 1). From the end of February, IAV RNA levels increased and then peaked at 1.15×10^5 copies/g on April 5, 2022. Overall IAV M1 gene concentrations ranged from nondetect to 1.27×10^5 copies/g (median nondetect), and a total of 42 student athlete cases were reported.

BCoV recoveries were >10%, and PMMoV concentrations were within the expected range for each plant, suggesting acceptable recovery of RNA. Wastewater data are available publicly through the Emory Dataverse for UM (10.15139/S3/TMOEDS) and through the Stanford Digital Repository for Stanford University (https://purl.stanford.edu/mb859sw3898). Case data are available in Figure S1.

At both UM and Stanford University, wastewater concentrations of IAV RNA closely reflect the IAV incidence rate (Figure 2). Daily IAV RNA concentrations were highly

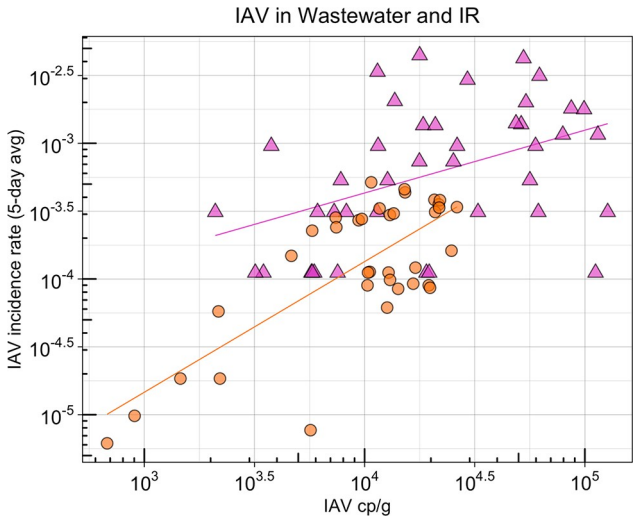


Figure 2. Association between the positive daily influenza A M1 gene concentration per gram of dry weight and the 5-day smoothed incidence rate for UM (orange circles) and Stanford University (purple triangles). The line represents the linear association between the two measurements for each site.

associated with both the 5-day smoothed incidence rate (for UM, $\tau = 0.58$, $p < 10^{-7}$, and $N = 45$; for Stanford University, $\tau = 0.67$, $p < 10^{-14}$, and $N = 83$). The results were not substantially different at either site when wastewater concentrations were normalized by an endogenous control (PMMoV) (see Table S1 and Figure S3). A 1 $\log_{10}$ increase in influenza A RNA in wastewater is associated with a 1.2 $\log_{10}$ increase in incidence rate at UM ($r^2 = 0.77$, $p < 10^{-14}$, and $N = 45$) and a 0.44 $\log_{10}$ increase in incidence rate at Stanford University ($r^2 = 0.60$, $p < 10^{-16}$, and $N = 94$). A trimmed 992-base sequence (see the Supporting Information) was generated from the November 11 AA WWTP sample and was identical to 212 IAV (H3N2) clinical samples that were sequenced during the 2021 IAV outbreak in Ann Arbor (see the Supporting Information for the sequence).

The strength of the association between IAV RNA concentrations and clinical cases at both sites is similar to those reported for SARS-CoV-2 measured across eight publicly owned treatment works [Kendall's τ of 0.66 ($p < 0.001$) compared to 0.58 ($p < 0.001$) and 0.67 ($p < 0.001$) for IAV at UM and Stanford University, respectively]. However, the linear relationship between wastewater measurements and incidence rate is different for SARS-CoV-2 and each IAV outbreak. A 1 $\log_{10}$ increase in the SARS-COV-2 RNA corresponded to a 0.59 $\log_{10}$ increase in incidence rate[6] compared to 1.2 $\log_{10}$ and 0.44 $\log_{10}$ increases in IAV incidence rate reported here for UM and Stanford University, respectively. This observation could be due to differences in shedding of the virus between SARS-CoV-2 and IAV, differences in wastewater infrastructure and travel times, or differences in clinical testing and reporting. IAV RNA, like SARS-CoV-2 RNA, appeared at higher rates and at higher magnitudes in solids than in liquid wastewater in a comparison of four samples from UM (details in the Supporting Information).

Previous research suggests that some viruses are highly concentrated in wastewater solids.[23,24] We compared concentrations of IAV RNA in four paired liquid influent and settled wastewater solid samples. We detected IAV RNA in all four of the solid samples and in only one of the four influent samples. IAV RNA concentrations were 10^3-fold higher on a mass equivalent basis (Figure S4) in the solid sample that matched the positive influent sample. Our results from the paired wastewater influent and solid samples suggest that IAV RNA is more frequently detected in the solid fraction than the liquid fraction of wastewater. We therefore focused on settled solids in the study. These results suggest that IAV wastewater surveillance will be more sensitive and effective if sampling and measurements focus on the solid portion of municipal wastewater.

This work has several limitations. Although the quality of the clinical data available from both UM and Stanford University is higher than typical influenza surveillance data (due to an outbreak investigation at UM and active student athlete surveillance at Stanford University), in both cases the wastewater catchment area does not exactly match the surveilled population. Thus, there is more work needed to clarify the relationship between IAV concentrations in wastewater and the number of cases in the contributing population. However, data from these two outbreaks together demonstrate that wastewater monitoring can effectively detect the rise and fall of cases during an outbreak of influenza A. Longitudinal studies of shedding through infection with different IAV types and subtypes in a range of populations (e.g., vaccine status, age, etc.) would help contextualize these results for IAV and indicate if the same relationships between wastewater concentrations and incidence would be expected for the other influenza types and subtypes and in different communities. Additional research is also necessary to characterize the potential leading or lagging of wastewater compared to influenza symptom onset, testing, and reporting dates.

Wastewater surveillance of influenza could aid clinical surveillance, especially when traditional systems are not fully engaged (e.g., outside of typical flu season). Relative levels of different co-circulating influenza strains and types could be measured simultaneously to provide early identification of drifted strains not covered by the vaccine, a situation that would prompt additional public health guidance for testing and

https://doi.org/10.1021/acs.estlett.2c00350
Environ. Sci. Technol. Lett. 2022, 9, 687−692

treatment.[25] Wastewater surveillance could also provide efficient monitoring for rare but impactful emergencies, such as introductions of new influenza strains at the animal–human interface with uncertain and potentially severe public health consequences.[26,27] Finally, distinguishing between influenza-like illnesses (ILIs) can be challenging; these outbreaks were detected due largely to equipment that facilitated frequent testing with a 4-plex virus panel at UM and active athlete surveillance at Stanford University. This type of in-house clinical testing and surveillance is not widely used. Wastewater testing for a panel of IAV, SARS-CoV-2, and other viruses would give public health entities the ability to monitor several respiratory diseases in the community simultaneously and anticipate disease occurrence and strain on local healthcare systems. We anticipate wastewater surveillance will ultimately play an important role in improving the health system and public health preparedness for IAV and other respiratory viruses as it has done for SARS-CoV-2.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.estlett.2c00350.

Additional methods, clinical data for UM and Stanford University (Figure S1), clinical data from county and university sources for the UM outbreak (Figure S2), raw IAV copies per gram in wastewater, IAV copies per gram normalized by PMMoV, and PMMoV measurements for UM and Stanford University (Figure S3), IAV copies per gram in solids versus liquids at UM (Figure S4), IAV copies per gram in pasteurized and unpasteurized samples at UM (Figure S5), and values of Kendall's τ for wastewater measurements and clinical data at UM and Stanford University (Table S1) (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Krista R. Wigginton − *Department of Civil & Environmental Engineering, University of Michigan, Ann Arbor, Michigan 48109, United States;* orcid.org/0000-0001-6665-5112; Email: kwigg@umich.edu

Alexandria B. Boehm − *Department of Civil & Environmental Engineering, Stanford University, Stanford, California 94305, United States;* orcid.org/0000-0002-8162-5090; Email: aboehm@stanford.edu

Authors

Marlene K. Wolfe − *Gangarosa Department of Environmental Health, Rollins School of Public Health, Emory University, Atlanta, Georgia 30322, United States;* orcid.org/0000-0002-6476-0450

Dorothea Duong − *Verily Life Sciences, South San Francisco, California 94080, United States*

Kevin M. Bakker − *Department of Epidemiology, University of Michigan, Ann Arbor, Michigan 48109, United States*

Michelle Ammerman − *Department of Civil & Environmental Engineering, University of Michigan, Ann Arbor, Michigan 48109, United States*

Lindsey Mortenson − *University Health Service, University of Michigan, Ann Arbor, Michigan 48109, United States*

Bridgette Hughes − *Verily Life Sciences, South San Francisco, California 94080, United States*

Peter Arts − *Department of Civil & Environmental Engineering, University of Michigan, Ann Arbor, Michigan 48109, United States*

Adam S. Lauring − *Division of Infectious Diseases, Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan 48109, United States*

William J. Fitzsimmons − *Division of Infectious Diseases, Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan 48109, United States*

Emily Bendall − *Division of Infectious Diseases, Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan 48109, United States*

Calvin E. Hwang − *Department of Orthopaedic Surgery, Stanford University School of Medicine, Stanford, California 94305, United States*

Emily T. Martin − *Department of Epidemiology, University of Michigan, Ann Arbor, Michigan 48109, United States*

Bradley J. White − *Verily Life Sciences, South San Francisco, California 94080, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.estlett.2c00350

Notes

The authors declare the following competing financial interest(s): B.H., D.D., and B.J.W. are employees of Verily Life Sciences, LLC. M.K.W. previously provided consulting for Verily Life Sciences.

■ ACKNOWLEDGMENTS

Funding: CDC Foundation, Stanford University Provost's Office, MDHHS, and UMich COE Skunkworks. The authors thank Miranda Delahoy (CDC), Kate Kusiak Galvin (UM), Earl Kenzie (AA WWTP), and Kyle Weinman (AA WWTP) for their contributions to and support of this work.

■ REFERENCES

(1) Lu, F. S.; Hou, S.; Baltrusaitis, K.; Shah, M.; Leskovec, J.; Sosic, R.; Hawkins, J.; Brownstein, J.; Conidi, G.; Gunn, J.; Gray, J.; Zink, A.; Santillana, M. Accurate Influenza Monitoring and Forecasting Using Novel Internet Data Streams: A Case Study in the Boston Metropolis. *JMIR Public Health Surveillance* **2018**, *4* (1), No. e4.

(2) DeJonge, P. M.; Monto, A. S.; Malosh, R. E.; Petrie, J. G.; Segaloff, H. E.; McSpadden, E.; Cheng, C.; Bazzi, L.; Callear, A.; Johnson, E.; Truscon, R.; Martin, E. T. Distinct Influenza Surveillance Networks and Their Agreement in Recording Regional Influenza Circulation: Experience from Southeast Michigan. *Influenza Other Respir. Viruses* **2021**, DOI: 10.1111/irv.12944.

(3) Miller, A. C.; Peterson, R. A.; Singh, I.; Pilewski, S.; Polgreen, P. M. Improving State-Level Influenza Surveillance by Incorporating Real-Time Smartphone-Connected Thermometer Readings Across Different Geographic Domains. *Open Forum. Infect. Dis.* **2019**, *6* (11), No. ofz455.

(4) Salathé, M.; Freifeld, C. C.; Mekaru, S. R.; Tomasulo, A. F.; Brownstein, J. S. Influenza A (H7N9) and the Importance of Digital Epidemiology. *N. Engl. J. Med.* **2013**, *369* (5), 401–404.

(5) Shaman, J.; Karspeck, A.; Yang, W.; Tamerius, J.; Lipsitch, M. Real-Time Influenza Forecasts during the 2012–2013 Season. *Nat. Commun.* **2013**, *4* (1), 2837.

(6) Wolfe, M. K.; Topol, A.; Knudson, A.; Simpson, A.; White, B.; Vugia, D. J.; Yu, A. T.; Li, L.; Balliet, M.; Stoddard, P.; Han, G. S.; Wigginton, K. R.; Boehm, A. B. 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.

(7) Kirby, A. E. Notes from the Field: Early Evidence of the SARS-CoV-2 B.1.1.529 (Omicron) Variant in Community Wastewater — United States, November–December 2021. *Morb. Mortal. Wkly. Rep.* **2021**, DOI: 10.15585/mmwr.mm7103a5.
(8) Heijnen, L.; Elsinga, G.; de Graaf, M.; Molenkamp, R.; Koopmans, M. P. G.; Medema, G. Droplet Digital RT-PCR to Detect SARS-CoV-2 Signature Mutations of Variants of Concern in Wastewater. *Sci. Total Environ.* **2021**, *799*, No. 149456.
(9) Hirose, R.; Daidoji, T.; Naito, Y.; Watanabe, Y.; Arai, Y.; Oda, T.; Konishi, H.; Yamawaki, M.; Itoh, Y.; Nakaya, T. Long-Term Detection of Seasonal Influenza RNA in Faeces and Intestine. *Clin. Microbiol. Infect.* **2016**, *22* (9), 813.e1–813.e7.
(10) Al Khatib, H. A.; Coyle, P. V.; Al Maslamani, M. A.; Al Thani, A. A.; Pathan, S. A.; Yassine, H. M. Molecular and Biological Characterization of Influenza A Viruses Isolated from Human Fecal Samples. *Infect., Genet. Evol.* **2021**, *93*, No. 104972.
(11) To, K. K. W.; Chan, K.; Li, I. W. S.; Tsang, T.; Tse, H.; Chan, J. F. W.; Hung, I. F. N.; Lai, S.; Leung, C.; Kwan, Y.; Lau, Y.; Ng, T.; Cheng, V. C. C.; Peiris, J. S. M.; Yuen, K. Viral Load in Patients Infected with Pandemic H1N1 2009 Influenza A Virus. *J. Med. Virol.* **2010**, *82* (1), 1–7.
(12) Chan, M. C. W.; Lee, N.; Chan, P. K. S.; To, K. F.; Wong, R. Y. K.; Ho, W.-S.; Ngai, K. L. K.; Sung, J. J. Y. Seasonal Influenza A Virus in Feces of Hospitalized Adults. *Emerg. Infect. Dis.* **2011**, *17* (11), 2038–2042.
(13) Hughes, B.; Duong, D.; White, B. J.; Wigginton, K. R.; Chan, E. M. G.; Wolfe, M. K.; Boehm, A. B. Respiratory Syncytial Virus (RSV) RNA in Wastewater Settled Solids Reflects RSV Clinical Positivity Rates. *Environ. Sci. Technol. Lett.* **2022**, *9*, 173–178.
(14) Olsen, S. J.; Winn, A. K.; Budd, A. P.; Prill, M. M.; Steel, J.; Midgley, C. M.; Kniss, K.; Burns, E.; Rowe, T.; Foust, A.; Jasso, G.; Merced-Morales, A.; Davis, C. T.; Jang, Y.; Jones, J.; Daly, P.; Gubareva, L.; Barnes, J.; Kondor, R.; Sessions, W.; Smith, C.; Wentworth, D. E.; Garg, S.; Havers, F. P.; Fry, A. M.; Hall, A. J.; Brammer, L.; Silk, B. J. Changes in Influenza and Other Respiratory Virus Activity During the COVID-19 Pandemic — United States, 2020–2021. *Morb. Mortal. Wkly. Rep.* **2021**, *70* (29), 1013–1019.
(15) Delahoy, M. J.; Mortenson, L.; Bauman, L.; Marquez, J.; Bagdasarian, N.; Coyle, J.; Sumner, K.; Lewis, N. M.; Lauring, A. S.; Flannery, B.; Patel, M. M.; Martin, E. T. Influenza A(H3N2) Outbreak on a University Campus - Michigan, October–November 2021. *Morb. Mortal. Wkly. Rep.* **2021**, *70*, 1712–1714.
(16) Research Use Only CDC Flu SC2 Multiplex Assay Primers and Probes. Centers for Disease Control and Prevention. https://www.cdc.gov/coronavirus/2019-ncov/lab/multiplex-primer-probes.html (accessed 2021-12-17).
(17) Kim, S.; Kennedy, L. C.; Wolfe, M. K.; Criddle, C. S.; Duong, D. H.; Topol, A.; White, B. J.; Kantor, R. S.; Nelson, K. L.; Steele, J. A.; Langlois, K.; Griffith, J. F.; Zimmer-Faust, A. G.; McLellan, S. L.; Schussman, M. K.; Ammerman, M.; Wigginton, K. R.; Bakker, K. M.; Boehm, A. B. SARS-CoV-2 RNA Is Enriched by Orders of Magnitude in Primary Settled Solids Relative to Liquid Wastewater at Publicly Owned Treatment Works. *Environ. Sci.: Water Res. Technol.* **2022**, *8*, 757.
(18) Flood, M. T.; D'Souza, N.; Rose, J. B.; Aw, T. G. Methods Evaluation for Rapid Concentration and Quantification of SARS-CoV-2 in Raw Wastewater Using Droplet Digital and Quantitative RT-PCR. *Food Environ. Virol.* **2021**, *13* (3), 303–315.
(19) Kitajima, M.; Sassi, H. P.; Torrey, J. R. Pepper Mild Mottle Virus as a Water Quality Indicator. *Npj Clean Water* **2018**, *1* (1), 1–9.
(20) Symonds, E. M.; Nguyen, K. H.; Harwood, V. J.; Breitbart, M. Pepper Mild Mottle Virus: A Plant Pathogen with a Greater Purpose in (Waste)Water Treatment Development and Public Health Management. *Water Res.* **2018**, *144*, 1–12.
(21) McClary-Gutierrez, J. S.; Aanderud, Z. T.; Al-Faliti, M.; Duvallet, C.; Gonzalez, R.; Guzman, J.; Holm, R. H.; Jahne, M. A.; Kantor, R. S.; Katsivelis, P.; Kuhn, K. G.; Langan, L. M.; Mansfeldt, C.; McLellan, S. L.; Grijalva, L. M. M.; Murnane, K. S.; Naughton, C. C.; Packman, A. I.; Paraskevopoulos, S.; Radniecki, T. S.; Roman, F. A.; Shrestha, A.; Stadler, L. B.; Steele, J. A.; Swalla, B. M.; Vikesland, P.; Wartell, B.; Wilusz, C. J.; Wong, J. C. C.; Boehm, A. B.; Halden, R. U.; Bibby, K.; Vela, J. D. Standardizing Data Reporting in the Research Community to Enhance the Utility of Open Data for SARS-CoV-2 Wastewater Surveillance. *Environ. Sci.: Water Res. Technol.* **2021**, *7*, 1545–1551.
(22) Topol, A.; White, B.; Wigginton, K.; Boehm, A. B. B. High Throughput SARS-COV-2, PMMOV, and BCoV quantification in settled solids using digital RT-PCR. *protocols.io* **2021**, DOI: 10.17504/protocols.io.btywnpxe.
(23) Ye, Y.; Ellenberg, R. M.; Graham, K. E.; Wigginton, K. R. Survivability, Partitioning, and Recovery of Enveloped Viruses in Untreated Municipal Wastewater. *Environ. Sci. Technol.* **2016**, *50* (10), 5077–5085.
(24) Graham, K. E.; Loeb, S. K.; Wolfe, M. K.; Catoe, D.; Sinnott-Armstrong, N.; Kim, S.; Yamahara, K. M.; Sassoubre, L. M.; Mendoza Grijalva, L. M.; Roldan-Hernandez, L.; Langenfeld, K.; Wigginton, K. R.; Boehm, A. B. SARS-CoV-2 RNA in Wastewater Settled Solids Is Associated with COVID-19 Cases in a Large Urban Sewershed. *Environ. Sci. Technol.* **2021**, *55* (1), 488–498.
(25) HAN Archive - 00458 | Health Alert Network (HAN). https://emergency.cdc.gov/han/2021/han00458.asp (accessed 2022-02-10).
(26) Bowman, A. S.; Walia, R. R.; Nolting, J. M.; Vincent, A. L.; Killian, M. L.; Zentkovich, M. M.; Lorbach, J. N.; Lauterbach, S. E.; Anderson, T. K.; Davis, C. T.; Zanders, N.; Jones, J.; Jang, Y.; Lynch, B.; Rodriguez, M. R.; Blanton, L.; Lindstrom, S. E.; Wentworth, D. E.; Schiltz, J.; Averill, J. J.; Forshey, T. Influenza A(H3N2) Virus in Swine at Agricultural Fairs and Transmission to Humans, Michigan and Ohio, USA, 2016. *Emerg. Infect. Dis.* **2017**, *23* (9), 1551–1555.
(27) Oliver, I.; Roberts, J.; Brown, C. S.; Byrne, A. M.; Mellon, D.; Hansen, R. D.; Banyard, A. C.; James, J.; Donati, M.; Porter, R.; Ellis, J.; Cogdale, J.; Lackenby, A.; Chand, M.; Dabrera, G.; Brown, I. H.; Zambon, M. A Case of Avian Influenza A(H5N1) in England, January 2022. *Euro Surveillance* **2022**, *27* (5), 2200061.