

RESEARCH ARTICLE – Microbes & Disease

Predicting daily COVID-19 case rates from SARS-CoV-2 RNA concentrations across a diversity of wastewater catchments

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One sentence summary: Estimating COVID-19 case rates directly from SARS-CoV-2 RNA concentrations in domestic wastewater.

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ABSTRACT

We assessed the relationship between municipality COVID-19 case rates and SARS-CoV-2 concentrations in the primary sludge of corresponding wastewater treatment facilities. Over 1700 daily primary sludge samples were collected from six wastewater treatment facilities with catchments serving 18 cities and towns in the State of Connecticut, USA. Samples were analyzed for SARS-CoV-2 RNA concentrations during a 10 month time period that overlapped with October 2020 and winter/spring 2021 COVID-19 outbreaks in each municipality. We fit lagged regression models to estimate reported case rates in the six municipalities from SARS-CoV-2 RNA concentrations collected daily from corresponding wastewater treatment facilities. Results demonstrate the ability of SARS-CoV-2 RNA concentrations in primary sludge to estimate

Received: 26 August 2021; Accepted: 6 January 2022

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COVID-19 reported case rates across treatment facilities and wastewater catchments, with coverage probabilities ranging from 0.94 to 0.96. Lags of 0 to 1 days resulted in the greatest predictive power for the model. Leave-one-out cross validation suggests that the model can be broadly applied to wastewater catchments that range in more than one order of magnitude in population served. The close relationship between case rates and SARS-CoV-2 concentrations demonstrates the utility of using primary sludge samples for monitoring COVID-19 outbreak dynamics. Estimating case rates from wastewater data can be useful in locations with limited testing availability, testing disparities, or delays in individual COVID-19 testing programs.

Keywords: SARS-CoV-2; wastewater-based epidemiology; primary sludge; lagged regression analysis; case rate estimation; quantitative PCR

INTRODUCTION

Wastewater surveillance has the potential to identify and track outbreaks of human pathogens that demonstrate gut tropism, including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of COVID-19. (Xiao et al. 2020) Recent studies have demonstrated that SARS-CoV-2 RNA concentrations in domestic wastewater reflect the rise and fall of COVID-19 cases based on daily positive tests in a community, (Graham et al. 2021; Medema et al. 2020; Vallejo et al. 2020) have produced ratios of wastewater concentrations to cases across sewersheds, (Wolfe et al. 2021) and have shown how important epidemiological parameters such as the effective reproduction number can be estimated using wastewater SARS-CoV-2 concentrations and other epidemic indicators. (Kaplan et al. 2020) If SARS-CoV-2 wastewater results are rapidly reported, they could provide a leading indicator of community infection rates over COVID-19 case rates and hospital admissions data. (Kaplan et al. 2020; Nemudryi et al. 2020; Peccia et al. 2020)

Estimates of infection based on COVID-19 case rates from testing of human specimens have been the standard for applying community interventions aimed at decreasing morbidity and mortality. Alternative approaches to COVID-19 testing are necessary to estimate the number of infections in locations with disparities in testing practices (Souch and Cossman 2020), limited testing resources (Ondoa et al. 2020) or during outbreaks when testing capacity cannot meet demand. SARS-CoV-2 RNA concentration measurements in wastewater may resolve these issues, but quantitative relationships between SARS-CoV-2 concentration in wastewater and infection (or proxies of infection such as cases) are not well-resolved.

The following manuscript reports 10 months of monitoring daily primary sludge for SARS-CoV-2 concentrations across six different USA wastewater treatment facilities ranging in over one order of magnitude in flow rate, serving 18 municipalities and approximately 1 million residents in the State of Connecticut, USA. Lagged regression models were evaluated to estimate community COVID-19 reported case rates from a time-course of primary sludge SARS-CoV-2 RNA concentrations.

MATERIALS AND METHODS

Wastewater sampling

Primary sewage sludge samples of 40 to 45 ml in volume were collected daily from six wastewater treatment plants (WWTP) in the State of Connecticut, USA. Table 1 lists the cities and towns served, and details the specific plant processes from which primary sludge was produced and a total of 1698 samples were withdrawn. Samples from Norwich were collected daily from August 17, 2020 to June 1, 2021. Samples from Hartford were collected daily from August 10, 2020 to June 1, 2021. Samples from Stamford, Bridgeport, New Haven and New London were

collected daily from August 3, 2020 to June 1, 2021, with the exception that samples for 51 consecutive days in February and March 2021 were not collected at Stamford due to plant renovation. All samples were collected between 8 am and 9 am, stored at -20°C before being transported to Yale University laboratories on ice, and analyzed immediately upon arrival.

SARS-CoV-2 RNA primary sludge concentrations

To quantify SARS-CoV-2 RNA concentrations, the 40 to 45 ml sample was mixed on a vortexer for 1 minute and 0.5 ml of primary sludge was added to a commercial extraction kit optimized for the isolation of total RNA from raw wastewater (Zymo, Quick-RNA Fecal/Soil Microbe Microprep, wastewater protocol). Modifications to the extraction protocol included the addition of 0.1 ml of phenol/chloroform/isoamyl alcohol (25:24:1) in the initial bead beating step and eluting isolated RNA into 50 μl of ribonuclease-free water. Before September 20, 2020 and prior to the rapid rise in case rates in early October 2020, primary sludge RNA extraction was accomplished using the RNeasy PowerSoil Total RNA kit (Qiagen) as previously described (Peccia et al. 2020). For all extracts, total nucleic acid was measured by spectrophotometry, purity assessed by A_{260}/A_{280} absorbance ratio and concentration adjusted to 200 $\text{ng } \mu\text{L}^{-1}$ (NanoDrop, Thermo Fisher Scientific) prior to RT-qPCR to normalize all samples to a consistent nucleic acid concentration. All samples were diluted 5 times prior to uses as a template to ensure that no RT-qPCR inhibition occurred.

SARS-CoV-2 RNA concentration was estimated using one-step RT-qPCR with SARS-CoV-2 N1 and N2 primer sets that produce 71 and 76 bp amplicons, respectively. (U.S. Centers for Disease Control and Prevention 2020; Vogels et al. 2020) CrAssphage, a ubiquitous bacteriophage that is highly concentrated in the human gut, (Crank et al. 2020; Stachler et al. 2017; Wu et al. 2020) was quantified to indicate successful nucleic acid extraction and qPCR amplification. All SARS-CoV-2 samples reported demonstrated Ct values below 38 for the crAssphage assay PCR control. All primer sets were run in separate reactions and a total of 5 μl template containing 200 ng of nucleic acid was used for each reaction. Analysis was conducted using a one-step RT-qPCR kit (BioRad iTaqTM Universal Probes One-Step Kit). Triplicate 20 μL reactions using a 5x diluted template were run at 55°C for 10 min and 95°C for 1 min, followed by 40 cycles consisting of 95°C for 10 seconds and 55°C for 30 seconds (Peccia et al. 2020) and Ct values for triplicates were average. N1 and N2 primer set standards were constructed using ten-fold dilutions (5×10^1 to 5×10^8 copies per reaction) of the N gene transcripts for the SARS-CoV-2 Wuhan-Hu-1 strain. The limit of detection corresponds to 30 copies per μl of nucleic acid extract. Concentrations in primary sludge samples were calculated using these standard curves and are presented as SARS-CoV-2 RNA copies per ml of sludge. No template negative and SARS-CoV-2 genome positive controls

Table 1. Wastewater treatment plant characteristics.

WWTP Name	Municipalities served (total population)	Average flow rate (m ³ d ⁻¹)	Population served	Primary sludge process details
Stamford	Stamford and Darien, CT (151,528)	91 000	140 000	Bar screens→primary clarifier→de-gritting of primary sludge with hydrocyclones. Sample of thickened sludge collected after hydrocyclones.
Bridgeport West	Bridgeport, CT (144,900)	120 000	105 000	Bar screens→gravity thickener→primary clarifier. Sample collected at primary solids effluent pump.
New Haven	New Haven, Hamden, East Haven, Woodbridge, CT (228,862)	151 000	200 000	Bar screen→grit chamber→primary clarifier→gravity thickener. Sample collected at effluent pump of gravity thickener.
Hartford, South Meadows	Hartford, West Hartford, Newington, Bloomfield, Wethersfield, CT (263,021)	300 000	300 000	Bar screen→grit chamber→primary clarifier→gravity thickener. Sample collected at effluent pump of gravity thickener.
New London	New London and Waterford, CT (45,826)	23 000	40 000	Bar screen grit chamber→ primary sedimentation. Sample collected at primary solids pump.
Norwich	Norwich, Sprague, Franklin, Bozrah (46,495)	32 000	22 500	Bar screen→grit chamber→primary clarifier→gravity thickener. Sample collected at effluent pump from gravity thickener.

were run for each RT-qPCR. Negative responses were observed for all no template controls and positive responses ($Ct < 38$) were observed for all SARS-CoV-2 controls. In addition to the 51 days when the Stamford treatment plant did not provide samples, less than 1.5% of samples were not available from the treatment facilities. In these cases, SARS-CoV-2 concentrations used in regression models were estimated by interpolating between the prior and subsequent measured SARS-CoV-2 concentration.

COVID-19 test data for the cities served by the treatment facilities

The number of confirmed and probable COVID-19 cases per the Council of State and Territorial Epidemiologists case definition (Council of State and Territorial Epidemiologists 2020) was provided by the Connecticut Department of Public Health (CT DPH) and used in this analysis. The date for each case was assigned, in order of preference, as test specimen collection date (if available), symptom onset date (if available), or date of report to the CT DPH. Greater than 90% of case values were by date of clinical specimen collection. Cases were compiled from CT DPH data from the individual towns served by each wastewater treatment plant (Table 1) and adjusted per 100,000 population based on town census size.

Model development

Multiple regression analysis was used to fit COVID-19 reported cases per 100 000 population on the day of specimen collection to observed values of SARS-CoV-2 RNA copies per mL of primary

sludge. The model structure is

$$\text{case rate}_{i,t} = \sum_{j=0}^{\tau} (\beta_{i,j} \text{RNA}_{i,t-j}) + \sum_{d=1}^7 \gamma_{i,d} 1\{w_t = d\} + \epsilon_{it} \quad (1)$$

where the reported case rate is the number of new cases reported by date of specimen collection per 100 000 population, i represents municipality, t represents time (days), j is the number of lagged days, τ is the maximum number of lagged days, $\text{RNA}_{i,t-j}$ are RNA concentrations (SARS CoV-2 RNA copies mL⁻¹), w_t indicates the day of the week on day t , $\beta_{i,j}$ and $\gamma_{i,d}$ are regression coefficients, d is day of the week, and ϵ_{it} is the residual error associated with each observation. Day of the week addresses the known daily variations in testing behavior. (Bergman et al. 2020) Adjustment for population, which shows a strong collinearity with average treatment plant flow (simple linear regression: slope = 0.9 m³d⁻¹ person⁻¹, $R^2 = 0.93$, $p = 0.002$), was accomplished by considering the case rate per 100 000 residents as the dependent variable. A weighted least squares approach was used to weigh (by reciprocal of the variance) case rate contributions to the model and reduce heteroskedasticity.

The statistical significance for predictive parameters was analyzed through a two-tailed t-test performed on the regression coefficients for all RNA concentration lags, and day of the week offsets. Akaike Information Criteria (AIC), Bayesian Information Criteria (BIC) F test, and coverage probability (calculated as the proportion of times that measured case rates fell within the 95% prediction interval of the model-estimated case rates), were used to select the maximum number of lagged RNA concentrations included in the model.

While the model in equation 1 was applied to each town, we also conducted a leave-one-out cross validation to test the model's ability to estimate COVID-19 reported case rates for other wastewater catchments. The model developed in the leave-one-out cross validation used the same variables as the model presented in equation 1, but removed any dependence on municipality (represented by i in equation 1). The model was run six times, each time leaving out the treatment facility in which cases were being predicted and training the model on data from the remaining five cities. Through estimates of prediction accuracy, the model's fit was then determined on the city which had been omitted from the training data. This allowed us to analyze the generalizability of this modeling approach."

For this analysis, six regression models, similar to Equation 1, were trained by leaving out data from one of the six treatment facilities. The models contained the same variables and coefficients, but used the entire data set for training, excluding one municipality at a time. These models were then utilized to estimate case rates in the cities covered by the treatment facility excluded from the model training set.

RESULTS

SARS-CoV-2 concentrations

The SARS-CoV-2 RNA concentrations in primary sludge for the six treatment facilities are shown in Fig. 1 from August 2020 to June 2021 and reflect fall/winter/spring COVID-19 outbreaks in each city. The bottom row of Fig. 1 displays the reported case rates over the same time period in the municipalities served by the six treatment plants. The timing of SARS-CoV-2 RNA concentrations in primary sludge visually tracks the dynamics in reported case rates, with observed increases in primary sludge RNA concentrations at each plant coinciding with the start of the October 2020 outbreaks for the corresponding cities.

Model selection and validation

This study estimated COVID-19 case rates using the regression model presented in equation 1 which included lagged SARS-CoV-2 RNA wastewater concentrations and day of the week as variables. An initial analysis was conducted to determine the maximum number of prior days of wastewater RNA concentrations ($RNA_{i,t-j}$, lagged days) to use. Table 2 presents Akaike Information Criterion (AIC), Bayesian Information Criterion (BIC), F statistics with P values, and coverage probabilities for maximum lags ranging from 0 to 6 days and indicates optimal model fit with minimized model complexity when using lagged RNA concentrations from 0 to 4 days. Graphical comparisons of measured reported case rates versus model-estimated case rates are provided in Fig. 2 for each WWTP using models with lagged RNA concentrations from 0 to 4 days. When applied to the six individual municipalities, resulting coverage probabilities range from 0.93 to 0.96, with a root mean standard error of 13.1 cases per 100,000 population for the entire model (see Fig. S1 for model confidence intervals). Tables S1–S7 provide regression coefficients, intercepts, and their significance for the linear models in Equation 1 applied to each municipality. Regression coefficients were typically greatest at lags 0 and 1 day.

Results of the leave-one-out cross validation analysis revealed coverage probabilities (95% prediction intervals) of the estimated case rates that ranged from 0.90 to 0.97 for the six different municipalities considered, suggesting that the model can

provide estimates of measured cases from sludge SARS-CoV-2 RNA concentrations for a variety of wastewater catchments that contain treatment facilities that produce primary sludge (Table 3).

DISCUSSION

Estimating the number of infections directly from wastewater pathogen concentrations is a central goal of wastewater surveillance practice. The gut tropism of coronaviruses and widespread COVID-19 testing provide a unique opportunity to develop these tools. We monitored daily SARS-CoV-2 RNA concentrations in the primary sludge of six wastewater treatment plants that covered 18 USA municipalities and explored regression analyses to estimate COVID-19 cases rates on the day of specimen collection. Our results demonstrate the feasibility, utility, and simplicity of estimating COVID-19 case rates across a variety of different wastewater treatment catchments using a single model form.

Prior studies have noted a concordance in wastewater SARS-CoV-2 RNA concentrations with other indicators of infection. Early work on 2020/2021 COVID-19 outbreaks observed similar behavior between reported daily positive COVID-19 testing results in a community and the SARS-CoV-2 RNA concentrations in that community's wastewater, (Wurtzer et al. 2020) and relationships between wastewater RNA concentrations and COVID-19 reported cases have been reported for SARS-CoV-2 and polio virus. (Berchenko et al. 2017; Medema et al. 2020; Nemudryi et al. 2020; Wu et al. 2020) Several features of this study are unique to these prior studies and mark advances in the science underpinning wastewater-based epidemiology. We estimate COVID-19 case rates solely from current and prior days of primary sludge RNA concentrations and day of the week across treatment plants that range in more than one order of magnitude in size as measured by average flow rate and population served and utilized different primary treatment schemes. Inclusion of concentration data from prior days yielded a model that can account for the previously observed offsets (Nemudryi et al. 2020; Peccia et al. 2020; Wu et al. 2020) between wastewater RNA concentrations and reported case data. The regression model used revealed that concentration lags of 0 to 1 day best predict case rates and confirms the previously described 0 to 2 day lag between case rates by date of specimen collection and SARS-CoV-2 concentrations in primary sludge (Peccia et al. 2020). A single general regression model trained by pooled plant data in a leave-one-out analysis was able to accurately estimate case rates for communities served by different domestic treatment plants, suggesting that this model could be extended more broadly to a variety of communities. The approach for utilizing primary sludge instead of raw wastewater allows for rapid sampling of a mixed influent stream without the use of specialized sampling equipment. The concentration of SARS-CoV-2 in wastewater solids is greater than that in raw wastewater (Graham et al. 2021) and negates the need for concentration steps often required when using untreated wastewater. The 0.5 ml sample volume RNA extraction considered in this study can be automated. Finally, sludge SARS-CoV-2 concentrations are less impacted by precipitation events (infiltration and inflow) that might dilute concentrations of SARS-CoV-2 in the aqueous phase of raw wastewater.

The ability to estimate case rates from etiological agent concentrations in wastewater can be of significant epidemiological value. Case rates are commonly used as a proxy for changes in community infection, and have been used as the standard for implementing non-pharmaceutical interventions and policies

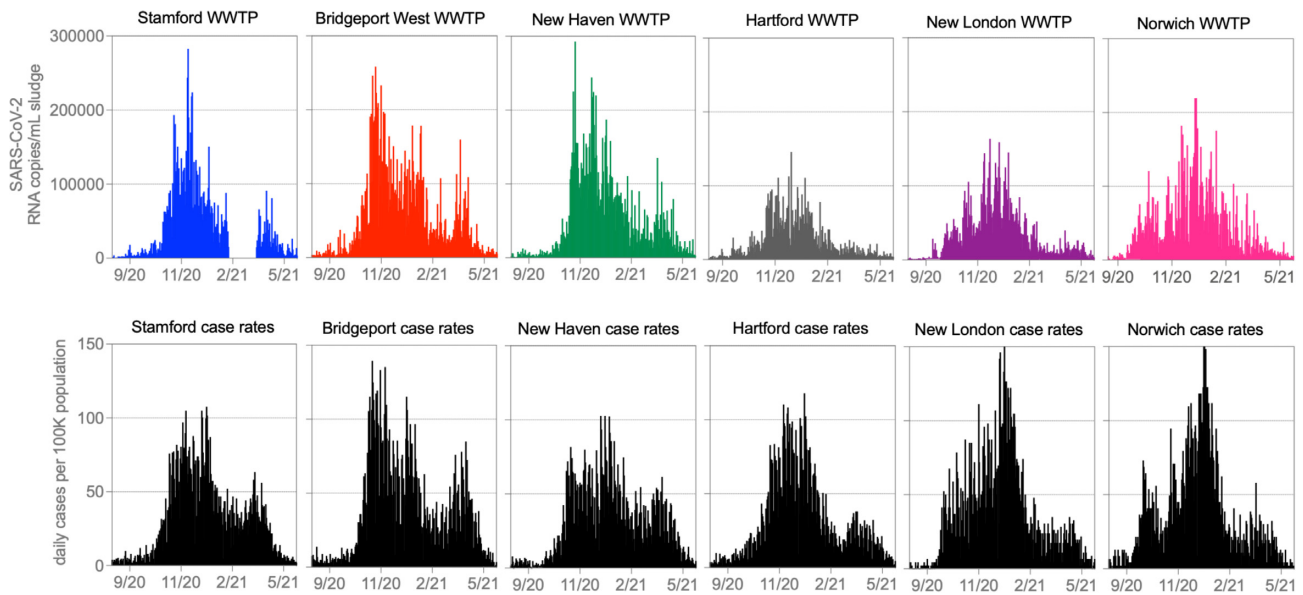


Figure 1. (top) Daily SARS-CoV-2 RNA concentrations in primary sludge of the six WWTP's considered in this study. (bottom) Daily COVID-19 cases by date of specimen collection per 100,000 residents for the cities and towns served by the above wastewater treatment plants.

Table 2. Resulting parameters for model selection to determine the maximum lagged RNA concentration.

Maximum lag τ	AIC	BIC	F statistic (P value)	Coverage Probability
0	19575	19867		0.93
1	19271	19603	56.38 (<0.01)	0.94
2	19137	19509	23.53 (<0.01)	0.95
3	19038	19450	17.501 (<0.01)	0.95
4	18945	19397	8.32 (<0.01)	0.95
5	18923	19406	6.3172 (<0.01)	0.95
6	18927	19473	7.123 (<0.01)	0.95

Table 3. Leave-one-out cross validation results for the general model (Xiao et al. 2020).

Town Omitted	F-statistic (P value)	Coverage Probability
Stamford	316.5 (P < 0.001)	0.97
Bridgeport West	298.7 (P < 0.001)	0.95
New Haven	367.1 (P < 0.001)	0.90
Hartford	301.2 (P < 0.001)	0.92
New London	323.3 (P < 0.001)	0.92
Norwich	292.1 (P < 0.001)	0.93

to reduce COVID-19 transmission and associated hospitalizations and deaths. In jurisdictions where testing is limited or does not exist, these models can be used as an independent estimate of infection rates. Wastewater RNA concentrations can be reported the same day the sample is collected, thus the statistical models used herein can be utilized to estimate up-to-date reported case rates in a community when COVID-19 testing data lags.

Limitations

This modeling approach relies on statistical relationships between wastewater primary sludge RNA concentrations and

COVID-19 case rates, which are primarily based on diagnostic test results. The reported prediction intervals reflect not only model fit but the variability in these reported case rates. While commonly used as a proxy, reported cases are believed to underestimate infection due to asymptomatic COVID-19 infections. (He et al. 2020) Clinical testing volumes in the municipalities considered were dynamic over the study period; responding to events such as school openings, holidays, and shifting of resources to locations with increasing case rates. The measured RNA concentrations in wastewater are not subject to variation in testing practice and should therefore exhibit a more direct relationship to the unobservable changes in SARS-CoV-2 infection in the community. While clinical testing data is considered

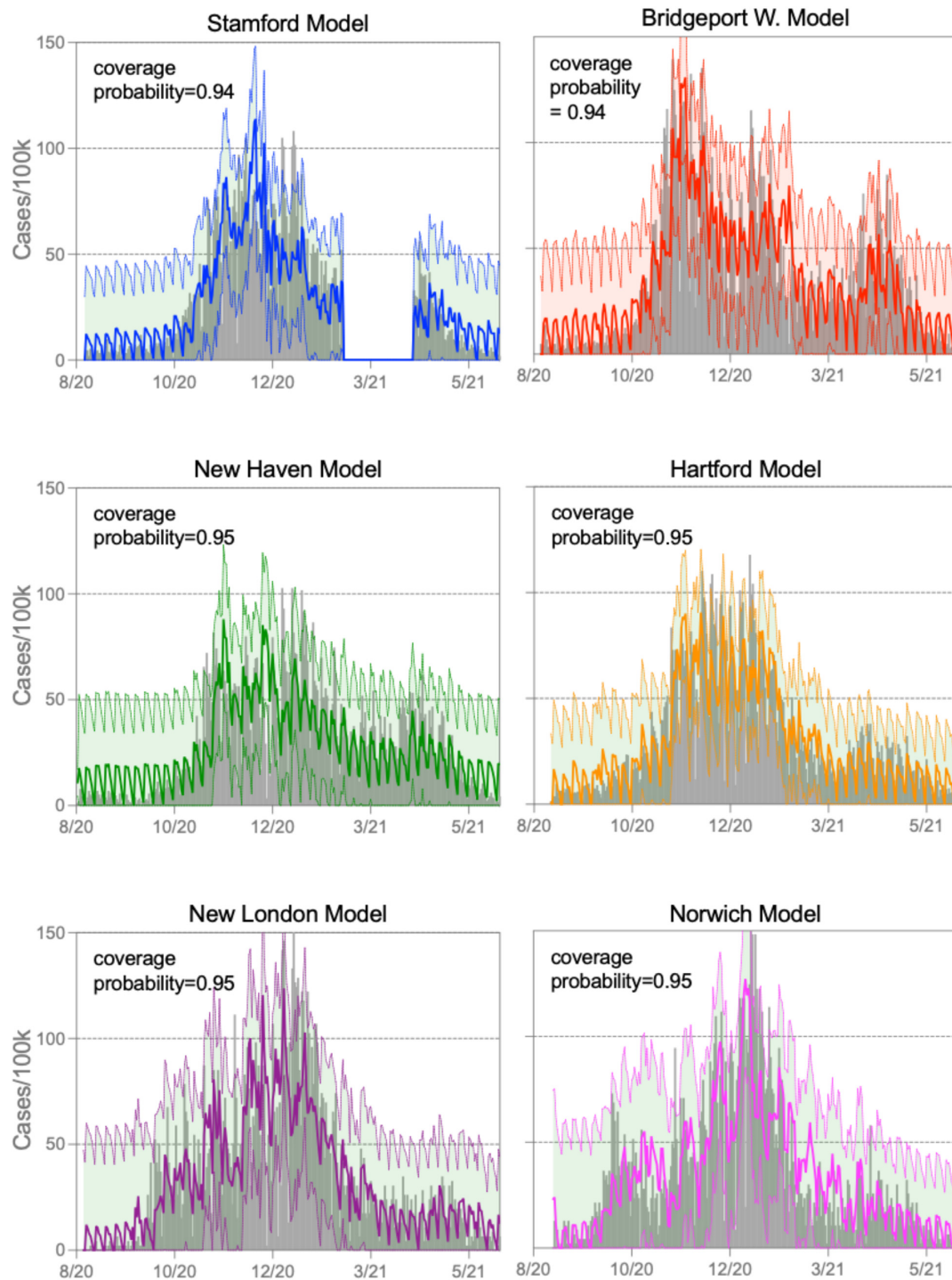


Figure 2. Comparison between COVID-19 case rates by day of clinical specimen collection and estimated case rates using RNA concentrations lagged from 0 to 4 days. The model is depicted by a solid line, measured cases are grey bars, and 95% prediction intervals are shown by shading and the dotted lines.

an imperfect measure of infection, even in locations with strong testing programs, we note that the data resulting from testing programs has been indispensable in understanding the progression of outbreaks and initiating action to stem the spread of COVID-19 throughout the world. That case results can be estimated from SARS-CoV-2 concentrations in sewage sludge provides an added measure of confidence in the use of reported cases to monitor the epidemic.

Summary

Measuring the concentration of pathogens in domestic wastewater can be a useful indicator of infection trends within a population. This study demonstrated that a regression model populated by daily lagged SARS-CoV-2 sewage sludge concentrations could estimate COVID-19 case rates across communities served by six different wastewater treatment facilities. Cross-validation by leave-one-out analysis suggests the regression model can

provide estimates of COVID-19 case rates for a broad variety of treatment facilities that produce primary sludge. Estimating case rates from wastewater pathogen concentrations can be useful in locations with limited or delayed COVID-19 testing programs or for infectious diseases where individual testing programs are not well-developed.

SUPPLEMENTARY DATA

Supplementary data are available at [FEMSMC](#) online.

ACKNOWLEDGMENTS

This project was supported by Cooperative Agreement no. 6NU50CK000524-01 from the Centers for Disease Control and Prevention using funds from the COVID-19 Paycheck Protection Program and Health Care Enhancement Act Response Activities. This activity was reviewed by CDC and was conducted consistent with applicable federal law and CDC policy. See e.g., 45 C.F.R. part 46.102(l) (2), 21 C.F.R. part 56; 42 U.S.C. 241(d); 5 U.S.C. 552a; 44 U.S.C. 3501 et seq. The findings and conclusions of this report are those of the author(s) and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

We also wish to thank the Stamford, Bridgeport West, New Haven, Hartford South Meadows, New London, and Norwich, CT wastewater treatment facilities for assistance in sampling and participation in the study and the Rothberg fund for equipment support.

Conflicts of interests. None declared.

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