



A multicenter study investigating SARS-CoV-2 in tertiary-care hospital wastewater. viral burden correlates with increasing hospitalized cases as well as hospital-associated transmissions and outbreaks

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ABSTRACT

SARS-CoV-2 has been detected in wastewater and its abundance correlated with community COVID-19 cases, hospitalizations and deaths. We sought to use wastewater-based detection of SARS-CoV-2 to assess the epidemiology of SARS-CoV-2 in hospitals. Between August and December 2020, twice-weekly wastewater samples from three tertiary-care hospitals (totaling > 2100 dedicated inpatient beds) were collected. Hospital-1 and Hospital-2 could be captured with a single sampling point whereas Hospital-3 required three separate monitoring sites. Wastewater samples were concentrated and cleaned using the 4S-silica column method and assessed for SARS-CoV-2 gene-targets (N1, N2 and E) and controls using RT-qPCR. Wastewater SARS-CoV-2 as measured by quantification cycle (Cq), genome copies and genomes normalized to the fecal biomarker PMMoV were compared to the total daily number of patients hospitalized with active COVID-19, confirmed cases of hospital-acquired infection, and the occurrence of unit-specific outbreaks. Of 165 wastewater samples collected, 159 (96%) were assayable. The N1-gene from SARS-CoV-2 was detected in 64.1% of samples, N2 in 49.7% and E in 10%. N1 and N2 in wastewater increased over time both in terms of the amount of detectable virus and the proportion of samples that were positive, consistent with increasing hospitalizations at those sites with single monitoring points (Pearson's $r = 0.679$, $P < 0.0001$, Pearson's $r = 0.799$, $P < 0.0001$, respectively). Despite increasing hospitalizations through the study period, nosocomial-acquired cases of COVID-19 (Pearson's $r =$

; **Abbreviations:** BCovV, Bovine coronavirus; HCW, Health care workers; IPC, Infection, Prevention and control; LOD, Limit of detection; NTC, No-template control; PMMoV, Pepper mild mottle virus; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; WWTP, wastewater-treatment plants.

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0.389, $P < 0.001$) and unit-specific outbreaks were discernable with significant increases in detectable SARS-CoV-2 N1-RNA (median 112 copies/ml) versus outbreak-free periods (0 copies/ml; $P < 0.0001$). Wastewater-based monitoring of SARS-CoV-2 represents a promising tool for SARS-CoV-2 passive surveillance and case identification, containment, and mitigation in acute-care medical facilities.

1. Introduction

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) RNA is present in the feces of most infected individuals – appearing just prior or concomitant with symptoms (Foladori et al., 2020). Accordingly, leaders in the field of wastewater-based epidemiology leveraged their expertise to study this emerging infectious disease (Gupta et al., 2020; Ling et al., 2020). Medema et al., (2020) first reported SARS-CoV-2-RNA in Dutch wastewater-treatment plants (WWTP). Several groups have since adapted this technology to understand community disease-burden (Ahmed et al., 2020a; D'Aoust et al., 2021; Peccia et al., 2020; Wurtzer et al., 2020). Recent studies suggest that SARS-CoV-2-RNA increases in WWTP precede clinically diagnosed cases by 0–2 days and associated hospitalizations by 1–4 days (Peccia et al., 2020).

Between 4–8% of individuals with COVID-19 will be hospitalized, with age and co-morbidities being key risk factors (Carrillo-Vega et al., 2020; Ioannou et al., 2020; Ko et al., 2020). Nosocomial-transmission and outbreaks affecting patients and health care workers (HCW) have occurred despite the best efforts of hospital infection, prevention and control (IPC) staff, and drivers remain to be fully understood (Carter et al., 2020; Long et al., 2020). While hospital-acquisition is rare (0.8–5 cases/10,000 patient-days in communities with high disease burden), public fear of acquiring COVID-19 from hospitals has resulted in reduced health-resource utilization and hospital avoidance, often to the detriment of patients (Czeisler et al., 2020; Hartnett et al., 2020). Accordingly, hospital-based detection tools are needed to understand the epidemiology of COVID-19 and potentially mitigate spread.

Hospitals hold great promise in understanding SARS-CoV-2 wastewater-generated data. Owing to their proximity to affected individuals in the municipal sewershed relative to WWTP (i.e., shorter transit time for signal degradation (Foladori et al., 2020)), hospitals may aid in understanding SARS-CoV-2 wastewater dynamics. Compared to the general community, hospitals are much more likely to comprehensively monitor and identify all cases within their populations. Furthermore, outbreaks in hospitals are rapidly and comprehensively investigated. For these reasons we embarked on this study to determine relationships between hospital SARS-CoV-2 wastewater dynamics and COVID-19 hospitalizations, nosocomial-transmissions and outbreaks.

2. Methods and materials

2.1. Acute-care hospitals and hospital information systems

We monitored SARS-CoV-2-RNA in the wastewater from three of Calgary's four adult tertiary-care hospitals, accounting for 89% of staffed-inpatient beds (see Supplementary Material). Daily prevalent-hospitalized cases were defined as all those with laboratory-confirmed COVID-19 within 14 days of their diagnosis, remaining on contact/droplet isolation (Alberta-Health-Services, 2020). Hospital-acquired cases were defined as patients who were admitted to hospital ≥ 7 days before COVID-19 symptom onset that were then confirmed by a positive RT-qPCR SARS-CoV-2 test; or a patient admitted to hospital for ≤ 7 days confirmed to have hospital-acquired COVID-19 infection based on an epidemiological link after detailed review by IPC staff. Hospital-acquired cases were separately adjudicated and recorded including the unit where they were acquired and are reported as hospital-wide signals for Hospital-1 and 2. Data for Hospital-3 are presented as 3A, 3B, 3C based on wastewater drainage outflows of different

buildings/units. COVID-19 outbreaks were defined as any unit with ≥ 1 confirmed hospital-acquired case(s) and/or ≥ 2 confirmed COVID-19 cases in HCWs linked to a unit. This research was approved by the University of Calgary's Conjoint Health Regional Ethics Board (REB-20-1252).

2.2. Wastewater sampling

Wastewater samples were collected from August 5th to December 17th, 2020 at three hospitals. Hospital-wide access through a single sampling point was not possible at Hospital-3 where an initial sampling point (Hospital-3A) captured the units predicted to be most relevant for COVID-19 (including intensive care units and dedicated COVID-19 care-units). Beginning October 1st two additional sites were added, Hospital-3B and Hospital-3C, expanding coverage to all inpatient-care buildings. ISCO GLS samplers (Lincoln, Nebraska) were installed by highly-trained personnel from the City of Calgary at the designated manholes and programmed to collect 100 ml of wastewater every 15 mins, for a total of 96 pooled samples over a period of 24 h. These 24 h composite samples were collected twice a week. Temperature readings of each sample were taken and recorded in the field, at the time of collection, as well in the laboratory during subsampling. Samples were transported to the City of Calgary's wastewater laboratory on ice. The 10 L carboy was well-mixed and poured off into five individual 500 ml sample bottles. Samples were stored at 4 °C, in accordance with standard methods, before being shipped to the Advancing Canadian Wastewater Assets (ACWA) lab for analysis.

2.3. Sample processing and RNA purification

Sample preparation and molecular analysis were performed at separate sites to prevent cross contamination. At ACWA, Bovine coronavirus (BCoV) aliquots were generated by resuspending a Bovilis® Coronavirus Vaccine dose (Merck, Catalogue #151921) in 2 ml of PBS (final concentration 5×10^5 50% tissue-culture-infective dose (TCID₅₀/ml)) and stored at -80 °C. Wastewater samples were processed with a 40 ml aliquot taken to comprise the sample. Each sample was spiked with 200 μ l of BCoV positive control (Final concentration of 2500 TCID₅₀/ml). Sample processing and RNA purification was conducted using the 4S (Sewage, Salt, Silica and SARS-CoV-2) method with a few modifications (Whitney et al., 2020). In brief, 40 ml aliquots of wastewater were transferred into 50 ml conical tubes where particle lysis and RNA preservation were conducted by the addition of 9.5 g of NaCl and 400 μ l of TE buffer, respectively. The mixture was filtered through a 5 μ m PVDF filter into 40 ml 70% EtOH to remove large particles and debris. Subsequent RNA binding, washing, and elution was performed using a silica spin column (Zymo III-P silica spin column, Zymo Research) attached to a custom vacuum manifold. Washing volumes for buffers 4S-WB1 and 4S-WB2 were adjusted to 10 ml and 20 ml respectively to minimize downstream inhibition. Nucleic acids were eluted in 100 μ l of 50 °C RNase free water and stored immediately at -80 °C. Purified nucleic acids were transported on dry ice to the Health Sciences center for molecular analysis. An extraction blank control was included for every processed sample batch to ensure no contamination occurred.

2.4. RT-qPCR analysis

We used RT-qPCR to quantify SARS-CoV-2-RNA and controls in wastewater. Specific primers and probes (Supplemental Table 1S) were

used to amplify two regions of the nucleocapsid gene (i.e., N1 and N2) and a region of the envelope gene (i.e., E). All amplification reactions for SARS-CoV-2 detection are described in Supplementary Materials. Samples were considered positive for the presence of SARS-CoV-2 RNA-target if amplification passed a detection cycle threshold in < 40 cycles for at least one of N1, N2 and/or E (Medema et al., 2020; Randazzo et al., 2020; Wu et al., 2020). Amplification of the Pepper mild mottle virus (PMMoV) was employed to incorporate a human fecal biomarker control in order to normalize SARS-CoV-2 for the relative bioburden in samples (D'Aoust et al., 2021). Amplification reactions to estimate the number of genomic copies of PMMoV and the number of copies of the internal control (i.e., BCoV) are described in Supplementary Materials. All RT-qPCRs were performed using a QuantStudio-5 Real-Time PCR System (Applied Biosystems) with each run including no-template controls (NTCs) in triplicate. For N1, N2 and E-assays three positive controls were included in each run. Samples where minimal signal for BCoV or PMMoV controls were recovered were excluded from the analysis to mitigate false-negative results.

To determine the limit of detection (LOD) of the N1 assay a similar approach described by La Rosa et al., 2021 was followed. Briefly, pure SARS-CoV-2 RNA was extracted from the NATrol™ SARS-Related Coronavirus 2 stock (ZeptoMetrix, Catalogue # NATSARS(COV2)-ST) using TRIzol™ Reagent treatment and RNeasy Mini Kit (Qiagen, Catalogue # 74104) following the manufacturer's instructions. Then, the SARS-CoV-2 pure RNA (quantified $\sim 5 \times 10^3$ genome copies/ μ l) was serially diluted to assess the sensitivity of the N1 assay. Similarly, to assess the sensitivity in wastewater nucleic acid extracts, the same dilutions of the pure target were performed in nucleic acids extracted from a hospital wastewater sample that had tested negative for SARS-CoV-2. Each diluted sample was analyzed by quadruplicate and the LOD (reported as genome copies of SARS-CoV-2 per ml wastewater) was determined as the last dilution where the relative repeatability standard deviation (RSDr) of the replicates was $\leq 33\%$ (Del Gaudio et al., 2012).

Genome copies per reaction were converted to copies per unit volume of wastewater (i.e., 40 ml) using the following dimensional analysis: Concentration (genomic copies/ml) = $C * V_{T \text{ eluted}} * 1 / V_{\text{per rxn}} * 1 / V_{\text{composite}}$, where C = number of copies exported from the QuantStudio 5 Real-Time PCR System, $V_{T \text{ eluted}}$ = total volume of nucleic acids eluted during silica column purification (μ l), $V_{\text{per rxn}}$ = volume of nucleic acids added to each RT-qPCR reaction (μ l) and $V_{\text{composite}}$ = volume of composite sample used to extraction (ml). To normalize the number of copies obtained for each of the three targets for SARS-CoV-2 detection (i.e., N1, N2 and E) by the detection of PMMoV in samples, we used the same approach described by D'Aoust et al., 2021.

2.5. Statistical analysis

To compare the surrogate BCoV and PMMoV signals between hospitals, pairwise Mann-Whitney tests were performed. Non-parametric Kruskal-Wallis tests were performed for multiple comparisons of assay sensitivity and surrogate organism signals between sampling locations. Pearson's correlation analyses were performed to determine the correlation of wastewater RNA-signal measured as (i). Cq, or (ii). genome copies/ml of wastewater or (iii). genome copies/genome of PMMoV, vs daily-hospitalized cases. To assess for correlation of wastewater RNA-signal with incident hospital-acquired cases, and to compensate for gaps owing to the twice-weekly sampling, incident cases occurring ± 3 days were compared to wastewater signals. To compare the SARS-CoV-2 wastewater N1-signal observed during and between unit-outbreaks, each sample was dichotomized as being collected within 3-days of a declared outbreak or not. Samples after a declared outbreak were excluded until 3-days after the last in-hospital linked case was identified. Statistical tests analyzed Hospital-1 and Hospital-2 together (given their capture of the entire hospital-facility) and separately. Statistical analyses were conducted with GraphPad's Prism-8 software (La Jolla, CA).

3. Results

3.1. Reproducibility of sample processing and RNA purification

The sampling timeframe spanned 18 weeks at Hospital-1 and Hospital-2, Hospital-3A and 11 weeks at each of Hospital-3B and Hospital-3C. To determine the reproducibility of sample processing among hospital samples, BCoV was used as an internal control for sample processing and RNA purification. Sample reproducibility was acceptable as similar BCoV concentrations in wastewater were observed among hospitals except for a few samples from Hospital-2 and Hospital-3A (Supplemental Fig. 1S). The identified median number of spiked BCoV copies per ml of wastewater samples differed between hospitals (Hospital-1, 9.5×10^5 [IQR, 3×10^5 – 2.1×10^6]; Hospital-2, 8.6×10^5 [IQR, 5.6×10^5 – 2.1×10^6]; Hospital-3A, 2×10^5 [IQR, 6.7×10^4 – 7.8×10^5]; Hospital-3B, 9.9×10^5 [IQR, 7.1×10^5 – 1.5×10^6]; and Hospital-3C, 8×10^5 [IQR, 2.8×10^5 – 1.2×10^6]; $P < 0.0001$). From the analysis, six samples were excluded owing to low BCoV signal (corresponding to samples collected on August 27th, September 3rd, 9th, 10th and 17th from Hospital-3A and September 15th from Hospital-2). Furthermore, there was no detectable signal for the PMMoV human fecal biomarker in these excluded samples, suggesting issues with sample integrity (Supplemental Fig. 2S). In general, the signal of BCoV recovery was lower from Hospital-3A location (Hospital-3A vs Hospital-3B, $P < 0.0001$; Hospital-3A vs Hospital-3C, $P = 0.0101$; Hospital-3A vs Hospital-1, $P = 0.0002$; Hospital-3A vs Hospital-2, $P < 0.0001$).

Chemical analysis of water from Hospital-3A indicated significant spikes in chloride concentration, as high as 1780 mg/l from a baseline average of 100–200 mg/l at all other processing locations. Additionally, sample turbidity was visually noted to be unusually low for primary municipal wastewater, and samples were observed to have a free-chlorine smell. Correspondingly, total chemical oxygen demand averaged 173 mg/l in Hospital-3A samples compared to average anticipated values greater than 500 mg/l in typical primary municipal wastewater, indicating substantial oxidation of the wastewater prior to sample collection. Inspection of building schematics showed that Hospital-3A contained wastewater from a medical reprocessing facility within the hospital, likely causing the observed spike in chloride concentration as well as apparent free-chlorine. The presence of a strong oxidant in the form of chlorine bleach in samples from Hospital-3A presents a high probability of degradation of SARS-CoV-2 in the water, though the level of such degradation cannot be determined. Wastewater fecal strength signal (i.e., PMMoV) varied from 0.5 to 11,015.2 genomic copies/ml (median 182.2 copies/ml [IQR:42.5–841.6]) among all hospitals (Supplemental Fig. 2S). The median number of PMMoV copies per ml of sample differed between hospitals (Hospital-1, 133 [IQR, 48.6–827]; Hospital-2, 143 [IQR, 43.7–625]; Hospital-3A, 42.4 [IQR, 5.15–241]; Hospital-3B, 1183 [IQR, 245–2753]; and Hospital-3C, 429 [IQR, 72.9–1383]; $P < 0.0001$).

3.2. Hospital SARS-CoV-2 wastewater-RNA kinetics

In total, 165 hospital wastewater samples were collected and 159 assessed through 135-days of bi-weekly observation (40 Hospital-1; 39 Hospital-2; 34 Hospital-3A; 23 Hospital-3B; 23 Hospital-3C). Six samples were excluded (Supplemental Fig. 1S-2S). SARS-CoV-2-RNA signal in wastewater increased over time in both the amount detectable and the proportion of samples that were positive, consistent with increasing cases and hospitalizations (Table 1, Supplemental Fig. 3S), coinciding with Calgary's COVID-19 'second wave'. Hospital-1 had a higher proportion of SARS-CoV-2-positive wastewater compared to Hospital-2 and Hospital-3, consistent with the higher burden of disease in NE Calgary (Table 1 and Fig. 1). Following a large outbreak in Hospital-3 involving 45 patients, 43 HCW and 5 visitors (beginning in a ward not monitored via Hospital-3A site and compounded by affected patients being transferred into different units through the hospital) that was declared on

Table 1
Descriptive statistics of SARS-CoV-2 RNA monitoring among wastewater hospital samples.

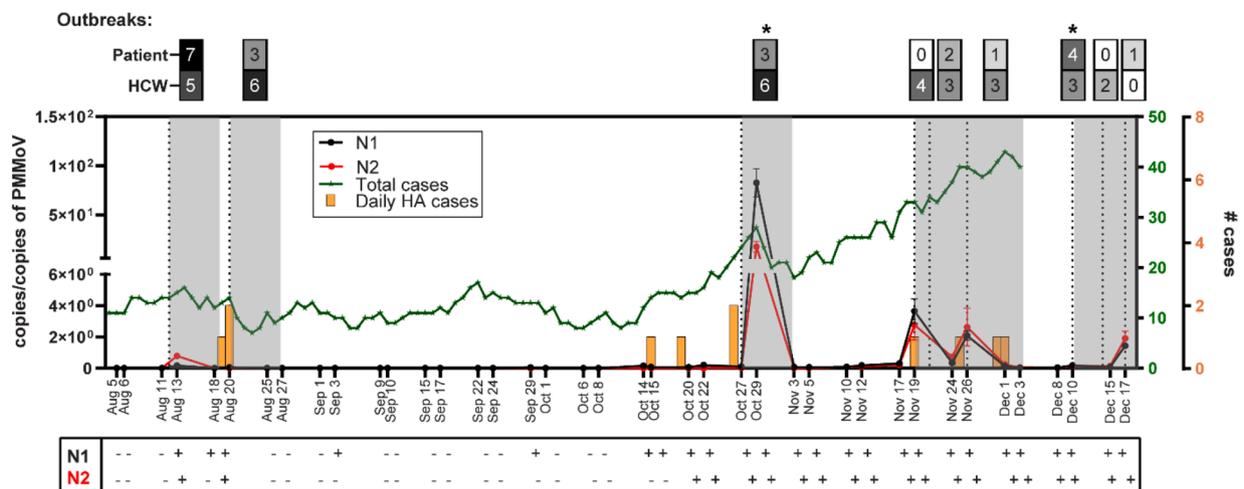
Hospital	Collection period	No. of tested samples	% of (+) SARS-CoV-2*	N1 Cq median (IQR)	Cq range†	% of (+) SARS-CoV-2*	N2 Cq median (IQR)	Cq range†	% of (+) SARS-CoV-2*	E ^β Cq median (IQR)	Cq range†
1	August 5 th to December 17 th	40	62.5	32.5 (30.6–36.4)	12.5	50	35.3 (33.5–37.7)	13.5	7.5	35.5 (31.3–37.3)	12.1
2	August 5 th to December 17 th	40	45	31.8–35.9	8.6	35	35.9 (34.5–37.8)	9.04	0	–	–
3A	August 5 th to December 17 th	39	51.3	32.8 (30.7–35.6)	11.9	48.7	36.2 (33.2–41.2)	16.3	12.8	30.6 (28.5–30.7)	5.7
3B	October 1 st to December 17 th	23	91.3	34.6 (33.8–36.5)	8.3	73.9	39.6 (38.1–41.7)	9.7	0	–	–
3C	October 1 st to December 17 th	23	78.3	36.4 (34.7–37.1)	11.07	52.2	41.1 (39.7–41.7)	13.08	4.3	34.9	–

* Percentage of samples positive for SARS-CoV2 (no. of positive samples/no. of tested samples).

† Range is the difference between the max and min value for Cq, quantification cycle.

β E assay was performed for samples collected from August 5th to October 29th.

A



B

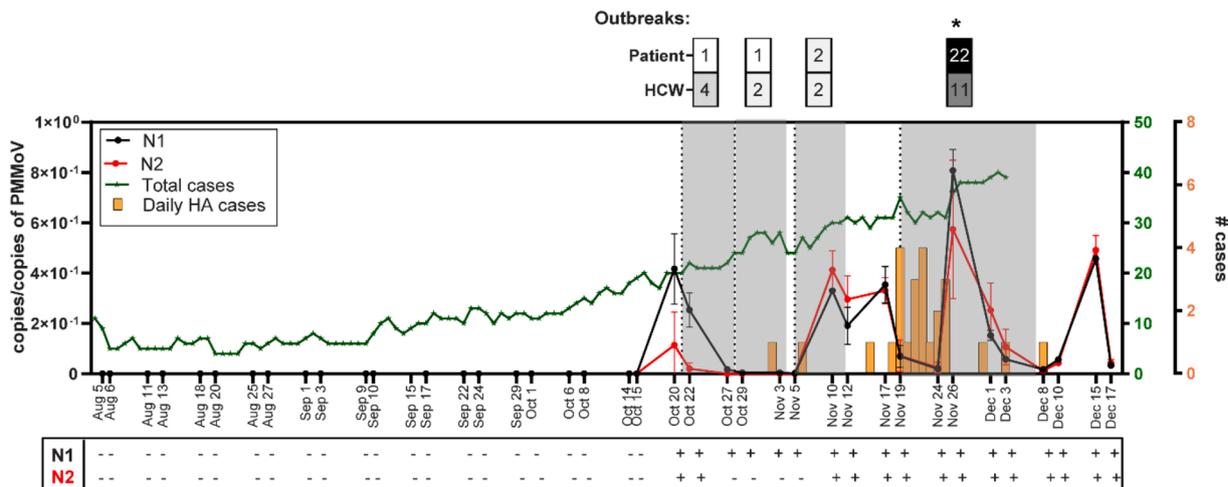


Fig. 1. Determination of SARS-CoV-2 RNA in wastewater samples from Hospital-1 and Hospital-2. Relative SARS-CoV-2 genomic copies compared to genomic copies of PMMoV from (A) Hospital 1 (August 5th to December 17th) and (B) Hospital 2 (August 5th to December 17th). Quantification of SARS-CoV-2 RNA in samples was determined by the N1 (black) and N2 (red) assays. Green line denotes the total daily number of active prevalent cases in the hospital. Orange bars denotes the number of daily hospital-acquired cases. Plots show the average of three technical replicates and error bars represent the standard deviation. Vertical dash lines correspond to days where outbreaks were declared (Table S3), where the number of patients and health care workers involved are indicated at the top each dotted dash line. Asterisk denotes that for a specific outbreak more than one unit was involved. Gray zones denote duration of the outbreak. Bottom individual boxed areas represent individual samples as positive (+) samples where SARS-CoV-2 signal was identified with a Cq<40, and negatives (-) had values ≥40. Please note that the scale is different in Figures. A and B. HA: hospital acquired, HCW: health care worker.

September 17th, wastewater sampling was expanded to include additional sites; Hospital-3B and Hospital-3C (Fig. 2) to enable complete capture of Hospital-3.

We observed that the N1-assay had the best sensitivity for detecting SARS-CoV-2. Of the 96 samples tested using N1, N2 and E-assays (i.e., those received between August 1st and October 29th), 9 samples were positive for all three targets, 28 were positive only for N1 and N2, and 39 were positive only for N1. After October 29th the E-assay was dropped, and 69 samples were analyzed, 51 were positive for both N1 and N2 and 63 were positive just with N1. Relative to N1 (considered for this study the gold-standard), N2 and E had a sensitivity of 77.4% and 23.07% respectively, – which did not differ across each site of sampling for N2 sensitivity (Hospital-3A: 80%, Hospital-3B: 81%, Hospital-3C: 66.7%, Hospital-1: 80% and Hospital-2: 77.8%, $P > 0.999$), or E (Hospital-3A: 55.6%, Hospital-3B: 0%, Hospital-3C: 14.3%, Hospital-1: 27.3% and Hospital-2: 0%, $P > 0.999$). N1 and N2-signals measured as Cq were positively correlated (Pearson's $r = 0.710$ Hospital-1, 0.762 Hospital-2, 0.792 Hospital-3A, 0.417 Hospital-3B and 0.491 Hospital-3C) across all sites (Table 2, Supplemental Fig. 4S). No-template and blank controls for sample processing and RNA purification were negative for all assays (i.e., N1, N2, E, BCoV and PMMoV). The LOD for the N1-target was found to be 1.025 genome copies of SARS-CoV-2 per ml wastewater. Standard curves for all RT-qPCR assays were within an acceptable range for efficiencies and R^2 (Supplemental Table 2S). Supplementary raw data for all the RT-qPCR is available in the Supplementary Material 2.

We validated our RT-qPCR detection of SARS-CoV-2 from hospital wastewater samples by Sanger sequencing a 127 bp PCR product from the N-gene of two wastewater samples (i.e., Hospital-1: October 29th and Hospital-3A: September 29th). The consensus alignment for the forward and reverse Sanger sequences for both samples analyzed confirmed a 100% identity match to the nucleocapsid phosphoprotein (N) gene of the SARS-CoV-2/human/CHN/Patient 12 isolate (GenBank: MW362756.1).

3.3. Wastewater SARS-CoV-2 signal correlates with total hospitalized COVID-19 cases

We assessed the correlation between the SARS-CoV-2 wastewater-N1 with active-COVID-19 patients on contact/droplet isolation at each hospital. When assessed together, Hospital-1 and Hospital-2, we observed that as prevalent cases increased, the wastewater-signal measured as N1-Cq also increased (Pearson's $r = 0.679$, CI: 0.529–0.787, $P < 0.0001$). This was also true when Hospital-1 and Hospital-2 were assessed separately (Table 2). The same was observed when SARS-CoV-2-N1 wastewater was normalized against copies of the PMMoV at Hospital-2, but only trended towards significance at Hospital-1 (Table 2). These same correlations are not as reliable at the Hospital-3 as we did not have access to prevalent cases as a function of sampling site. However, we continued to observe a positive correlation (Table 2) between prevalent cases vs N1-wastewater signal was measured as Cq at Hospital-3A (including dedicated COVID-19 care units and ICUs) (Pearson's $r = 0.717$) or measured as copies/ml and copies normalized to PMMoV at Hospital-3C (Pearson's $r = 0.503$ and 0.479, respectively). In general, those same trends observed with N1 were observed with N2 as well (Supplemental Table 3S).

3.4. Wastewater SARS-CoV-2 signal correlates with hospital-acquired infections and outbreaks

We observed a positive correlation between wastewater N1-signal and hospital-acquired cases at Hospital-1 and Hospital-2 when analyzed together (Pearson's $r = 0.389$, CI: 0.177–0.566, $P < 0.001$) and individually (Table 2). Hospital-3 data could not be fully analyzed as we did not have complete access to patient/HCW movements. Total SARS-CoV-2 as measured by Cq correlated with incident hospital-acquired cases at Hospital-3A when normalized relative to PMMoV. With

respect to whether peaks in SARS-CoV-2 in wastewater associated with outbreaks, we compared SARS-CoV-2 signal from wastewater samples collected within 3 days of an outbreak being declared with samples collected during outbreak-free periods. When Hospital-1 and Hospital-2 were analyzed together, we observed significant differences in median SARS-CoV-2 N-1 between outbreak-free periods vs outbreak periods when measured as copies/ml (0 [IQR: 0–6.6] vs 39 [IQR: 11–1592], $P < 0.0001$) and normalized for PMMoV (0 [IQR: 0–0.05] vs 0.17 [IQR: 0.06–0.89], $P < 0.0001$). We observed that at each of Hospital-1, Hospital-2 and Hospital-3A there were significant differences in median SARS-CoV-2-N1 measured using copies/ml between outbreak and outbreak-free periods (Table 3). Similarly, the same trend was observed at Hospital-1 and Hospital-3A when wastewater SARS-CoV-2-N1 was normalized for PMMoV (Table 3). In general, those same trends associating N1 with incident cases and outbreaks were observed with N2 (Supplemental Table 3S and 4S).

3.5. SARS-CoV-2 in the city of Calgary during the study period

During the period of observation, cases identified in the City of Calgary remained relatively stable from August at ~40–60 new cases/day (3.1–4.6 incident cases/100,000 residents per day) until mid-October when they began to increase, peaking in mid-December at ~600–700 cases/day (46.7–54.4 incident cases/100,000 residents per day). The absolute number of people in area hospitals on contact/droplet precautions generally mirrored community incident data (Supplemental Fig. 3S).

4. Discussion

Hospital-associated outbreaks of COVID-19 are increasingly being reported. Early data suggests that patients with hospital-acquired COVID-19 may fare worse than those with community-acquired disease, experiencing longer hospital stays but not increased mortality (Carter et al., 2020). This observation balances the opposing impacts of increased co-morbidities and medical acuity in hospitalized-individuals on one-hand, with the potential for earlier detection and more rapid supportive care/treatment on the other.

Preventing hospital-associated transmission of COVID-19 is challenging for a myriad of reasons (Harada et al., 2020). In addition to its highly infectious nature, accurate identification, triage, and effective isolation of cases is exceedingly difficult. While COVID-19 has a typical incubation period of 5–7 days, it can take as long as 14 days to manifest such that identifying evolving symptoms in previously admitted patients is challenging (Backer et al., 2020; Wang et al., 2020). Furthermore, up to 40% of individuals (including patients and HCW) may be asymptomatic, pauci-symptomatic, or pre-symptomatic-each just as likely to transmit infection as symptomatic individuals (Arons et al., 2020; Johansson et al., 2021; Yanes-Lane et al., 2020). Despite rigorous infection control protocols, hospital-acquired infections continue to occur. Novel strategies to understand the epidemiology of SARS-CoV-2 in hospitals are therefore urgently required. One such strategy may be the monitoring of hospital wastewater (Gonçalves et al., 2021).

To date, most wastewater-based SARS-CoV-2 RNA surveillance has focused on monitoring community burden of disease by sampling WWTP (Ahmed et al., 2020a; D'Aoust et al., 2021; Peccia et al., 2020; Wurtzer et al., 2020; Wannigama et al., 2021). More recently, moving sampling 'upstream' in the wastewater-network, closer to patients, is actively being explored. The most granular data comes from single-facility assessments. Passive wastewater surveillance could hold promise as an early warning strategy, adaptable to both low- and high-risk facilities. Wong et al. (2021) showed the utility of wastewater surveillance for the detection of SARS-CoV-2 RNA signal in wastewaters of a high-risk apartment buildings. Similarly, others have used wastewater SARS-CoV-2 surveillance to monitor resident populations of university dormitories to aid in active case finding – including those that

Table 2

Correlation analyses between SARS-CoV-2 RNA signal from N1 assay in wastewater from Calgary Hospitals with daily-prevalent cases of patients hospitalized with COVID-19 and those incident hospital-acquired infection.

Hospital	Comparison	Cq			Copies/ml			Copies/Copies PMMoV [†]		
		r	P-value	95% CI	r	P-value	95% CI	r	P-value	95% CI
1	N1 vs N2	0.710	<0.0001	0.511 – 0.836	–	–	–	–	–	–
	N1 vs prevalent	0.486	0.0027	0.187 – 0.702	0.290	0.0858	–0.0422 – 0.565	0.187	0.2737	–0.150 – 0.486
	N1 vs incident*	0.537	0.0005	0.263 – 0.731	0.279	0.0896	–0.0445 – 0.550	0.277	0.0920	–0.0466 – 0.548
2	N1 vs N2	0.762	<0.0001	0.587 – 0.868	–	–	–	–	–	–
	N1 vs prevalent	0.862	<0.0001	0.742 – 0.929	0.542	0.0008	0.255 – 0.741	0.534	0.0009	0.245 – 0.736
	N1 vs incident*	0.474	0.0030	0.177 – 0.692	0.255	0.1273	–0.0750 – 0.535	0.283	0.0900	–0.0455 – 0.556
3A	N1 vs N2	0.792	<0.0001	0.620 – 0.892	–	–	–	–	–	–
	N1 vs prevalent#	0.717	<0.0001	0.481 – 0.856	0.202	0.2840	–0.171 – 0.524	0.156	0.4115	–0.217 – 0.489
	N1 vs incident*	0.350	0.0492	0.00201 – 0.623	0.463	0.0077	0.136 – 0.699	0.444	0.0109	0.113 – 0.686
3B	N1 vs N2	0.417	0.0478	0.00565 – 0.708	–	–	–	–	–	–
	N1 vs prevalent#	0.139	0.5709	–0.337 – 0.558	0.135	0.5821	–0.340 – 0.555	–0.139	0.5707	–0.558 – 0.337
	N1 vs incident*	0.0654	0.7783	–0.377 – 0.483	–0.0396	0.8645	–0.463 – 0.399	0.187	0.4179	–0.267 – 0.572
3C	N1 vs N2	0.491	0.0174	0.0987 – 0.751	–	–	–	–	–	–
	N1 vs prevalent#	–0.0929	0.7052	–0.525 – 0.377	0.503	0.0282	0.0632 – 0.779	0.479	0.0379	0.0318 – 0.767
	N1 vs incident*	0.266	0.2433	–0.187 – 0.626	–0.0481	0.8361	–0.470 – 0.392	0.00604	0.9793	–0.427 – 0.437

Cq, quantification cycle.

* Average of incident hospital acquired cases (-/+3 days from sample collection).

† Relative SARS-CoV2 genomic copies compared to genomic copies of PMMoV.

Prevalent cases at Hospital-3 are reported for the entire facility – as opposed to those cases directly housed in 3A, 3B, 3C.

Table 3

SARS-CoV-2 RNA detection measured with the N1 assay in hospital-wastewater samples as a function of proximity to a declared outbreak.

Hospital	Measurement	Outbreak-free periods vs outbreaks (Median (IQR))	P-value
1	Copies/ml	0 (0–7.9) vs 280 (35–1768)	<0.0001
	Copies/Copies PMMoV	0 (0–0.07) vs 0.18 (0.09–1.8)	0.0001
2	Copies/ml	0 (0–0) vs 8 (1.1–17)	0.022
	Copies/Copies PMMoV	0 (0–0) vs 0.035 (0–0.21)	0.453
3A	Copies/ml	0 (0–12) vs 122 (5.9–408)	0.031
	Copies/Copies PMMoV	0 (0–0.11) vs 0.5 (0.01–10)	0.026
3C	Copies/ml	2.4 (0–8.8) vs 26 (1.1–3179)	0.212
	Copies/Copies PMMoV	0.013 (0–0.08) vs 0.1 (0.0003–6.9)	0.253

were asymptomatic (Gibas et al., 2021). Importantly, if an incipient signal is detected in facility-wide wastewater samples, in-building plumbing systems can be strategically sampled in a nested manner in order to confirm an outbreak location.

Here we demonstrate that both the frequency of positive samples and the abundance of SARS-CoV-2 RNA in hospital wastewater systems correlated with increasing hospitalized cases – analogous to WWTP levels correlating with the COVID-19 community-diagnosed cases (Ahmed et al., 2020a; D'Aoust et al., 2021; Peccia et al., 2020; Wurtzler et al., 2020). This was most evident using raw SARS-CoV-2 Cq values but was also evident when normalized against PMMoV levels. We observed the N1-region of the nucleocapsid gene to be more sensitive than N-2, and E so low as to be dropped from our protocol. Other groups have reported similar trends in that the N1-target is the most sensitive marker in WWTP studies (Medema et al., 2020) and cruise ships (Ahmed et al., 2020b). Recently, other study showed a positive correlation as well between the prevalent cases COVID-19 with the SARS-CoV-2 RNA signal in the wastewater in a major hospital in the city of Toledo, OH, USA, that at the time of the study was known to be treating COVID-19 patients (Spurbeck et al., 2021).

Despite nosocomial cases and outbreaks representing a small fraction of the overall population of patients hospitalized with COVID-19, these events were discernable by wastewater testing. The natural history of SARS-CoV-2-RNA presence in the gastrointestinal tract remains incompletely understood (Gupta et al., 2020; Ling et al., 2020). Although,

some studies have addressed the SARS-CoV-2 RNA load dynamics in fecal samples (Cevik et al., 2021; Huang et al., 2020; Tan et al., 2020; Wölfel et al., 2020; Zhang et al., 2021; Zheng et al., 2020), changes in fecal viral loads over the course of the disease is still not well understood. Extrapolating from our hospital-based wastewater data, it appears that peak fecal viral shedding may occur prior to around symptom onset, given the congruence of wastewater RNA-signal and nosocomial cases and outbreaks identified in hospitals. Based on the rapid decline in wastewater signal thereafter, it is likely that fecal SARS-CoV-2-RNA drops significantly after initial presentation. Indeed, early in the pandemic when fewer patients with COVID-19 were hospitalized, wastewater samples routinely tested negative. This critical observation suggests that wastewater-based monitoring of SARS-CoV-2 may be most sensitive for identifying acutely occurring incident cases. Accordingly, wastewater-based monitoring of individual high-risk facilities (i.e., hospitals, nursing homes and industrial meat plants) – providing more granular data - through to WWTP may have great merit.

A key limitation to the identification of SARS-CoV-2 in wastewater samples, relative to clinical samples (e.g., swabs) is the massive volume of water in which samples are diluted. This necessitates sample concentration. While procedures for the efficient recovery of non-enveloped viruses exist, researchers continue to search for satisfactory protocols for enveloped viruses such as SARS-CoV-2 (Alygizakis et al., 2021). Many groups have explored ways to improve the sensitivity of wastewater SARS-CoV-2 detection. Diagnostic platforms with improved sensitivity and more impervious to impurities in the wastewater matrix (i.e., digital droplet RT-PCR) also show considerable promise (Falzone et al., 2020; Gonzalez et al., 2020). Sampling in the proximal sewershed may lead to day-to-day variance resulting in signal noise. This is particularly true for single-facility studies where potential extremes in individual virus shedding could confound results; limited data suggests significant variations in fecal viral load occur (from $10^{3.4}$ to $10^{7.6}$) (Cheung et al., 2020). This is a key challenge that remains to be solved if wastewater data is to be used in a meaningful way. Similarly, issues arise with attempts to normalize SARS-CoV-2 based on the contributing population. We chose to use the PMMoV as a fecal biomarker to control for variations in fecal loading – a particular risk when sampling takes place 'upstream' in the sewershed. While this marker has been validated in WWTP samples where large and diverse populations contribute to sewage (Kitajima et al., 2018), hospitals are a much smaller collection of individuals and variations in PMMoV excretion owing to differences in diet could have a much larger impact (Colson et al., 2010).

By capturing longitudinal data from three tertiary-care hospitals (>

2100 inpatient beds) we have demonstrated that passive wastewater monitoring is indeed possible at a range of hospital-facilities. Whereas Hospital-1 and Hospital-2 had a single municipal access point enabling surveillance of the entire facility-Hospital-3 required three locations to capture fully. As patients were frequently moved from one unit/building to another, either based on attending services geographic location, COVID-positive patient cohorting or need for intensive care support, attributing SARS-CoV-2 signal in the wastewater of this facility was more complicated but nonetheless correlations were evident. Furthermore, while nursing staff is often assigned to individual units, many allied health workers and physicians work or consult throughout the entire facility. If passive wastewater monitoring is to be adapted for other aspects of nosocomial surveillance (i.e., antibiotic consumption, emergence of antimicrobial resistant organisms, etc.) – a keen insight into the collection network is required.

There are limitations of our work that merit discussion. Given the complexity involved in sample collection, we were limited to twice-weekly sampling. Knowing how quickly SARS-CoV-2 can spread and outbreaks can occur, a daily monitoring strategy would have much greater capacity to identify and mitigate secondary cases of COVID-19. Incident cases are likely to be clinically diagnosed in hospital far faster than in other high-risk facilities owing to greater resources and heightened suspicion – potentially leading to an even greater lead-time associated with a positive wastewater-signal of outbreaks than observed herein. Hospitals pose unique challenges in wastewater monitoring owing to these facilities' high use of chemical disinfectants and detergents (Zotesso et al., 2017) that could interfere with molecular assays; this may explain why 3.6% of our samples spiked controls and PMMoV were not detected by RT-qPCR. To minimize the risk of false negatives, rigorous protocols that use internal controls (such as our BCoV spike) are necessary. The role of PCR-inhibitors in the wastewater matrix within the proximal sewerhed is an area that deserves considerable study if this field is to expand.

Wastewater-based monitoring can only effectively monitor those individuals that contribute fecal matter to the sewerhed. Importantly, hospitalized patients – those most vulnerable to COVID-19 adverse events – are often unable to self-toilet. Rather, these sick and often elderly individuals are dependent on continence aids, adult diapers, sanitary pads and nursing cleanup; this results in fecal matter from these individuals being disposed into biohazard solid waste. Accordingly, wastewater-based sampling could miss between 10 and 20% of patients in general hospital patients (Condon et al., 2019; Toba et al., 1996). This proportion is expected to be even higher in intensive care units where immobilization necessitated through ventilatory support further heightens toileting assistance requirements. Adapting wastewater surveillance technology to other high-risk settings like long-term care will encounter this same limitation. Finally, – tracking workers through passive wastewater monitoring poses inherent challenges. There is data that suggests that 52–56% of employees are uncomfortable defecating at work (Bennet, 2019; MacKenzie, 2016).

5. Conclusion

In a five-month observational study we were able to detect SARS-CoV-2 from the wastewater of Calgary's three largest tertiary-care hospitals.

The rate of SARS-CoV-2 wastewater test-positivity and RNA-abundance increased over time, concomitant with the increasing proportion of patients hospitalized with COVID-19.

Despite persistent low levels of SARS-CoV-2-RNA in wastewater resulting from patients being treated for and recovering from COVID-19 acquired in the community, we detected spikes attributable to hospital-acquired infections and outbreaks.

This study reveals that wastewater-based monitoring of SARS-CoV-2 RNA holds promise for early detection, monitoring and containment of incident infections.

CRedit authorship contribution statement

Nicole Acosta: Formal analysis, Writing - original draft, Writing - review & editing. **María A. Bautista:** Writing - review & editing. **Jordan Hollman:** Writing - review & editing. **Janine McCaldler:** Writing - review & editing. **Alexander Buchner Beaudet:** Writing - review & editing. **Lawrence Man:** Writing - review & editing. **Barbara J. Waddell:** Formal analysis, Writing - review & editing. **Jianwei Chen:** Writing - review & editing. **Carmen Li:** Writing - review & editing. **Darina Kuzma:** Writing - review & editing. **Srijak Bhatnagar:** Writing - review & editing. **Jenine Leal:** Writing - review & editing. **Jon Meddings:** Writing - review & editing. **Jia Hu:** Writing - review & editing. **Jason L. Cabaj:** Writing - review & editing. **Norma J. Ruecker:** Writing - review & editing. **Christopher Naugler:** Writing - review & editing. **Dylan R. Pillai:** Writing - review & editing. **Gopal Achari:** Writing - review & editing. **M. Cathryn Ryan:** Writing - review & editing. **John M. Conly:** Writing - review & editing. **Kevin Frankowski:** Writing - review & editing. **Casey RJ Hubert:** Writing - review & editing. **Michael D. Parkins:** Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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References

- Ahmed, W., Angel, N., Edson, J., Bibby, K., Bivins, A., O'Brien, J.W., Choi, P.M., Kitajima, M., Simpson, S.L., Li, J., Tscharke, B., Verhagen, R., Smith, W.J.M., Zaugg, J., Dierens, L., Hugenholtz, P., Thomas, K.V., Mueller, J.F., 2020a. First confirmed detection of SARS-CoV-2 in untreated wastewater in Australia: a proof of concept for the wastewater surveillance of COVID-19 in the community. *Sci. Total Environ.* 728, 138764.
- Ahmed, W., Bertsch, P.M., Angel, N., Bibby, K., Bivins, A., Dierens, L., Edson, J., Ehret, J., Gyawali, P., Hamilton, K.A., Hosegood, I., Hugenholtz, P., Jiang, G., Kitajima, M., Sichani, H.T., Shi, J., Shimko, K.M., Simpson, S.L., Smith, W.J.M., Symonds, E.M., Thomas, K.V., Verhagen, R., Zaugg, J., Mueller, J.F., 2020b. Detection of SARS-CoV-2 RNA in commercial passenger aircraft and cruise ship wastewater: a surveillance tool for assessing the presence of COVID-19 infected travellers. *J. Travel Med.* 27 (5).
- Alberta-Health-Services, 2020. Contact and Droplet Precautions. Health, A. Edmonton, AB.

- Alygizakis, N., Markou, A.N., Rousis, N.I., Galani, A., Avgeris, M., Adamopoulos, P.G., Scorilas, A., Lianidou, E.S., Paraskevis, D., Tsiodras, S., Tsakris, A., Dimopoulos, M. A., Thomaidis, N.S., 2021. Analytical methodologies for the detection of SARS-CoV-2 in wastewater: protocols and future perspectives. *Trends Anal. Chem.* 134, 116125.
- Arons, M.M., Hatfield, K.M., Reddy, S.C., Kimball, A., James, A., Jacobs, J.R., Taylor, J., Spicer, K., Bardossy, A.C., Oakley, L.P., Tanwar, S., Dyal, J.W., Harney, J., Chisty, Z., Bell, J.M., Methner, M., Paul, P., Carlson, C.M., McLaughlin, H.P., Thornburg, N., Tong, S., Tamin, A., Tao, Y., Uehara, A., Harcourt, J., Clark, S., Brostrom-Smith, C., Page, L.C., Kay, M., Lewis, J., Montgomery, P., Stone, N.D., Clark, T.A., Honein, M. A., Duchin, J.S., Jernigan, J.A., 2020. Presymptomatic SARS-CoV-2 Infections and Transmission in a skilled nursing facility. *N. Engl. J. Med.* 382 (22), 2081–2090.
- Backer, J.A., Klinkenberg, D., Wallinga, J., 2020. Incubation period of 2019 novel coronavirus (2019-nCoV) infections among travellers from Wuhan, China, 20-28 January 2020. *Eur. Surveill.* 25 (5).
- J.a.A.M Bennet. 2019 Women Poop. Sometime at work. Get over it. .**
- Carrillo-Vega, M.F., Salinas-Escudero, G., Garcia-Pena, C., Gutierrez-Robledo, L.M., Parra-Rodriguez, L., 2020. Early estimation of the risk factors for hospitalization and mortality by COVID-19 in Mexico. *PLoS ONE* 15 (9), e0238905.
- Carter, B., Collins, J.T., Barlow-Pay, F., Rickard, F., Bruce, E., Verduri, A., Quinn, T.J., Mitchell, E., Price, A., Vilches-Moraga, A., Stechman, M.J., Short, R., Einarsson, A., Braude, P., Moug, S., Myint, P.K., Hewitt, J., Pearce, L., McCarthy, K., CollaboratorsC.S., 2020. Nosocomial COVID-19 infection: examining the risk of mortality. the COPE-nosocomial study (COVID in older PEople). *J. Hosp. Infect.* 106 (2), 376–384.
- Cevik, M., Tate, M., Lloyd, O., Maraolo, A.E., Schafers, J., Ho, A., 2021. SARS-CoV-2, SARS-CoV, and MERS-CoV viral load dynamics, duration of viral shedding, and infectiousness: a systematic review and meta-analysis. *Lancet Microbe* 2 (1), e13–e22.
- Cheung, K.S., Hung, I.F.N., Chan, P.P.Y., Lung, K.C., Tso, E., Liu, R., Ng, Y.Y., Chu, M.Y., Chung, T.W.H., Tam, A.R., Yip, C.C.Y., Leung, K.H., Fung, A.Y., Zhang, R.R., Lin, Y., Cheng, H.M., Zhang, A.J.X., To, K.K.W., Chan, K.H., Yuen, K.Y., Leung, W.K., 2020. Gastrointestinal manifestations of SARS-CoV-2 infection and virus load in fecal samples from a hong kong cohort: systematic review and meta-analysis. *Gastroenterology* 159 (1), 81–95.
- Colson, P., Richet, H., Desnues, C., Balique, F., Moal, V., Grob, J.J., Berbis, P., Lecoq, H., Harle, J.R., Berland, Y., Raoult, D., 2010. Pepper mild mottle virus, a plant virus associated with specific immune responses, fever, abdominal pains, and pruritus in humans. *PLoS ONE* 5 (4), e10041.
- Condon, M., Mannion, E., Molloy, D.W., O’Caoimh, R., 2019. Urinary and faecal incontinence: point prevalence and predictors in a university hospital. *Int. J. Environ. Res. Public Health* 16 (2), 194.
- Czeisler, M.E., Marynak, K., Clarke, K.E.N., Salah, Z., Shakya, I., Thierry, J.M., Ali, N., McMillan, H., Wiley, J.F., Weaver, M.D., Czeisler, C.A., Rajaratnam, S.M.W., Howard, M.E., 2020. Delay or avoidance of medical care because of COVID-19-related concerns—United States. June 2020 MMWR Morb. Mortal. Wkly. Rep. 69 (36), 1250–1257.
- D’Aoust, P.M., Mercier, E., Montpetit, D., Jia, J.J., Alexandrov, I., Neault, N., Baig, A.T., Mayne, J., Zhang, X., Alain, T., Langlois, M.A., Servos, M.R., MacKenzie, M., Figeys, D., MacKenzie, A.E., Graber, T.E., Delatolla, R., 2021. Quantitative analysis of SARS-CoV-2 RNA from wastewater solids in communities with low COVID-19 incidence and prevalence. *Water Res.* 188, 116560.
- Del Gaudio, S., Cirillo, A., Di Bernardo, G., Galderisi, U., Cipollaro, M., 2012. Verification of real-time PCR methods for qualitative and quantitative testing of genetically modified organisms. *J. Food Qual.* 35 (6), 442–447.
- Falzone, L., Musso, N., Gattuso, G., Bongiorno, D., Palermo, C.I., Scalia, G., Libra, M., Stefani, S., 2020. Sensitivity assessment of droplet digital PCR for SARS-CoV-2 detection. *Int. J. Mol. Med.* 46 (3), 957–964.
- Foladori, P., Cutrupi, F., Segata, N., Manara, S., Pinto, F., Malpei, F., Bruni, L., La Rosa, G., 2020. SARS-CoV-2 from faeces to wastewater treatment: what do we know? a review. *Sci. Total Environ.* 743, 140444.
- Gibas, C., Lambirth, K., Mittal, N., Juel, M.A.I., Barua, V.B., Roppolo Brazell, L., Hinton, K., Lontai, J., Stark, N., Young, I., Quach, C., Russ, M., Kauer, J., Nicolosi, B., Chen, D., Akella, S., Tang, W., Schlueter, J., Munir, M., 2021. Implementing building-level SARS-CoV-2 wastewater surveillance on a university campus. *Sci. Total Environ.* 782, 146749.
- Gonçalves, J., Koritnik, T., Mioč, V., Trkov, M., Bolješić, M., Berginc, N., Prosenč, K., Kotar, T., Paragi, M., 2021. Detection of SARS-CoV-2 RNA in hospital wastewater from a low COVID-19 disease prevalence area. *Sci. Total Environ.* 755, 143226.
- Gonzalez, R., Curtis, K., Bivins, A., Bibby, K., Weir, M.H., Yetka, K., Thompson, H., Keeling, D., Mitchell, J., Gonzalez, D., 2020. COVID-19 surveillance in southeastern Virginia using wastewater-based epidemiology. *Water Res.* 186, 116296.
- Gupta, S., Parker, J., Smits, S., Underwood, J., Dolwani, S., 2020. Persistent viral shedding of SARS-CoV-2 in faeces—a rapid review. *Colorectal Dis.* 22 (6), 611–620.
- Harada, S., Uno, S., Ando, T., Iida, M., Takano, Y., Ishibashi, Y., Uwamoto, Y., Nishimura, T., Takeda, A., Uchida, S., Hirata, A., Sata, M., Matsumoto, M., Takeuchi, A., Obara, H., Yokoyama, H., Fukunaga, K., Amagai, M., Kitagawa, Y., Takebayashi, T., Hasegawa, N., 2020. Control of a nosocomial outbreak of COVID-19 in a university hospital. *Open Forum Infect. Dis.* 7 (12), ofaa512.
- Hartnett, K.P., Kite-Powell, A., DeVies, J., Coletta, M.A., Boehmer, T.K., Adjemian, J., Gundlapalli, A.V., National Syndromic Surveillance Program Community of, P., 2020. Impact of the COVID-19 pandemic on emergency department visits—United States, January 1, 2019–May 30, 2020. *MMWR Morb. Mortal. Wkly. Rep.* 69 (23), 699–704.
- Huang, J., Mao, T., Li, S., Wu, L., Xu, X., Li, H., Xu, C., Su, F., Dai, J., Shi, J., Cai, J., Huang, C., Lin, X., Chen, D., Lin, X., Sun, B., Tang, S., 2020. Long period dynamics of viral load and antibodies for SARS-CoV-2 infection: an observational cohort study. *MedRxiv*, 2020.2004.2022.20071258.
- Ioannou, G.N., Locke, E., Green, P., Berry, K., O’Hare, A.M., Shah, J.A., Crothers, K., Eastment, M.C., Dominitz, J.A., Fan, V.S., 2020. Risk factors for hospitalization, mechanical ventilation, or death among 10131 US veterans with SARS-CoV-2 infection. *JAMA Netw. Open* 3 (9), e2022310.
- Johansson, M.A., Quandelacy, T.M., Kada, S., Prasad, P.V., Steele, M., Brooks, J.T., Slayton, R.B., Biggerstaff, M., Butler, J.C., 2021. SARS-CoV-2 transmission from people without COVID-19 symptoms. *JAMA Netw. Open* 4 (1), e2035057.
- Kitajima, M., Sassi, H.P., Torrey, J.R., 2018. Pepper mild mottle virus as a water quality indicator. *NPJ Clean W.* 1 (1), 19.
- Ko, J.Y., Danielson, M.L., Town, M., Derado, G., Greenlund, K.J., Daily Kirley, P., Alden, N.B., Yousey-Hindes, K., Anderson, E.J., Ryan, P.A., Kim, S., Lynfield, R., Torres, S.M., Barney, G.R., Bennett, N.M., Sutton, M., Talbot, H.K., Hill, M., Hall, A. J., Fry, A.M., Garg, S., Kim, L., Team, C.-N.S., 2020. Risk factors for COVID-19-associated hospitalization: COVID-19-associated hospitalization surveillance network and behavioral risk factor surveillance system. *Clin. Infect. Dis.*
- La Rosa, G., Mancini, P., Bonanno Ferraro, G., Veneri, C., Iaconelli, M., Bonadonna, L., Lucentini, L., Suffredini, E., 2021. SARS-CoV-2 has been circulating in northern Italy since December 2019: evidence from environmental monitoring. *Sci. Total Environ.* 750, 141711.
- Ling, Y., Xu, S.B., Lin, Y.X., Tian, D., Zhu, Z.Q., Dai, F.H., Wu, F., Song, Z.G., Huang, W., Chen, J., Hu, B.J., Wang, S., Mao, E.Q., Zhu, L., Zhang, W.H., Lu, H.Z., 2020. Persistence and clearance of viral RNA in 2019 novel coronavirus disease rehabilitation patients. *Chin. Med. J.* 133 (9), 1039–1043 (Engl).
- Long, D.R., O’Reilly-Shah, V., Rustagi, A.S., Bryson-Cahn, C., Jerome, K.R., Weiss, N.S., Sunshine, J.E., 2020. Incidence of health care-associated COVID-19 during universal testing of medical and surgical admissions in a large US health system. *Open Forum Infect. Dis.* 7 (10), ofaa435.
- M. MacKenzie 2016 This is how many people refuse to poop at work-Are you one of them?**
- Medema, G., Heijnen, L., Elsinga, G., Italiaander, R., Brouwer, A., 2020. Presence of SARS-coronavirus-2 RNA in sewage and correlation with reported COVID-19 prevalence in the early stage of the epidemic in the Netherlands. *Environ. Sci. Technol. Lett.* 7, 511–516.
- Peccia, J., Zulli, A., Brackney, D.E., Grubaugh, N.D., Kaplan, E.H., Casanovas-Massana, A., Ko, A.L., Malik, A.A., Wang, D., Wang, M., Warren, J.L., Weinberger, D. M., Arnold, W., Omer, S.B., 2020. Measurement of SARS-CoV-2 RNA in wastewater tracks community infection dynamics. *Nat. Biotechnol.* 38 (10), 1164–1167.
- Randazzo, W., Truchado, P., Cuevas-Ferrando, E., Simón, P., Allende, A., Sánchez, G., 2020. SARS-CoV-2 RNA in wastewater anticipated COVID-19 occurrence in a low prevalence area. *W. Res.* 181, 115942-115942.
- Spurbeck, R.R., Minard-Smith, A., Catlin, L., 2021. Feasibility of neighborhood and building scale wastewater-based genomic epidemiology for pathogen surveillance. *Sci. Total Environ.* 789, 147829.
- Tan, W., Lu, Y., Zhang, J., Wang, J., Dan, Y., Tan, Z., He, X., Qian, C., Sun, Q., Hu, Q., Liu, H., Ye, S., Xiang, X., Zhou, Y., Zhang, W., Guo, Y., Wang, X.-H., He, W., Wan, X., Sun, F., Wei, Q., Chen, C., Pan, G., Xia, J., Mao, Q., Chen, Y., Deng, G., 2020. Viral kinetics and antibody responses in patients with COVID-19. *MedRxiv*, 20042382, 2020.2003.2024.
- Toba, K., Ouchi, Y., Orimo, H., Iimura, O., Sasaki, H., Nakamura, Y., Takasaki, M., Kuzuya, F., Sekimoto, H., Yoshioka, H., Ogiwara, T., Kimura, I., Ozawa, T., Fujishima, M., 1996. Urinary incontinence in elderly inpatients in Japan: a comparison between general and geriatric hospitals. *Aging* 8 (1), 47–54 (Milano).
- Wang, X., Zhou, Q., He, Y., Liu, L., Ma, X., Wei, X., Jiang, N., Liang, L., Zheng, Y., Ma, L., Xu, Y., Yang, D., Zhang, J., Yang, B., Jiang, N., Deng, T., Zhai, B., Gao, Y., Liu, W., Bai, X., Pan, T., Wang, G., Chang, Y., Zhang, Z., Shi, H., Ma, W.L., Gao, Z., 2020. Nosocomial outbreak of COVID-19 pneumonia in Wuhan, China. *Eur. Respir. J.* 55 (6).
- Wannigama, D.L., Amarasiri, M., Hurst, C., Phattharapornjaroen, P., Abe, S., Hongsing, P., Rad, S.M.A.H., Pearson, L., Saethang, T., Luk-in, S., Kueakulpattana, N., Storer, R.J., Ounjai, P., Jacquet, A., Leelahavanichkul, A., Chatsuwat, T., 2021. Tracking COVID-19 with wastewater to understand asymptomatic transmission. *Int. J. Infect. Dis. Press.*
- O.N. Whitney, B. Al-Shayeb, A. Crits-Cristoph, M. Chaplin, V. Fan, H. Greenwald, A. Hinkle, R. Kantor, L. Kennedy, A. Maurer, R. Tjian, K.L. Nelson and U.B.W.-b.e. consortium 2020 V.4 -Direct wastewater RNA capture and purification via the "sewage, salt, silica and SARS-CoV-2 (4S). <https://dx.doi.org/10.17504/protocols.io.bpdfmi3n>.**
- Wong, J.C.C., Tan, J., Lim, Y.X., Arivalan, S., Hapuarachchi, H.C., Mailepessu, D., Griffiths, J., Jayarajah, P., Setoh, Y.X., Tien, W.P., Low, S.L., Koo, C., Yenamandra, S. P., Kong, M., Lee, V.J.M., Ng, L.C., 2021. Non-intrusive wastewater surveillance for monitoring of a residential building for COVID-19 cases. *Sci. Total Environ.* 786, 147419.
- Wölfel, R., Corman, V.M., Guggemos, W., Seilmaier, M., Zange, S., Müller, M.A., Niemeyer, D., Jones, T.C., Vollmar, P., Rothe, C., Hoelscher, M., Bleicker, T., Brünink, S., Schneider, J., Ehmann, R., Zwirgmaier, K., Drosten, C., Wendtner, C., 2020. Virological assessment of hospitalized patients with COVID-2019. *Nature* 581 (7809), 465–469.
- Wu, F., Zhang, J., Xiao, A., Gu, X., Lee, W.L., Armas, F., Kauffman, K., Hanage, W., Matus, M., Ghaeli, N., Endo, N., Duvallet, C., Poyet, M., Moniz, K., Washburne, A.D., Erickson, T.B., Chai, P.R., Thompson, J., Alm, E.J., 2020. SARS-CoV-2 Titers in wastewater are higher than expected from clinically confirmed cases. *mSystems* 5 (4), e00614–e00620.
- Wurtzer, S., Marechal, V., Mouchel, J., Maday, Y., Teysou, R., Richard, E., Almayrac, J., Moulin, L., 2020. Evaluation of lockdown effect on SARS-CoV-2 dynamics through viral genome quantification in waste water, greater Paris, France, 5 March to 23 April 2020 *Eurosurveillance* 25 (50), 2000776.

- Yanes-Lane, M., Winters, N., Fregonese, F., Bastos, M., Perlman-Arrow, S., Campbell, J. R., Menzies, D., 2020. Proportion of asymptomatic infection among COVID-19 positive persons and their transmission potential: a systematic review and meta-analysis. *PLoS ONE* 15 (11), e0241536.
- Zhang, N., Gong, Y., Meng, F., Shi, Y., Wang, J., Mao, P., Chuai, X., Bi, Y., Yang, P., Wang, F., 2021. Comparative study on virus shedding patterns in nasopharyngeal and fecal specimens of COVID-19 patients. *Sci. China Life Sci.* 64 (3), 486–488.
- Zheng, S., Fan, J., Yu, F., Feng, B., Lou, B., Zou, Q., Xie, G., Lin, S., Wang, R., Yang, X., Chen, W., Wang, Q., Zhang, D., Liu, Y., Gong, R., Ma, Z., Lu, S., Xiao, Y., Gu, Y., Zhang, J., Yao, H., Xu, K., Lu, X., Wei, G., Zhou, J., Fang, Q., Cai, H., Qiu, Y., Sheng, J., Chen, Y., Liang, T., 2020. Viral load dynamics and disease severity in patients infected with SARS-CoV-2 in Zhejiang province, China, January-March 2020: retrospective cohort study. *BMJ* 369, m1443.
- Zotoso, J.P., Cossich, E.S., Janeiro, V., Tavares, C.R.G., 2017. Treatment of hospital laundry wastewater by UV/H₂O₂ process. *Environ. Sci. Pollut. Res. Int.* 24 (7), 6278–6287.