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Epidemiological evaluation of sewage surveillance as a tool to detect the presence of COVID-19 cases in a low case load setting

Jim Black⁠,⁎, Phyo Aung⁠, Monica Nolan⁠, Emma Roney⁠, Rachael Poon⁠, Daneeta Hennessy⁠, Nicholas D. Crosbie⁠, Dan Deere⁠, Aaron R. Jex⁠, Nijoy John⁠, Louise Baker⁠, Peter J. Scales⁠, Shane P. Usher⁠, David T. McCarthy⁠, Christelle Schang⁠, Jonathan Schmidt⁠, Steven Myers⁠, Natacha Begue⁠, Christine Kaucner⁠, Bruce Thorley⁠, Julian Druce⁠, Paul Monis⁠, Melody Lau⁠, Suzie Sarkis

a Department of Health and Human Services, State Government of Victoria, 50 Lonsdale Street, Melbourne, Victoria 3000, Australia
b Melbourne Water Corporation, 990 La Trobe St., Docklands, Victoria 3001, Australia
c Water Research Australia, Level 2, 250 Victoria Square, Adelaide, South Australia 5000, Australia
d Population Health and Immunity Division, Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3010, Australia
e Faculty of Veterinary and Agricultural Sciences, University of Melbourne, Parkville, Victoria 3010, Australia
f Department of Chemical Engineering, University of Melbourne, Victoria 3010, Australia
g Department of Civil Engineering, Monash University, Wellington Road, Clayton, Victoria 3800, Australia
h South East Water Corporation, 101 Wells Street, Frankston, Victoria 3199, Australia
i Australian Laboratory Services, 22 Dalmore Drive, Scoresby, Victoria 3179, Australia
j Victorian Infectious Diseases Reference Laboratory, Royal Melbourne Hospital, Peter Doherty Institute for Infection and Immunity, Melbourne, Victoria 3000, Australia
k Australian Water Quality Centre, SA Water Corporation, Adelaide, South Australia 5000, Australia

HIGHLIGHTS
• Sewage surveillance as a diagnostic test for infected people in low prevalence settings was not previously quantified.
• The location of infected persons, from 2 days before onset till 55 after, was related to the catchments and sampling sites.
• The odds of detection were ~20 times higher in the first two weeks, and ~5 times higher at 55 days, at distances to 34 km.
• Sensitivity was moderate at all distances but fell with time after onset. Specificity was high at all distances and times.
• The probability of detection was ~10% with one infected person, and ~60% with 5 people within 5 km and 1 week of onset.

Abstract
In low prevalence settings the development of sensitive and specific quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) tests to detect SARS-CoV-2 (the virus causing COVID-19) in sewage presents the possibility of using sewage sampling as a diagnostic test for the presence of infected people in the catchment of the sampled sewer. However, the usefulness of such surveillance has not been quantified. In this study in the Australian state of Victoria between August and October 2020 the location of each known SARS-CoV-2-infected person was determined on each day from two days before onset to 55 days after, in 46 metropolitan and rural...
1. Introduction

The use of wastewater-based epidemiology to inform the control of infectious diseases has a long history. In the 1940s Moore and colleagues used it in England to find typhoid carriers by culturing sewage and tracing positive cultures back up the branches of the sewer system (Moore, 1951; Moore, et al., 1952). Poliovirus has been detected in sewage since the 1930s (Trask and and Paul, 1941), and from the 1990s the World Health Organization’s Global Polio Eradication Initiative has relied on viral culture from sewage as evidence of the absence of cases and to determine the extent and serotype of new outbreaks (Chen et al., 2020a; Zhao et al., 2020a; Ashgar et al., 2014).

The development of highly sensitive and specific quantitative Reverse Transcript Polymerase Chain Reaction (qRT-PCR) tests to detect SARS-CoV-2 (the coronavirus causing the COVID-19 pandemic) in sewage samples, presents the potential to use wastewater sampling as a diagnostic test for the presence of infectious individuals with SARS-CoV-2 in the community (Larson et al., 2020; Randazzo et al., 2020). This may allow rapid and localised public health actions to reduce the ultimate size of COVID-19 outbreaks. Sewage sampling does not depend on individual action such as recognising the symptoms and seeking nasopharyngeal tests, and could add useful information about spread that cannot be obtained any other way, including for asymptomatic, pre-symptomatic or undiagnosed individuals.

However, previous successful sewage surveillance programmes have concentrated on enteric organisms that survive well in the human gut and in sewage. People infected with SARS-CoV-2 may shed the virus, or its RNA, via saliva, sputum, stools or urine (Wang et al., 2020; Fontana et al., 2020; Zheng et al., 2020), all of which have the potential to enter wastewater through showers, baths, bowls, sinks, toilets and drains. Viral shedding to wastewater may come directly (e.g. from coughing in a shower or use of a toilet) or indirectly (e.g. from discarding used tissues or washing of hands or clothes) (Sinclair et al., 2008). The concentration of viral RNA measured is typically higher in respiratory than stool samples (Zheng et al., 2020) and shedding may last for many weeks beyond the infectious period (Xu et al., 2020b).

Being enveloped, coronaviruses degrade relatively rapidly in sewage, especially at higher ambient temperatures and in the presence of detergents and other substances commonly found in wastewater (Silverman and Boehm, 2020). Further, sewage is a difficult medium in which to perform reliable qRT-PCR testing, and qRT-PCR inhibition is a serious issue (Schraeder et al., 2012). In addition, not all COVID-19 patients excrete the virus in their faeces, with variation in point prevalence estimates from 35% to 83% (Fontana et al., 2020; Zheng et al., 2020; Zhang et al., 2020; Chen et al., 2020c; Wu et al., 2020; Parasa et al., 2020), a higher proportion of paediatric versus adult faecal shedding (Xu et al., 2020a), and a higher proportion of faecal shedding earlier in the infection cycle (Zhao et al., 2020b), and orders of magnitude variation between individuals in the amount of virus shed. Finally, the ‘grab sample’ method most commonly used depends on either fortuitous timing or wide dispersal of the virus in the wastewater stream. So there is a possibility of many false negative tests, which would limit the utility of sewage surveillance (O’Reilly et al., 2020).

However, in this period of COVID-19 detection and containment, the utility of wastewater surveillance for a diagnostic test for the presence of infected people in the relevant sewage catchment, this study aimed to characterise its sensitivity and specificity for a given sewage catchment area. In this context sensitivity means the proportion of days with known infected people residing in a catchment that had positive sewage samples by qRT-PCR, and specificity is the proportion of days with no known infected people residing in a catchment that had negative sewage samples. This is not the same as the sensitivity and specificity of the qRT-PCR as a test for the presence of SARS-CoV-2 in a sample, but a broader version considering its use at population level.

This study also aimed to answer a range of basic questions to ascertain whether sewage sampling can reliably be used to diagnose the presence of infectious COVID-19 cases in a community:

- Does the presence of people known to be infected with SARS-CoV-2 in the catchment from which a sewage sample is taken influence the odds of detection of the virus in a sewage sample?
- What stage in the course of SARS-CoV-2 infection can best be detected by sewage sampling?
- How far upstream from the sampling site can infected people be detected?
- What is the minimum number of infected people in a catchment needed for reliable detection (and how does this relate to distance from the sampling site)?
- As a diagnostic test for the presence of infected people in the relevant sewage catchment, what is the sensitivity and specificity of sewage testing for SARS-CoV-2?
- Will it be best used to detect cases where none are known to exist, or to provide reassurance that there really are no cases, or both, or neither?

This study attempts an initial answer to the above questions in the context of a low case load setting (notified cases peaked at 148 per 100,000 persons in Victoria during July 2020 (Australian Government Department of Health, 2020)), using data from much of the ‘second wave’ of COVID-19 disease and SARS-CoV-2 infections in the South-
Eastern Australian State of Victoria beginning in the third quarter of 2020.

Specific questions addressed are:

• What combination of definitions of ‘infected people’ and ‘in the catchment’ most increases the odds of detection of virus on a given day?
  ○ ‘Infected people’ refers to known persons with a diagnosis of SARS-CoV-2 infection and within a defined time before and/or after the onset of symptoms (or the date of testing in the case of asymptomatic infection, in which case they were isolated for 10 days). A range of time windows were evaluated, from two days before to 6 days after onset (i.e. −2 to +6 days), −2 to +13 etc., up to −2 to +55 days.
  ○ ‘In the catchment’ refers to the distance in kilometres from each known infected person’s residence on that day to the sampling site. A range of distances were evaluated, from 0 to 4 km, 0 to 9 km etc., up to 0 to 34 km.

• What is the sensitivity and specificity of each combination of these definitions as a diagnostic test?
• What is the probability of detecting SARS-CoV-2 in a sample, for different numbers of infected people, at various distances in the catchment?

2. Methods

2.1. Epidemiological methods

The questions related to odds of detection were addressed using a case control study method. The same data were then used to calculate the sensitivities and specificities.

In the case control approach:

• A ‘case’ was a sewage catchment-sampling-day with positive detection of the SARS-CoV-2 subgenome in the sewage sample.
• A ‘control’ was a sewage catchment-sampling-day with a negative qRT-PCR result for SARS-CoV-2 subgenome in the sewage sample.
• ‘Exposure’ was the presence of known infected people for each of the multiple time-period definitions of ‘infected people’ and distance-based definitions of ‘in the catchment’. E.g. Known infected people −2 to 6 days from ‘onset’, and residing within 5 km of the sampling site, −2 to 13 days and within 9 km, and so on for all combinations up to 55 days and within 35 km. For the primary analysis ‘exposure’ was the presence of one or more people in the category. For the logistic regression models the number of known infected people was used.
• For individuals infected with SARS-CoV-2, ‘onset’ was defined as the date of onset of symptoms when these were present and the date of sample collection for the first positive test for those with no symptoms.

The resulting Odds Ratios can thus be interpreted as the change in odds of detecting virus in a sewage sample when there is one or more infected people in the catchment within the relevant band of time and distance from the sampling site. All Odds Ratios were calculated using Stata/MP 15.1 for Windows (StataCorp, 2017).

For sensitivity and specificity, the ‘diagnostic test’ was the qRT-PCR test of each sewage sample, and the ‘gold standard’ test was the presence of at least one known infected person in the catchment (with multiple definitions as above). Sensitivity was the ‘true positive’ proportion: the count of days with infected people in the catchment and a positive sewage qRT-PCR test, divided by the total count of days with infected people in the catchment. Specificity was the ‘true negative’ proportion: the count of days with no infected people in the catchment and a negative sewage qRT-PCR test, divided by the total count of days with no infected people in the catchment. In each case a 95% Confidence Interval (95%CI) was calculated using a logit method for single proportions (using the Stata ‘proportion’ command).

To estimate the probability of virus detection in sewage for different numbers of infected people within a given distance of a sampling point, logistic regression models were made, and post-estimation done using the Stata predict, xb and stdp commands. 95% Confidence Intervals for these probability estimates were made using (Inlow, n.d.):

\[
95\% CI = \frac{\text{Inverse Logit(Predicted Value)}}{+/-\text{Normal Quantile(0.975)}} + \text{Standard Error}
\]

The data were collected by the Victorian State Government’s Department of Health and Human Services (DHHS), through its COVID-19 case-finding and contact-tracing processes and its SARS-CoV-2 sewage surveillance programme. The study included all samples taken as part of the sewage surveillance programme between 25 August 2020 and 27 October 2020. All the sampled catchments were included except the Western Treatment Plant and the Eastern Treatment Plant, (both serving metropolitan Melbourne and including the studied metro catchments within them, and both tested using composite samples rather than grab sampling) and three small catchments at individual public hospitals (which had no other residences inside them). Most of the sampling was done by a ‘grab’ method and on a weekly basis. The laboratory methods and testing algorithm to define a SARS-CoV-2 subgenome detection were standardised and consistent throughout the study period. All test results were included except any which were classified as inconclusive after a single technical replicate was positive by RT-qPCR but could not be confirmed by sequencing.

The allocation of ‘exposure’ on each catchment-sampling-day was a multi-step process:

• A line list was extracted from the DHHS Azure Data Model for SARS-CoV-2 infection for the period 25 August 2020 to 27 October 2020, with the latitude and longitude of the residence of each known infected person from day −2 to day +55 from onset. Latitude and longitude were assigned using the recorded address and the Geo-coded National Address File (G-NAF) (Commonwealth Government of Australia Department of Industry Science Energy and Resources, 2020).
• Addresses that were recorded as having been altered (e.g. corrected or updated) were adjusted for the relevant dates using an extract from the DHHS Public Health Event Surveillance System (PHESS) public health database that underpins the Azure Data Model.
• The catchment for each sampling site was provided by the relevant water authority from its own database, as polygons of latitude and longitude points. Each represented the total area in which a person using a toilet, bathroom, kitchen or laundry might have contributed wastewater to the sample.
• The location of each infected person on each day was allocated to any addresses that were recorded as having been altered (e.g. corrected or updated) were adjusted for the relevant dates using an extract from the DHHS Public Health Event Surveillance System (PHESS) public health database that underpins the Azure Data Model. The catchment for each sampling site was provided by the relevant water authority from its own database, as polygons of latitude and longitude points. Each represented the total area in which a person using a toilet, bathroom, kitchen or laundry might have contributed wastewater to the sample.
• For those locations found to be inside a catchment, distance from the residence to the sampling site was calculated via the same application, using the Haversine formula (Evans, 1919) as implemented at the Earth’s surface between two latitude/longitude points.
• The number of known infected people in each catchment on each case date and control day was calculated for each definition of ‘infected people’ and ‘in the catchment’, using Microsoft Access.

The final spreadsheet for analysis in Stata/MP 15.1 had one record for each catchment-sampling-day, its status as a case or control, and whether each catchment was ‘exposed’ as defined above.
Univariable logistic regression was used to estimate the change in odds for each extra known infected person in a catchment, and for each extra kilometre of mean distance. Multivariable logistic regression was then used to adjust each for the effect of the other: that is, when calculating the change in odds for each extra infected person these were adjusted for the mean distance from the sampling site, and when calculating the change in odds for each extra kilometre of distance these were adjusted for the number of known infected people in the same distance/time category.

Most of the analyses presented use ‘inclusive’ definitions of both time and distance. That is, the time category ‘1–2 to 13 days’ includes the people in the ‘1–2 to 6 days’ category (and so on for later times), and the distance category ‘0 to 9 km’ includes the people in the ‘0 to 4 km’ category, and so on for further distances. However, calculation of the probability of virus detection in sewage was made using inclusive distances but ‘exclusive’ times (in which ‘14–20 days’ does not include ‘1–2 to 6 days’, and so on).

All analyses compared the presence of known infected people in the catchment on the same day the catchment was sampled. Sewage moves quickly through the study catchments. In accordance with the applicable standard (Water Services Association of Australia, 2014), conventional gravity sewers are designed to ensure a self-cleansing flow velocity is achieved on a typical day, and pressure sewers are also designed to minimize retardation of sewage flow. A sewage sample on a given day therefore mostly contains wastewater put into the sewers on the same day. The severe ‘lockdowns’ across the State during the study period also meant that there was little movement from one day to the next: the borders between Victoria and the neighbouring states of New South Wales and South Australia were closed; workers who could work from home were obliged to do so; face coverings were mandatory; hospitality and retail establishments were closed; from 2 August to the next: the borders between Victoria and the neighbouring states was banned. Case ascertainment was restricted to within 5 km of home (changing to 25 km on 18 October);

2.2.1. Sample collection and filtration

Grab or composite (up to 24 h) samples of raw sewage were collected and transported on ice to the testing laboratory. Samples were generally received by the laboratory on the same day or next business day from collection, and either processed the same day or stored overnight at 2 to 8 °C for processing the next working day.

Briefly, 50 mL samples were supplemented with 2.5 M MgCl₂ to a final concentration of 25 mM MgCl₂ and then passed through a 0.45 μm 47 mm GN-6 mixed cellulose ester membrane (Pall Corporation) using a vacuum manifold (Ahmed et al., 2020). For every batch of samples processed, a negative control sample comprising the equivalent volume of sterile reagent water and a positive control sample comprising the equivalent volume of sewage spiked with approximately 10⁸ copies of gamma irradiated SARS-CoV-2 (provided by the Virus Identification Laboratory at the Doherty Institute, Melbourne, Australia) was included to monitor for sample contamination and evaluate the performance of the method, respectively. The recovery of the gamma irradiated SARS-CoV-2 was calculated as the difference between the number of copies of gamma irradiated SARS-CoV-2 detected in the spiked sewage sample and the number of copies of indigenous SARS-CoV-2 detected in the same sewage sample processed in parallel, divided by the number of copies of gamma irradiated SARS-CoV-2 in the spike, with the result expressed as a percentage. The number of copies of gamma irradiated and indigenous SARS-CoV-2 was based on the average number of copies of the N gene target from the replicate qRT-PCR assays that gave the highest average number of copies for the volume of template tested.

2.2.2. Nucleic acid extraction

Total RNA was extracted from the membrane using the MagMAX™ Microbiome Ultra Nucleic Acid Isolation Kit (Thermo Fisher Scientific) with modification of the lysis step. Lysis was performed by transferring the membrane to a 7 mL bead tube containing 1 g of 0.15 mm garnet beads and 0.5 g of 0.7 mm garnet beads (Omni International) and 1 mL lysis buffer. The preparation was then homogenised at a liquid velocity of 4 m/s for 15 min using the Bead Ruptor Elite (Omni International) and the lysate was processed as described by the manufacturer using the KingFisher™ Duo Prime robotic workstation (Thermo Fisher Scientific) in a final elution volume of 100 μL. The preparation was also supplemented with 5 μL of the MS2 bacteriophage (MS2) internal positive control as supplied in the PerkinElmer SARS-CoV-2 Nucleic Acid Detection Kit (RTO) (PerkinElmer).

2.2.3. qRT-PCR

RNA samples were analysed using the PerkinElmer® SARS-CoV-2 Nucleic Acid Detection Kit (RTO). This kit is a multiplex assay using primers and TaqMan® probes targeting the N gene and ORF1ab region of the SARS-CoV-2 genome and an unspecified region of the MS2 genome. qRT-PCR assays were performed using two replicates of 10 μL template and two replicates of 1 μL template, in a total reaction volume of 60 μL.

The negative qRT-PCR control was prepared using nuclease-free molecular grade water. The Twist Synthetic SARS-CoV-2 RNA Control 1 (Twist Bioscience) was used as a positive qRT-PCR control and to prepare the standard curve for quantification. The quantification of the Twist Synthetic SARS-CoV-2 RNA Control 1 was supplied by the manufacturer. The qRT-PCR assay was performed as described by the manufacturer using the Bio-rad CFX96 Touch™ real-time PCR system (Bio-rad). The cycle threshold and baseline adjustment were automatically calculated by the instrument software.

The cycle threshold values (Ct) for the MS2 internal positive control in the qRT-PCR assays using 10 and 1 μL of template were used to calculate the relative abundance of MS2 in the qRT-PCR assay using 10 μL of template compared to 1 μL template based on the formula below.

\[
\text{MS2 relative abundance} = \frac{10^{\Delta Ct}}{10}
\]

where \(\Delta Ct = \text{average MS2 Ct in the qRT-PCR assay using 10 μL of template} - \text{average MS2 Ct in the qRT-PCR assay using 1 μL of template} \).
number of copies of the N gene target and ORF1ab region that could be detected in 80% of the replicates tested. The LOD was expressed as the lowest detectable concentration of the N gene target and ORF1ab region in sewage based on the equivalent volume of sewage analysed in each qRT-PCR assay, not adjusting for any potential loss through the processing of the sewage sample or any potential inhibition of the qRT-PCR assay.

2.2.4. Sequence confirmation

Amplicons from SARS-CoV-2 positive samples were confirmed by sequencing on an Illumina MiSeq using the ‘overhang’ primer and dual-index barcoding method (Penington et al., 2018). Overhang forward and reverse primers were synthesized by a commercial service (Bioneer Pacific) for the CCDC N and CCDC ORF1b primer pairs as per (Chinese Center for Disease Control and Prevention, 2020) and (Niu et al., 2020). These extension primers include a universal oligonucleotide overhang at the 5' end (5' GTGACCTATGAACTCAGGAGTC for CCDC-N-F and CCDC-ORF1ab-F; 5' CTGAGACTTGCAATGGCACGC for CCDC-N-R and CCDC-ORF1ab-R). Amplicons were cleaned prior to overhang-PCR using either the ExoSAP-IT™ Express PCR Product Cleanup Kit (Applied Biosystems, USA) or the Machery-Nagel PCR cleanup kit. Successful amplification was assessed by Agilent 4200 Tapestation (Agilent) using D1000 screentape. PCR negative controls and extraction blanks (PBS) were also amplified and processed in parallel for each extension PCR run and carried through to amplicon sequencing and analysis. Following overhang-PCR, each amplicon was index-barcoded and prepared from sequencing on the Illumina MiSeq platform as per the manufacturer’s instructions (Illumina, USA).

All reads were demultiplexed and filtered for a minimum Phred quality of 30 (1 error per 1000 bases) using Pipeline Pilot (Dassault Systèmes). Filtered reads were loaded into Geneious v.2020.2.2, adapter trimmed, allowing up to 4 mismatches, and then aligned using the Geneious Mapper to the specific amplicon region (positions 28,881–28,980 for N gene and positions 13,341 to 13,460 for ORF1ab) of the SARS-CoV-2 Wuhan reference genome (NCBI Accession number NC045512), with each alignment refined over 5 iterations. For the N gene amplicon a minimum overlap of 99 base pairs was required, with a minimum 94% sequence identity, allowing a maximum of 5% gaps with a maximum gap size of 5 bp, a minimum alignment seed word length and index word length of 14 and 12 nt respectively. For ORF1ab, the same alignment conditions were used, but requiring a minimum overlap of 110 bp.

2.3. Ethical review

The study was approved by the Department of Health and Human Services Human Research Ethics Committee (ERM ID 67569).

3. Results

3.1. Laboratory method performance

The laboratory method was able to recover an average of 38% (SD = 25 percentage points) of gamma irradiated SARS-CoV-2 from spiked sewage samples (N = 20) from different regional and metropolitan catchments. The LOD was 5 copies per qRT-PCR for the N gene and ORF1ab target. Based on the analysis of 10 μL of template, which corresponds to an equivalent volume of 5 mL of sewage, the limit of quantification for the qRT-PCR assay was 1 copy/mL for the N gene or ORF1ab target.

The average relative abundance of the MS2 internal positive control in the qRT-PCR assays using 10 μL of template compared to 1 μL of template was 43% (N = 237, SD = 25 percentage points). Inhibition is generally considered to be significant if the Ct value for a target gene in a spiked sample compared to spiked nuclease-free molecular grade water differs by 2 or more cycles, which is equivalent to a relative abundance of 25% or less. As the average relative abundance of the MS2 internal positive control was 43% there was no significant difference observed in the inhibition of the MS2 internal positive control in the qRT-PCR assays using 10 and 1 μL of template. However, there were some sample sites where the relative abundance of the MS2 internal positive control was significantly and consistently below the average, indicating that the inhibition of the qRT-PCR assay may be site specific. It should be noted that the MS2 internal positive control appeared to be more sensitive to inhibition than the SARS-CoV-2 N gene and ORF1ab region targets, based on the observation that the recovery of gamma irradiated SARS-CoV-2 in spiked sewage samples where 10 μL of template was tested was on average greater than the relative abundance of the MS2 internal positive control (data not shown).

3.2. The study period in relation to the outbreak in Victoria

The State of Victoria suffered its ‘second wave’ of COVID-19 cases between June and October 2020, with a peak of nearly 700 cases per day in early August. This means that infected people contributed one or more days of their infection course to the data set beginning early in the wave, and new cases contributed days in the latter part of the falling epidemic curve. Fig. 1 illustrates the COVID-19 epidemic in Victoria from January to November 2020, as determined by positive nasopharyngeal swabs.

3.3. Sampling

A total of nine weeks of sampling were included, from 46 catchments across the state (10 metropolitan Melbourne (‘metro’) and 36 regional Victorian). Between two and 15 samples were collected per catchment, with a median of eight. Most of the samples were collected by the standard ‘grab’ method (Environmental Monitoring and Support Laboratory Cincinnati Ohio, 1982; International Organization for Standardization (ISO), 2018), and most were taken once per week.

Fig. 2 shows the distribution of sampling sites across the state. There was some overlap between the metro catchments – two are subcatchments of one larger one – but none between any of the regional ones. Sampling points included wastewater treatment plants in the regional catchments, and main sewer pipes via manhole covers in metropolitan Melbourne. ‘Catchment’ in this study therefore does not always refer to the full catchment of a wastewater treatment plant, but to the geographical area upstream from each sampling point from which sewage drains to that sampling point – that is, the area in which any wastewater entering the sewer system may have contributed to the sample.

The amount of industrial input and the proportion of household wastewater vary across the state, and it was not possible to measure these for this study.

3.4. Cases and controls

Table 1 shows the distribution of the 346 samples by case/control status and by type of catchment. Overall, there were 3.9 control catchment-sampling-days per case catchment-sampling-day, although the ratio for metro catchments was closer to 1:1.

3.5. Exposure

A total of 486,193 person-location days were recorded, of which 354,155 (72.8%) were within –2 to +55 days of illness onset and within a study catchment; 93.9% were in metropolitan Melbourne catchments. 26,298 (5.3%) of the person-location-days were changed after cross-checking with the PHESS database.

As many infected people stayed at the same location for multiple days, 18,835 unique person-locations were recorded in the data set.
3.6. Odds ratios

Table 2 is a heatmap displaying the Odds Ratios for the different definitions of ‘infected people’ (time) and ‘in the catchment’ (distance). No Odds Ratio was close to 1.0 for any time or distance. The Odds Ratios ranged from 5.1 to 20.5, and all were statistically significant ($p < 0.05$). The highest Odds Ratios were for the earliest times and shortest distances – especially less than two weeks from onset and within 15 km of the sampling sites.
Table 1

|        | Cases | Controls | Total |
|--------|-------|----------|-------|
| Metro  | 41    | 46       | 87    |
| Regional | 30   | 229      | 259   |
| Total  | 71    | 275      | 346   |

3.7. Sensitivity and specificity

Table 3 presents a heatmap of the sensitivity (‘true positive’ proportion) and specificity (‘true negative’ proportion) of a sewage sample as a diagnostic test for the presence of one or more known infected people in the catchment, for various times in the illness course and distances from the sampling site. Sensitivity was highest 76% (95%CI 60%–87%) for the earliest period and shortest distance and progressively diminished with greater time since onset and distance to 31% (95%CI 25%–38%) at the furthest distance and longest time category. There was a clear tendency to lower sensitivity with increasing time, although for a given time range there was little change with increasing distance. Specificity was generally high, ranging from 87% (95%CI 83%–90%) to 94% (95%CI 90%–96%). There was a slight tendency for specificity to increase with increasing time, but little change with respect to distance.

All the above analyses used the presence of one or more known infected people in each time/distance category as the outcome variable.

The heatmap in Table 4 shows the results of logistic regression analysis using both the number of known infected people and the mean distance in kilometres as independent variables, and case/control status as the dependent variable. This emphasises the 0 to 4 km category as the one where distance most increases the odds of detection in a sewage sample for each extra kilometre of mean distance from the sampling site. The greatest effect is in the 0 to 4 km and — 2 to 6 days category, where the odds of detecting virus are more than doubled by each extra person. For most categories beyond the second week of illness there is very little effect of additional infected people.

3.8. Probability of detection

Table 5 is a heatmap illustrating the change in odds of detecting virus in a sewage sample by the number of known infected people at different times since onset and at different distances from the sampling site. Probability of detection varied widely with the number of known infected people: where only one was present the probability ranged from 0.098 (95%CI 0.069–0.139) to 0.112 (95%CI 0.077–0.159), but where 100 known infected people were present the probability ranged from 0.004 (95%CI 4.02 × 10⁻⁷–0.978) to 1.000 (95%CI 0.0002–1.000). The wide range for the latter reflects small numbers in some categories.

4. Discussion

The results of this study show that sewage surveillance can be applied to detect the presence of COVID-19 cases in a low case load setting. The Odds Ratios for all combinations of the time in the illness course and distance from the sampling site were well above 1.0. The highest Odds Ratios were for the presence of known infected people in the first 2 weeks of the illness, which corresponds to the period where they are most likely to exhibit elevated shedding (Sethuraman et al., 2020), and are of highest infectiousness – i.e. the cases that most need to be detected. However, the presence of such people even at the end of eight weeks still increased the odds of detection, which is surprising given

Table 2

Heatmap of the unadjusted Odds Ratios for the full range of time and distance categories. (Italic means p < 0.05.)

|        | -2 to 6 days | -2 to 13 days | -2 to 20 days | -2 to 27 days | -2 to 34 days | -2 to 41 days | -2 to 48 days | -2 to 55 days |
|--------|--------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| 0 to 4 km | 20.5         | 17.5          | 16.0          | 14.4          | 14.0          | 11.9          | 8.3           | 6.8           |
| 0 to 9 km | 17.8         | 13.8          | 12.8          | 13.0          | 11.9          | 9.5           | 6.7           | 5.4           |
| 0 to 14 km | 17.6         | 13.8          | 12.8          | 13.0          | 11.9          | 9.5           | 6.7           | 5.4           |
| 0 to 19 km | 13.2         | 11.6          | 11.3          | 11.6          | 10.9          | 8.7           | 6.3           | 5.1           |
| 0 to 24 km | 13.2         | 12.3          | 11.3          | 11.6          | 10.9          | 8.7           | 6.3           | 5.1           |
| 0 to 29 km | 13.2         | 12.3          | 11.3          | 11.6          | 10.9          | 8.7           | 6.3           | 5.1           |
| 0 to 34 km | 13.2         | 12.3          | 11.3          | 11.6          | 10.9          | 8.7           | 6.3           | 5.1           |
studies of the duration of shedding suggesting that more than 8 weeks is an extreme outlier (Sun et al., 2020). The odds of detection did not decrease appreciably with distance from the sampling site, although they did when adjusted for the number of known infected people. When adjusted for distance, the number of known infected people had the greatest effect within 5 km.

Sensitivity was moderate at best, and fell with longer time into the illness course, though not with distance. However, specificity was high and sustained throughout the illness course and for longer distances.

The probability of detection of SARS-CoV-2 in grab samples taken within 15 km of the known infected people’s residences was close to 100% for large numbers of cases (more than 50 in a time/distance

Table 3
Heatmap of the sensitivity and specificity of a sewage test as a diagnostic test for the presence of one or more known infected people in the catchment.

|        | -2 to 6 days | -2 to 13 days | -2 to 20 days | -2 to 27 days | -2 to 34 days | -2 to 41 days | -2 to 48 days | -2 to 55 days |
|--------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| Sensitivity |              |              |              |              |              |              |              |              |
| 0 to 4km | 0.76         | 0.67         | 0.61         | 0.54         | 0.49         | 0.44         | 0.38         | 0.35         |
| 0 to 9km | 0.71         | 0.60         | 0.54         | 0.50         | 0.45         | 0.40         | 0.35         | 0.32         |
| 0 to 14km | 0.70        | 0.60         | 0.54         | 0.50         | 0.45         | 0.40         | 0.35         | 0.32         |
| 0 to 19km | 0.64         | 0.56         | 0.51         | 0.48         | 0.43         | 0.38         | 0.34         | 0.31         |
| 0 to 24km | 0.64         | 0.57         | 0.51         | 0.48         | 0.43         | 0.38         | 0.34         | 0.31         |
| 0 to 29km | 0.64         | 0.57         | 0.51         | 0.48         | 0.43         | 0.38         | 0.34         | 0.31         |
| 0 to 34km | 0.64         | 0.57         | 0.51         | 0.48         | 0.43         | 0.38         | 0.34         | 0.31         |
| Specificity |              |              |              |              |              |              |              |              |
| 0 to 4km | 0.87         | 0.90         | 0.91         | 0.92         | 0.94         | 0.94         | 0.93         | 0.93         |
| 0 to 9km | 0.88         | 0.90         | 0.92         | 0.93         | 0.94         | 0.94         | 0.93         | 0.92         |
| 0 to 14km | 0.88        | 0.90         | 0.92         | 0.93         | 0.94         | 0.94         | 0.93         | 0.92         |
| 0 to 19km | 0.88         | 0.90         | 0.92         | 0.93         | 0.93         | 0.93         | 0.93         | 0.92         |
| 0 to 24km | 0.88         | 0.90         | 0.92         | 0.93         | 0.93         | 0.93         | 0.93         | 0.92         |
| 0 to 29km | 0.88         | 0.90         | 0.92         | 0.93         | 0.93         | 0.93         | 0.93         | 0.92         |
| 0 to 34km | 0.88         | 0.90         | 0.92         | 0.93         | 0.93         | 0.93         | 0.93         | 0.92         |
Second, most samples were grab samples, and the sensitivity in low prevalence settings is likely to improve with other methods that are less affected by the time of sampling, such as repeat grab samples, composite and/or passive sampling methods. Grab samples may be least limited for parts of the catchment where the sewage is well mixed at the point of sampling. Where there are small numbers of infected people close to the sampling site the viral fragments may pass the sampling site as a bolus, and the timing of grab samples in relation to the time these enter the sewer may have a major effect on the likelihood of detection.

Third, although the sensitivity and specificity of a clinical diagnostic test are relatively unaffected by the prevalence of the condition in the population studied, they may have been affected in this study by the point in the State of Victoria’s second wave in which the study took place. The number of known infected people was high at the start of the study period but fell steadily as the study progressed.

Fourth, mis-classification may have occurred, especially for the location of infected people beyond the end of the second week of illness, after which time most cases are cleared from isolation and therefore may have increased their movements. However, this mis-classification is likely to have been non-differential, and thus its effect would have been to bring the Odds Ratios closer to 1.0, not further away as seen in this study. Case locations were based on the ‘residence’ address recorded in the database for the day of sampling. This is not always correct, especially where the infected people were actually in hotels or hospitals, where such information was not captured as reliably. Nor is it always where the people used toilets or bathrooms on that day. However, the state was under quite severe lockdown during much of the study period and this would be expected to reduce such errors.

Fifth, ‘distance’ measures more than just linear distance. It is an amalgam of the effects of distance, hydraulic travel time in the sewer pipes, dilution from other sources (including industrial effluents), and the population served in the catchment. Although this should not influence the interpretation of the results of this study, the separate effects of these cannot be estimated from the currently available data. Future studies might usefully include a more detailed consideration of the sewer pipeline networks, catchment area and population, and typical travel times of wastewater in the sewer network.

Finally, a binary detection/non-detection definition was used in this study with a testing algorithm explicitly seeking to be as sensitive as possible. This results in the equivalence of sewage detections at very low and very high levels. Consideration of quantitative PCR results would provide a greater understanding of the detection of viral fragments in sewage at a point in time with no known infected person in the catchment and/or non-resident infected residents. It may have increased their movements. However, this mis-classification is likely to have been non-differential, and thus its effect would have been to bring the Odds Ratios closer to 1.0, not further away as seen in this study. Case locations were based on the ‘residence’ address recorded in the database for the day of sampling. This is not always correct, especially where the infected people were actually in hotels or hospitals, where such information was not captured as reliably. Nor is it always where the people used toilets or bathrooms on that day. However, the state was under quite severe lockdown during much of the study period and this would be expected to reduce such errors.

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Finally, a binary detection/non-detection definition was used in this study with a testing algorithm explicitly seeking to be as sensitive as possible. This results in the equivalence of sewage detections at very low and very high levels. Consideration of quantitative PCR results

| Category | -2 to 6 days | -2 to 13 days | -2 to 20 days | -2 to 27 days | -2 to 34 days | -2 to 41 days | -2 to 48 days | -2 to 55 days |
|----------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| 0 to 4 km | 2.36         | 2.19         | 2.12         | 2.01         | 2.01         | 1.91         | 1.78         | 1.71         |
| 0 to 9 km | 1.69         | 1.54         | 1.46         | 1.47         | 1.43         | 1.38         | 1.33         | 1.29         |
| 0 to 14 km| 1.52         | 1.46         | 1.41         | 1.38         | 1.33         | 1.29         | 1.26         | 1.22         |
| 0 to 19 km| 1.21         | 1.21         | 1.19         | 1.20         | 1.18         | 1.17         | 1.17         | 1.16         |
| 0 to 24 km| 1.19         | 1.19         | 1.17         | 1.17         | 1.16         | 1.15         | 1.14         | 1.14         |
| 0 to 29 km| 1.19         | 1.17         | 1.17         | 1.16         | 1.15         | 1.14         | 1.14         | 1.14         |
| 0 to 34 km| 1.19         | 1.17         | 1.17         | 1.16         | 1.15         | 1.14         | 1.14         | 1.14         |
adjusted for sewage flow and population size could be used in further analyses to adjust for key variables and further inform sensitivity and specificity estimates.

5. Conclusions

This study provides objective evidence that sewage surveillance can be used as a diagnostic test for the presence of people with SARS-CoV-2 infection in both metropolitan and rural sewer catchments. It is best at detecting cases within the first 2–3 weeks of illness, but it also detects cases to at least 8 weeks, and possibly longer. Specifically, the Odds Ratios never fell to 1.0 or close to it, even at 8 weeks. It seems less affected by distance from the source to the sampling point than time in since onset, with the exception of the closest distance. It can be used in all catchments in Victoria that are similar to those in the study.

Single negative tests of sewage at a given site provide little reassurance that there are no cases in the catchment. However, sewage tests are specific enough to suggest that all positive sewage samples should be followed up with a search for cases, e.g. by increased clinical testing. Because the people putting virus into the sewers are not necessarily new or infectious cases, it may be useful to seek clinical samples, including conventional oropharyngeal and deep nasal swabs as well as anal swabs, from all the known cases of SARS-CoV-2 infection in the catchment (for at least the previous eight weeks, if not longer), to see whether they may still be shedding the virus.

Detection is influenced by the number of cases, especially early in the illness course and close to the sampling site. The estimates of probability of detection for different numbers of infected people suggest that a single person passing through the catchment (such as a truck driver) could spark a detection, but this is not likely, and highly dependent on the time and method of sampling.

Drawing on these findings, sewage surveillance efforts in Victoria and similar low prevalence settings should continue as part of the COVID-19 surveillance toolkit, complementing clinical testing in the

| Change in odds of a sewage detect for each extra kilometre of mean distance, or for each extra known infected person, in each case adjusted for the other variable. (Italic means $p < 0.05$.) |
|---|---|---|---|---|---|---|---|
| -2 to 6 days | -2 to 13 days | -2 to 20 days | -2 to 27 days | -2 to 34 days | -2 to 41 days | -2 to 48 days | -2 to 55 days |
| Change in odds for each extra kilometre of mean distance, adjusted for case count |
| 0 to 4 km | 1.26 | 1.47 | 1.51 | 1.47 | 1.52 | 1.47 | 1.35 | 1.29 |
| 0 to 9 km | 1.26 | 1.12 | 1.15 | 1.15 | 1.13 | 1.08 | 1.05 | 1.02 |
| 0 to 14 km | 1.24 | 1.12 | 1.12 | 1.09 | 1.06 | 1.02 | 1.00 | 0.99 |
| 0 to 19 km | 1.01 | 0.97 | 0.99 | 0.99 | 1.00 | 0.99 | 1.00 | 1.00 |
| 0 to 24 km | 1.00 | 1.01 | 0.99 | 0.99 | 0.99 | 0.98 | 0.98 | 0.98 |
| 0 to 29 km | 1.00 | 1.01 | 0.99 | 0.99 | 0.99 | 0.98 | 0.98 | 0.98 |
| 0 to 34 km | 1.00 | 1.01 | 0.99 | 0.99 | 0.99 | 0.98 | 0.98 | 0.98 |
| Change in odds for each extra person, adjusted for mean distance |
| 0 to 4 km | 2.59 | 1.44 | 1.21 | 1.14 | 1.08 | 1.06 | 1.05 | 1.04 |
| 0 to 9 km | 1.44 | 1.29 | 1.12 | 1.08 | 1.05 | 1.04 | 1.03 | 1.02 |
| 0 to 14 km | 1.27 | 1.21 | 1.10 | 1.06 | 1.04 | 1.03 | 1.02 | 1.01 |
| 0 to 19 km | 1.34 | 1.20 | 1.10 | 1.06 | 1.04 | 1.02 | 1.02 | 1.01 |
| 0 to 24 km | 1.31 | 1.14 | 1.08 | 1.05 | 1.03 | 1.02 | 1.01 | 1.01 |
| 0 to 29 km | 1.31 | 1.14 | 1.08 | 1.05 | 1.03 | 1.02 | 1.01 | 1.01 |
| 0 to 34 km | 1.31 | 1.14 | 1.08 | 1.05 | 1.03 | 1.02 | 1.01 | 1.01 |
most cost-effective combination. Health departments should seek to continue to improve the sensitivity of sewage testing, and its performance close to the sampling site, by exploring alternative sampling methods and strategies; developing traceback strategies so that cases can be better localised in the catchment with public health actions directed to appropriate areas; further characterising performance with low numbers of cases and late in the illness course, during post-wave periods; and improving the integration of the wastewater surveillance programmes and data with clinical surveillance programmes and data, to better account for the movement of infected individuals and their disease progression so that wastewater detects are classified more reliably as ‘expected’ or ‘unexpected’.

CRediT authorship contribution statement

Jim Black: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Supervision, Visualization, Writing – original draft, Writing – review & editing. Phyo Aung: Conceptualization, Formal analysis, Supervision, Writing – review & editing. Monica Nolan: Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. Emma Roney: Conceptualization, Formal analysis, Methodology, Writing – review & editing. Rachael Poon: Conceptualization, Data curation, Funding acquisition, Supervision, Writing – review & editing. Daneeta Hennessy: Data curation, Formal analysis, Methodology, Software.

| Infected people | Distance   | -2-6 days | 7-13 days | 14-20 days | 21-27 days | 28-34 days | 35-41 days | 42-48 days | 49-55 days |
|-----------------|------------|-----------|-----------|------------|------------|------------|------------|------------|------------|
| 0 to 4 km       | 0.15       | 0.13      | 0.08      | 0.12       | 0.10       | 0.09       | 0.10       | 0.09       |
| 0 to 9 km       | 0.12       | 0.11      | 0.10      | 0.10       | 0.10       | 0.09       | 0.10       | 0.09       |
| 0 to 14 km      | 0.11       | 0.11      | 0.11      | 0.11       | 0.10       | 0.10       | 0.10       | 0.10       |
| 0 to 19 km      | 0.11       | 0.11      | 0.10      | 0.11       | 0.10       | 0.10       | 0.10       | 0.10       |
| 0 to 24 km      | 0.11       | 0.12      | 0.10      | 0.12       | 0.10       | 0.10       | 0.11       | 0.10       |
| 0 to 29 km      | 0.12       | 0.12      | 0.10      | 0.12       | 0.10       | 0.10       | 0.11       | 0.10       |
| 0 to 34 km      | 0.13       | 0.13      | 0.11      | 0.11       | 0.11       | 0.11       | 0.11       | 0.11       |
| 0 to 4 km       | 0.64       | 0.43      | 0.04      | 0.31       | 0.10       | 0.09       | 0.11       | 0.10       |
| 0 to 9 km       | 0.26       | 0.17      | 0.12      | 0.11       | 0.14       | 0.07       | 0.13       | 0.08       |
| 0 to 14 km      | 0.16       | 0.16      | 0.13      | 0.13       | 0.13       | 0.09       | 0.12       | 0.09       |
| 0 to 19 km      | 0.15       | 0.17      | 0.10      | 0.17       | 0.11       | 0.09       | 0.12       | 0.09       |
| 0 to 24 km      | 0.16       | 0.20      | 0.09      | 0.18       | 0.11       | 0.09       | 0.12       | 0.09       |
| 0 to 29 km      | 0.17       | 0.20      | 0.09      | 0.18       | 0.10       | 0.10       | 0.11       | 0.10       |
| 0 to 34 km      | 0.25       | 0.23      | 0.11      | 0.11       | 0.11       | 0.11       | 0.11       | 0.11       |
| 0 to 4 km       | 1.00       | 1.00      | 0.00      | 0.97       | 0.15       | 0.07       | 0.17       | 0.12       |
| 0 to 9 km       | 0.93       | 0.62      | 0.26      | 0.19       | 0.36       | 0.03       | 0.30       | 0.05       |
| 0 to 14 km      | 0.51       | 0.47      | 0.26      | 0.24       | 0.26       | 0.06       | 0.19       | 0.07       |
Writing – review & editing. Nicholas D. Crosbie: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. Dan Deere: Conceptualization, Funding acquisition, Project administration, Validation, Writing – review & editing. Aaron R. Jex: Validation, Writing – review & editing. Nijoy John: Validation, Writing – review & editing. Louise Baker: Validation, Writing – review & editing. Steven Myers: Data curation, Investigation, Methodology, Writing – review & editing. Natacha Begue: Data curation, Investigation, Methodology, Writing – review & editing. Steve P. Usher: Validation, Writing – review & editing. Natacha Begue: Data curation, Investigation, Methodology, Writing – review & editing. Christelle Schang: Conceptualization, Methodology, Supervision, Validation, Writing – review & editing. Jonathan Schmidt: Conceptualization, Methodology, Supervision, Validation, Writing – review & editing. Shane P. Usher: Validation, Writing – review & editing. Natacha Begue: Data curation, Investigation, Methodology, Writing – review & editing. Andrew L. Strong: Validation, Writing – review & editing. Christelle Schang: Conceptualization, Methodology, Supervision, Validation, Writing – review & editing. J. Black, P. Aung, M. Nolan et al. Science of the Total Environment 786 (2021) 147469

| Distance | 0 to 4 km | 0 to 9 km | 0 to 14 km | 0 to 19 km | 0 to 24 km | 0 to 29 km | 0 to 34 km |
|----------|-----------|-----------|------------|-----------|-----------|-----------|-----------|
|          | 1.00      | 1.00      | 0.00       | 1.00      | 0.27      | 0.05      | 0.39      | 0.19      |
|          | 1.00      | 0.99      | 0.67       | 0.44      | 0.88      | 0.01      | 0.77      | 0.02      |
|          | 0.97      | 0.95      | 0.67       | 0.58      | 0.67      | 0.02      | 0.42      | 0.04      |
|          | 0.94      | 0.99      | 0.09       | 0.98      | 0.18      | 0.06      | 0.38      | 0.02      |
|          | 0.95      | 0.99      | 0.03       | 0.98      | 0.14      | 0.04      | 0.33      | 0.03      |
|          | 0.96      | 0.99      | 0.03       | 0.98      | 0.09      | 0.07      | 0.25      | 0.04      |
|          | 1.00      | 1.00      | 0.11       | 0.11      | 0.11      | 0.11      | 0.11      | 0.11      |

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.

Acknowledgements

The authors gratefully acknowledge the contribution to the study of the staff of DHHS, especially in the contact tracing and intelligence teams; the collaborating water authorities (Barwon Water, Central Highlands Water, City West Water, Coliban Water, East Gippsland Water, Gippsland Water, Goulburn Valley Water, Grampians Wimmera Mallee Water, Lower Murray Water, Melbourne Water, North East Water, South East Water, South Gippsland Water, Wannon Water, Westernport Water, Western Water, and Yarra Valley Water); and Australian Laboratory Services, the University of Melbourne, Melbourne Water, Monash University, Victorian Infectious Diseases Reference Laboratory, the Walter and Eliza Hall Institute, SA Water and Sydney Water, for methods development, qRT-PCR testing and sequencing. All the collaborating groups are members of the Collaboration on Sewage Surveillance for SARS-CoV-2 (ColoSSoS) project, coordinated by Water Research Australia.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. Louise Baker was supported by a Melbourne Water Centenary Fellowship, and Aaron Jex by Water Research Australia Project 2064, NHMRC Career Development Fellowship APP1164534, and The Victorian State Government Operational Infrastructure Support and Australian Government National Health and Medical Research Council Independent Research Institute Infrastructure Support Scheme.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.147469.

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