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Wastewater surveillance demonstrates high predictive value for COVID-19 infection on board repatriation flights to Australia

Warish Ahmed a,*, Aaron Bivins b, Stuart L. Simpson c, Paul M. Bertsch a, John Ehret d, Ian Hosegood d, Suzanne S. Metcalfe a, Wendy J.M. Smith a, Kevin V. Thomas e, Josh Tynan e, Jochen F. Mueller e

a CSIRO Land and Water, Ecosciences Precinct, 41 Boggo Road, Brisbane, QLD 4102, Australia
b Department of Civil and Environmental Engineering & Earth Science, University of Notre Dame, 156 Fitzpatrick Hall, Notre Dame, IN 46556, USA
c CSIRO Land and Water, Lucas Heights, NSW 2234, Australia
d Qantas Airways Limited, 10 Bourke Rd Mascot, 2020, NSW, Australia
e Queensland Alliance for Environmental Health Sciences (QAEHS), The University of Queensland, 20 Cornwall Street, Woolloongabba, QLD 4103, Australia

ABSTRACT

Controlling importation and transmission of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) from overseas travelers is essential for countries, such as Australia, New Zealand, and other island nations, that have adopted a suppression strategy to manage very low community transmission. Wastewater surveillance of SARS-CoV-2 RNA has emerged as a promising tool employed in public health response in many countries globally. This study aimed to establish whether the surveillance of aircraft wastewater can be used to provide an additional layer of information to augment individual clinical testing. Wastewater from 37 long-haul flights chartered to repatriate Australians was tested for the presence of SARS-CoV-2 RNA. Children 5 years or older on these flights tested negative for coronavirus disease 19 (COVID-19) (deep nasal and oropharyngeal reverse-transcription (RT)-PCR swab) 48 h before departure. All passengers underwent mandatory quarantine for 14-day post arrival in Howard Springs, NT, Australia. Wastewater from 24 (64.9 %) of the 37 flights tested positive for SARS-CoV-2 RNA. During the 14 day mandatory quarantine, clinical testing identified 112 cases of COVID-19. Surveillance for SARS-CoV-2 RNA in repatriation flight wastewater using pooled results from three RT-qPCR assays demonstrated a positive predictive value (PPV) of 87.5 %, a negative predictive value (NPV) of 76.9 % and 83.7% accuracy for COVID-19 cases during the post-arrival 14-day quarantine period. The study successfully demonstrates that the surveillance of wastewater from aircraft for SARS-CoV-2 can provide an additional and effective tool for informing the management of returning overseas travelers and for monitoring the importation of SARS-CoV-2 and other clinically significant pathogens.

1. Introduction

Wastewater surveillance of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) genetic fragments (i.e., RNA) has proven to be an effective tool for assessing COVID-19 infections at the community level (Ahmed et al., 2020a; Peccia et al., 2020; Prado et al., 2021). SARS-CoV-2 RNA is shed in feces and other body fluids of pre-symptomatic, and asymptomatic infected individuals, and can be detected in wastewater several days before clinical COVID-19 cases are detected (D’Aoust et al., 2021; Nemudryi et al., 2020; Park et al., 2021). Over the course of community transmission, the RNA concentration in wastewater has been found to correlate with COVID-19 epidemiological curves derived from individual clinical testing (Fernandez-Casti et al., 2021; Graham et al., 2021). Wastewater surveillance can be applied to monitor COVID-19 infection status and trends within populations of varying sizes and locations (scalable for catchment, postcode level, building) (Huisman et al., 2021). This approach can be particularly useful in locations with low or no existing active caseloads, including facilities such as nursing homes, prisons, college campuses, and student dormitories, as well as ships, aircraft, resort hotel locations, and regional and remote vulnerable communities (Betancourt et al., 2021; Hassard et al., 2021; Karkikeyan et al., 2021; Spurbeck et al., 2021; Davo et al., 2021).

* Corresponding author at: Ecosciences Precinct, 41 Boggo Road, Dutton Park 4102, Queensland, Australia.
E-mail address: Warish.Ahmed@csiro.au (W. Ahmed).

https://doi.org/10.1016/j.envint.2021.106938
Received 14 July 2021; Received in revised form 18 September 2021; Accepted 12 October 2021
Available online 14 October 2021
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Early in the pandemic, we demonstrated that SARS-CoV-2 RNA could be detected in wastewater collected from international flights and cruise ships (Ahmed et al., 2020b). A recent study by Albastaki et al. (2021) detected SARS-CoV-2 RNA in 13.6 % of wastewater samples from 198 commercial aircrafts that arrived at Dubai Airport. Consequently, aircraft wastewater surveillance could offer a cost-effective method for screening onboard passengers to inform public health officials for managing passenger and crew well-being and prioritizing clinical testing. The application of wastewater surveillance at international travel entry points, such as airports, coupled with point of care clinical testing and well-coordinated open reporting, could provide public health officials with additional means of assessing the presence or absence of SARS-CoV-2 and other infections among people arriving at border entry points.

Australia historically has a low risk of importation of infectious diseases due to its geographic remoteness and, as an island nation, the ability to apply strict controls at entry points, including the use of mandatory quarantine, which has been an important part of minimizing COVID-19 impacts in Australia (Potheringham et al., 2021).

However, the COVID-19 pandemic has revealed Australia’s vulnerability despite its geographic isolation and caused significant economic and social damages despite comparatively low national case numbers compared to other countries. The stringent border measures, including border closures and long periods of quarantine on arrival, contribute to these direct impacts, thereby causing significant disruption to the travel, tourism, and education sectors. Australia’s policies for suppressing all community transmissions of COVID-19 show that the identification of even a single new instance of community transmission is epidemiologically significant due to highly contagious variants such as delta. In the absence of broad immunity, one of the best lines of defense against COVID-19 is preparedness, early detection, and aggressive response to isolate the identified cases.

Currently, international travel to Australia is limited. Many Australians are being repatriated via facilitated commercial flights operated by the airline Qantas on behalf of the Government. Upon arrival in Australia, passengers on these international flights must undertake a mandatory 14-day quarantine in a facility designated by the Government. Under the current program, to be accepted onto these flights, potential travelers are required to provide verified negative test results for COVID-19 (deep nasal and oropharyngeal RT-PCR swabs) 48 h before departure. Children under five are exempt. Strict application of personal protective equipment (PPE) and other measures are required during the flight. Each passenger is provided with a sanitizer, face masks, and a disposal bag for used masks. Face masks must be worn during the flight, and every passenger must change masks every-two hours throughout the flight.

In this study, we describe the data collected from flight passengers upon arrival at dedicated facilities in Howard Springs, NT, Australia, where they were quarantined. We report the application of wastewater surveillance for SARS-CoV-2 RNA in wastewater from de-identified repatriation flights and its correlation with de-identified clinical data for subsequent detection of SARS-CoV-2 among passengers during the mandatory quarantine period (usually 14 days). Typically, COVID-19 clinical testing for travelers during quarantine includes an early deep nasal and oropharyngeal RT-PCR swab test (days 0–3 of quarantine), and a late deep nasal and oropharyngeal swab test (day 11 or 12). For some repatriating passengers, additional tests were conducted between days 3 and 11 for public health purposes. Once the international travel restrictions ease, wastewater analysis may be used for surveillance for the presence of SARS-CoV-2 infections, information on novel variants, and COVID-19 case importation.

2. Materials and methods

2.1. Wastewater sampling from the lavatory

A total of 37 aggregated wastewater samples (500 mL to 1 L in volume) were collected from the wastewater exiting lavatories of 37 repatriation flights landing at Darwin International Airport (DRW), Northern Territory, Australia, between 17/12/2020 and 27/03/2021. Wastewater samples were collected from flights exceeding 7 h duration. This duration was selected with the expectation that the repatriation passengers would consume food on one or more occasions while on these long-haul flight-legs (arriving at Darwin, Australia), and were more likely to use the lavatory during the flight. Wastewater was sampled using a specifically designed sample trap capable of retaining an aliquot of bulk wastewater sample exiting the aircraft lavatory before entering a waste service truck system (Supplementary Fig. SF1). This minimized any potential health risks associated with wastewater collection and prevented sample cross-contamination from multiple flights (Ahmed et al., 2020b). The sample trap was designed and fabricated based on an alloy version of the standard WYE 4° connector (Supplementary Fig. SF1). When the aircraft reached the parking stand, the sample trap inlet was connected to the aircraft wastewater removal valve and the outlet to the lavatory waste truck using a hose. An aircraft may have up to three wastewater tanks, depending on the model. During sampling, the first tank was emptied, and once the wastewater flow passed into the lavatory waste truck, the ball valve was opened to drain approximately 250 mL sample into a sterile sample bottle. Similarly, the second and third tanks were drained into the same bottle for more samples. After wastewater sampling, a sanitizing line was connected to the aircraft, and each tank was dosed with aircraft toilet deodorant and viricidal/bactericidal gel (Novirusac Gel Bulk, Aero Defence Pty. Ltd., Southport, Queensland, Australia) before departure. The sample trap was steam cleaned before sampling the next aircraft.

2.2. Post-lavatory wash sampling

To assess whether SARS-CoV-2 fragments remained in aircraft wastewater tanks (after positive wastewater detection), we collected 28 lavatory wash samples (i.e., post-lavatory wash) from pre-departure flights to confirm the presence/absence of SARS-CoV-2 RNA. After emptying the wastewater tanks, the waste drain valve was left open, and approximately 200 L of water was pumped into each tank and drained into the lavatory waste truck. The waste drain valve was then closed, and an additional 40 L of water was pumped into each tank, followed by opening the waste drain valve to flush out 40 L of water into the lavatory waste truck. An aliquot (i.e., 500 mL to 1 L) of wash water sample was collected from the sampling trap, as wash water exited the aircraft into a lavatory waste service truck system. After wash water sampling, each aircraft tank was sanitized according to the procedure described above. PPE (face masks, gloves, goggles, and safety boots) was used during sampling. Samples were transported on ice to the laboratory, stored at 4 °C, and processed within 24–72 h after collection.

2.3. RT-qPCR inhibition test

Before the sample concentration, known numbers (10^5 gene copies (GC)/50 mL) of murine hepatitis virus (MHV) were seeded into each wastewater sample as a process control (Ahmed et al., 2020c). The same numbers of MHV were added to distilled water and subjected to RNA extraction. The reference quantification cycle (Cq) values obtained for the MHV-seeded distilled water were compared with the Cq values of the MHV-seeded wastewater to obtain information on potential RT-qPCR inhibition. If the Cq value of the wastewater RNA sample was > 2 – Cq value difference compared to the reference Cq value for distilled water, the RNA sample was considered to have PCR inhibitors (Ahmed et al., 2020b). The presence of PCR inhibition in RNA/DNA samples extracted from wastewater was assessed using an MHV RT-qPCR assay detailed elsewhere (Bessels et al., 2002). All samples were analyzed alongside three non-template controls.
2.4. Sample concentration and RNA extraction

Viruses were concentrated from each aggregated wastewater sample (50 mL) using an automated, rapid concentrator instrument (Concentrating Pipette Select™, CP Select™, InnovaPrep, Drexel, MO) designed for concentrating bacteria, protozoa, and viruses from water matrices simultaneously. The rationale for using 50 mL wastewater is that aircraft wastewater is more concentrated than primary influent samples of community wastewater treatment plants since much less water is used for flushing and per person water use is very low onboard aircraft. Hence, 50 mL wastewater sample was used for virus concentration. This instrument was used because it provides a faster concentration step than most other methods (e.g., adsorption-extraction, centrifugation, precipitation; (Ahmed et al., 2020b)) and achieves relatively high recovery efficiency; reported to range from 11 to 39 % (for SARS-COV-2) and ~48 % (for OC43 Betacoronavirus) (Ahmed et al., 2021b; McMinn et al., 2021). Before concentration, the sample was first centrifuged at 4000 g for 30 min to pellet the suspended solids and toilet paper using a Beckman Coulter Avanti J-15R Centrifuge. The supernatant (~45 mL) was then concentrated using an unirradiated 0.05 μm PS hollow fiber filter concentrating pipette tip (Cat. No. CC08004-200) with the CP Select™. Optimized wastewater application settings provided in the instrument instructions were used. Upon concentration, the tip was eluted twice using CP elution fluid (HC08001) containing 0.075 % Tween 20-and 25-mM Tris (Cat. No. HC08001). The eluates (0.8 mL) were collected in sterile 15-mL polypropylene tubes. Immediately after virus concentration, RNA was extracted from 280 μL of the eluate using the QIamp Viral RNA Mini Kit (Cat. No. 52905) (Qiagen) with a minor modification. In a final step, a 100 μL volume of buffer AVE was used to elute the RNA instead of 60 μL. All RNA samples were stored at −80 °C for 3–5 days and subjected to RT-qPCR analysis.

2.5. RT-qPCR analysis

Previously published RT-qPCR assays that target the N gene of SARS-CoV-2 (US CDC N1, US CDC N2, and China CDC N) were used for SARS-CoV-2 RNA detection and quantification in wastewater samples. The primer and probe sequences, along with qPCR cycling parameters for all the RT-qPCR assays used, including MHV, are shown in Supplementary Table S1. RNA extracted from gamma-irradiated SARS-CoV-2 hCoV 19/ Australia/VIC01/2020 with QIamp Viral RNA mini kit was measured with dPCR and used as the RT-qPCR standard for SARS-CoV-2 US CDC N1, N2, and China CDC N assays. US CDC N1, CDC N2, and China CDC N standard dilutions ranged from 1 × 10^3 to 1 GC/μL. For MHV, gBlocks gene fragments were purchased from Integrated DNA Technologies (Integrated DNA Technology Coralville, IA, US) and used as a positive control. MHV and SARS-CoV-2 N gene assays were performed in 20-μL reaction mixtures using TaqMan™ Fast Virus 1-Step Master Mix (Applied Biosystem, California, USA). MHV RT-qPCR mixture contained 5 μL of Supermix, 300 nM of forward primer, 300 nM of reverse primer, 400 nM of probe, and 5 μL of template RNA. US CDC N1 and N2 RT-qPCR mixture contained 5 μL of Supermix, 2019-nCoV Kit (500 nM of forward primer, 500 nM of reverse primer, and 125 nM of probe) (Catalogue No. 10006666), and 5 μL of template RNA. China CDC N RT-qPCR mixture contained 5 μL of Supermix, 400 nM of forward primer, 400 nM of reverse primer, 250 nM of probe (Catalogue No. 10006666), and 5 μL of template RNA. All RT-qPCR reactions were performed in triplicate. For each RT-qPCR run, triplicate negative controls were included. The RT-qPCR assays were performed using a Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratories, Richmond, CA), with manual settings for baseline threshold (50 fluorescent units). For each sample, the SARS-CoV-2 RNA concentration (GC/50 mL) was calculated from the standard curve and accounts for the difference in eluate volumes.

For US CDC N1, N2, and China CDC N assays, the assay limit of detection (ALOD) is defined as the minimum GC number with a 95 % probability of detection and determined as previously described (Verbyla et al., 2016). The sample limit of detection (SLOD) was calculated by dividing the ALOD by the RNA template volume added to the PCR well and then multiplying this number by the total volume of RNA extracted from each sample to yield the total RNA gene copies that could be detected with 95 % probability. This number was then normalized to the total sample volume processed to yield the SLOD of SARS-CoV RNA GC/50 mL.

2.6. Quality control

To minimize RT-qPCR contamination, RNA extraction and RT-qPCR setup were performed in separate laboratories. A concentration method negative control was included for each batch of wastewater samples. A reagent negative control was also included during RNA extraction to account for any contamination during extraction. For each RT-qPCR run, triplicate negative controls were included. All concentration method, reagent and RT-qPCR negative controls were negative for the targets analyzed. All RT-qPCR experimental details are reported in compliance with the Minimum Information for Publication of Quantitative Real-Time PCR experiments as summarized in Table S4.

2.7. Statistical analysis

Positive and negative predictive values (PPV and NPV) were estimated for wastewater surveillance of repatriation flights and subsequent COVID-19 cases during a mandatory 14-day quarantine period (Parikh et al., 2008). In this case, PPV is the probability of an incident COVID-19 case among the passengers of an aircraft following a positive wastewater result, and NPV is the probability of no incident COVID-19 cases following a negative wastewater result. We also calculated accuracy: the overall probability that wastewater samples correctly classified the presence or absence of COVID-19 clinical cases in quarantine. In addition to predictive values and accuracies, the probability of an RT-qPCR-positive wastewater sample for a given number of cumulative COVID-19 cases during the quarantine period was described by fitting a cumulative Gaussian distribution to paired wastewater RT-qPCR replicates and subsequent COVID-19 cases from each flight. This is synonymous with the methods previously described to determine 95 % ALOD (Bivins et al., 2021). All graphs and statistical analyses were prepared and performed using GraphPad Prism Version 9.0.0 (GraphPad Software, LaJolla, CA, USA).

2.8. Ethics approval

Low-risk approval as defined by the National Statement on Ethical Conduct in Human Research was obtained from the CSIRO Ethics Committee (reference number 2020_031_LR). Flight numbers and passenger information were de-identified.

3. Results

3.1. PCR inhibition, performance characteristics of RT-qPCR assays and ALOD

All RNA samples were free from PCR inhibition as determined by Cq values from the MHV RT-qPCR assay and were therefore used for downstream RT-qPCR analysis for SARS-CoV-2 RNA. The amplification efficiencies of US CDC N1, CDC N2, and China CDC N assays were 95.9, 91.1, and 99.0 %, respectively, within acceptable bounds (Bustin et al., 2009). The correlation coefficient (R²) values for all three assays were between 0.973 and 0.997. The slopes of the standard curves were −3.424 (US CDC N1), −3.556 (US CDC N2), and −3.346 (China CDC N). The y-intercept values were between 39.32 (US CDC N1) and 41.68 (US CDC N2). ALOD values ranged between 1.8 (US CDC N1 and China CDC N) to 2.7 GC/μL of RNA (US CDC N2), while SLOD values ranged between 180
3.2. SARS-CoV-2 RNA in lavatory wastewater samples

Of the 37 flights, 21 (56.7 %), 12 (32.4 %), and 13 (35.1 %) had wastewater samples that were positive for at least one of three RT-qPCR replicates for US CDC N1, N2, and China CDC N assays, respectively. For the US CDC N1 assay, 15 (40.5 %) and 8 (21.6 %) wastewater samples that were positive for two of three and three of three RT-qPCR replicates, respectively. For US CDC N2, these figures were 8 (21.6 %) (at least two of three replicates) and 7 (18.9 %) samples (three of three replicates) positive. For China CDC 2, these figures were 10 (27 %) (at least two of three replicates) and 8 (21.6 %) samples (three of three replicates) positive. When pooling the results for all three assays (with at least one of three replicates positive), wastewater from 24 (64.9 %) unique flights was positive for SARS-CoV-2 RNA. Of the 24 flights with SARS-CoV-2 positive wastewater, 10 were positive for all three assays, two were negative, and two were positive for COVID-19.

Table 1

| Assay          | Efficiency (E) (%) | Linearity ($R^2$) | Slope | Y-intercept | ALOD for SARS-CoV-2 RNA (GC/µL reaction) | SLOD for SARS-CoV-2 RNA (GC/50 mL) |
|----------------|-------------------|------------------|-------|-------------|-----------------------------------------|-----------------------------------|
| US CDC N1      | 95.9              | 0.997            | –3.424| 39.32       | 1.8                                     | 180                               |
| US CDC N2      | 91.1              | 0.973            | –3.556| 41.68       | 2.7                                     | 270                               |
| China CDC N    | 99.0              | 0.996            | –3.346| 39.63       | 1.8                                     | 180                               |

Table 2

Detection of SARS-CoV-2 RNA in wastewater samples collected from repatriation flights at Darwin International Airport (DRW).

| Aircraft ID | Departure airport | Sampling date* | Number of passengers | Mean ± SD log10 GC/50 mL of wastewater | Incidence of COVID-19 during the 14-day mandatory quarantine period |
|-------------|-------------------|----------------|----------------------|---------------------------------------|---------------------------------------------------------------|
| A1R         | CDG               | 17/12/2020     | 161                  | DNQ                                  | <ALOD                                                        |
| A2R         | DEL               | 27/10/2020     | 188                  | 3.10 ± 0.18 3.72 ± 0.13 3.07 ± 0.09 | 7                                                            |
| A3R         | DEL               | 11/11/2020     | 160                  | 2.92 ± 0.27 2.44 ± 0.57 2.32 ± 0.61 | 6                                                            |
| A4R         | DEL               | 24/11/2020     | 172                  | 3.33 ± 0.39 3.23 ± 0.22 2.72 ± 0.28 | 6                                                            |
| A5R         | DEL               | 28/11/2020     | 152                  | 2.36 ± 0.68 <ALOD <ALOD 7            |                                                               |
| A6R         | DEL               | 08/01/2021     | 120                  | 2.43 ± 0.40 <ALOD <ALOD 2            |                                                               |
| A7R         | DEL               | 19/01/2021     | 165                  | DNQ 2.48 ± 0.67 3.01 ± 0.40 2       |                                                              |
| A8R         | DEL               | 20/01/2021     | 197                  | <ALOD <ALOD <ALOD 0                 |                                                              |
| A9R         | DEL               | 04/02/2021     | 196                  | <ALOD <ALOD <ALOD 4                 |                                                              |
| A10R        | DEL               | 14/03/2021     | 200                  | DNQ DNQ DNQ 1                      |                                                              |
| A11R        | DEL               | 25/03/2021     | 198                  | DNQ DNQ DNQ 4                      |                                                              |
| A12R        | DEL               | 17/04/2021     | 192                  | 4.10 ± 0.02 3.88 ± 0.22 3.87 ± 0.30 | 23                                                           |
| A13R        | DEL               | 15/05/2021     | 150                  | DNQ <ALOD <ALOD 1                  |                                                              |
| A14R        | FRA               | 13/12/2020     | 170                  | 2.60 ± 0.73 <ALOD <ALOD 3           |                                                              |
| A15R        | FRA               | 03/01/2021     | 193                  | 3.96 ± 0.30 3.68 ± 0.31 3.33 ± 0.27 | 3                                                            |
| A16R        | FRA               | 21/02/2021     | 195                  | <ALOD <ALOD <ALOD 0                 |                                                              |
| A17R        | FRA               | 12/03/2021     | 194                  | <ALOD <ALOD <ALOD 0                 |                                                              |
| A18R        | FRA               | 31/03/2021     | 175                  | <ALOD <ALOD <ALOD 0                 |                                                              |
| A19R        | FRA               | 07/05/2021     | 173                  | <ALOD <ALOD <ALOD 0                 |                                                              |
| A20R        | JNB               | 11/04/2021*    | 190                  | <ALOD <ALOD <ALOD 1                 |                                                              |
| A21R        | LHR               | 08/11/2021     | 175                  | 5.54 ± 0.01 5.77 ± 0.07 5.09 ± 0.02 | 2                                                            |
| A22R        | LHR               | 12/11/2020     | 161                  | 2.58 ± 0.53 DNQ 2.97 ± 0.96 0        |                                                              |
| A23R        | LHR               | 30/11/2020     | 171                  | 2.32 ± 0.31 <ALOD <ALOD 1           |                                                              |
| A24R        | LHR               | 16/01/2021     | 213                  | <ALOD <ALOD DNQ 3                   |                                                              |
| A25R        | LHR               | 30/12/2020     | 199                  | <ALOD <ALOD DNQ 1                   |                                                              |
| A26R        | LHR               | 02/02/2020     | 202                  | <ALOD <ALOD <ALOD 0                 |                                                              |
| A27R        | LHR               | 17/02/2021     | 204                  | <ALOD <ALOD <ALOD 0                 |                                                              |
| A28R        | LHR               | 19/02/2021     | 207                  | 3.25 ± 0.20 3.29 ± 0.24 2.88 ± 0.47 1 |                                                              |
| A29R        | LHR               | 23/02/2021     | 208                  | 2.43 ± 0.42 <ALOD <ALOD 0           |                                                              |
| A30R        | LHR               | 08/03/2021     | 194                  | 2.52 ± 0.48 <ALOD <ALOD 0           |                                                              |
| A31R        | LHR               | 10/03/2021     | 200                  | <ALOD <ALOD <ALOD 0                 |                                                              |
| A32R        | LHR               | 02/05/2021     | 105                  | <ALOD <ALOD <ALOD 0                 |                                                              |
| A33R        | LHR               | 04/05/2021     | 98                   | <ALOD <ALOD <ALOD 1                 |                                                              |
| A34R        | MAA               | 15/12/2020     | 175                  | DNQ <ALOD <ALOD 4                   |                                                              |
| A35R        | MAA               | 06/02/2021     | 196                  | <ALOD <ALOD <ALOD 0                 |                                                              |
| A36R        | MAA               | 15/04/2021     | 185                  | 4.04 ± 0.14 3.62 ± 0.20 3.86 ± 0.15 24 |                                                              |
| A37R        | YVR               | 27/03/2021     | 138                  | <ALOD <ALOD <ALOD 0                 |                                                              |

CDG: Charles-de-Gaulle Airport; DEL: Indira Gandhi International Airport; FRA: Frankfurt Airport; LHR: London Heathrow Airport; MAA: Chennai International Airport; YVR: Vancouver International Airport; DNQ: Did not quantify; ALOD: Assay limit of detection; *all samples were collected from DRW. Green color denotes aircraft wastewater positive and the positive incidence of COVID-19 during the 14-day mandatory quarantine period; Light blue color denotes aircraft wastewater negative and the negative incidence of COVID-19; light orange color denotes aircraft wastewater negative and the positive incidence of COVID-19; grey color denotes wastewater positive and the negative incidence of COVID-19.
positive for two assays (US CDC N1 and N2), and 12 were positive for only a single assay (US CDC N1 = 9, N2 = 0, China CDC N = 3). Of the positive samples, 15, 9, and 11 were quantifiable by US CDC N1, N2, and China CDC N assays, respectively. Among the quantifiable samples, the SARS-CoV-2 RNA concentration by US CDC N1 ranged between 2.32 and 5.54 log₁₀ GC/50 mL of wastewater, with a mean of 3.17 ± 0.90 log₁₀ GC/50 mL wastewater (Table 2). The mean concentrations by US CDC N2 and China CDC were also similar—3.56 ± 0.97 (CDC N2) to 3.22 ± 0.81 (China CDC N) log₁₀ GC/50 mL wastewater.

3.3. SARS-CoV-2 RNA in post-lavatory wash samples

Among the 28 post-lavatory wash samples collected, only two samples (7.14 %) were RT-qPCR positive for SARS-CoV-2 RNA by the US CDC N1 assay, but they were below the quantifiable range. US CDC N2 and China CDC assays yielded non-detections in all post-lavatory wash samples (Supplementary Table S2).

3.4. Wastewater surveillance and COVID-19 cases

Among the 6,570 passengers repatriated by the 37 flights included in the study, 112 COVID-19 cases (1.70 %) were detected by clinical testing during the mandatory quarantine period (Supplementary Table S3). These infections were detected among passengers from 24 (64.9 %) flights, leaving only 13 (35.1 %) of 37 flights without subsequent detection of COVID-19 cases on board. For repatriation flights with positive COVID-19 cases, the number of incident cases ranged from 1 to 24 with an average and standard deviation (SD) of 4.62 ± 6.19 (95 % CI = 2.15 to 7.11) COVID-19 cases/positive flight. COVID-19 incidence rates among the passengers of individual flights during the quarantine period ranged from a maximum of 12.9 % (24/185) to a minimum of 0.48 % (1/207). Of the 108 COVID-19 cases, 58 (53.7 %) were detected on day 0, and 97 (89.8 %) were detected on day 7.

Surveillance for SARS-CoV-2 RNA in repatriation flight wastewater using pooled results from all three RT-qPCR assays demonstrated a PPV of 87.5 % and an NPV of 76.9 % for COVID-19 cases during the post-arrival 14-day quarantine period (83.7 % accuracy) (Fig. 1). The China CDC N assay yielded the highest PPV (92.3 %), while the US CDC N1 assay yielded the highest NPV (62.5 %) and highest accuracy (75.7 %) for subsequent COVID-19 cases. Among the 37 repatriation flights, wastewater samples from three flights were RT-qPCR negative (i.e., < ALOD) for SARS-CoV-2 in wastewater when COVID-19 cases were subsequently detected. Four passengers...
from flight A9R tested positive over a 14-day quarantine period (two on day 0, one on day 1, and one on day 12). Furthermore, two passengers (one from each flight A20R and A33R) tested positive on day 7 and day 0, respectively, by deep nasal and oropharyngeal swab testing. Similarly, among the 37 flights, wastewater samples from three flights were RT-qPCR positive, but clinical testing failed to detect any COVID-19 cases over the 14-day mandatory quarantine. There were six flights with 1 case/flight, which resulted in wastewater RT-qPCR positive detections.

A cumulative Gaussian distribution was fit to the proportion of positive RT-qPCR replicates given an observed number of COVID-19 cases to estimate the analytical sensitivity of wastewater surveillance using RT-qPCR assay data obtained in this study (Fig. 2). This model, based on the empirical data, indicates the number of COVID-19 cases required to yield a given probability of a single positive RT-qPCR reaction. The US CDC N1 assay was the most sensitive, with a 50% probability of a positive reaction at just 2.8 COVID-19 cases and 95% of replicates positive for 9.7 cases ($R^2 = 0.72$). The US CDC N2 and China CDC N assays demonstrated more variable performance, with $R^2$ values of 0.59 and 0.48, respectively. Both were less analytically sensitive than US CDC N1, with 6.3 COVID-19 cases required for a 50% probability of a single N2 replicate to be positive and 5.8 cases for a 50% probability of a single China CDC N replicate to be positive.

As shown in Supplementary Fig. SF2, Spearman correlations between SARS-CoV-2 RNA $\log_{10}$ GC/50 mL by RT-qPCR and COVID-19 cases during the 14-day quarantine period were positive but not statistically significant. For US CDC N1 ($n = 15$), the correlation was moderate ($r = 0.47, p = 0.076$), while China CDC N ($n = 11$) had a weak correlation ($r = 0.37, p = 0.26$) and US CDC N2 ($n = 9$) had a very weak correlation ($r = 0.04, p = 0.92$).

4. Discussion

Until global COVID-19 cases reach a manageable level, restrictions on international travel to certain countries may continue for an extended period. Such restrictions, along with reduction in demand for passenger travel, will continue to significantly impact tourism and aviation industries that rely heavily on people moving across national and international borders. International airports are important COVID-19 control points, specifically for nations such as Australia and New Zealand, but also for many other small island nations that are following an elimination strategy. A significant number of COVID-19 infections recorded in Australia have been acquired overseas and detected in quarantine (Price et al., 2020). It is therefore critical to screen incoming passengers for COVID-19 at points of entry.

In our previous proof-of-concept study, we demonstrated that SARS-CoV-2 RNA could be detected in aircraft wastewater and could provide information on the presence of COVID-19 on-board passengers.
However, there were some limitations in the previous study (Ahmed et al., 2020b), including that wastewater was sampled from a lavatory waste truck that collected wastewater from multiple aircraft; creating the likelihood of false-positive results. Most importantly, in the absence of clinical testing results for on-board passengers, it was not possible to establish a link between the wastewater surveillance data and COVID-19 infected passengers. Such information is critical for determining the usefulness of wastewater surveillance as a complementary tool to clinical testing.

In this study, a new sample trap was designed to allow direct wastewater sampling from the aircraft before it enters the lavatory waste truck, thereby reducing the likelihood of false-positive results that may occur if lavatory waste truck is sampled. The lavatory wastewater chambers are cleaned after landing. However, it is possible that traces of SARS-CoV-2 may remain in the wastewater chambers and may yield false-positive wastewater results for aircraft with no COVID-19 on-board passengers. To obtain information on the likelihood of false-positive results occurring between destinations, many aircraft waste tanks were cleaned twice before departure, and the wash samples were also analyzed for the presence of SARS-CoV-2 RNA. Among the 28 wash samples tested, two aircraft were indeed positive by US CDC N1 assay, but these samples were not quantifiable and not all replicates were positive. Furthermore, these samples were RT-qPCR negative by US CDC N2 and China CDC N assays, suggesting that a low level of cross-contamination may have occurred. The application of multiple RT-qPCR assays, such as those used in this study, and an increased number of PCR replicates (n = 6 replicates if possible) may be needed to distinguish true positive results from low levels of false-negatives. Consequently, we recommend that each aircraft tank be cleaned, and wash samples be archived and analyzed later if the returning flight yields RT-qPCR positive signals. If the returning flight is RT-qPCR negative, then analyzing the wash samples will not be required. If wash samples (departing flight) and wastewater samples (returning flight) both yield RT-qPCR positive signals, then the Cq values (i.e., 3 Cq difference between wash and wastewater samples) or GC numbers (i.e., one log difference) need to be examined carefully to classify a wastewater sample as positive or negative.

Currently used SARS-CoV-2 assays for wastewater surveillance are not equally sensitive (Ahmed et al., 2020b). Therefore, three different assays were used in parallel to increase the analytical sensitivity and reduce the likelihood of negative results. When the concentration of SARS-CoV-2 is low in wastewater (due to excretion dynamics by infected individuals), SARS-CoV-2 RNA may be difficult to detect consistently with one RT-qPCR assay due to sub-sampling error (Ahmed et al., 2021a). However, unlike domestic toilets, which use several liters of water per flush, aircraft lavatories use a substantially reduced volume of water per flush, resulting in less dilution, which could be an added advantage for detecting small quantities of SARS-CoV-2 RNA.

In this study, even with the reduced dilution associated with aircraft sanitation systems, three of 37 wastewater samples were RT-qPCR negative for all three assays, but clinical testing identified COVID-19-positive cases in the mandatory 14-day quarantine. This is not completely unexpected since not every infected individual will shed SARS-CoV-2 RNA in their feces or through other body fluids (Cevik et al., 2021). Additionally, there is a non-negligible likelihood that passengers on an international flight may not avail themselves of the lavatory. The lavatory behavior of passengers on international and long-haul flights is understandable. SARS-CoV-2 wastewater samples were RT-qPCR positive, but clinical testing did not detect any COVID-19 positive cases in passengers from these flights in pre-screening (before boarding) and in the mandatory 14-day quarantine. No SARS-CoV-2 RNA was detected in post lavatory wash samples collected from these flights. The reason for such discrepancies is unclear. It has been reported that SARS-CoV-2 viral load in the upper respiratory tract appears to peak in the first week of illness (Cevik et al., 2021), and people can shed the virus in feces for five to seven weeks (Gupta et al., 2020). Therefore, it is possible that asymptomatic or recovered individuals shed the virus in the lavatory at a detectable level, but the viral load in the deep nasal and oropharyngeal samples was too low to be detected (Chen et al., 2020; Gupta et al., 2020). It is also possible that children four years old and younger may have shed the virus who are exempt from the pre-departure clinical testing.

Overall, the three RT-qPCR assays collectively achieved an accuracy of 83.7 % for detecting COVID-19 cases in quarantine using aircraft wastewater, which was 8 to 21.5 % better than any single assay performed alone. The 87.5 % PPV and 76.9 % NPV (for all the three assays combined) were 6 % greater and 12 % lower, respectively, than those reported during wastewater surveillance of college dormitories for COVID-19 using the US CDC N1 and CDC N2 assays (Betancourt et al., 2021). This demonstrates the superior performance of multiple assays in parallel rather than a single assay to maximize the predictive value of wastewater surveillance. Since, assay sensitivity issues appear to occur when SARS-CoV-2 RNA concentrations are at or below 10 GC/µL of RNA eluate (Vogels et al., 2020), we recommend the application of multiple assays and RT-qPCR replicates to improve detection sensitivity.

A Gaussian model was used to determine the analytical sensitivity of the RT-qPCR data obtained in this study. Regarding the Gaussian model results, a greater than 95 % probability of a positive RT-qPCR reaction for US CDC N2 and China CDC N assays would require 19.5 and 22.2 COVID-19 cases, respectively. These probabilities are premised on a single RT-qPCR reaction. However, in this study, all assays were run in triplicate. The probability of detection estimated for a single reaction can be used to assess the probability of detection by triplicate reactions by cubing the probability of non-detection in a single reaction. For example, for the US CDC N1 assay, there is a 50 % (0.5) probability of a non-detection in a single reaction at 2.8 COVID-19 cases; thus, the probability of non-detection associated with triplicate reactions would be 0.5^3 (0.125 or 12.5 %), and the probability of detection would be 0.875 (87.5 %). Using this mathematical basis, the probability of detecting a single COVID-19 case via aircraft wastewater for US CDC N1, N2, and China CDC, and all assays performed in triplicate could be as high as 70.4 %, 58.6 %, 67.6 %, and 73.2 %, respectively. If the number of replicates for each assay were doubled to six, the probabilities of detection could be as high as 91.2 %, 82.8 %, 89.4 %, and 92.8 %, respectively.

These probabilities are based on the current detection sensitivity of the RT-qPCR method. The analytical sensitivity of the SARS-CoV-2 assays when applied to wastewater could be further improved by increasing the sample volume from 50 mL to 200 mL wastewater, increasing the number of RT-qPCR replicates, and/or increasing the volume of RNA template added to each RT-qPCR reaction or using digital PCR. Droplet digital PCR offered substantially greater analytical sensitivity for the detection of SARS-CoV-2 in wastewater, yielding fewer false-negative results compared to RT-qPCR (Ciesielski et al., 2021). However, the effects of increasing the sample volume or template volume on RT-PCR inhibition should be carefully monitored.

Importantly, all repatriation flight passengers (age greater than 5 years) tested negative for COVID-19 via both deep nasal and oropharyngeal testing during the 48 h prior to the flight. Yet, there were 112 COVID-19 cases during the mandatory 14-day quarantine, many of which were predicted by wastewater surveillance. While not every passenger will defecate on a long-haul flight, and not every infected passenger will shed SARS-CoV-2 in their feces, most passengers will use the lavatory on these flights, and nasal secretions of SARS-CoV-2 via sputum and cough may also enter wastewater via the lavatory sink (Crank et al., 2022). SARS-CoV-2 RNA has been detected in all these bodily fluid samples (Peng et al., 2020). It has been reported that there is the possibility of a lag in viral detection in deep nasal and oropharyngeal samples; therefore, the inclusion of wastewater surveillance will enhance detection sensitivity (Lo et al., 2020). Analysis of aircraft wastewater samples will provide valuable information regarding the emergence of imported cases, especially for countries with a high
prevalence of COVID-19 and minimal quarantine requirements. SARS-CoV-2 RNA surveillance in aircraft wastewater has demonstrated the potential to detect onboard infections and prioritize clinical testing of all passengers to maximize the efficient use of resources. Although not performed in this study, there is also the possibility of screening wastewater for variants of concern via sequencing or RT-qPCR assays (Bar-Or et al., 2021; Wilton et al., 2021). Further studies are required regarding the public health implications of both positive and negative RT-qPCR results in the context of surveillance. However, the approach presented in this study is valuable alongside clinical testing to provide multiple lines of evidence of the COVID-19 infection status of passengers during international travel. New approaches, such as wastewater surveillance applied to transportation-based sanitation systems, provide an additional layer of data that can be integrated with clinical testing, travel, border restrictions, and quarantine to robustly manage SARS-CoV-2 transmission during the COVID-19 pandemic. Surveillance of wastewater from aircraft offers an effective, convenient, and cost-effective means of monitoring infectious agents that could be globally scaled to manage the importation of disease during pandemics. However, such efforts at airports will require further method optimization, seeking to achieve rapid on-site analysis, and we are assessing the feasibility of these requirements.

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 feasibility of these requirements.

Acknowledgments

We thank Qantas ground staff for collecting wastewater samples. Our thanks to Prof. Martyn Kirk from Australian National University for providing feedback on the manuscript draft.

Funding

The authors did not receive any funding for this project.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2021.106938.

References

Ahmed, W., Angel, N., Edson, J., Bibby, K., Bibvis, A., O’Brien, J.W., Choi, P.M., Kitajima, M., Simpson, S.L., Li, J., Tscharke, B., Verhagen, R., Smith, W.J.M., Zaug, J., Dieren, L., Hneguelhoz, 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, 136764. https://doi.org/10.1016/j.scitotenv.2020.136764.

Ahmed, W., Bertisch, P.M., Angel, N., Bibvis, K., Bibby, A., Dieren, J., Edson, J., Ehet, J., Gyawali, P., Hamilton, K.A., Hosegood, I., Hneguelhoz, 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., Zaug, 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 (tasa116).

Ahmed, W., Bertisch, P.M., Bibvis, K., Bibby, K., Farcard, K., Gatherfore, A., Hanamoto, E., Gyawali, P., Korajika, A., McMinn, B.R., Mueller, J.F., Simpson, S.L., Smith, W.J.M., Symonds, E.M., Thomas, K.V., Verhagen, R., Kitajima, M., 2020c. Comparison of virus concentration methods for the RT-qPCR-based recovery of murine hepatitis virus, a surrogate for SARS-CoV-2 from untreated wastewater. Sci. Total Environ. 739, 139960.

Ahmed, W., Simpson, S., Bertisch, P., Bibby, K., Bibvis, A., Blackall, L., Bobill-Mas, S., Bosch, A., Brandao, J., Choi, P., Ciesielski, M., Donner, E., D’Souza, N., Farlanezner, A., Gerrity, D., Gonzalez, R., Griffith, J., Gyawali, P., Haas, C., Hamilton, K., Hapuarachchi, C., Harwood, V., Haque, R., Jackson, G., Khan, S., Khan, W., Kitajima, M., Korajika, A., La Rosa, G., Layton, B., Lipp, E., McElman, S., McMinn, B., Medema, G., Metcalfe, S., Meijer, W., Mueller, J., Murphy, H., Naughton, C., Noble, R., Poyyappat, S., Peterson, S., Fikkanen, T., Rajai, V., Reineke, B., Roman, F., Rose, J.B., Rusinol, M., Sadowsky, M.J., Sala-Comorera, L., Setoh, Y.X., Sherrahan, S., Srivankunchana, K., Smith, W., Steele, J., Subbarg, R., Symonds, E., Tan, P., Thom, J., Tyn, K., Yoon, K., Zaug, J., Sano, D., Wuertz, S., Xagoraraki, I., Zhang, Q., Zimmer-Faust, A., Shaks, O., 2021a. Minimizing Errors in RT-PCR Detection and Quantification of SARS-CoV-2 RNA for Wastewater Surveillance. Sci. Total Environ. 797, 149877.

Ahmed, W., Bartisch, P., Bertsch, P., Thomas, L., Thom, J., Whitey, R., Wong, J., Yang, A., Wong, J., Sano, D., Wuertz, S., Xagoraraki, I., Zhang, Q., Zimmer-Faust, A., Shaks, O., 2021b. Minimizing Errors in RT-PCR Detection and Quantification of SARS-CoV-2 RNA for Wastewater Surveillance. Sci. Total Environ. 797, 149877.
Karthikeyan, S., Ronquillo, N., Belda-Ferre, P., Alvarado, D., Javidi, T., Longhurst, C.A., Knight, R., 2021. High-throughput wastewater SARS-CoV-2 detection enables forecasting of community infection dynamics in San Diego County. mSystems. 6, e00045-21.

McMinn, Brian R., Korajkic, Asja., kelleher, Julie., Herrmann, Michael P., Pemberton, Adin C., Ahmed, Warish., Villegas, Eric N., Oshima, Kevin, 2021. Development of a large volume concentration method for recovery og coronavirus from wastewater. Sci. Total Environ. 774, 145727.

Nemudryi, Artem, Nemudriaia, Anna, Wiegand, Tanner, Surya, Kevin, Buyakryoruk, Murat, Cicha, Calvin, Vanderwood, Karl K., Wilkinson, Royce, Wiedenheft, Blake, 2020. Temporal Detection and Phylogenetic Assessment of SARS-CoV-2 in Municipal Wastewater. Cell Rep. Med. 1 (6), 100098.

Lo, Iek Long, Lio, Chon Fu, Cheong, Hou Hou, Lei, Chin Ion, Cheong, Tak Hong, Zhong, Yu, Tian, Yakun, Sin, Nin Ngan, 2020. Evaluation of SARS-CoV-2 RNA shedding in clinical specimens and clinical characteristics of 10 patients with COVID-19 in Macau. Int. J. Biol. Sci. 16 (10), 1698–1707.

Park, S.-K., Lee, C.-W., Park, D.I.I., Woo, H.-Y., Cheong, H.S., Shin, H.C., Ahn, K., Kwon, M.-J., Joo, E.-J., 2021. Detection of SARS-CoV-2 in fecal samples from patients with asymptomatic and mild COVID-19 in Korea. Clin. Gastroenterol. Hepatol. 19 (7), 1387–1394.

Price, D.J., Shearer, F.M., Meehan, M.T., McBryde, E., Moss, R., Golding, N., Conway, E. J., Dawson, P., Cromer, D., Wood, J., Abbott, S., McVernon, J., McCaw, J.M., 2020. Early analysis of the Australian COVID-19 epidemic. Elife. 9, e58785.

Wilton, T., Bujaki, E., Klapsa, D., Majumdar, M., Zambon, M., Fritzsche, M., Mate, R., Martin, J., 2021. Rapid increase of SARS-CoV-2 variant B.1.1.7 detected in sewage samples from England between October 2020 and January 2021. mSystems. 6 (3), e003521.