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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

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Abstract

Background: Wastewater-based epidemiology (WBE) for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) can be an important source of information for coronavirus disease 2019 (COVID-19) management during and after the pandemic. Currently, governments and transportation industries around the world are developing strategies to minimize SARS-CoV-2 transmission associated with resuming activity. This study investigated the possible use of SARS-CoV-2 RNA wastewater surveillance from airline and cruise ship sanitation systems and its potential use as a COVID-19 public health management tool.

Methods: Aircraft and cruise ship wastewater samples ($n = 21$) were tested for SARS-CoV-2 using two virus concentration methods, adsorption–extraction by electronegative membrane ($n = 13$) and ultrafiltration by Amicon ($n = 8$), and five assays using reverse-transcription quantitative polymerase chain reaction (RT-qPCR) and RT-droplet digital PCR (RT-ddPCR). Representative qPCR amplicons from positive samples were sequenced to confirm assay specificity.

Results: SARS-CoV-2 RNA was detected in samples from both aircraft and cruise ship wastewater; however concentrations were near the assay limit of detection. The analysis of multiple replicate samples and use of multiple RT-qPCR and/or RT-ddPCR assays increased detection sensitivity and minimized false-negative results. Representative qPCR amplicons were confirmed for the correct PCR product by sequencing. However, differences in sensitivity were observed among molecular assays and concentration methods.

Conclusions: The study indicates that surveillance of wastewater from large transport vessels with their own sanitation systems has potential as a complementary data source to prioritize clinical testing and contact tracing among disembarking passengers. Importantly, sampling methods and molecular assays must be further optimized to maximize detection sensitivity. The potential for false negatives by both wastewater testing and clinical swab testing suggests that the two strategies could be employed together to maximize the probability of detecting SARS-CoV-2 infections amongst passengers.

Key words: SARS-CoV-2, COVID-19, WBE, wastewater, enveloped viruses, cruise ship, aircraft

Introduction

The ongoing pandemic of severe pneumonia and other associated health effects known as coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has resulted in >10 million diagnosed cases of COVID-19 and >500 000 deaths globally to date.[1,2] In response, governments throughout the world have implemented stringent measures, including complete lockdowns, border closures and social distancing to suppress transmission of the virus.[3] However, these measures are having tremendous negative impacts on local and global economies.[4,5] Particularly impacted industries include commercial air travel and the cruise liner industry, which have been forced to reduce or cease operation when virus transmission restrictions have been put in place. Cruise ships and aircraft present a confined environment for transmission of infections from human-to-human and numerous outbreaks including SARS-CoV-2 have been reported.[35–37]

The International Air Transport Association estimates that international air travel curtailment and restrictions will result in a USD $113 billion loss across the industry (https://www.iata.org/en/iata-repository/publications/economic-reports/third-impact-assessment/). As social distancing measures are being implemented throughout the world to reduce COVID-19 outbreaks, governments and industries are now developing plans for a COVID-19-safe society.[6] However, as normal operations resume, passengers of air travel and cruise line travel could play a significant role in importing new COVID-19 cases, with several epidemics of COVID-19 observed on cruise ships during the first wave of the pandemic.[7] The transport industry would benefit from objective matrices for monitoring the potential risk of SARS-CoV-2 transmission associated with their operations.[8]

One potential approach is afforded by the observation that SARS-CoV-2 infection is frequently accompanied by prolonged shedding of viral RNA in the stool and naso-oral fluid of both symptomatic and asymptomatic individuals.[9,10] SARS-CoV-2 RNA has been successfully detected in municipal wastewaters during clinically documented outbreaks of COVID-19 throughout the world.[11–14] Wastewater-based epidemiology (WBE) could provide useful information on COVID-19 infection status and trends in the community that informs risk management decisions.[15,16] For example, WBE could be used as an early warning tool to monitor the appearance and resurgence of COVID-19, because it allows for the detection of viral RNA derived from mild, subclinical or even asymptomatic infections. In countries monitoring wastewater for SARS-CoV-2 RNA, the viral signal was detected in wastewater days to weeks before the first clinically confirmed case.[13,17,18]

During both air travel and cruises, passengers are provided with onboard sanitation facilities. Monitoring the wastewater from these facilities for SARS-CoV-2 RNA could provide public health officials with an additional means of assessing the presence or absence of SARS-CoV-2 infections among the passengers since at least one COVID-19 patient has been observed to be positive by faecal specimen despite being negative by pharyngeal and sputum samples.[38] Wastewater-based COVID-19 surveillance could be a cost-effective method for screening of a large proportion of the passenger population to inform and prioritize clinical testing of nasopharyngeal samples. Furthermore, the duration of faecal shedding is longer, and therefore the probability of detecting SARS-CoV-2 RNA in wastewater containing faeces may be greater than clinical screening. However, little has been reported on the presence of SARS-CoV-2 RNA in wastewater from precisely bounded environments, such as aircraft, cruise ships, prisons, aged care facilities and remote vulnerable communities. Establishing the feasibility, performance specifications and limitations of testing wastewater originating from aircraft and cruise ships is critical to rationally leverage WBE within the existing public health framework.

The aim of this study was to investigate whether SARS-CoV-2 RNA can be detected and quantified in wastewater collected from inbound commercial passenger aircraft and a cruise ship docked in Australia, thereby enhancing our understanding of how SARS-CoV-2 monitoring in wastewater can be included in COVID-19 safe society plans, particularly those related to the transportation industry. Implementation of WBE on aircraft and cruise ships could facilitate the resumption of travel via these modes of transport with appropriate precautions for the ongoing COVID-19 pandemic.

Materials and Methods

Wastewater sampling

Two wastewater grab samples (1 L) were collected from the influent and effluent of the membrane bioreactor of a cruise ship on 23 April 2020. Sample collection occurred over a month

after passenger disembarcation with only crew on board on the ship on its last day berthed in Australia. Unconfirmed reports suggested as many as 24 infected persons may have been on board in the days prior to sample collection. A total of three aircraft wastewater samples (1 L each) were collected. These were collected from a valve at the bottom of the vacuum-truck that collects the wastewater tanks of the aircraft immediately after landing. The tanks of the aircraft and the vacuum trucks were emptied but not cleaned between flights. Wastewater grab sample (1 L each) were collected from passenger aircraft flight (i) Los Angeles–Brisbane (arr. 26/04/20; 117 passengers plus crew duration 13 h and 52 min), (ii) Hong Kong–Brisbane (arr. 07/05/20; 19 passengers plus crew duration 8 h and 10 min) and (3) New Delhi–Sydney (arr. 10/05/20; 185 passengers plus crew duration 11 h and 23 min). Standard personal protective equipment was used during sample collection. Samples were transported on ice to the laboratory and stored at 4°C and processed within 6–24 h after collection.

Sample concentration and RNA extraction

A total of 21 replicate samples (volume ranging from 50 to 200 ml) were aliquoted from the five wastewater samples (Table 2). Viruses were concentrated from these wastewater samples (50–200 ml) using two previously published virus concentration methods[19,20]: (i) adsorption–extraction with electronegative membrane and (ii) ultrafiltration with Amicon® Ultra-15 centrifugal filter unit (30 kDa). In total, 13 samples (seven from aircraft and six from cruise ship) were concentrated using the adsorption–extraction method and the remaining eight samples (three from aircraft and five from cruise ship) were concentrated using Amicon® Ultra-15 centrifugal filter (Merck Millipore Ltd).

RNA was directly extracted from the electronegative membrane using a combination of two kits (RNeasy PowerWater Kit and RNeasy PowerMicrobiome Kit; Qiagen, Hilden, Germany). Briefly, a 5-ml bead tube from the RNeasy PowerWater Kit was used to accommodate the electronegative membrane followed by adding 990 µl of buffer PM1 and 10 µl of β-mercaptoethanol (Sigma-Aldrich, Australia). A tissue homogenizer (Precellys Evolution 24, Bertin Technologies, France) was used to homogenize the samples, in which homogenization occurred for 3×20 s cycles at 10 000 rpm with a 10 s pause between cycles. After homegenization, tubes were further centrifuged at 10 000 *g* for 5 min to pellet the filter debris and beads. RNA was extracted from 450 µl of lysate using the RNeasy PowerMicrobiome and the QIAcube Connect platform (Qiagen) to obtain a final RNA extract volume of 100 µl.

The ultrafiltration method began with the centrifugation of the sample at 4500 *g* for 10 min at 4°C to remove debris and larger particles from the sample. The resulting supernatant was concentrated using an Amicon® Ultra-15 centrifugal filter, which was centrifuged at 4750 *g* for 10 min at 4°C. This centrifugal concentration step was repeated multiple times to pass the entire supernatant volume through the filter.[19,21,22] The concentrated sample (200–300 µl) was collected from the sample reservoir with a pipette and transferred to a 2 ml-bead beating tube followed by adding 650 µl of PM1 and 6.5 µl of β-mercaptoethanol. The tissue homogenizer was used to homogenize the samples (5 ml and 2 ml bead beating tubes) and RNA was extracted as described above. All RNA samples were stored at −80°C and subjected to reverse-transcription quantitative polymerase chain reaction (RT-qPCR) analysis within the 1–3 days of RNA extraction to avoid losses associated with storing, as well as freezing and thawing RNA preparations.

RT-qPCR analysis

Recently published RT-qPCR assays that target different regions of the SARS-CoV-2 genome, specifically N and E genes (CDC N1, CDC N2, N_Sarbeco, NIID_2019-nCOV_N and E_Sarbeco), were used for SARS-CoV-2 RNA detection in wastewater samples.[23–25]

The primers and probes sequences, along with qPCR cycling parameters, are shown in Supplementary Table S1. For RT-qPCR assays, double-stranded DNA gene fragment containing the assay target (gBlocks gene fragments) and 2019-nCoV_N plasmid control (Catalogue No. 10006625) were purchased from the Integrated DNA Technologies (Coralville, IA, USA) and used to generate the standard curves (copy/µl). CDC N1 and N2 standard dilutions ranged from 1×10^5 to 1 copy/µl. N_Sarbeco, NIID_2019-nCOV_N, and E_Sarbeco standard dilutions, also ranging from 1×10^5 to 1 copy/µl, were prepared from the gBlocks gene fragments as per the manufacturer's instructions. All RT-qPCR amplifications were performed in 20 µl reaction mixtures using iTaq™ Universal Probes One-Step Reaction Mix (Bio-Rad Laboratories, Richmond, CA, USA).

Each CDC N1 and N2 RT-qPCR mixture contained 10 µl of Supermix, 2019-nCoV Kit (500 nM of forward primer, 500 nM of reverse primer and 125 nM of probe) (Catalogue No. 10006606), 50 ng/µl of bovine serum albumin (BSA), 0.4 µl of iScript reverse transcriptase and 3 µl of template RNA. N_Sarbeco RT-qPCR mixtures contained 10 µl of Supermix, 600 nM of forward primer, 800 nM of reverse primer, 200 nM of probe, 1 µg of BSA, 0.4 µl of iScript reverse transcriptase and 3 µl of template RNA. NIID_2019-nCOV_N RT-qPCR mixtures contained 10 µl of Supermix, 500 nM of forward primer, 700 nM of reverse primer R2, 700 nM of reverse primer R2-Ver3, 200 nM of probe, 50 ng/µl of BSA, 0.4 µl of iScript reverse transcriptase and 3 µl of template RNA. E_Sarbeco RT-qPCR mixtures contained 10 µl of Supermix, 400 nM of forward primer, 400 nM of reverse primer, 200 nM of probe, 50 ng/µl of BSA, 0.4 µl of iScript reverse transcriptase and 3 µl of template RNA. The RT-qPCR assays were performed using a Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratories). All RT-qPCR reactions were performed in triplicate. For each RT-qPCR run, a series of three positive and no template controls were included.

All RT-qPCR data were generated using default settings for baseline and threshold. Data were only collected from instrument runs in which the positive control was positive and the no template control was negative. All instrument runs passed these criteria. A master standard curve with 95% upper and lower confidence intervals was generated for each assay. The $\log_{10}$-linear regression of copy number and corresponding quantification cycle (Cq) values (derived from the 6-point, assay gBlock 1:10 serial dilution series) measured in triplicate from three qPCR

instrument runs were used to generate the master standard curve and 95% confidence intervals.

For each sample replicate, the SARS-CoV-2 RNA concentration (copies/reaction), with the 95% confidence intervals, was calculated from the master standard curve and accounts for the difference in nucleic acid type between the double-stranded oligonucleotide used to generate the standard curve and the single-stranded genome of SARS-CoV-2 (i.e. divide by two).[26] For each assay in this study, the assay limit of detection (ALOD) defined as the minimum copy number/µL of template with a 95% probability of detection, was determined as previously described[27] and also takes into account the difference in nucleic acid type between the standard curve material and the SARS-CoV-2 genome. The sample limit of detection (SLOD) was calculated by multiplying the ALOD 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 total sample volume processed to yield the SLOD of SARS-CoV RNA/100 ml.

RT-ddPCR analysis

CDC N1 RT-droplet digital PCR (RT-ddPCR) mixture contained 5 µl of One-Step RT-Supermix 900 nM of forward primer, 900 nM of reverse primer and 250 nM of probe, 2 µl of reverse transcriptase, 1 µl of 300 mM DTT and 2 µl of template RNA in a final volume of 22 µl. The reaction mixture and 70 µl droplet generation oil were used to form droplets using an automated droplet generator. About 40 µl of droplet-partitioned samples were then transferred to a 96-well plate, sealed and placed on a Bio-Rad C1000 Touch Thermal Cycler (ramping speed 2.5°C/s) using the following conditions: 45°C for 60 min, followed by 40 cycles of 95°C for 10 s, 95°C for 30 s and 55°C for 1 min and 98°C for 10 min. The plate was then transferred to a QX 200 droplet reader (Bio-Rad) for automatic measurement of fluorescence in each droplet. For each RT-ddPCR run, a series of three positive and no template controls were included. All samples were run in triplicate.

qPCR inhibition and quality control

An experiment was conducted to determine the presence of qPCR inhibition in RNA extracted from wastewater samples using a Sketa22 real-time PCR assay.[28] A known copy (10^4/reaction) of *Oncorhynchus keta* (*O. keta*) was added in the DNase- and RNase-free water and the Cq value obtained acted as a reference point. If the Cq value of a wastewater sample increases compared with the reference Cq value, the sample is considered to have PCR inhibitors. Wastewater samples with a 2-Cq delay was considered to have qPCR inhibition.[11,29]

With respect to quality control, a reagent blank and extraction blank were included for each batch of RNA extraction to ensure no carryover contamination occurred during RNA extraction. No carryover contamination was observed in reagent blank samples. To minimize potential contamination, RNA extraction and RT-qPCR setup were performed in separate laboratories.

Sequencing and bioinformatics

For NextSeq Illumina sequencing, representative RT-qPCR products were cleaned with 1× ratio of AmpureXP (BeckmanCoulter, USA) and eluted in 15 µl of DNase- and RNase-free water. Amplicons were prepared for sequencing using the NEB UltraII Total RNA kit (New England Biolabs, USA) according to the manufacturer's protocol but modified to begin at the end repair step. PCR indexing of libraries PCR was undertaken using the NEBNExt Multiplex Oligos Unique Dual indices for Illumina using 10 cycles of PCR. Samples were pooled in equimolar amounts for sequencing and sequenced as a 150-bp paired end run using a 300 cycle v2 NextSeq kit (Illumina, USA).

Primer sequences were removed from de-multiplexed reads using cutadapt (ver. 2.9), with reads not containing primers discarded (−discard-untrimmed). Poor quality reads were identified and removed with trimmomatic (ver. 0.39) using a sliding window of four bases with an average quality of 15 (SLIDINGWINDOW:4:15). Reads were cropped to 140 bp (CROP:140), with any <100 bp in length discarded (MINLEN:100). Overlapping forward and reverse reads were merged using bbmerge from the BBMap suite (ver. 38.71, https://sourceforge.net/projects/bbmap/;mininsert = 100). Quality-controlled, merged reads were then mapped to the reference genome (GenBank accession number MT276598.1) using CoverM 'make' (ver 0.4.0, B. Woodcroft, unpublished, https://github.com/wwood/CoverM). Low quality read mappings were removed with CoverM 'filter' (minimum identity 95% and minimum aligned length of 90%). Read depth profiles for each sample were calculated using samtools (ver. 1.9).

Effects of aircraft wastewater tank disinfectant

The effect of aircraft toilet deodorant and viricidal/bactericidal (Novirusac Gel Bulk, Aero Defence Pty. Ltd, Southport, Queensland, Australia), which is typically dosed into the tank of an aircraft before departure, on coronavirus (i.e. murine hepatitis virus) stability was assessed. Novirusac Gel Bulk comprised of hexylene glycol, benzalkonium chloride, didecylmethylammonium propionate ethhoxylated, N-(3-aminopropyl)-N-dodecyl-1,3-propanediamine, ethanolamine and water. Briefly, 100 µl of Novirusac Gel Bulk was mixed with 100 µl of untreated wastewater. Murine hepatitis virus (10 µl) was seeded into the mixture in triplicates. Before, seeding the Cq value of the MHV RNA was determined using RT-qPCR. Two sets of samples were incubated at 15°C (typical temperature of wastewater in an aircraft) for 48 h. RNA was extracted from the incubated samples after 24 (set 1) and 48 h (set 2). The Cq values obtained for 24-hour and 48-hour incubated samples were compared with the Cq value obtained for the seeded murine hepatitis virus stock to determine the shift in Cq values over the incubation period. RNA extraction and RT-qPCR of MHV was performed according to a recent study.[20]

Ethics approval

Low-risk approval as defined by the National Statement on Ethical Conduct in Human Research was obtained from CSIRO Ethics Committee (reference number 2020_031_LR).

Table 1. RT-qPCR performance characteristics and assay limit of detection (ALOD) and sample limit of detection (SLOD)

Assay	Performance characteristic (range)					
	Efficiency (E) (%)	Linearity (R^2)	Slope	Y-intercept	ALOD for SARS-CoV-2 RNA (copies/µl RNA Template)	SLOD for SARS-CoV-2 RNA (copies/100 ml)
CDC N1	98.6–106	0.98–0.99	−3.197 to −3.357	36.60–37.63	1	100
CDC N2	94.6–103	0.99–0.99	−3.247 to −3.458	36.69–38.32	2	200
N_Sarbeco	96.9–116	0.97–0.99	−3.129 to −3.399	39.80–40.25	3	300
NIID_2019-nCOV_N	90.9–104	0.99–0.99	−3.226 to −3.562	37.34–38.71	4	400
E_Sarbeco	89.6–96.5	0.97–0.98	−3.412 to −3.417	39.99–40.44	2	200

Results

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

All RNA samples were free from PCR inhibition as determined by Sketa22 qPCR, and therefore used for downstream SARS-CoV-2 RT-qPCR analysis. The amplification efficiencies of CDC N1, CDC N2 and NIID_2019-nCOV_N assays were within the prescribed range (90–110%) of MIQE guidelines.[26] However, the amplification efficiencies of N_Sarbeco (116%) and E_Sarbeco (89.6%) were slightly outside the recommended range. The correlation coefficient (R^2) values for all assays were between 0.996 and 0.998. The slope of the standard curves, Y-intercepts, ALOD and SLOD values are shown in Table 1.

SARS-CoV-2 RNA in wastewater samples

Of the five replicate wastewater samples collected from Aircraft 1 that were processed using both virus concentration methods, four samples yielded a positive signal for SARS-CoV-2 RNA using two different assays (N_Sarbeco and E_Sarbeco) (Table 2). The positive ratio (i.e. 3 of 5) of E_Sarbeco in Aircraft 1 replicate wastewater samples was greater than N_Sarbeco (i.e. 1 of 5). The RT-qPCR amplifications were not consistent for all RT-qPCR replicates; Cq values of the positive samples ranged from 36.3 to 39.0 (E_Sarbeco assay, Table 2). Samples from both adsorption–extraction with an electronegative membrane and ultrafiltration with an Amicon® Ultra-15 centrifugal filter unit recovered SARS-CoV-2 RNA from aircraft wastewater. CDC N1, CDC N2 and NIID_2019-nCoV N assays did not produce any amplification for these samples in two consecutive RT-qPCR runs. All three replicate wastewater samples from Aircraft 2 and two replicate wastewater samples from Aircraft 3 using adsorption–extraction method were negative for all five RT-qPCR assays (i.e. consistently no detection of SARS-CoV-2). All eight replicate wastewater samples from Aircrafts 1 and 2 were negative for CDC N1 RT-ddPCR assay. Samples from Aircraft 3 were not tested with RT-ddPCR due to shortage of supplies.

For the untreated wastewater collected from the cruise ship, all six replicate samples prepared using both virus concentration methods yielded a positive signal for SARS-CoV-2 RNA using the CDC N1 assay (Table 2). The CDC N2 and NIID_2019-nCoV N assays detected SARS-CoV-2 RNA in four replicate samples. The E_Sarbeco assay appeared to be less analytically-sensitive (i.e. greater ALOD); only one of six replicates were RT-qPCR positive. The N_Sarbeco assay did not produce any amplification for these samples in two consecutive RT-qPCR runs. The CDC N1 and CDC N2 assays were consistently positive in replicate RT-qPCR reactions.

When results from all five assays were combined for each virus concentration method, the adsorption–extraction method yielded a greater number ($n = 9$) of positive samples compared with ultrafiltration with the Amicon® Ultra-15 centrifugal filter device ($n = 6$) (Table 2). For the adsorption–extraction method, the mean Cq value (Cq = 33.5) of the CDC N1 assay was much lower than the mean Cq value (Cq = 38) of CDC N2, E_Sarbeco, and NIID_2019-nCoV N. For ultrafiltration with the Amicon® Ultra-15, the mean Cq value (Cq = 36.5) of the CDC N1 assay was slightly lower than the mean Cq value (Cq = 37.15) of CDC N2, E_Sarbeco and NIID_2019-nCoV N assays. Among the replicate cruise ship untreated wastewater samples, four of six replicate samples were positive by the CDC N1 RT-ddPCR assay.

Of the five replicate cruise ship effluent wastewater (after treatment) samples prepared using both virus concentration methods, two replicate samples (adsorption–extraction method) yielded a positive signal for SARS-CoV-2 RNA using E_Sarbeco and NIID_2019-nCoV N assays (Table 2). The E_Sarbeco assay detected SARS-CoV-2 RNA in two of three replicate samples, and the NIID_2019-nCoV N assay detected in one of three replicate samples. The RT-qPCR amplifications were not consistent for all RT-qPCR replicates; Cq values ranged from 36.0 to 38.7. Samples processed with the adsorption–extraction method were positive, whereas samples processed through the Amicon® Ultra-15 centrifugal filter unit were negative. Among the five replicate cruise ship treated wastewater samples, two of five replicate samples were positive by the CDC N1 RT-ddPCR assay. Among the 21 replicates tested, seven samples were negative for all assays, and the remaining 14 samples were positive for at least one assay (Table 2).

The concentrations of SARS-CoV-2 RNA (copies/100 ml) in wastewater samples are shown in Table 3. Of the replicate RT-qPCR measurements for positively identified SARS-CoV-2 samples, 15 of 37 (41%) had concentrations above the ALOD for SARS-CoV-2. Greater concentrations were observed in the influent from the cruise ship in comparison to the single positive sample from an aircraft (Aircraft 1) and also the effluent of the cruise ship. Concentrations ranged from approximately 596 copies/100 ml (recovery uncorrected) wastewater to concentrations less than the SLOD for SARS-CoV-2 RNA (Table 1).

Table 2. Detection of SARS-CoV-2 RNA in wastewater samples collected from three aircraft and a cruise ship

Sources (sampling dates)	Virus concentration methods used	Sample ID (volume concentrated)	RT-qPCR positive results (Cq)					RT-ddPCR results
			CDC N1	CDC N2	N_Sarbeco	E_Sarbeco	NIID_2019-nCoV N	CDC N1
Aircraft 1	Adsorption–extraction	A1a-1 (100 ml)	–	–	–	+(36.3)	–	–
Los Angeles–Brisbane (26 April 2020)		A1a-2 (100 ml)	–	–	+(38.7)	—	–	–
	Amicon® Ultra-15	A1b-1 (50 ml)	–	–	–	+(37.7)	–	–
		A1b-2 (50 ml)	–	–	–	–	–	–
		A1b-3 (50 ml)	–	–	–	+(39.0)	–	–
Aircraft 2	Adsorption–extraction	A2a-1 (100 ml)	–	–	–	–	–	–
Hong Kong–Brisbane (07 May 2020)		A2a-2 (100 ml)	–	–	–	–	–	–
		A2a-3 (100 ml)	–	–	–	–	–	–
Aircraft 3	Adsorption–extraction	A3a-1 (100 ml)	–	–	–	–	–	NT
New Delhi–Sydney (10 May 2020)		A3a-2 (100 ml)	–	–	–	–	–	NT
Cruise ship-influent (23 April 2020)	Adsorption–extraction	CSI1a-1 (100 ml)	+(33.6) +(33.2) +(33.4)	+(35.8) +(37.6)	–	–	–	+
		CSI1a-2 (100 ml)	+(35.0) +(34.5) +(34.4)	+(38.6) +(39.0) +(41.3)	–	–	+(42.1)	+
		CSI1a-3 (100 ml)	+ (32.5) +(33.1) +(32.1)	+(38.2) +(37.9) +(37.9)	–	+(37.3) +(37.3)	+(36.9) +(37.0) +(35.1)	+
	Amicon® Ultra-15	CSI1b-1 (50 ml)	+(36.1)	–	–	–	+(35.7)	+
		CSI1b-2 (50 ml)	+(36.6)	–	–	–	+(36.3)	–
		CSI1b-3 (50 ml)	+(36.8)	+(38.2) +(38.4)	–	–	–	–
Cruise ship-effluent (23 April 2020)	Adsorption–extraction	CSE1a-1 (200 ml)	–	–	–	+(38.7)	–	–
		CSE1a-2 (200 ml)	–	–	–	–	–	+
		CSE1a-3 (200 ml)	–	–	–	+(37.8)	+(36.0)	–
	Amicon® Ultra-15	CSE1b-1 (100 ml)	–	–	–	–	–	+
		CSE1b-2 (100 ml)	–	–	–	–	–	–

–, not detected; +, positive; NT, not tested due to unavailability of reagents.

Replicate measurements for a given sample were typically within one order of magnitude for a given assay. Differing assays estimated variable concentrations for a given sample. For example, the concentrations estimated for cruise ship influent concentrated using adsorption–extraction method differed by as much as an order of magnitude depending upon the RT-qPCR assay used (Table 3). Minimal differences were observed between the concentrations estimated by RT-qPCR and RT-ddPCR using the CDC N1 assay; nevertheless, the frequency of SARS-CoV-2 RNA detection was slightly greater using the CDC N1 RT-ddPCR than RT-qPCR. Representative amplicons were confirmed through sequencing and mapping to their corresponding positions in the SARS-CoV-2 genome (Supplementary Figure F1).

The mean Cq value (29.2) of obtained for MHV RNA in untreated wastewater in the presence of high concentration of Novirusac Gel Bulk after 24 h was similar to the mean Cq (28.9) value of seeded MHV RNA. However, a 2 Cq value increase was observed after 48 h.

Discussion

Until an effective global SARS-CoV-2 vaccination becomes available, restrictions on domestic and international travel may continue for an extended period of time. Such restrictions have had and will continue to have a significant impact on the commercial airline and cruise line industries, and consequently on tourism and many other industries that depend heavily on people moving across national and international borders. Travel is an important COVID-19 control point. For example, among the COVID-19 infections in Australia, the majority of cases (62.8%) were acquired overseas (https://www.health.gov.au/resources/australian-covid-19-cases-by-source-of-infection). Therefore, it is of utmost importance to identify potential carriers of COVID-19 at points of entry. Screening wastewater samples from incoming aircraft or cruise ships could support clinical testing by providing site-specific, population-level information that can be used to guide passenger screening and contact tracing in a resource efficient and prioritized manner. Given that false negatives are possible through both clinical surveillance and wastewater surveillance, using the two in parallel could maximize the sensitivity of identification SARS-CoV-2 infections upon entry.

In our previous study, we demonstrated that SARS-CoV-2 RNA could be detected in municipal wastewater and has the potential to provide information on the prevalence of COVID-19 in Australian communities.[11] In this study, SARS-CoV-2 RNA was analysed and detected in wastewater samples collected from passenger aircraft and a cruise ship that was docked in Australia. To screen wastewater samples for SARS-CoV-2 RNA, we used two virus concentration methods (adsorption–extraction and Amicon® Ultra-15 (30 kDa) Centrifugal Filter Device), five RT-qPCR assays (four targeting N gene and one targeting E gene) and one RT- ddPCR assay (targeting N gene).

Although we were able to detect SARS-CoV-2 RNA in the multiple replicate wastewater sample collected from the vacuum truck that emptied wastewater from Aircraft 1, the RNA fragments were not consistently detected in all replicate wastewater samples and/or corresponding replicate RNA samples. This may be attributed to several factors, including low SARS-CoV-2 RNA concentrations and varying analytical sensitivity among the assays.[11,13] It was also postulated that the disinfectants used in the aircraft may accelerate the decay of SARS-CoV-2 RNA. However, only 1.6–2 Cq increase was observed after 48 h for murine hepatitis virus, suggesting that Novirusac Gel Bulk has little impacts on the decay of SARS-CoV-2 for the flight duration 8–13 h. It has to be also noted that for the MHV RNA decay experiment, we used a high concentration of Novirusac Gel (i.e. 1:1 ratio Novirusac Gel:wastewater), however, the ratio of Novirusac Gel to wastewater is typically 100–1000 times lower in the aircraft and will have little impact on the decay of SARS-CoV-2 RNA.

In addition, the wastewater collected from the airplane contains large particulate matter (i.e. toilet paper) when compared with regular wastewater. This heterogeneity can contribute to difficulties in obtaining representative wastewater samples. Additionally, it is possible that the virus concentration, RNA extraction, and RT efficiencies varied among sample replicates given the inherent stochastic variability of these methods.[11,13,30] As viral RNA concentrations become lower, it is expected that subsampling errors will increase because only a small portion of the sample (i.e. 3 µl from a total of 100 µl) is used in the RT-qPCR reaction. Although we detected RNA in the wastewater from the vacuum truck, quarantine isolation for 14 days and nasopharyngeal swab testing did not identify infected passengers. Thus, it is possible that the SARS-CoV-2 RNA detected could be carry over from other flights or residuals left in the vacuum truck. Alternatively, the positive detection in the sample from Aircraft 1 could be from a person on board that shed virus particles and did not develop symptoms. At least one study has identified a COVID-19 patient that was positive for SARS-CoV-2 RNA in stool despite repeated negatives in pharyngeal and sputum samples.[38] Wastewater samples from Aircrafts 2 and 3 were negative, which corroborated clinical testing and quarantine isolation of passengers from those aircraft. To avoid possible carry over from other flights due to mixing in the vacuum truck Qantas is designing a new sample extraction system allowing direct sampling from the plane before it enters the vacuum truck.

In contrast, SARS-CoV-2 RNA was detected more frequently in untreated cruise ship wastewater samples. It is possible that a greater proportion of passengers had COVID-19; thus, this contributed to the higher concentrations in wastewater. When the cruise ship wastewater samples were collected, there were 24 cases on board immediately prior to wastewater sampling. Additionally, the cruise ship passenger capacity is an order of magnitude greater than that of a commercial aircraft, which could increase the probability of passengers actively shedding SARS-CoV-2 RNA in their faeces. Finally, cruise ship passengers remain on board for several days to months and all passengers will defecate onboard during their trip; whereas aircraft passengers may not defecate in flight, particularly on short flights. Follow-up testing of the cruise ship wastewater was not possible as the ship departed following sample collection.

The frequency of SARS-CoV-2 RNA detection in treated cruise ship effluent wastewater was low in replicate RT-qPCR reactions compared with the cruise ship influent sample; this indicates that SARS-CoV-2 removal occurred in the wastewater treatment process. A recent study in Paris, France reported 2-log removal of SARS-CoV-2 RNA through the WWTP processes.[14] The SARS-CoV-2 RNA copies were low, except for cruise ship

Table 3. SARS-CoV-2 RNA concentrations (copies/100 mL wastewater), with 95% confidence interval (CI), in wastewater samples collected from aircrafts and a cruise ship

Sources (sampling dates)	Virus concentration methods used	Sample ID (volume concentrated)	Copies/100 ml of wastewater (95% CI)					RT-ddPCR Copies/100 ml of wastewater
			CDC N1	CDC N2	N_Sarbeco	E_Sarbeco	NIID_2019-nCoV N	CDC N1
Aircraft 1 (26 April 2020) Los Angeles–Brisbane	Adsorption–extraction	A1a-1 (100 ml)	–	–	–	272 (492–158)	–	–
		A1a-2 (100 ml)	–	–	36.3 (72.9–18.6)[a]	–	–	–
	Amicon® Ultra-15	A1b-1 (50 ml)	–	–	–	211 (366–127)	–	–
		A1b-2 (50 ml)	–	–	–	–	–	–
		A1b-3 (50 ml)	–	–	–	87.2 (146–54.6)[a]	–	–
Aircraft 2 (07 May 2020) Hong Kong–Brisbane	Adsorption–extraction	A2a-1 (100 ml)	–	–	–	–	–	–
		A2a-2 (100 ml)	–	–	–	–	–	–
		A2-a-3 (100 ml)	–	–	–	–	–	–
Aircraft 3 (10 May 2020) New Delhi–Sydney	Adsorption–extraction	A3a-1 (100 ml)	–	–	–	–	–	NT
		A3a-2 (100 ml)	–	–	–	–	–	NT
Cruise ship-influent (23 April 2020)	Adsorption–extraction	CSI1a-1 (100 ml)	208 (663–76.9)	60.9 (353–14.0)[a]	–	–	–	387
			275 (896–98.9)	7.6 (91.6–4.32)[a]				103
			239 (769–86.9)					
		CSI1a-2 (100 ml)	77.6 (230–30.3)[a]	8.99 (43.3–2.33)[a]	–	–	0.66 (2.33–0.33)[a]	175
				6.66 (31.9–1.66)[a]				201
			110 (333–42.3)	1.33 (5.66–0.33)[a]				398
			118 (363–42.3)					
		CSI1a-3 (100 ml)	450 (1518–157)	11.7 (58.3–2.99)[a]	–	138	34.3 (145–11.0)[a]	880
			295 (966–106)	14.3 (73.3–3.66)[a]		(243–82.6)	32.0 (118–10.3)[a]	445
			596 (2054–203)	14.3 (73.3–3.66)[a]		138 (243–82.6)	117 (596–33.3)[a]	258
	Amicon® Ultra-15	CSI1b-1 (50 ml)	71.9 (199–29.3)[a]	–	–	–	156 (740–46.6)	–
		CSI1b-2 (50 ml)	50.6 (136–21.3)[a]	–	–	–	103 (465–33.6)[a]	–
		CSI1b-3 (50 ml)	43.9 (118–18.6)[a]	23.3 (117–6.00)[a]	–	–	–	–
				20.0 (100–5.32)[a]				
Cruise ship-effluent (23 April 2020)	Adsorption–extraction	CSE1a-1 (200 ml)	–	–	–	53.6 (90.2–32.9)[a]	–	–
		CSE1a-2 (200 ml)	–	–	–	–	–	94.5
		CSE1a-3 (200 ml)	–	–	–	98.6 (171–59.6)[a]	63.6 (279–19.3)[a]	–
	Amicon® Ultra-15	CSE1b-1 (100 ml)	–	–	–	–	–	83.5
		CSE1b-2 (100 ml)	–	–	–	–	–	–

[a] Indicates that SARS-CoV-2 was positively identified and the calculated copy numbers is below the sample limit of detection (SLOD): 100 copy for CDC N1, 200 copies for CDC N2 and E_Sarbeco, 300 copies for N_Sarbeco and 400 copies for NIID_2019-nCoV N; NT, not tested due to unavailability of reagents.

influent wastewater samples as determined by the CDC N1 assay. The combined results indicate that when SARS-CoV-2 RNA concentrations are high, they are readily detectable by RT-qPCR. However, when the concentration is low or at concentrations near the SLOD, SARS-CoV-2 RNA will be difficult to detect consistently with one RT-qPCR assay. This present study indicated that the use of only a single assay (rather than a suite of 3–5), and/or not testing a sufficient number of replicate subsamples, may yield false-negative results that can negatively impact SARS-CoV-2 RNA risk mitigation and management decisions.

To maximize the probability of detection of SARS-CoV-2 RNA in wastewater samples, we have used five different RT-qPCR assays (targeting different regions of RNA from SARS-CoV-2 genome). Among the five assays tested, overall, the CDC N1 assay was the most sensitive and the N_Sarbeco assay was the least sensitive. Medema *et al.*[13] also noted discrepancies between CDC N1, CDC N2, CDC N3 and E_Sarbeco assays for several wastewater samples.[13] Assay sensitivity issues have also been documented for nasopharyngeal or oropharyngeal samples[31,32] and appear to occur when SARS-CoV-2 RNA concentrations are at or below 10 copies/µl of RNA extract.[32]

In this study, the Cq values of SARS-CoV-2 in RT-qPCR positive samples were near the ALOD (i.e. amplified between 37–40 cycles). This may have partially contributed to the inconsistent results among the assays tested. CDC N1 and N2 assays consistently produced amplifications for cruise ship influent samples. Meanwhile, the N_Sarbeco assay yielded negative results for two consecutive runs for the cruise ship influent samples; thus this assay may be less sensitive. Alternatively, it is possible that nucleotide mutations are occurring in the genomic region targeted by the N_Sarbeco assay, which may have affected the detection.[33]

Nonetheless, the analytical sensitivity of the five assays warrants further cross-validation using untreated wastewater samples seeded with low levels of SARS-CoV-2. The analytical sensitivity of the SARS-CoV-2 assays in wastewater may also be improved significantly by employing a concentration method which is able to recover >50% SARS-CoV-2 RNA from wastewater.[20] Furthermore, detection sensitivity can be improved by increasing the number of RT-qPCR replicates or sample volume from 50–100 ml to 300–500 ml wastewater, or by using digital PCR which has been reported to be one- to two-logs more sensitive than conventional qPCR platforms.[1]

Wastewater surveillance has a potential role to play in the management of COVID-19. The monitoring of SARS-CoV-2 RNA in aircraft and cruise ship wastewater could help these industries return to full operation sooner. Many countries, including Australia, have adopted a 'Controlled Adaptation Strategy'; a reality of accepting that there are ongoing international infections, potential asymptomatic transmission, and a limit to the duration of social distancing measures.[34] This strategy requires extensive testing and contact tracing to actively manage public health responses. Although not every passenger will use the toilet on a long-haul flight, the duration of a cruise means that they will on board of a cruise ship. SARS-CoV-2 RNA surveillance in aircraft and cruise ship wastewater has the potential to detect an onboard infection and prioritize clinical testing of all passengers to maximize the efficient use of resources. 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 and border restrictions, as well as quarantine, to robustly manage SARS-CoV-2 transmission during the COVID-19 pandemic.

In conclusion, we show that monitoring vessel wastewater may potentially be a convenient and cheap means of monitoring for viruses during pandemics. This is the first study that reports SARS-CoV-2 RNA detection and concentrations in wastewater from aircraft and a cruise ship in Australia using multiple RT-qPCR assays and an RT-ddPCR assay. All positive RT-qPCR products were confirmed by sequencing. When the concentration of SARS-CoV-2 is low in wastewater samples, application of multiple RT-qPCR or RT-ddPCR assays increase detection sensitivity and minimize false-negative results. The detection sensitivity can be further enhanced by selecting an appropriate virus concentration and RNA extraction method. The analytical sensitivity of detecting low infection prevalences could be greatly increased by reductions in the SLOD through improvements in SARS-CoV-2 concentration and detection methods. It may also be possible to make small modifications of wastewater collection systems within aircraft and ships to improve sample capture. We also acknowledge that the presence of SARS-CoV-2 RNA in wastewater samples does not provide information regarding the infectivity or viability of SARS-CoV-2. 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 along with the clinical testing to provide multiple lines of evidence of the COVID-19 infection status of passengers during travel.

Finally, although not assessed in this study, there is potential for alternative samples to be collected from passengers to assist with monitoring for viruses during pandemics. For example, pooled saliva samples or faecal samples might be collected from passengers before boarding, and test results from these samples could be available by the time passengers reach their destination. In this way, it will be possible to provide information on potential infections on-board. Thus, we recommend that such alternatives to wastewater testing also be explored.

Conflict of Interest

None declared.

Authors' contribution

Warish Ahmed performed the designing and analysis of the study. Paul M. Bertsch did the designing of the study. Nicola Angel and Julian Zaugg performed the data analysis. Kyle Bibby, Aaron Bivins, Pradip Gyawali, Kerry Hamilton and Masaaki Kitajima wrote the manuscript. Leanne Dierens, Janette Edson, Philip Hugenholtz did the analysis of data. John Ehret, Ian Hosegood, Guangming Jiang, Jiahua Shi and Rory Verhagen did the sampling. Homa T. Sichani, Katja M. Shimko did the sample analysis. Stuart L. Simpson performed writing and designing. Wendy J.M. Smith did the analysis. Erin M. Symonds and Kevin V. Thomas performed the writing and analysis. Jochen F. Mueller did the designing of study and performed sampling.

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References

1. Dong E, Du H, Gardner L. An interactive web-based dashboard to track COVID-19 in the real-time. *Lancet Infect Dis* 2020; **20**:533–4.
2. Zhou P, Yang XL, Wang XG *et al.* A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* 2020; **579**:270–3.
3. Lewnard JA, Lo NC. Scientific and ethical basis for social-distancing interventions against COVID-19. *Lancet Infect Dis* 2020; **30190-0**:S1473–3099.
4. Barrot JN, Grassi B, Julien S. Sectoral effects of social distancing. *SSRN* 2020. doi: 10.2139/ssrn.3569446.
5. Thunstorm L, Newbold S, Finnoff D *et al.* The benefits and costs of using social distancing to flatten the curve for COVID-19. *J Benefit Cost Anal* 2020. doi: 10.1017/bca.2020.12.
6. Tae Kwon K, Hoon Ko J, Shin H *et al.* Drive-through screening center for COVID-19: a safe and efficient screening system against massive community outbeak. *J Korean Med Sci* 2020; **35**:e123.
7. Mizumoto K, Kagaya K, Zerebski A *et al.* Estimating the asymptomatic proportion of coronavirus disease 2019 (COVID-19) cases on board the diamond princess cruise ship, Yokohama, Japan, 2020. *Euro Surveill* 2020; **25**:2000180.
8. Boldog P, Tekeli T, Vizi Z *et al.* Risk assessment of novel coronavirus COVID-19 outbreaks outside China. *J Clin Med 2020* 2020; **9**: 571.
9. Wölfel R, Corman VM, Guggemos W *et al.* Virological assessment of hospitalized cases of coronavirus disease 2019. *Nature* 2020; **581**:465–9.
10. Zheng S, Fan J, Yu F *et al.* Viral load dynamics and disease severity in patients infected with SARS-CoV-2 in Zhejiang Province, China, January-march 2020: retrospective cohort study. *BMJ 2020* 2020; **m1443**:369.
11. Ahmed W, Angel N, Edson J *et al.* 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* 2020; **728**:138764.
12. La Rosa G, Laconelli M, Mancini P *et al.* First detection of SARS-CoV-2 in untreated wastewaters in Italy. *Sci Total Environ* 2020; **736**:139652.
13. Medema G, Heijnen L, Elsinga G *et al.* Presence of SARS-Coronavirus-2 in sewage and correlation with reported COVID-19 prevalence in the early stage of the epidemic in the Netherlands. *Environ Sci Technol Lett* 2020. doi: 10.1021/acs.estlett.0c00357.
14. Wurtzer S, Marechal V, Mouchel J-M *et al.* Time course quantitative detection of SARS-CoV-2 in Parisian wastewaters correlated with COVID-19 confirmed cases. *medRxiv* 2020. doi: 10.1101/2020.04.12.20062679.
15. Kitajima M, Ahmed W, Bibby K *et al.* SARS-CoV-2 in wastewater: state of the knowledge and research needs. *Sci Total Environ* 2020; 139076.
16. Lodder W, de Roda Husman AM. SARS-CoV-2 in wastewater: potential health risk, but also data source. *The Lancet Gastro Hepatol* 2020; **5**:533–4.
17. Peccia J, Zulli A, Brackney DE *et al.* SARS-CoV-2 RNA concentrations in primary municipal sewage sludge as a leading indicator of COVID-19 outbreak dynamics. *medRxiv* 2020. doi: 10.1101/2020.05.19.20105999.
18. Randazzo W, Truchado P, Ferrando CE *et al.* SARS-CoV-2 RNA titers in wastewater anticipated COVID-19 occurrence in a low prevalence area. *Water Res* 2020; **181**:115942.
19. Ahmed W, Harwood VJ, Gyawali P *et al.* Comparison of concentration methods for quantitative detection of sewage-associated viral markers in environmental waters. *Appl Environ Microbiol* 2015; **81**:2042–9.
20. Ahmed W, Bertsch P, Bivins A *et al.* 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* 2020b; **739**:139960.
21. Symonds EM, Griffin DW, Breitbart M. Eukaryotic viruses in wastewater samples from the United States. *Appl Environ Microbiol* 2009; **75**:402–1409.
22. Ikner LA, Soto-Beltran M, Bright KR. New method using a positively charged microporous filter and ultrafiltration for concentration of viruses from tap water. *Appl Environ Microbiol* 2011; **77**:3500–6.
23. US Centers for Disease Control and Prevention. *2019-Novel coronavirus (2019-nCoV) real-time rRT-PCR panel primers and probes*. 2019. https://www.cdc.gov/coronavirus/2019-ncov/downloads/rt-pcr-panel-primer-probes.pdf.
24. Corman VM, Landt O, Kaiser M *et al.* Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Euro Surveill* 2020; **25**:2000045.
25. Shirato K, Nao N, Katano H *et al.* Development of genetic diagnostic methods for novel coronavirus 2019 (nCoV-2019) in Japan. *Jpn J Infect Dis* 2020. doi: 10.7883/yoken.JJID.2020.061.
26. Bustin SA, Benes V, Garson JA *et al.* The MIQE guidelines: minimum information for publication of quantitative real-time pCR experiments. *Clin Chem* 2009; **55**:611–22.
27. Verbyla ME, Symonds EM, Kafle RC *et al.* Managing microbial risks from indirect wastewater reuse for irrigation in urbanizing watersheds. *Environ Sci Technol* 2016; **50**:6803–13.
28. Haugland RA, Varma M, Kelty CA *et al.* Evaluation of genetic markers from the 16S rRNA V2 region for use in quantitative detection of selected *Bacteroides* species and human fecal waste by real-time PCR. *Syst Appl Microbiol* 2010; **33**:348–57.
29. Staley C, Gordon KV, Schoen ME. Performance of two quantitative PCR methods for microbial source tracking of human sewage and implications for microbial risk assessment in recreational waters. *Appl Environ Microbiol* 2013; **78**:7317–26.
30. Gerba CP, Betancourt WQ, Kitajima M. How much reduction of virus is needed for recycled water: a continuous changing need for assessment. *Water Res* 2017; **108**:25–31.
31. Nalla AK, Casto AM, Huang MW *et al.* Comparative performance of SARS-CoV-2 detection assays using seven different primer/probe sets and one assay kit. *J Clin Microbiol* 2020; pii:JCM.00557-20.
32. Vogels CBF, Brito AF, Wyllie AL. Analytical sensitivity and efficiency comparisons of SARS-CoV-2 qRT-PCR assays. *medRxiv* . doi: 10.1101/2020.03.30.20048108.
33. Yan C, Cui J, Huang L *et al.* Rapid and visual detection of 2019 novel coronavirus (SARS-CoV-2) by a reverse transcription loop-mediated isothermal amplification assay. *Clin Microbiol Infection* 2020; **26**:773–9.

34. COVID-19 Roadmap to Recovery. *A report for the nation*. Group of Eight Australia.
35. Brotherton JML, Delpech VC, Gilbert GL *et al.* A large outbreak of influenza a and B on a cruise ship causing widespread morbidity. *Epdemiol Infect* 2003; **130**:263–71.
36. Rogers KB, Roohi S, Uyeki TM *et al.* Laboratory-based respiratory virus surveillance pilot project on select cruise ships in Alaska, 2013-15. *J Travel Med* 2017; **24**:tax069.
37. Röcklov J, Sjödin H, Wilder-Smith A. COVID-19 outbreak on the diamond princess cruise ship: estimating the epidemic potential and effectiveness of public health countermeasures. *J Travel Med* 2020; **27**:taaa030.
38. Chen L, Lou J, Bau Y *et al.* COVID-19 disease with positive fecal and negative pharyngeal and sputum viral tests. *Am J Gastroenterol* 2020; **115**. doi: 10.14309/ajg.0000000000000610.