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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 travelers

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Running title: SARS-CoV-2 RNA in wastewater from different modes of travel

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Abstract

Background: Wastewater-based epidemiology (WBE) for the 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 minimise 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: Airline and cruise ship wastewater samples (n = 21) were tested for SARS-CoV-2 RNA using two virus concentration methods, adsorption-extraction by electronegative membrane (n = 13) and ultrafiltration by Amicon (n = 8), and five assays using reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) and RT-droplet digital PCR (RT-ddPCR). Representative 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 minimised false-negative results. Representative amplicons were confirmed for the correct PCR product by sequencing. However, differences in sensitivity were observed among 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 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.

Keywords: SARS-CoV-2; COVID-19; WBE; Wastewater; Human health risks; Enveloped viruses; Cruise Ship; Aircraft

71 **Introduction**

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

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

93 One potential approach is afforded by the observation that SARS-CoV-2 infection is
94 frequently accompanied by prolonged shedding of viral RNA in the stool and naso-oral fluid of
95 both symptomatic and asymptomatic individuals.[9,10] SARS-CoV-2 RNA has been successfully
96 detected in municipal wastewaters during clinically documented outbreaks of COVID-19
97 throughout the world.[11-14] Wastewater-based epidemiology (WBE) could provide useful
98 information on COVID-19 infection status and trends in the community that informs risk
99 management decisions.[15,16] For example, WBE could be used as an early warning tool to
100 monitor the appearance and resurgenece of COVID-19, because it allows for the detection of

101 viral RNA derived from mild, subclinical, or even asymptomatic infections. In countries
102 monitoring wastewater for SARS-CoV-2 RNA, the viral signal was detected in sewage days to
103 weeks before the first clinically confirmed case.[13,17,18]

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

119 The aim of this study was to investigate whether SARS-CoV-2 RNA can be detected and
120 quantified in wastewater collected from inbound commercial passenger aircrafts and a cruise
121 ship docked in Australia, thereby enhancing our understanding of how SARS-CoV-2 RNA
122 monitoring in wastewater can be included in COVID-19 safe society plans, particularly those
123 related to the transportation industry. Implementation of WBE on aircraft and cruise ships
124 could facilitate the resumption of travel via these modes of transport with appropriate
125 precautions for the ongoing COVID-19 pandemic.
126

127 Materials and methods

128 *Wastewater sampling*

129 Two wastewater grab samples (1 L) were collected from the influent and effluent of the
130 membrane bioreactor of a cruise ship on 23/04/2020. Sample collection occurred over a

131 month after passenger disembarcation with only crew on board on the ship on its last day
132 berthed in Australia. Unconfirmed reports suggested as many as 24 infected persons may
133 have been on board in the days prior to sample collection. A total of three aircraft wastewater
134 samples (1 L each) were collected. These were collected from a valve at the bottom of the
135 vacuum-truck that collects the wastewater tanks of the aircraft immediately after landing.The
136 tanks of the aircraft and the vacuum trucks were emptied but not cleaned between flights.
137 Wastewater grab sample (1 L) were collected from passenger aircraft flight 1) Los Angeles –
138 Brisbane (arr. 26/04/20; 117 passengers plus crew duration 13 h and 52 min), 2) Hongkong –
139 Brisbane (arr. 07/05/20; 19 passengers plus crew duration 8 h and 10 min) and 3) New Delhi
140 – Sydney (arr. 10/05/20; 185 passengers plus crew duration 11 h and 23 min). Standard
141 personal protective equipment was used during sample collection. Samples were transported
142 on ice to the laboratory and stored at 4ºC and processed within 6-24 h after collection.
143
144 *Sample concentration and RNA extraction*
145 A total of 21 replicate samples (volume ranging from 50-200 mL) were aliquoted from the five
146 wastewater samples (Table 2). Viruses were concentrated from these wastewater samples
147 (50-200 mL) using two previously published virus concentration methods:[19,20] (A) adsorption-
148 extraction with electronegative membrane and (B) ultrafiltration with Amicon® Ultra-15
149 centrifugal filter unit. In total, 13 samples (seven from aircrafts and six from cruise ship) were
150 concentrated using the adsorption-extraction method and the remaining eight samples (three
151 from aircrafts and five from cruise ship) were concentrated using Amicon® Ultra-15
152 centrifugal filter (Merck Millipore Ltd.).
153 RNA was directly extracted from the electronegative membrane using a combination of
154 two kits (RNeasy PowerWater Kit and RNeasy PowerMicrobiome Kit; Qiagen, Hilden,
155 Germany). Briefly, a 5-mL bead tube from the RNeasy PowerWater Kit was used to
156 accommodate the electronegative membrane followed by adding 990 µL of buffer PM1 and
157 10 µL of β-mercaptoethanol (Sigma-Aldrich, Australia). A tissue homogenizer (Precellys 24,
158 Bertin Technologies, France) was used to homogenize the samples, in which homogenization
159 occured for 3 × 20 s cycles at 10,000 rpm with a 10 s pause between cycles. After
160 homegenization, tubes were further centrifuged at 10,000 *g* for 5 min to pellet the filter debris

161 and beads. RNA was extracted from 450 µL of lysate using the RNeasy PowerMicrobiome
162 and the QIAcube Connect platform (Qiagen) to obtain a final RNA elution volume of 100 µL.
163 The ultrafiltration method began with the centrifugation of the sample at 4,500 *g* for 10
164 min at 4°C to remove debris and larger partcles from the sample. The resulting supernatant
165 was concentrated using an Amicon® Ultra-15 (30 kDa) centrifugal filter, which was
166 centrifuged at 4,750 *g* for 10 min at 4°C. This centrifugal concentration step was repeated
167 multiple times to pass the entire supernatant volume through the filter.[19,21,22] The concentrated
168 sample (200-300 µL) was collected from the sample reservoir with a pipette and transferred to
169 a 2 mL-bead beating tube followed by adding 650 µL of PM1 and 6.5 µL of β-
170 mercaptoethanol. The tissue homogenizer was used to homogenize the samples (5 mL and 2
171 mL bead beating tubes) and RNA was extracted as described above. All RNA samples were
172 stored at -80°C and subjected to RT-qPCR analysis within the 1-3 days of RNA extraction to
173 avoid losses associated with storing, as well as freezing and thawing RNA preparations.
174

175 *RT-qPCR analysis*

176 Recently published RT-qPCR assays that target different regions of the SARS-CoV-2
177 genome, specifically N and E genes (CDC N1, CDC N2, N_Sarbeco, NIID_2019-nCOV_N,
178 and E_Sarbeco), were used for SARS-CoV-2 RNA detection in wastewater samples.[23,24,25]
179 The primers and probes sequences, along with qPCR cycling parameters, are shown in
180 Supplementary Table T1. For RT-qPCR assays, double-stranded DNA gene fragment
181 containing the assay target (gBlocks gene fragments) and 2019-nCoV_N plasmid control
182 (Catalogue No. 10006625) were purchased from the Integrated DNA Technologies
183 (Coralville, IA, USA) and used to generate the standard curves (copy/mL). CDC N1 and N2
184 standard diutions ranged from 1×10^5 to 1 copy/µL. N_Sarbeco, NIID_2019-nCOV_N, and
185 E_Sarbeco standard dilutions, also ranging from 1×10^5 to 1 copy/µL, were prepared from the
186 gBlocks gene fragments as per the manufacturer's instructions. All RT-qPCR amplifications
187 were performed in 20 µL reaction mixtures using iTaq™ Universal Probes One-Step Reaction
188 Mix (Bio-Rad Laboratories, Richmond, CA).
189 Each CDC N1 and N2 RT-qPCR mixtures contained 10 µL of Supermix, 2019-nCoV Kit
190 (500 nM of forward primer, 500 nM of reverse primer and 125 nM of probe) (Catalogue No.

191 10006606), 50 ng/μL of bovine serum albumin (BSA), 0.4 μL of iScript reverse transcriptase
192 and 3 μL of template RNA. N_Sarbeco RT-qPCR mixtures contained 10 μL of Supermix, 600
193 nM of forward primer, 800 nM of reverse primer, 200 nM of probe, 1 μg of BSA, 0.4 μL of
194 iScript reverse transcriptase and 3 μL of template RNA. NIID_2019-nCOV_N RT-qPCR
195 mixtures contained 10 μL of Supermix, 500 nM of forward primer, 700 nM of reverse primer
196 R2, 700 nM of reverse primer R2-Ver3, 200 nM of probe, 50 ng/μL of BSA, 0.4 μL of iScript
197 reverse transcriptase and 3 μL of template RNA. E_Sarbeco RT-qPCR mixtures contained 10
198 μL of Supermix, 400 nM of forward primer, 400 nM of reverse primer, 200 nM of probe, 50
199 ng/μL of BSA, 0.4 μL of iScript reverse transcriptase and 3 μL of template RNA. The RT-
200 qPCR assays were performed using a Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratories).
201 All RT-qPCR reactions were performed in triplicate. For each RT-qPCR run, a series of three
202 positive and no template controls were included.

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

211 For each sample replicate, the SARS-CoV-2 RNA concentration (copies/reaction), with
212 the 95% confidence intervals, was calculated from the master standard curve and accounts
213 for the difference in nucleic acid type between the double-stranded oligonucleotide used to
214 generate the standard curve and the single-stranded genome of SARS-CoV-2 (i.e., divide by
215 2).[26] For each assay in this study, the assay limit of detection (ALOD) defined as the minimum
216 copy number with a 95% probability of detection, was determined as previously described[27]
217 and also takes into account the difference in nucleic acid type between the standard curve
218 material and the SARS-CoV-2 genome. The sample limit of detection (SLOD) was calculated
219 by dividing the ALOD by the RNA template volume added to the PCR well and then
220 multiplying this number by the total volume of RNA extracted from each sample to yield the

221 total RNA gene copies that could be detected with 95% probability. This number was then
222 normalised to total sample volume processed to yield the SLOD of SARS-CoV RNA/100 mL.
223
224 *RT-ddPCR analysis*
225 CDC N1 RT-ddPCR mixture contained 5 µL of One-Step RT-Supermix 900 nM of forward
226 primer, 900 nM of reverse primer and 250 nM of probe, 2 µL of reverse transcriptase, 1 µL of
227 300 mM DTT and 2 µL of template RNA in a final volume of 22 µL. The reaction mixture and
228 70 µL droplet generation oil were used to form droplets using an automated droplet generator.
229 40 µL of droplet-partitioned samples were then transferred to a 96-well plate, sealed and
230 placed on a Bio-Rad C1000 Touch Thermal Cycler (ramping speed 2.5°C/s) using the
231 following conditions: 45°C for 60 min, followed by 40 cycles of 95°C for 10 s, 95°C for 30 s
232 and 55°C for 1 min and 98°C for 10 min. The plate was then transferred to a QX 200 droplet
233 reader (Bio-Rad) for automatic measuremet of fluorescence in each droplet. For each RT-
234 ddPCR run, a series of three positive and no template controls were included. All samples
235 were run in triplicate.
236
237 *qPCR inhibition and quality control*
238 An experiment was conducted to determine the presence of qPCR inhibition in RNA extracted
239 from wastewater samples using a Sketa22 real-time PCR assay.[28] A known copy
240 (10^4/reaction) of *Oncorhynchus keta* (*O. keta*) was added in the DNase- and RNase-free
241 water and the Cq value obtained acted as a reference point. If the Cq value of a wastewater
242 sample increases compared to the reference Cq value, the sample is considered to have
243 PCR inhibitors. Wastewater samples with a 2-Cq delay was considered to have qPCR
244 inhibition.[11.29]
245 With respect to quality control, a reagent blank and extraction blank were included for
246 each batch of RNA extraction to ensure no carryover contamination occurred during RNA
247 extraction. No carryover contamination was observed in reagent blank samples. To minimize
248 potential contamination, RNA extraction and RT-qPCR setup were performed in separate
249 laboratories.
250

251 *Sequencing and bioinformatics*

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

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

272

273 *Effects of aircraft wastewater tank desinfectant*

274 The effect of aircraft toilet deodorant and viricidal/bactericidal (Novirusac Gel Bulk, Aero
275 Defence Pty. Ltd., Southport, Qld, Australia), which is typically dosed into the tank of an
276 aircraft before departure, on coronavirus (i.e., murine hepatitis virus) stability was assessed.
277 Novirusac Gel Bulk comprised of hexylene glycol, benzalkonium chloride,
278 didecylmethylammonium propionate ethhoxylated, N-(3-aminopropyl)-N-dodecyl-1,3-
279 propanediamine, ethanolamine and water. Briefly, 100 μL of Novirusac Gel Bulk was mixed
280 with 100 μL of untreated wastewater. MHV (10 μL) was seeded into the mixture in triplicates.

281 Before, seeding the Cq value of the MHV RNA was determined using RT-qPCR. Two sets of
282 samples were incubated at 15°C (typical temperature of wastewater in an aircraft) for 48 h.
283 RNA was extracted from the incubated samples after 24 (set 1) and 48 h (set 2). The Cq
284 values obtained for 24-h and 48-h incubated samples were compared to the Cq value
285 obtained for the seeded MHV stock to determine the shift in Cq values over the incubation
286 period. RNA extraction and RT-qPCR of MHV was performed according to a recent study.[20]

287 *Ethics approval*

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

290 Results

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

292 All RNA samples were free from PCR inhibition as determined by Sketa22 qPCR, and
293 therefore, used for downstream RT-qPCR analysis. The amplification efficiencies of CDC N1,
294 CDC N2 and NIID_2019-nCOV_N assays were within the prescribed range (90 to 110%) of
295 MIQE guidelines.[26] However, the amplification efficiencies of N_Sarbeco (116%) and
296 E_Sarbeco (89.6%) were slightly outside the recommended range. The correlation coefficient
297 (R^2) values for all assays were between 0.996 and 0.998. The slope of the standard curves,
298 Y-intercepts, ALOD and SLOD values are shown in Table 1.
299

300 *SARS-CoV-2 RNA in wastewater samples*

301 Of the five replicate wastewater samples collected from aircraft-1 that were processed using
302 both virus concentration methods, four samples yielded a positive signal for SARS-CoV-2
303 RNA using two different assays (N_Sarbeco and E_Sarbeco) (Table 2). The positive ratio
304 (i.e., 3 of 5) of E_Sarbeco in aircraft-1 replicate wastewater samples was greater than
305 N_Sarbeco (i.e., 1 of 5). The RT-qPCR amplifications were not consistent for all RT-qPCR
306 replicates; Cq values of the positive samples ranged from 36.3 to 39.0 (E_Sarbeco assay,
307 Table 2). Samples from both adsorption-extraction and ultrafiltration with an Amicon® Ultra-
308 15 centrifugal filter unit recovered SARS-CoV-2 RNA from aircraft wastewater. CDC N1, CDC
309 N2, and NIID_2019-nCoV N assays did not produce any amplification for these samples in

310 two consecutive RT-qPCR runs. All three replicate wastewater samples from aircraft-2 and
311 two replicate wastewater samples from aircraft-3 using adsorption-extraction method were
312 negative for all five RT-qPCR assays (i.e. consistently no detection of SARS-CoV-2). All eight
313 replicate wastewater samples from aircrafts 1 and 2 were negative for CDC N1 RT-ddPCR
314 assay. Samples from aircraft 3 were not tested with RT-ddPCR due to shortage of supplies.
315 For the untreated wastewater collected from the cruise ship, all six replicate samples
316 prepared using both virus concentration methods yielded a positive signal for SARS-CoV-2
317 RNA using the CDC N1 assay (Table 2). The CDC N2 and NIID_2019-nCoV N assays
318 detected SARS-CoV-2 RNA in four replicate samples. The E_Sarbeco assay appeared to be
319 less analytically-sensitive (i.e., greater ALOD); only one of six replicates were RT-qPCR
320 positive. The N_Sarbeco assay did not produce any amplification for these samples in two
321 consecutive RT-qPCR runs. The CDC N1 and CDC N2 assays were consistently positive in
322 replicate RT-qPCR reactions.
323 When results from all five assays were combined for each virus concentration method, the
324 adsorption-extraction method yielded a greater number (n = 9) of positive samples compared
325 to ultrafiltration with the Amicon® Ultra-15 centrifugal filter device (n = 6) (Table 2). For the
326 adsorption-extraction method, the mean Cq value (Cq = 33.5) of the CDC N1 assay was
327 much lower than the mean Cq value (Cq = 38) of CDC N2, E_Sarbeco, and NIID_2019-nCoV
328 N. For ultrafiltration with the Amicon® Ultra-15, the mean Cq value (Cq = 36.5) of the CDC N1
329 assay was slightly lower than the mean Cq value (Cq = 37.15) of CDC N2, E_Sarbeco, and
330 NIID_2019-nCoV N assays. Among the replicate cruise ship untreated wastewater samples,
331 four of six replicate samples were positive by the CDC N1 RT-ddPCR assay.
332 Of the five replicate cruise ship effluent wastewater (after treatment) samples prepared
333 using both virus concentration methods, two replicate samples (adsorption-extraction method)
334 yielded a positive signal for SARS-CoV-2 RNA using E_Sarbeco and NIID_2019-nCoV N
335 assays (Table 2). The E_Sarbeco assay detected SARS-CoV-2 RNA in two of three replicate
336 samples, and the NIID_2019-nCoV N assay detected in one of three replicate samples. The
337 RT-qPCR amplifications were not consistent for all RT-qPCR replicates; Cq values ranged
338 from 36.0 to 38.7. Samples processed with the adsorption-extraction method were positive,
339 while samples processed through the Amicon® Ultra-15 centrifugal filter unit were negative.

340 Among the five replicate cruise ship treated wastewater samples, two of five replicate
341 samples were positive by theCDC N1 RT-ddPCR assay. Among the 21 replicates tested,
342 seven samples were negative for all assays, and the remaining 14 samples were positive for
343 at least one assay (Table 2).

344 The concentrations of SARS-CoV-2 RNA (copies/100 mL) in wastewater samples are
345 shown in Table 3. Of the replicate RT-qPCR measurements for positively identified SARS-
346 CoV-2 samples, 15 of 37 (41%) had concentrations above the ALOD for SARS-CoV-2.
347 Greater concentrations were observed in the influent from the cruise ship in comparison to
348 the single positive sample from an aircraft (aircraft 1) and also the effluent of the cruise ship.
349 Concentrations ranged from approximately 596 copies/100 mL (recovery uncorrected)
350 wastewater to concentrations less than the SLOD for SARS-CoV-2 RNA (Table 1). Replicate
351 measurements for a given sample were typically within one order of magnitude for a given
352 assay. Differing assays estimated variable concentrations for a given sample. For example,
353 the concentrations estimated for cruise ship influent concentrated using adsorption-extraction
354 method differed by as much as an order of magnitude depending upon the RT-qPCR assay
355 used (Table 3). Minimal differences were observed between the concentrations estimated by
356 RT-qPCR and RT-ddPCR using the CDC N1 assay; nevertheless, the frequency of SARS-
357 CoV-2 RNA detection was slightly greater using the CDC N1 RT-ddPCR than RT-qPCR.
358 Representative amplicons were confirmed through sequencing and mapping to their
359 corresponding positions in the SARS-CoV-2 genome (Supplementary Fig. F1).

360 The mean Cq value (29.2) of obtained for MHV RNA in untreated wastewater in the
361 presence of high concentration of Novirusac Gel Bulk after 24 h was similar to the mean Cq
362 (28.9) value of seeded MHV RNA. However, a 2 Cq value incraese was observed after 48 h.
363

364 **Discussion**

365 Until an effective global SARS-CoV-2 vaccination is available, restrictions on domestic and
366 international travel may continue for an extended period of time. Such restrictions have had
367 and will continue to have a significant impact on the commerical airline and cruise line
368 industries, and consequently on tourism and many other industries that depend heavily of
369 people moving across national and international borders. Travel is an important COVID-19

370 control point. For example, among the COVID-19 infections in Australia, the majority of cases
371 (62.8%) were acquired overseas (https://www.health.gov.au/resources/australian-covid-19-
372 cases-by-source-of-infection). Therefore, it is of utmost importance to identify potential
373 carriers of COVID-19 at points of entry. Screening wastewater samples from incoming aircraft
374 or cruise ships could support clinical testing by providing site-specific, population-level
375 information that can be used to guide passenger screening and contact tracing in a resource
376 efficient and prioritized manner. Given that false negatives are possible through both clinical
377 surveillance and wastewater surveillance, using the two in parallel could maximize the
378 sensitivity of detecting of SARS-CoV-2 infections upon entry.

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

387 While we were able to detect SARS-CoV-2 RNA in the multiple replicate wastewater
388 sample collected from the vacuum truck that emptied wastewater from aircraft 1, the RNA
389 fragments were not consistently detected in all replicate wastewater samples and/or
390 corresponding replicates RNA samples. This may be attributed to several factors, including
391 low SARS-CoV-2 RNA concentrations and varying analytical sensitivity among the
392 assays.[11,13] It was also postulated that the disinfectants used in the aircraft may accelerate
393 the decay of SARS-CoV-2 RNA. However, only 1.6-2 Cq increase was observed after 48 h for
394 MHV suggesting that Novirusac Gel Bulk has little impacts on the decay of SARS-CoV-2 for
395 the flight duration 8-13 h. It has to be also noted that for the MHV RNA decay experiment, we
396 used a high concentration of Novirusac Gel (i.e., 1:1 ratio Novirusac Gel:wastewater),
397 however, the ratio of Novirusac Gel to wastewater is typically 100 to 1,000 times lower in the
398 aircraft and will have little impact on the decay of SARS-CoV-2 RNA.

399 In addition, the wastewater collected from the airplane contains large particulate matter
400 (i.e., toilet paper) when compared to regular wastewater. This heterogeneity can contribute to
401 difficulties in obtaining representative wastewater samples. Additionally, it is possible that the
402 virus concentration, RNA extraction, and reverse transcription efficiencies varied among
403 sample replicates given the inherent stochastic variability of these methods.[11,13,30] As viral
404 RNA concentrations become lower, it is expected that sub-sampling errors will increase
405 because only a small portion of the sample (i.e., 3 μL from a total of 100 μL) is used in the
406 RT-qPCR reaction. Although we detected RNA in the wastewater from the vacuum truck,
407 quarantine isolation for 14 days and nasopharyngeal swab testing did not identify infected
408 passengers. Thus, it is possible that the SARS-CoV-2 RNA detected could be carry over from
409 other flights or residuals left in the vacuum truck. Alternatively, the positive detection in the
410 sample from aircraft 1 could be from somebody on board that shed virus particles and did not
411 develop symptoms. At least one study has identified a COVID-19 patient that was positive for
412 SARS-CoV-2 RNA in stool despite repeated negatives in pharyngeal and sputum samples.[38]
413 Wastewater samples from aircrafts 2 and 3 were negative, which corroborated clinical testing
414 and quarantine isolation of passengers from those aircraft. To avoid possible carry over from
415 other flights due to mixing in the vacuum truck Qantas is designing a new sample extraction
416 system allowing direct sampling from the plane before it enters the vacuum truck.
417 In contrast, SARS-CoV-2 RNA was detected more frequently in untreated cruise ship
418 wastewater samples. It is possible that a greater proportion of passengers had COVID-19;
419 thus, this contributed to the higher concentrations in wastewater. When the cruise ship
420 wastewater samples were collected, there were 24 cases on board immediately prior to
421 wastewater sampling. Additionally, the cruise ship passenger capacity is an order of
422 magnitude greater than that of a commercial aircraft, which could increase the probability of
423 passengers actively shedding SARS-CoV-2 RNA in their faeces. Finally, cruise ship
424 passengers remain on board for several days to months and all passengers will defecate
425 onboard during their trip; whereas, aircraft passengers may not defecate in flight, particularly
426 on short flights. Follow-up testing of the cruise ship wastewater was not possible as the ship
427 departed following sample collection.

428 The frequency of SARS-CoV-2 RNA detection in treated cruise ship effluent wastewater
429 was low in replicate RT-qPCR reactions compared to the cruise ship influent sample; this
430 indicates that SARS-CoV-2 RNA removal occurred in the wastewater treatment process. A
431 recent study in Paris, France reported 2-log removal of SARS-CoV-2 RNA through the
432 WWTP processes.[14] The SARS-CoV-2 RNA copies were low, except for cruise ship influent
433 wastewater samples as determined by the CDC N1 assay. The combined results indicate that
434 when SARS-CoV-2 RNA concentrations are high, they are readily detectable by RT-qPCR.
435 However, when the concentration is low or at concentrations near the SLOD, SARS-CoV-2
436 RNA will be difficult to detect consistently with one RT-qPCR assay. This present study
437 indicated that the use of only a single assay (rather than a suite of 3-5), and/or not testing a
438 sufficient number of replicate subsamples, may yield false-negative results that can
439 negatively impact SARS-CoV-2 RNA risk mitigation and management decisions.

440 To maximise the probability of detection of SARS-CoV-2 in wastewater samples, we have
441 used five different RT-qPCR asays (targeting different regions of RNA from SARS-CoV-2
442 genome). Among the five assays tested, overall, the CDC N1 assay was the most sensitive
443 and the N_Sarbeco assay was the least sensitive. Medema and colleagues[13] also noted
444 discrepancies between CDC N1, CDC N2, CDC N3 and E_Sarbeco assays for several
445 wastewater samples.[13] Assay sensitivity issues have also been documented for
446 nasopharyngeal or oropharyngeal samples[31,32] and appear to occur when SARS-CoV-2 RNA
447 concentrations are at or below ten copies/µL of RNA eluate.[32]

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

456 Nonetheless, the analytical sensitivity of the five assays warrants further cross-validation
457 using untreated wastewater samples seeded with low levels of SARS-CoV-2. The analytical

458 sensitivity of the SARS-CoV-2 assays in wastewater may also be improved significantly by
459 employing a concentration method which is able to recover >50% SARS-CoV-2 RNA from
460 wastewater.[20] Furthermore, detection sensitivity can be improved by increasing the number of
461 RT-qPCR replicates or sample volume from 50-100 mL to 300-500 mL wastewater, or by
462 using digital PCR which has been reported to be one- to two-logs more sensitve than
463 conventional qPCR platforms.[1]
464 Wastewater surveillance has a potential role to play in the management of COVID-19.
465 The monitoring of SARS-CoV-2 RNA in aircraft and cruise ship wastewater could help these
466 industries return to full operation sooner. Many countries, including Australia, have adopted a
467 "Controlled Adaptation Strategy"; a reality of accepting that there are ongoing international
468 infections, potential asymptomatic transmission, and a limit to the duration of social distancing
469 measures.[34] This strategy requires extensive testing and contact tracing to actively manage
470 public health responses. While not every passenger will use the toilet on a long-haul flight, the
471 duration of a cruise means that they will on board of a cruise ship. SARS-CoV-2 RNA
472 surveillance in airline and cruise ship wastewater has the potential to detect an onboard
473 infection and prioritize clinical testing of all passengers to maximize the efficient use of
474 resources. New approaches, such as wastewater surveillance applied to transportation-based
475 sanitation systems, provide an additional layer of data that can be integrated with clinical
476 testing, travel and border restrictions, as well as quarantine, to robustly manage SARS-CoV-2
477 transmission during the COVID-19 pandemic.
478 In conclusion, we show that monitoring vessel wastewater may potentially be a
479 convenient and cheap means of monitoring for viruses during pandemics. This is the first
480 study that reports SARS-CoV-2 RNA detection and concentrations in wastewater from aircraft
481 and a cruise ship in Australia using multiple RT-qPCR assays and an RT-ddPCR assay. All
482 positive RT-qPCR were confirmed by sequencing. When the concentration of SARS-CoV-2 is
483 low in wastewater samples, application of multiple RT-qPCR or RT-ddqPCR assays increase
484 detection sensitivity and minimize false-negative results. The detection sensitivity can be
485 further enhanced by selecting an appropriate virus concentration and RNA extraction method.
486 The analytical sensitivity of detecting low infection prevalences could be greatly increased by
487 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 collections 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, while 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.

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518 **Authors statements**

519 **Conflict of interest**

520 The authors have declared no conflicts of interest

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523 **Author contribution**

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527 Kyle, Bibby - Writing manuscript
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669 **Table 1**

670 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 reaction)	SLOD for SARS-CoV-2 RNA (copies/100 mL)
CDC N1	98.6 to 106	0.98 to 0.99	-3.197 to -3.357	36.60 to 37.63	1	100
CDC N2	94.6 to 103	0.99 to 0.99	-3.247 to -3.458	36.69 to 38.32	2	200
N_Sarbeco	96.9 to 108	0.97 to 0.99	-3.129 to -3.399	39.80 to 40.25	3	300
NIID_2019-nCOV_N	90.9 to 104	0.99 to 0.99	-3.226 to -3.562	37.34 to 38.71	4	400
E_Sarbeco	96.2 to 96.5	0.97 to 0.98	-3.412 to -3.417	39.99 to 40.44	2	200

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Table 2

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

Sources (sampling dates)	Virus concentration methods used	Sample ID (volume analysed)	RT-qPCR positive results (Cq)/reaction					RT-ddqPCR positive results/reaction
			CDC N1	CDC N2	N_Sarbeco	E_Sarbeco	NIID_2019-nCoV N	CDC N1
Aircraft 1 Los Angeles-Brisbane (26/04/20)	Adsorption-extraction	A1a-1 (100 mL)	- - -	- - -	- - -	- + (36.3) -	- - -	- - -
		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 Hong Kong-Brisbane (07/05/20)	Adsorption-extraction	A2a-1 (100 mL)	- - -	- - -	- - -	- - -	- - -	- - -
		A2a-2 (100 mL)	- - -	- - -	- - -	- - -	- - -	- - -
		A2a-3 (100 mL)	- - -	- - -	- - -	- - -	- - -	- - -
Aircraft 3 New Delhi-Sydney (10/05/20)	Adsorption-extraction	A3a-1 (100 mL)	- - -	- - -	- - -	- - -	- - -	NT NT NT
		A3a-2 (100 mL)	- - -	- - -	- - -	- - -	- - -	NT NT NT
Cruise ship - influent (23/04/2020)	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 (23/04/2020)	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.

Table 3
SARS-CoV-2 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 analysed)	Copies/100 mL of wastewater (95% CI)					RT-ddqPCR Copies/100 mL of wastewater
			CDC N1	CDC N2	N_Sarbeco	E_Sarbeco	NIID_2019-nCoV N	CDC N1
Aircraft 1 Los Angeles-Brisbane (26/04/20)	Electronegative membrane	A1a-1 (100 mL)	- - -	- - -	- - -	- 272 (492-158) -	- - -	- - -
		A1a-2 (100 mL)	- - -	- - -	- - 36.3 (72.9-18.6)*	- - -	- - -	- - -
	Ultrafilter device	A1b-1 (50 mL)	- - -	- - -	- - -	- - 211 (366-127)	- - -	- - -
		A1b-2 (50 mL)	- - -	- - -	- - -	- - -	- - -	- - -
		A1b-3 (50 mL)	- - -	- - -	- - -	- - 87.2 (146-54.6)*	- - -	- - -
Aircraft 2 Hong Kong-Brisbane (07/05/20)	Electronegative membrane	A2a-1 (100 mL)	- - -	- - -	- - -	- - -	- - -	- - -
		A2a-2 (100 mL)	- - -	- - -	- - -	- - -	- - -	- - -
		A2-a-3 (100 mL)	- - -	- - -	- - -	- - -	- - -	- - -
Aircraft 3 New Delhi-Sydney (10/05/20)	Electronegative membrane	A3a-1 (100 mL)	- - -	- - -	- - -	- - -	- - -	NT NT NT
		A3a-2 (100 mL)	- - -	- - -	- - -	- - -	- - -	NT NT NT
Cruise ship	Electronegative	CSI1a-1	208 (663-76.9)	60.9 (353-14.0)*	-	-	-	387

- influent (23/04/2020)	membrane	(100 mL)	275 (896-98.9) 239 (769-86.9)	- 17.6 (91.6-4.32)*	- -	- -	- -	103 -
		CSI1a-2 (100 mL)	77.6 (230-30.3)* 110 (333-42.3) 118 (363-42.3)	8.99 (43.3-2.33)* 6.66 (31.9-1.66)* 1.33 (5.66-0.33)*	- - -	- - -	0.66 (2.33-0.33)* - -	175 201 398
		CSI1a-3 (100 mL)	450 (1518-157) 295 (966-106) 596 (2054-203)	11.7 (58.3-2.99)* 14.3 (73.3-3.66)* 14.3 (73.3-3.66)*	- - -	138 (243-82.6) - 138 (243-82.6)	34.3 (145-11.0)* 32.0 (118-10.3)* 117 (596-33.3)*	880 445 258
	Ultrafilter device	CSI1b-1 (50 mL)	71.9 (199-29.3)* - -	- - -	- - -	- - -	156 (740-46.6) - -	- - -
		CSI1b-2 (50 mL)	- 50.6 (136-21.3)* -	- - -	- - -	- - -	- - 103 (465-33.6)*	- - -
		CSI1b-3 (50 mL)	- 43.9 (118-18.6)* -	- 23.3 (117-6.00)* 20.0 (100-5.32)*	- - -	- - -	- - -	- - -
Cruise ship - effluent (23/04/2020)	Electronegative membrane	CSE1a-1 (200 mL)	- - -	- - -	- - -	- 53.6 (90.2-32.9)* -	- - -	- - -
		CSE1a-2 (200 mL)	- - -	- - -	- - -	- - -	- - -	94.5 - -
		CSE1a-3 (200 mL)	- - -	- - -	- - -	- 98.6 (171-59.6)* -	- - 63.6 (279-19.3)*	- - -
	Ultrafilter device	CSE1b-1 (100 mL)	- - -	- - -	- - -	- - -	- - -	- - 83.5
		CSE1b-2 (100 mL)	- - -	- - -	- - -	- - -	- - -	- - -

* 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_Shirato; NT: not tested due to unavailability of reagents.

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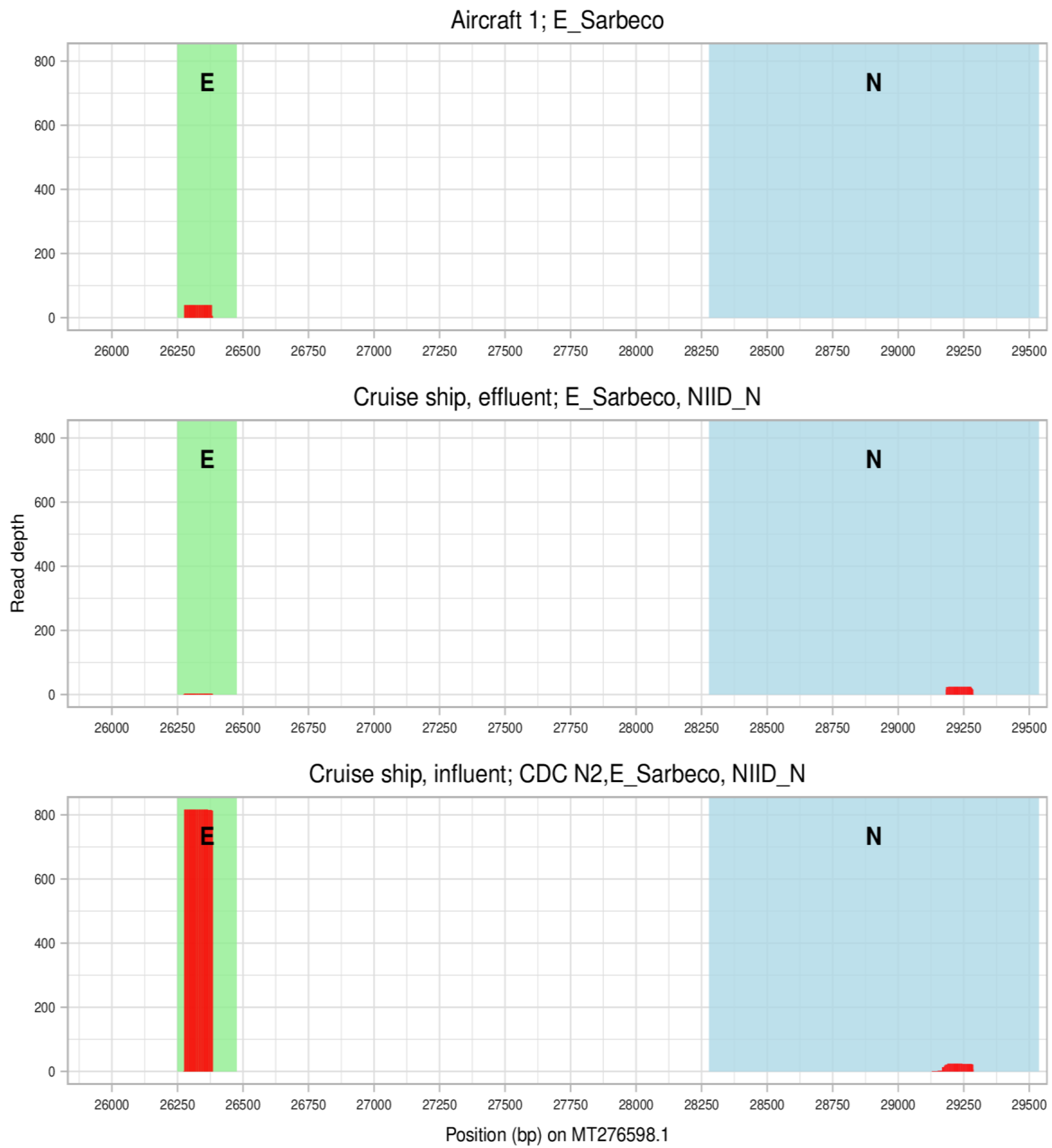
Supplementary Materials

Supplementary Table S1
Primers and probes used in this study

Organisms	Target gene	Assay name	Sequence (5'–3')	Cycling parameters	Reference
Oncorhynchus keta	-	Sketa22	F-GGTTTCCGCAGCTGGG R-CCGAGCCGTCCTGGTCTA P-FAM-AGTCGCAGGCGGCCACCGT-TAMRA	95°C for 10 min; 40 cycles of 95°C for 15 s, 63°C for 45 s.	Haugland et al., 2005
SARS-CoV-2	N protein	CDC N1	F-GACCCCAAAATCAGCGAAAT R-TCTGGTTACTGCCAGTTGAATCTG P-FAM- ACCCCGCATTACGTTTGGTGGACC-BHQ1	50°C for 10 min for RT; 95°C for 5 min and 45 cycles of 95°C for 10 s, 55°C for 30 s.	US CDC, 2020
		CDC N2	F-TTACAAACATTGGCCGCAAA R-GCGCGACATTCCGAAGAA P-FAM- ACAATTTGCCCCCAGCGCTTCAG-BHQ1	50°C for 10 min for RT; 95°C for 5 min and 45 cycles of 95°C for 10 s, 55°C for 30 s.	US CDC, 2020
		N_Sarbeco	F-CACATTGGCACCCGCAATC R-GAGGAACGAGAAGAGGCTTG P-FAM-ACTTCCTCAAGGAACAACATTGCCA-BHQ1	50°C for 10 min for RT; 95°C for 3 min and 45 cycles of 95°C for 15 s, 58°C for 30 s.	Corman et al., 2020
		NIID_2019-nCOV_N	F-AAATTTTGGGGACCAGGAAC R2-TGGCAGCTGTGTAGGTCAAC R2Ver3-TGGCACCTGTGTAGGTCAAC P-FAM-ATGTCGCGCATTGGCATGGA-BHQ1	50°C for 10 min for RT; 95°C for 15 min; and 45 cycles of 95°C for 15 s and 60°C for 1 min.	Shirato et al., 2020
	E protein	E_Sarbeco	F-ACAGGTACGTTAATAGTTAATAGCGT R-ATATTGCAGCAGTACGCACACA P- FAM-ACACTAGCCATCCTTACTGCGCTTCG-BHQ1	50°C for 10 min for RT; 95°C for 3 min and 45 cycles of 95°C for 15 s, 58°C for 30 s.	Corman et al., 2020

FAM: 6-carboxyfluorescein ; BHQ1: Black Hole Quencher-1



Supplementary Figure F1

Read depths of merged reads from Aircraft 1, cruise ship effluent and influent samples mapping (minimum identity 95% and minimum aligned length of 90%) to the SARS-CoV-2 reference genome MT276598.1. E and N gene regions are annotated and highlighted in green and blue, respectively.