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Keywords: Aircraft; Wastewater; Surveillance; Indicator viruses; Enteric viruses; Respiratory viruses

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Application of Adsorption-Extraction and Nanotrap® Microbiome A Particles Workflows for Detection and Quantification of Indicator, Enteric, and Respiratory Viruses in Aircraft Lavatory Wastewater

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Abstract: Background: The effective detection of viruses in aircraft wastewater is crucial to establish surveillance programs for monitoring virus spread via aircraft passengers. The presence of SARS-CoV-2 in aircraft lavatory wastewater has been studies, but the existence of other diseases causing viruses is not well-studied. **Methods:** This study aimed to compare the performance of two virus concentration workflows, adsorption-extraction (AE) and Nanotrap® Microbiome A Particles (NMAP), in detecting the prevalence and concentrations of 15 endogenous viruses in aircraft lavatory wastewater samples. qPCR and RT-qPCR assays were used to detect two indicator viruses, four enteric viruses, and nine respiratory viruses. **Results:** The results showed that cross-assembly phage (crAssphage), human polyomavirus (HPyV), rhinovirus A (RhV A), and rhinovirus B (RhV B) were detected in all wastewater samples using both workflows. However, enterovirus (EV), human norovirus (HNoV GII), human adenovirus (HAdV), bocavirus (BoV), parechovirus (PeV), epstein-barr virus (EBV), influenza A virus (IAV), and respiratory syncytial virus B (RsV B) were infrequently detected by both workflows. The results showed that CrAssphage and HPyV had the greater mean concentrations than enteric and respiratory viruses using both workflows. The mean concentrations of CrAssphage, HPyV, RhV A, and RhV B between the two workflows were statistically significant ($p < 0.05$). **Conclusions:** The present study provides valuable insights into the performance of virus concentration workflows in detecting and quantifying different viruses in aircraft lavatory wastewater samples. The findings can aid in the selection of an appropriate concentration workflow for virus surveillance studies and contribute to the development of efficient and reliable virus detection methods.

Keywords: aircraft; wastewater; surveillance; indicator viruses; enteric viruses; respiratory viruses

Introduction

Urban wastewater surveillance has been employed as a critical tool in eradicating polio globally, [1,2] and to monitor a wide array of enteric and respiratory viruses at a population level [3–7]. The concept of aircraft wastewater surveillance is similar to urban wastewater surveillance, which has been demonstrated as a valuable tool in managing the COVID-19 pandemic [8,9]. For many infectious diseases a significant number of passengers who are infected with mild or no symptoms may continue to travel by air, posing a potential risk for the spread of viruses across borders. Despite their asymptomatic or pre-symptomatic status, these individuals can contribute pathogens to the aircraft's wastewater system through bodily excretions such as feces, urine, nasal mucus, sputum, and gargling water with mouthwash while using the lavatory [10–12]. Therefore, screening the wastewater produced

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by aircraft passengers and crews during their flight can play a role in public health surveillance at international borders, and help track the circulation of pathogens across borders [8,10–12].

A Danish research team demonstrated remarkable foresight in early 2019 when they employed a metagenomic technique to detect respiratory and enteric viruses in aircraft wastewater [13]. The authors were able to detect and measure enteric, respiratory, and latent viruses in aircraft wastewater from 19 international flights that arrived at Copenhagen Airport. Recent studies have demonstrated the application of aircraft wastewater monitoring for SARS-CoV-2 genetic fragments in Australia [8], United Arab Emirates [14], USA [12] and France [13]. Ahmed et al. [10] found that aircraft wastewater surveillance provided 84% accuracy in predicting SARS-CoV-2 infections among passengers during subsequent quarantine, all of whom had tested negative for COVID-19 by nasal swab prior to boarding. Ahmed and colleagues [16] detected the presence of the Omicron variant in an aircraft wastewater sample from a flight that travelled from Johannesburg, South Africa to Darwin, Australia in 2021. The putative detection was made using RT-qPCR and confirmed by genomic sequencing. The Australian Northern Territory Health Department confirmed the presence of Omicron in one passenger who was abroad the same flight, as determined by sequencing of a nasopharyngeal swab samples. The study demonstrated the significance of using aircraft wastewater as an independent and unobtrusive surveillance measure for infectious disease, such as COVID-19.

A critical step in monitoring low concentrations of viral pathogens in wastewater, including wastewater derived from aircraft, is the efficient concentration of viruses from diluted wastewater samples. Therefore, there is a need for identifying rapid and efficient virus concentration methods that are cost-effective for achieving sensitive detection and/or quantification of a variety viruses in aircraft wastewater samples [13,17]. Aircraft wastewater analyses for SARS-CoV-2 genetic fragments have used a range of concentration methods, including Concentrating Pipette Select™ (CP Select™), adsorption-extraction (AE) [8], ultrafiltration [8], Nanotrap® Microbiome A Particles (NMAP) [12], filtration and centrifugation [14] and direct extraction [13] for metagenomics analysis. Each of these methods has advantages and disadvantages, and a concentration method that is universally efficient for a wide range of different virus targets is yet to be identified for aircraft wastewater surveillance.

Ahmed et al. [18] conducted a study that compared the efficiency of two different concentration methods, AE and NMAP, and commercially available extraction kits to measure endogenous pepper mild mottle virus (PMMoV) and SARS-CoV-2 in nucleic acid extracted from 48 municipal wastewater samples. The results showed that in 58% of individual wastewater samples, the concentrations of PMMoV were greater from the NMAP workflow compared to the AE workflow. However, in 69% of individual samples, the concentrations of SARS-CoV-2 were greater in the AE workflow compared to the NMAP workflow. Based on the findings, the authors suggested that the turbidity or suspended solids concentration of wastewater samples, as well as the target virus for analysis, should be considered when determining optimal workflows for virus surveillance in wastewater.

Aircraft lavatory wastewater is less diluted compared to municipal wastewater as a substantially reduced volume of water is used per toilet flush and the absence of many sources of dilution in municipal wastewater collection systems such as infiltration and inflow and industrial discharges. Another characteristic of aircraft wastewater is the presence of substantial amounts of toilet paper, which makes the isolation of viruses difficult. To enable the establishment of effective surveillance programs for virus detection in aircraft wastewater, consistent, well-optimized, rapid, efficient (high recovery), and cost-effective virus concentration workflows are needed worldwide. The present study aimed to compare the performance of two virus concentration workflows, AE and NMAP, in detecting the prevalence and concentrations of 15 endogenous viruses in aircraft lavatory wastewater samples. These viruses include—two indicator viruses (CrAssphage, human polyomavirus (HPyV), four enteric viruses (enterovirus (EV), hepatitis A virus (HAV), human adenovirus (HAdV), human norovirus GII (HNoV GII)), and nine respiratory viruses (bocavirus (BoV), epstein-barr virus (EBV), influenza A virus (IAV), influenza B virus (IBV), parechovirus (PeV), rhinovirus subtype A (RhV A), rhinovirus subtype B (RhV B), RSV subtype A virus (RSV A), and RSV subtype B virus (RSV B)). The findings from this study can aid in selecting an optimal concentration workflow for aircraft wastewater surveillance studies and contribute to efficient virus surveillance.

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Methods

Sources of aircraft wastewater samples and virus concentration

The study used 24 archived aircraft lavatory wastewater samples that were stored at -20 °C. These samples were thawed at 4 °C before virus concentration was carried out using the AE and NMAP concentration methods, as described in. [18] The use of archived wastewater samples allowed for the retrospective analysis of these samples and comparison of the performance of the two concentration methods.

The AE workflow used in this study started by centrifuging 25 mL of the wastewater sample at 4,000× *g* for 2 mins. The pellet that contained mostly toilet paper and suspended solids was discarded, and the supernatant was transferred to a new 50 mL centrifuge tube. To achieve a final concentration of 25 mM $MgCl_2$, each wastewater supernatant sample was supplemented with $MgCl_2$ (Sigma-Aldrich, St. Louis, Missouri, USA). Immediately after the $MgCl_2$ amendment, the wastewater samples were filtered through a 0.80-µm pore-size, electronegative HA membrane (47-mm diameter AAWG04700; Merck Millipore Ltd., Sydney, Australia) using a magnetic filter funnel (Pall Corporation, Port Washington, New York, USA) and filter flask (Merck Millipore Ltd.) [18]. Following filtration, the membrane was removed from the filter funnel using aseptic technique, rolled, and inserted into a 5-mL-bead-beating tube (Qiagen, Valencia, CA, USA) for nucleic acid extraction.

The NMAP workflow involves several steps to concentrate viruses from the wastewater sample. In this specific application, 12.5 mL of the wastewater sample was transferred into a 15 mL sterile conical falcon tube (Eppendorf, Hamburg, Germany). The tube was then centrifuged at 4,000× *g* for 2 mins. After centrifugation, the pellet containing toilet paper and suspended solids was discarded. The supernatant, which was presumed to contain the virus particles, was transferred into a new 15 mL tube. Each wastewater supernatant sample was amended with 100 µL of Nanotrap® Enhancement Reagent 2 (ER2) (Ceres Nanosciences SKU 10112) and then inverted twice to achieve homogeneity of the sample, and then 150 µL of Nanotrap Microbiome A particles were added (Ceres Nanosciences SKU 44202). The samples were then mixed by inverting the tubes twice and incubated at room temperature (24 ± 1ºC) for 10 min with an additional inversion at 5 mins.

Next the tubes were placed on a magnetic rack (Dynal MPC™-6) to pelletize the NMAP at the bottom of the tube. Next, the supernatant was carefully removed via pipette and discarded without disturbing the NMAP pellet. The pellet was then resuspended by adding 1 mL of molecular grade water into each tube. The resulting virus particle suspension was transferred into a sterile 1.5 mL microcentrifuge tube using a pipette and placed on a magnetic rack (Invitrogen™ DynaMag™-2 Magnet) to create a final NMAP pellet. Finally, the supernatant was carefully discarded without disturbing the NMAP pellet for nucleic acid extraction.

Nucleic acid extraction

After membrane filtration in the AE workflow, the membrane was immediately removed, rolled, and inserted into a 5-mL bead-beating tube of the RNeasy PowerWater Kit (Cat. No. 14700-50-NF) (Qiagen, Hilden, Germany) to directly extract nucleic acid from the membrane. Next, 990 µL of buffer PM1 and 10 µL of β-Mercaptoethanol (Cat. No. M6250-10 mL) (Sigma-Aldrich, St. Louis, Missouri, USA) were added into each bead-beating tube. Bead beating tubes were homogenized using a Precellys 24 tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). The homogenization step involved shaking the tubes at 10,000 rpm for three cycles of 15 s each, with a 10 s interval between each cycle. After homogenization, the tubes were centrifuged at 4,000× *g* for 5 min to pellet the filter debris and beads. Nucleic acid extraction was carried out with the RNeasy PowerWater Kit (Qiagen) according to the manufacturer's protocol, with two modifications. First, DNase I solution was omitted from the protocol to isolate nucleic acid that includes both DNA and RNA since viruses analysed in this study included both DNA and RNA viruses. Second, the nucleic acid was eluted 200 µL of DNase and RNase free water.

In the NMAP workflow, 500 µL of buffer PM1 and 5 µL of β-Mercaptoethanol were added to the Nanotrap A particles and resuspended using a pipette. The tubes were then incubated on a

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heating block at 95 °C for 10 min. After incubation, the microcentrifuge tubes were placed on a DynaMag-2 magnetic rack to separate the Nanotrap particles from the sample for 2 min. The supernatant/lysate was transferred to a new 2 mL collection tube, while the pellet consisting of Nanotrap A particles pellet was discarded. Then, 150 µL of Qiagen Solution IRS was added to the lysate and briefly vortexed to ensure adequate mixing. To isolate nucleic acid (both DNA and RNA), the RNeasy PowerWater Kit (Qiagen) was used for extraction according to the manufacturer's protocol, with two modifications: (i) the DNase I solution was excluded, and the nucleic acid was eluted with 200 µL of DNase and RNase free water. The purity of the nucleic was confirmed by measuring the $A_{260/280}$ ratio utilizing a DeNovix Spectrophotometer & Fluorometer (DeNovix, Wilmington, DE, USA) and was found to be within the acceptable limits [19].

PCR inhibition assessment

An experiment was conducted to determine the presence of PCR inhibition in the nucleic acids extracted from aircraft wastewater samples using the Sketa22 PCR assay [20] and MHV RT-PCR assays [21]. To accomplish this, a controlled experiment was conducted, whereby, a known copy number (3×10^4/reaction) of *Oncorhynchus keta* (*O. keta*) and MHV was added to each PCR reaction, including controls consisting of DNase- and RNase-free water rather than extracted wastewater. The Cq value for the controls was used as a reference point. If the Cq value of a wastewater nucleic acid sample was greater than 2 cycles higher than the reference Cq value, the sample was considered to contain PCR inhibitors [22].

RT-PCR, qPCR and RT-qPCR analysis

The detection of *O. keta* and MHV was carried out using PCR and RT-PCR assays that had been previously published by Haugland et al. [20] and Besselsen et al. [21], respectively. Similarly, previously published qPCR and RT-qPCR assays were utilized for quantifying CrAssphage, HPyV, EV, HAdV, HAV, and HNoV GII [23–28]. Positive controls or standards were obtained in the form of gBlocks gene fragments from Integrated DNA Technologies (Integrated DNA Technology Coralville, IA, USA). Custom TaqMan® qPCR and RT-qPCR assays from Thermofisher Scientific (Thermofisher Scientific, Waltham, MA, USA) were used to quantify respiratory viruses, including BoV (assay ID Vi9999003_po), EBV (assay ID Vi06439675_s1), IAV (assay ID Vi99990011_po), IBV (assay ID Vi99990012_po), PeV (assay ID Vi99990006_po), RhV A (assay ID Vi99990016_po), RhV B (Vi99990017_po), RSV A (assay ID Vi99990014_po) and RSV B (assay ID Vi99990015_po). A linearized multi-target plasmid pool (Cat. No. A50382) containing all target sequences was purchased from Applied Biosystems (Applied Biosystems, Waltham, MA, USA) and used as qPCR/RT-qPCR standards for the respiratory viruses.

PCR, RT-PCR, qPCR, and RT-qPCR analyses were performed in 20 µL reaction mixtures using QuantiNova Probe PCR Kit (Qiagen) and TaqMan™ Fast Virus 1-Step Master Mix (Applied Biosystems), respectively. The components of PCR, RT-PCR, qPCR, and RT-qPCR reactions were described in our previous studies [29,30]. For each run, a series of standard (3×10^5 to 3 GC/reaction or positive controls) and no template controls ($n = 3$) were included. The PCR assays were performed on a Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratories, Hercules, California, USA) using manual settings for threshold and baseline. Sketa 22 and MHV detection and CrAssphage, HPyV, EV, HAV, HAdV and HNoV GII qPCR and RT-qPCR reactions were performed in triplicate reactions. All respiratory virus qPCR and RT-qPCR reactions were performed in duplicate reactions.

qPCR and RT-qPCR assay limit of detection

Assay limits of detection (ALODs) for each qPCR and RT-qPCR were determined in our recent studies [29,30]. We defined the 95% ALOD by using an exponential survival model to fit the proportion of positive PCR replicates at each step along the gradient as outlined in Verbyla et al. [31].

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Quality control

To reduce the potential of PCR contamination, two separate laboratories were used for nucleic acid extraction and PCR set up. A sample negative control was included in the concentration process, and an extraction negative control was included during nucleic acid extraction to identify any contamination during the extraction process. All sample and extraction negative controls tested negative for the analyzed targets.

Data analysis

Samples were classified as positive (virus detected) for qPCR and RT-qPCR if amplification was detected in at least two out of three replicates for indicator and enteric viruses, and in at least one out of the two replicates for respiratory viruses, within 45 cycles. Samples were deemed quantifiable if amplification was observed in all replicates with concentrations exceeding the ALOD values. To facilitate a quantitative comparison between the two workflows, concentrations derived from wastewater volume of 25 mL using the AE workflow were converted to an equivalent value of 12.5 mL using the NMAP workflow. A student's t-test was conducted to compare the means of CrAssphage, HPyV, RhV A and RhV B. T-test was not conducted for other viruses due to low detection frequencies.

Results

qPCR and RT-qPCR assay performance and relevant QA/QC

The slopes of the standard curves varied between -3.56 (for RSV B) and -3.10 (for both RhV A and RhV B) as shown in Supplementary Table ST2 [29]. The amplification efficiencies (ranging from 91.0 to 110%) and y-intercepts (ranging from 35.40 to 40.68) of the standard curves were within the prescribed range of the MIQE guidelines [32]. The correlation coefficients (r^2) across the standard curves for all assays were observed to be between 0.97 to 1.00. The ALODs for the qPCR and RT-qPCR assays ranged from 5.60 and 9.30 GC/reaction as presented in Supplementary Table ST2. All positive controls or standard curve samples were amplified in each PCR run. No PCR inhibition was detected in any nucleic acid samples based on the seeded GC of *O. keta* and MHV, which were all within 2 Cq values of the reference Cq value.

Prevalence of indicator, enteric and respiratory viruses in aircraft wastewater samples

Among the 15 indicator, enteric and respiratory viruses, crAssphage, HPyV, RhV A and RhV B were detected in all wastewater samples (Table 1) using both AE and NMAP workflows. EV, HNoV GII, HAdV, BoV, PeV, EBV, IAV and RsV B were infrequently detected by both workflows (ranging from 4.20 to 41.7%). HAV, IBV and RsV A were not detected in aircraft wastewater samples by both workflows. The detection rates of EV (41.7%), PeV (12.5%) and RsV B (25.0%) determined using the NMAP workflow were greater than those determined using the AE workflow (EV = 37.5%; PeV = 8.30%; RsV B = 16.7%). In contrast, the detection rates of HAdV (54.2%), BoV (29.2%), EBV and (20.8%) determined using the AE workflow were greater than those determined using the NMAP workflow (HAdV = 16.7%; BoV = 8.30%; EBV = 16.7%). When combining detection rates for both AE and NMAP workflows, the detection rates of crAssphage, HPyV, HNoV GII, HAdV, RhV A, and RhV B remained the same. However, the detection rates of EV, BoV, PeV, EBV, IAV, and RsV B increased when detection rate results from two workflows were combined.

Concentrations of indicator, enteric and respiratory viruses in aircraft wastewater samples

Not all viruses detected through PCR could be quantified, with varying percentages of samples for different viruses being quantifiable with both AE and NMAP workflows. Specifically, 100% of samples were quantifiable for CrAssphage and HPyV, 8.33% for EV, 4.16% for HNoV GII, 20.8% for HAdV, 8.33% for BoV, 4.16% for PeV, 100% for RhV A and RhV B, and 8.33% for RsV B, out of 24 aircraft wastewater samples using the AE workflow. Similarly, 100% of samples were quantifiable

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for CrAssphage and HPyV, 29.2% for EV, 4.16% for HNoV GII, 100% for RhV A and 100% for RhV B using the NMAP workflow. The Supplementary Tables ST3 and ST4 display the concentrations of indicator, enteric, and respiratory viruses in individual aircraft wastewater samples, as determined by the AE and NMAP workflows. The quantifiable samples for qPCR and RT-qPCR along with their minimum, maximum, mean, standard deviation, and 95% CI of means were calculated for aggregated aircraft wastewater samples for the AE and NMAP workflows (Supplementary Tables ST5 and ST6). Boxplots of the concentration of each quantifiable virus by AE and NMAP workflows are shown in Figure 1. CrAssphage had the highest mean concentration (6.76 $\log_{10}$ GC/12.5 mL) followed by HPyV (5.46 $\log_{10}$ GC/12.5 mL using the AE workflow, while the mean concentrations of enteric and respiratory viruses ranged from 2.48 to 3.63 $\log_{10}$ GC/12.5 mL. Using the NMAP workflow, the mean concentration of crAssphage was 5.18 $\log_{10}$ GC/12.5 mL and the mean concentration of HPyV was 4.20 $\log_{10}$ GC/12.5 mL, while mean concentrations of enteric and respiratory viruses ranged from 2.55 to 3.74 $\log_{10}$ GC/12.5 mL. Supplementary Tables ST3 to ST6 provide further details on the concentration of each virus. The mean concentrations of CrAssphage ($p < 0.0001$), HPyV ($p = 0.0001$), RhV A ($p = 0.0059$) and RhV B ($p = 0.0002$) between the two workflows were statistically significant.

Table 1. Prevalence of indicator, enteric and respiratory viruses in wastewater samples collected from the lavatories.

Viruses	No. of positive/no. of samples tested (%)		
	AE	NMAP	HA and NMAP combined
CrAssphage	24/24 (100)	24/24 (100)	24/24 (100)
HPyV	24/24 (100)	24/24 (100)	24/24 (100)
HAV	0/24 (0)	0/24 (0)	0/24 (0)
EV	9/24 (37.5)	10/24 (41.7)	14/24 (58.3)
HNoV GII	1/24 (4.20)	1/24 (4.20)	1/24 (4.20)
HAdV	13/24 (54.2)	4/24 (16.7)	13/24 (54.2)
BoV	7/24 (29.2)	2/24 (8.30)	8/24 (33.3)
PeV	2/24 (8.30)	3/24 (12.5)	4/24 (16.7)
RhV A	24/24 (100)	24/24 (100)	24/24 (100)
RhV B	24/24 (100)	24/24 (100)	24/24 (100)
EBV	5/24 (20.8)	4/24 (16.7)	7/24 (29.2)
IAV	2/24 (8.30)	2/24 (8.30)	4/24 (16.7)
IBV	0/24 (0)	0/24 (0)	0/24 (0)
RsV A	0/24 (0)	0/24 (0)	0/24 (0)
RsV B	4/24 (16.7)	6/24 (25.0)	9/24 (37.5)

CrAssphage: Cross-assembly phage; HPyV: human polyomavirus; EV: Enterovirus; HAdV: Human adenovirus; HAV: Hepatitis A virus; HNoV GII: Human norovirus GII, BoV: Bocavirus; EBV: Epstein-Barr Virus; IAV: Influenza A virus; IBV: Influenza B virus; PeV: Parechovirus; RhV A: Rhinovirus A; RhV B: Rhinovirus B; RSV A: Respiratory syncytial virus A; RSV B: Respiratory syncytial virus B.

Discussion

Surveillance of municipal wastewater has proven to be a robust method for tracking COVID-19 across a wide range of communities [33,34]. Similarly, surveillance of wastewater from long-haul aircraft has proven useful for predicting subsequent COVID-19 among passengers during quarantine and even detecting single infections of emerging variants of concern such as Omicron [10,16]. Despite its promise, wastewater-based public health surveillance of both municipal and aircraft wastewater faces many challenges including the lack of optimized methods, expansion to include a broader range of infectious agents, and uncertainty in translating the surveillance data to an epidemiological frame [33,35]. To inform our consideration of these challenges, in the current study, we have utilized two workflows to measure a diverse array of viruses (two indicator viruses, four enteric viruses, and nine respiratory viruses) in archived aircraft wastewater samples. While interpreting the findings, it

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should be noted that these archived samples were stored at -20 °C for ~18 months, and therefore, degradation of the nucleic acids over that time frame is possible. For this reason, the PCR-based measurement performance should be interpreted as a conservative lower bound throughout the discussion. However, the potential degradation (if any) may not affect evaluation of virus concentration workflows used in this study.

The CrAssphage and HPyV have been proposed as indicators of human waste (feces and/or urine) in the environment [36,37]. More recently, CrAssphage has been widely applied as a metric for the adjustment of wastewater surveillance results to account for variation in human fecal content from sample to sample [38,39]. Both workflows yielded detection of both CrAssphage and human polyomavirus in all 24 samples; however, the AE workflow yielded mean $\log_{10}$ concentrations (6.76 and 5.46 $\log_{10}$ GC/12.5 mL, respectively) that were more than one order of magnitude higher for each virus compared to Microbiome A particles (5.18 and 4.20 GC/12.5 mL). The reason for concentration discrepancies between the two workflows for DNA viruses is unclear and requires further investigations. The consistent detections of these indicator viruses suggest the reliable presence of human waste, specifically human feces and urine, in aircraft wastewater samples. The mean concentration of CrAssphage observed in the aircraft wastewater samples is comparable with the concentration range in municipal wastewater in Kentucky, USA, [40] and mean concentrations in wastewater from cities throughout Italy [41]. The mean HPyV concentration in aircraft wastewater samples was also consistent with mean concentrations observed in Italy [41] and Spain [43]. Although there remain uncertainties concerning bathroom behaviour in aircraft lavatories, our observation supports the notion that some portion of passengers onboard long-haul aircraft are consistently defecating and urinating in the lavatory [11]. Many viruses have been reported to be present in the human urinary tract of healthy and infected individuals such as herpesvirus, papillomavirus, adenovirus, cytomegalovirus, Zika virus, West Nile virus and SARS-CoV-2 [43]. Therefore, aircraft wastewater may also be appropriate for tracking viruses that are associated with human urinary tract.

Enteric viruses are obvious candidates for wastewater-based public health surveillance since they are shed in high densities in the feces of those infected and were among the first infectious agents studied via wastewater [44–46]. In this study, detection rates of EV and HNoV GII were comparable between the two workflows, but the NMAP yielded a greater number of RT-qPCR quantifiable enterovirus results (7/24) than the AE workflow (2/24), although the resulting mean concentration was approximately one order of magnitude greater for the two quantifiable AE compared to NMAP results. For HAdV on the other hand, the AE workflow yielded 13 positive detections with five quantifiable results while the NMAP workflow produced only 4 detections and none of them were quantifiable. The prevalence of HAdV (13/24) and EV (10/24) in the 24 aircraft wastewater samples also adds more evidence in support of consistent defecation in aircraft toilets among long-haul passengers.

A key observation in the use of wastewater for public health surveillance of COVID-19 is the shedding of a respiratory virus in body fluids which are likely to be captured in wastewater collection systems, such as feces and urine [47]. More recently, public health surveillance using municipal wastewater has expanded to include a diverse array of respiratory viruses including influenza A, RSV, RHV, and others [29,48]. Among the 24 aircraft wastewater samples in the current study, the AE and NMAP yielded comparable detection frequencies for all but BoV and RSV B. The AE workflow yielded seven BoV detections with two quantifiable results compared to only two detections with no quantifiable results for the NMAP workflow. Just as was observed for the indicator and enteric viruses, the AE workflow results deviated from the NMAP results for a non-enveloped virus with a DNA genome. RhV A and B were detected and quantifiable in all 24 samples by both workflows with the NMAP yielding greater mean concentrations. In this case both RhV are characterized by non-enveloped morphology and RNA genomes. However, for another non-enveloped RNA virus (EV), AE yielded mean concentrations that were greater than NMAP workflow, although this result was from only two quantifiable samples compared to seven for NMAP workflow. The mixed results continued for RSV B (enveloped, ssRNA genome) where NMAP workflow resulted in a slightly greater detection frequency, but AE yielded more quantifiable results.

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There are many factors that may have attributed such discrepancy between workflows such as sub-sampling error, different sample volume and type and the efficacy of each concentration method.

Our comparisons continue to indicate a universal method that performs "best" for all viruses in all wastewater types is yet to be found. Given the consistently mixed results among viruses of different morphologies and genome types, our experience in the current study strongly suggests that virus characteristics alone are not sufficient for methodological development. Wastewater characteristics, for example suspended solids, are important drivers of virus detection and quantification using PCR-based methods.[18] Further development and characterization toward a standard method will require integration of systematic wastewater characterization with PCR-based measurements to produce further insight. In the meantime, the "best" method should be carefully considered in light of the end point required (e.g., qualitative or quantitative) and the relevant infectious agents for public health decision making. Importantly, in this paper, we did not consider sequencing endpoints, but such analysis could be critical for maximizing public health intelligence derived from aircraft wastewater.[12,16] No method has yet provided a systematic advantage over others, which raises a more fundamental question—how good is good enough? Nonetheless, the prevalence of indicator and enteric viruses in the current study suggests long haul passengers are reliably depositing biological materials useful for public health surveillance in toilets in aircraft lavatories. Further, the detection of a wide variety of respiratory viruses within aircraft wastewater suggests the approach could be applied for global surveillance of a broad array of infectious agents.

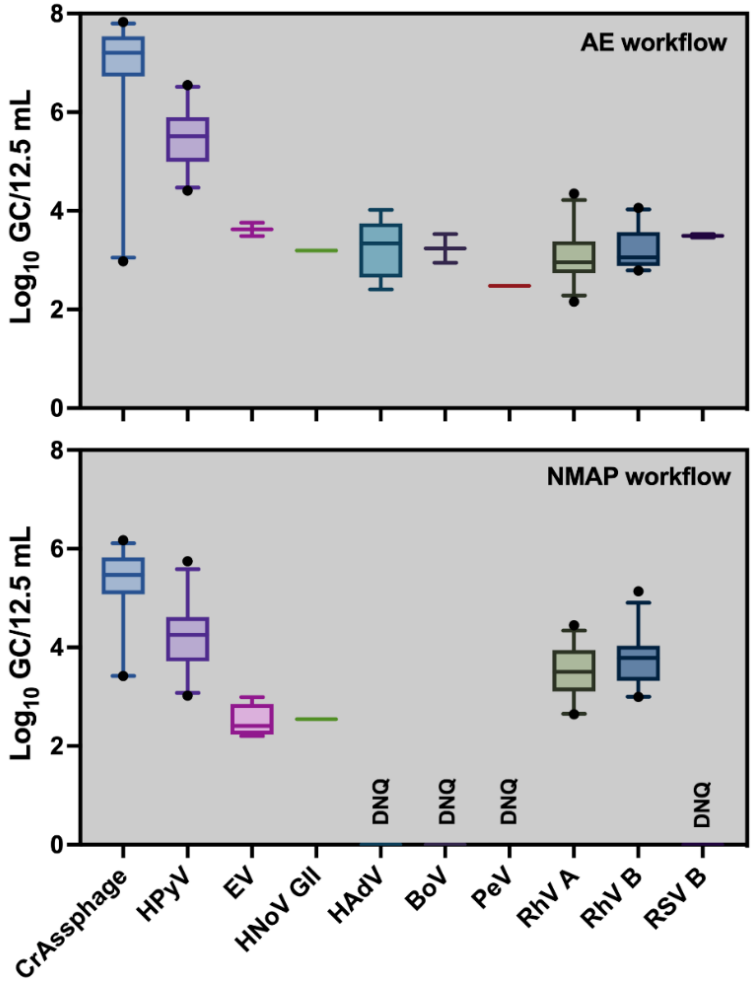

Figure 1. Box-and-whisker plots of the concentrations (log_{10} GC) of indicator, enteric and respiratory viruses in 24 aircraft wastewater samples determined using the AE and NMAP workflows. The lower and upper boxes denote 25th and 75th percentiles. The lower and upper bars represent the 5th and 95th percentiles. CrAssphage: Cross-assembly phage; HPyV: human polyomavirus; EV: Enterovirus;

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HNoV GII: Human norovirus GII; HAdV: Human adenovirus; BoV: Bocavirus; Parechovirus; RhV A: PeV: Rhinovirus A; RhV B: Rhinovirus B; RSV B: Respiratory syncytial virus B.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

Author Contributions: W.A. designed the study and conducted laboratory work. W.J.M. conducted laboratory work. W.A., A.T., A.B. and S.S. wrote the manuscript. All the authors critically reviewed the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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