Quantification of SARS-CoV-2 and cross-assembly phage (crAssphage) from wastewater to monitor coronavirus transmission within communities

Hyatt Green[1*], Maxwell Wilder[1], Mary Collins[2], Ariana Fenty[1], Karen Gentile[3], Brittany L. Kmush[4], Teng Zeng[5], Frank A. Middleton[3], David A. Larsen[4*]

[1]Department of Environmental and Forest Biology, SUNY-ESF, Syracuse, NY 13210
[2]Department of Environmental Studies, SUNY-ESF, Syracuse, NY 13210
[3]Department of Neuroscience and Physiology, Upstate Medical University, Syracuse, NY 13210
[4]Department of Public Health, Syracuse University, Syracuse, NY 13244
[5]Department of Civil and Environmental Engineering, Syracuse University, Syracuse, NY 13244

*Corresponding author: hgreen@esf.edu, 315-470-4814

Abstract

Wastewater surveillance of SARS-CoV-2 has become an attractive tool for combating the spread of COVID-19 by assessing the presence or levels of the virus shed in a population. However, the methods to quantify viral RNA and to link those quantities to the level of infection within the community vary. In this study, we sought to identify and optimize scalable methods for recovery of viral nucleic acids from wastewater and attempted to use a constitutive member of the gut virome, human-specific crAssphage, to help account for unknown levels of SARS-CoV-2 decay and dilution in the wastewater infrastructure. Results suggest that ultracentrifugation of a small volume of wastewater through a 50% sucrose cushion followed by total nucleic acid extraction yielded quantifiable virus in an area with a modest number of COVID-19 cases. Further, the ratio of log_{10}(SARS-CoV-2):log_{10}(crAssphage) appears to be associated with the cumulative incidence of COVID-19 in the Syracuse, NY area. In areas where ultracentrifuges are available, these methods may be used to link SARS-CoV-2 quantities in wastewater to levels of transmission within communities with sewer service.

Introduction

While the primary mechanism of transmission of Sudden Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), the virus that causes the respiratory disease Coronavirus Disease 2019 (COVID-19), is through respiratory droplets, a significant number of inactive viral particles are shed in the feces of infected persons[1-3]. At the very least, the prospect of surveilling possible disease burden via surveillance of untreated wastewater offers opportunities for monitoring the emergence of SARS-CoV-2 transmission[4] and trends over time[5] including any

eventual reduction in transmission. When coupled with information from standard surveillance methodology, such a surveillance system could conceivably and efficiently inform decisions about where to focus resources (e.g., individual swab testing, contact tracing), where to target interventions such as social distancing, as well as how and at what rate to reduce broad scale social distancing and reopen local economies[6]. The tool could also be used to efficiently monitor potentially vulnerable facilities, such as jails, schools, or assisted-living facilities.

Methods to recover SARS-CoV-2 from wastewater have varied widely in the few recent reports with implications for their cost, scalability, and susceptibility to disruptions in supply chains. Medema and colleagues used moderate speed centrifugation methods with centrifuge filters (Centricon® Filters) to successfully concentrate and purify viral particles/genomes[4]. This method was somewhat successfully replicated by another group that also had some success by filtration through electronegative membranes[7]. However, the cost and availability of centrifuge filters can be limiting for some laboratories, especially considering to likelihood of supply chain disruptions during a pandemic. Perhaps the most scalable method that requires very little specialized equipment was proposed by Wu and colleagues where 0.2 micron filtrates were PEG-precipitated and then Trizol™ extracted to obtain purified RNA[8]. Among the simplest procedures reported, assuming the equipment is available, is the ultracentrifugation of small volumes (11 ml) of wastewater for one hour followed by extracting nucleic acids from the pellet[5]. Aluminum-driven flocculation followed by nucleic acid extraction has also been used to successfully detect the virus in wastewater[9]. Tangential flow filtration followed by PEG-precipitation has also been used to concentrate and purify viruses from wastewater, but might pose problems in scaling-up due to the length of time needed to filter a single sample[10]. In the anticipation that wastewater surveillance for SARS-CoV-2 and other pathogens will become widespread, methods that are sensitive, scalable, and cost-effective will be needed by regional and local laboratories.

Methodological differences aside, the question of how SARS-CoV-2 concentrations in wastewater can have a significant bearing on public health decisions remains. An attractive prospect is to use viral concentrations in wastewater to estimate the number of infected individuals "upstream" in the represented catchment area. Pairing results from wastewater with shedding rates to arrive at the estimated number of infected individuals in the represented area provided a higher than expected number of cases in the Boston area[8] and highly uncertain estimates in Southeast Queensland[7]. Factors that remain problematic to this approach and others are the low concentrations of virus obtained, which thereby increases the uncertainty in the quantification process, as well as issues regarding viral fate and transport throughout the wastewater infrastructure.

In this study, our goals were to: a) detect and, if possible, quantify SARS-CoV-2 in wastewaters of the upstate New York area (Onondaga County, NY, US), b) improve scalable methods of wastewater sample processing to maximize the recovery of viral nucleic acids, and c) integrate the measurements of both SARS-CoV-2 and an abundant gut-derived bacteriophage (crAssphage) which may help account for decay and dilution in wastewater infrastructure. Since we also sought an inexpensive, effective method that could be brought to scale,

ultracentrifugation seemed attractive because few supplies were needed, which allowed us to bypass some supply chain issues, and many research institutions in the region have the required equipment on site. Our results indicate that ultracentrifugation incorporating a 50% sucrose cushion was successful at concentrating viral nucleic acids while removing impurities. As a result, we were able to detect, and in most cases, quantify SARS-CoV-2 in small volumes (20 ml) of wastewater collected from an area with a moderate number of COVID-19 cases. We also show that the ratio of SARS-CoV-2:crAssphage visually correlates with the cumulative incidence of COVID-19.

Methods

Wastewater Ultracentrifugation

Twenty-four hour composite wastewater samples (1.9 L) were collected from 11 access points (i.e., wastewater treatment plants, influent pump stations, or interceptor lines) in Syracuse, NY and other locations in Onondaga County, NY on May 6th and 13th, 2020 (Table 1). Samples were stored at 4 °C following collection and transported on ice to Upstate Medical University (Syracuse, NY) for processing the next morning. Upon receipt, samples were mixed to resuspend particulates and 20 ml was aliquoted to a 38.5 ml ultracentrifuge tube (ThermoFisher, #750000471). A 12 ml sucrose cushion (50% sucrose in TNE buffer [20 mM Tris-HCl (pH 7.0), 100 mM NaCl, 2 mM EDTA]) was carefully added underneath the wastewater with a 10 ml disposable serological pipette so that two distinct layers were formed (Figure 1A). Fifty percent sucrose yielded higher crAssphage DNA concentrations than 20% or 70% (Table S1). In batches of six, samples were then purified by centrifugation at 150,000 x g at 4°C for either 90 minutes (May 6) or 45 minutes (May 13) on a Sorvall WX Ultra series with a Sorvall Surespin™ 630 rotor (ThermoFisher). Centrifugation times of 45 and 90 minutes provided roughly the same recovery of viral nucleic acids (Table S2). Supernatant was then decanted, and pellets (Figure 1B) were resuspended in 200 µL 1X PBS. Pellets were stored at -20°C for <12 hours prior to nucleic acid extraction. Distilled water (20 ml) was used as a processing blank.

Table 1. Facility characteristics and average flow (millions of gallons per day) on May 6 and 13, 2020.

Facility/Catchment ID	Facility Type	Population Served	Flow May 6, 2020 (M.G.D.)	Flow May 13, 2020 (M.G.D.)
600	Contributor to 604	3,841	1.25	0.95
601	WWTP	25,300	5	4.5
603	Contributor to 604	47,688	11.16	9.36
604	WWTP	223,900	70.3	62.2
605	WWTP	25,600	4.1	4.7
606	WWTP	37,800	5.4	4.9
610	Contributor to 604	112,747	29.1	25.7
617	WWTP	20,500	3.4	3.5
619	WWTP	12,800	1.9	1.7
725	Contributor to 604	39,262	11.2	10.01
1700	Contributor to 604	20,347	17.58	16.16

Nucleic Extraction and Synthesis of crAssphage cDNA

Total nucleic acids were extracted using the AllPrep® PowerViral® DNA/RNA Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. Samples were eluted in 50 µL RNase free water. Extraction blanks using distilled water were performed in each extraction batch to assess contamination. To assess the recovery of crAssphage RNA through the methods previously described, cDNA was generated using the QuantiTect Reverse Transcription Kit (Qiagen) according to manufacturer's protocol.

CrAssphage qPCR

qPCR was used to detect the presence of crAssphage using the previously developed CPQ_056 assay[11] (Table 2). Reactions consisted of 12.5 µL TaqMan® Environmental MasterMix (ThermoFisher), 1 µM primers, 80 nM probe, molecular grade water, and 2 µL template DNA for a total reaction volume of 25 µL. Each reaction was run on either a QuantStudio3™ or QuantStudio5™ (ThermoFisher) under the following thermal cycling conditions: 10 minutes at 95 °C, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. A DNA standard was generated by purification of amplified PCR product using Roche High Pure PCR Template

Preparation Kit. Standard DNA quantity was assessed on NanoDrop spectrophotometer and Qubit® fluorometer. A standard curve, consisting of purified amplicons ranging from 1×10^6 to 5 copies/reaction, was used to convert Ct values to gene copies per reaction (Table 3). A CPQ_056 assay modified for SYBR Green chemistry was used in some optimization trials (see Supplementary).

Table 2. qPCR assays used.

Assay Name	Target	Oligonucleotide Name	Oligonucleotide Sequence (5'-3')	Amplicon length	Reference
IP2IP4 Multiplex	SARS-CoV-2	nCoV_IP2-12669Fw	ATGAGCTTAGTCCTGTTG	108 bp	12
		nCoV_IP2-12759Rv	CTCCCTTTGTTGTGTTGT		
		nCoV_IP2-12696bProbe(+)	[5']Hex AGATGTCTT[Zen]GTGCTGCCGGTA [3'] IABkFQ		
		nCoV_IP4-14059Fw	GGTAACTGGTATGATTTCG	107 bp	
		nCoV_IP4-14146Rv	CTGGTCAAGGTTAATATAGG		
		nCoV_IP4-14084Probe(+)	[5'] Hex TCATACAAA[Zen]CCACGCCAGG [3'] IABkFQ		
CPQ_056	CrAssphage	056F1	CAGAAGTACAAACTCCTAAAAAACGTAGAG	126 bp	11
		056R1	GATGACCAATAAACAAGCCATTAGC		
		056P1	[FAM] AATAACGATTTACGTGATGTAAC[MGB]		

SARS-CoV-2 RT-qPCR

To detect SARS-CoV-2, RT-qPCR was used with a multiplex reaction containing the previously published IP2 and IP4[12] assays (Table 2). Reactions consisted of 6.25 uL Reliance One-Step Multiplex RT-qPCR Supermix, 0.4 µM each primer, 0.16 µM probes, molecular grade water, and 2.5 uL template total nucleic acids for a total reaction volume of 25 µL. Thermal cycling conditions were 10 minutes at 50 °C, 10 minutes at 95 °C, followed by 45 cycles of 95 °C for 10 seconds and 59 °C for 30 seconds. A standard curve, consisting of amplicons ranging from 250 to 2.5 copies/reaction, was used to convert Ct values to gene copies per reaction (Table 3). All no-template controls for both crAssphage and IP2IP4 (n > 40) were negative throughout the entire study.

Table 3. qPCR assay performance parameters.

Assay	R^2	Intercept	Slope	Efficiency
CPQ_056	0.974	39.568	-3.406	0.97
IP2IP4	0.849	41.026	-3.508	0.93

Recovery of crAssphage and SARS-CoV-2

To assess the proportion of viral nucleic acids remaining in each phase (including the pellet) after centrifugation, wastewater samples (2 x 20 ml) were spiked with SARS-CoV-2 (resulting in approximately 580 gene copies per ml wastewater). Pellets were generated by ultracentrifugation at 150,000 x g for 45 minutes and the following layers were removed; aqueous upper (top 10 ml), aqueous lower (second 9 ml), cushion interface (1.5 ml, specifically targeting visible suspended particles on top of sucrose layer), sucrose upper (6 ml), sucrose lower (6 ml), and pellet (appx. 200 µL). A 200 µL aliquot of each layer was extracted using the PowerViral kit (Qiagen) and extracts were analyzed with the IP2IP4 assay in 25 µL reaction volumes under the conditions described previously (Table S6).

Cumulative Incidence of COVID-19

To correlate evidence of SARS-CoV-2 transmission in wastewater with cases of COVID-19 in the wastewater catchment area we retrieved the total number of COVID-19 cases by ZIP code from the Upstate Hospital electronic medical record system. These records reflect approximately 40% of the total COVID-19 cases in Onondaga County. We standardized the number of COVID-19 cases by ZIP code to the ZIP code population to calculate a cumulative incidence per 1,000 residents. We then visualized cumulative incidence alongside the ratio of $\log_{10}$(SARS-CoV-2):$\log_{10}$(crAssphage).

Results

Recovery of Viral Nucleic Acids

Direct ultracentrifugation of a wastewater sample through a %50 sucrose cushion resulted in the formation of a translucent, but visible, pellet often bordered by darker, lower density residue, presumably organics, metal sulfides, and/or other impurities in the wastewater (Figure 1). Depending on the wastewater sample, lower density impurities were often resting at the cushion interface. Recovery trials with inactive SARS-CoV-2 spiked samples indicated that no quantifiable SARS-CoV-2 RNA, crAssphage RNA, or crAssphage DNA remained in the upper aqueous phase or sucrose layer after centrifugation (Table 4). Given that only a portion of each layer was tested, it's possible there may have been some non-pelleted residual viral nucleic acids at concentrations below the limit of detection, but it is clear that the vast majority of SARS-Cov-2 and crAssphage viral nucleic acids are pelleted under these conditions. After nucleic acid extraction and qPCR, we estimated recovery of SARS-CoV-2 based on the addition of a known quantity of SARS-CoV-2 to be about 12% attributing some loss to non-pelleted viral RNA, but most to the subsequent nucleic acid extraction procedure.

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Yield and Quality Assessment of Total RNA

The direct ultracentrifugation and purification of wastewater resulted in recovery of a considerable amount of total RNA. In the samples that were collected on May 6 the average yield was 26.3 ng/μL (std. dev. = 11.7) (Figure S2, top panel). However, these estimates were likely affected by the presence of considerable DNA carryover. Thus, for the May 13 samples, the addition of DNase produced lower yields, with an average of 2.5 ng/μL (std. dev. = 2.3) (Figure S2, bottom panel). The RNA integrity numbers of the samples were not significantly different in either batch, however, with May 6 average of 4.3 ng/μL (std. dev. = 0.8) and a May 13 average of 3.4 ng/ μL (std. dev. = 1.9) (Figure S2). Notably, these RNA integrity values are similar to ones obtained from human biofluid waste products, such as saliva, urine, or fecal matter.
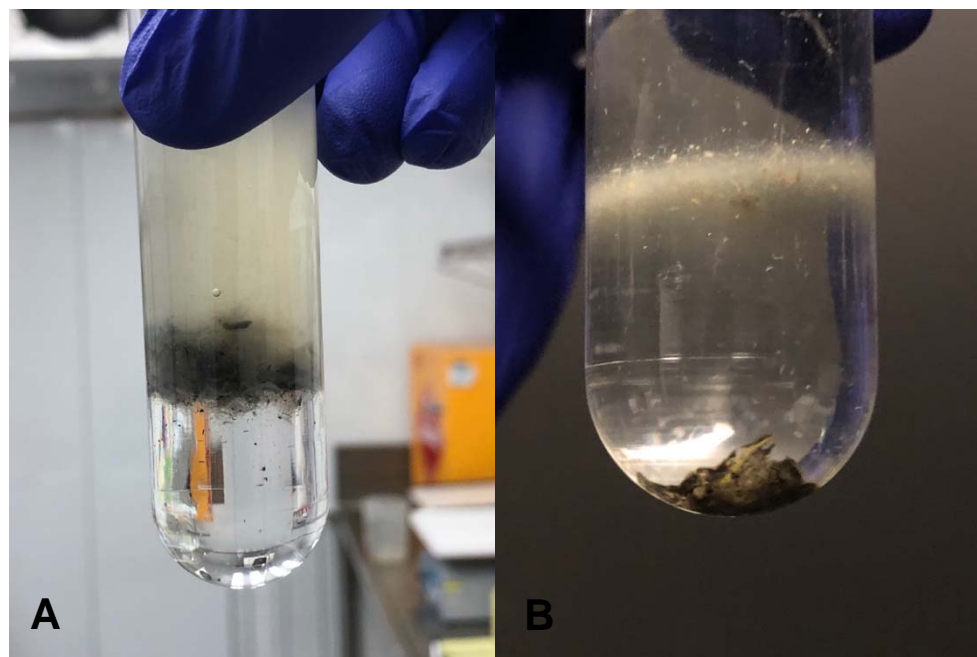

Figure 1. (**A**) Raw wastewater above a 50% sucrose solution prior to ultracentrifugation. (**B**) Pellet produced by ultracentrifugation and residual debris on top of the 50% sucrose cushion.

Table 4. Recovery of SARS-CoV-2 RNA through a sucrose cushion.

Layer	Replicate Tube	Mean Copies SARS-CoV-2 (+/- SD)	SARS-CoV-2 Rxns Positive (out of three)
Aqueous Upper	1	<LOQ	0
	2	<LOQ	0
Aqueous Lower	1	<LOQ	0
	2	<LOQ	0
Cushion Interface	1	<LOQ	0
	2	<LOQ	2
Sucrose Upper	1	<LOQ	0
	2	<LOQ	0
Sucrose Lower	1	<LOQ	0
	2	<LOQ	0
Pellet	1	1.37E3 (7.39E2) per pellet	3
	2	1.42E3 (7.19E2) per pellet	3

Detection and Quantification of Viral Nucleic Acids in Wastewater

Over a two-week period, we detected some level of SARS-CoV-2 in 18 out of 22 samples, 13 of which were in the quantifiable range (Table 5). SARS-CoV-2 was more prevalent in the May 13 samples (detected in 11 out of 11 samples) compared to the May 6 samples (7 out of 11), possibly due to a significantly lower flow on May 13 (paired t-test, p=0.045). If any of the three reaction wells crossed the fluorescence threshold, the sample was interpreted as positive due to all negative controls throughout the study testing negative. Likewise, SARS-CoV-2 fell within the quantifiable range in 9 out of 11 May 13 samples and only 4 out of 11 May 6 samples. The average number of SARS-CoV-2 genome copies within quantifiable samples over the two week period was 42.7 (std.dev = 32.9) genomes/ml while the highest observed was 112.35 (std. dev. = 8.01) genome/ml of wastewater.

In contrast, crAssphage DNA was abundant in every sample analyzed with an average of 1.11 x 10^5 copies/ml across the two week period with no significant difference between the two sample sets. crAssphage RNA was detected in every sample except the sample from Facility 610 on May 13. crAssphage RNA was much less abundant than crAssphage DNA with an average of 1.68 x 10^2 copies/ml. Interestingly, while there was no significant difference in crAssphage DNA between the two sample sets, crAssphage RNA was significantly lower on May 13 than May 6 (paired and unpaired t-tests, $p < 0.001$).

Table 5. Concentrations of SARS-CoV-2 and crAssphage DNA and RNA in sampled catchments.

Date Collected	Facility/ Catchment	Mean copies crAssphage (CPQ_056)/mL wastewater (+/- SD)		SARS-CoV-2	
		DNA	RNA	Mean Copies SARS-CoV-2 / mL (+/- SD)	Rxns Positive (out of three)
May 6	600	5.79E4 (3.58E3)	2.76E2 (4.50E0)	<LOQ	0
May 6	601	1.76E5 (3.36E4)	5.33E2 (1.33E1)	1.12E2 (8.01E0)	3
May 6	603	5.44E4 (4.03E3)	1.65E2 (3.75E1)	1.51E1 (7.89E0)	3
May 6	604	9.29E4 (5.65E3)	3.95E2 (5.08E1)	4.59E1 (1.29E1)	3
May 6	605	1.19E5 (1.26E4)	3.31E2 (7.55E1)	<LOQ	0
May 6	606	7.36E4 (1.47E3)	2.48E2 (4.61E1)	<LOQ	2
May 6	610	3.33E4 (1.19E3)	1.37E2 (1.59E1)	2.25E1 (1.45E1)	3
May 6	617	1.47E5 (8.78E3)	2.73E2 (5.12E1)	<LOQ	2
May 6	619	7.58E4 (1.18E4)	1.62E2 (2.35E1)	<LOQ	0
May 6	725	1.01E5 (8.28E3)	2.32E2 (2.99E1)	<LOQ	0
May 6	1700	2.50E4 (2.25E3)	1.02E2 (2.30E1)	<LOQ	1
May 13	600	1.07E5 (6.97E3)	1.03E2(2.44E1)	7.50E0(2.94E0)	3
May 13	601	8.44E4 (7.8E33)	1.40E2 (3.44E1)	2.82E1 (4.67E0)	3
May 13	603	6.43E4 (1.43E3)	1.32E1 (6.52E0)	3.04E1 (3.14E1)	3
May 13	604	6.74E4 (1.03E3)	5.50E1 (1.57E1)	5.72E1 (2.62E1)	3
May 13	605	9.15E4 (1.81E3)	3.94E1 (1.57E1)	1.20E1 (2.39E0)	3
May 13	606	9.65E4 (1.38E3)	4.00E1 (1.60E1)	4.90E1 (6.33E0)	3
May 13	610	2.21E4 (1.02E3)	<LOQ (0/3)	6.92E1 (7.89E0)	3
May 13	617	9.81E4 (3.15E3)	4.90E1 (1.17E1)	<LOQ	1
May 13	619	1.54E5 (7.35E3)	4.21E1 (8.88E0)	<LOQ	1
May 13	725	1.95E5 (7.73E3)	1.13E2 (7.16E0)	1.30E1 (8.34E0)	3
May 13	1700	4.96E5 (3.66E3)	8.29E1 (3.43E1	9.41E1 (2.00E1)	3

Association Between crAssphage Loads and Population Served

While crAssphage DNA concentrations were not significantly associated with population served, flow, SARS-CoV-2 concentrations, or crAssphage RNA concentrations, there was a significant linear relationship between the loads of both crAssphage DNA ($p < 0.001$) and RNA ($p < 0.01$) and population served in each catchment (Figure 2).

Spatial Association Between SARS-CoV-2:crAssphage Ratios in Wastewater and COVID-19 Incidence

Although the number of cases in each catchment would allow a better assessment of the relationship between viral concentrations in wastewater and the level of transmission in the respective community, visual inspection suggests a spatial correlation between the cumulative incidence of cases by zip code from the Upstate hospital system and the ratio of SARS-CoV-2:crAssphage in wastewater with higher ratios occurring in areas of higher incidence (Figure 3).
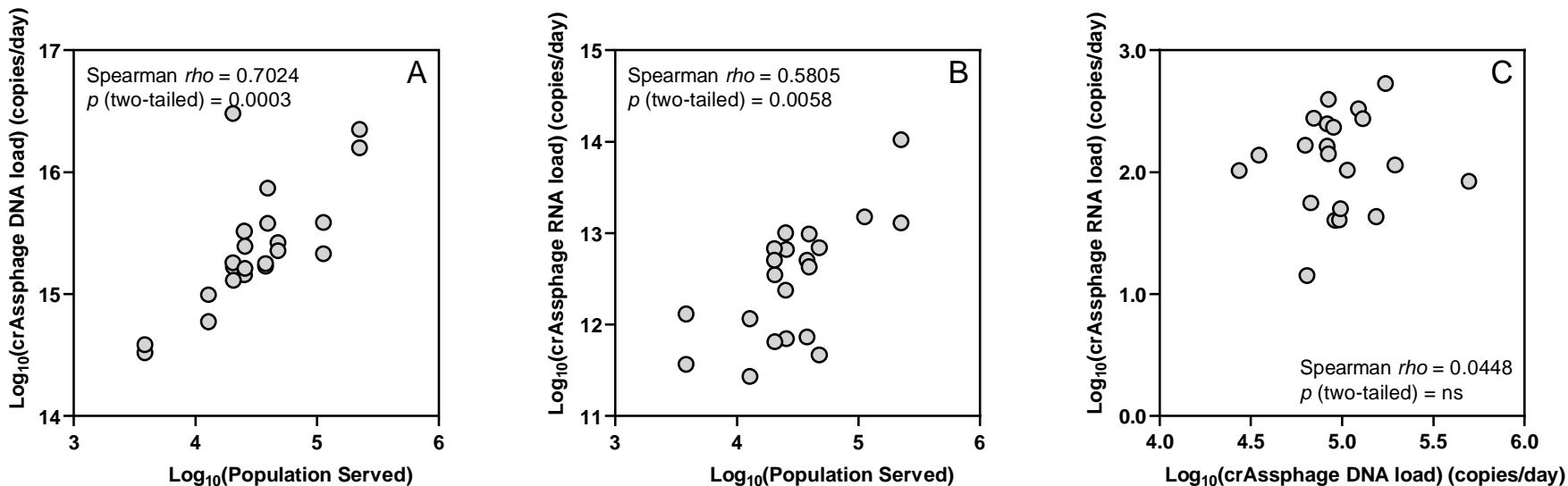
Figure 2. Relationship between crAssphage DNA load and population served (A), crAssphage RNA load and population served (B), crAssphage RNA load and crAssphage DNA load (C). Loads are simply calculated as the concentration x the flow rate.
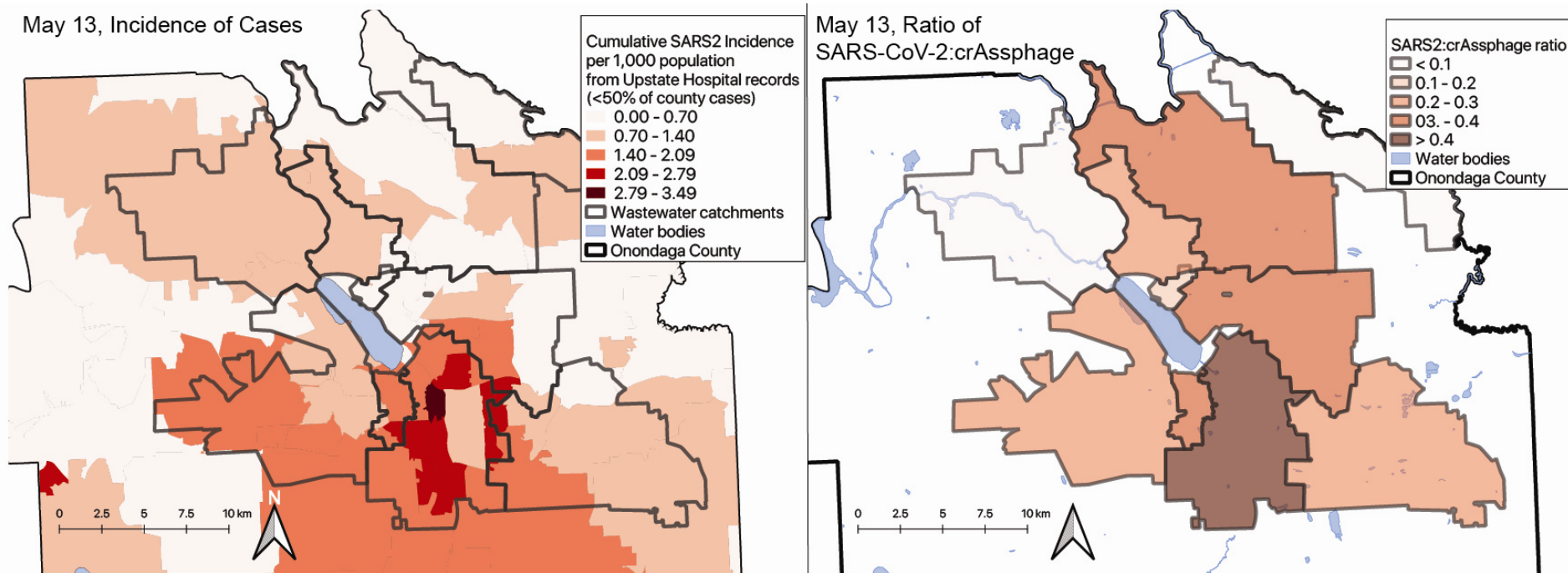

Figure 3. Maps of incidence of cases by zip code (left) and SARS-CoV-2:crAssphage DNA ratios (right) on May 13th, 2020.

Discussion

The first demonstration that surveillance of SARS-CoV-2 in wastewater could be used to inform the public health response to COVID-19 instantly expanded the tools available to fight the pandemic[4]. However, many recent reports of SARS-CoV-2 detection in wastewater are limited by the low levels of viral RNA recovered which can limit quantitative interpretation[4, 7]. Here, we report the optimization of a simple sucrose cushion ultracentrifugation method for the recovery of SARS-CoV-2 from wastewater that provided quantitative results from a relatively small sample size (20 ml) in about 8 hours depending on the number of samples processed at one time. We understand that the requirement of an ultracentrifuge is unrealistic for many and that other approaches will be required in the absence of this equipment. Nonetheless, using a single centrifuge, we estimate a modest throughput of about 60 samples in 24 hours with these methods.

Much of the prior work on crAssphage, specifically those targeted by the CPQ_056 assay, has been focused on its utility as an indicator of human fecal pollution in natural waterbodies[11, 13-15]. Many of the same attributes that make crAssphage an attractive indicator organism, such as its prevalence and abundance in the human population as well as its scarcity in other hosts[11, 16], are also helpful in gauging the degree of SARS-CoV-2 transmission within the community. In addition to using crAssphage as an abundant surrogate for SARS-CoV-2 during method development and optimization, crAssphage can be used to ensure sufficient viral recovery, which may become an important quality assurance measure when comparing wastewater surveillance data within and between labs. Furthermore, like SARS-CoV-2, crAssphage is subject to decay and dilution within the wastewater infrastructure and while concentrations of SARS-CoV-2 alone are difficult to interpret, the ratio of SARS-CoV-2:crAssphage is likely more robust to processes that contribute to the loss of viral nucleic acids during transport. It is conceivable that these ratios could then be used to rank catchment areas by their relative degree of transmission independent of mass-balance calculations.

Enveloped RNA viruses, like SARS-CoV-2, are known to be less resilient under environmental conditions than non-enveloped DNA viruses, like crAssphage[17]. Furthermore, DNA released from lysed viral particles is thought to be more resilient to degradation than RNA. The hypothesized rapid decay of SARS-CoV-2 compared to crAssphage would likely result in the underestimation of SARS-CoV-2 transmission within the community when using this approach. Decay rates of the two viruses and their genetic material within water infrastructure are needed to further refine predictions of transmission within the population using this approach. Decay may also play a larger role in larger service areas with longer average wastewater transit times and may explain why only low levels of SARS-CoV-2 were detected in some areas with known cases.

In summary, we demonstrated that an ultracentrifugation method using a sucrose cushion can be used for quantitative environmental surveillance of SARS-CoV-2 transmission. While a more quantitative analysis is underway, we further showed that the ratio of SARS-CoV-2

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RNA:crAssphage DNA found in wastewater may be spatially associated with incidence of the disease and could potentially be used to guide public health and economic intervention strategies. Regional or national surveillance of wastewater, in conjunction with clinical testing, may provide a robust decision-making platform that authorities can use to continue restarting local economies while prioritizing public health. Furthermore, frequent and widespread wastewater surveillance has the potential to indicate when and where a resurgence of SARS-CoV-2 or outbreaks of future pathogens might occur.

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Acknowledgments

Supplementary Material

Sucrose Cushion Optimization

Concentration and Centrifugation Time Assessment

To optimize the sucrose cushion purification method, we assessed recovery of crAssphage markers with varying sucrose concentrations and ultracentrifugation times. Three sucrose concentrations were used; 20%, 50%, and 70% (in TNE buffer [20 mM Tris-HCl (pH 7.0), 100 mM NaCl, 2 mM EDTA]) with two total replicates of each treatment. Prior to cushion purification, wastewater samples were centrifuged at 2,000 x g for 25 mins to remove large particles and debris (sample clarification). Six 20 ml aliquots were then distributed to 38.5 ml ultracentrifuge tubes. To the bottom of each tube, 12 ml of a solution of sucrose in TNE was slowly added with a 10 ml serological pipette so that the sucrose formed a distinct layer below the wastewater. A pellet was generated by ultracentrifugation at 4 °C at 150,000 x g for between 20 and 150 minutes depending on the sucrose concentration (Table S1). Supernatant was then decanted, and pellets were resuspended in 200 µL 1X PBS (20% treatment) or RNA*later* (50% and 70% treatments). Resuspended pellets were stored at -20 °C for <12 hours prior to nucleic acid extraction using the methods previously described.

A 50% sucrose cushion combined with a 90 minute ultracentrifugation spin time resulted in the highest concentration of recovered crAssphage markers and was thus used for subsequent optimization and wastewater sample processing until it was clear shorter centrifuge times resulted in approximately the same recovery of viral nucleic acids.

Table S1. Recovery of crAssphage DNA under different sucrose concentrations and centrifugation times.

Sucrose Concentration	Spin Time (Minutes)	Replicate	Mean DNA Copies crAssphage /mL Initial WW Source (+/- SD)
20%	20	1	1.95E4 (4.40E2)
20%	20	2	2.53E4 (5.95E2)
50%	90	1	9.04E4 (3.56E3)
50%	90	2	1.27E5 (1.88E3)
75%	150	1	3.26E4 (3.41E2)
75%	150	2	7.52E4 (8.55E2)

50% Sucrose Concentration Centrifuge Time Assessment

Further optimization was performed by assessing varying spin times with a 50% sucrose cushion (Table S2). Six samples were prepared using the methods previously described, with the omission of the initial 2,000 x g clarification spin. Pellets were generated by ultracentrifugation at 4 °C at 150,000 x g for 30, 45, and 75 minutes (2 replicates per centrifuge time). Pellets were examined for physical differences (Figure S1) under the assumption that darker pellets indicated a greater concentration of impurities in the sample. Pellets were

resuspended in 200 µL PBS (1X) and stored at -20 °C for <12 hours prior to nucleic acid extraction and cDNA generation using the methods previously described.

A 45 minute centrifugation time resulted in the highest concentration of recovered crAssphage cDNA markers and approximately the same amount of crAssphage DNA markers as longer spins. Due to these recovery data, and the benefit of increased processing throughput provided by a shorter spin time, wastewater samples were purified with 45 minutes of ultracentrifugation starting on May 13th.

Table S2. Recovery of crAssphage from the pellet after 30, 45, and 75 minute centrifugation times.

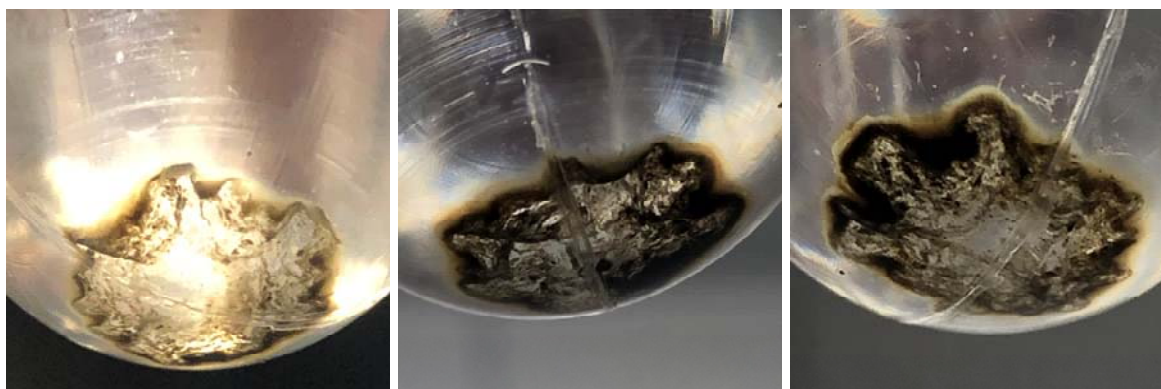| Spin Time (Minutes)	Replicate	Mean DNA Copies crAssphage /mL Initial WW Source (+/- SD)	Mean cDNA Copies crAssphage /mL Initial WW Source (+/- SD)
30	1	6.74E4 (1.57E3)	1.40E1 (3.68E0)
30	2	7.21E4 (4.47E3)	<LOQ
45	1	9.89E4 (4.16E3)	3.46E1 (6.72E0)
45	2	9.90E4 (2.82E3)	3.42E1 (1.36E1)
75	1	9.39E4 (5.86E3)	<LOQ
75	2	1.19E5 (3.80E3)	1.14E1 (5.77E0)

Figure S1. Pellets generated by 30 (left), 45 (center), and 75 (right) minutes of ultracentrifugation at 150,000 x g. Darker regions may indicate that longer spin times resulted in more impurities being pelleted.

Quantification of crAssphage using SYBR Green Chemistry

For optimization of our sample processing approach, SYBR Green qPCR was used to assess recovery of crAssphage markers. Reactions consisted of 12.5 µL TaqMan Environmental MasterMix, 1 µM CPQ_056 primers, 0.25 µL 10X SYBR Green dye, molecular grade water, and 2 µL template DNA for a total reaction volume of 25 µL. Thermal cycling conditions were 10 minute at 95 °C, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute followed by a melt curve. A standard curve, consisting of purified amplicons ranging from 1×10^6 to 5 copies/reaction, was used to convert Ct values to gene copies per reaction (Table S3).

Table S3. qPCR assay performance for CPQ_056 with SYBR Green detection chemistry

Assay	Detection Chemistry	R^2	Intercept	Slope	Efficiency
CPQ_056	SYBR Green	0.997	35.626	-3.343	0.99

Bioanalyzer Results – May 6 Flow
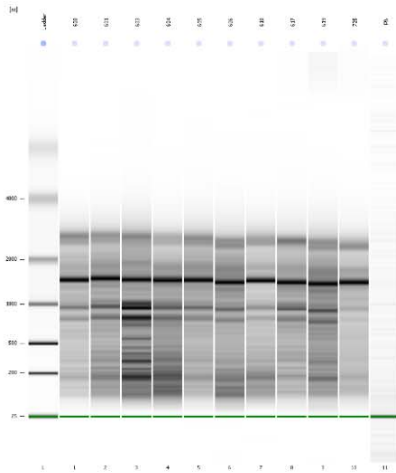
Overall Results for sample 1 : 600
RNA Area: 9,396.5
RNA Concentration: 25,363 pg/µl
rRNA Ratio [28s / 18s]: 1.0
RNA Integrity Number (RIN): 5.1 (B.02.08)

Overall Results for sample 2 : 601
RNA Area: 13,559.3
RNA Concentration: 36,599 pg/µl
rRNA Ratio [28s / 18s]: 0.7
RNA Integrity Number (RIN): 4.3 (B.02.08)

Overall Results for sample 3 : 603
RNA Area: 10,159.0
RNA Concentration: 27,421 pg/µl
rRNA Ratio [28s / 18s]: 0.8
RNA Integrity Number (RIN): 3.6 (B.02.08)

Overall Results for sample 4 : 604
RNA Area: 20,326.6
RNA Concentration: 54,866 pg/µl
rRNA Ratio [28s / 18s]: 0.6
RNA Integrity Number (RIN): 3.8 (B.02.08)

Overall Results for sample 5 : 605
RNA Area: 7,841.5
RNA Concentration: 21,166 pg/µl
rRNA Ratio [28s / 18s]: 0.7
RNA Integrity Number (RIN): 4.3 (B.02.08)

Overall Results for sample 6 : 606
RNA Area: 7,838.8
RNA Concentration: 21,159 pg/µl
rRNA Ratio [28s / 18s]: 0.1
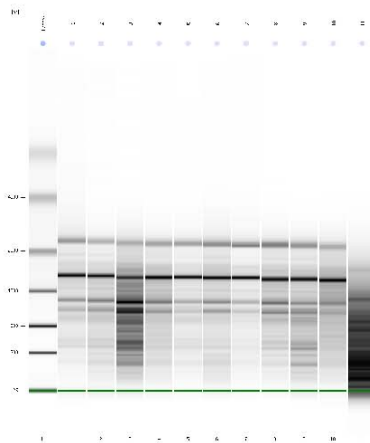RNA Integrity Number (RIN): 3 (B.02.08)

Overall Results for sample 7 : 610
RNA Area: 6,175.2
RNA Concentration: 16,668 pg/µl
rRNA Ratio [28s / 18s]: 0.6
RNA Integrity Number (RIN): 5 (B.02.08)

Overall Results for sample 8 : 617
RNA Area: 9,679.5
RNA Concentration: 26,127 pg/µl
rRNA Ratio [28s / 18s]: 0.7
RNA Integrity Number (RIN): 4.8 (B.02.08)

Overall Results for sample 9 : 619
RNA Area: 5,267.9
RNA Concentration: 14,219 pg/µl
rRNA Ratio [28s / 18s]: 1.0
RNA Integrity Number (RIN): 3.5 (B.02.08)

Overall Results for sample 10 : 725
RNA Area: 7,396.4
RNA Concentration: 19,964 pg/µl
rRNA Ratio [28s / 18s]: 0.7
RNA Integrity Number (RIN): 5.2 (B.02.08)

Bioanalyzer Results – May 13 Flow (with DNAse)

Overall Results for sample 1 : 1
RNA Area: 204.2
RNA Concentration: 742 pg/µl
rRNA Ratio [28s / 18s]: 0.5
RNA Integrity Number (RIN): 2 (B.02.08)

Overall Results for sample 2 : 2
RNA Area: 319.8
RNA Concentration: 1,162 pg/µl
rRNA Ratio [28s / 18s]: 0.3
RNA Integrity Number (RIN): 3 (B.02.08)

Overall Results for sample 3 : 3
RNA Area: 533.2
RNA Concentration: 1,938 pg/µl
rRNA Ratio [28s / 18s]: 0.9
RNA Integrity Number (RIN): 2.5 (B.02.08)

Overall Results for sample 4 : 4
RNA Area: 632.6
RNA Concentration: 2,299 pg/µl
rRNA Ratio [28s / 18s]: 0.3
RNA Integrity Number (RIN): 5.8 (B.02.08)

Overall Results for sample 5 : 5
RNA Area: 440.0
RNA Concentration: 1,599 pg/µl
rRNA Ratio [28s / 18s]: 0.4
RNA Integrity Number (RIN): 1.6 (B.02.08,

Overall Results for sample 6 : 6
RNA Area: 277.2
RNA Concentration: 1,008 pg/µl
rRNA Ratio [28s / 18s]: 0.5
RNA Integrity Number (RIN): 1.7 (B.02.08,

Overall Results for sample 7 : 7
RNA Area: 409.3
RNA Concentration: 1,488 pg/µl
rRNA Ratio [28s / 18s]: 0.7
RNA Integrity Number (RIN): 1.6 (B.02.08,

Overall Results for sample 8 : 8
RNA Area: 761.3
RNA Concentration: 2,767 pg/µl
rRNA Ratio [28s / 18s]: 0.8
RNA Integrity Number (RIN): 5.8 (B.02.08)

Overall Results for sample 9 : 9
RNA Area: 686.2
RNA Concentration: 2,494 pg/µl
rRNA Ratio [28s / 18s]: 0.5
RNA Integrity Number (RIN): 5.7 (B.02.08)

Overall Results for sample 10 : 10
RNA Area: 803.3
RNA Concentration: 2,920 pg/µl
rRNA Ratio [28s / 18s]: 0.3
RNA Integrity Number (RIN): 5.4 (B.02.08)

Overall Results for sample 11 : 11
RNA Area: 2,522.5
RNA Concentration: 9,169 pg/µl
rRNA Ratio [28s / 18s]: 0.0
RNA Integrity Number (RIN): 2.2 (B.02.08)

Figure S2. Nucleic acid quality and quantity from May 6 and May 13 samples.