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CrAssphage abundance and correlation with molecular viral markers in Italian wastewater

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ABSTRACT

Current fecal indicators for environmental health monitoring are primarily based on fecal indicator bacteria (FIB) which do not accurately represent viral pathogens. There is a need for highly abundant, human-associated viral fecal indicators to represent viral pathogens in sewage-contaminated water. In the present study, we evaluate the abundance of the emerging viral fecal indicator crAssphage in 156 Italian wastewater samples collected between 2014 and 2018. Samples were collected using two separate viral concentration methods, glycine-CF and PEG−dextran and qPCR assays were run for crAssphage (CPQ56) and Human Polyomavirus (HPyV) and endpoint PCR assays were run for Human Bocavirus (HBoc) and Hepatitis E Virus (HepE). CrAssphage was detected in 96% of samples and no statistically significant difference was observed in crAssphage abundance between concentration methods ($p = 0.39$). CrAssphage concentrations also did not correlate with location (latitude) or size (load and capacity) of the wastewater treatment plant. HPyV detection rates with the glycine-CF and PEG−dextran methods were 64% and 100%, respectively, and the concentrations of HPyV were statistically significantly influenced by the concentration method ($p < 0.0001$). CrAssphage was measured at significantly higher concentrations than HPyV for both concentration methods ($p < 0.0001$). The observed concentration ranges were 3.84−7.29 $\log_{10}$GC/100 mL for crAssphage and 3.45−5.17 $\log_{10}$GC/100 mL for HPyV. There was a strong positive correlation between crAssphage and HPyV abundance for both concentration methods; however, the slope of the correlation depended on the concentration method. CrAssphage presence correlated with the presence of HBoc in samples concentrated with glycine-CF, but did not correlate with the presence of HBoc concentrated with the PEG−dextran method or with the presence of HepE. Overall, these results demonstrate that crAssphage is an abundant viral fecal indicator in wastewater with statistically significant correlation with human viral pathogens (e.g., HPyV) and viral concentration methods influence the interpretation of fecal viral indicator detection.

1. Introduction

Gastrointestinal diseases resulting from exposure to wastewater-impacted water account for an estimated 842,000 deaths globally per year (WHO, 2014) and viruses are predicted to account for the majority of gastrointestinal infections from exposure to sewage contaminated water (Boehm et al., 2015; Crank et al., 2019; McBride et al., 2013). Current water quality monitoring criteria utilize fecal indicator bacteria (FIB), which do not adequately represent risk from infectious pathogenic viruses. For example, in recreational water exposure scenarios viral outbreaks have occurred when FIB were at or below acceptable levels (Hauri et al., 2005; Rose et al., 1987; Sinclair et al., 2009). Viruses enter the water environment through the release of untreated or poorly treated wastewater; thus, evaluation of potential viral indicators in wastewater is a necessary first step in supporting their use for environmental monitoring. Improved methods to monitor viral pathogens in wastewater impacted waters are vital to informing regulatory agencies and engineering efforts to protect human

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health.

The bacteriophage crAssphage, short for 'cross-assembly phage', was discovered in 2014 in human fecal metagenomes and was an order of magnitude more abundant than previously known gut bacteriophages (Dutilh et al., 2014) and was subsequently suggested as a viral fecal pollution indicator (Stachler and Bibby, 2014b). CrAssphage has a dsDNA genome, an isolate has recently been cultured using *Bacteroides intestinalis* as a host (Shkoporov et al., 2018), and a recent metagenomic survey demonstrated that crAssphage is globally distributed (Edwards et al., 2019). In a 2014 metagenomic study assessing crAssphage relative abundance in sewage from the United States and Europe alongside Pepper Mild Mottle Virus, adenovirus, human polyomavirus, Torque teno virus, and norovirus, crAssphage was found to be significantly more abundant than the other viruses studied (Stachler and Bibby, 2014b). We note that although novel crass-like phages have recently been described (Guerin et al., 2018), here we use 'crAssphage' to refer to prototypical crAssphage (i.e., *p*-crAssphage or crAssphage *sensu stricto*). CrAssphage is abundant in wastewater, which facilitates the detection of low-level wastewater pollution in the environment (Stachler et al., 2017, 2018b, 2019). CrAssphage appears to be human-associated; however, it has been detected in a limited number of seagull, dog, duck, pig and chicken fecal samples (Ahmed et al., 2018a; Malla et al., 2019b; Stachler et al., 2017). CrAssphage has also been successfully deployed to detect fecal pollution in environmental waters in the United States, Australia, the United Kingdom, Nepal, Japan and Thailand (Ahmed et al., 2018b; Farkas et al., 2019; Kongprajug et al., 2019a; Malla et al., 2019a, ; Stachler et al., 2018b, 2019). CrAssphage, along with Pepper Mild Mottle Virus, has also been proposed for application in Quantitative Microbial Risk Assessment to predict the probability of illness from swimming in wastewater polluted water, with the potential to lower the detectable risk threshold by detecting wastewater concentrations that are below the detection limit of FIB-based recreational water quality indicators (Crank et al., 2019). The driving factor in the ability to lower risk thresholds is the high abundance of the studied indicators in wastewater, and it is expected that highly abundant alternative molecular indicators would perform similarly in the studied model. Despite the promise of crAssphage as a viral water quality indicator, large-scale evaluation efforts in wastewater, are necessary to support its continued development and eventual application.

There is a great diversity of viral pathogens that may exist in wastewater as demonstrated by previous metagenomic surveys (Bibby and Peccia, 2013; Bibby et al., 2011). Thus, targeted surveys typically select a subset of potential human pathogens with increased relevance or abundance in wastewater. Human Polyomavirus (HPyV) is a double-stranded DNA virus of the family *Polyomaviridae.* This pathogen does not normally cause symptomatic infections in healthy individuals but can cause serious infections in immunocompromised populations. HPyV occurs globally in wastewater and has been widely proposed as a viral fecal pollution indicator (Albinana-Gimenez et al., 2006; Bofill-Mas et al., 2000, 2013; Cantalupo et al., 2011; Iaconelli et al., 2015). Human Bocavirus (HBoc) is a single-stranded DNA virus of the family *Parvoviridae.* HBoc is an emerging infectious disease first identified in 2005 that can cause both enteric and respiratory infections, having been isolated from both stool samples from patients with gastroenteritis as well as respiratory tract samples of patients with respiratory infections (Allander et al., 2005; Iaconelli et al., 2016). The role of HBoc as a pathogen is still not fully understood as it co-occurs in stool samples with other known viral pathogens (Ong et al., 2016). HBoc is also globally distributed (Bibby et al., 2019; Iaconelli et al., 2016). Hepatitis E virus (HepE) is a RNA virus of the family *Hepeviridae*. This fecal-oral pathogen causes acute liver infections in symptomatic cases, with an estimated 3.3 million symptomatic cases of HepE reported in 2005 (WHO, 2019). HepE is also globally distributed (La Rosa et al., 2011).

The ideal viral indicator of human pathogenic viruses has three characteristics: specificity to human wastewater, high abundance in human wastewater, and known geographic variability. Additional considerations also include ease of detection, speed, and cost effectiveness. In this study we address crAssphage abundance in wastewater and geographic variability. Greater understanding of crAssphage quantitative occurrence and abundance across geographic regions was recently highlighted as a research needs to inform crAssphage application for fecal source monitoring (Bivins et al., 2020b). Specifically, we survey 156 previously collected Italian wastewater samples to demonstrate the presence and abundance of crAssphage in Italian wastewater and compare it to the occurrence of three human viral pathogens: HBoc, HepE, and HPyV. These viruses were chosen so as to include a human pathogen that has also been suggested as an indicator, HPyV, an enteric pathogen, HBoc, and an RNA viral pathogen, HepE. This study informs the prevalence of crAssphage in Italian wastewater, the influence of wastewater treatment plant (WWTP) location and size on crAssphage concentration, and the co-occurrence of crAssphage with representative human pathogens in Italian wastewater. Ultimately, this data allows the evaluation of the relationship between crAssphage and human viral pathogens, as well as the abundance of crAssphage across Italy, which furthers the application of crAssphage as a viral fecal pollution indicator.

2. Materials and methods

2.1. Sample collection

Grab samples of wastewater influent were collected as a part of a large sampling effort from 25 WWTPs across Italy in 2014 ($n = 43$), 2015 ($n = 34$), and 2016 ($n = 49$). Composite samples representing a 24hr period of time were collected in 2017 ($n = 26$), and 2018 ($n = 4$) as previously described (Iaconelli et al., 2016). Sampling locations are shown in Fig. 1. Sample details are available in Table S1.

2.2. Sample concentration and DNA extraction

Samples collected between 2014 and 2016 were concentrated and extracted as previously described (La Rosa et al., 2014), which we refer to as the glycine-CF method. Briefly, a 20 mL aliquot of wastewater was treated with 2 mL of 2.5M glycine at pH 9.5 and incubated on ice for 30 min. The solution was then treated with 2.2 mL chloroform and centrifuged at 2300 g for 10 min. The viral nucleic acids were extracted from 10 mL of chloroform-treated samples using the NucliSENS miniMAG semi-automated extraction system with magnetic silica, according to the manufacturer's instructions (BioMerieux, Marcy l'Etoile, France). 2017–2018 samples were concentrated using a two-phase separation as detailed in the 2003 WHO Guidelines for Environmental Surveillance of Poliovirus protocol and will be referred to as the PEG-dextran method (WHO, 2003). In brief, the wastewater sample (250 mL) was centrifuged to pellet the wastewater solids. The pellet was stored at 4 °C to be combined with the concentrated supernatant. The clarified wastewater was mixed with dextran T40 and polyethylene glycol (PEG) 6000 and the mixture was left to stand overnight at 4 °C in a separation funnel. The bottom layer and the interphase were then collected drop-wise. The pellet from the initial centrifugation was suspended in this concentrate, which was then treated with 1:20 (v/v) chloroform. ~10 mL of clear supernatant was recovered, and 5 mL was subjected to extraction. Eluates

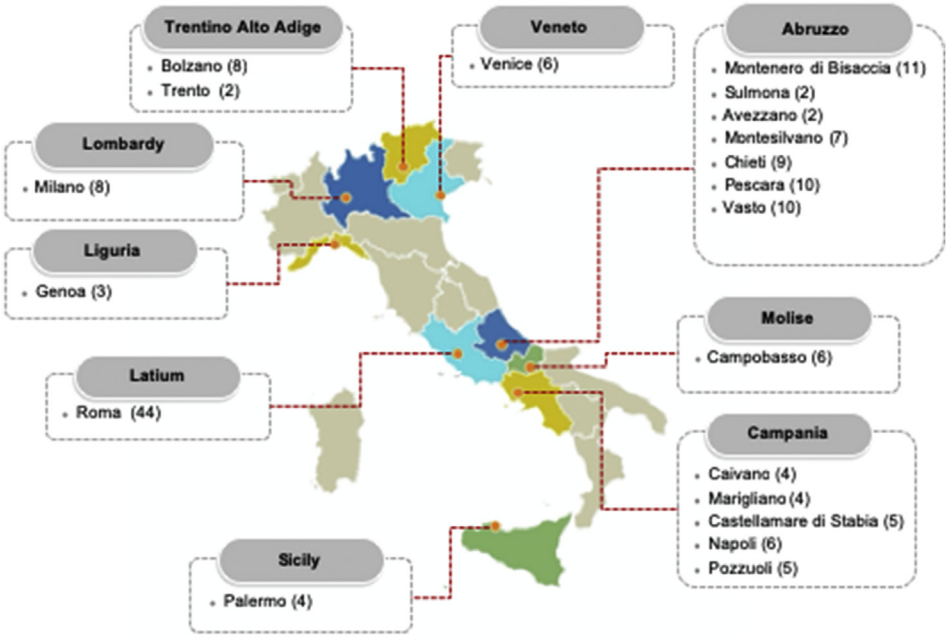

Fig. 1. Sampling locations by administrative region. Names of the municipalities are noted with the number of samples collected.

(100 μl each) were divided into small aliquots and subsequently frozen at −70 °C until analyzed. The samples were tested using endpoint PCR for the presence/absence of HBoc and HepE and were quantitatively tested for crAssphage and HPyV using qPCR. For the PEG-dextran concentration method, samples underwent spiking with a known quantity of murine norovirus (MNV-1), used as a control process in order to monitor the efficiency of the entire procedure. Recovery efficiency, calculated on selected samples, showed an average MNV recovery of 1.5%. Recovery efficiency was not calculated for the glycine-CF method.

2.3. Endpoint PCR

PCR assay information is shown in Table 1. HBoc presence/absence was determined with nested PCR. PCR was performed using 2 μL of DNA and 22 pmol of each primer in a 25 μL reaction. Cycling profile included an initial denaturing at 94 °C (10 min), followed by 40 cycles of denaturing at 94 °C (30 s), annealing at 50 °C (30 s),72 °C (1 min), a final extension at 72 °C (5 min), and a hold at 4 °C. Two microliters of the PCR product were used as a template in the nested PCR assay, under the same cycling conditions. HepE presence/absence was determined with a nested RT-PCR assay targeting the Methyltransferase (Mtase) gene in ORF1. The universal primers amplify a 172 bp region of all HepE genotypes (Fogeda et al., 2009). PCR amplification was performed using a MyTaq One-Step RT-PCR Kit (Bioline) in a T100 Thermal Cycler (Bio-Rad). PCR reactions were prepared in a 25 μL mixture containing 12.5 μL of PCR One Step Mix, 1 μL (10 pmol) of each primer, 2 μL of extracted RNA, and 9.5 μL molecular water. PCR cycling conditions included reverse transcription at 45 °C (20 min), 95 °C (1 min), followed by 40 cycles of 95 °C (10 s), 51 °C (10 s), and 72 °C (30 s), and a final step at 72 °C (5 min). After the first round of PCR amplification, one μL of PCR product underwent nested amplification (35 cycles), performed using MyTaq red mix kit (Bioline). Cycling profile included an initial denaturing at 95 °C (1 min), followed by 35 cycles of 95 °C (15 s), 48 °C (15 s),72 °C (10 s), and a final elongation step at 72 °C (5 min).

2.4. qPCR standard curves

Primers and probes for Human Polyomavirus (HPyV) and crAssphage (CPQ56) are shown in Table 1. Synthetic DNA strands

Table 1
Primers and probes used for qPCR and PCR assays.

Target	Type	Primer	Primer sequence (5′–3′)	Reference
Human Bocavirus (general)	Endpoint PCR	2028 (+)	GAAATGCTTTCTGCTGYTGAAAG	Iaconelli et al. (2016)
		2029 (−)	GTGGATATACCCACAYCAGAA	
		2030 (+)	GGTGGGTGCTTCCTGGTTA	
		2031 (−)	TCTTGRATTTCATTTTCAGACAT	
Hepatitis E Virus	Endpoint PCR	ORF1F (+)	CCAYCAGTTYATHAAGGCTCC	Fogeda et al. (2009)
		ORF1R (−)	TACCAVCGCTGRACRTC	
		ORF1FN (+)	CTCCTGGCRTYACWACTGC	
		ORF1FR (−)	GGRTGRTTCCAIARVACYTC	
CrAssphage (CPQ56)	qPCR	056F1	CAGAAGTACAAACTCCTAAAAAACGTAGAG	Stachler et al. (2017)
		056R1	GATGACCAATAAACAAGCCATTAGC	
		056P1	[FAM] AATAACGATTTACGTGATGTAAC [MGB]	
Human Polyomavirus	qPCR	SM2	AGTCTTTAGGGTCTTCTACCTTT	Staley et al. (2012)
		P6	GGTGCCAACCTATGGAACAG	
		KGJ3	[FAM] TCATCACTGGCAAACAT [MGBNFQ]	

Table 2
qPCR parameters for assays used in this study.

Assay	Slope	Y-Intercept	*E*	R^2	LLOQ (Cq)
CrAssphage (CPQ56)	−3.604	42.669	0.894	0.989	39.539
Human Polyomavirus	−3.475	42.165	0.940	0.996	39.172

LLOQ, lower limit of quantification. The LLOQ is displayed as the highest Cq (qPCR cycle number) that allowed quantification.

were synthesized as gBlocks Gene Fragments (Integrated DNA Technologies, Coralville, IA). The fragments were suspended in 1× TE buffer (Affymetrix, Cleveland, OH) and diluted to concentrations of 10, 10^2, 10^3, 10^4, 10^5 GC/2 μL using a NanoDrop ND-1000 UV spectrophotometer (NanoDrop Technologies, Wilmington, DE). Three independent calibration standard runs were performed to generate an average calibration standard curve for each of the assays. Lower limit of quantification (LLOQ, 95% CI with 10 GC/2 μL) values were calculated using Rstudio (Version 1.1.442) by assuming the corresponding quantification cycle (Cq) at 10 GC/2 μL was normally distributed. The LLOQ for the CPQ56 assay corresponded to 3.86 $\log_{10}$GC/100 mL and 2.77 $\log_{10}$GC/100 mL for the glycine-CF and PEG–dextran methods respectively. The LLOQ for the HPyV assay corresponded to 3.86 $\log_{10}$GC/100 mL and 2.76 $\log_{10}$GC/100 mL for the glycine-CF and PEG–dextran methods respectively. qPCR performance standards are shown in Table 2.

2.5. qPCR assays

One quarter of the volume of extracted DNA from all samples was lyophilized for sample shipment. Samples were rehydrated in 50 μL TE buffer and stored at −20 °C before analysis. Each 25 μL qPCR reaction contained 1× TaqMan Environmental Master Mix (Thermo Fisher Scientific), 0.2 mg/mL bovine serum albumin (Sigma-Aldrich, St. Louis, MO), 1 μM of each primer, 80 nM of the 6-carboxyfluorescein (FAM)-labeled probe, molecular water, and 2 μL of DNA (calibration standards or extracted DNA) (Li et al., 2019). HPyV samples below the LLOQ were re-run with 6 μL of extracted DNA, and molecular water was reduced accordingly to maintain a 25 μL reaction volume and the resulting data was normalized accordingly. All qPCR reactions were performed in triplicate on a CFX96 Touch™ Real-Time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA) with the RFU (relative florescent units) manually set to 100 for both assays (Cao et al., 2015). The thermocycling conditions for all reactions were as follows: initial denaturation for 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s, with a final annealing and elongation step for 1 min at 60 °C. For each run, six no template controls (NTCs) were included. A detect was defined as amplification of one or more of the three replicates. Concentrations were calculated from the standard curve in Microsoft Excel (Redmond, CA) as described previously (Stachler et al., 2018b). Three samples were excluded from the HPyV assay due to laboratory handling issues.

2.6. Statistical analysis

qPCR concentrations were calculated from raw data using Microsoft Excel. The results were then exported to GraphPad Prism for descriptive statistics (GraphPad Software, La Jolla, CA). The results were not normally distributed, so a non-parametric approach was taken by using the Mann-Whitney *U* test with GraphPad Prism. Linear regressions and Spearman's rank analyses were performed between the qPCR concentrations and latitude, capacity of the WWTP, and load. These analyses were also performed using GraphPad Prism. The level of significance was alpha = 0.05.

3. Results

3.1. Abundance of crAssphage and HPyV in wastewater

In this study, 156 previously collected DNA samples from untreated wastewater were analyzed for crAssphage (CPQ56) and Human Polyomavirus (HPyV) molecular markers. Two separate viral concentration methods, namely the glycine-CF and PEG–dextran methods, were used during the sample collection period. Samples collected between 2014 and 2016 were concentrated with the glycine-CF method (n = 126) and samples collected between 2017 and 2018 were concentrated with the PEG–dextran method (n = 30). Sample concentration data for positive crAssphage and HPyV detections is shown in Fig. 2.

CrAssphage detection rates with the glycine-CF and PEG–dextran concentration methods were 95% (n = 120/126) and 100% (n = 30/30), respectively, with a combined detection rate of 96%. HPyV detection rates with the glycine-CF and PEG–dextran methods were 64% (n = 79/123) and 100% (n = 30/30), respectively. Positive detection concentration ranges were 3.84–7.29 $\log_{10}$GC/100 mL for crAssphage and 3.45–5.17 $\log_{10}$GC/100 mL for HPyV. The mean concentrations of crAssphage were 5.62 ± 0.74 $\log_{10}$GC/100 mL and 5.72 ± 0.71 $\log_{10}$GC/100 mL for the glycine-CF and PEG–dextran methods, respectively. No statistically significant difference was observed in crAssphage concentration between concentration methods ($p = 0.38$) (Fig. 2a). The mean concentrations of HPyV were 4.13 ± 0.38 $\log_{10}$GC/100 mL and 4.89 ± 0.72 $\log_{10}$GC/100 mL for the glycine-CF and PEG–dextran methods, respectively. The concentrations of HPyV between the glycine-CF and the PEG–dextran methods were significantly different ($p < 0.0001$) (Fig. 2b). CrAssphage concentrations were significantly higher than HPyV for both concentration methods ($p < 0.0001$, glycine-CF; $p < 0.0001$, PEG–dextran).

3.2. WWTP location, capacity, load, and abundance of viral markers

The relationship between the WWTP location as measured by latitude and virus concentration was examined using Spearman's rank analysis to investigate a potential relationship between WWTP location and the abundance of crAssphage and HPyV (Fig. 3). Latitude was selected as a surrogate of WWTP location as latitude has been previously suggested to be a driven of human gut microbiome diversity (Dikongué and Ségurel, 2017). No statistically significant correlations were observed between either crAssphage or HPyV concentration and the latitude of the WWTP. In addition, we investigated correlations between crAssphage or HPyV concentration and the size of the WWTP. The capacity of the WWTP in liters served as a surrogate measure of maximum population that can be served, and load in liters served as a surrogate measure of the population being served at time of sampling. Neither measure demonstrated a statistically significant correlation with the concentration of crAssphage or HPyV. All p-values generated by the Mann-Whitney non-parametric statistical test are shown in Fig. 3.

3.3. CrAssphage correlation with molecular viral markers

We further evaluated the correlation between crAssphage and the concentration of HPyV using Spearman's rank analysis and a linear regression. Since concentration method was found to affect HPyV concentrations, the comparison was separated by concentration method. Fig. 4 shows the results of a linear regression with Spearman's rank and significance levels. There was a strong positive correlation between crAssphage and HPyV abundance for both concentration methods (Fig. 4); however, the slope for the correlation with the glycine-CF concentration method was 0.43, whereas

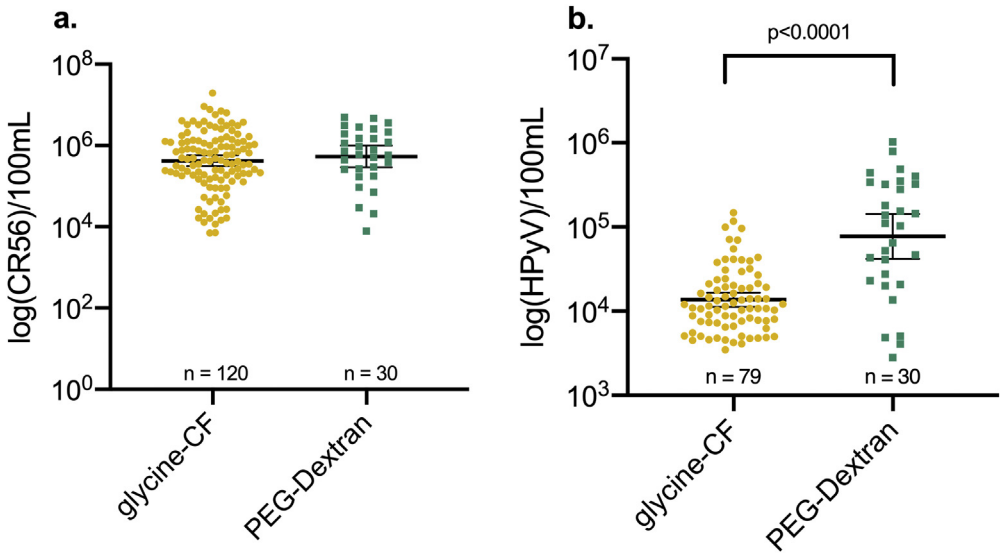

Fig. 2. Concentrations of (a) crAssphage and (b) HPyV concentrated using glycine-CF and PEG−dextran methods. Only positive detections within the quantifiable range are shown. The black horizontal line represents the mean and whiskers represent the 95% confidence interval.

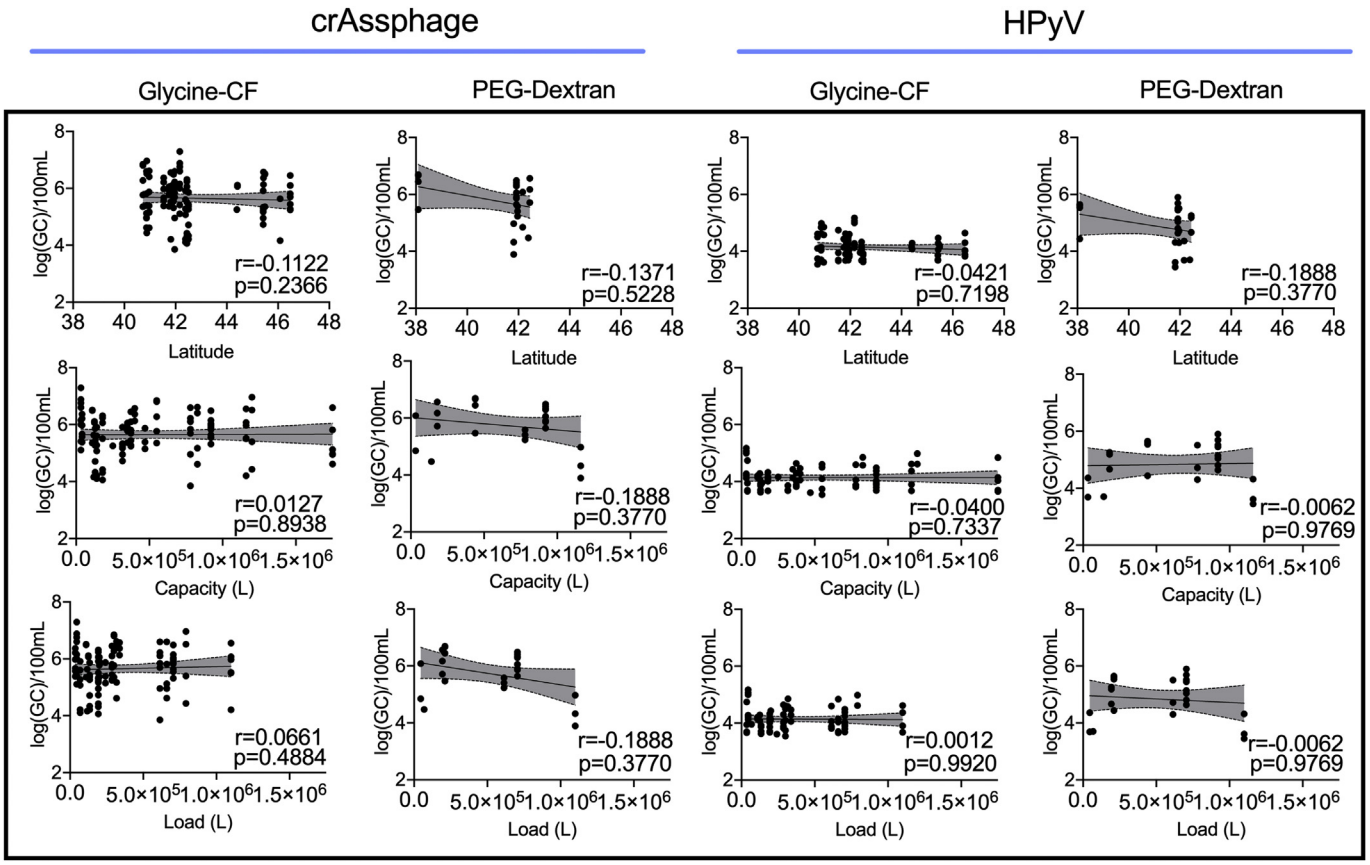

Fig. 3. Concentrations of crAssphage and HPyV versus latitude, load, and capacity of the WWTP. Spearman's rank correlation coefficient (r) and significance (p) are shown.

the slope for the correlation with the PEG−dextran concentration method was 0.89. This demonstrates the importance of sample concentration method in the interpretation of the correlation between crAssphage and HPyV.

We also evaluated the relationship between crAssphage concentration and presence/absence of HBoc and HepE as measured by endpoint PCR. We note that endpoint PCR assays were not carried out on all samples; samples without endpoint PCR measurements were excluded from this analysis. Comparisons were separated by concentration method as the influence of concentration method on HBoc and HepE detection is unknown.

Fig. 5a shows the concentrations of crAssphage for HBoc positive

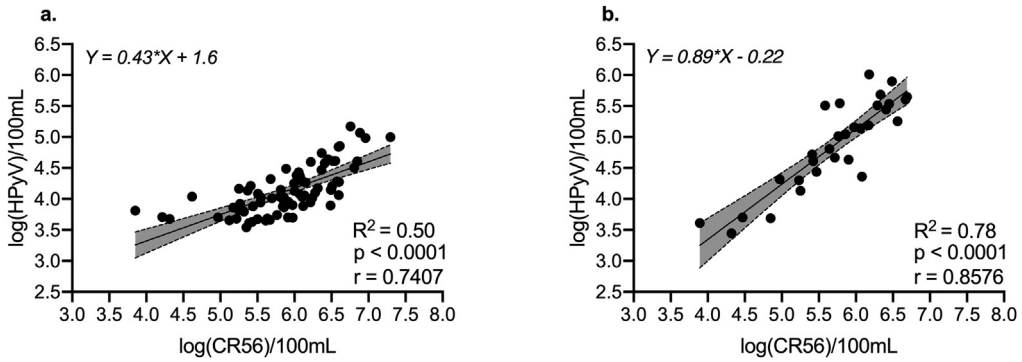

Fig. 4. Linear regression of HPyV molecular viral marker and crAssphage separated by (a) glycine-CF (n = 79) and (b) PEG–dextran (n = 30) concentration methods. The linear regression equations are as shown. Spearman's rank correlation coefficient (r), significance of the Spearman's test (p), and the linear regression R^2 are shown.

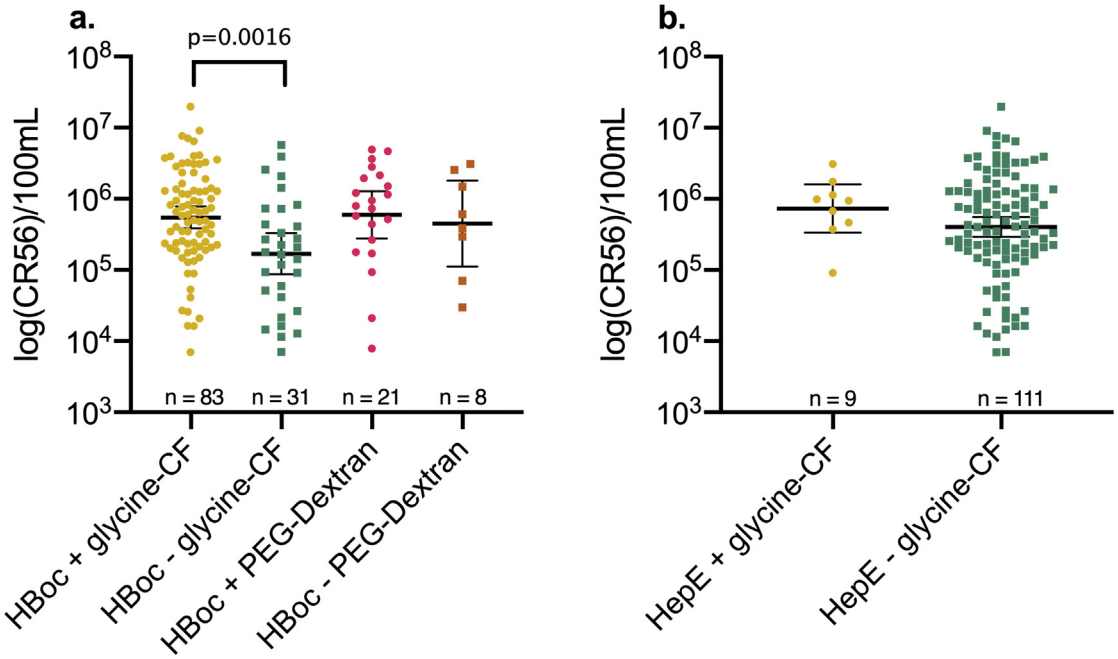

Fig. 5. Concentrations of crAssphage molecular viral marker for which (a) HBoc and (b) HepE are measured as either present or absent. HBoc is separated by concentration method, HepE is only shown for glycine-CF. The black horizontal line represents the mean and whiskers represent the 95% confidence interval.

and HBoc negative samples. HBoc detection rates with the glycine-CF and PEG–dextran methods were 72.8% (n = 83/114) and 72.4% (n = 21/29), respectively, with a combined detection rate of 72.7%. For samples that were concentrated using the glycine-CF method, there was a statistically significant increase (p = 0.0016) in crAssphage concentrations in HBoc positive samples than in HBoc negative samples. This was not observed in samples concentrated with the PEG-dextran method, which showed no statistically significant difference (p = 0.65) in crAssphage concentrations between HBoc positive and negative samples.

Fig. 5b shows the concentrations of crAssphage for HepE positive and HepE negative samples concentrated with the glycine-CF method. We note that this comparison was not possible for samples concentrated using the PEG–dextran method as there was a single HepE positive sample in that dataset. HepE detection rates with the glycine-CF and PEG–dextran methods were 7.5% (n = 9/120) and 3.3% (n = 1/30), respectively, with a combined detection rate of 6.7%. CrAssphage concentration was not statistically significantly different between HepE positive and negative samples for samples concentrated using the glycine-CF method (p = 0.32).

4. Discussion

4.1. CrAssphage presence and abundance

Prospective viral human fecal source tracking organisms must be highly abundant in wastewater to facilitate their detection once released and diluted in the environment. In the present study, we assess crAssphage abundance in 156 Italian wastewater samples, as well as crAssphage correlation with other molecular viral markers. CrAssphage was present in 150 out of 156 samples, with a 96% overall detection rate. This is notable, as prior studies have shown a 100% crAssphage detection rate in untreated wastewater (Ahmed et al., 2018a, 2018b; García-Aljaro et al., 2017; Stachler et al., 2017).

The current study has significantly expanded the number of described crAssphage concentration samples in untreated wastewater, summarized in Fig. 6 (Farkas et al., 2019; Kongprajug et al., 2019a) (Wu et al., 2020) (García-Aljaro et al., 2017; Malla et al.,

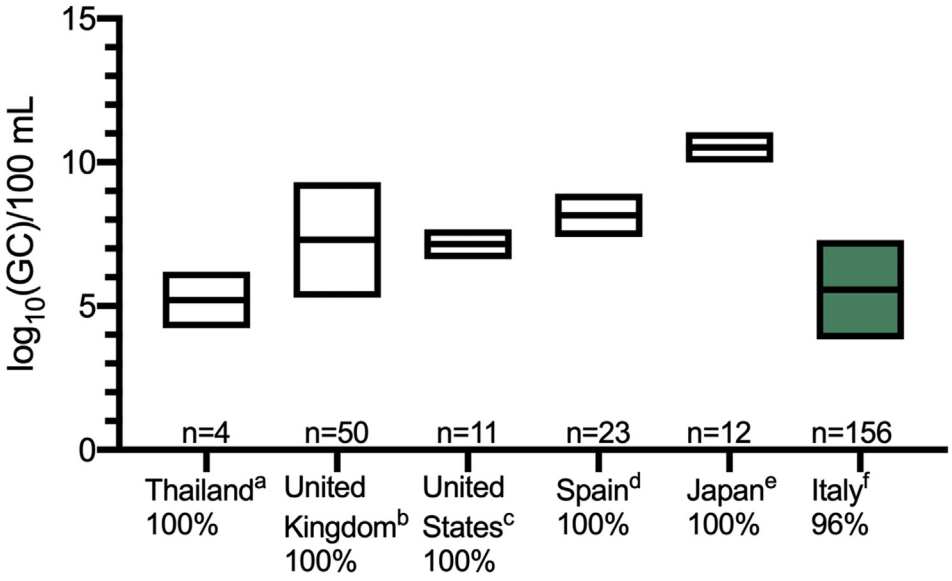

Fig. 6. Comparative ranges of crAssphage concentrations in wastewater from a selection of global studies. The center line indicates the median. Green is the current study. Percentages represent percent of samples positive for crAssphage. [a](Kongprajug et al., 2019a), [b](Farkas et al., 2019), [c](Wu et al., 2020), [d](García–Aljaro et al., 2017). This study used primers targeting a different genomic region than the rest of the studies visualized here, [e](Malla et al., 2019a), [f]The current study. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2019a). CrAssphage concentrations in crAssphage-positive samples in the current study ranged between 3.84 and 7.29 $\log_{10}$GC/100 mL, a wider range than previous studies. A recent study in the United States examining fecal source tracking marker removal efficiencies of a WWTP found 100% of untreated wastewater samples (n = 11) were positive for crAssphage at an average of 7.23 ± 0.36 $\log_{10}$GC/100 mL (Wu et al., 2020). Using the same crAssphage assay, Ahmed et al. found that 100% of tested United States wastewater samples (n = 8) were positive for crAssphage with a range of 6.08–6.98 $\log_{10}$ GC/100 mL (Ahmed et al., 2018a). Similarly, 100% of Australian tested raw wastewater samples (n = 12) were positive for crAssphage using the same assay, with an average of 6.43 ± 0.14 $\log_{10}$GC/100 mL (Ahmed et al., 2018b). In Spain, primers targeting a different crAssphage genomic region were utilized on 23 samples of wastewater and all were positive for crAssphage at a range of 5.4–6.9 $\log_{10}$GC/100 mL (García-Aljaro et al., 2017). In Thailand, 100% of wastewater samples (n = 23) were positive for crAssphage with concentrations ranging from 5.28 to 7.38 $\log_{10}$GC/100 mL. crAssphage concentrations reported here are similar to previously reported concentrations of alternative human fecal indicators adenovirus and F-specific coliphage in untreated wastewater. Pouillot et al. performed a meta-analysis of F-specific coliphage concentrations in untreated wastewater across 62 WWTPs and found the average concentration to be 5.2 $\log_{10}$ PFU/100 mL (95% CI; 5.1, 6.4 $\log_{10}$ PFU/100 mL) (Pouillot et al., 2015). In 2019, Verani et al. found adenovirus concentrations in wastewater at an average of 7.78 ± 1.19 $\log_{10}$ GC/100 mL with 100% of samples testing positive(Verani et al., 2019). Ultimately, when compared to culturable bacteriophages, crAssphage as a molecular indicator has the strong advantage of being more rapid, human specific, and more strongly associated with human pathogens (Bivins et al., 2020).

In this study, we demonstrate a lower detection rate and a wider range of crAssphage concentration in Italian wastewater than has been shown previously. This may be due to multiple factors. First, the scope of this study vastly exceeds other similar studies using direct PCR measurement of viral markers and pathogens in wastewater. The expanded scope, encompassing five years of sampling efforts, may have captured natural variability in the crAssphage concentration that was not observed in other studies. Second, we have previously identified variability in concentration due to the quantification method (Stachler et al., 2019) – a multi-laboratory effort would be useful to exclude potential variability due to laboratory handling or quantification approach. This variability may also extend to the application of emerging viral quantification approaches and additional efforts are necessary to establish concordance between concentration methods (Farkas et al., 2020; Hamza and Bibby, 2019). Third, differing studies use variable concentration approaches which may influence observed crAssphage concentrations. Fourth, this study uses historical samples. While every effort was made for sample preservation, the possibility that some 'negative detections' were due to sample processing cannot be excluded. It should be noted that 'negative detections' may also include samples containing crAssphage but with concentrations below the study detection limit. Finally, recent studies have highlighted the diversity of crAss-like phages in human gut metagenomes. It is possible that natural variability in crAss-like phages was not captured by the CPQ56 assay used here, which is targeted at the prototypical crAssphage. Ultimately, while further data would be beneficial to confirm these observations, this study at a minimum suggests caution when assuming the ubiquitous presence of crAssphage in wastewater.

4.2. Geographic and population variation

Fecal pollution indicators may vary geographically and based on the population size served, as lifestyles and diets affect the makeup of the gut microbiome (Kau et al., 2011). Examining the geographic and population distribution of prospective viral indicator crAssphage can facilitate its implementation across geographically diverse areas. Geographic variability was measured by latitude and population size was represented by WWTP capacity and load. None of these parameters had a statistically significant effect on crAssphage abundance.

Most WWTPs represented in this study are in populous, urban areas, and so results presented here are representative of a large mixture of diets and potential fecal pathogens in wastewater. We

note that local verification is necessary for application in new geographic areas, especially less populous areas. Future research should aim to assess crAssphage and human viral pathogen concentrations in wastewater from communities with non-westernized diets or lifestyles.

4.3. CrAssphage correlation with viral markers

Viral human fecal indicators must correlate with viral pathogens in wastewater to adequately represent the viral risk posed by human fecal pollution. In this study, crAssphage concentration was compared with HPyV concentration and HBoc and HepE presence.

HPyV abundance correlated with crAssphage abundance for both concentration methods ($p < 0.0001$). CrAssphage was also measured at significantly higher concentrations than HPyV ($p < 0.0001$), suggesting that measuring crAssphage in a dilute environment (e.g. water contaminated by wastewater) as an indicator is more favorable than directly measuring HPyV. This is consistent with a report of crAssphage concentration correlating with HPyV through a WWTP (Wu et al., 2020). Conversely, a study examining a wastewater-impacted stream found poor crAssphage and HPyV correlation, suggesting that further work may be necessary to examine the comparative fates of crAssphage and HPyV in the environment (Stachler et al., 2018b).

CrAssphage concentrations were significantly higher in samples concentrated with the glycine-CF method where HBoc was detected, suggesting that crAssphage abundance correlates with HBoc presence. CrAssphage concentration did not correlate with HepE presence. Thus, crAssphage correlates with the DNA viruses evaluated in this study (HPyV and HBoc) but not the RNA virus evaluated (HepE). We note that our ability to make conclusions about the suitability of crAssphage as an indicator for HepE was limited by the low number of samples in which HepE was tested positive ($n = 9$). Further work is necessary to evaluate the suitability of crAssphage as an indicator for HepE and other RNA viruses. Additionally, future work should study other commonly established human fecal indicators, alongside crAssphage, to assess which indicators perform strongest for individual pathogens and pathogen groupings.

4.4. Importance of concentration method

The results of this study highlight the importance of concentration method when quantifying viruses in wastewater, similar to recent reports on norovirus and adenovirus (Maunula et al., 2019). CrAssphage concentrations did not vary significantly by concentration method; however, HPyV did show a significant difference in abundance between concentration methods ($p < 0.0001$). The differing behavior of viral targets by concentration method implies that viral fecal indicator suitability is dependent on the viral concentration method used. Assessing the efficiency of specific concentration methods is outside the scope of this study; however, more research is needed to determine which concentration methods best fits each scenario. Perhaps more importantly, the specific concentration method should be considered when evaluating viral indicator suitability, as performance metrics may vary based upon the specific concentration method used. The two methods used in this study were the glycine-CF and PEG-dextran methods. The glycine-CF method has the advantage of being faster and requires a low amount of wastewater (20 mL), which is good for limited samples. However, the PEG-dextran method, while more laborious and time consuming, is the standard concentration method recommended by WHO for viral sewage monitoring. There are tradeoffs when determining a viral concentration method. Researchers must consider downstream applications, quantity of available wastewater sample, target, available laboratory resources, and the time it takes to concentrate. For previous viral molecular experimentation, the ideal concentration method has been determined as they are needed, rather than referring to a collective resource of concentration methods (Bivins et al., 2020). However, two recent reviews have examined viral concentration methods. Hjelmsø et al. examine viral concentration methods in the context of metagenomic sequencing as a downstream application, and Haramoto et al. focus more broadly on the concentration of viruses from a water matrix (Haramoto et al., 2018; Hjelmsø et al., 2017). Both provide useful, scenario-specific recommendations and can inform future research. In addition to these studies, the current study suggests using caution when combining quantitative viral abundance data that has been collected using different concentration methods and that the most appropriate approach may be to separate data based upon the concentration method used.

5. Conclusions

The high abundance of crAssphage as well as its co-occurrence with pathogenic DNA viruses HPyV and HBoc suggests crAssphage has the strong potential to serve as viral indicator of human fecal pollution. While crAssphage has been shown to be globally distributed by metagenomic analysis of fecal samples, a full global survey of molecular concentration data is needed to aid in application of this bacteriophage as a fecal indicator. Here, we contribute to the growing literature of crAssphage and pathogen quantitative detection by showing that crAssphage is abundant in Italian wastewater and that its presence correlates with the presence of two viral human pathogens.

- CrAssphage is highly abundant in Italian wastewater.
- CrAssphage abundance correlates with DNA viral pathogens HPyV and HBoc in wastewater.
- Concentration methods have a significant effect on the concentrations of viruses and future studies should take care to standardize concentration methods
- CrAssphage concentration did not correlate with latitude, load, or capacity of the WWTP, suggesting that crAssphage concentration did not vary geographically or by population size.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: K.B. is a co-inventor on a US patent application entitled “Cross-Assembly Phage DNA Sequences, Primers and Probes for PCR-based Identification of Human Fecal Pollution Sources” (Application Number: 62/386,532). United States universities and non-profit researchers interested in using this technology must obtain a research license from the US EPA. To apply for a research license, please request additional information from ftta@epa.gov. The authors declare no other conflict of interest.

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Appendix A. Supplementary data

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