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Evaluation of the novel crAssphage marker for sewage pollution tracking in storm drain outfalls in Tampa, Florida

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ABSTRACT

CrAssphage are recently-discovered DNA bacteriophages that are prevalent and abundant in human feces and sewage. We assessed the performance characteristics of a crAssphage quantitative PCR (qPCR) assay for quantifying sewage impacts in stormwater and surface water in subtropical Tampa, Florida. The mean concentrations of crAssphage in untreated sewage ranged from 9.08 to 9.98 $\log_{10}$ gene copies/L. Specificity was 0.927 against 83 non-human fecal reference samples and the sensitivity was 1.0. Cross-reactivity was observed in DNA extracted from soiled poultry litter but the concentrations were substantially lower than untreated sewage. The presence of the crAssphage marker was monitored in water samples from storm drain outfalls during dry and wet weather conditions in Tampa, Florida. In dry weather conditions, 41.6% of storm drain outfalls samples were positive for the crAssphage marker and the concentrations ranged from 3.60 to 4.65 $\log_{10}$ gene copies/L of water. After a significant rain event, 66.6% of stormwater outlet samples were positive for the crAssphage marker and the concentration ranged from 3.62 to 4.91 $\log_{10}$ gene copies/L of water. The presence of the most commonly used *Bacteroides* HF183 marker in storm drain outfalls was also tested along with the crAssphage. Thirteen samples (55%) were either positive (i.e., both markers were present) or negative (i.e., both markers were absent) for both the markers. Due to the observed cross-reactivity of this marker with DNA extracted from poultry litter samples, it is recommended that this marker should be used in conjunction with additional markers such as HF183. Our data indicate that the crAssphage marker is highly sensitive to sewage, is adequately specific, and will be a valuable addition to the MST toolbox.

1. Introduction

Microbial pollutants released via untreated or improperly treated sewage discharge (Winterbourn et al., 2016), fractured sewer pipes (Andersson and Stenstrom, 1987), on-site septic systems (Sowah et al., 2017), storm water run-off (Rajal et al., 2007), and combined sewer overflows (CSOs) (Jalliffier-Verne et al., 2016) are known to pollute recreational water or beach sand (Bonilla et al., 2007; Hlavasa et al., 2015). As a result, recreational water users may be exposed to a number of pathogens. The economic burden of such illnesses can be quite high (Dwight et al., 2005). To protect human health and remediate pollution, it is important for regulators to differentiate between human and other animal sources of fecal pollution. However, the current regulatory paradigm of monitoring fecal indicator bacteria (FIB) such as *Escherichia coli* (*E. coli*) and/or *Enterococcus* spp (Ahmed et al., 2016; Harwood et al., 2014). cannot achieve this goal due to the broad distribution of FIB across many host species.

A variety of quantitative PCR (qPCR) assays have been frequently used to detect and quantify host-associated molecular markers in environmental waters. Sewage-associated molecular markers such as *Bacteroides* HF183 (Green et al., 2014), human adenovirus (HAdV) (Rusiñol et al., 2014), human polyomaviruses (HPyV) (Ahmed et al., 2010; McQuaig et al., 2009) and pepper mild mottle viruses (PMMoV) (Rosario et al., 2009) have been most commonly used in field studies. Sewage-associated molecular markers, especially bacterial markers, lack the desired host-specificity for unequivocal identification of sewage pollution in water. In contrast,

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sewage-associated viral markers (i.e., HAdV and HPyV), although highly host-specific, generally lack sensitivity due to comparatively low levels in sewage (Harwood et al., 2013). An ideal sewage marker (i.e., highly host-specific and sensitive) is yet to be discovered.

A novel bacteriophage called "crAssphage" was reported to be present in the majority of published human fecal metagenomes (Dutilh et al., 2014). This newly discovered phage was predicted to be a *Bacteroides* phage by co-occurrence profiling. In a subsequent study, crAssphage was found to be highly abundant in sewage and biosolids from the USA and Europe, and less abundant in sewage from Asia and Africa. The crAssphage was not detected in animal fecal samples with the exception of bat guano (Stachler and Bibby, 2014). Therefore, Stachler and Bibby (2014) concluded that a crAssphage based microbial source tracking (MST) marker may be suitable for sewage pollution tracking in environmental studies. Consequently, Stachler et al. (2017) developed a crAssphage assay by designing 284 primer sets along the length of the crAssphage genome. From these primer sets, two novel crAssphage qPCR assays (CPQ_056 and CPQ_064) were developed and evaluated for the quantitative detection of sewage pollution in water. Both assays exhibited high specificity (98.6%) when tested against a panel of a large number of non-human fecal samples and were highly abundant in untreated sewage and sewage-impacted water (Stachler et al., 2017).

The primary objective of this study was to evaluate the host-sensitivity and -specificity of the newly designed crAssphage qPCR assay CPQ_056 (i.e., marker) in fecal samples collected from various human and non-human host groups in Tampa, Florida, USA. Between the two assays developed by Stachler et al. (2017), CPQ_056 was chosen as both assays showed similar performance characteristics. The accuracy of the crAssphage marker to detect sewage pollution was further evaluated by testing against blind samples (source unknown to the analyst) seeded with human and non-human feces. Finally, environmental water samples were also collected from an accidental sewage spill and several stormwater drain outfalls during dry and wet weather periods to test for the presence of crAssphage marker, and co-occurrence with HF183. The occurrence of the crAssphage marker was used to provide evidence of sewage pollution in stormwater outlfalls in Tampa, Florida.

2. Materials and methods

2.1. Animal fecal and sewage sampling and DNA extraction

To determine the host-sensitivity and -specificity of the crAssphage marker, individual fecal and sewage samples were collected from nine non-human hosts and a sewage treatment plant (STP) in Tampa, Florida (Table 1). Eight composite human (i.e., untreated sewage) samples were collected from the primary influent of the Falkenburg Advanced STP in Tampa. Individual fecal sample was collected for each animal species giving a total number of 73 fecal samples from alligator ($n = 10$), bird ($n = 5$), cat ($n = 10$), cattle ($n = 10$), deer ($n = 8$), dog ($n = 10$), duck ($n = 10$), and horse ($n = 10$). Five composite poultry litter samples were collected from a poultry farm. All samples were transported on ice to the laboratory, stored at 4 °C for 24 h, and processed within 24–72 h.

DNA was extracted from the 240–300 mg of individual animal fecal samples using the DNeasy Power Soil Kit (Qiagen, Valencia, CA, USA). Sewage samples (i.e., 20 mL of primary influent mixed with 480 mL of phosphate buffer saline) were filtered through 47-mm, 0.45 μm pore size nitrocellulose membrane (Fisherbrand, ThermoFisher Scientific, Waltham, MA, USA). The DNeasy Power Soil Kit was also used to extract DNA directly from the membrane. DNA concentrations were determined using a Qubit DNA BR assay kit according to the protocol provided by the manufacturer (ThermoFisher Scientific). All DNA samples were stored at −80 °C until qPCR analysis.

2.2. qPCR assays

A recently published TaqMan qPCR chemistry was used for the analysis of the crAssphage marker (Stachler et al., 2017). CrAssphage was amplified using the forward primer (5′-CAG AAG TAC AAA CTC CTA AAA AAC GTA GAG-3′), the reverse primer (5′-GAT GAC CAA TAA ACA AGC CAT TAG C-3′) and the probe (FAM- AAT AAC GAT TTA CGT GAT GTA AC-TAMRA). A synthetic DNA fragment, containing a 125 bp crAssphage qPCR target (CAG AAG TAC AAA CTC CTA AAA AAC GTA GAG GTA GAG GTA TTA ATA ACG ATT TAC GTG ATG TAA CTC GTA AAA AGT TTG ATG AAC GTA C TG ATT GTA ATA AAG CTA ATG GCT TGT TTA TTG GTC AT) was purchased from Integrated DNA Technologies (IDTDNA.com, Coralville, IA). Standards were prepared from the synthetic DNA, ranging from 10^6 to 1 gene copies/μL of DNA. Quantitative PCR amplifications were performed in 20 μL reaction mixtures using SsoAdvanced Universal Probes Supermix (Bio-Rad Laboratories, Richmond, CA). The crAssphage qPCR mixtures contained 10 μL of Supermix, 900 nM of each primer, 80 nM probe and 3 μL of template DNA. The qPCR cycling parameters consisted of 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 60 s at 60 °C. The crAssphage qPCR assay performance criteria such as efficiency (E), slope, intercept, r^2 and assay limit of detection (ALOD) were determined by analysis of the standards over the course of the study. The HF183 qPCR assay was performed according to the assay conditions described elsewhere (Green et al., 2014). The qPCR assays were performed using an ABI 7500 thermal cycler. All qPCR reactions were performed in triplicate. For each qPCR run, a set of standards and a no-template control were included. No-template controls did not show any amplification.

2.3. qPCR method limit of quantification (MLOQ) and process limit of quantification (PLOQ)

qPCR MLOQ and PLOQ were determined according to previous studies (Staley et al., 2012; Symonds et al., 2016). The MLOQ is the lowest concentration of a marker which can be quantified by qPCR in diluted sewage DNA samples. For the MLOQ assay, 5 mL of untreated sewage sample was added into 295 mL phosphate buffered saline (PBS) and serially diluted (10 fold) in PBS. DNA was extracted from each dilution and tested with the crAssphage qPCR assay to determine the MLOQ. The PLOQ is the lowest concentration of a marker which can be quantified by qPCR in serially diluted environmental water samples seeded with untreated sewage. PLOQ incorporates loss of marker due to sample processing steps. PLOQ was determined in a similar fashion except for 5 mL of untreated sewage was added to 295 mL of river water (Hillsborough River; 28.0549° N, 82.3635° W, Tampa, FL) and serially diluted in river water, followed by DNA extraction and qPCR analysis. MLOQ and PLOQ analysis were undertaken in triplicate.

2.4. Recovery efficiency

The concentrations of crAssphage were quantified in untreated sewage samples in triplicate using qPCR assay as described above. In brief, 10 mL, 1 mL and 100 μL volumes of untreated sewage were seeded into 290 mL, 299 mL and 299.9 mL of river water samples (final volume of 300 mL) in triplicate. Water samples seeded with untreated sewage were filtered through 0.45 μm mixed cellulose esters membrane (Fisherbrand, Thermo Fisher Scientific). A DNeasy Power Soil Kit was used to extract DNA directly from the membrane and subjected to qPCR analysis. The recovery efficiency of the