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Concentrations of host-specific and generic fecal markers measured by quantitative PCR in raw sewage and fresh animal feces

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ABSTRACT

We measured the concentrations of four host-specific (human, dog, cow, and horse *Bacteroidales*), four generic fecal (16S total *Bacteroidales* and *Escherichia coli*, 23S *Enterococcus* and *uidA E. coli*,) and two universal bacterial (16S universal and *rpoB* universal) DNA targets by qPCR in raw sewage and pooled fecal samples from dogs, cows, horses, and Canada Geese. A spiking protocol using the non-fecal bacterium *Pseudomonas syringae* pph6 was developed to estimate the recovery of DNA from fecal and environmental samples. The measured fecal marker concentrations were used to calculate baseline ratios and variability of host-specific to generic indicators for each host type. The host-specific markers were found in high concentrations (8–9 $\log_{10}$ copies/g dry wt.) in their respective hosts' samples, which were equal to or greater than the concentrations of generic *E. coli* and *Enterococcus* markers, lending support to the use of host-specific and generic *Bacteroidales* as sensitive indicators of fecal pollution. The host-specific markers formed a consistent percentage of total *Bacteroidales* in target host feces and raw sewage, with human-specific comprising 82%, dog-specific 6%, cow-specific 4% and horse-specific 2%. Based on this limited data set, the measurement of host-specific indicators by qPCR has several promising applications. These applications include determining the percentage of total *Bacteroidales* contributed by a specific host type, using the ratios of host-specific markers to *E. coli* or *Enterococcus* to estimate the contribution of each source to these regulated fecal indicator bacteria, and estimating the mass of feces from each host type in environmental samples.

1. Introduction

Fecal pollution is a serious environmental and public health problem that affects many of the surface waters in the US and around the world. Despite efforts to reduce or eliminate fecal pollution, bacterial indicators for pathogens remain the most common cause of impairment for streams and rivers in the US, primarily due to failure to identify and mitigate non-point sources of contamination (USEPA, 2002; Santo Domingo and Stoeckel, 2003). Traditional methods used to quantify fecal pollution levels, such as culturing of fecal coliform bacteria, do not distinguish among human and other animal sources of pollution. Efforts to address water quality impairments through the total maximum daily load (TMDL) process require determining the various sources of a contaminant in a watershed so that discharge limits can be allocated. Recent research has focused on developing new fecal source tracking (FST) methods, particularly on host-specific genetic markers in

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non-pathogenic enteric bacteria like *Bacteroidales* (Bernhard and Field, 2000a,b; Bernhard et al., 2003; Dick et al., 2005b,a; Soule et al., 2006; Kildare et al., 2007). However, there are still no fully validated methods for quickly and accurately quantifying the sources of fecal contamination in impaired watersheds (Scott et al., 2002; Simpson et al., 2002; Santo Domingo et al., 2007).

Bernhard and Field (2000a,b) pioneered the development of host-specific markers in *Bacteroidales* using length heterogeneity-PCR (LH-PCR) and terminal restriction length polymorphism (T-RFLP) phylogenetic analyses to identify human-specific and ruminant-specific markers in the 16S ribosomal DNA of *Bacteroides-Prevotella* and *Bifidobacterium*. Initial identification of the markers requires constructing phylogenetic 16S rDNA clone libraries, but once a host-specific marker has been identified, routine detection involves a straightforward PCR assay.

Since then, others have used similar methods and genetic sequences available in GenBank to develop additional host-specific markers for cow, horse, dog and swine fecal contamination (Simpson et al., 2004; Layton et al., 2006; Reischer et al., 2007, 2006; Kildare et al., 2007; Okabe et al., 2007). Additionally, some of the host-specific markers have been used to develop real-time quantitative PCR (qPCR) methods for determining the concentrations of host-specific bacterial DNA in environmental samples. Quantitative PCR has also been used to measure the concentrations of generic indicator bacteria, including total *Bacteroidales* (Dick and Field, 2004; Kildare et al., 2007), *Escherichia coli* (Huijsdens et al., 2002; Silkie et al., 2008), and *Enterococcus* (Ludwig and Schleifer, 2000; He and Jiang, 2005). Thus, it is now possible to measure both host-specific and generic fecal indicators in environmental samples using qPCR.

However, the relative contributions from different fecal sources cannot be calculated directly from the quantitative information produced by qPCR (Santo Domingo et al., 2007). Because not all fecal bacteria are host-specific, the concentration of host-specific targets does not necessarily represent the proportional contribution of a given host's feces. In addition, different hosts have different fecal bacterial community compositions. For example, *Bacteroidales* are approximately 30% of the fecal bacteria in humans (van Tongeren et al., 2005; Delgado et al., 2006), but are rarely detected in gull feces. Similarly, the ratio of fecal coliforms to fecal streptococci (FC:FS ratio) was previously advocated as a method for distinguishing human from non-human fecal pollution, with a ratio greater than 4.0 corresponding to a predominantly human source and a ratio less than 0.7 corresponding to a predominantly non-human source (APHA, 1976; Geldreich, 1978; Coyne and Howell, 1994). However, differential survival rates of streptococcal species and fecal coliforms in the environment make this method unreliable (APHA, 1999). Differential survival rates of host-specific markers from generic indicator bacteria may also complicate attempts to quantify the relative contributions from a given host in a watershed (Field and Samadpour, 2007).

The goal of this research was to determine the concentrations of host-specific and generic indicator qPCR targets in fresh fecal matter from several different types of hosts (human, dog, cow, horse, and Canada Goose). We discuss how this information may be useful for determining the relative contributions of fecal pollution from different hosts in a watershed. We also consider two unresolved issues affecting the accuracy of measuring cell concentrations with qPCR. First, we investigate how the copy number of the target gene and its proximity to the origin of replication affects qPCR results. This question is addressed by targeting two single copy and two multi-copy genes in a growing pure culture of *E. coli*. Second, a surrogate spiking method is presented to reduce uncertainty associated with DNA extraction efficiency and PCR inhibition and to improve the accuracy of estimating initial cell concentrations from qPCR.

2. Methods

DNA was extracted from raw sewage as well as pooled fecal samples of dogs, cows, horses, and Canada Geese. Each DNA sample was tested by qPCR for presence and quantity of two generic bacterial indicators (16S Uni and *rpoB* Uni), four fecal bacteria indicators (16S total *Bacteroidales*, *Enterococcus* and *E. coli*, and *uidA E. coli*) and four host-specific fecal bacteria indicators (BacHum, BacCan, BacCow, and HorseBact). A spike surrogate, *Pseudomonas syringae* pv. *phaseolica* race 6 (pph6) was used to assess recovery of bacteria through the DNA extraction process. Additionally, DNA was extracted from a pure culture of *E. coli* (ATCC 700891) over a time-series and tested for two *E. coli*-specific markers (16S *E. coli* and *uidA*) and two universal markers (16S Uni and *rpoB*).

2.1. Design and preparation of spike

P. syringae pv. *phaseolica* race 6 (pph6) was chosen as a spike to measure whole assay DNA recovery. Like *E. coli* and *Bacteroidales* it is Gram-negative, and so is expected to behave similarly during the DNA extraction process. However, it may not as accurately represent DNA extraction efficiency for a Gram-positive target like *Enterococcus*. The *P. syringae* primers and probe were modified from the assay targeting the *avr PphE* gene published by Thwaites et al. (2004) to make a minor-groove binding (MGB) qPCR assay that would be more specific and amenable to future multiplexing. This gene was selected on the basis of being very specific to plant pathogens and not likely to be common in environmental water or feces. *P. syringae* cells were obtained from Professor Kate Field (Oregon State University, Corvallis, OR) and cultured in Kings B media. For standardized spike aliquots, freshly cultured cells were centrifuged, the supernatant discarded and then rinsed three times by resuspending with phosphate buffered saline, centrifuging and discarding the supernatant. After the final wash, the cells were resuspended in 15% glycerol–0.01 M $MgSO_4$. An aliquot of the *P. syringae* cell supension was enumerated by filtering and staining with Acridine Orange (Molecular Probes, Eugene, OR) using the protocols 9-41 and 9-42 outlined in Standard Methods (APHA,1999). Cells were counted under 1000× magnification on a microscope (Olympus) with a 100 W mercury lamp. The remaining cell suspension was divided into single-use aliquots of 5×10^8 cells in 200 μL, and stored at −20 °C until use. *P. Syringae* DNA for