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Evaluating Human-Associated *Escherichia coli* Marker Genes for Microbial Source Tracking Applications in Florida

by

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Biology
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Date of Approval:
October 24, 2018

Keywords: E. coli, H genes, Fecal Indicator Bacteria, MST

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Abstract:

Fecal pollution of surface water can lead to human health issues because waterborne transmission of pathogens is a common cause of gastroenteritis. Genetic markers developed for human-associated *Escherichia coli* (H8, H12, H14, and H24) are promising tools for microbial source tracking (MST) of sewage in environmental waterbodies but are untested in the US. H gene marker performance characteristics (specificity, sensitivity, and prevalence) were assessed, with the goal of developing a quantitative PCR assay for the markers with the best performance. Individual *E. coli* isolates from reference samples were tested to determine end-point PCR assay performance. Three hundred *E. coli* strains isolated from sewage and 900 obtained from animal fecal samples were screened for H8, H12, H14, and H24. Specificity values against animal fecal isolates were 92%, 97%, 86%, and 81% for H8, H12, H14, and H24, respectively. Sensitivity values against sewage were 100%, 90%, 100%, and 100%. Prevalence values for the marker genes in *E. coli* isolates were 16.6%, 7.7%, 16.3%, and 21.3%. Two new TaqMan qPCR assays were developed for the highest performing markers, H8 and H12. Specificity and sensitivity for the qPCR assays were determined against reference fecal samples. Host specificity for the H8 and H12 qPCR assays was 96% and 93%, respectively. The H8 and H12 qPCR assays were positive in all sewage samples. Mean H8 qPCR concentrations were higher in sewage compared to H12, ranging from $3.2x10^7$ to $3.9x10^7$ gene copies/100 mL, while H12 qPCR concentrations varied from $1.5x10^6$ to $1.7x10^6$ gene copies/100 mL. Concentrations in sewage were significantly and positively

correlated with the human-associated *Bacteroides* marker HF83.These results indicate that the H8 TaqMan assay developed in this study for quantification of human host-associated *E. coli* is a promising addition to the MST toolbox due to high specificity for sewage, abundance in untreated and partially-treated wastewater, and correlation with the well-established HF183 marker. While the H12 assays also showed strong performance characteristics, the gene's concentration was over ten-fold lower than that of H8, making it less likely to be detected in diluted sewage. Host-associated *E. coli* markers are a tool that can improve water quality management decisions by allowing investigators to determine whether human sources contribute to elevated *E. coli* densities in surface waters, which can inform total maximum daily load plans and risk assessment.

Introduction:

Fecal Pollution in the Environment:

Fecal pollution of surface water can lead to human health issues because waterborne transmission of pathogens is a common cause of gastroenteritis (Harwood *et al.*, 2014). Fecal contaminants enter surface water through combined sewers overflows (CSOs) (Fong *et al.*, 2010), sanitary sewer overflows (SSOs) (USEPA, 2004), leaking septic tanks (Iverson *et al.*, 2017, Sowah *et al.*, 2017), agricultural and urban stormwater runoff (Rajal *et al.*, 2007), and direct deposition from wildlife and domestic animals (Nguyen *et al.*, 2018). Common fecal pathogens detected in human and animal wastes include *Escherichia coli* O157:H7 and *Campylobacter jejuni* (Wery *et al.*, 2008, Li *et al.*, 2015), viruses including norovirus or enterovirus (Harwood *et al.*, 2005, Montazeri *et al.*, 2015), and protozoa including *Giardia* and *Cryptosporidium* (Cox et al., 2005, Harwood et al., 2005). Ensuring the safety of surface water for human uses requires monitoring for pathogens, but testing for the presence of every potential pathogen is not practical or affordable (Harwood *et al.*, 2005). Instead, regulatory agencies worldwide recommend measuring fecal indicator bacteria (FIB) densities in surface water as a surrogate for fecal pathogens (WHO, 2003, USEPA, 2012). FIB include *Escherichia coli* and enterococci, commensal bacteria found in the gastrointestinal tract of warm-blooded animals, which are shed in feces along with any potential fecal pathogens.

Widespread adoption of FIB as a tool for estimating pathogen risk is due to their proven risk assessment utility. Epidemiological investigations conducted since the late 1970s have reported a positive correlation between gastrointestinal illness rates among swimmers and FIB densities in surface water (USEPA, 1986, USEPA, 2012). While useful as a general measure of fecal contamination, one limitation to FIB is that their ubiquity among various host groups makes fecal source identification based on culturable FIB alone an impossible task. This inability is important because waterbodies impacted by fecal contaminants from multiple host groups (human and/or animals) may represent varying levels of risk to human health (Soller *et al.*, 2010). Additionally, an inability to accurately identify fecal sources responsible for elevated FIB levels hinders remediation efforts in watersheds characterized by mixed land-use with many potential sources (Nguyen *et al.*, 2018).

Microbial Source Tracking:

Microbial source tracking (MST) refers to a suite of techniques developed to discriminate between sources of fecal contamination in environmental water samples. These techniques are based on the premise that differences in the gut conditions and diet of various animal host groups drive the development of detectable host-associated genes in microbes (Santo Domingo & Sadowsky, 2007). Gene fragments unique to host-associated microorganisms are referred to as "markers". Over the past decade advances in DNA sequencing technology have resulted in increased identification of host-associated markers in fecal microbes that can be detected using molecular techniques including polymerase chain reaction (Harwood *et al.*, 2014). Validation studies are recommended for newly developed genetic markers in any region where they have not been previously utilized due to geographic variation in the composition of the microbial flora

of various host GI tracts (USEPA, 2005). MST markers are assessed using multiple metrics including specificity, sensitivity and prevalence (Stoeckel & Harwood, 2007). These metrics are important measures of source tracking utility in a given watershed and require extensive sample collection from target and potential non-target host groups representative of those found within the watershed (Santo Domingo & Sadowsky, 2007).

Sensitivity refers to the frequency of marker detection among target (host species whose waste a method is designed to detect) samples and depends on marker distribution in the host population and its concentration in waste (Stoeckel & Harwood, 2007). Sensitivity is determined by testing waste samples (e.g. feces, sewage) from the target host, and is calculated by dividing the number of true positive test results by the total number of tested host samples (Stoeckel & Harwood, 2007); low marker sensitivity increases the likelihood of false negatives, wherein risk due to exposure to waterborne pathogens may be underestimated (Stoeckel & Harwood, 2007). For example, if 100 sewage samples were tested and only 80 were positive for human marker presence, the sensitivity value would be 80%. Specificity refers to the ability of a marker to correctly produce a negative test result when a source is not present (true negative). Detection of host-associated markers in non-target host groups indicates lower specificity and an increased likelihood of obtaining a false positive result in environmental samples (Harwood & Stoeckel, 2011). Specificity is calculated by dividing the number of true negative results with the total number of true negatives and false positives. For example, if 100 animal fecal samples were tested and 10 were found to contain the marker, the specificity value would be 90%. Prevalence refers to frequency of marker possession within the target organism population and is usually measured on an isolate-by-isolate basis. For example, if 100 *E. coli* isolates from the target

organism were tested and resulted in 20 *E. coli* positive for marker presence, the marker prevalence would be 20%.

One of the most widely used MST markers for detection of human fecal contamination is HF183, an assay targeting the host-associated 16S rRNA gene sequence found in alternative indicator organism *Bacteroides*. While specific and sensitive to human waste the major drawback to this assay is that the target organism is not regulated by established water quality criteria and lacks consistent correlation with FIB or pathogen concentrations in wastewater or contaminated surface water (Harwood *et al.*, 2014, Napier *et al.*, 2017). As a result, reliance on alternative fecal indicators for microbial source tracking represents a dilemma as the bacteriological quality of freshwater is based on monitoring *E. coli* densities. In such a scenario, it is more ideal to track the sources of *E. coli* due to their established correlation with GI illness and widespread utilization as indicators of fecal contamination.

Despite many attempts at developing a technique for MST based on *E. coli*, this has proven to be a difficult task due to the ubiquity of *E. coli* among different host groups (Santo Domingo & Sadowsky, 2007). Various genes, including those encoding for serotype and toxin production, have previously been targeted to identify host-associated *E. coli* but featured various drawbacks to their use One research group (Clermont *et al.*, 2008) reported that members of the *E. coli* B2 clonal group with an O81 serotype were human host specific. A PCR-based assay indicated that this clone was not detected among 904 fecal isolates from non-human animals and that it was found in people living in Africa, Europe and South America. One major limitation to using serotypes as a target for MST is that genes encoding for serotype can be highly variable, and as a result, temporally unstable as markers for host-associated microbes. Oshiro and Olson

found that the STIb enterotoxin gene in *E. coli* was associated with sewage (Oshiro & Olson, 1997). One major limitation of a toxin gene marker is that their prevalence is usually low in the host population. Markers found at low concentration and prevalence in host feces or sewage are difficult to detect in environmental waters due to dilution and are typically not useful as MST markers (Harwood *et al.*, 2014).

Analysis of *E. coli* population structure has revealed multiple lineages associated with various functions and lifestyles (i.e., commensal vs pathogenic strains) (Luo *et al.*, 2011). While these lineages are not strongly host-associated, the evidence indicates that *E. coli* not only survive outside of the GI tract but are capable of colonizing and persisting in matrices including freshwater sediment and on the surface of aquatic vegetation (Badgley *et al.*, 2011). Comparison of environmental and fecal *E. coli* isolates indicates that the fate of different strains of *E. coli* is dependent upon differences in the genetic makeup of the organism (van Elsas *et al.*, 2011). Comparative genome analysis of numerous commensal and pathogenic *E. coli* revealed a conserved core genome size of approximately 2,300 genes, while the pangenome reservoir size was estimated at more than 13,000 genes (Rasko *et al.*, 2008). This evidence suggests that novel human-associated markers could be identified and utilized for MST (Harwood *et al.*, 2014).

H-Genes: Novel Genetic Markers for Human Host-Associated *E. coli*

In Japan a series of human-associated *E. coli* genetic markers were identified following whole genome sequencing of 22 unique E. coli strains from four different host groups: nine from human sewage, two from cows, six from pigs, and five from chickens (Gomi *et al.*, 2014). Discarding the core genome shared by all isolates allowed for identification of human-associated

accessory genes with various cellular functions (Table 1) deemed H8, H12, H14 and H24 (Gomi *et al.*, 2014). Marker performance studies conducted in Japan (Gomi *et al.*, 2014) and Australia (Warish *et al.*, 2015) found H8 and H12 had high specificity (>90%) compared to H14 and H24 (≤90%), but that all four genes were sensitive (100%) to sewage and prevalent (≥20%) among sewage *E. coli* isolates for both studies. Based on these promising results, it was determined that marker validation studies should be performed in Florida to assess the utility of all four markers for MST applications.

In this study sensitivity, specificity, and prevalence testing was performed on an isolate-by-isolate basis to determine whether any of the four H-gene markers could potentially be used for microbial source tracking applications in Florida. High performing markers (specificity and sensitivity >90%) were then used as the basis for developing two TaqMan qPCR assay which were then subjected to specificity and sensitivity testing using a reference fecal DNA library. Following identification of the TaqMan assay with the highest performance characteristics *E. coli* TaqMan marker concentrations were measured in environmental water samples impacted by fecal contamination in comparison to HF183 concentrations in order to assess whether or not these markers correlate with each other (Figure 1).

Table 1: *E. coli* H gene marker names and targeted gene sequences

Marker Name:	Target Size (Base Pairs)	Gene/Feature
H8	177	Sodium/Hydrogen Exchanger Precursor
H12	213	Hypothetical Phage Protein
H14	271	ATP/GTP Binding Protein
H24	229	Methyl-thioribulose-1-phosphate dehydratase

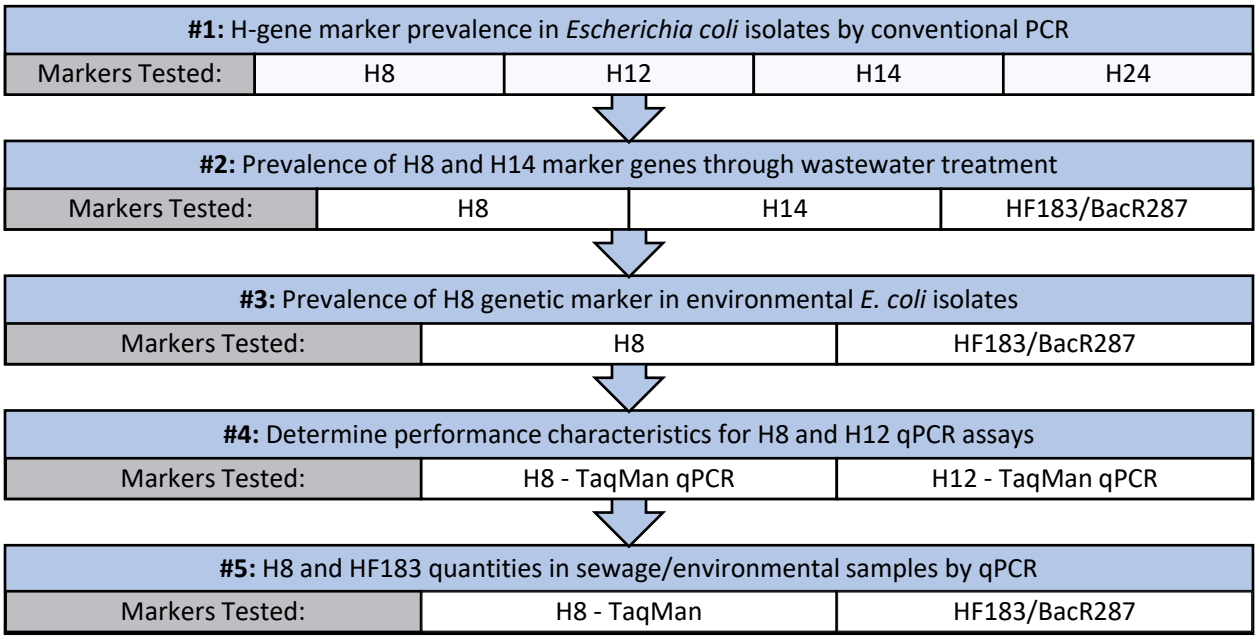

Figure 1. Study overview with markers tested for each sub-study:

Methods:

Sewage and fecal samples tested for H-gene marker prevalence*:* Forty-six fecal samples were collected for specificity and sensitivity testing by binary (conventional) PCR. Samples represented eight host groups (alligator, cat, cattle, dog, feral pig, horse, duck, and human), and were collected between May 2016 and June 2017. Ten untreated sewage samples (i.e., human host) were collected from three wastewater treatment facilities in the greater Tampa Bay area: the Falkenburg Advanced Wastewater Treatment Plant (6 million gallons daily (MGD)), the William E. Dunn Water Reclamation Facility (6.5 MGD), and the South Cross Bayou Wastewater Treatment Plant (22 MGD). Thirty-six fresh fecal samples weighing approximately 1-30 g were collected from seven non-human animal species (Table 3). One alligator fecal sample was collected from an individual alligator at a meat processing plant. One cat fecal sample was collected from a household. Nine cow fecal samples were collected from a beef cattle farm located in Plant City, Florida. Six dog fecal samples were collected from various households within the Tampa Region. Ten duck fecal samples were collected from a pond near the University of South Florida Campus. Three feral pig fecal samples were collected from a Florida State Park. Six horse fecal samples were collected from a farm located in Plant City. Each fecal sample was deposited by a different individual and was collected aseptically with a sterile tongue depressor and inserted into a sterile 50 mL centrifuge tube. The tubes were transported on ice to the laboratory and processed within 4-6 h.

To determine host-sensitivity and host-specificity for the *E. coli* H8 and H12 TaqMan qPCR markers developed in this study, 81 fecal and sewage samples were collected from nine non-human hosts and a wastewater treatment plant (WWTP) in Tampa, Florida (Table 4). Three untreated sewage samples (i.e., human host) samples were collected from the primary influent of the Falkenburg Advanced WWTP in Tampa, FL. Individual fecal samples were collected from each animal for 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 extraction from sewage and fecal samples: DNA was extracted from 240-300 mg of each fecal sample using a DNeasy PowerSoil Kit (Qiagen, Valencia, CA, USA). For the human samples 10 mL of primary influent and 490 mL of phosphate buffered saline (PBS) were mixed and filtered through 0.45 µm (47 mm diameter) nitrocellulose membranes (ThermoFisher Scientific, Waltham, MA, USA). A DNeasy PowerSoil Kit (Qiagen, Valencia, CA, USA) was used to extract DNA directly from the membrane and DNA concentrations were determined using a Qubit DNA HSBR assay kit according to the protocol provided by the manufacturer (ThermoFisher Scientific). All DNA samples were stored at -20°C until qPCR analysis.

Environmental water sample collection and processing: Three sites each were chosen in Bullfrog and Sweetwater creek and its tributaries under different land uses and runoff conditions. Sweetwater Creek originates from residential Lake Magdalene and flows southwest through urban areas. Three consecutive sampling sites (designated as SWC1, SWC2 and SWC3) were chosen along Sweetwater Creek. Bullfrog Creek (BFC) is comprised of one major and one minor

tributary. The major tributary is characterized by agricultural land use and the minor tributary is characterized by residential development. Both tributaries merge into the main stem of Bullfrog Creek. Out of the three sites chosen at BFC for water sampling, one was in the major Bullfrog Tributary (BFT1), one in minor Bullfrog Tributary (BFT2) and one in Bullfrog Creek (BFC3). Twenty-seven water samples were collected between August 2016 and April 2017 in 2L sterile containers and placed on ice for transport to the laboratory. Five hundred ml of water were processed from each sample by membrane filtration (Staley *et al.*, 2013) within 6 hours of collection using 0.45 µM (47 mm diameter) nitrocellulose filter paper. DNA was directly extracted from the filter paper using a DNeasy PowerWater Kit (Qiagen, Valencia, CA, USA). Each sample was tested for the presence of potential PCR inhibitors using a Sketa22 assay (Haugland *et al.*, 2005). The samples were PCR inhibitor free.

***E. coli* isolation and DNA extraction for PCR:** *E. coli* cultures representing various host groups (Table 3) were isolated using the following procedure: One mL of sewage or 1 g of fresh feces was added into 9 mL of PBS followed by preparation of serial dilutions. Serial dilutions were filtered through 0.45 µM (47 mm diameter) nitrocellulose filter membranes (ThermoFisher Scientific, Waltham, MA, USA) and plated on membrane thermotolerant *Escherichia coli* agar plates (mTEC) (Difco, Detroit MI) for isolation of *E. coli* (USEPA, 2009). Plates were incubated at 35°C for two hours to recover stressed cells, followed by incubation at 44.5° C for 18-20 hours in a water bath. *E. coli* colonies from the mTEC plates were then streaked for isolation on tryptic soy agar (TSA) (Difco, MD, USA) and incubated at 37°C for 18 h to obtain pure cultures. Individual *E. coli* isolates (*n* = 35-40 from each sample) were picked with a sterile toothpick and placed into a 2 mL microcentrifuge tubes containing 200 µL of reagent grade water (Fisher Diagnostics, VA,

USA), and boiled at 100°C in a thermal cycler for 10 mins to release DNA (Abberton *et al.*, 2016). A total of 1,290 *E. coli* isolates were obtained and tested (Table 3). DNA samples extracted from putative *E. coli* isolates were screened for the presence of the *E. coli* β-glucoronidase *uidA* gene (Chern *et al.*, 2009) to confirm presence of *E. coli*. A small number of *E. coli* isolates from certain samples did not amplify for the *uidA* gene and were excluded from the further analysis.

Conventional PCR for H-genes from *E. coli* isolates: Thirty *E. coli* isolates from each fecal and water sample were tested for *E. coli* H8, H12, H14, and H24 markers using conventional PCR. Previously published primers and cycling parameters were used for PCR detection of H-genes (Table 2). Conventional PCR amplifications were performed in 25 µL reaction mixtures using GoTaq Green Master Mix (Promega, WI, USA). The PCR reaction mixtures contained 12.5 µL of master mix, 1000 nM of each primer, 8.5 µL of nuclease free water (Promega), and 2 µL of DNA template. For each conventional PCR assay, positive (genomic DNA from control strain) and negative (nuclease free water) controls were included. PCR products were visualized on 2% agarose gel with 2X sodium borate buffer solution with ethidium bromide.

Quantitative PCR (qPCR) assay development for H8 and H12: H-gene markers with the highest performance characteristics (host-specificity and host-sensitivity) were H8 and H12. Probe design was carried out using the IDT PrimerQuest tool. Quantitative PCR amplifications were performed in 20 µL reaction mixtures using SsoAdvanced Universal Probes Supermix (Bio-Rad Laboratories, Richmond, CA). H8 and H12 qPCR reaction mixtures contained 10 µL of Supermix, 900 nM of each primer, 200 nM probe and 3 µL of template DNA. qPCR cycling parameters consisted of 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 60 s at 58°C (Table 2). Assays were performed using an ABI 7500 thermal cycler. All qPCR reactions were performed in triplicate. For each qPCR run,

a set of standards prepared from *E. coli* genomic DNA and a no-template control were included. No-template controls did not show any amplification.

H8 and H12 qPCR standards were analyzed over the course of this study to determine the reaction efficiencies (E), slopes, and correlation coefficients. For H8 the amplification efficiencies ranged from 92.61 to 96.02% while for H12 they ranged from 86.8 to 93.8%. The slope of the standards for H8 ranged from −3.40 to −3.51 and the correlation coefficient (r^2) ranged from 0.95 to 0.99. The slope of the standards for H12 ranged from −3.48 to −3.68 and the correlation coefficient (r^2) ranged from 0.98 to 0.99. The qPCR assay limit of quantification was determined to be 30 gene copies per qPCR reaction for both H8 and H12 assays.

qPCR in Environmental Samples and Statistical Analysis: DNA extracted from all environmental water samples impacted by sewage contamination were tested for the quantity of the H8 TaqMan marker developed in this study compared to the human-associated marker HF183/BacR287 using previously published methods (Green *et al.*, 2014). For all qPCR analyses, samples were run in triplicate and marker quantities were measured by comparison to a standard curve. Pearson correlation of qPCR marker quantities in environmental samples contaminated with sewage was performed using R-studio.

Table 2: PCR and qPCR primers, probes and cycling conditions

Assays	Sequences	Cycling parameters	Reference
E. coli uidA	F: CAACGAACTGAACTGGCAGA	10 min at 95 °C, followed by 40	Chern et
	R: CATTACGCTGCGATGGAT	cycles of 15 s at 95 °C, 30 s at 60°C	al. 2009
Sketa22	F: GGTTTCCGCAGCTGGG	2 min at 50 °C then 10 min at 95 °C	Haugland
	R: CCGAGCCGTCCTGGTCTA	followed by 40 cycles of 15 s at 95	et al. 2005
	P: FAM-AGTCGCAGGCGGCCACCGT-TAMRA	°C, 60 s at 60°C	
E. coli H8	F: ACAGTCAGCGAGATTCTTC	5 min at 95 °C, followed by 40	Gomi et
	R: GAACGTCAGCACCACCAA	cycles of 15 s at 94 °C, 30 s at 60°C	al. 2014
E. coli H12	F: GTAAAAGGACTGCCGGGAAA	5 min at 95 °C, followed by 40	Gomi et
	R: TCAGATCGTCCTTTACCAG	cycles of 15 s at 94 °C, 30 s at 60°C	al. 2014
E. coli H14	F: CAGCCTGAGCGTCTTTTAC	5 min at 95 °C, followed by 40	Gomi et
	R: CGGTGGGAAAAGAAGTTGAA	cycles of 15 s at 94 °C, 30 s at 60°C	al. 2014
E. coli H24	F: CTGGTCTGGCTTTATAACAC	5 min at 95 °C, followed by 40	Gomi et
	R: ATCATTTCCACTTGTCGGG	cycles of 15 s at 94 °C, 30 s at 60°C	al. 2014
H8 - qPCR	F: ACAGTCAGVGAGATTCTTC	5 min at 95 °C, followed by 40	This study
	R: GAACGTCAGCACCACCAA	cycles of 15 s at 94 °C, 30 s at 58°C	
	P: FAM-ACTGGCATCGGCATGGAACAC-BHQ		
H12 - qPCR	F: GTAAAAGGACTGCCGGGAAA	5 min at 95 °C, followed by 40	This study
	R: TCAGATCGTCCTTTACCAG	cycles of 15 s at 94 °C, 30 s at 58°C	
	P: FAM-AGAGTAAACGCTTCGCC CTTAGCC-BHQ		
HF183/BacR287	F: ATCATGAGTTCACATGTCCG	5 min at 95 °C, followed by 40	Green et
	R: CTTCCTCTCAGAACCCCTATCC	cycles of 15 s at 94 °C, 30 s at 60°C	al. 2014
	P: FAM-CTAATGGAACGCATCCC-MGB		

Table 3: Fecal and sewage samples collected for conventional PCR analysis

Host groups	# of fecal samples collected	Total # of *E. coli* isolates tested
Alligator	1	30
Cat	1	30
Cattle	9	270
Dog	6	180
Horse	6	180
Duck	10	300
Sewage	10	300
Total	43	1290

Table 4: Fecal and sewage samples collected for qPCR analysis

Host groups	Number of samples	Sample type
Alligator	10	Individual
Bird	5	Individual
Cat	10	Individual
Cattle	10	Individual
Deer	8	Individual
Dog	10	Individual
Duck	10	Individual
Horse	10	Individual
Poultry	5	Composite
Sewage	3	Composite
Total	81	-

Results:

Marker prevalence in *E. coli* isolates by conventional PCR: Prevalence, or the frequency of marker occurrence among individual *E. coli* isolates as measured by PCR, was 16.6%, 7.7%, 16.3%, and 21.3% for H8, H12, H14, and H24, respectively (Table 5, Figure 2). Sensitivity values, or the percentage of target fecal samples (sewage) with one or more *E. coli* isolates positive for H-gene marker presence, was 100%, 90%, 100%, and 100% for H8, H12, H14, and H24 marker genes, respectively. Absence of *E. coli* isolates positive for H12 in one sewage sample resulted in a lower sensitivity value for H12 (Table 5, Figure 2). Specificity, or the percentage of *E. coli* colonies isolated from non-target host groups correctly classified as PCR negative, was 92%, 97%, 86%, and 81% for H8, H12, H14, and H24 (Table 5). False-positive results were obtained for the H8 marker in three isolates from the feces of two different host species, one alligator (one isolate) and two horse samples (one isolate each). False positives for H12 were limited to *E. coli* isolated from one duck sample (seven isolates). False positives for H14 were detected in one dog sample (one isolate), one alligator sample (one isolate), two horse samples (three isolates), three duck samples (three isolates), and one feral pig sample (two isolates). False positives for H24 were detected in three dog samples (71 isolates), one duck sample (five isolates), and two feral pig samples (four isolates) (Figure 2).

Prevalence of H8 and H14 marker genes through wastewater treatment: Detection of fecal coliforms in wastewater lagoons prompted the need to determine if *E. coli* and other sewage-

associated bacteria were surviving the wastewater treatment process. Two methods were utilized to perform this task: conventional PCR for H8 and H14 marker prevalence in *E. coli* isolates and quantitative PCR (qPCR) to measure human host-associated *Bacteroides* marker HF183/BacR287 and H8. Marker prevalence for H8 and H14 genetic markers was highest in primary influent (untreated sewage) with 11 isolates (37%) positive for H8 and 13 isolates (43%) positive for H14, followed by the secondary clarifier effluent with three isolates (10%) positive for H8 and six isolates (20%) positive for H14, and finally the wastewater lagoon with two isolates (7%) positive for H8 and three isolates (10%) positive for H14 (Table 6). HF183 gene copies/100 mL ranged from $5.0x10^1$ to $1.0x10^9$, with the highest values observed in primary influent and the lowest values observed in the wastewater lagoon (Table 6). H8 gene copies/100 mL ranged from $5.7x10^1$ to $8.2x10^6$, with the highest values observed in primary influent and the lowest values observed in the wastewater lagoon (Table 6)

Prevalence of H8 in environmental *E. coli* isolates: Conventional PCR analysis of environmental *E. coli* isolates sampled two freshwater streams resulted in a 0% H8 marker prevalence out of 810 tested isolates (Table 7). HF183 was detected in 11.1% of samples from two sampling sites, Bullfrog Tributary 1 (BFT1) and Bullfrog Creek (BFC3). HF183 marker concentrations ranged from $7.1x10^1$ to $4.5x10^2$ gene copies/100 mL (Table 7).

qPCR assays specificity and host-sensitivity: Sensitivity and Specificity values for the H8 and H12 qPCR assays were determined by testing target and non-target fecal DNA samples for marker presence using qPCR. All three sewage (target) samples were positive for H8 and H12 marker presence, resulting in a host-sensitivity value of 100% (Table 8, Figure 3). Mean H8 qPCR marker concentrations in sewage ranged from $3.2x10^7$ to $3.9x10^7$ gene copies/100 mL. Mean H12 qPCR

marker concentrations in sewage ranged from $1.5x10^6$ to $1.7x10^6$ gene copies/100 mL. Host-specificity for the H8 qPCR assay was 96.2%. Three out of 78 fecal DNA samples from non-target sources had quantifiable amounts of the H8 marker (one bird, one duck, and one alligator) ranging from $8.5x10^2$ to $4.9x10^3$ gene copies/g feces. Host-specificity for the H12 qPCR assay was 93%. Six out of 78 fecal DNA samples from non-target sources had quantifiable amounts of the H12 marker in three alligator samples ($7.6x10^3$ to $7.4x10^4$ gene copies/1g), one chicken sample ($6.9x10^4$ gene copies/1g), and two dog samples ($4.3x10^1$ gene copies/1g) (Table 8, Figure 3).

H8 proportion of *E. coli* in sewage: Culturable *E. coli* concentrations in untreated sewage influent were $7.0x10^6$ CFU/100 mL (Figure 4A). H8 marker prevalence, or the percentage of *E. coli* isolates PCR positive for H8 presence by conventional PCR, was 20% (6 out of 30 *E. coli* isolates tested positive). QPCR for *E. coli* uidA and *E. coli* H8 marker genes showed $4.5x10^7$ and $1.5x10^7$ gene copies/100 mL of influent for uidA and H8, respectively. H8 qPCR marker prevalence was 33.64%, measured as the ratio of H8 (host-specific *E. coli*) marker gene copies to uidA (general *E. coli*) marker gene copies (Figure 4B).

H8 and HF183 in Spilled Sewage: In an environmental water sample collected near the site of accidental untreated wastewater discharge caused by a lift station failure the H8 qPCR marker gene concentration was $8.2x10^4$ gene copies/100 mL while HF183 was measured at $1.5x10^8$ gene copies/100 mL (Figure 5). In a sample of wastewater illegally dumped on a Florida highway the H8 qPCR marker gene concentration was $4.3x10^5$ gene copies/100 mL and the HF183 concentration was $2.8x10^6$ gene copies/100 mL (Figure 5). A positive correlation (r^2=0.89, P-value= 2.2e-16) was found between the H8 TaqMan and HF183 assays.

TABLE 5: Conventional PCR results for human wastewater associated *E. coli* genetic markers in *E. coli* isolates from untreated sewage and animal feces in Tampa, Florida

Host Groups	# of Fecal Samples	# of *E. coli* Isolates	# of Isolates PCR Positive for Genetic Markers			
			H8	H12	H14	H24
Cat	1	30	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Cattle	9	270	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Dog	6	180	0 (0.00)	0 (0.00)	1 (0.55)	71 (39.4)
Gator	1	30	1 (3.33)	0 (0.00)	1 (3.33)	0 (0.00)
Horse	6	180	2 (1.11)	0 (0.00)	3 (1.66)	0 (0.00)
Duck	10	300	0 (0.00)	7 (2.33)	3 (1.00)	5 (1.66)
Feral Pig	3	90	0 (0.00)	0 (0.00)	2 (2.22)	4 (4.44)
Human	10	300	50 (16.6)	23 (7.66)	49 (16.3)	64 (21.3)
Host-Specificity:			92%	97%	86%	81%
Host-Sensitivity:			100%	90%	100%	100%
Marker Prevalence (Sewage *E. coli*)			16.6%	7.66%	16.3%	21.3%

TABLE 6: Prevalence of H8 and H14 marker genes and HF183 concentrations through wastewater treatment

Treatment Stage	# of *E. coli* Isolates Tested	# of Isolates PCR Positive for Marker Presence		qPCR Marker Concentrations: Log10 Gene Copies/100 mL	
		H8	H14	EC-H8	HF183
Primary Influent #1	30	11 (37%)	13 (43%)	6.91	9.02
Primary Influent #2	N/A	N/A	N/A	5.41	7.79
Clarifier Effluent #1	30	3 (10%)	6 (20%)	1.90	3.69
Clarifier Effluent #2	N/A	N/A	N/A	2.12	3.02
Lagoon #1	30	2 (7%)	3 (10%)	1.76	2.49
Lagoon #2	N/A	N/A	N/A	2.46	1.70

TABLE 7: Prevalence of *E. coli* H8 markers in environmental *E. coli* isolates and the concentration of the HF183 marker in water samples collected from Sweetwater and Bullfrog Creek

Sampling Sites	Sampling Event (Month/Year)	# of *E. coli* Isolated	Isolates PCR Positive for H8	HF183 qPCR log_{10} Per 100 mL of Water
Sweetwater Creek				
SWC1	Aug. 2016	30	0	ND
	Nov. 2016	30	0	ND
	Jan. 2017	30	0	ND
	Mar. 2017	30	0	ND
SWC2	Aug. 2016	30	0	ND
	Nov. 2016	30	0	ND
	Jan. 2017	30	0	ND
	Mar. 2017	30	0	ND
SWC3	Aug. 2016	30	0	ND
	Nov. 2016	30	0	ND
	Jan. 2017	30	0	ND
	Mar. 2017	30	0	ND
Bullfrog Creek				
BFT1	Sep. 2016	30	0	2.13
	Jan. 2017	30	0	ND
	Apr. 2017	30	0	ND
BFT2	Sep. 2016	30	0	ND
	Jan. 2017	30	0	ND
	Apr. 2017	30	0	ND
BFC3	Sep. 2016	30	0	ND
	Jan. 2017	30	0	1.85
	Apr. 2017	30	0	2.65

ND: Non-detect.

TABLE 8: QPCR analysis of *E. coli* H8 and H12 marker gene concentrations in animal feces and sewage. Host-specificity and host-sensitivity are also shown.

Host Groups	H8 Marker Results:		H12 Marker Results:	
	# of qPCR Positive Results/# of Samples Tested	Log_{10} Gene Copies (Range) Per g Wet Weight Feces or per 100 mL Sewage	# of qPCR Positive Results/# of Samples Tested	Log_{10} Gene Copies (Range) Per g of Wet Weight Feces or per 100 mL of Sewage
Alligator[a]	1/10	3.55	3/10	3.88-4.87
Bird[a]	1/5	3.91	0/5	ND
Cat[a]	0/10	ND	0/10	ND
Chicken[a]	0/10	ND	1/10	4.84
Cattle[a]	0/10	ND	0/10	ND
Deer[a]	0/8	ND	0/8	ND
Dog[a]	0/10	ND	2/10	1.63-2.20
Duck[a]	1/10	3.15	0/10	ND
Horse[a]	0/10	ND	0/10	ND
Human[b]	3/3	7.51-7.59	3/3	6.17-6.23
Host-Specificity	96%		93%	
Host-Sensitivity	100%		100%	

[a]:Non-human host group; [b]:Human host group (sewage); ND = Non-Detect

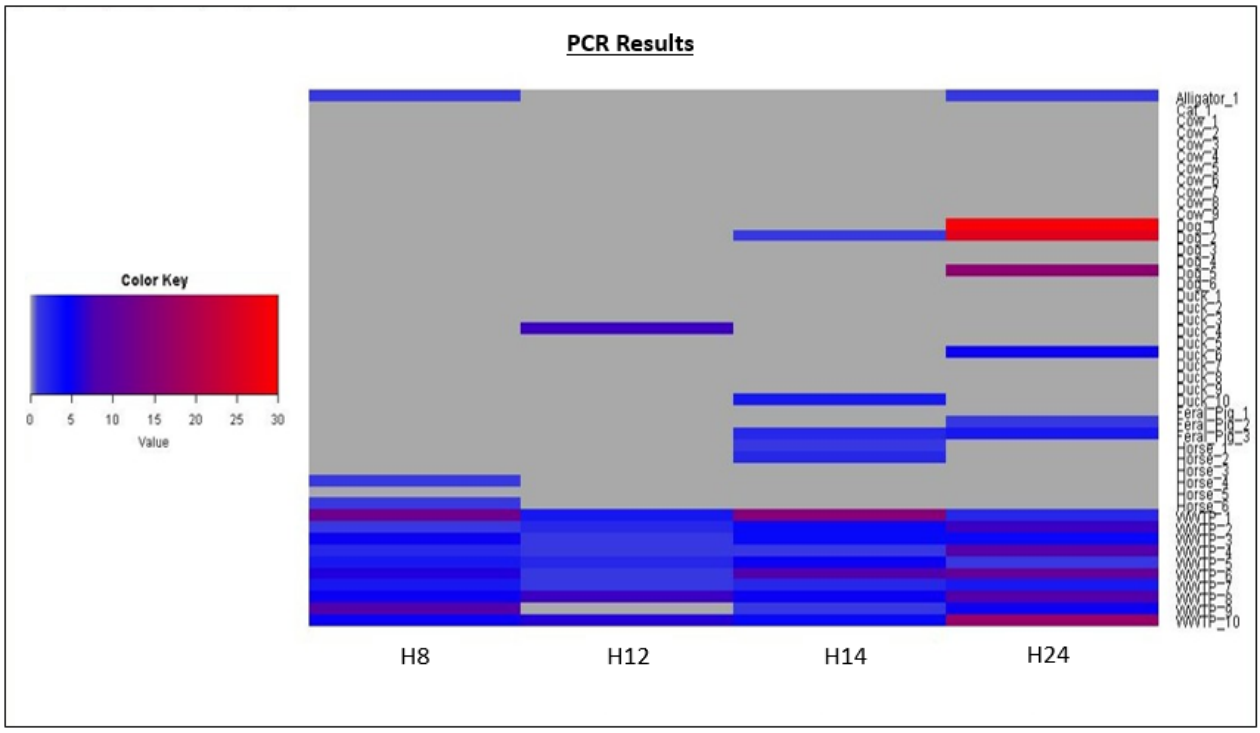

FIGURE 2: Heat map of conventional PCR results for human wastewater associated *E. coli* genetic markers in *E. coli* isolates from sewage and animal feces in Tampa, Florida. Heat map shows higher $\log_{10}$ concentrations in red, lower in blue.

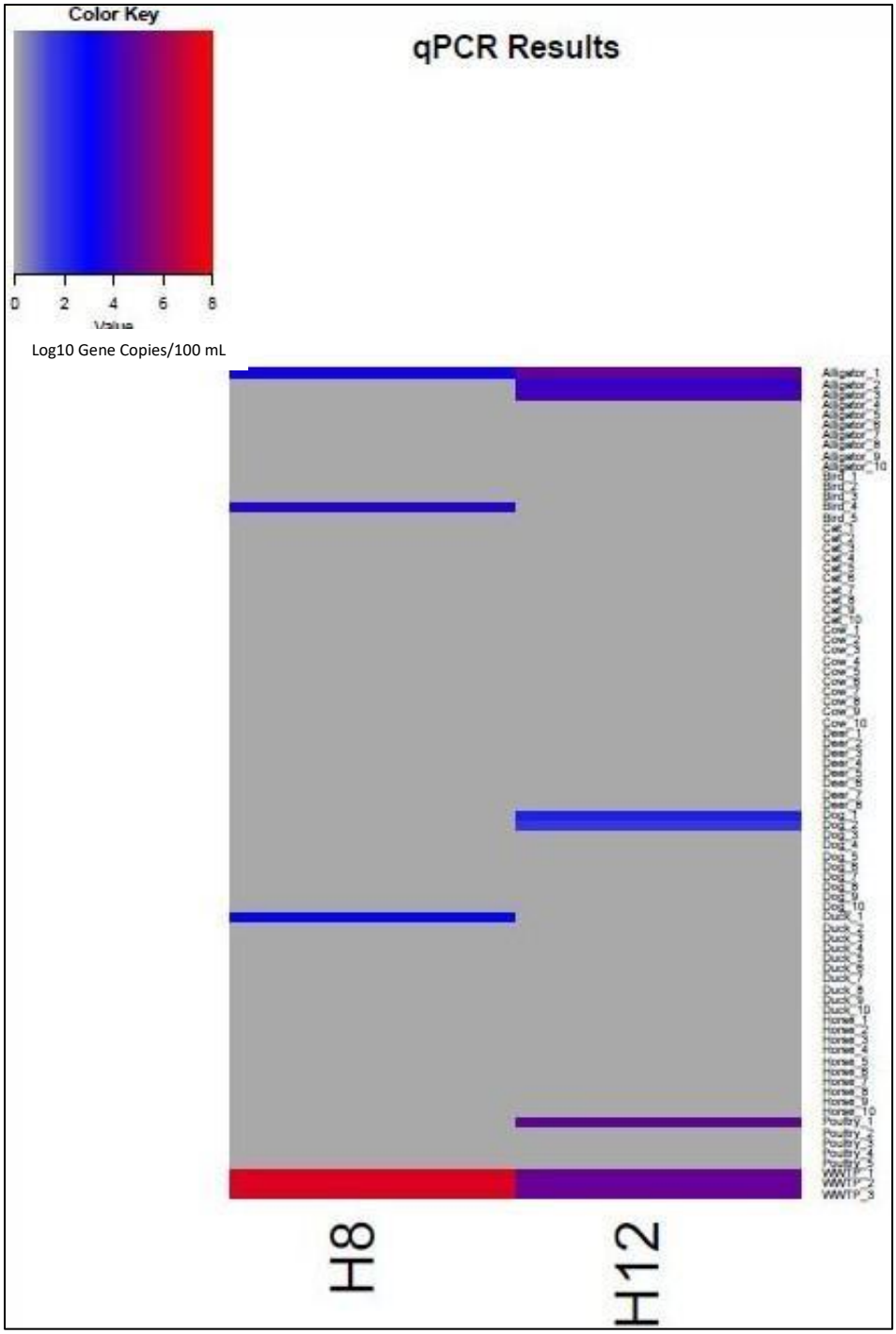

FIGURE 3: Heat map of *E. coli* H8 and H12 log10 marker gene concentrations per 100 mL of raw sewage or 1 g of wet weight animal feces. Heat map shows higher $\log_{10}$ concentrations in red, lower in blue.

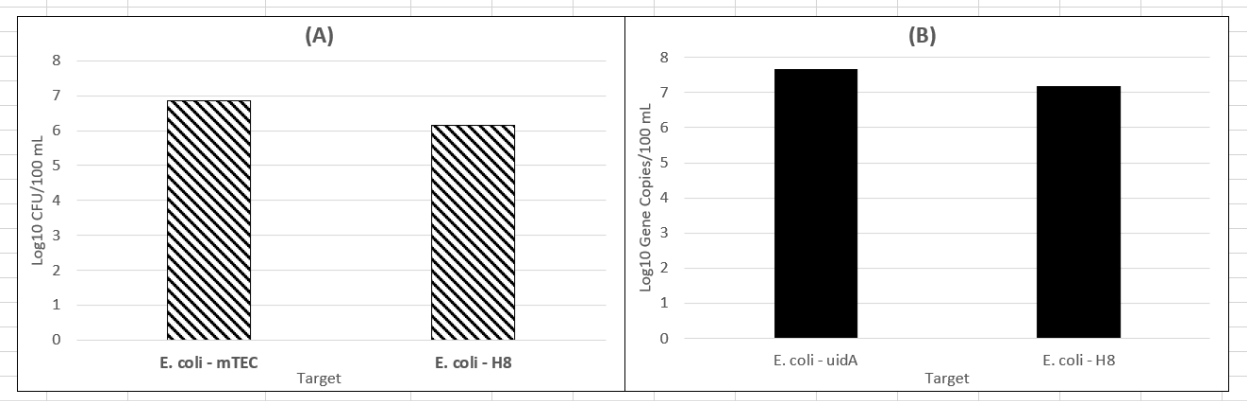

Figure 4A and 4B: (A) Left – Total culturable *E. coli* concentrations in untreated sewage compared to H8 prevalence among *E. coli* isolates. (B) Right – Total *E. coli* (uidA) and human-associated (EC-H8) marker concentrations in untreated sewage obtained using qPCR.

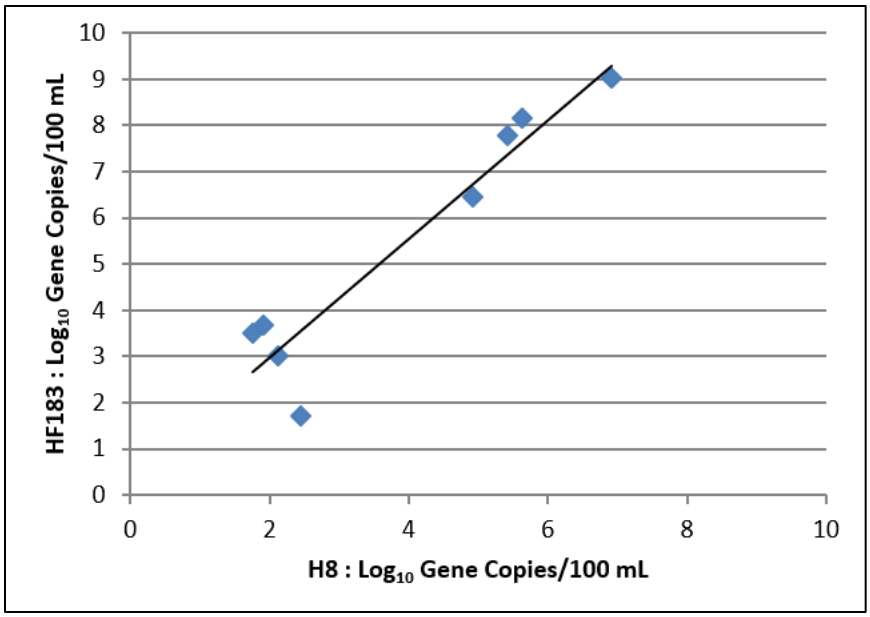

Figure 5: H8 and HF183 $\log_{10}$ marker concentrations in spilled and treated sewage using qPCR

Discussion:

A drawback of the most useful MST methods, including the various versions of the *Bacteroides dorei*-like HF183 (Haugland *et al.*, 2010, Harwood *et al.*, 2014), is that they have no direct connection to any FIB that has a demonstrated predictive relationship with human health risk associated with recreational water use. The *E. coli* H genes evaluated in this study, on the other hand, originate from a FIB species recognized as a regulatory tool in the U.S. (USEPA, 2012) and worldwide (WHO, 2003). This is the first study in the U.S. to assess four recently developed genetic markers (H8, H12, H14, and H24) for human host-associated *E. coli* markers for microbial source tracking applications and introduces two probe-based TaqMan qPCR assays for quantification of sewage contamination in environmental waterbodies.

Prior to using a marker for microbial source tracking, marker performance should be assessed in any region where they have not been previously utilized (USEPA, 2005, Harwood *et al.*, 2014). In this study, as well as in the prior studies conducted in Japan and Australia, *E. coli* marker genes H8 and H12 exhibited greater specificity than H14 or H24. Prevalence, or frequency of marker presence among target organisms (Stoeckel & Harwood, 2007), for H8 and H12 in this study was lower than previously reported (Gomi *et al.*, 2014, Ahmed *et al.*, 2015), possibly due to the smaller sample sizes tested in those studies (<100 isolates). Previous studies also utilized different methods to determine marker performance; in Japan, *E. coli* isolates were obtained from human and non-human sources, then grown in batch culture for DNA extraction and tested for marker presence using SYBR green qPCR. In Australia, isolate-by-isolate testing was not

performed for fecal samples from non-target hosts; instead, fecal DNA extracts from non-target hosts were tested using SYBR green qPCR, demonstrating the lower specificity of H14 and H24 markers (Ahmed *et al.*, 2015). In the current study, marker performance was initially assessed by conventional PCR for *E. coli* isolates obtained from target (300 isolates) and non-target (1080 isolates) host groups. The results of performance testing indicate that H8 and H12 markers are geographically stable, commonly found among sewage *E. coli* isolates and largely absent or at levels too low to detect in non-target animal fecal samples, indicating suitability for MST applications.

Based on the results of isolate-by-isolate testing, TaqMan QPCR assays were developed in order to quantify H8 and H12 markers. Performance for each marker was assessed against a reference library of fecal DNA extracts from target and non-target host sources. Both assays exhibited high specificity and sensitivity for sewage, but H8 quantities were found at an average of two orders of magnitude higher concentration than H12. These results suggest that H12 is the less suitable candidate for environmental applications since lower concentrations may result in failure to detect diluted sewage in environmental samples. An additional advantage of the H8 TaqMan assay is that specificity testing does not indicate a cross-reaction with deer feces, while HF183 has a known issue with false positive detection of deer feces (Layton *et al.*, 2013, Nguyen *et al.*, 2018). Comparison of HF183 (human-associated *Bacteroides dorei*) and H8 concentrations in treated and untreated wastewater found comparable levels that were strongly correlated. In a separate Australian study (Hughes *et al.*, 2017), H8 and HF183 concentrations in untreated wastewater measured by SYBR green qPCR were observed at similar levels. Furthermore, in the

current study, HF183 and H8 were correlated in environmental water samples impacted by human sewage.

These results indicate that the H8 TaqMan assay developed in this study for quantification of human host-associated *E. coli* is a promising addition to the MST toolbox due to high specificity for sewage, abundance in untreated and partially-treated wastewater, and correlation with the well-established HF183 marker. Host-associated *E. coli* markers are a tool that can improve water quality management decisions by allowing investigators to determine whether elevated *E. coli* densities in surface water are caused by anthropogenic inputs or are a part of natural background FIB levels (Goodwin *et al.*, 2017). Furthermore, the ability to quantify and compare the percentages of human-specific *E. coli* present in environmental water samples to the total (non-host specific) *E. coli* may allow for the development of alternative site specific criteria for recreational water quality (Nguyen *et al.*, 2018) facilitating the identification and removal of FIB from anthropogenic sources associated with higher risk of illness.

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