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ARTICLE *in* ENVIRONMENTAL SCIENCE & TECHNOLOGY · FEBRUARY 2015

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Quantitative PCR measurements of *Escherichia coli* including Shiga Toxin-Producing *E. coli* (STEC) in Animal Feces and Environmental Waters

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ABSTRACT: Quantitative PCR (qPCR) assays were used to determine the concentrations of *E. coli* including shiga toxin-producing *E. coli* (STEC) associated virulence genes (*eaeA*, stx_1, stx_2, and *hlyA*) in ten animal species (fecal sources) and environmental water samples in Southeast Queensland, Australia. The mean Log_{10} concentrations and standard deviations of *E. coli* 23S rRNA across fecal sources ranged from 1.3 ± 0.1 (horse) to 6.3 ± 0.4 (cattle wastewater) gene copies at a test concentration of 10 ng of DNA. The differences in mean concentrations of *E. coli* 23S rRNA gene copies among fecal source samples were significantly different from each other ($P < 0.0001$). Among the virulence genes, stx_2 (25%, 95% CI, 17−33%) was most prevalent among fecal sources, followed by *eaeA* (19%, 95% CI, 12−27%), stx_1 (11%, 95% CI, 5%−17%) and *hlyA* (8%, 95% CI, 3−13%). The Log_{10} concentrations of STEC virulence genes in cattle wastewater samples ranged from 3.8 to 5.0 gene copies at a test concentration of 10 ng of DNA. Of the 18 environmental water samples tested, three (17%) were positive for *eaeA* and two (11%) samples were also positive for the stx_2 virulence genes. The data presented in this study will aid in the estimation of quantitative microbial risk assessment (QMRA) from fecal pollution of domestic and wild animals in drinking/recreational water catchments.

INTRODUCTION

Escherichia coli (*E. coli*) is commonly used as an indicator of the microbiological quality of drinking and surface waters. Their presence in water indicates the occurrence of fecal pollution along with the presence of pathogens that are capable of causing illnesses in humans. *E. coli* are commonly found in the gastrointestinal tracts of warm-blooded animals, and are often characterized as harmless or commensal bacteria.[1] However, certain strains of *E. coli* can be pathogenic and responsible for both intestinal and extraintestinal infections.[2,3] For example, shiga toxin-producing *E. coli* (STEC) are important zoonotic pathogens associated with bloody diarrhea and sometimes hemolytic uraemic syndrome (HUS) in humans.[3,4]

It has been reported that dairy and beef cattle are the major sources of STEC. Global testing of beef cattle feces indicated variable prevalence rates for O157 STEC (0.2−49%) and non-O157 STEC (0.4−74%).[5,6] In addition, pets (cats and dogs), ruminants (deer, sheep, and goats), and wildlife (ducks) are also known to harbor STEC.[7,8] STEC may enter surface waters or drinking water supplies by direct defecation from animals or as a result of stormwater runoff from grazing areas.[9] The ability of STEC to cause severe diseases in humans is mainly associated with the production of at least one shiga toxin-producing virulence gene (encoded by stx_1 and stx_2).[10] Many STEC also produce intimin, a 97-kDA attachment-and-effacement protein (encoded by *eaeA* virulence gene), and hemolysin (encoded by *hlyA* virulence gene).[11,12]

The majority of data available on the concentrations of fecal indicator bacteria in animal feces and surface waters worldwide has been generated using culture-based methods. These methods are widely accepted because of their ease of use and affordability.[13] The sample processing time, however, is lengthy, ranging from 18 to 96 h, which is not practical for a situation that demands quick assessment of water quality, such as beach closure due to fecal pollution.[14] Other limitations include underestimation of the concentration due to presence of injured or stressed cells, and the fact that *E. coli* can be viable but not culturable (VBNC).[15,16] The recent advances in quantitative PCR (qPCR) assays enable rapid, specific, and sensitive detection of various microorganisms in fecal, wastewater, and water matrices. These assays eliminate the incubation step by quantifying DNA from any microorganism of interest, and can yield results within 2−4 h.[17] Over the past

Received: August 11, 2014
Revised: January 26, 2015
Accepted: February 3, 2015

DOI: 10.1021/es505477n
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

Table 1. Number of Fecal Samples Tested, Their Sources, and Concentration of DNA Extract from Fecal Samples

	no. of samples	types of samples	sources of samples	weight/volume used for DNA extraction	range (ng) DNA/μL of extract
cat	10	individual	veterinary hospital	180−220 mg	15−88
cattle wastewater	16	composite	abattoir	10 mL	10−46
deer	10	individual	sanctuary	180−220 mg	18−68
dog	10	individual	veterinary hospital and parks	180−220 mg	11−229
emu	10	individual	emu farm	180−220 mg	7.2−64
horse	10	individual	horse racecourse	180−220 mg	10−74
human wastewater	10	composite	WWTPs[a]	10 mL	11−56
kangaroo	10	individual	wild/sanctuary	180−220 mg	17−78
possum	10	individual	wild	180−220 mg	19−64
waterfowl	10	individual	wild	100−150 mg	10−268

[a]Wastewater treatment plants.

few years, the application of qPCR assays has been used to measure the fecal indicator bacteria in environmental waters. Several studies have found correlations between qPCR assays and corresponding culture-based methods used for measuring fecal indicator bacteria in environmental waters.[18−20]

The presence of STEC associated virulence genes namely *eaeA*, stx_1, and stx_2 in environmental waters in Southeast Queensland, Australia has been reported.[21−23] End-point and multiplex-PCR assays have been widely used to detect STEC strains from variety of animal feces and surface waters.[11,22−24] However, in order to conduct a quantitative microbial risk assessment (QMRA) for the estimation of potential illness associated with polluted environmental waters, there is a necessity for estimating the concentrations of *E. coli* including STEC in the feces of various animals and environmental waters. The primary aim of this study was to identify animals that shed high concentrations of *E. coli* 23S rRNA gene along with STEC virulence genes (*eaeA*, stx_1, stx_2, and *hlyA*) in their feces in Southeast Queensland, Australia. Environmental water samples were also collected from the six sites in Brisbane River and the concentrations of *E. coli* 23S rRNA and STEC virulence genes were determined using qPCR assays. Once the concentrations of these STEC virulence genes in the feces/wastewater and receiving environmental waters are known then the predictive mathematical model of transport, and fate along with exposure scenarios (potable or swimming) can be developed to predict the probability of human infections.

MATERIALS AND METHODS

Collection of Feces and Wastewater. Fecal samples were collected from 10 animal species (Table 1). Individual animal fecal samples were collected from the veterinary hospital (University of Queensland, Gatton), animal sanctuaries, park, farm, and racecourse. Beef cattle composite wastewater (mixture of feces and urine) samples were collected from two different abattoirs located on the outskirts of Brisbane. Human wastewater samples were collected from the primary influent of four wastewater treatment plants (WWTPs) serving 50 000 to 500 000 people in Brisbane and Sunshine Coast regions. A fresh fecal sample was collected from the defecation of each individual animal. All samples were transported on ice to the laboratory, stored at 4 °C, and processed within 12−24 h.

Environmental Water Sampling. Environmental water samples were collected from six sites (designated BR1−BR6) from the Brisbane River, Qld, Australia. Three samples were collected from each site on one occasion, giving a total number of 18 samples. Site BR1 is located upstream of the Brisbane River. This site receives overflow of water from the Wivenhoe Reservoir. The suspected sources of fecal pollution include waterfowl and wildlife. The site is used for swimming and fishing by local residents. Site BR2 is located in peri-urban nonsewered catchment. The potential sources of contamination include cattle, horse, septic systems, and wildlife. Site BR3 is a major tributary of the Brisbane River and is tidally influenced. The catchment where the site is located has residential and industrial developments and is serviced by a WWTP. The elevated levels of fecal indicator bacteria in site BR3 has been a major water quality issue identified by the catchment water quality monitoring group. Site BR4 is located in a highly urban area and is also tidally influenced. This site receives urban runoff through a stormwater drain. Sites BR5 and BR6 are located downstream of the river in the highly urbanized areas. The potential sources of contamination in these sites (BR5 and BR6) include waterfowl and recreational boats. Water samples were collected from each site in 10-L sterile carboy containers (Nalgene Labware, Rochester, NY) at 30 cm below the water surface. The water samples were transported on ice to the laboratory and processed within 6−8 h.

Enumeration of Fecal Indicator Bacteria. The membrane filtration method was used for the isolation and enumeration of culturable *E. coli*. Sample serial dilutions were made in sterile Milli-Q water, and filtered through 0.45-μm pore size (47-mm diameter) nitrocellulose membranes (Millipore, Tokyo, Japan), and placed on Chromocult coliform agar (Merck, Germany). Plates were incubated at 37 °C for 24 h and then typical colony forming units (CFUs) were enumerated and expressed per 100 mL of water sample.

Sample Processing. The human and cattle wastewater samples were concentrated with Amicon Ultra-15 (30 K) Centrifugal Filter Devices (Merck Millipore Ltd.). In brief, 10 mL of wastewater sample was added to the Amicon device and centrifuged at 4750*g* for 10 min. Concentrated samples (180−200 μL) were collected from the filter device sample reservoir using a pipet.[25] Each water sample (2 L) collected from the Brisbane River was filtered through 0.45-μm pore size (47-mm diameter) nitrocellulose membranes. In case of membrane clogging during filtration, multiple membranes were used. The filter(s) was immediately transferred into a 15-mL sterile tube containing phosphate buffer saline. The tube was vortexed for 5 min to detach the bacteria from the filter, followed by centrifugation at 4,500*g* for 15 min at 4 °C to obtain a pellet.[50] All processed samples were stored at −20 °C for a maximum of 24 h prior to DNA extraction.

DNA Extraction and Standardization. DNA was extracted from the concentrated wastewater samples using DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). A

DOI: 10.1021/es505477n
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Table 2. Target, Primer Sequences, and Amplification Conditions for End-Point PCR and qPCR Assays Used in This Study

assay	target	primer or probe sequence (5′−3′)[b]	amplification conditions	refs
general bacteria	16S rRNA	F: ACT CCT ACG GGA GGC AGC AG R: ATT ACC GCG GCT GCT GG	5 min at 95 °C, 40 cycles of 15 s at 95 °C, 30 s at 53 °C, and 60 s at 60 °C	26
Sketa22	*Oncorhynchus keta*	F: GGT TTC CGC AGC TGG G R: CCG AGC CGT CCT GGT CTA P: FAM-AGT CGC AGG CGG CCA CCG T-TAMRA	10 min at 95 °C, 40 cycles of 15 s at 95 °C, 45 s at 63 °C	35
E. coli	23S rRNA	F: GGT AGA GCA CTG TTT TGG CA R: TGT CTC CCG TGA TAA CTT TCTC P: FAM-TCA TCC CGA CTT ACC AAC CCG-TAMRA	10 min at 95 °C, 40 cycles of 15 s at 95 °C, 60 s at 60 °C	18
enterohemorrhagic *E. coli* associated virulence genes	*eaeA*[a]	F: GAC CCG GCA CAA GCA TAA GC R: CCA CCT GCA GCA ACA AGA GG	8 min at 95 °C, 40 cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C	12
	hlyA	F: GCA TCA TCA AGC GTA CGT TCC R: AAT GAG CCA AGC TGG TTA AGC T		
	stx_1	F: ATA AAT CGC CTA TCG TTG ACT AC R: AGA ACG CCC ACT GAG ATC ATC		
	stx_2	F: GGC ACT GTC TGA AAC TGC TCC R: TCG CCA GTT ATC TGA CAT TCT G		

[a]Also found in enteropathogenic *E. coli* pathotype. [b]F, forward primer; R, reverse primer; P, probe; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

QIAamp Stool DNA kit (Qiagen) was used to isolate DNA from 100−220 mg of fresh animal feces from each fecal sample. DNA was extracted from the pellet obtained from the Brisbane River water samples using the MO Bio PowerSoil DNA Isolation Kit (Mo Bio Laboratories). All extracted DNA samples were quantified using a NanoDrop spectrophotometer (ND-1000; NanoDrop Technology, Wilmington, DE). Because different fecal source and water samples had different DNA concentrations (Table 1), all DNA samples were standardized to a mass of 5 ng/μL.

PCR Inhibition. An experiment was conducted to determine the effects of PCR inhibitors on the detection of *E. coli* 23S rRNA gene and STEC virulence genes in all standardized DNA samples. DNA samples were spiked with 10 pg of *Oncorhynchus keta* DNA (Sigma Chemical Co., St. Louis, Mo.). The threshold cycle (C_T) values of the 10 pg *O. keta* spiked DNA samples were compared to those of distilled water spiked with 10 pg of *O. keta* DNA to obtain information on the level of PCR inhibition.

Preparation of qPCR Standards. Standards for qPCR assays were prepared from the genomic DNA of *E. coli* ATCC 35150. A single *E. coli* colony was inoculated into nutrient broth (Oxoid, London, U.K.), and incubated at 37 °C overnight. Two mL of this overnight culture was centrifuged at 10 000*g* to obtain a pellet. DNA was extracted from the pellet using DNeasy Blood and Tissue Kit (Qiagen). The concentration of extracted genomic DNA was determined using the NanoDrop ND-1000 spectrophotometer (Nanodrop Technology). The gene copies were calculated using the mean mass of the *E. coli* genome. Ten-fold serial dilution ranging from 10^5 to 10^0 copies/μL of DNA extract was prepared and stored at −20 °C. A 2-μL template from each dilution was used to prepare standard curve for each qPCR assay. The amplification efficiency (E) was determined by analysis of the standards and was estimated from the slope of the standard curve as $E = 10^{-1/\text{slope}}$.

End-Point PCR and qPCR Assays. The primer sequences and amplification conditions for end-point and qPCR assays used in this study are shown in Table 2. Each standardized DNA sample was amplified using an end-point 16S rRNA general bacterial PCR assay to confirm successful DNA extraction process.[26] End-point PCR amplification was performed in 20-μL reaction mixtures using SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Richmond, CA). The general bacteria PCR mixtures contained 10 μL of Supermixes, 300 nM of each primer, and 10 ng (2 μL) of template DNA. All the samples tested in this study gave PCR positive amplification for the 16S rRNA general bacteria. End-point Sketa22 PCR amplification for *O. keta* was performed in 25-μL reaction mixtures using iQ Supermixes (Bio-Rad Laboratories). The PCR assay mixtures contained 12.5 μL of Supermixes, 300 nM of each primer, 400 nM of probe, 10 ng (2 μL) of template DNA, and 10 pg of *O. keta* DNA. None of the samples tested in this study showed signs of PCR inhibition. *E. coli* 23S rRNA qPCR amplification was performed in 25-μL reaction mixtures using iQ Supermixes (Bio-Rad Laboratories). The qPCR mixtures contained 12.5 μL of Supermixes, 800 nM of each primer, 80 nM of probe, and 10 ng (2 μL) of template DNA. STEC virulence genes *eaeA*, stx_1, stx_2, and *hlyA* qPCR amplifications were performed in 20-μL reaction mixtures using SsoFast EvaGreen Supermix (Bio-Rad Laboratories). The qPCR mixtures contained 10 μL of Supermixes, 300 nM each primer, and 10 ng (2 μL) of template DNA. For PCR/qPCR assays, positive (genomic DNA and standards for each qPCR run) and negative (sterile water) controls were included. The end-point and qPCR assays were performed using the Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratories). All end-point and qPCR reactions were performed in triplicate.

DOI: 10.1021/es505477n
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qPCR Assays Lower Limit of Quantification (LLOQ). Lower limit of quantification (LLOQ) provides a threshold value where a quantitative number can be determined. To determine qPCR LLOQ, 10-fold serial dilutions of standards (2×10^5 to 2×10^0 gene copies) were tested in replicates ($n = 4$). The lowest amount of diluted standards detected in at least 75% replicate assays was considered qPCR LLOQ.

Statistical Analysis. The concentrations of *E. coli* 23S rRNA gene and STEC virulence genes at a test concentration of 10 ng of DNA were Log_{10} transformed for fecal and environmental water samples. One-way analysis of variance (ANOVA) was also used to investigate whether the concentrations of *E. coli* 23S rRNA genes significantly differed among the fecal sources. A P value of <0.05 was considered to be significant.

RESULTS

qPCR Standards and Lower Limit of Quantification (LLOQ). qPCR standards were analyzed in order to determine the reaction efficiencies. The standards had a linear range of quantification from 2×10^5 to 2×10^1 gene copies/μL of DNA extracts. The slope of the standards ranged from −3.213 to −3.456. The amplification efficiencies ranged from 95% to 105%, and the correlation coefficient (r^2) ranged from 0.94 to 0.99. LLOQ of qPCR assays were determined using the standards. The qPCR LLOQ was determined to be 20 gene copies (for *E. coli* 23S rRNA assay) and 200 gene copies (for STEC virulence genes assays).

Concentrations of *E. coli* 23S rRNA in Fecal Source Samples. All fecal source samples were determined to be above qPCR LLOQ for *E. coli* 23S rRNA gene. For comparison among fecal sources, the concentrations of *E. coli* 23S rRNA gene copies obtained from each individual feces/wastewater samples within each fecal source were pooled. The mean Log_{10} concentrations and standard deviations across the entire fecal source samples ranged from 1.3 ± 0.1 (horse) to 6.3 ± 0.4 (cattle) gene copies/10 ng of DNA (Figure 1). Cattle wastewater DNA had more than 3 orders of magnitude higher *E. coli* 23S rRNA gene copies than combined mean concentrations of the remaining fecal sources. ANOVA results indicated that the differences in mean concentrations of *E. coli* 23S rRNA gene copies among fecal source samples were significantly different from each other ($df = 9$, $P < 0.0001$).

Prevalence and Concentrations of STEC Virulence Genes in Fecal Source Samples. The prevalence of STEC virulence genes in fecal source samples is shown in Table 3.

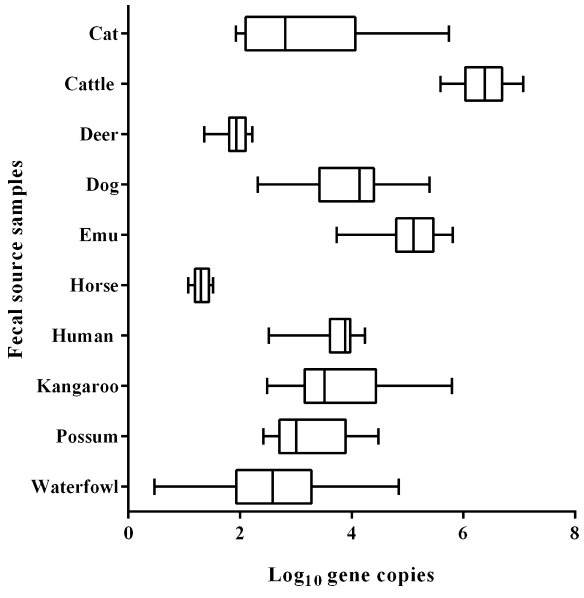

Figure 1. Box-and-whisker plots of the Log_{10} concentrations of *Escherichia coli* 23S rRNA gene copies in fecal source samples at a test concentration of 10 ng of DNA. The inner box lines represent the medians, while the outer box lines represent 25th and 75th percentiles.

Table 3. Prevalence of Shiga Toxin-Producing *E. coli* Virulence Genes in Fecal Source Samples

	no. of PCR positive samples/no. of samples tested (%)			
fecal source samples	*eaeA*	stx_1	stx_2	*hlyA*
cat	0/10 (0)	0/10 (0)	9/10 (90)	0/10 (0)
cattle	14/16 (86)	12/16 (75)	10/16 (63)	9/16 (56)
deer	0/10 (0)	0/10 (0)	1/10 (10)	0/10 (0)
dog	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)
emu	1/10 (10)	0/10 (0)	0/10 (0)	0/10 (0)
horse	0/10 (0)	0/10 (0)	4/10 (40)	0/10 (0)
human	4/10 (40)	0/10 (0)	0/10 (0)	0/10 (0)
kangaroo	0/10 (0)	0/10 (0)	2/10 (20)	0/10 (0)
possum	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)
waterfowl	1/10 (10)	0/10 (0)	1/10 (10)	0/10 (0)
total	20/106 (19)	12/106 (11)	27/106 (25)	9/106 (8)

Among the virulence genes, stx_2 was most prevalent (25%, 95% CI, 17−33%) among fecal sources, followed by *eaeA* (19%, 95% CI, 12−27%), stx_1 (11%, 95% CI, 5%−17%), and *hlyA* (8%, 95% CI, 3−13%). Among the fecal source samples, cattle wastewater harbored all four STEC virulence genes. The remaining fecal sources (cat, deer, emu, horse, human wastewater, kangaroo, and waterfowl) carried at least one virulence gene (either *eaeA* or stx_2). Fecal DNA samples from three cats, one deer, one emu, four horses, and one waterfowl gave qPCR positive amplifications but the C_T values of these samples fell below the LLOQ (<200 gene copies), and therefore could not be quantified.

The mean Log_{10} concentration and standard deviation of *eaeA* in cattle wastewater was 5.0 ± 0.4 gene copies at a test concentration of 10 ng of DNA. The concentrations of stx_1 (3.8 ± 0.6), stx_2 (4.8 ± 0.3), and *hlyA* (4.3 ± 0.8) were also high in cattle wastewater (Figure 2). The Log_{10} concentrations and standard deviations of stx_2 in cat (2.3 ± 0.3), kangaroo (3.0), and human wastewater (2.9 ± 0.5) were 2−3 orders of magnitude lower than those of cattle wastewater. To obtain information on the shedding variability, we also estimated the STEC virulence genes as a percentage of total *E. coli* 23S rRNA

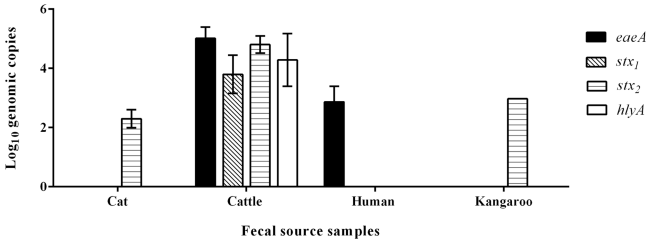

Figure 2. Mean Log_{10} concentrations of shiga toxin-producing *Escherichia coli* (STEC) associated virulence genes in fecal source samples at a test concentration of 10 ng of DNA.

DOI: 10.1021/es505477n
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gene copies in feces and wastewater samples. The percentage of stx_2 virulence gene over *E. coli* 23S rRNA gene in individual cat fecal DNA sample ranged from 0.01 to 29% suggesting high variability in shedding rates. Similarly, the percentage of *eaeA*, stx_1, stx_2, and *hlyA* in cattle ranged 0.7−3.8%, 3.2−43%, 0.4−41%, and 0.008−10%, respectively. The percentage of *eaeA* and stx_2 over total *E. coli* 23S rRNA gene copies in human wastewater ranged from 4.0 to 21% and 4.9%, respectively. The percentage of stx_2 virulence gene in kangaroo fecal DNA samples ranged from 27 to 42%.

Prevalence and Concentrations of *E. coli* and STEC Virulence Genes in Environmental Water Samples. The concentrations of culturable *E. coli* in environmental water samples collected from the six sites of the Brisbane River ranged from 1.0 to 2.7 Log_{10} CFU/100 mL of water (Table 4).

Table 4. Log_{10} Concentrations of Culturable *E. coli* and 23S rRNA Gene and the Prevalence of Shiga Toxin-Producing *E. coli* in Environmental Water

			shiga toxin-producing *E. coli*			
sampling site[a]	range of *E. coli* (CFU/100 mL)	range of *E. coli* 23S rRNA (gene copies/100 mL)	*eaeA*	stx_1	stx_2	*hlyA*
BR1	1.0−1.2	2.1−2.6	ND	ND	ND	ND
BR2	1.7−1.9	3.4−3.8	+	ND	+	ND
BR3	2.4−2.5	3.2−3.5	+	ND	+	ND
BR4	2.6−2.7	4.1−4.3	+	ND	ND	ND
BR5	2.1−2.2	2.9−3.1	ND	ND	ND	ND
BR6	1.8−2.0	2.9−3.1	ND	ND	ND	ND

[a]Triplicate samples were collected from each site. ND: Not detected.

The concentrations of culturable *E. coli* were generally much higher in samples collected from sites BR3 and BR4 compared to others. Among the 18 samples tested, 9 (50%) samples from sites BR3−BR5 exceeded the Australian and New Zealand Environment and Conservation Council (ANZECC) recreational water quality guidelines for fresh and marine waters of 150 faecal coliforms/100 mL for primary contact. The concentrations of *E. coli* 23S rRNA genes were 1−2 Log higher than culture-based methods. Of the 18 samples tested, three (17%) samples were positive for *eaeA* and two (11%) samples were also positive for the stx_2 virulence genes. The concentrations of these genes could not be determined because the C_T values of these samples fell below the LLOQ.

DISCUSSION

Several studies have reported the concentrations of fecal indicator bacteria such as *E. coli* and *Enterococcus* spp. in various fecal sources (colony forming units/g of feces) using culture-based methods.[9,27−32] However, these methods may underestimate the cell numbers due to the presence of injured/stressed cells and VBNC state of microorganisms.[33,34] For these reasons, qPCR-based methods began to be used to detect a wide range of fecal indicator bacteria and pathogens in clinical and environmental samples.[18,35,36] In view of this, in the current study, we also used qPCR assays to quantify *E. coli* and STEC virulence genes in fecal source and environmental water samples.

An important factor that may influence the estimation of fecal indicator bacteria in fecal sources is the unit of measure.[37] A number of strategies such as (i) fecal wet mass, (ii) fecal dry mass, (iii) copies of genetic markers, and (iv) a mass of total DNA have been tested.[32,37−40] The latter approach (mass of total DNA) has some advantages such as the avoiding the need to measure and correct for DNA extraction efficiencies and error introduced by sample variability (solid or liquid phases). The standardization process may also relieve PCR inhibition.[37] Finally, if required, the concentration of target gene/gram of feces can be extrapolated from the known (10 ng) concentration of DNA. However, little information is available on what mass of DNA should be used in PCR amplifications. A recent study determined the concentrations of fecal indicator bacteria and genetic markers in primary influent and 20 animal fecal samples across the United States. The authors used 1 ng of DNA in their qPCR analysis.[37] However, several samples at the test concentration fell below the LLOQ. Because of this, we have standardized all DNA samples at a test concentration of 10 ng for PCR analysis. This concentration was chosen to minimize nondetects and increase the chance of detecting STEC virulence genes in fecal sources that may occur in low concentrations. For qPCR assays of *E. coli*, we also targeted multiple 23S rRNA gene copies as opposed to single gene copy (*uidA* gene) to increase the detection sensitivity. Nonetheless, 23S rRNA gene copies can be converted to equivalent cell numbers by dividing total gene copies by 23S rRNA gene (seven for *E. coli*). All the samples tested for the *E. coli* 23S rRNA gene in this study were above the qPCR LLOQ, suggesting the factors such as concentration of DNA mass and assay types should be carefully selected.

The concentration of *E. coli* 23S rRNA gene was highest in cattle composite wastewater samples. This could be due to the fact that composite wastewater samples contain *E. coli* possibly from hundreds of cattle. In addition, the concentrations of *E. coli* 23S rRNA gene among the cattle wastewater samples were remarkably consistent across all samples. A little variation was also observed in concentrations of *E. coli* 23S rRNA gene in cattle wastewater samples collected from two abattoirs. We also extrapolated the concentrations of *E. coli* 23S rRNA gene/g of feces or 100 mL of wastewater from the concentrations obtained at the test concentration of 10 ng of DNA. The mean concentrations of *E. coli* 23S rRNA/g feces or 100 mL of wastewater showed trends similar to those measured at a test concentration of 10 ng of DNA. Caution should be exercised when comparing the concentrations of *E. coli* 23S rRNA gene copies across studies because we determined the concentrations of *E. coli* in DNA samples from individual animals and wastewater while the other studies pooled the DNA extracted from individual fecal samples or used composite fecal samples for DNA extraction. The disadvantage of pooling DNA or fecal samples is a single positive individual sample may result in the whole pooled samples being positive.[40] In addition, DNA extraction, qPCR efficiencies, and DNA mass measurement techniques across studies may also influence the concentrations of target microorganism detected.[41]

The variable concentrations of *E. coli* 23S rRNA in different fecal sources at a test concentration of 10 ng suggest that some fecal sources may contribute more microbial load in environmental waters. This information can be particularly important to managers because best management practices can be employed to improve the environmental water quality. In this study, we have also estimated the concentrations of STEC associated virulence genes in cattle wastewater, human wastewater, emu, and waterfowl fecal DNA samples using qPCR assays. The concentrations of STEC associated virulence genes in cattle wastewater were high. This is particularly

DOI: 10.1021/es505477n
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important because a recent study reported the high risks of gastrointestinal illness from cattle feces contaminated water.[42] The percentage of STEC virulence gene over *E. coli* 23S rRNA gene in fecal source samples also indicated that the concentrations of these virulence genes could be as high as 42% in individual fecal samples, indicating some animals harbor more virulence genes than others.

The numbers of *E. coli* measured by qPCR assay were 1−2 Log higher than culture-based methods for most of the environmental water samples. Previous method comparison studies also reported high numbers of qPCR cell equivalents compared to culture-based numbers in recreational waters.[35,43,44] Water samples collected from sites BR2−BR4 were PCR positive for *eaeA* and stx_2 virulence genes. Site BR2 is characterized by agricultural practices including cattle farming. The presence of *eaeA* and stx_2 in site BR3 could have originated from upstream cattle farms. It has to be noted that all water samples were collected during low tide condition, and, probably for this reason, STEC virulence genes were also detected in downstream sites. Nonetheless, the presence of STEC virulence genes in water samples from sites BR2 and BR3 which are used for recreational activities indicates potential health risks because the infectious dose of STEC could be as low as 10−100 cells.[12]

We acknowledge that the PCR measurements may have overestimated the concentrations of target microorganism in this study because the assays quantified DNA from both viable and nonviable cells.[45,46]

However, STEC *E. coli* can survive in feces for a long period of time (1−18 weeks at a temperature 15 °C) and retain its ability to produce toxins.[47] From our results, it can be postulated that kangaroo, cattle, and human wastewater may be the sources of these STEC virulence genes in *E. coli* isolates from environmental waters in Southeast Queensland. This is further supported by the fact that in a recent study we have reported the co-occurrence (6−11%) of cattle feces associated markers with *E. coli* O157 *rfbE* gene.[21]

In conclusion, we provide quantitative data on the concentrations of *E. coli* along with STEC virulence genes in different fecal sources including human wastewater. We have also shown the presence of STEC virulence genes in environmental water samples. Prevalence and concentrations of STEC virulence genes in different fecal sources not only provide important information on their potential sources but also the magnitude of potential risks. The data presented in this study indicating that health risks may be higher in catchments dominated by livestock or intensive cattle farming. On the basis of the STEC concentration in different animal feces and wastewater, the dose can be estimated by multiplying the volume of water ingested via drinking or swimming. Feeding the dose into the QMRA model would yield probability of infection which can be multiplied by a morbidity factor to estimate the probability of illness.[42,48,49] We acknowledged that this assumption is likely to overestimate the risk of infection by the QMRA analysis because qPCR generated concentrations represent both viable and nonviable cells. Nonetheless, a highly conservative "worst-case" scenario may be acceptable if the environmental water is used as drinking water or for recreational activities. The results from the current study may provide valuable information to water quality managers in terms of minimizing the risk from STEC in environmental waters impacted by fecal pollution.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This research presented in this paper was funded by the CSIRO Water for a Healthy Country Flagship Program. We thank Dr. Jatinder Sidhu for providing technical advice. We thank Lyell Deer Sanctuary, Lone Pine Koala Sanctuary, and "Peter the Possum" possum removalist for providing animal fecal samples.

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