Water Research 88 (2016) 613–622

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

Utility of *Helicobacter* spp. associated GFD markers for detecting avian fecal pollution in natural waters of two continents

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ARTICLE INFO

Article history:
Received 6 July 2015
Received in revised form
18 October 2015
Accepted 26 October 2015
Available online xxx

Keywords:
Microbial source tracking
Fecal indicator bacteria
Avian fecal pollution
Molecular markers
Wastewater
Quantitative PCR

ABSTRACT

Avian fecal droppings may negatively impact environmental water quality due to the presence of high concentrations of fecal indicator bacteria (FIB) and zoonotic pathogens. This study was aimed at evaluating the performance characteristics and utility of a *Helicobacter* spp. associated GFD marker by screening 265 fecal and wastewater samples from a range of avian and non-avian host groups from two continents (Brisbane, Australia and Florida, USA). The host-prevalence and -specificity of this marker among fecal and wastewater samples tested from Brisbane were 0.58 and 0.94 (maximum value of 1.00). These values for the Florida fecal samples were 0.30 (host-prevalence) and 1.00 (host-specificity). The concentrations of the GFD markers in avian and non-avian fecal nucleic acid samples were measured at a test concentration of 10 ng of nucleic acid at Brisbane and Florida laboratories using the quantitative PCR (qPCR) assay. The mean concentrations of the GFD marker in avian fecal nucleic acid samples (5.2×10^3 gene copies) were two orders of magnitude higher than non-avian fecal nucleic acid samples (8.6×10^1 gene copies). The utility of this marker was evaluated by testing water samples from the Brisbane River, Brisbane and a freshwater creek in Florida. Among the 18 water samples tested from the Brisbane River, 83% ($n = 18$) were positive for the GFD marker, and the concentrations ranged from 6.0×10^1–3.2×10^2 gene copies per 100 mL water. In all, 92% ($n = 25$) water samples from the freshwater creek in Florida were also positive for the GFD marker with concentrations ranging from 2.8×10^1–1.3×10^4 gene copies per 100 mL water. Based on the results, it can be concluded that the GFD marker is highly specific to avian host groups, and could be used as a reliable marker to detect the presence and amount of avian fecal pollution in environmental waters.

1. Introduction

Microbial source tracking (MST) is a process of determining the sources of fecal pollution in waters. A range of MST tools have been developed to identify sewage and animal-derived fecal pollution in global waters. The initially developed MST methods were library-dependent, requiring fingerprint matching of fecal indicator bacteria (FIB) from different animals (host groups) to compare with patterns of FIB isolated from environmental waters (Scott et al., 2002; Stoeckel and Harwood, 2007). Library-dependent methods can be costly and time consuming due to the requirement for developing a representative library of isolates from host groups and environmental waters. In addition, the performance of a library is influenced by several factors such as geographical stability, temporal stability, and complexity in statistical analysis (Field and Samadpour, 2007; Gordon et al., 2002; Hartel et al., 2002; Harwood et al., 2003).

In contrast, another set of methods known as "library-independent methods" primarily involve identifying a specific DNA sequence or target gene (known as molecular marker) of a bacterial species or virus in host groups, and identifying the same marker in environmental water samples using polymerase chain reaction (PCR) assays. Many PCR and quantitative PCR (qPCR) based assays have been developed to identify (and in some cases quantify) a

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http://dx.doi.org/10.1016/j.watres.2015.10.050
0043-1354/

wide array of molecular markers in host groups and environmental waters (Ahmed et al., 2008, 2010b; Harwood et al., 2014). The application of these markers for MST studies depends on several performance characteristics such as host-specificity, host-prevalence, evenness, persistence, and relevance to health risks (Harwood et al., 2014; Stoeckel and Harwood, 2007). It has been recommended that the performance characteristics of a newly developed marker(s) must be assessed prior to field application in new geographical areas (Stoeckel and Harwood, 2007; US EPA, 2005).

Fecal pollution from avian wildlife may impact the microbiological quality of environmental waters (Converse et al., 2012; Edge and Hill, 2007; Lee et al., 2013; Lévesque et al., 1993; Lu et al., 2008; Shibata et al., 2010; Wither et al., 2005). Avian waste is known to contain high concentrations of FIB (*Escherichia coli* or *Enterococcus* spp.) (Alderisio and DeLuca, 1999; Fogarty et al., 2003; Ge et al., 2010). For example, *E. coli* concentrations in gull feces may range from 10^5–10^9 per g of feces (Fogarty et al., 2003). In addition, the presence of pathogenic *E. coli* (Ahmed et al., 2012; Wallace et al., 1997), *Campylobacter* spp. (Fallacara et al., 2004; Kinzelman et al., 2008), *Salmonella* spp. (Fallacara et al., 2004; Kinzelman et al., 2008), *Giardia lamblia* (Kuhn et al., 2002), *Cryptosporidium parvum* (Kuhn et al., 2002), and antibiotic resistance genes (Cizek et al., 2007; Middleton and Ambrose, 2005; Simões et al., 2010) have been reported in avian feces. Humans can come in contact with avian waste by recreational activities or consuming shellfish contaminated with pathogenic microorganism from different avian species. Avian species are highly mobile and display varied feeding habits. Therefore, they can be considered important vehicles for spreading pathogens that may present significant human health risks.

A recent study has reported the development of two qPCR assays for the identification of gull, Canada goose, duck and chicken feces-associated genetic markers in various host groups in the United States, Canada and New Zealand (Green et al., 2012). For the identification of host-specific unique fecal sequences, the authors used microplate subtractive hybridization, which is able to differentiate between very closely related hosts, and between hosts that live in close contact. Based on the 16S rRNA gene fragments, the authors identified *Catellicoccus marimammalium* associated GFC and unclassified *Helicobacter* spp. -associated GFD markers. The distributions of these markers in avian feces across USA, Canada and New Zealand suggested they might have broad applicability for MST studies in other parts of the world (Green et al., 2012).

The primary aim of this study was to evaluate the host-specificity and -prevalence of avian feces-associated GFD marker by testing fecal samples from a variety of avian and non-avian host groups at the Commonwealth Industrial Scientific Research Organization (CSIRO) laboratory in Brisbane, Australia, and the University of South Florida (USF), Florida, USA. Environmental water samples (potentially affected with avian feces) were collected from the Brisbane River (Brisbane) and a freshwater creek (Florida). All environmental water samples were tested for the presence of the GFD marker using a qPCR assay along with FIB enumeration using culture-based methods. The host-specificity and -prevalence of the GFD marker along with their presence in environmental waters were then used to validate the presence of avian fecal pollution in the studied water bodies.

2. Materials and methods

2.1. Avian and non-avian host group sampling

To determine the host-specificity and -prevalence of the avian feces-associated GFD markers, individual and composite fecal and wastewater samples were collected from various avian and non-avian host groups in Brisbane and Florida (Table 1). Additional information on the fecal and wastewater samples is given in the Supplementary note 1. All samples were transported on ice to the respective laboratories, stored at 4 °C, and processed within 6–24 h.

2.2. Concentration of cattle, human and pig wastewater samples

The wastewater samples collected from host groups in Brisbane 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 4,750 *g* for 10 min. Entire volumes (180–200 μL) of concentrated samples were collected from the filter device sample reservoir using a pipette (Ahmed et al., 2010a). The concentrated samples were stored at −20 °C for a maximum of 24 h prior to nucleic acid extraction.

2.3. Environmental water sampling in Brisbane and Florida

Water samples were collected from six sites on one occasion in December 2013 (designated B1 to B6) along the Brisbane River, Brisbane, Australia. Each site was sampled in triplicate giving a total number of 18 water samples. From each site, grab water samples were collected in 10 L sterile containers from 30 cm below the water surface, and transported on ice to the laboratory where they were processed within 6 h of collection. Site B1 is located upstream of the Brisbane Central Business District (CBD). This site receives overflow of water from the Wivenhoe Reservoir. The suspected sources of fecal pollution at this site include waterfowl and wildlife. Local residents also use this site for swimming and fishing. Site B2 is located in a peri-urban, non-sewered catchment feeding into the Brisbane River. The potential sources of pollution at this site include cattle, horses, septic systems and wildlife. Site B3 is a major tributary of the Brisbane River and is tidally influenced. The catchment where the site B3 is located has residential and industrial developments, and is serviced by a wastewater treatment plant (WWTP) which discharges treated wastewater into the Brisbane River. The elevated levels of FIB in this site have been a major water quality issue identified by the local catchment water quality-monitoring program. Site B4 is located in a highly urban area and is also tidally influenced. This site receives urban runoff through a stormwater drain. Sites B5 and B6 are located downstream of the CBD in highly urbanized areas. Potential sources of fecal pollution at these sites include waterfowl and recreational boats. Sampling sites, their location, suspected sources of fecal pollution and physico-chemical water quality parameters have been provided in the Supplementary Table 1.

Water samples in Florida were collected monthly from five sites (designated F1 to F5) for five consecutive months in a freshwater creek in Kissimmee, Florida, USA. From each site, grab water samples were collected in 1 L sterile containers from 30 cm below the water surface, and transported on ice to the laboratory and processed within 6–8 h.

The watershed is a channelized wetland area managed for wildlife, with frequent regulatory exceedances of water quality criteria for FIB. The area is rich in waterfowl and other wildlife, and adjacent to some suburban development though there are no known point sources of sewage contamination. Samples were collected mid-channel from a research boat. Sampling sites, suspected sources of fecal pollution and physico-chemical water quality parameters have been provided in the Supplementary Table 2.

Table 1
Background information on the fecal samples from avian and non-avian host groups.

Host groups	No. of samples	Sample type	Sources of samples	Weight or volume used for nucleic acid extraction	Range (ng) nucleic acid per μL of extract
Brisbane					
Avian[a]	36	Individual	Wild, farm	100–220 mg	4.10–252
Cat	14	Individual	Veterinary hospital	180–220 mg	14.8–87.6
Cattle[b]	12	Individual and composite	Farms/abattoir	180–220 mg per 10 mL	10.2–32.3
Deer	12	Individual	Sanctuary	180–220 mg	18.3–68.1
Dog	20	Individual	Veterinary hospital and parks	180–220 mg	11.3–229
Goat	10	Individual	Veterinary hospital	180–220 mg	5.80–12.5
Horse	20	Individual	Horse racecourse	180–220 mg	10.4–74.2
Human[c]	27	Individual and composite	Wastewater treatment plants	180–220 mg per 10 mL	10.7–55.8
Kangaroo	16	Individual	Sanctuary	180–220 mg	17.3–77.6
Koala	12	Individual	Sanctuary	180–220 mg	2.50–16.1
Pig	12	Composite	Abattoir	10 mL	10.0–18.1
Possum	21	Individual	Wild	180–220 mg	19.4–64.1
Sheep	14	Individual	Veterinary hospital	180–220 mg	1.50–12.1
Florida					
Alligator	7	Individual	Wild, sanctuary	300 mg	4.00–283
Avian[d]	10	Individual and composite	Wild, farm	300 mg	1.40–65.3
Bobcat	1	Individual	Wild	300 mg	15.5
Cat	5	Individual	Domestic	300 mg	5.60–17.6
Cattle	2	Individual	Farm	300 mg	4.10–5.20
Coyote	1	Individual	Wild	300 mg	125.6
Deer	3	Individual	Wild	300 mg	4.30–89.5
Dog	7	Individual	Domestic	300 mg	3.80–24.8
Fox	1	Individual	Wild	300 mg	2.30
Gopher Tortoise	1	Individual	Wild	300 mg	3.70
Raccoon	1	Individual	Wild	300 mg	33.8

[a] Brisbane avian group include samples from chicken, emu, ducks and ibis.
[b] Comprises four individual cattle fecal samples and eight composite cattle wastewater samples.
[c] Comprises three individual human fecal samples and 24 wastewater samples.
[d] Florida avian comprises chicken, goose, poultry litter, zebra finch, vulture, seagull and one unknown species.

2.4. Enumeration of fecal indicator bacteria (FIB)

The membrane filtration method was used for the isolation and enumeration of FIB from water samples at the CSIRO laboratory. Sample serial dilutions were made in sterile MilliQ water, and filtered through 0.45 μm pore size (47-mm diameter) nitrocellulose membranes (Millipore, Tokyo, Japan), and placed on modified membrane-thermotolerant *E. coli* agar (modified mTEC agar) (Difco, Detroit, MI, USA) and membrane-*Enterococcus* indoxyl-D-glucoside (mEI) agar (Difco) for isolation of *E. coli* and *Enterococcus* spp., respectively. 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. USEPA Standard Methods SM9221E for most probable number (MPN) and IDEXX Enterolert (IDEXX Laboratories, Inc., Westbrook, ME) were used to enumerate fecal coliforms and *Enterococcus* spp., respectively in the Florida samples.

2.5. Biomass collection from water sample

Water samples (2 L) collected from the sites B1–B6 were filtered through 0.45-μm pore size (47-mm diameter) nitrocellulose membranes (Millipore) at the CSIRO laboratory in Brisbane. In case of membrane clogging due to turbidity, multiple membranes were used. The membrane(s) were immediately transferred into a 15 mL sterile tube containing 10 mL phosphate buffer saline (Sigma–Aldrich, St. Louis, MO). The sample tube was vortexed for 5 min to detach the microbial biomass from the membrane followed by centrifugation at 4,500 × *g* for 15 min at 4 °C to obtain a pellet as per the method previously described (Ahmed et al., 2009). Water samples (500 mL) collected from the sites F1–F5 were filtered through 0.45-μm pore size (47-mm diameter) nitrocellulose membranes (Millipore) at the USF laboratory. Membranes were immediately transferred to PowerWater microbead tubes (Mo Bio Laboratories, USA), and stored at −80 °C until processing for nucleic acid extraction.

2.6. Nucleic acid extraction and standardization

Nucleic acid was extracted from the concentrated wastewater samples using DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) at the CSIRO laboratory. A QIAamp Stool DNA Kit (Qiagen) was used to extract nucleic acid from 100 to 220 mg of fresh animal fecal samples, whereas Mo Bio PowerSoil DNA Isolation Kit (Mo Bio Laboratories, USA) was used to extract nucleic acid from water samples. At the USF laboratory, nucleic acid was extracted from fecal samples using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories). For water samples, a PowerWater DNA Isolation Kit (Mo Bio Laboratories) was used.

Nucleic acid samples were quantified using a NanoDrop spectrophotometer (ND-1000 or ND-2000; NanoDrop Technology, Wilmington, DE) at both laboratories. Since nucleic acid samples extracted from the feces of host groups had different concentrations (ranging from 1.40 to 283 ng per μL of nucleic acid) (Table 1), all nucleic acid samples were standardized to a mass of 10 ng at both laboratories.

2.7. GFD qPCR assay

For the qPCR analysis of the avian feces-associated GFD marker, a previously published assay was used at both laboratories (Green et al., 2012). GFD was amplified in a SYBR Green-based assay with forward primer (5′-TCG GCT GAG CAC TCT AGG G-3′) and reverse primer (5′-GCG TCT CTT TGT ACA TCC CA-3′). A gene fragment was amplified from avian feces and cloned into the pGEM-T Easy vector system II (Promega, Madison, WI) at the CSIRO laboratory. Plasmid DNA was isolated using the Plasmid Mini Kit (Qiagen). Standards were prepared from the plasmid DNA, ranging from 10^6 to 1 gene copies per μL.

At the Florida laboratory, purified recombinant plasmids, containing 160 bp GFD qPCR target (GTC CTT AGT TGC TAA CAG TTC GGC TGA GCA CTC TAG GGA GAC TGC CTT CGT AAG GAG GAG GAA GGT GAG GAC GAC GTC AAG TCA TCA TGG CCC TTA CGC CTA GGG CTA CAC ACG TGC TAC AAT GGG ATG TAC AAA GAG ACG CAA TAC CGC GAG GTG GAG C) were produced by Integrated DNA Technologies (pIDTSmart with ampicillin; IDT), and cloned into a vector followed by plasmid extraction (IDTDNA.com; Coralville, IA). The purified recombinant plasmids were serially diluted to create a standard ranging from 10^6 to 1 gene copies per 5 μL.

Quantitative PCR amplifications were performed in 20 μL reaction mixtures using SsoFast EvaGreen® Supermix (Bio-Rad Laboratories, Richmond, CA) at the CSIRO laboratory. The qPCR mixtures contained 10 μL of Supermix, 100 nM of each primer and 2 μL of template nucleic acid. qPCR amplifications at USF laboratory were performed in 25 μL reaction mixtures using GoTaq qPCR Master Mix (Promega, Madison, WI), and each mixture contained 12.5 μL of GoTaq MasterMix, 0.25 μL of 10 mM of each primer, 10 μL of nuclease free water, and 2 μL of template nucleic acid. The qPCR cycling parameters consisted of 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 30 s at 57 °C at both laboratories. To separate the specific product from non-specific products, including primer dimers, a melting curve analysis was performed for each qPCR run. During melt curve analysis, the temperature was increased from 65 to 95°C at 0.5°C increment. Melting curve analysis showed a distinct peak at temperature 84 ± 0.2 °C, indicating a positive and correct amplification. The qPCR assays were performed using the Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratories) at the CSIRO laboratory, and an ABI 7500 thermal cycler at the USF laboratory. All qPCR reactions were performed in triplicate. For each qPCR run, a set of standards, an extraction control, and a no-template control were included. Extraction and non-template controls did not show any amplifications. The qPCR assay performance criteria such as efficiency (E), slope, intercept, r^2 and lower limit of quantification (LLOQ) were determined by analysis of the standards over the course of the study.

2.8. PCR inhibition

To obtain information on the level of PCR inhibition, undiluted and standardized nucleic acid samples extracted from avian and non-avian host groups and water samples were spiked with 10 pg of *Oncorhynchus keta* DNA at the CSIRO laboratory (Sigma Chemical Co., St. Louis, Mo.), and tested with Sketa22 real-time PCR assay as previously described (Ahmed et al., 2015; Haugland et al., 2005). All undiluted and standardized Florida nucleic acid samples were analysed for inhibition using a reaction mix composed of 2.5 μL of 200 mg per mL bovine serum albumin (BSA), 12.5 μL of Taqman 2 × universal PCR mastermix No AmpErase UNG, and 3 μL of multiplex primer-probe mix, which included primers for general *Bacteroidales* (GenBac) and probes for both GenBac and a synthetic internal amplification control (IAC) (Staley et al., 2012). Reactions were carried out in 96-well plates with each well consisting of 18 μL of mastermix, 2 μL of IAC plasmid and either 5 μL plasmid standards for GenBac or 5 μL of template nucleic acid. Two sets of negative controls were run in triplicate: one set contained the IAC plasmid (uninhibited control) and one set did not (true negative). The mean C_T value for amplification of IAC in uninhibited controls was used as the expected C_T value in uninhibited samples. Reactions with C_T values greater than three standard deviations of the mean IAC C_T were considered inhibited (Staley et al., 2012). None of the reactions were deemed inhibited since the C_T values for all samples were lower than the mean C_T value for amplification of IAC in uninhibited controls.

2.9. Data analysis

The host-prevalence and -specificity of the GFD marker were determined as follows: host-prevalence = $a/(a + b)$ and -specificity = $c/(c + d)$, where a is true positive (samples were positive for avian host group), b is false negative (samples were negative for avian host group), c is true negative (samples were negative for non-avian host groups), and d is false positive (samples were positive for non-avian host groups). Fecal and wastewater samples were considered negative for the GFD marker in the absence of PCR amplifications at CSIRO and USF laboratories. Samples were considered positive when the GFD levels were above the qPCR LLOQ at both laboratories.

Bayes' theorem was used to calculate the conditional probability that the detection of the GFD marker in Brisbane River water samples originated from avian feces rather than feces from the non-avian host groups. The following formula was used to calculate the conditional probability (Kildare et al., 2007; Weidhaas et al., 2011): $P(H|T) = [P(T|H)P(H)]/[P(T|H)P(H) + P(T|H')P(H')]$, where $P(H|T)$ is the probability (P) of avian fecal pollution (H) in a water sample given a positive test result (T) for the sample, $P(T|H)$ is the true positive, $P(H)$ is the background probability of detecting a marker in a water sample, $P(T|H')$ is the false positive, and $P(H')$ is the background probability that a marker was not detected in a water sample. The value of $P(H')$ is $1 - P(H)$. However, reliable estimates for prior $P(H)$ are often difficult to ascertain due to the geographic, spatial, and temporal variability of the GFD marker. Although previous approaches have defined $P(H)$ using the total marker frequency for the entire catchment (Kildare et al., 2007), estimating the relative loads of fecal contamination from various sources in relation to total fecal load (Reisher et al., 2011), or incorporating expert judgement regarding the probability of host-associated fecal contamination (Åström et al., 2015), these approaches may still be limited for obtaining an accurate value for $P(H)$. Here, the approach by Kildare et al. (2007) was used with minor modifications (Ryu et al., 2012; Lamandella et al., 2009) by evaluating the posterior probability $P(T|H)$ for various hypothetical values of the prior probability $P(H)$ ranging from a low level pollution scenario (0, all negative samples, 0% avian fecal pollution) to a high level pollution scenario (1, all positive samples, 100% avian fecal pollution). This was used to determine the potential influence of the percentage of avian-associated fecal loading in a water body on the positive predictive value of the GFD assay for the observed host-prevalence and -specificity values in the current study. Bayes' theorem was not undertaken for Florida water samples because the GFD marker could not be detected in non-avian fecal samples. Pearson's correlation was performed using SPSS Statistical software ver. 19.2 (IBM, Armonk, NY). All statistics were evaluated at $\alpha = 0.05$.

3. Results

3.1. PCR performance characteristics

qPCR standards were analysed at both laboratories in order to determine the reaction efficiencies. The standards had a linear range of quantification from 2×10^6-2 gene copies per 2 μL of DNA (CSIRO laboratory) and 10^6-1 gene copies per 5 μL DNA extracts (USF laboratory). The slope of the standard ranged from −3.327 to −3.412 (CSIRO) and −3.416 to −3.474 (USF). The amplification efficiencies ranged from 98.6% to 103% (CSIRO) and 94.0%–96.2% (USF), and the correlation coefficient (r^2) ranged from 0.996 to 0.997 (CSIRO) and 0.966 to 0.974 (USF). LLOQ of the qPCR was determined to be 20 and 10 gene copies at CSIRO and USF laboratories, respectively. The results of this study indicated that the qPCR assay performance characteristics were similar at both CSIRO and USF laboratories.

3.2. Host-prevalence and -specificity of the GFD marker

To determine the host-prevalence of the GFD marker, undiluted samples were used at both laboratories. Among the 36 individual avian fecal samples tested from Brisbane, Australia, 21 (58%) were PCR positive for the GFD marker (Table 2). The host-prevalence value of the GFD marker in nucleic acid samples from avian feces was 0.58 (maximum value of 1). Among the 10 individual avian fecal nucleic acid samples tested from Florida, 3 (30%) were PCR positive for the GFD marker. The host-prevalence value of the GFD marker was determined to be 0.30. The combined host-prevalence of this marker in fecal samples from both Brisbane and Florida was 0.52.

Similarly, for the host-specificity assay, undiluted samples were used at both laboratories. Among the 190 non-avian fecal nucleic acid samples tested from Brisbane, 179 (94.2%) samples were negative for the GFD marker. Dog ($n = 4$), kangaroo ($n = 4$), possum ($n = 2$) and sheep ($n = 1$) undiluted fecal nucleic acid samples, however, were positive for the GFD marker. These samples were also positive for the GFD marker at a test concentration of 10 ng per μL nucleic acid. The host-specificity value of the GFD marker in nucleic acid samples for non-avian host groups was 0.94 (maximum value of 1.0). None of the non-avian fecal nucleic acid samples from Florida were positive for the GFD marker. Therefore, the host-specificity value of the GFD marker determined to be 1.0. The combined host-specificity of the GFD marker in both Brisbane and Florida was 0.96.

Table 2
Percentage of avian and non-avian fecal nucleic acid samples positive for the avian-associated GFD marker in Brisbane, Australia and Florida, USA.

Host groups	No. of samples tested	No. of PCR positive samples	
		Undiluted nucleic acid	10 ng per μL nucleic acid
Brisbane			
Cat	14	0	–[e]
Cattle[a]	12	0	–
Chicken	1	0	–
Deer	12	0	–
Dog	20	4[d]	4
Duck	12	7	–
Emu	14	10	–
Goat	10	0	–
Horse	20	0	–
Human[b]	27	0	–
Kangaroo	14	4[d]	4
Koala	12	0	–
Pig	12	0	–
Possum	21	2[d]	2
Sheep	14	1[d]	1
Ibis	9	4	–
Florida			
Alligator	7	0	–
Bobcat	1	0	–
Cat	5	0	–
Cattle	2	0	–
Chicken	2	2	–
Coyote	1	0	–
Deer	3	0	–
Dog	7	0	–
Fox	1	0	–
Goose	1	0	–
Gopher Tortoise	1	0	–
Poultry[c]	2	0	–
Raccoon	1	0	–
Seagull	2	0	–
Unknown bird	1	0	–
Vulture	1	0	–
Zebra Finch	1	1	–

[a] Comprises four individual cattle fecal samples and eight composite cattle wastewater samples.
[b] Comprises three individual human fecal samples and 24 wastewater samples.
[c] Comprises two composite poultry litter samples.
[d] Nucleic acid diluted and tested at 10 ng concentration.
[e] Not tested.

3.3. Concentrations of the GFD markers in avian and non-avian host groups

The concentrations of all GFD positive fecal samples were determined at a test concentration of 10 ng of nucleic acid at both laboratories. All fecal nucleic acid samples except two (one from an ibis and another one from emu) were determined to be above qPCR LLOQ for the GFD marker assay. The concentrations of the GFD marker in avian fecal nucleic acid samples (from Brisbane) were highly variable at the standardized test concentration (Fig. 1). The concentrations in these samples ranged from 1.9×10^1–1.2×10^4 (ibis), 3.2×10^1–7.2×10^4 (duck), and 6.6×10^1–1.5×10^3 (emu) gene copies per 10 ng of nucleic acid. The concentration of the GFD markers in non-avian host groups ranged from 1.3×10^1–5.0×10^2 (possum), 10^1–1.3×10^2 (kangaroo), 1.9×10^1–4.3×10^1 (dog). Only one sheep sample was positive for the GFD marker, and the concentration was 1.9×10^1. The mean concentration of the GFD marker in avian host groups was 5.2×10^3, two orders of magnitude higher than the non-avian host groups (8.6×10^1). The concentrations of the GFD in avian host groups in Florida ranged from 1.1×10^2–3.1×10^3 (chicken) and 6.4×10^4 (zebra finch).

3.4. Concentrations of fecal indicator bacteria (FIB) and GFD marker in environmental water samples

Among the 18 samples (from the six sites) tested from the Brisbane River, all yielded culturable *E. coli* and *Enterococcus* spp., respectively (Fig. 2a). The mean concentrations of *E. coli* and *Enterococcus* spp. in the water samples ranged from 1.5×10^1–4.7×10^2 CFU and 4.0×10^1–5.5×10^2 per 100 mL of water, respectively. Among the 18 samples tested 15 (83%) were positive for the GFD marker and the concentrations in these positively identified samples ranged from 6.0×10^1–3.2×10^2 gene copies per 100 mL of water. Pearson's correlation was used to test the relationship between FIB concentrations (*E. coli* and *Enterococcus* spp.), and with the GFD marker concentrations. The concentrations of *E. coli* were found to correlate with concentrations of *Enterococcus* spp. ($r_p = 0.93$; $P < 0.0001$), however, the concentrations of *E. coli* ($r_p = -0.07$; $P = 0.776$) or *Enterococcus* spp. ($r_p = 0.02$, $P = 0.933$) did not correlate with the GFD marker for the Brisbane

samples.

Among the 25 samples tested from Florida, all samples also yielded fecal coliforms and *Enterococcus* spp. The mean concentrations of fecal coliforms and *Enterococcus* spp. in the water samples ranged from 10^2 to 6.3×10^2 MPN and 3.6×10^2–5.3×10^2 MPN per 100 mL of water, respectively (Fig. 2b). Among the 25 samples tested, 23 (92%) were positive for the GFD marker and of the 23 samples 22 were qPCR quantifiable. The concentrations of the GFD marker in those quantifiable samples ranged from 2.8×10^1–1.3×10^4 gene copies per 100 mL of water. The concentrations of fecal coliform were also found to correlate with concentrations of *Enterococcus* spp. ($r_p = 0.42$; $P = 0.03$) for the Florida samples. However, the concentrations of fecal coliform ($r_p = -0.13$; $P < 0.516$) or *Enterococcus* spp. ($r_p = -0.18$, $P < 0.367$) did not correlate with the GFD marker.

3.5. Application of Bayes' theorem to estimate the conditional probability of accurately detecting the presence of avian fecal pollution in the Brisbane River water samples

Bayes' theorem was used to estimate the conditional probability of accurately detecting avian fecal pollution in the Brisbane River

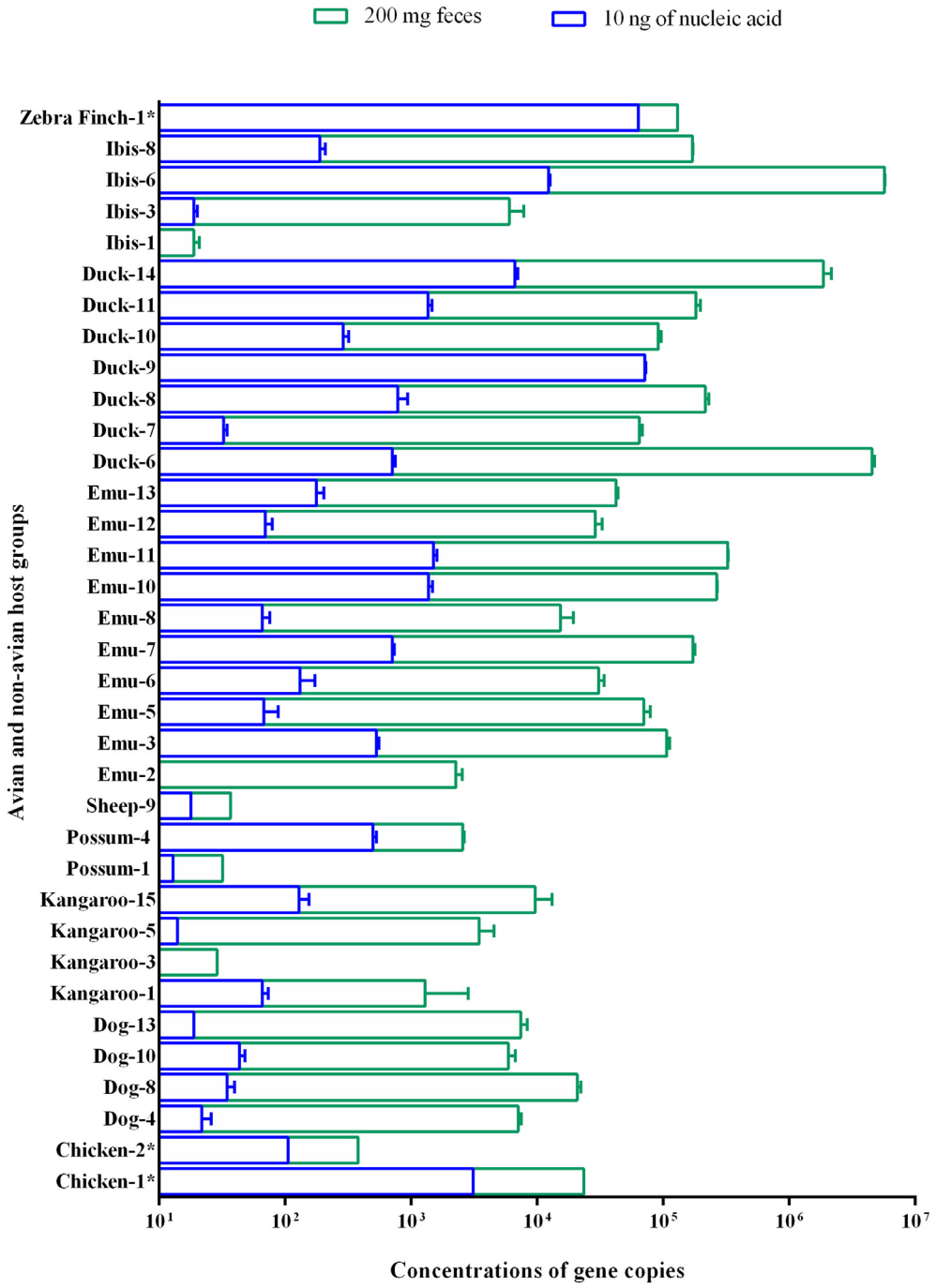

Fig. 1. Concentration of avian feces-associated GFD markers in avian and non-avian fecal (200 mg) and fecal nucleic acid (10 ng) samples in Brisbane and Florida. * denotes samples from Florida.

water samples for the GFD marker, since it was detected in fecal nucleic acid samples from non-avian host groups. The impact of varying values for the prior probability, $P(H)$ on the posterior distribution for $P(T|H)$, given the observed host-prevalence and -specificity values for Brisbane of 0.58 ($P(T|H)$, the true-positive rate of the assay) and 0.06 ($P(T|H')$, the false positive rate or 1–0.94), respectively, is shown in Fig. 3. Using the approach of Kildare et al. (2007) of assuming the observed GFD-positive proportion of Brisbane River water samples, 0.83, is equal to $P(H)$ would correspond to a 98% probability that the detection of the GFD marker in a river water sample was due to true avian fecal pollution and not from non-avian hosts.

4. Discussion

The distributions of avian feces associated GFD marker in North America and New Zealand indicated its potential application for tracking avian fecal pollution in natural waters (Green et al., 2012). It has to be noted that, however, little is known regarding the host-specificity and -prevalence of this marker in different geographical locations. In this study, the performance characteristics of the GFD marker were rigorously evaluated by testing fecal samples from a wide range of avian and non-avian host groups from two continents (Brisbane, Australia and Florida, USA). The performance characteristics of the GFD marker and qPCR assay, and the presence in natural waters were then used to validate the usefulness of this marker to identify avian fecal pollution.

Host-specificity and -prevalence are considered as the two most important performance characteristics of MST molecular markers. Non-specific and less prevalent marker may yield false positive or negative detection of fecal pollution in environmental studies (Ahmed et al., 2013; Stoeckel and Harwood, 2007). Although, high host-specificity and -prevalence values (maximum value of 1.00) are desirable, most of the bacterial MST markers do not exhibit absolute host-specificity and -prevalence (Ahmed et al., 2013; Carson et al., 2005; McQuaig et al., 2009). It has been recommended that a marker with host-specificity value > 0.80 may be suitable for MST field studies (US EPA, 2005). In view of this, we evaluated the host-specificity of the GFD marker by testing 219 fecal and wastewater samples from a range of non-avian host groups.

In order to prevent false negative signals during host-specificity PCR assay, all undiluted fecal and wastewater nucleic acid samples were tested for the presence of PCR inhibitors at both laboratories. None of the samples showed inhibition. The GFD marker showed 100% host-specificity for non-avian fecal nucleic acid samples tested at the USF laboratory. The high host-specificity of the GFD marker in North America and New Zealand has been reported in the original study (Green et al., 2012). The host-specificity value of the marker in Australia was not absolute but the value 0.94 was well above the recommended value of 0.80. The marker was detected in fecal nucleic acid samples from four dogs, four kangaroos, two possums and one sheep. These nucleic acid samples were standardized and further tested with PCR at a concentration of 10 ng. Despite that, all the samples were PCR positive for the GFD marker. In contrast, the dog fecal nucleic acid samples tested at the USF

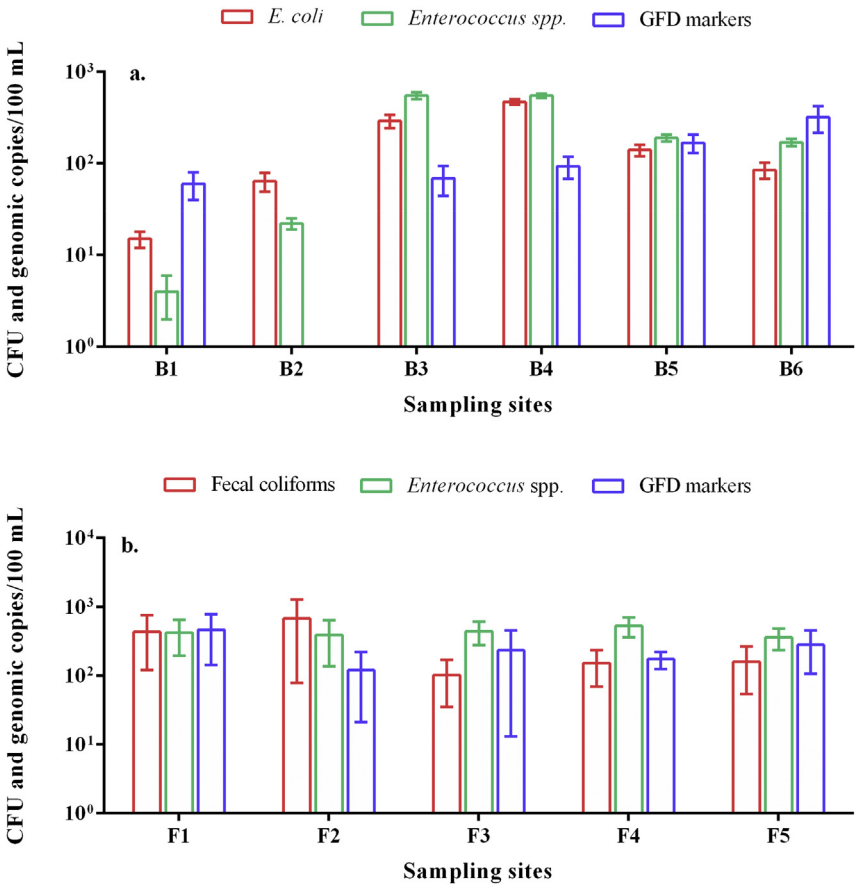

Fig. 2. - (a) Concentrations of *E. coli*, *Enterococcus* spp. and avian feces-associated GFD markers in environmental waters samples in Brisbane, Australia. Error bars are standard deviations. Values are the mean of triplicate samples at each site (B1–B6), (b) Concentrations of fecal coliforms, *Enterococcus* spp. and avian feces-associated GFD markers in environmental water samples in Florida, USA. Error bars are standard deviations. Values are the mean of five sampling dates at each site (F1–F5, monthly for five months).

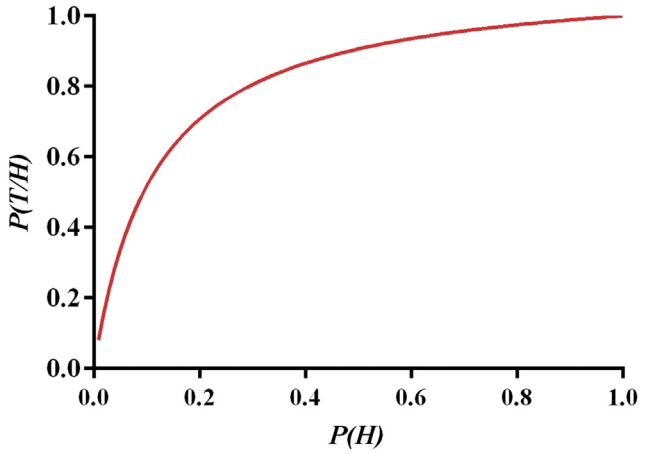

Fig. 3. - Posterior probability of avian fecal pollution *P(T|H)* given a positive PCR result over a range of prior probabilities *P(H)*.

laboratory were negative for the marker. Green and colleagues also reported the absence of this marker in dog and sheep fecal nucleic acid samples in USA and New Zealand (Green et al., 2012). This could be due to the fact that horizontal transfer of fecal bacteria is possible among animal species, which may have attributed the occurrence of the GFD marker in small number of non-avian fecal nucleic acid samples in Brisbane (Carson et al., 2005). This phenomenon may not be a critical issue as long as the concentrations remain low in non-avian host groups. The mean concentrations of the GFD marker in non-avian fecal nucleic acid samples were 2–3 orders of magnitude lower than those in avian fecal nucleic acid samples.

In this study, the host-prevalence value of the GFD marker was determined to be 0.58 for avian fecal samples in Brisbane. This value corresponds with the study undertaken by Green and colleagues in fecal nucleic acid samples from USA and New Zealand (Green et al., 2012). The host-prevalence value obtained for the avian nucleic acid fecal samples in Florida was lower (0.30). Data obtained in the current and previous studies suggest that the GFD marker may not be widely distributed among avian feces. Therefore, the absence of this marker in a water sample may not rule out the possibility of avian fecal pollution and subsequent associated health risks. To increase the detection sensitivity of avian fecal pollution in a water sample, multiple avian marker(s) should be used if possible. For example, combining GFD marker with GFC (Green et al., 2012) or other avian markers such as gull3 (Ryu et al., 2012) may provide additional information on the occurrence of avian fecal pollution in environmental waters.

For the estimation of the concentrations of the GFD marker in fecal samples from avian and non-avian host groups, we used a constant mass of total nucleic acid (10 ng) from each sample. This approach has some advantages such as the need to measure and correct for nucleic acid extraction efficiencies and error introduced by sample variability (solid or liquid phases) can be avoided. The nucleic acid standardization process may also relieve PCR inhibition (if any) (Kelty et al., 2012), and the concentrations obtained in this way may be comparable among studies. Finally, if required, the concentration of target gene can be estimated on a wet weight basis from the known (10 ng) concentration of nucleic acid (see Fig. 1). A mass of 10 ng nucleic acid per PCR reaction was chosen based on the results from a recent study, which indicated that testing 10 ng could yield better results over 1 ng (Ahmed et al., 2015). The concentrations of the GFD marker in avian fecal samples from Brisbane and Florida were highly variable within and among species. The previous study also noted the high variability of the GFD marker in individual gull fecal samples (Green et al., 2012). This could be attributed to factors such as diet, which may vary both regionally and seasonally (Shanks et al., 2011; Turnbaugh et al., 2009). This has implications because a marker with variable concentrations in its host(s) can be difficult to detect in the environment waters due to factors such as dilution, turbidity and inactivation potential. Further study would be required to shed some light on the variability of this marker in avian feces.

In all, 50% and 66% of the Brisbane River water samples exceeded the Australian and New Zealand Environment Conservation Council (ANZECC) water quality guideline values for primary contact of 150 fecal coliforms and 35 *Enterococcus* spp. per 100 mL of water, respectively (ANZECC, 2000). The low concentrations of FIB were expected as the samples were collected from the Brisbane River during low tide and dry weather conditions. Similarly 24% and 100% of the Florida water samples exceeded Florida Department of Environmental Protection (FDEP) and United States Environmental Protection Agency (USEPA) water quality guideline values for recreational contact of 400 fecal coliforms or 130 *Enterococcus* spp. per 100 mL of water, respectively (FDEP, 2013; US EPA, 2012).

The GFD marker was highly prevalent in water samples from Brisbane and Florida, indicating the occurrence of chronic avian fecal pollution. The possibility that feces from non-avian host groups may have contributed this marker in environmental water samples in Brisbane River was considered. Using the Bayes' theorem approach of Kildare et al. (2007) with the assumption that the tested water samples are indicative of background avian fecal pollution, there was a 98% probability that the detection of the GFD marker in a Brisbane water sample was due to true avian fecal pollution and not due to fecal pollution from non-avian hosts such as dog, kangaroo, possum, and sheep. Varying the true prevalence of avian fecal impact demonstrates that the assay would perform well (greater than 90% predictive value) when avian fecal source contributions to the water body are greater than 49%.

Strong correlations between *E. coli* and *Enterococcus* were observed in water samples from both Brisbane and Florida. Concentrations of *E. coli* and *Enterococcus* spp. did not correlate with the concentrations of the GFD marker in water samples from both Brisbane and Florida. Lack of a relationship between FIB and MST markers likely reflect differences in methodology where FIB analysis provides viable concentration of *E. coli* or *Enterococcus* spp., and MST marker detection provides the information on the presence or absence of host-specific fecal pollution and their magnitude. Also MST markers come from a specific host group, whereas FIB come from most warm-blooded animals. In addition, the fate (inactivation) of the GFD marker could be different than *E. coli* and *Enterococcus* spp. (Lu et al., 2011). Similar lack of correlations has been reported for other MST marker concentrations with *E. coli* and *Enterococcus* spp. concentrations (Ahmed et al., 2013; Lee et al., 2013; McQuaig et al., 2012).

5. Conclusions

- In conclusion, the GFD marker appears to be highly avian host-specific in Brisbane and Florida but has low host-prevalence. Since its prevalence was low and the concentrations were variable among avian feces, the application of this marker may not be sensitive enough to provide the total evidence of avian fecal pollution alone, and therefore, it is recommended that multiple avian feces-associated markers should be used if possible.
- The high prevalence of the GFD marker in water samples collected from Brisbane River and the freshwater creek in

Florida suggests that the quality of water is affected by avian fecal pollution.
- No correlations were observed between the concentrations of FIB and the GFD marker, thus indicating that FIB could not be relied upon alone to obtain information on the microbiological quality of environmental water polluted with avian feces.
- An important area for further research is to understand the decay rates of the GFD marker in environmental water samples in relation to FIB and pathogens. In addition, the correlations between GFD and pathogens need to be investigated.

Acknowledgements

This research presented in this paper was funded by the CSIRO Water for a Healthy Country Flagship Program. Our thanks to Australian-American Fulbright Commission for supporting Valerie J Harwood's and Kerry Hamilton's visit to CSIRO Lab, Brisbane Australia.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2015.10.050.

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