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Genetic Markers for Rapid PCR-Based Identification of Gull, Canada Goose, Duck, and Chicken Fecal Contamination in Water

Hyatt C. Green,[a] Linda K. Dick,[b] Brent Gilpin,[c] Mansour Samadpour,[d] and Katharine G. Field[a]

Department of Microbiology, Oregon State University, Corvallis, Oregon, USA[a]; School of Environment and Natural Resources, The Ohio State University, Columbus, Ohio, USA[b]; Institute of Environmental Science and Research, Christchurch, New Zealand[c]; and Institute for Environmental Health, Lake Forest Park, Washington, USA[d]

Avian feces contaminate waterways but contribute fewer human pathogens than human sources. Rapid identification and quantification of avian contamination would therefore be useful to prevent overestimation of human health risk. We used subtractive hybridization of PCR-amplified gull fecal 16S RNA genes to identify avian-specific fecal rRNA gene sequences. The subtracters were rRNA genes amplified from human, dog, cat, cow, and pig feces. Recovered sequences were related to *Enterobacteriaceae* (47%), *Helicobacter* (26%), *Catellicoccus* (11%), *Fusobacterium* (11%), and *Campylobacter* (5%). Three PCR assays, designated GFB, GFC, and GFD, were based on recovered sequence fragments. Quantitative PCR assays for GFC and GFD were developed using SYBR green. GFC detected down to 0.1 mg gull feces/100 ml (corresponding to 2 gull enterococci most probable number [MPN]/100 ml). GFD detected down to 0.1 mg chicken feces/100 ml (corresponding to 13 *Escherichia coli* MPN/100 ml). GFB and GFC were 97% and 94% specific to gulls, respectively. GFC cross-reacted with 35% of sheep samples but occurred at about 100,000 times lower concentrations in sheep. GFD was 100% avian specific and occurred in gulls, geese, chickens, and ducks. In the United States, Canada, and New Zealand, the three markers differed in their geographic distributions but were found across the range tested. These assays detected four important bird groups contributing to fecal contamination of waterways: gulls, geese, ducks, and chickens. Marker distributions across North America and in New Zealand suggest that they will have broad applicability in other parts of the world as well.

Contamination from gulls, Canada geese, ducks, and other birds negatively impacts water quality (5, 16, 24, 33a, 49, 56). Their feces are sources of fecal coliforms, enterococci, and *Escherichia coli*, and their presence is correlated with elevated fecal indicator bacteria (FIB) and beach closures (2, 22, 23, 38). Pathogenic *E. coli* and *Campylobacter*, *Salmonella*, *Giardia*, and *Cryptosporidium* spp. occur in bird feces (11, 18, 19, 42) and can infect domestic poultry and humans (27, 41) and contaminate shellfish (1). Bird feces are also a source of antibiotic resistance genes (34, 39, 50). Recently, because of avian influenza, concerns have risen about pathogen movement due to bird migration (8, 10, 17, 28, 30).

Although pathogens occur in bird feces, exposure to bird feces is considered less harmful to humans than exposure to other sources of fecal contaminants, especially that of humans (43, 51). For example, molecular evidence indicates that genotypes of certain parasites in birds, such as *Giardia* and *Cryptosporidium*, are host adapted and cannot cross-infect among different hosts (20, 57). The relative human health risks of bird and human fecal contamination will be more amenable to measurement once reliable methods are developed to distinguish them quantitatively. The ability to rapidly identify and quantify fecal contamination from birds will improve our ability to estimate human health risk from contaminated waters. Although reliable methods can identify human fecal contamination in water without cultivating indicator bacteria (7), tools for bird fecal source identification are less widely tested (12, 21, 26, 35–37).

We previously developed PCR-based fecal source tracking assays that target 16S rRNA gene sequences from fecal anaerobes in the order *Bacteroidales* (6, 7, 13–15). However, an analysis of gull feces uncovered many gull *Bacteroidales* sequences that were closely related to sequences from human, dog, and cat feces, suggesting horizontal acquisition of bacteria between hosts and affording no useful targets for source tracking (13). Cloning and sequencing of near full-length 16S rRNA fragments generated using general bacterial primers suggest that the *Bacteroidales* group accounts for only a small fraction of gull fecal bacteria (37).

We previously showed that microplate subtractive hybridization (58) could empirically identify unique fecal sequences that differentiate between very closely related hosts (cow versus elk) and between hosts that live in close contact and undergo horizontal transfer of fecal bacteria (human versus dog) (15). In microplate subtractive hybridization, subtracter DNA is fixed to the bottom of a microplate well. Target DNA is added in solution and allowed to hybridize to the attached subtracter DNA. Unhybridized target fragments that remain in solution are removed from the microplate well, amplified, cloned, and sequenced. The method has allowed recovery of unique sequences not present in the subtracters (58).

Since 16S rRNA genes provide attractive and well-studied targets for molecular identification, we performed microplate subtractive hybridization to identify unique 16S rRNA gene fragments found in gull feces but not in other species, including humans, dogs, cats, cows, and pigs. These sequences formed the basis of three new PCR assays that identify fecal contamination from gulls, ducks, geese, and chickens. We modified two of the assays for quantitative PCR (qPCR) and tested their ability to quantify avian contaminants in natural water sources.

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Address correspondence to Katharine G. Field, kate.field@oregonstate.edu.

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TABLE 1 Target bacterial groups, primer sequences, conventional PCR conditions, and LODs by conventional PCR for GFB, GFC, and GFD

Assay	Target bacterial group	Primer sequence[e]	$MgCl_2$ concn[a] (mM)	Annealing temp (°C)	Product size (bp)	LOD		
						Plasmid[b] (no. of copies/reaction)	Gull feces[c] (mg feces/100 ml)	Enterococci (MPN/100 ml)[d]
GFB	Unclassified *Fusobacterium* spp.	F 5′-TCA TGA AAG CTA TAT GCG CCA AAA R 5′-TCC ATT GTC CAA TAT TCC CCA C	1.5	64	176	2,000	1	30
GFC	*Catellicoccus marimammalium*	F 5′-CCC TTG TCG TTA GTT GCC ATC ATT C R 5′-GCC CTC GCG AGT TCG CTG C	2.0	69	162	20	1	30
GFD	Unclassified *Helicobacter* spp.	F 5′-TCG GCT GAG CAC TCT AGG G R 5′-GCG TCT CTT TGT ACA TCC CA	1.0	57	123	20	10	194

[a] Final $MgCl_2$ concentration in the reaction mixture.
[b] Defined as the lowest number of plasmids at which all PCR replicates amplified.
[c] Defined as the lowest fecal dilution at which all PCR replicates amplified.
[d] FIB in the lowest fecal dilution at which all PCR replicates amplified.
[e] F, forward; R reverse.

MATERIALS AND METHODS

Sample collection and DNA preparation. We utilized fecal samples or fecal DNA collected around Columbus, OH; Seattle, WA; Corvallis, OR; and the Oregon Coast; in New Zealand around Christchurch; and from the Institute for Environmental Health (Lake Forest Park, WA) collections or donated by collaborators in the United States (California, Texas, Florida, and North Carolina), and Canada (British Columbia, Alberta, Saskatchewan, and New Brunswick). Marine mammal samples came from the Marine Mammal Center, CA, and Monterey Bay Aquarium, CA. Many fecal samples had been identified by common name only. Sources of gull fecal samples included western, California, herring, laughing, and ring-billed gulls; sources of goose fecal samples included Canada, cackling, brant, and domestic geese; sources of duck fecal samples included mallard, black, wood, and domestic ducks; and sources of chicken samples were broiler and egg operations. Donated gull fecal samples from California were used with Oregon samples in the target for subtractive hybridization; otherwise, fecal and DNA samples donated by outside collaborators were used for prevalence and geographic distribution assays only, and the integrity of fecal DNA samples that did not amplify was checked by reamplifying with rRNA gene primers 27F (bacterial) and 1492R (bacterial/archaeal) (31). DNA samples that did not amplify with these rRNA gene primers were excluded from the study. In the authors' laboratories, fecal samples were collected in sterile containers and stored at −80°C. The FastDNA kit for Soils (Q-Biogene, Carlsbad, CA) was used to extract DNA from the fecal samples used in the initial subtractive hybridization. We used a DNeasy blood and tissue kit (Qiagen, Inc., Valencia, CA) for subsequent DNA isolation and a PowerWater DNA kit (MoBio Laboratories, Inc., Carlsbad, CA) for DNA extractions from chicken fecal dilutions.

Environmental water samples were collected in August 2011 from 5 sites in Oregon: Willamette River, Corvallis; Starker Pond, Corvallis; Oak Creek, Corvallis; freshwater seep into tide pools, Seal Rock; and Yaquina Bay, Newport. Samples from the first four sites were freshwater, and that from the fifth was marine water. Ducks were present in the first two sites. The fourth and fifth sites had gulls present. Two water samples at each site were separately collected in sterile containers and held on ice as previously described (47). Subsamples were used for FIB enumeration with the Colilert reagent (Idexx Laboratories, Westbrook, ME), and 100 ml of each sample was filtered as previously described (47). DNA was extracted from filters with the PowerWater DNA kit (MoBio Laboratories) according to the manufacturer's directions.

Subtractive hybridization and sequence identification. The target was a gull fecal DNA pool containing an equal mixture of DNA from 12 Oregon and 12 California gull samples (3 ng/ml). For the subtracter, fecal DNA extracts from 10 to 30 individual samples from human, dog, cat, cow, and pig (from Oregon) were combined and mixed in equal amounts (3 ng/μl) for each species. DNA was quantified by PicoGreen assay (Molecular Probes, Inc., Eugene, OR). The experimental design was adapted from a study by Zwirglmaier and colleagues (58). Subtractive hybridization was performed as described in a previous paper (15), except that instead of using fecal *Bacteroidales* 16S rRNA genes, we used fecal rRNA genes amplified with 27F and 1492R primers (31) extended with AciI restriction sites: Aci27F (5′ AAT ATA AAC CGC AGR GTT TGA TYM TGG CTC AG) and Aci1492R (5′ AAT ATA AAC CGC GGT TAC CTT GTT ACG ACT T). After hybridization, subtracted (nonhybridized) target DNA fragments were removed, diluted 10,000-fold, and reamplified as previously described (15). Two different-sized PCR products were separately gel purified (QIAquick gel purification kit; Qiagen) and cloned (TOPO TA; Invitrogen, Carlsbad, CA). Five clones from each amplified band were randomly selected for sequencing on an ABI 3730 capillary sequence machine. Sequences were identified and aligned with related sequences using NCBI/BLAST (3). Short (60-bp) overlapping regions within each sequence were analyzed separately in order to uncover chimeric sequences.

Assay design, optimization, and performance testing. Unique regions in sequences were identified by comparison to related sequences and used to design PCR primers. Primer sequences were tested *in silico* using NCBI/BLAST, Oligo Analyzer (version 3.0; Integrated DNA Technologies, Coralville, IA), and the Probe Match program of the Ribosomal Database Project (9). Primers were optimized for annealing temperature and magnesium concentration and tested for host specificity using PCR beads (Institute for Environmental Health, Lake Forest Park, WA) containing buffer, *Taq* DNA polymerase, deoxynucleoside triphosphates (dNTPs), trehalose, and 1.0 mM $MgCl_2$. PCR mixtures contained one PCR bead, 2.5 μM each primer, additional $MgCl_2$ as determined for each primer pair by optimization, and 2 μl DNA in a 25-μl reaction volume. Cycling parameters were as follows: 4°C for 2 min and 95°C for 10 min, followed by 35 cycles of 95°C for 10 s, the primer-specific annealing temperature for 30 s, and 72°C for 20 s (Table 1). Host pools were constructed from fecal DNA samples from 5 to 12 individuals of the same species; these were used for initial testing of host specificity. Once primers were optimized, fecal DNA samples were tested individually.

We used the GFC and GFD primer sets for SYBR green qPCR. Twenty-five-microliter reaction mixtures consisted of 2.0 mM $MgCl_2$, PCR buffer I (ABI, Foster City, CA), 2 mM each dNTP, 100 nM each primer, 0.04 μg/μl bovine serum albumin, 0.625 U *Taq* polymerase (AmpliTaq poly-

merase; ABI), 50 μM carboxy-X-rhodamine dye (Invitrogen), 0.1× SYBR green nucleic acid gel stain (supplied at 10,000× by Invitrogen), and 2 μl template. SYBR green reactions were cycled for 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and the primer-dependent annealing temperature for 32 s (Table 1). Melt curve analysis with a resolution of 0.3°C was used to determine amplification specificity. We tested reference fecal DNA pools, as described above, to ensure that the host range of qPCR assays did not change due to modifications in reaction chemistry. The fluorescence threshold was set at 0.8. Reactions were cycled on an ABI 7300 real-time PCR system. Triplicate standard curves were used to convert threshold cycle (C_T) values to copy numbers for each run.

We measured the limits of detection (LODs) of each new conventional PCR assay and GFC and GFD qPCR assays as (i) target copy number, using 11 replicate marker-specific plasmid dilutions, and (ii) feces wet weight in either marine water or freshwater, with accompanying FIB counts. The LOD was defined as the lowest number of plasmids or the fecal dilution at which all PCR replicates amplified. For plasmid LODs, plasmids containing marker fragments were purified using a QIAprep spin miniprep kit (Qiagen) and quantified with a NanoDrop-1000 apparatus (Thermo Scientific, Wilmington, DE). Serial dilutions from 10^6 copies/μl to 1 copy/μl were used as PCR templates.

For gull fecal dilutions, equal weights of 12 different fresh fecal samples from Oregon coast gulls were combined, and 1 g of the mix was diluted in 1 liter of seawater and stirred vigorously. This emulsion was serially diluted in either marine water or marine water containing human sewage. Dilution blanks with sewage contained 119 enterococci most probable number (MPN)/100 ml before the addition of gull feces. One hundred milliliters of each dilution was filtered through 0.22-μm-pore-size filters (Supor 200; Pall Life Sciences, Ann Arbor, MI) in parallel for a total of six filtration replicates for each dilution. We extracted DNA using the DNeasy blood and tissue kit (Qiagen) according to the manufacturer's directions and eluted the DNA in 100 μl Qiagen AE buffer. These dilutions were used to find LODs of conventional GFB and GFD assays as well as both conventional and quantitative GFC assays.

For chicken fecal dilutions, equal weights of 10 fresh individual fecal samples from chicken were mixed, and 1 g of the mix was diluted in 1 liter of stream water (Oak Creek, Corvallis, OR) and stirred vigorously. This emulsion was serially diluted prior to filtration in duplicate. DNA was extracted using the PowerWater DNA kit (MoBio Laboratories) and eluted in 100 μl supplied elution buffer. We substituted this extraction method to decrease variance between extraction replicates. These dilution extracts were used to find the LODs of the GFD qPCR assay.

We enumerated MPN enterococci (the fecal indicator recommended for marine waters [54]) in the gull fecal dilutions (Enterolert; Idexx) and MPN coliforms and *E. coli* (the fecal indicator recommended for freshwaters [54]) (Colilert-18; Idexx) in chicken fecal dilutions in order to relate the PCR LODs to FIB. FIB MPNs were rounded to the nearest whole cell.

Environmental water samples were analyzed by qPCR for GFC and GFD and also by qPCR for HF183 (human [7]) and CF128 (ruminant [7]) as previously described (55).

To compare the concentrations of GFC and GFD in gull feces, DNA was extracted from fresh individual gull fecal DNA samples from Oregon using the DNeasy blood and tissue kit (Qiagen), quantified by a PicoGreen kit, and used in qPCR as described above.

Analysis of covariance was performed using R (52).

Nucleotide sequence accession numbers. Sequences that met the minimum size criterion of 200 bases were deposited in GenBank (accession numbers JN084061 to JN084064). The remaining sequence set, including sequences shorter than 200 bases, is available upon request.

RESULTS

Sequences recovered from subtractive hybridization. After subtractive hybridization targeting rRNA genes in gull feces, we obtained two different-sized bands, which were separately eluted, cloned, and sequenced. Sequences from the smaller band ranged from 227 to 303 bp; sequences from the larger band ranged from 303 to 459 bp.

Six of the 10 sequences were chimeras. We separated the chimeric sequences into their component sequences, when possible, resulting in 19 separate sequences. Sequence diversity was low. Sequences fell into 5 taxonomic groups: 9 related to *Enterobacteriaceae* (47%), 5 related to *Helicobacter* (26%), 2 perfectly matched to *Catellicoccus marimammalium* (11%), 2 related to *Fusobacterium* (11%), and 1 related to *Campylobacter* (5%).

Eleven of the sequences recovered were perfect matches to sequences found in the GenBank database, including sequences for members of the *Enterobacteriaceae* such as *E. coli*, *Shigella*, *Salmonella*, *Enterobacter*, *Klebsiella*, and *Serratia* and *C. marimammalium* (*Lactobacillales*, *Firmicutes*). Some of these were components of chimeras, but three sequences that matched *Enterobacteriaceae* and one that matched *C. marimammalium* comprised an entire clone sequence.

TABLE 2 GFC and GFD qPCR assay performance characteristics based on 11 standard curves separately run over a 6-month period

Assay	Range of values for:				
	Slope	Intercept	Efficiency[a]	r^2	ROQ[b] (no. of copies)
GFC	−4.06, −3.34	34.55, 39.54	0.88, 1.00	0.990, 0.996	20-2 × 10^5
GFD	−3.51 −3.02	32.52, 38.26	0.96, 1.07	0.993, 0.999	200-2 × 10^5

[a] Defined as $10^{(1/-\text{slope})}/2$.

[b] Range of quantification: plasmid concentration range that remained linear to C_T values ($r^2 > 0.98$) in all 11 standard curves over the 6-month period.

Assay development and performance testing. Potential primer pairs were designed on the basis of unique sequences. These were first tested *in silico* to search for matches to known sequences; unique primer pairs (those exclusively found in their target bacterial sequences) were optimized. Conventional PCR assays, termed GFB, GFC, and GFD, were based on 16S rRNA sequence fragments matching *Fusobacterium* spp., *C. marimammalium*, and *Helicobacter* spp., respectively (Table 1). In tests for specificity against fecal DNA pools, GFB, GFC, and GFD amplified fecal DNA from birds but not from human, dog, cat, cow, horse, deer, pig, rodent, or sea mammals. Both GFB and GFC detected gull fecal dilutions in marine water when the fecal enterococcus concentration in the dilution was 30 MPN/100 ml, well below the regulations for recreational water.

We developed quantitative assays for GFC and GFD, based on detection with SYBR green. We did not use GFB for qPCR because its sensitivity for gull fecal DNA samples (0.26) was lower than the sensitivities of GFC and GFD (0.64 and 0.58, respectively) and because the GFB assay formed primer dimers, interfering with SYBR green detection. qPCR performance characteristics based on plasmid dilutions are shown in Table 2. In marine water and marine water with added human sewage (119 enterococcus MPN/100 ml), the GFC qPCR consistently detected down to 0.1 mg gull feces/100 ml, which corresponded to 2 gull enterococcus MPN/100 ml (Fig. 1 and Table 3). In dilutions with 0.01 mg gull feces, the marker was detected in 10 of 12 qPCR replicates. Analysis of covariance indicated that the addition of human sewage did not significantly change the assay's ability to quantify gull feces in marine water within fecal LODs ($P > 0.10$, $n = 96$). In addition, estimated marker quantities within each dilution were not significantly different between marine water and marine water with

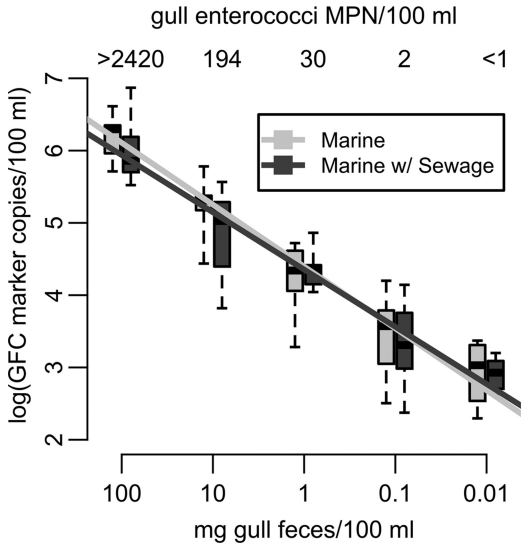

FIG 1 GFC qPCR assay performance on gull fecal dilutions in marine water and marine water with added sewage. Duplicate PCRs were performed on six replicate filters for each dilution. Regression lines were formed for each matrix using fecal concentrations within the fecal LOD. Box plot whiskers extend to data extremes. The number of enterococci (MPN/100 ml) measured in each dilution is shown across the top.

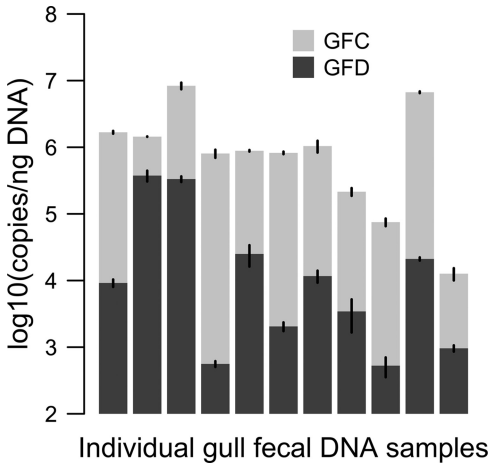

FIG 2 GFC and GFD marker abundance per nanogram Oregon gull fecal DNA from individual samples. Error bars represent standard deviations of qPCR replicates.

human sewage, as shown by one- or two-sided *t* tests ($P > 0.068$, $n = 24$). In freshwater, GFD detected down to 0.1 mg chicken feces/100 ml, corresponding to 87 coliform MPN/100 ml or 13 *E. coli* MPN/100 ml (Table 3).

Because with conventional PCR we were able to detect gull feces at a 10 times more dilute concentration with GFC than GFD (Table 1), we used the GFC and GFD qPCR assays to estimate marker concentrations in individual fecal samples. On average, the GFC marker occurred in Oregon gull fecal samples at about 100 times the concentration of GFD per ng DNA (Fig. 2).

Species prevalence and geographic distribution. We used DNA from 635 individual fecal samples to establish the prevalence of the markers within host species (Table 4). Although the markers were designed from sequences recovered from gull feces, all three occurred in fecal DNA from other species. Two (GFB and GFC) were far more common in gulls than in other species tested (97% and 94% specificity, respectively). GFB amplified 2 out of 12 New Zealand rabbit samples. GFC was present in 1 of 12 New Zealand sheep and 11 of 22 Oregon sheep. The third marker, GFD, was 100% avian specific and amplified fecal DNA from gulls, geese, ducks, and chickens, as well as from a variety of other seabirds. GFB and GFC each occurred in several beach/seaside bird species besides gull, although the number of individual samples tested was very low. GFB detected only 26% of gull samples tested, whereas GFC and GFD had 64% and 58% gull sample sensitivities, respectively, and GFD detected 57% of all bird fecal DNA samples. The total percentages of individual avian samples detected with at least one assay were 70% for gulls, 69% for geese, 78% for ducks, and 45% for chickens.

Because the GFC conventional PCR assay amplified some sheep fecal DNA, we used qPCR to measure the relative concentrations of the marker in Oregon gull and sheep fecal samples. In sheep where we could not detect the marker, we assumed that it was present at concentrations just below limits of detection. The GFC marker occurred at concentrations approximately 100,000 times lower in those sheep with detectable GFC ($2.9 \times 10^1 \pm 9.6 \times 10^1$ copies/ng DNA) than in gulls ($2.0 \times 10^6 \pm 2.8 \times 10^6$ copies/ng DNA).

Although the number of samples from some locations was very small, we found evidence suggesting that the three markers differed in their geographic distributions (Fig. 3). GFB occurred in 50 to 100% of gulls from the West Coast (British Columbia, Washington, Oregon, and California) but was rare or absent in gulls from Ohio, Florida, and New Brunswick. GFC occurred in 71 to 100% of gull samples from the West Coast and Ohio but in 38% of samples from the Gulf Coast and was absent in New Brunswick samples. GFD occurred in 90 to 100% of gull samples from British Columbia, Oregon, and Ohio and at an 18 to 52% frequency in Washington, California, and Florida and was absent in gulls in New Brunswick. GFD was a good indicator for both goose and duck feces in all the samples, occurring in 68% and 76% of these samples, respectively, and was found in about half of the chicken samples in most areas.

Environmental water samples. GFC markers were detected in 4 of 4 coastal samples thought to be impacted by gull fecal contamination. Trace levels of the marker were detected in the Willamette River, consistent with occasional observations of gulls in this waterway. GFD markers were detected in both the inland and coastal sites. Other fecal contamination markers detected in the samples included trace levels of HF183 (human) in the Willamette River and Oak Creek samples and CF128 (ruminant) in the Starker Pond samples.

TABLE 3 GFC and GFD qPCR performance on fecal dilutions

Assay	Fecal source	Slope	r^2	LOD	
				Fecal (mg feces/100 ml)	Indicator (MPN indicator/100 ml)
GFC	Gull[a]	0.86	0.89	0.1	2 enterococci
GFD	Chicken[b]	1.01	0.96	0.1	87 coliforms or 13 *E. coli* cells

[a] For GFC, gull feces were diluted in unfiltered marine water.
[b] For GFD, chicken feces were diluted in unfiltered stream water.

TABLE 4 Species distribution of markers

Fecal source	No. of samples		No. (%) of samples that amplified with the following marker:			
	Total	From New Zealand	GFB	GFC	GFD	Total[a]
Gull	73	12	19 (26)	47 (64)	43 (59)	51 (70)
Goose	106	12	1 (1)	3 (3)	72 (68)	73 (69)
Duck	76	12	0 (0)	3 (4)	58 (76)	59 (78)
Chicken	98	8	6 (6)	4 (4)	42 (43)	44 (45)
Human	11		0 (0)	0 (0)	0 (0)	0 (0)
Sewage	11	11	0 (0)	0 (0)	0 (0)	0 (0)
Cat	9	4	0 (0)	0 (0)	0 (0)	0 (0)
Dog	16	12	0 (0)	0 (0)	0 (0)	0 (0)
Cow	24	12	0 (0)	0 (0)	0 (0)	0 (0)
Horse	18	6	0 (0)	0 (0)	0 (0)	0 (0)
Sheep	34	12	0 (0)	12 (35)	0 (0)	12 (35)
Goat	12	12	0 (0)	0 (0)	0 (0)	0 (0)
Deer	9		0 (0)	0 (0)	0 (0)	0 (0)
Pig	5		0 (0)	0 (0)	0 (0)	0 (0)
Rabbit	12	12	2 (17)	0 (0)	0 (0)	2 (17)
Rodent	3		0 (0)	0 (0)	0 (0)	0 (0)
Possum	12	12	0 (0)	0 (0)	0 (0)	0 (0)
Sea lion	22		0 (0)	0 (0)	0 (0)	0 (0)
Dolphin	9		0 (0)	0 (0)	0 (0)	0 (0)
Elephant seal	10		0 (0)	0 (0)	0 (0)	0 (0)
Harbor seal	1		0/0	0 (0)	0 (0)	0 (0)
Godwit	2		1 (50)	1 (50)	0 (0)	2 (100)
Sandpiper	13		1 (8)	3 (23)	4 (31)	7 (54)
Coot	2		1 (50)	0 (0)	2 (100)	2 (100)
Pigeon	13		1 (8)	0 (0)	2 (15)	3 (23)
Cormorant	3		0 (0)	0 (0)	2 (67)	2 (67)
Egret	3		0 (0)	0 (0)	2 (67)	2 (67)
Pelican	4		0 (0)	1 (25)	4 (100)	4 (100)
Tern	3		0 (0)	0 (0)	1 (33)	1 (33)
Crow	1		0 (0)	0 (0)	1 (100)	1 (100)
Swan	8	8	4 (50)	1 (13)	8 (100)	8 (100)
Pukeko	10	10	0 (0)	9 (90)	0 (0)	9 (90)
Avian specificity[b]			0.99	0.98	1.00	
Avian sensitivity[c]			0.08	0.17	0.57	
Gull specificity[d]			0.97	0.94	0.64	
Gull sensitivity[e]			0.26	0.64	0.58	

[a] The total number (percentage) of each source that was detected by at least one marker.
[b] Avian specificity = true negative$_{nonavian}$/(true negative$_{nonavian}$ + false positive$_{nonavian}$).
[c] Avian sensitivity = true positive$_{avian}$/(true positive$_{avian}$ + false negative$_{avian}$).
[d] Gull specificity = true negative$_{nongull}$/(true negative$_{nongull}$ + false positive$_{nongull}$).
[e] Gull sensitivity = true positive$_{gull}$/(true positive$_{gull}$ + false negative$_{gull}$).

DISCUSSION

The PCR assays described here detected fecal DNA from 70% of all gulls, 69% of all geese, 78% of all ducks, and 45% of all chickens and could detect fecal DNA in environmental waters. These new assays will therefore allow rapid and sensitive detection over a wide geographic range of the most important avian groups contaminating environmental waters. In addition, combining quantitative GFC and GFD assays could provide information on relative contributions of gulls versus other birds.

The GFC assay was highly specific for gulls, with the ability to detect gull fecal contamination at a level representing only 2 enterococcus MPN/100 ml. The identification of a *Helicobacter* sp. sequence common to gulls, geese, ducks, and chickens was a serendipitous result of the gull subtractive hybridization, allowing design of the GFD assay to detect all of these groups. The GFD assay detected 68% and 76% of goose and duck samples, respectively, and showed 100% specificity for avian fecal samples.

Differences between LODs of plasmid copy number and feces (wet weight) of GFC and GFB (Table 1) in conventional PCR suggested that each targeted bacterial group occurred at different concentrations in feces. Similarly, the GFD assay had the same plasmid copy number limit of detection as the GFC assay, but its limit of detection in feces was 10 times higher. Quantitative comparison of GFC and GFD in individual gull fecal samples (Fig. 2) demonstrated that GFC occurred at a higher concentration. The presumed difference in ribosomal gene copy number (2 in *Helicobacter*; 6 in *Lactococcus*, closely related to *Catellicoccus*; http://rrndb.mmg.msu.edu/index.php) is not enough to explain this difference, suggesting that differences in target bacterial cell concentrations in gull feces also occur.

Many assays to identify the sources of fecal contamination have been based on *Bacteroides* and relatives (7, 29, 33), as the *Bacteroides* group is common in mammalian feces and amenable to detection in the environment. However, past studies have demonstrated both the relative paucity of *Bacteroides* in gulls (37) and the likelihood of horizontal transfer from human to gull (13). We did not recover any sequences in this group following subtractive hybridization and thus utilized sequences matching *Fusobacterium*, *Catellicoccus*, and *Helicobacter* for our assays. However, since the ability to apportion fecal contamination among sources is dependent on a similar persistence of host group-specific fecal markers (25), it is important to measure the persistence of these new avian markers, in comparison with each other, FIB, *Bacteroides* markers, and pathogens.

Previous isolation and investigation of *C. marimammalium* found the organism in marine mammals (32). Marine mammal fecal samples from this and another study (37) did not contain detectable sequences matching *C. marimammalium.* In this study, marine mammal fecal samples were collected from confined animals, which could have limited horizontal acquisition from gulls in comparison to their wild counterparts. Further investigations into the occurrence of *C. marimammalium* in wild marine mammals may be necessary if they are to be ruled out as contributors of fecal bacteria in recreational waters.

Geographic and species distributions of the markers were inferred from low numbers of samples in some cases, as noted in Fig. 3, and should be repeated with larger sample sizes. We utilized donated samples, a few of which had been extracted in other laboratories, and eliminated samples that could not be amplified with 16S rRNA gene primers. This could have led to an underestimate of the markers' prevalence, if the DNA in a sample was sufficient to amplify 16S rRNA but not to amplify the less common markers.

In addition, we noted very large differences in DNA quantities obtained from Canada goose feces depending on the time of year and diet, underlining the importance of surveys of the seasonal/temporal prevalence of these markers. Since the substrates for fecal bacteria are host dietary compounds, proportions of fecal bacteria in a given host species will vary according to diet (45, 53), which for birds could change both regionally and seasonally.

The limits of detection of fecal source tracking assays have been reported in plasmid copy numbers (37, 46) or amounts of fecal

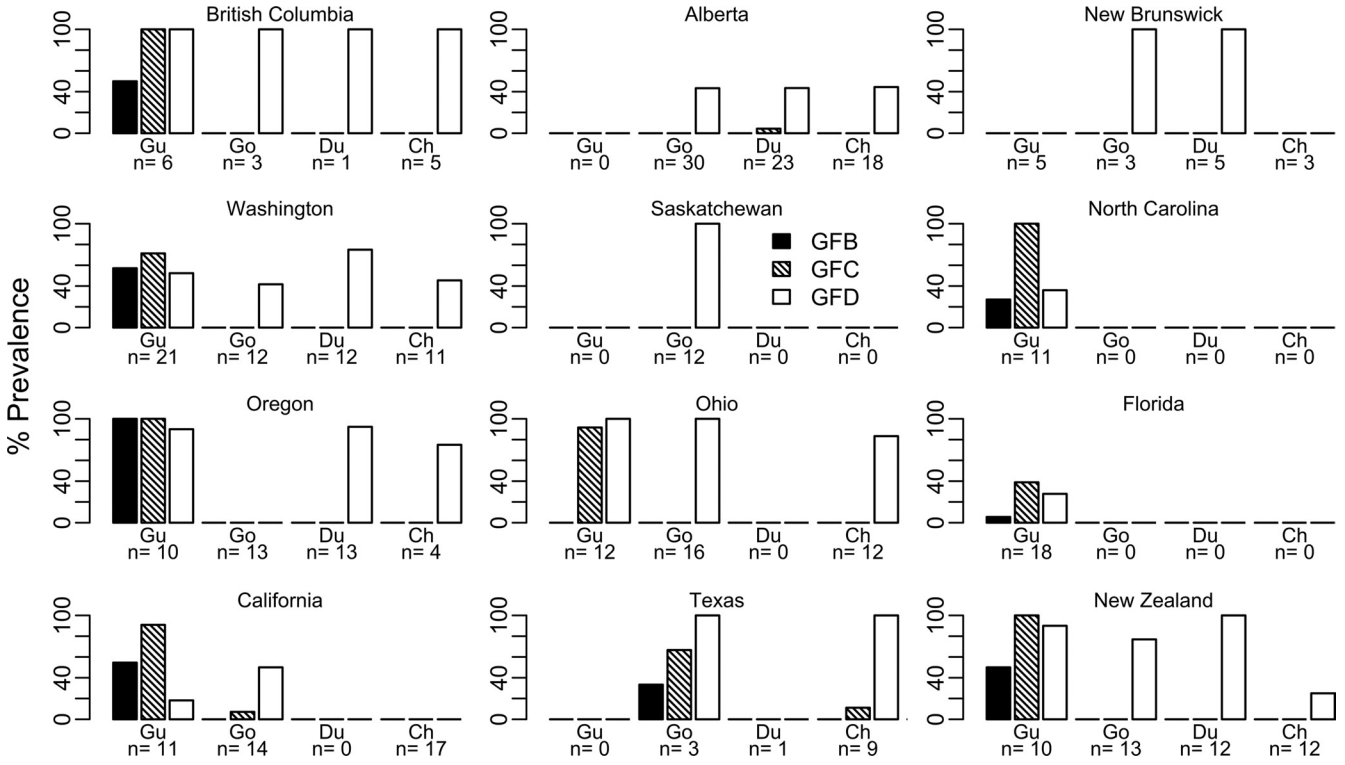

FIG 3 Marker prevalence and geographic distribution. Prevalence and geographic distribution of 3 bird fecal PCR markers in gull (Gu), goose (Go), duck (Du), and chicken (Ch) fecal DNA samples in the United States, Canada, and New Zealand. *n* is the number of individual samples tested. The distribution of the three markers in New Zealand gull, goose, and duck samples was very similar to their distributions on the West Coast of the United States and Canada. GFD occurred at a somewhat lower frequency in New Zealand chickens (25%) than in U.S./Canadian chickens and was absent in samples of California chickens. GFC occurrence in sheep was 50% in Oregon ($n = 22$) but 8% in New Zealand ($n = 12$).

DNA (37), units that are not informative for field applications. We expressed our assays' limits of detection in terms of mg of feces but related these to counts of indicator bacteria per mg feces, a standard that is both familiar and more useful to regulators. These FIB counts supported the compatibility of the GFC and GFD assays with environmental applications, as assay limits of detection were below the EPA-mandated FIB cutoffs for recreational waters. However, because FIB counts in bird feces are expected to vary according to diet, season, geographic location, and bird species, our estimates of FIB should be repeated for specific sites.

The number of chimeric sequences was high, suggesting that the subtractive hybridization procedure systematically produced and then selected for chimeric sequences. Components of the chimeras included sequences that were expected to occur in subtracters (e.g., perfect matches to *E. coli* and *Enterobacter*). In future studies, using linkers that contain the appropriate overhang to ligate to the target fragments but that do not contain the entire restriction recognition sequence would protect the connection between linkers and target fragments, allowing chimeras to be removed with a second restriction digestion.

The subtractive hybridization technique used by Shanks and colleagues (35, 44, 48) to enrich for host-specific sequences for source tracking did not appear to produce chimeras. However, because their studies targeted metagenomic sequences, many of which are likely to be single-copy genes or pseudogenes, whereas we targeted multicopy 16S rRNA, our assays are likely to have a lower limit of detection. Our subtractive hybridization technique provided enough unique sequence data to obtain three different host-associated assays with a very small, targeted amount of sequencing compared to the amount required for clone library or metagenomic analyses.

Modification of the GFC and GFD conventional PCR assays to use on a qPCR platform permitted more favorable limits of detection and quantification of these molecular markers. The assays consistently detected markers down to levels below recreational FIB cutoffs in natural water sources. The presence of multiple contaminant sources might be expected to decrease the accuracy of molecular discrimination tools by introducing interfering particulates, organics, or bacteria previously untested for specificity (25). We showed that the addition of sewage did not significantly affect our estimates of gull contamination. However, we did observe unexplained variability over the expected linear range ($r^2 = 0.89$). This variability was reduced in the chicken fecal dilution experiment, where we used an improved extraction protocol ($r^2 = 0.96$). Optimized extraction protocols and accurate methods to account for loss of nucleic acids during processing may increase method precision in future studies. Despite this variability, the ability of the qPCR assays to detect down to FIB levels well below common recreational water quality standards supports their utility as source-tracking markers in areas potentially impaired by avian fecal contamination. However, an understanding of marker survival (25), as well as site-specific information on sensitivity and specificity (29), is needed in order to better interpret quantitative results.

Some sheep in the United States and New Zealand tested positive for the GFC marker. However, the very low concentration of GFC in sheep feces means that the marker could not be detected at less than 0.2 g sheep feces/100 ml, assuming negligible extraction loss. On the basis of estimates of enterococcus concentrations in sheep feces (40), we calculated that initial sheep contaminant inputs would have to contain about 1.6×10^5 sheep enterococcus MPN/100 ml to be detected by the GFC assay. In contrast, high concentrations of the marker in gulls allow as few as 2 gull enterococcus MPN/100 ml (0.1 mg gull feces/100 ml) to be detected. The low limit of detection and the high dosage of this marker in feces make it a valuable tool for estimating gull fecal inputs.

In conclusion, this paper describes three new assays for bird fecal contamination with broad distributions, both geographically and among bird species. Together, these assays detect four of the most important bird groups contributing to fecal contamination of waterways: gulls, geese, ducks, and chickens. Although additional studies are needed to validate these assays across a range of conditions, the assays are useful across North America and in New Zealand to estimate amounts of bird feces, even in water with small amounts of traditional fecal indicator bacteria. This distribution suggests that they will have broad applicability in other parts of the world as well.

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