Molecular detection of *Campylobacter* spp. in California Gull (*Larus Californicus*) excreta

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Abstract

This study examined the prevalence, quantity, and diversity of *Campylobacter* species in the excreta of 159 California Gull (*Larus californicus*) samples using culture, PCR- and qPCR-based detection assays. While *Campylobacter* spp. prevalence and abundance was relatively high in the gull excreta examined, molecular data indicated that most *L. californicus* campylobacters were unique and not closely related to species commonly associated with human illness. *Campylobacter* spp. estimates were positively related with those of fecal indicators, including a gull fecal marker based on the *Catellicoccus marimammalium* 16S rRNA gene.

Introduction

Campylobacter is a leading cause of bacterial gastroenteritis in developed regions, mostly resulting in sporadic infections (Whelan *et al*. 1988). For example, in the United States (US), over 6,033 cases of campylobacteriosis were reported in 2009 (cdc.gov/mmwr/PDF/wk/mm5914.pdf), with a foodborne rate of 13.02 per 100,000, ranking it as the second highest cause of foodborne disease (after *Salmonella*). In California, the incidence of campylobacteriosis per 100,000 population was highest (29.4) among other pathogens reported in the US. While most infections are foodborne, major waterborne outbreaks have also been reported (Clark *et al*. 2003). From a public health perspective, *C. jejuni*, *C. coli* and *C. lari* are the *Campylobacter* spp. most frequently implicated in human illness (Butzler 2004). Wild birds are a recognized source, particularly for infection in young children (French *et al*. 2009). In the environment, *Campylobacter* is normally associated with poultry (Müllner *et al*. 2009), although other environmental sources find their way into water (Kemp *et al*. 2005, Leatherbarrow *et al*. 2007). A number of studies have also documented the presence of campylobacters in the excreta of gulls from different regions, such as herring gulls in Scotland (Whelan *et al*. 1988), ring-billed gulls (*Larus delawarensis*) in Canada (Lévesque *et al*. 2000, Quessy and Messier 1992), and seagulls (*Larus* spp.) from three coastal locations of Northern Ireland (Moore *et al*. 2002). Gulls can be major contributors of fecal contamination in recreational beaches, with loadings of up to 10^8 *Escherichia coli* per fecal dropping (Alderisio and DeLuca 1999); however, in areas predominantly impacted by seagulls, the level of contamination appears to be unassociated with health risks (Colford *et al*. 2007). This suggests that many of the *Campylobacter* species contaminating beaches may not be infectious to humans.

The objectives of this study were to examine the prevalence, quantity, and diversity of *Campylobacter* species in California Gull excreta, as key inputs to undertake quantitative microbial risk assessments

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of bather risks (Schoen and Ashbolt 2010). Most studies screening for the presence of *Campylobacter* in waterfowl have used cumbersome culture-based methods that are known to have low recovery yields and to potentially underestimate the number of species in a given sample (Van Dyke *et al*. 2010). As a result, it is unclear what the prevalence and densities of different *Campylobacter* spp. are in gull excreta. In particular, there is no published data for California Gulls (*Larus californicus*) even though they are ubiquitous in western coastal areas of the United States, and have been suspected to be an important source of fecal pollution for recreational beaches (Colford *et al*. 2007). Quantification of potentially human infectious *Campylobacter* spp. in gull excreta is an important step in estimating risks posed by gull excreta (Schoen and Ashbolt 2010).

Methods

Gull excreta samples (n = 159) were collected from southern California beaches over 19 sampling dates from July to September of 2009. Samples were collected just after defecation and immediately suspended in 3 ml of phosphate buffered saline solution (pH 7.5, fecal concentration: 0.1 ~ 0.5 g/ml), stored and shipped overnight on ice to the laboratory at Cincinnati (OH) for further processing. Aliquots (1 ml) of triplicate ten-fold dilutions (1, 0.1 and 0.01 ml) from each gull excreta slurry (~0.3 g wet fecal mass) was added to 4 ml of Bolton liquid medium (CM0983, Oxoid, USA) that included supplements (SR0183, Oxoid, USA: Cefoperazone, Trimethoprim lactate, Vancomycin and Cycloheximide) and incubated for 48 hour at 42 °C under microaerophilic conditions (7% O_2, 10% CO_2, 83% N_2). Aliquots (100 µl) were taken from presumptive positive Bolton broths and spread onto Karmali agar (SR0205, Oxoid, USA) plates that contained supplements (SR0167 Oxoid: sodium pyruvate, Cefoperazone, Vancomycin and Amphotericin B) and incubated under the same microaerophilic condition at 42°C for 48 to 72 hours. Presumptive positive colonies (4 - 16 per sample) were picked and transferred into cryopreservative vials containing 15% glycerol and stored at -80°C prior to PCR identification.

An aliquot (1 ml) from each gull excreta slurry was also transferred to microcentrifuge tubes and concentrated by centrifugation 16,000 × *g* for 9 minutes. Total community DNA extraction of the resulting pellets and of reference strains used as positive controls (*C. jejuni* LMG 8842, *C. coli* ATCC 33559, and *C. lari* ATCC 35221) was carried out using MO BIO Power Soil kit (MO BIO Laboratories Inc., Carlsbad, CA) per the manufacturer's instructions. The *Campylobacter* genus-specific PCR assay developed by Linton *et al*. (1996) was used to determine the presence of members of this genus and selected amplification products were used in cloning reactions. Positive PCR products from the same sampling date were pooled, cloned into pCR4.1 TOPO (Invitrogen, Carlsbad, CA) per the manufacturer's instructions and sequenced to determine the molecular diversity of this bacterial group. Sequences were analyzed using Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and software MEGA 4.1 (Tamura *et al*. 2007). Representative *Campylobacter* spp. sequences from cloning experiments were deposited in GenBank with accession numbers HQ402729 to HQ402898. For extracts showing positive amplification results, species-specific PCR assays for *C. jejuni*, *C. coli,* and *C. lari* were conducted using 16S-23S rRNA gene primer sets (Khan and Edge 2007). In addition, PCR assays targeting the functional genes *map*A and *ceu*E were used to further confirm the presence of *C. jejuni* and *C. coli*, respectively (Gonzalez *et al*. 1997, Stucki *et al*. 1995).

A genus-specific *Campylobacter* qPCR assay was also performed on the extracted samples using CampF2 and CampR2 primers as described by Lund *et al*. (2004) with minor modifications. Specifically, 4 pmol of probe P2 was used and qPCR assays were conducted using 2 µl of DNA extracts on a 7900 HT Fast Real-Time Sequence Detector (Applied Biosystems, Foster City, USA). Data was analyzed with the Sequence Detection Systems software (version 2.3). The baseline cycles were set from 3 to 15 and the threshold value at the point where fluorescence exceeded 10 times the standard deviation of the mean baseline emission. All samples were run in duplicate. Samples in which both duplicates had a threshold cycle (C_t) value below 40 were regarded as positive. Pooled PBS-excreta supernatants (n = 10) that tested negative for *Campylobacter* (as determined by selective enrichment culture and by genus-specific PCR assay) were used to determine detection limits and PCR inhibition. Four replicates of serially diluted *C. jejuni* LMG 8842 were spiked into each pooled PBS-excreta supernatant (0.39 g/ml). All DNA extracts were examined in two sets: an original

and a 10-fold dilution in duplicate. The inhibition of gull excreta supernatant was determined by comparing 1) the differences of C_t values between the original DNA extracts and 10-fold dilutions, and 2) the correlations between C_t values and log *C. jejuni* spiked into previously *Campylobacter*-negative excreta supernatant. If there was no or low-inhibition from the supernatant, there was a highly significant correlation between C_t values and log *C. jejuni* concentrations or a slope between -3.58 and -3.10. The data recorded from the experiments without significant inhibition were used to generate standard curves**,** and the qPCR detection limit was estimated**.**

Other real-time qPCR assays were conducted to determine the levels of other fecal indicator bacteria (FIB). A gull qPCR assay was conducted using SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) according to the protocol described by Lu *et al*. (2008). Data points with artifacts that resulted in signal overestimation were not included. Signal intensity values were recorded for those reactions showing one corresponding amplification peak within the disassociation curves. Serial dilutions of *Catellicoccus marimammalium* DNA (ng to 10 fg) in duplicate were used to generate a standard curve for the gull-targeted fecal assay. Real-time PCR (qPCR) units were calculated as fg/g wet excreta. qPCR analyses for *Enterococcus* were performed as described by Haugland *et al*. (2005) with minor modifications. The standard curve was estimated through independent DNA extracts in duplicate cell suspensions of *E. faecalis* ATCC 29212 spiked in the sample supernatants (10^1 to 10^8 CFU). Target cells in the extracts were reported as spiked cell equivalents (CE). Ten-fold dilutions of the extracts were routinely analyzed to signal potential PCR inhibitors. General *Bacteroidetes* levels were estimated as described previously (Dick and Field 2004). A standard curve was created from serial dilutions of plasmid DNA containing known copy numbers of the template. For data management and calculations, Microsoft Excel 2003 and SAS Systems version 8.2 (SAS, Cary, N.C.) were used. Quantitative analyses were conducted using the following SAS procedures: PROC GLM, PROC CORR and PROC LOESS.

Results

Out of the 159 gull samples, 45% were positive for *Campylobacter* at the genus-level PCR (Supplemental Information (SI) Table SI-1), indicating high occurrence in California Gull excreta. However, the incidence of known cultured species was low. Specifically, using the *map*A- and ITS-based assays, *C. jejuni* and *C. lari* were detected in less than 2% of the isolates and DNA extracts from the fecal samples that tested positive, while none of the fecal samples or isolates were positive for *C. coli* using *ceu*E- *and* ITS-based assays (Tables SI-1 and SI-2). A total of 255 sequences (812 bp) were generated in this study. BLAST results indicated that clones were closely related to known *Campylobacter* spp. (Table SI-3). The majority of sequences analyzed showed sequence identity of 95 to 97% to *C. coli* (55%), *C. rectus* (24%), *C. upsaliensis* (7%) and *C. hyointestinalis* (4%), thus not considered to represent these species. A smaller proportion of the sequences (i.e., 2 to 5%) were closely related to pathogenic species at a 99% or greater sequence identity, such as *C. lari* and *C. jejuni*, which was in agreement with the species-specific PCR results (Table SI-3). Within the sequences closely related to *C. jejuni*, some were nearly identical to *C. insulaenigrae or C. subantarcticus*, species that share a high level of 16S rRNA sequence identity with *C. jejuni* (Table SI-4). The 812 bp PCR product encompasses three of the major variable regions within the *Campylobacter* 16S rRNA gene sequence (i.e., Vc2, Vc5 and Vc6) described by Gorkiewicz *et al*. (2003). These regions enabled the identification of the sequences obtained in this study as members of the *Campylobacter* genus. Since we used partial 16S rRNA gene sequences, taxa identified by BLAST may not accurately discriminate between different *C. coli*, *C. lari*, and *C. jejuni* related strains. Thus, in order to further classify the sequences from California Gull excreta, the sequences were aligned at 99% identity level with sequences from *Campylobacter* reference strains (n = 104; Table SI-4) and species from closely related genera (i.e., *Helicobacter*, *Arcobacter*, and *Geospirilium*). Representative gull fecal sequences (n = 13) and reference strain sequences (n = 29) were selected to determine phylogenetic relatedness of the fecal sequences. The fecal clone sequences fell within four distinct clades (A-D; Figure 1). A small portion (8%) of the fecal sequences were associated to *C. lari* or *C. jejuni* (clades C-D), while most of the fecal sequences (85%) constitued a distinct clade (A) away from *C. coli* (Figure 1), suggesting potentially novel species within the *Campylobacter* genus.

The detection limit of the *Campylobacter* spp. assay was approximately 300 CE in 1 ml

PBS-excreta supernatants (0.33 g wet mass; R^2 = 0.98), indicating that the assay could be useful at detecting low *Campylobacter* levels. These results compare favorably with the *Campylobacter* detection limits (250 to 500 CFU/g of excreta) estimated in chicken excreta (Lund *et al*. 2004), cloacal swabs (Fukushima *et al*. 2003), and for detecting *C. jejuni* in poultry, milk and environmental water (Yang *et al*. 2003). Estimated cell equivalents (CE) of *Campylobacter* spp. ranged from 340 to 1 x 10^8 CE/g with a mean of 6.7 x 10^6 CE/g, but only four samples exceeded 1 x 10^7 CE/g (Table 1). Using this qPCR method, 54% of the gull excreta samples were positive for *Campylobacter*, which is in close agreement with the data generated using the conventional PCR genus-specific method. The relative concentration of *Enterococcus* spp., *Bacteroidetes* and *C. marimammalium* ranged from 0 to 3.7 x 10^7 CE/g (mean of 1 x 10^6 CE/g) and 0 to 2.9 x 10^7 CE/g (mean of 1.9 x 10^5 CE/g), and 0 to 3.6 x 10^8 pg DNA g^{-1} (mean of 9.6×10^6 pg DNA g^{-1}), respectively. *Campylobacter* abundance among samples showed a consistent trend with other fecal indicator bacteria (Table 1). Additionally, statistical analysis showed moderate, yet significant positive correlations ($p<0.0001$) between $\log_{10}$ qPCR values of *Campylobacter* and the $\log_{10}$ qPCR values of the gull marker, *Enterococcus* and *Bacteroidetes* (R^2 = 0.45, 0.55 and 0.33, respectively, $p<0.0001$). The positive relationship of qPCR values between the gull marker and *Campylobacter* spp. (Figure 2) implies potential to use the *C. marimammalium* gull-marker to predict the presence and relative abundance of *Campylobacter* in gull excreta.

Discussion

A number of studies have documented the presence of *Campylobacter* spp. in gull excreta. The occurrence of *Campylobacter* spp. appears to vary from 13 - 97% among gull species, depending on the locations and methods used in previous studies (Whelan 1988, Quessy and Messier 1992, Lévesque *et al*. 2000, Moore *et al*. 2002). In comparison, the occurrence estimated in this study for California Gull (*L. californicus*) appears to be in the upper range. Species-specific assays used in this study showed a lower occurrence of commonly human-pathogenic campylobacters in California Gulls compared to previous studies of other gull species based on culture techniques. For example, using culturing techniques Whelan *et al*. (1988) reported that two-thirds of the herring gulls (*L. argentatus*) tested were positive to *Campylobacter* spp., with *C. laridis* as the dominant species (55%), followed by *C. jejuni* (30%) and *C. coli* (15%). In contrast, out of 13 fecal strains tested, Hughes *et al*. (2009) identified one strain as *C. lari* and no *C. jejuni* nor *C. coli* in black-headed gull (*L. ridibundus*) using different functional and ribosomal gene (i.e., 16 and 23S rRNA gene) PCR assays. Based on previous culture estimations, *Campylobacter* has been estimated to range from 3.0 x 10^3 to 1.7 x 10^7 CFU/wet g in the ring-billed gull (*L. delawarensis*) droppings (Lévesque *et al*. 2000), with lower estimates from herring gull and common black-headed gull (*L. ridibundus*) excreta (1.8 x 10^2 to 4.9 x 10^6 CFU/g and from 7.4 x 10^2 to 1.7 x 10^5 CFU/g, respectively; Fenlon *et al*. 1982). The differences between the higher estimates by qPCR assays compared to culture-based numbers may in part reflect active but non-culturable campylobacters (Lund *et al*. 2004, Van Dyke *et al*. 2010). The unidentified campylobacters from California Gulls (which represented nearly 92% of the clones) require further examination, and may represent novel species that are problematic to culture using currently available media.

FIB have also been documented for gull excreta (Converse *et al*. 2009, Fogarty *et al*. 2003, Jeter *et al*. 2009). Total *Enterococcus* spp., *E. coli*, *E. faecalis*, and *E. casseliflavus* are the prominent FIB reported from gull excreta (Converse *et al*. 2009), and enterococci from gull excreta have been considered a major contributor of recreational water enterococci at Great Lakes areas (Fogarty *et al*. 2003) and a cause of beach closures in southern California. Our study also showed that enterococci were higher than the quantity of *Bacteroidetes*, with *Bacteroidetes* being consistently low in California Gulls, as has also been reported for ring-billed gulls (*L. delawarensis*) from the Great Lakes (Jeter *et al*. 2009). Further, *Campylobacter* spp. were positively associated with FIB, suggesting that *Campylobacter* spp. are a likely normal component of gull excreta, rather than being sporadic. Many of the campylobacters might be a commensal component of the digestive tract of gulls as suggested by Hatch (1996). *Campylobacter* spp. are known to colonize the intestinal mucus layer in the crypts of the intestinal epithelium and may be commensal to the gastrointestinal tract of poultry (Beery 1988). The commensalism is supported by high densities of *Campylobacter* spp. in the gut of

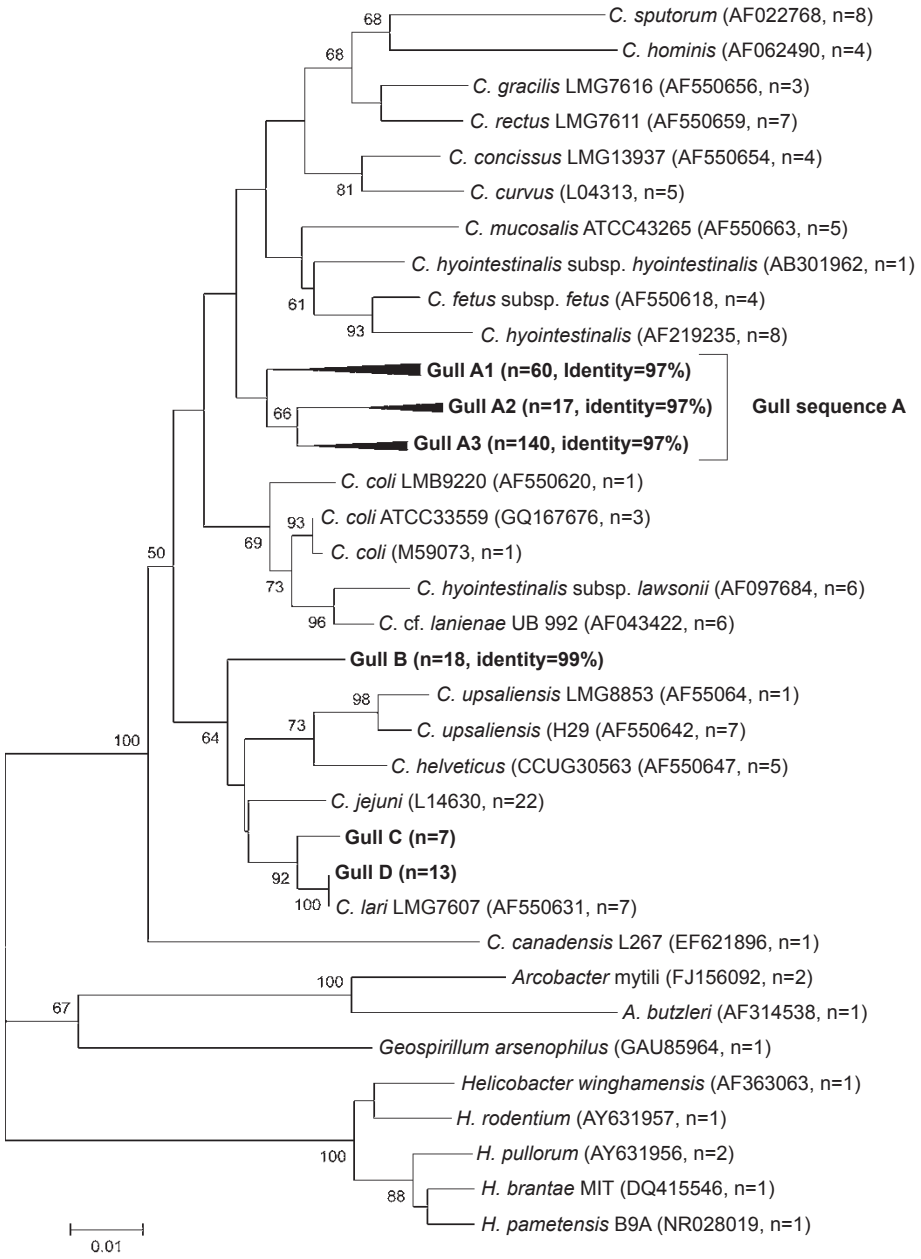

Figure 1. Unrooted neighbor-joining tree of 255 Campylobacter 16S rRNA gene sequences obtained in this study. The sequences were obtained from individual clone libraries developed from excreta collected at 15 different dates. The tree was built using MEGA 4.1 and representative California Gull fecal sequences of different operation taxonomic units (97% identity for Clade A and 99% identity for other clades) and sequences from bacterial reference strains. Number of sequences for each group is included within parentheses (n). Bootstrap values obtained from 1000 bootstrap replicates are reported as percentages greater than 50%. The scale bar corresponds to 0.01 change per nucleotide.

chickens (up to 10^9 CFU/g of cecal content) that do not show symptoms of disease (Evans 1997).

Schoen and Ashbolt (2010) estimated the risk of gull fecal contamination in recreational water causing human illness based on an assumed human-infectious *Campylobacter* spp. composition of 20%. According to their estimate with enterococci at the swimming criteria limit (35 enterococci per 100 ml), the probability of illness was estimated to be well under the benchmark illness risk of 0.01. Based on the species identified and sequence analysis in the current study, with <8 % of campylobacters belonging to possibly human-infectious species, bather risk from waters impacted by California

Table 1. Summary of qPCR detection of *Campylobacter* and fecal indicators (*Enterococcus*, and *Bacteroidetes*) in gull excreta.

Sampling Date	Number of Samples (*Campylobacter* spp. positive samples)	*Campylobacter* spp. (CE/g)[1] (STD)	*Enterococcus* (CE/g)[1] (STD)	*Bacteroidetes* (CE/g)[1] (STD)	*Campylobacter/Enterococcus* *Campylobacter/Bacteroidetes* Ratios[2]
7/23/09	5 (3)	1.5e+4 (2.6e+4)	1.2e+6 (1.9e+6)	1.9e+3 (2.1+3)	0.01, 7.95
7/24/09	8 (5)	2.9e+6 (6.9e+6)	5.2e+5 (1.3e+6)	559 (859)	5.61, 5261.16
7/25/09	7 (1)	73 (193)	8.5e+3 (2e+4)	754 (824)	0.01, 0.09
7/26/09	9 (7)	1.0e+5 (2.7e+5)	7.6e+5 (1.5e+6)	537 (351)	0.13, 187.48
8/8/09	10 (7)	2.2e+3 (3.7e+3)	570 (845)	1.1e+3 (1.9e+3)	3.84, 1.93
8/9/09	10 (3)	2.3e+5 (7e+5)	3.9e+4 (1.1e+5)	298 (587)	5.82, 757.45
8/15/09	10 (6)	7.6e+4 (1.3e+5)	1.7e+5 (3.3e+5)	4.4e+3 (1.2e+4)	0.44, 17.18
8/16/09	4 (2)	2.2e+5 (4.4e+5)	4.5e+5 (8.9e+5)	1.9e+3(1e+3)	0.49, 118.84
8/18/09	10 (7)	2.4e+5 (5.2e+5)	1.8e+5 (4.4e+5)	1.5e+4 (4.2e+4)	1.38, 16.76
8/19/09	10 (1)	128 (405)	0 (0)	0 (0)	
8/20/09	10 (7)	6.8e+7 (2.2e+8)	8.6e+5 (1.4e+6)	1.1e+4 (3.e+4)	78.61, 6102.14
8/22/09	10 (0)	0 (0)	41 (131)	239 (506)	
8/23/09	7 (3)	2e+7 (5.2e+7)	2.1e+4 (4.4e+4)	3e+3 (5.3e+3)	954.44, 6565.18
8/29/09	10 (7)	1.3e+5 (2.2e+5)	4.5e+6 (1.2e+7)	3.2e+3 (8e+3)	0.02, 41.33
8/30/09	9 (8)	2.7e+6 (2.4e+6)	4.6e+5 (1.1e+6)	1.e+3 (3e+3)	5.77, 1618.00
9/5/09	8 (7)	2e+7 (4.7e+7)	3.4e+6 (6.7e+6)	8e+3 (1.4e+4)	5.76, 2455.40
9/6/09	9 (6)	7.4e+5 (2e+6)	3.7e+6 (1.1e+7)	3.2e+6 (9.7e+6)	0.19, 0.22
9/12/09	8 (6)	4.7e+5 (8.9e+5)	1.2e+6 (2e+6)	2.1e+3 (2.8e+3)	0.40, 226.61
9/13/09	5 (0)	0 (0)	784 (612)	1.1 e+3 (998)	
Total	**159 (86)**				

[1]*Each value is an average of the replicates of gull samples processed for a given date. Relative abundance was estimated using cell equivalents (CE) per wet gram.*

[2]*Average value of each group was used to calculate the ratios.*

Gulls could be even lower than estimated by Schoen and Ashbolt (2010). The very low occurrence of *Campylobacter* species with high sequence identity (≥99%) to species commonly infectious to humans detected in this study (i.e., *C. jejuni, C. lari*) supports the view that California Gulls are unlikely to be a direct source of infection to humans. However gulls could feed on sewage ponds or dump sites, which could increase their sporadic carriage rate for human-infectious campylobacters and possibly other pathogens. Indeed, Monaghan *et al*. (1985) suggested that due to their increased use of sewage and refuse as a food source, other gull species may provide a good indicator of the extent of contamination of the environment with human waste products.

In summary, this study showed high prevalence of campylobacters in California Gull excreta, consistent with previous results for other gull species that mostly used culture-based methods. However, sequence analysis revealed the majority to be unique *Campylobacter* members. These potentially novel campylobacters were readily detected by qPCR at levels that are similar to fecal indicator bacteria. Many of these *Campylobacter* populations may be part of the normal gull intestinal microbiota and more importantly might not present major public health risks. The sequences obtained in this study could be used to develop assays to further determine the source and distribution of *Campylobacter* spp. associated with gull fecal contamination and other waterfowl sources. This in turn could be used to

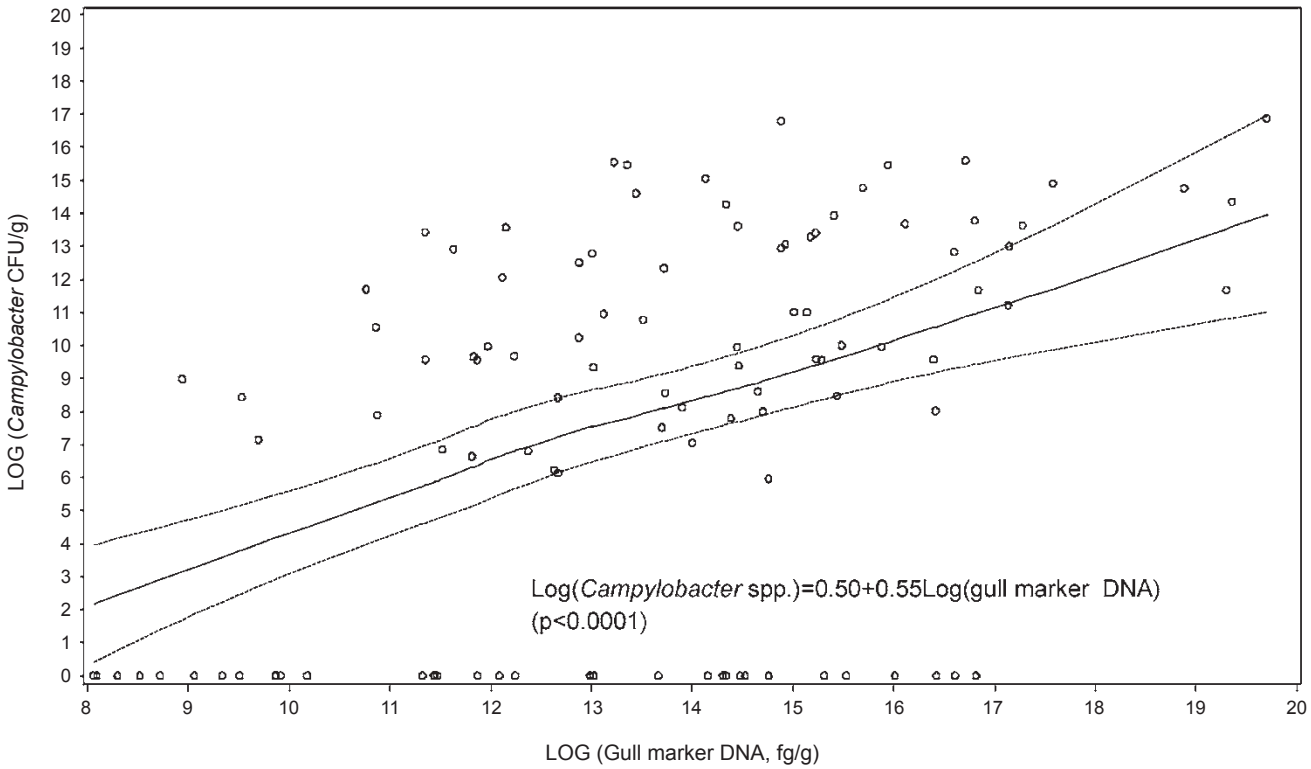

Figure 2. The relationship between the quantity of *Campylobacter* spp. and the gull marker, C. *marimammalium*. Least squares linear regression for predictions of *Campylobacter* spp. (*solid lines*) and 95% prediction intervals (*dashed lines*) are from qPCR assays of *C. marimammalium* (*circles*) against *Campylobacter* spp. and computed using SAS 8.2

estimate possible bather risk at coastal southern Californian recreational sites and describe their relationship with other fecal indicators, information that is needed to improve current quantitative microbial risk assessment models assessing waterfowl risk to recreators.

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Supplemental Information

Supplemental information available online at ftp://ftp.sccwrp.org/pub/download/DOCUMENTS/AnnualReports/2011AnnualReport/ar11_SupplementalInfo_GullExcreta.pdf