Identification of human enteric pathogens in gull feces at Southwestern Lake Michigan bathing beaches

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Abstract: Ring-billed (*Larus delawarensis* Ord, 1815) and herring (*Larus argentatus* Pontoppidan, 1763) gulls are predominant species of shorebirds in coastal areas. Gulls contribute to the fecal indicator burden in beach sands, which, once transported to bathing waters, may result in water quality failures. The importance of these contamination sources must not be overlooked when considering the impact of poor bathing water quality on human health. This study examined the occurrence of human enteric pathogens in gull populations at Racine, Wisconsin. For 12 weeks in 2004 and 2005, and 7 weeks in 2006, 724 gull fecal samples were examined for pathogen occurrence on traditional selective media (BBL CHROMagar-Salmonella, Remel Campy-BAP, 7% horse blood agar) or through the use of novel isolation techniques (*Campylobacter*, EC FP5-funded CAMPYCHECK Project), and confirmed using polymerase chain reaction (PCR) for pathogens commonly harbored in gulls. An additional 226 gull fecal samples, collected in the same 12-week period in 2004, from a beach in Milwaukee, Wisconsin, were evaluated with standard microbiological methods and PCR. Five isolates of *Salmonella* (0.7%), 162 (22.7%) isolates of *Campylobacter*, 3 isolates of *Aeromonas hydrophila* group 2 (0.4%), and 28 isolates of *Plesiomonas shigelloides* (3.9%) were noted from the Racine beach. No occurrences of *Salmonella* and 3 isolates of *Campylobacter* (0.4%) were found at the Milwaukee beach. A subset of the 2004 samples was also examined for *Giardia* and *Cryptosporidium* and was found to be negative. Information as to the occurrence of human pathogens in beach ecosystems is essential to design further studies assessing human health risk and to determine the parameters influencing the fate and transport of pathogens in the nearshore environment.

Key words: gulls, bathing beach, *Campylobacter*, *Salmonella*, *Plesiomonas*.

Résumé : Le goéland à bec cerclé (*Larus delawarensis* Ord, 1815) et le goéland argenté (*Larus argentatus* Pontoppidan, 1763) sont des espèces d'oiseaux marins des zones côtières. Les goélands contribuent à la charge fécale du sable de plages qui, lorsque transportée dans les eaux de baignade, peut diminuer la qualité de l'eau. L'importance de ces sources de contamination ne doit pas être ignorée lorsque l'on considère l'impact d'une mauvaise qualité de l'eau de baignade pour la santé humaine. Cette étude s'est penchée sur la présence de pathogènes entériques humains chez la population de goélands à Racine, WI. Pendant 12 semaines, en 2004 et 2005, et pendant 6 semaines en 2006, 724 échantillons de fèces de goélands ont été examinés relativement à la présence de pathogènes sur des milieux sélectifs traditionnels (BBL CHROMagar-salmonella, Remel Campy-BAP, gélose au sang de cheval 7 %), ou à l'aide de nouvelles méthodes d'isolement (*Campylobacter*, projet CAMPYCHECK FP-5 de la CE), et confirmée par des PCR pour détecter les pathogènes communément transportés par les goélands. Deux cent vingt-six échantillons supplémentaires de fèces de goélands récoltés pendant la même période de 12 semaines, en 2004, sur une plage de Milwaukee, Wisconsin, ont été évalués par des méthodes microbiologiques standards et par PCR. Cinq isolats de *Salmonella* (0,7 %), 162 isolats de *Campylobacter* (22,7 %), trois isolats d'*Aeromonas hydrophila* du groupe 2 (0,4 %) et 28 isolats de *Plesiomonas shigelloides* (3,9 %) ont été répertoriés à la plage de Racine. Aucun isolat de *Salmonella* et 3 isolats de *Campylobacter* (0,4 %) ont été trouvés à la plage de Milwaukee. Un sous-ensemble d'échantillons de 2004 a aussi été examiné quant à la présence de *Giardia* et de *Cryptosporidium* et s'est révélé négatif. L'information quant à la présence de pathogènes humains dans l'écosystème des plages est essentielle afin d'élaborer des études plus poussées visant à évaluer le risque pour la santé humaine et pour déterminer les paramètres qui influencent la destinée et le transport des pathogènes dans l'environnement côtier.

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Mots-clés : goélands, plage de baignade, *Campylobacter*, *Salmonella*, *Plesiomonas*.

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Introduction

In the United States, *Escherichia coli* is used as an indicator of recreational water quality at freshwater beaches (United States Environmental Protection Agency (USEPA) 1986). As a surrogate for human pathogens, it is implied that this organism will only be present in the environment when pathogens are present, and absent when pathogens are absent (Jones and Smith 2004). However, recent research indicates that *E. coli* and other fecal bacteria indicators of bathing water quality may be found autochthonous in the environment, rather than being solely distributed as a result of an influx of fecal matter from human and animal sources, including waterfowl and shorebirds (Oshiro and Fujioka 1995; Belant 1997; Belant et al. 1998; Jones and Obiri-Danso 1999; Lévesque et al. 2000; Obiri-Danso and Jones 2000; Grant et al. 2001; McLellan et al. 2001; Fogarty et al. 2003; Haack et al. 2003; Lebuhn et al. 2003; Yorio and Caille 2004; Wither et al. 2005). It may be that these microorganisms, once deposited in the environment under varying ambient conditions with sufficient nutrients (via scavenging), or mutation under starvation conditions, have the ability to persist (detectable or in the viable-but-not-culturable state) or even replicate, independent of a host (Smith et al. 1994; Farrell and Finkel 2003; Lu et al. 2004; Signoretto et al. 2004). Contact with surfaces, such as sediments, may also facilitate favorable growth conditions, through the formation of biofilms (Lee et al. 2006) or contact with pooled or flowing water, which may occur in the subsurface beach environment (He et al. 2007).

In Racine, Wisconsin, nearshore beach sands have been proven to be a reservoir for *E. coli*, with precipitation (as surface runoff) and waves acting as vehicles to transport these microorganisms to surface water (Kinzelman et al. 2004). Fecal loading from beach sands frequently results in the posting of swimming advisories (Alm et al. 2003; Kinzelman et al. 2004; Whitman et al. 2001; Whitman and Nevers 2003; Beversdorf et al. 2007). With a large resident population of ring-billed and herring gulls (*Larus argentatus* Pontoppidan, 1763 and *Larus delawarensis* Ord, 1815, respectively), the constant deposition of avian fecal material is thought to contribute to the bacterial burden on bathing waters (Lévesque et al. 2000; Jones 2002; Fogarty et al. 2003; Kleinheinz et al. 2006; Wright et al. 2006). These observational data have been supported by antibiotic resistance analysis (ARA), which indicates that the pattern of resistance seen in *E. coli* isolates collected from nearshore surface water is generally nonhuman in nature (Edge and Schaefer 2005; Waldenström et al. 2005).

The risk to human health from contact with recreational waters affected by nonhuman, nonwastewater sources of contamination remains ill defined. Early epidemiological studies, which form the basis of current regulatory limits for monitoring United States coastal bathing waters, recognized the need to consider the nature of the etiological agent (responsible for gastrointestinal illness in exposed individuals), as well as the difficult question of health risk posed by stormwater runoff and other nonpoint sources of pollution (Cabelli et al. 1983). Furthermore, a recent study in California suggested that elevations in traditional fecal indicators (USEPA 1986) were not associated with increased health risk at beaches where the dominant source of pollution was nonpoint in nature (Colford et al. 2007). While the Colford et al. (2007) study has shown poor correlation between nonpoint source pollution (as determined by currently approved fecal indicator organism densities) and human health risk, the nature of the contamination may still be a concern, and should be determined before any definitive statement regarding exposure can be made. Previous British studies have isolated *Campylobacter* strains known to be of the types associated with human infections from sand on bathing beaches (Bolton et al. 1999). Literature has shown that potential health risks may be associated with nonhuman fecal contamination (Pell 1997; Guan and Holley 2003). Additionally, avian species, such as gulls, have been known to carry human pathogens, such as *Salmonella* and *Campylobacter* (Ferns and Mudge 2000; Duarte et al. 2002; Newell 2002; Haag-Wackernagel and Moch 2004; Jones 2004; Tizard 2004; Jones 2005; Dixon 2007).

Gulls, particularly ring-billed gulls, are noted carriers of *Salmonella* spp., *Campylobacter* spp., *Listeria monocytogenes*, and *Yersinia* spp. (Kapperud and Rosef 1983; Hill and Grimes 1984; Yogasundram et al. 1989; Fallacara et al. 2001). A survey in Quebec of 264 ring-billed gulls reported infection rates of 8.7%, 15.9%, and 9.5% for *Salmonella* spp., *Campylobacter* spp., and *Listeria monocytogenes*, respectively (Quessy and Messier 1992). In another study, approximately 200 strains of *Salmonella* spp. were isolated, of which 42 serovars were identified, including 7 serovars that are pathogenic in humans (Lévesque et al. 1993). Further, a study in Sweden found *Campylobacter* spp. in 117 (27%) of 419 black-headed gulls sampled one year, and 133 (36.2%) of 367 gulls sampled the following year; 92%–95% of the *Campylobacter* isolates were found to be *Campylobacter jejuni* (Broman et al. 2002). In light of that information, the purpose of this study was to examine gull feces for the presence of a suite of enteric pathogens (*Salmonella*, *Campylobacter*, *Helicobacter*, *Plesiomonas*, *Giardia*, and *Cryptosporidium*), using conventional and experimental (or novel) microbiological (*Campylobacteraceae*) (Lastovica et al. 1998; Keevil et al. 2006) and molecular methods to determine whether gulls pose a health risk to bathers at these Great Lakes public beaches. The development of microbiological and molecular techniques capable of assessing the prevalence of human pathogens from nonhuman sources, coupled with an estimate of their presence from the perspective of infective doses capable of affecting human health, will aid researchers in developing risk-based standards for the management of recreational waters (Jones and Smith 2004; Edge and Schaefer 2005).

Material and methods

Sample collection

Gull feces

For 12 weeks during the summer of 2004, 8 weeks during

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the summer of 2005, and 7 weeks during the summer of 2006, 724 fresh gull fecal samples were collected from North Beach in Racine, and analyzed for the presence of *Salmonella* spp. and *Campylobacter/Helicobacter* spp. (2004, n = 313; 2005, n = 300; 2006, n = 111). An additional 226 samples were collected from Bradford Beach in Milwaukee, Wisconsin. Samples for microbiological and polymerase chain reaction (PCR) analyses were collected in Cary-Blair fecal transport media, using the sampling device included in the sample container cap (Remel, Inc.; Lenexa, Kansas), directly from beach sands; care was taken to transfer only the fecal matter to the sampling container. Samples designated for PCR analysis only were collected in 15 mL centrifuge tubes, containing 0.5 mL sterile saline. All samples were transported in a cooler on ice packs to the laboratory and processed within 60 min of collection. In the laboratory, an aliquot of a subset of samples (75 from Racine and 75 from Milwaukee) were transferred to 2% dichromate solution for protozoan analysis. Gull fecal samples for PCR were frozen until DNA was extracted.

Isolation of Campylobacter or Helicobacter spp. from gull feces

All gull fecal samples were either plated from the Cary-Blair transport media directly to Campy-BAP (Remel, Inc.) and incubated under microaerophilic conditions for 48 ± 3 h at 42.0 ± 2.0 °C (all 2004 samples and 200 samples from 2005) (Forbes et al. 1998), or processed using an abbreviated form of the Cape Town protocol, as described by Lastovica et al. (1998, revised 2004), and with advice from the members of the EC FP5-funded CAMPYCHECK project (QLK CT 2002 02201) (Keevil et al. 2006) (100 samples from 2005 and all 2006 samples). The Cape Town protocol uses a membrane filtration technique to isolate suspected *Campylobacter* spp. (Steele and McDermott 1984; Allos et al. 1995; le Roux and Lastovica 1998) on tryptose blood agar (TBA) plates (without antibiotics and enriched, in this case, with 7% horse blood), which are incubated in an increased H_2-rich microaerophilic atmosphere, and held for an extended period (up to 6 days) at 42 °C (Lastovica et al. 1998, revised 2004).

From the Campy-BAP plates, characteristic colonies (flat to convex, grayish-white, yellow, or pink) were Gram stained. On a Gram stain, *Campylobacter* spp. appear as faint, curved, slender gram-negative rods (GNCR). All oxidase positive GNCR were further identified using an agglutination test specific for the isolation of *Camplylobacter* spp. (*Campylobacter coli*, *C. jejuni*, *Campylobacter lari*, *Campylobacter upsaliensis*, and *Campylobacter fetus*) (Dryspot *Campylobacter* test kit, Oxoid; Hampshire, United Kingdom). Agglutination tests have been demonstrated to detect antigens specific to several *Campylobacter* spp. (Nachamkin and Barbagallo 1990). Positive control organisms, *C. jejuni* ATCC No. 33291 and *C. lari* ATCC No. 35221, were processed in the same manner to ensure test performance.

For those gull fecal samples processed with the abbreviated Cape Town protocol, a 0.6 μm pore size, 47 mm membrane filter (Millipore Corp., Billerica, Massachusetts) was placed directly on a TBA (Oxoid) plate with 7% defibrinated horse blood (Remel Inc.), using sterile forceps. The central area of the filter was then flooded twice with a well-mixed stool emulsion, using a sterile transfer pipette, and discarded within 15 min of application (Lastovica et al. 1998, revised 2004). After specimen application, the plates were incubated in an H_2-rich environment for 48 h at 42 °C (Oxoid anaerobic system BR0038B, no catalyst). After the initial incubation, the plates were examined and the colony characteristics recorded. Isolated colonies appearing as potential pathogens were Gram stained; if no faintly stained GNCR were found on the initial plates, they were reincubated at 42 °C (for up to 6 d, re-examined every 2 d). Isolated colonies resembling *Campylobacter* spp. from the initial plates were subcultured to new TBA plates to obtain pure growth. One H_2-rich plate and 1 CO_2-rich plate were incubated for 48–72 h at 42 °C. After this incubation period, both plates were examined, colony characteristics were recorded, the Gram stain was repeated, and additional rapid biochemical tests (oxidase, catalase, indoxyl acetate, hippurate, and nitrate reduction) were conducted (The glycine, aryl sulfatase, and rapid H_2S steps were omitted, as it was not necessary to distinguish between *C. jejuni* and *C. fetus* subtypes for the purpose of this study.) The Dryspot *Campylobacter* (Oxoid) test was performed as a confirmatory step (for *C. coli*, *C. jejuni*, *C. lari*, *C. upsaliensis*, and *C. fetus)* when isolates were presumptively positive on rapid biochemical testing.

Isolation of Salmonella spp. from gull feces

Fecal material was transferred from Cary-Blair transport media to selenite broth (Remel, Inc.), and incubated aerobically for 24 h at 35.0 ± 2.0 °C to enhance the recovery of *Salmonella* spp. (Forbes et al. 1998). After 24 h, all tubes of selenite broth exhibiting growth were subcultured to BBL CHROMagar Salmonella (BD; Franklin Lakes, New Jersey), and incubated for an additional 24 h at 35.0 ± 2.0 °C. Most *Salmonella* strains develop mauve-colored colonies on this medium within 18–24 h (Eigner et al. 2001; Maddocks et al. 2002). All characteristic colonies were Gram stained and verified using API 20E (bioMerieux, Durham, North Carolina). Two quality-control organisms, *Salmonella hadar* ATCC No. 51956 (positive) and *E. coli* ATCC No. 25922 (negative), were used to ensure test performance. *Plesiomonas shigelloides* ATCC No. 51572 was used to confirm the colonies appearing as *Salmonella* spp. on the BBL CHROMAGAR media, which were subsequently identified as such using API 20E.

Detection of Campylobacter spp. and Salmonella spp. by PCR in gull fecal samples

DNA was extracted from gull fecal samples using a QIAamp DNA Stool Mini kit (Qiagen; Valencia, California). PCR analysis was used to detect *C. coli* and *C. jejuni*, specifically based on primers for the 16S rRNA gene, yielding an 833 bp product (Linton et al. 1997): CCCJ609 (F) 5′AATCTAATGGCTTAACCATTA3′ and CCCJ1442 (R) 5′GTAACTAGTTTAGTATTCCGG3′, with an annealing temperature of 52 °C. Genus-specific primers targeting *Campylobacter* spp. (851 bp product) were also employed, as described by Eyers et al. (1993) and Fermér and Engvall (1999): Therm1 (F) 5′AATCTAATGGCTTAACCATTA3′ and Therm4 (R) 5′GTAACTAGTTTAGTATTCCGG3′, with an annealing temperature of 54 °C. To detect *Salmonella*,

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primers targeting 2 virulence genes, *spvC* (570 bp product) and *invA* (243 bp product), were used (Chiu and Ou 1996): spvC (F) 5′ACTCCTTGCACAACCAAATGCGGA3′ and spvC (R) 5′TGTCTTCTGCATTTCGCCACCATCA3′, with an annealing temperature of 68 °C; invA (F) 5′ACA GTGCTCGTTTACGACCTGAAT3′ and invA (R) 5′AGA CGACTGGTACTGATCGATAAT3′, with an annealing temperature of 56 °C. *Escherichia coli* control primers targeted a 151 bp product of the *β-glucuronidase* gene: uidA1663 (F) 5′GCGACCTCGCAAGGCATA3′ and uidA1790 (R) 5′GATTCATTGTTTGCCTCCCTGCTGCG3′, with an annealing temperature of 60 °C. PCR was carried out using 12.5 μL of 2× PCR master mix (Qiagen), 9.5 μL of water, and 2 μL of DNA template, with cycling conditions of 1 min at 95 °C, 1 min at each specified annealing temp, and 1 min at 72 °C for 30 cycles, with a final 5 min extension at 72 °C. All products were visualized on a 1.5% agarose gel following staining with ethidium bromide.

Control organisms *C. jejuni* ssp. *jejuni* (ATCC No. 35920) and *Salmonella enterica* ssp. *enterica* serovar *Typhimurium* (provided by the Milwaukee Health Department (MHD)) were each spiked into gull fecal samples at a level of approximately 1×10^4 or 1×10^5 cells per sample, which corresponds to 10 and 100 cells per μL, respectively, in the final DNA extraction (20 or 200 cells in each reaction); each primer pair produced the expected size product. Three of 5 samples with 1×10^4 cells were positive for *C. jejuni*, and 5 of 5 samples with 1×10^5 cells were positive. We therefore determined that 1×10^5 cells per fecal sample was our limit of detection. To test for inhibition in extracted DNA, as well as to ensure that bacterial DNA was adequately recovered, each gull fecal sample was tested with primers specific for *E. coli*, an organism that is expected to be present in all samples. Only 4 of 420 samples tested were negative for *E. coli*; these were, therefore, not included in this study.

PCR and sequence confirmation of isolates

Presumptive *Campylobacter* isolates were screened with *C. coli* and *C. jejuni* specific primers (Linton et al. 1997). In some cases, 16S rRNA gene sequencing was carried out on presumptive *Campylobacter* isolates with PCR amplification, using 8F and 1492R primers. PCR products were purified using a QIAquick PCR purification kit or a QIAquick gel extraction kit (Qiagen). Sequencing was carried out using a BigDye Terminator version 3.1 cycle sequencing kit, according to the manufacturer's instructions, and sequencing reactions were run on an ABI Prisim 3730 (Applied Biosystems, Foster City, California). Single-sequence reads were trimmed for quality, using PHRED (Ewing and Green 1998), which provided approximately 700 bp reads for further analysis. Isolate identification was made using the BLAST algorithm (Altschul et al. 1990).

Detection of Giardia and Cryptosporidium from gull feces

Gull fecal samples were evaluated by the MHD laboratory for the presence of *Cryptosporidium* and *Giardia*. Samples were collected in Carey-Blair transport medium and refrigerated at 0–4 °C until tested by the formalin – ethyl acetate sedimentation concentration procedure (Crede 1992). Concentrates were then stained with a fluorescent antibody staining method (Merifluor, Meridian Bioscience, Inc.; Cincinnati, Ohio) specific for *Crytposporidium* and *Giardia*. (Garcia et al. 1987, Garcia et al. 1992; Garcia and Shimizu 1997). Positive and negative kit controls were run with each batch of samples.

Detection of human enterovirus and human and avian adenovirus from gull feces

Gull fecal samples were screened by the MHD laboratory for human entero- and adenoviruses, as well as for avian adenoviruses. To detect human viruses in avian fecal specimens, fecal swabs were processed and inoculated onto the following cell types used by the MHD Virus Laboratory for clinical specimens (Sedmak et al. 2003): Rhesus monkey kidney primary, HEp-2, human foreskin diploid, human embryonic lung diploid, RD, Caco-2, and BGM. These cells were all propagated in 24-well plastic plates. After a 90–120 min adsorbtion period, the inoculum was removed, and the cultures were refed and incubated at 35.5 °C in a CO_2 incubator. Cultures were observed daily, during a 14 day period, for the development of viral cytopathic effect. For avian adenoviruses, the specimens were also inoculated onto 24-well plates of a chicken hepatoma continuous cell line (CH-SAH, ATCC) (Alexander et al. 1998).

Results

Culture-based microbiological analyses

2004

Of the 313 fecal samples collected at the Racine beach, 5 isolates of *Salmonella* (1.6%), 45 isolates of *Campylobacter* (14.4%), and 22 isolates of *P. shigelloides* (7.0%) were confirmed for the target organism. Of the 110 isolates containing GNCR (presumptive), 45 were verified as *Campylobacter* spp., which translates to a 40.5% accuracy rate using Campy-BAP (Remel, Inc.) for the recovery of *Campylobacter* spp. from gull feces. None of the gull fecal samples were positive when *C. jejuni* and *C. coli* were used as specific primers. A subset of the presumptive isolates were also negative on PCR, indicating that they were neither *C. jejuni* nor *C. coli*, suggesting that gulls carry *Campylobacter* spp. other than these 2 common human pathogens.

The frequent occurrence of *Plesiomonas*, seen when screening for *Salmonella*, can be attributed to similarities in colonial characteristics on CHROMagar *Salmonella*. While not initially intending to screen for *P. shigelloides*, mauve colonies appearing on CHROMOagar Salmonella were frequently confirmed as such, indicating that there is some cross reactivity with this organism and the chromogenic indicator present in the media.

In addition to *Campylobacter*, *Salmonella*, and *Plesiomonas*, several other microorganisms were occasionally isolated from gull feces, including *Shigella* spp. (1.3%) (unconfirmed), *Aeromonas hydrophila* (0.6%), *Hafnia alvei* (0.6%), and *Pasteurella pneumotropica* or *Pasteurella haemolytica* (0.9%). Many of these pathogen isolates came from diarrrheal stool samples, indicating that these organisms were potentially pathogenic, rather than commensal, in the gull population.

Of the 224 gull fecal samples collected from the Milwau-

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kee beach, 100 were screened for *Salmonella* and *Campylobacter*. No *Salmonella* was detected. *Campylobacter coli* was found in 2 samples, and *C. jejuni* was found in 1 sample. PCR results from these 3 samples were also positive for *C. jejuni* and *C. coli*.

2005

In 2005, 200 gull fecal samples were processed using the same culture-based methods employed in 2004. An additional 100 samples were processed in the same manner, except for the isolation of *Campylobacter*, which was accomplished using a slight modification of the Cape Town protocol, as described by Lastovica et al. (1998, revised 2004), and with advice from the members of the EC FP5-funded CAMPYCHECK project (QLK CT 2002 02201) to enhance the recovery of nontraditional *Campylobacter* spp. Of the 200 samples processed in the traditional manner, 21.5% (43 of 200) had confirmed positive *Campylobacter* isolates. Only 3 of these were confirmed as either *C. jejuni* or *C. coli* by PCR of gull feces. Of the 100 samples processed using the Cape Town protocol, 68% (68 of 100) were biochemically indicative of *Campylobacter*, including species *C. coli* (59), *C. lari* (4), *C. upsaliensis* (1), *Campylobacter sputorum* (biovar *sputorum*) (2), *Campylobacter hyointestinalis* (1), and *C. jejuni* (1). Of these presumptive isolates, 38% (26 of 68) were confirmed using the Oxoid Dryspot *Campylobacter* test. Overall, 3 gull fecal samples were confirmed as either *C. jejuni* or *C. coli* by PCR. There were 2 isolates of *P. shigelloides* (0.7%), 2 isolates of *A. hydrophila* (0.7%), but no *Salmonella* spp., recovered in 2005.

When using the Cape Town protocol (TBA plates with 7% defibrinated horse blood and an H_2-rich atmosphere), subcultured plates generally exhibited growth (with the exception of 2 plates). The majority of the isolates were indoxyl acetate positive and hippurate negative (indicating species *C. coli*, *C. upsaliensis*, *C. fetus*, *C. lari*, or *Arcobacter*); many were nitrate positive and catalase positive (*C. jejuni*). The subcultured plates that were incubated in the CO_2 atmosphere frequently did not exhibit the same growth as those incubated in the H_2-rich atmosphere, i.e., the colony characteristics were not comparable, indicating potential co-infection.

Of the 100 isolates processed using biochemical testing, as delineated in the Cape Town protocol, 68 were presumptively positive for *Campylobacter*, representing 6 different species (Table 1). The total number of presumptive isolates using biochemical tests that were also confirmed with the Oxoid Dryspot *Campylobacter* test was 26 of 68 or 38%, leaving the number of presumptive isolates confirmed using biochemical tests that were not confirmed with the Dryspot test at 36 of 68 or 53%.

2006

In 2006, 111 additional gull fecal samples were screened for the presence of human enteric pathogens. Culture-based microbiological methods were utilized, in the same manner as in the previous 2 years, with the Cape Town protocol entirely supplanting the more common Campy-BAP as the primary isolation protocol for *Camplylobacter* spp. Of the 111 samples, 11 presumptive isolates of *Campylobacter*, representing 5 different species (*C. upsaliensis* (1), *C. coli* (4), *C. fetus* (2), *C. jejuni* (1), and *C. lari* (3)) were identified (10%). There were 5 isolates of *Plesiomonas shigelloides* (4.5%), but no *Salmonella* spp. were recovered.

Table 1. Breakdown of total number of presumptive *Campylobacter* isolates (n = 68) by species, using a modification of the Cape Town protocol in 2005.

Campylobacter species	No. of isolates	Percentage of total
C. coli	59	87.0
C. lari	3	4.0
C. upsaliensis	1	1.5
C. lari, *C. coli**	1	1.5
C. sputorum (biovar *sputorum*)	2	3.0
C. lari, *C. hyointestinalis**	1	1.5
C. jejuni	1	1.5

*Biochemical tests indicated the possibility of both.

Molecular analyses

Campylobacter spp

Overall, 416 gull fecal samples were extracted for DNA and tested using pathogen-specific primers. PCR analysis for pathogens did not appear to be more sensitive for detecting *C. coli* and *C. jejuni* (Table 2, above results). Control experiments using cultures of *C. jejuni* spiked into gull fecal samples demonstrated that the detection limit for PCR was between 1×10^4 and 1×10^5 cells per fecal sample. The 2004 and 2005 Racine and Milwaukee gull fecal samples that were positive for *C. coli* or *C. jejuni* on PCR were also presumptively identified as *Campylobacter* spp. by culture. In addition, culture methods detected other presumptive isolates where PCR results were negative. This suggests that the culture may be more sensitive or, alternatively, that *Campylobacter* spp. other than *C. jejuni* and *C. coli* are present in the gulls.

To further investigate the differences in detection of presumptive *Campylobacter* spp. by culture, compared with *C. jejuni* and *C. coli* PCR results, PCR for *Campylobacter* spp. (in general), using the Therm1 and Therm4 primers, was carried out on the gull fecal samples (Table 2); these results demonstrated that *Campylobacter* spp. were present at a higher frequency than was found with primers targeting *C. coli* and *C. jejuni*.

A subset of the 22 isolates that were identified as *Campylobacter* spp., using the Dryspot *Campylobacter* test, were also tested by PCR and by sequencing. Eight of the 20 were viable following resuscitation from frozen stocks. Two isolates in the subset were positive using primers targeting *C. jejuni* or *C. coli*. Six additional isolates were sequenced. Four were identified as *Helicobacter* spp., with 2 unidentified suggesting that *Helicobacter* spp. carried in gulls may be presumptively isolated by traditional and enriched methods of *Campylobacter* isolation, and may also cross react with the Dryspot test.

Salmonella spp

We detected very few *Salmonella* spp. with culture-based methods or PCR-based methods that target 2 virulence genes, *spvC* and *invA*. In all, the Racine Health Department

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Table 2. *Campylobacter* spp. detected in gull feces during the summers of 2004 and 2005

	Total no. of gull fecal samples for 2004 and 2005	
Testing results	Milwaukee gulls (n = 226)	Racine gulls (n = 613)
Total gulls submitted for microbiological analysis, n	100	613
Percent positive for *Campylobacter* spp., based on biochemical testing	Not reported	25.4
Percent positive using Dryspot *Campylobacter* test*	3.0	21.5†
Total gulls analyzed by PCR, n	226	190
Percent positive for *C. jejuni* or *C. coli* on PCR	1.0	1.3
Percent positive for *Campylobacter* spp. on PCR	4.0	6.8

*The Dryspot test detects the following *Campylobacter* spp.: *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*.

†A modification of the Cape Town proctocol was used in 21.5% of the subset of samples that were analyzed. The DNA extracted from gull feces of these same samples yielded 1.3% of the samples positive for *C. jejuni* or *C. coli*. The samples presented here do not include those analyzed by the Racine Health Department (RHD) using the Cape Town protocol.

(RHD) identified *Salmonella choleraesuis* in 3 gull samples in 2004; all other 2005 samples were negative (n = 200). The MHD did not detect *Salmonella* in any 2004 samples (n = 100). All PCR analyses were negative, with the exception of 2 samples in which *invA* was detected, but not *spvC*. These samples could not be verified by culture methods.

Parasitology

More than 150 gull fecal samples collected in 2004 were screened for *Giardia* and *Cryptosporidium* by concentrating gull feces (collected in transport media), using the formalin ethyl–acetate concentration method followed by identification with specific and sensitive fluorescent antibody staining. Neither *Giardia* nor *Cryptosporidium* were detected. Positive and negative kit controls for the fluorescent antibody method were run with each batch.

Virology

One hundred gull fecal samples were screened by the MHD for culturable viruses, such as adenoviruses and enteroviruses. Gull fecal samples were processed and screened using 7 cell lines (G. Sedmak, personal communication, 2008). No viruses were detected using cell culture techniques. Routine positive sewage and clinical samples run in the MHD laboratory served as positive controls for the cell culture system. Direct PCR to detect avian adenovirus on DNA extracted from gull feces also produced no positive samples.

Discussion

In Racine, it was demonstrated that gulls have the capability to carry human pathogens. Of the total number of samples collected (n = 713), 211 (30%) were found to contain potential bacterial pathogens, most notably *Campylobacter*, *Salmonella*, and *Plesiomonas* spp. *Salmonella* has the ability to cause gastroenteritis, affecting approximately 1.4 million people annually in the United States and, because of the communal nature of resident populations of gulls, it was anticipated that several fecal samples would test positive for this common enteric pathogen. However, very few isolates of *Salmonella* spp. were detected using culture-based methods or PCR-based methods targeting 2 virulence genes, *spv*C and *inv*A. In all, *S. choleraesuis* was isolated from 5 gull fecal samples in 2004. Compared with other reports regarding the prevalence of *Salmonella* (Lévesque et al. 1993; Quessy and Messier 1992), we found a much lower occurrence of *Salmonella* by both culture-based methods and PCR. *Plesiomonas shigelloides* also causes gastroenteritis, and has been implicated in disease outbreaks associated with fresh water environments (Krovacek et al. 2000; Lee et al. 2002).

Of the potential pathogens noted, the majority were presumptively identified as *Campylobacter* or related organisms or genera, e.g., *Helicobacter* spp. (167 of 211 or 79%). *Campylobacter*, although found throughout the course of the study, followed a seasonal pattern of distribution; more isolates were detected later in the summer (data not shown). *Campylobacter* spp. is the most common bacterial cause of gastroenteritis, and an estimated 2.4 million people are affected each year (Centers for Disease Control and Prevention online: http://www.cdc.gov/nczved/dfbmd/disease_listing/campylobacter_gi.html). *Campylobacter jejuni* grows best at body temperature within the digestive tract of birds (Food and Drug Administration online: http://www.cfsan.fda.gov/~mow/chap4.html), and has the ability to cause disease in both humans and animals, as evidenced by its frequent recovery from diarrheal stool.

Microbiological results noted frequent presumptive *Campylobacter* spp., as defined by growth on *Campylobacter* media (Campy blood agar, Remel Inc.) under microaerophilic conditions at 42 °C and a positive agglutination test (Dryspot *Campylobacter* test kit). In 2005, a comparative study was conducted against a novel method for the isolation of *Camplylobacter* spp. (Cape Town protocol). The total confirmed positive isolates using traditional media, such as CAMPY-BAP and atmospheric conditions (CO_2, microaerophillic), yielded a confirmation rate of 21.5% (43 of 200) with the Dryspot *Campylobacter* test. The confirmation rate improved to 38% using the Cape Town protocol (Dryspot test positive) including an additional 36 isolates presumptively positive using the novel biochemical testing scheme, but demonstrating a negative Dryspot test. If these presumptively positive isolates were *Campylobacter* spp. not detected by this agglutination test, this would improve the overall confirmation rate to 68%, a significant increase over 2004.

Several of the positive gull fecal samples also had DNA extracted for analysis by PCR, however, PCR analysis using primers specific for *C. coli* and *C. jejuni* rarely produced a positive result. A likely explanation for the high occurrence

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of presumptive *Campylobacter* spp. is that gulls carry species that cross react with the Dryspot *Campylobacter* test, but that are not *C. coli* or *C. jejuni*. For example, the 16S rRNA gene from 6 presumptive isolates was sequenced. Four of these isolates were identified as *Helicobacter* spp. There are several other reasons that might account for the high recovery possibilities, including that the organisms in the gull feces were at low levels, or degraded, and insufficient DNA was recovered. Control experiments demonstrated that a minimum of 1×10^4, and in some cases 1×10^5, cells per sample (20–200 cells per reaction) needed to be present to detect the organisms by PCR. These results demonstrate that microbiological methods, while more cumbersome, might be more sensitive for determining pathogen occurrence in gulls.

Attempts to identify presumptive *Campylobacter* isolates to the species level by sequencing the 16S rRNA gene gave mixed results. In the sequencing of presumptive *Campylobacter*, 80% were found to be *Helicobacter*. It is suspected that the non-*Campylobacter* isolates, e.g., *Helicobacter*, were more easily recovered, and the nonviable cultures may have been primarily *Campylobacter* spp., since several isolates (16 of 22) were not viable after resuscitation from frozen stocks. This would be consistent with what other laboratories have experienced (C.W. Keevil, personal communication, 2007). Work is underway to resuscitate frozen stocks of the isolates so that the various primers can be retested against the organism itself, rather than the DNA extracted from the feces of these same samples. Future work may target *Campylobacter* spp., using a general primer to amplify either *Campylobacter* spp. or epsilon Proteobacteria, coupled with sequencing to identify their organisms. Further screening of gull populations at beaches could then be carried out using family-specific and genus-specific primers targeting *Campylobacter* species that are commonly found in gulls. Specific primers are being designed for *Helicobacter* spp., and the entire repository of gull DNA samples will be screened for *Helicobacter* as well, since this organism appears to be commonly carried by gulls. The human health implications of contamination with *Campylobacter* spp. or *Helicobacter* spp. is unknown, and warrants further work.

Overall, traditional microbiological techniques appeared to be an adequate assessment tool for examining Great Lakes gull populations for the presence of potential human enteric pathogens, such as *Salmonella* spp. However, re-evaluating conventional microbial isolation methods during the second year of the study (2005) potentially improved the detection rate of *Campylobacter* spp., as noted when identifying underdiagnosed species and related organisms in a clinical setting (Engberg et al. 2000). Novel microbial isolation techniques (i.e., the Cape Town protocol), which allow for enhanced identification of under-represented microorganisms, such as various *Campylobacter* spp., may prove of benefit to researchers seeking to determine the prevalence of those microorganisms in coastal environments.

Recreational water quality is a public health priority in Great Lakes communities, given the high density of gulls and gull feces in coastal waterways. Targeting avian fecal contamination will clarify the presence and types of potential human pathogens present, as well as their probability of occurrence and availability for transport to recreational waters. Furthermore, findings from this research are important in determining whether a risk to health is posed by the beach face (sand) itself (as a source of contamination) (Bolton et al. 1999). Information about the occurrence of human pathogens in beach ecosystems is essential to design studies that assess human health risk from nonsewage sources, to determine parameters that influence the fate and transport of these pathogens in the nearshore environment, and, finally, to develop assessment tools that can definitively discern sources of fecal contamination. This becomes especially true in light of the controversy regarding the ability of currently approved bacterial indicator organisms to credibly ascertain the health risk posed to bathers from nonpoint, nonhuman sources of fecal contamination, such as gulls.

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