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ENUMERATION AND ANTIBIOTIC RESISTANCE PATTERNS OF FECAL INDICATOR ORGANISMS ISOLATED FROM MIGRATORY CANADA GEESE (*BRANTA CANADENSIS*)

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ABSTRACT: Thermotolerant fecal indicator organisms carried by migratory waterfowl may serve as reservoirs of antibiotic resistance. To determine the extent to which such antibiotic resistance markers were present in migratory Canada geese (*Branta canadensis*) on the Maryland Eastern Shore, we isolated *Enterococcus* spp. and *Escherichia coli* from fresh feces and examined the antibiotic resistance profiles of these bacteria. Samples were obtained in October 2002, January 2003, and March 2003. Thermotolerant *E. coli* counts ranged from 0 to 1.0×10^7 colony forming units (CFU)/0.1g (g^{-1}) wet weight of feces, whereas *Enterococcus* spp. counts ranged from 1.0×10^2–1.0×10^7 CFU g^{-1} wet weight of feces. Primary isolates of each indicator organism were tested against a panel of 10 antibiotics. Greater than 95% of *E. coli* isolates were resistant to penicillin G, ampicillin, cephalothin, and sulfathiazole; no *E. coli* were resistant to ciprofloxacin. Enterococcal isolates showed highest resistance to cephalothin, streptomycin, and sulfathiazole; no enterococci were resistant to chloramphenicol. The tetracyclines, streptomycin, and gentamycin provided the greatest discrimination among *E. coli* isolates; chlortetracycline, cephalothin, and gentamycin resistance patterns provided the greatest discrimination between enterococcal strains. Multiple antibiotic resistance (MAR) profiles were calculated: fall (*E. coli* = 0.499; enterococci = 0.234), winter (*E. coli* = 0.487; enterococci = 0.389), and spring (*E. coli* = 0.489; enterococci = 0.348). *E. faecalis* and *E. faecium,* which are recognized human nosocomial pathogens, were cultured from winter (44 and 56%, respectively) and spring (13 and 31%, respectively) fecal samples.

Key words: *Branta canadensis,* Canada goose, *Enterococcus* spp.; *Escherichia coli;* multiple antibiotic resistance.

INTRODUCTION

Antibiotics have become commonplace in our environment (Col and O'Connor, 1987). They are widely used in medical therapy, animal husbandry, and agriculture (Houndt and Ochman, 2000; Vidaver, 2002). Microbes may develop resistance to antibiotics under selective pressure, or they may acquire antibiotic resistance determinants without direct exposure to an antibiotic (Koshland, 1994). Most antibiotic resistance genes reside on horizontally mobile elements (HMEs). These HMEs, which include viruses, conjugative plasmids, integrons, and transposons, can readily transfer antibiotic resistance genes from one organism to another (Heinemann, 1998), and can persist in bacterial genomes in the absence of selective pressure by antibiotics (Jabes et al., 1989).

The thermotolerant bacteria *Escherichia coli* and *Enterococcus* spp. are routinely used by public health authorities as indicators of fecal pollution in recreational waters (United States Environmental Protection Agency, 1986). These fecal indicator organisms are found in the digestive tracts of many homeothermic animals, and *E. faecalis* and *E. faecium* are potential nosocomial pathogens. Conjugative exchange of antibiotic resistance plasmids in *E. coli* from migratory waterfowl other than Canada geese (*Branta canadensis*) has been demonstrated (Tsubokurea et al., 1995). Genetic exchange of virulence determinants had been shown to occur between food and medical enterococcal isolates (Eaton and Gasson, 2001).

We were interested in determining whether the thermotolerant fecal indicator organisms carried by migratory Canada geese might serve as reservoirs of antibiotic resistance. Because these migratory water-

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fowl have a large flight range, it is possible that they may be effective disseminators of antibiotic resistance determinates. To determine the extent to which these geese carry bacteria with antibiotic resistance markers, we assessed the prevalence and combinations of antibiotic resistance determinants in the fecal indicator organisms of migratory Canada geese.

Several researchers have enumerated fecal *E. coli* from Canada geese (Alderisio and DeLuca, 1999; Kullas et al., 2002), but little is known about the fecal levels of enterococci in these birds. Therefore, we also evaluated levels of the thermotolerant *E. coli* and *Enterococcus* spp. present in feces from these birds.

MATERIALS AND METHODS

Sample collection

Goose feces were collected from winter wheat or soybean fields utilized by migratory Canada geese on the Oxford peninsula on Maryland's Eastern Shore (38°69′N, 76°14′W); all samples were collected within a 1.6-km radius. Samples were collected along a single transect through large flocks of geese ($n>200$) to provide maximum assurance that each sample was from a different individual bird. Samples were collected in late October 2002 (fall), late January 2003 (winter), and early March 2003 (spring); air temperatures at sampling were 8.8 C, 0.3 C, and 8.8 C, respectively.

Fresh goose feces (within 5 min of defecation) were collected from the ground into an everted sterile sampling bag (Fisher Scientific, Pittsburgh, Pennsylvania, USA) placed over the hand of the collector. Care was taken to collect only the fresh fecal sample, avoiding soil and grass contaminants.

Microbial enumeration and identification

All samples (fall, $n = 21$; winter, $n = 25$; spring, $n = 17$) were processed within 4 hr. Each sample was weighed, diluted 1:10 (w/v) in phosphate buffered saline (pH 7.0), shaken vigorously for 3–5 min, and then placed on a platform shaker at 150 rpm for 30 min. After these steps, the sample was allowed to stand for 10 min so that solids could settle. Serial dilutions were made in phosphate buffered saline, and dilutions were spread plated on Levine Eosin Methylene Blue Agar (EMB) and on Enterococcosel Agar (ENT) (BBL®, Becton, Dickinson, and Company, Sparks, Maryland, USA). Plates were incubated at 44.5 C for 24–48 hr, and characteristic colonies (*E. coli* on EMB; enterococci on ENT) were counted. Samples exhibiting no growth on spread plates were streak plated from the original 1:10 dilution, which had been held at 24 C overnight, to detect recovery of stressed cells.

All thermotolerant *E. coli* were verified by growth at 44.5 C and o-Nitrophenyl-β-D-galactopyranoside/methylumbelliferyl-β-D-glucuronide positive reactions (Colilert®, Idexx, Westbrook, Maine, USA). Thermotolerant enterococci were verified by lack of catalase production, esculin hydrolysis, and growth at 44.5 C. Species identification of selected nonpigmented enterococci from winter and spring samples was performed according to the method of Manero and Blanch (1999).

Antibiotic resistance testing

Colonies derived from primary spread plates were used to assess antibiotic resistance patterns. Isolated colonies of each species were picked from the EMB or ENT isolation plates into sterile 96-well microtiter plates containing 180 μl of M-FC broth (for *E. coli* isolation) or Enterococcosel broth (for isolation of enterococci) (both BBL®, Becton, Dickinson, and Company, Sparks, Maryland, USA) and incubated at 44.5 C for 24–48 hr. A maximum of 24 individual colonies from each positive *E. coli* and enterococci sample were picked, but for some samples, fewer than 24 distinct colonies were present. Only samples containing more than six colonies of either genus were evaluated. All isolates (*E. coli*: $n = 447$, 264, 211; enterococci: $n = 367$, 542, 355) from fall, winter, and spring samples, respectively, were replica-plated onto antibiotic and control plates and incubated at 37 C for 48–72 hr (Wiggins, 1996). Each isolate was tested against a panel of 10 antibiotics on Trypticase Soy Agar (BBL®) plates supplemented with tetracycline (ICN Biochemicals, Aurora, Ohio, USA), chlortetracycline hydrochloride (ICN Biochemicals), cephalothin (Sigma-Aldrich, St. Louis, Missouri, USA) (each at 25 μg/ml), ampicillin (Sigma), streptomycin (Fisher Scientific, Fair Lawn, New Jersey, USA), gentamycin (Fisher Scientific) (each at 10 μg/ml), ciprofloxacin (ICN Biochemicals) (5 μg/ml), or sulfathiazole (ICN Biochemicals) (200 μg/ml); control plates were Trypticase Soy Agar. The antibiotic panel was chosen to include antibiotics with potential efficacy against both *E. coli* and the enterococci. The antibiotic concentrations used were those that have been shown to allow discrimination between isolates on the basis of susceptibility patterns differences (Kaspar et al., 1990; Wig-

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TABLE 1. Colony-forming units (CFU) of thermotolerant enterococci and *Escherichia coli* isolated from Canada goose feces during 2002–3.

	Fall	Winter	Spring
Enterococcus spp.			
Mean[a]	6.1±5.1	6.1±5.0	5.2±4.4
Range[b]	2.0–6.7	3.4–7.0	3.3–5.2
E. coli			
Mean[a]	4.8±3.9	6.1±4.9	5.7±4.9
Range[b]	2.0–5.8	0–7.0	0–6.9

[a] Log_{10} CFU/0.1 g (g^{-1}) wet weight of feces ± SE.
[b] Log_{10} CFU g^{-1} wet weight of feces.

gins, 1996) or to correspond to concentrations used in clinical in vitro susceptibility agar disc diffusion testing.

Multiple antibiotic resistance evaluation

Multiple antibiotic resistance (MAR) values for each isolate were calculated by summing the number of antibiotics to which the isolate was resistant and dividing by the total number of antibiotics assayed (Kaspar et al., 1990). The MAR values for each sample were calculated by summing the MAR values of all individual isolates and dividing by the total number of isolates per sample.

Statistical analysis

Changes in the number of birds carrying indicator organisms resistant to specific antibiotics over sampling dates were evaluated by using the G-log likelihood ratio; individual birds were the sampling unit. Changes in the proportion of individual isolates resistant to specific antibiotics were evaluated by using the two-tailed Z test; the total number of organisms resistant to a given antibiotic (fall, winter, spring) were the sampling units. The two-tailed t-test for two samples with unequal variances was used to evaluate differences between MAR values (Daniel, 1998). Statistical tests were performed by using SPSS software (SPSS, Base 10, Chicago, Illinois, USA).

RESULTS

The thermotolerant fecal indictor organism concentration for each sample was determined (Table 1). Enterococci were isolated from all 63 samples (fall, $n = 21$; winter, $n = 25$; spring, $n = 17$). The mean enterococcal concentration was 7.3×10^5 CFU/0.1g (g^{-1}) wet weight of feces, with counts ranging from 1×10^2–1×10^7 CFU g^{-1} wet weight of feces. *E. coli* were isolated from 47 samples (fall, $n = 21$; winter, $n = 14$; spring, $n = 12$). The mean *E. coli* concentration was 3.6×10^5 CFU g^{-1} wet weight of feces, with counts ranging from 0–1×10^7 CFU g^{-1} wet weight of feces. Of the 63 initial culture attempts, *E. coli* was not isolated from one fall (5%), 11 winter (44%), and seven spring (41%) samples. After overnight resuscitation in phosphate buffered saline, *E. coli* was isolated from the previously negative fall sample and from two each of the initially negative winter and spring samples.

The percentage of individual birds carrying antibiotic-resistant thermotolerant *Enterococcus* spp. and *E. coli* was evaluated (Table 2). The only significant differences in antibiotic resistance between

TABLE 2. Percentage of individual birds carrying antibiotic resistant thermotolerant fecal enterococci and *Escherichia coli* during 2002–3.

	Fecal enterococci			*E. coli*		
	Fall	Winter	Spring	Fall	Winter	Spring
Tetracycline	0	8	6	33	8	0
Chlortetracycline	62	42	63	100	83	100
Penicillin G	14	25	31	100	100	100
Ampicillin	5	29	38	100	100	100
Cephalothin	95	100	100	100	100	100
Streptomycin	100	100	100	67	92	0
Gentamycin	90	96	100	10	58	0
Ciprofloxacin	5	8	0	0	0	0
Sulfathiazole	100	100	100	100	100	100
Chloramphenicol	0	0	0	14	8	0

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sampling periods were among birds carrying enterococci resistant to penicillin G ($G = 12.95$, $df = 2$, $P = 0.002$) or ampicillin ($G = 7.53$, $df = 2$, $P = 0.023$). The number of birds carrying *E. coli* resistant to the following antibiotics exhibited significant variation among sampling dates: gentamycin ($G = 15.08$, $df = 2$, $P = 0.001$), chloramphenicol ($G = 12.61$, $df = 2$, $P = 0.002$), streptomycin, and tetracycline (each $G = 8.11$, $df = 2$, $P = 0.017$).

The percentage of total *Enterococcus* spp. and *E. coli* isolates resistant to each antibiotic is shown in Figure 1. Enterococcal resistance to cephalothin, streptomycin, gentamycin, and sulfathiazole increased from fall to winter ($P<0.001$ for each). The percentage of isolates resistant to streptomycin declined significantly from winter to spring ($P<0.001$); *E. coli* resistant to chlortetracycline decreased from fall to winter and then increased from winter to spring ($P<0.001$ for each).

The mean MAR value, the total number of MAR patterns observed, the mean number of MAR patterns, and the most frequently isolated patterns for *Enterococcus* spp. and *E. coli* are shown in Table 3. The enterococcal mean MAR value decreased from fall to winter ($df = 35$, $P<0.001$) but was unchanged from winter to spring ($P = 0.45$). The variation in the number of different MAR patterns for enterococci on different sampling dates was insignificant. There were no differences in the mean MAR values for *E. coli* over the three sampling dates. The number of observed *E. coli* MAR patterns decreased between winter and spring ($df = 24$, $P = 0.001$).

The most common MAR pattern exhibited by enterococcal isolates was resistance to cephalothin, streptomycin, gentamycin, and sulfathiazole (47.4% of total enterococcal isolates). Resistance to the four previous antibiotics plus chlortetracycline accounted for an additional 7.8% of isolates. Four birds carried enterococci resistant to seven of the 10 antibiotics; 16 birds carried isolates resistant to six antibiotics. Not all isolates from birds carrying enterococci resistant to a given antibiotic were resistant to that antibiotic. Five birds carrying multiresistant enterococci also carried enterococci sensitive to all antibiotics; 22 birds carried enterococci resistant to only one antibiotic.

The most common MAR pattern shown by *E. coli* was multiple resistance to chlortetracycline, penicillin G, ampicillin, cephalothin, and sulfathiazole (55.8% of total *E. coli* isolates). An additional 16.9% of total isolates were resistant to the above combination minus chlortetracycline. Only 0.02% of total *E. coli* isolates were resistant to less than four antibiotics, whereas 0.77% of total isolates were simultaneously resistant to seven different antibiotics.

Enterococcal colonies may be classified as either pigmented (yellow) or nonpigmented (white). The recognized human nosocomial pathogens *E. faecalis* and *E. faecium* are both nonpigmented. We identified all nonpigmented isolates from all winter and spring samples to determine whether *E. faecalis* or *E. faecium* were present. Only nine of 24 winter samples contained nonpigmented enterococci, whereas all spring samples contained these organisms. Of nonpigmented winter isolates, 44% were identified as *E. faecalis* and 56% were *E. faecium.* In the spring sample, 13% of nonpigmented isolates were *E. faecalis* and 31% were *E. faecium.* Other nonpigmented spring isolates were identified as *E. durans* and *E. hirae.* The fall samples were not evaluated for enterococcal species identity.

DISCUSSION

The mean concentration of *E. coli* in our sample (47 geese) was 6.6×10^5 CFU g^{-1} wet weight of feces, with seasonal averages ranging from 7.0×10^4–1.3×10^6 CFU g^{-1} wet weight of feces. Of 236 geese sampled in Westchester, New York, USA, in 1995–97, the mean fecal coliform (FC) level was 1.53×10^4 FC g^{-1} wet weight of feces, with seasonal averages ranging from 5.2×10^4–1.2×10^7 FC g^{-1} wet weight of fe-

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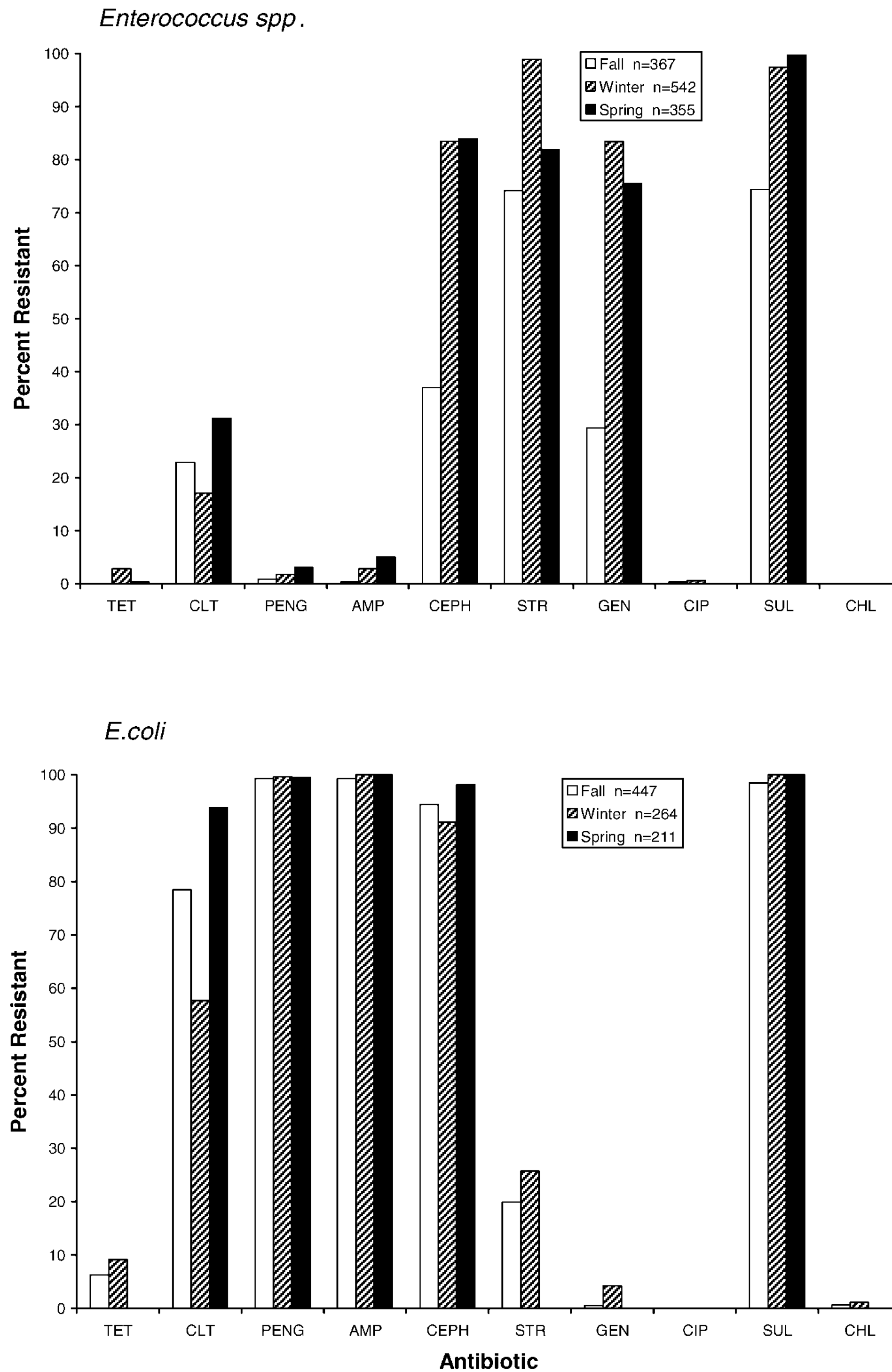

FIGURE 1. Percentage of individual *Enterococcus* spp. and *Escherichia coli* isolates from migratory Canada geese resistant to 10 antibiotics, 2002–3. TET = tetracycline; CLT = chlortetracycline; PENG = penicillin G; AMP = ampicillin; CEPH = cephalothin; STR = streptomycin; GEN = gentamycin; CIP = ciprofloxacin; SUL = sulfathiazole; CHL = chloramphenicol.

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TABLE 3. Multiple antibiotic resistance (MAR) values and patterns for enterococci and *Escherichia coli* isolated from migratory Canada goose feces during 2002–3.

	Fecal enterococci			*E. coli*		
	Fall	Winter	Spring	Fall	Winter	Spring
Mean MAR value/sample	0.487	0.391	0.371	0.499	0.487	0.467
Range	0.408–0.608	0.211–0.500	0.093–0.560	0.432–0.578	0.062–0.608	0.012–0.500
SD	0.062	0.046	0.093	0.039	0.062	0.012
Total number MAR patterns	27	32	21	19	16	4
Mean number MAR						
Patterns/sample	3.6	4.0	4.2	3.6	3.6	1.5
Range	1–6	1–9	1–10	2–7	1–6	1–2
SD	1.6	2.3	2.8	1.3	1.6	0.5
Most frequent MAR patterns[a] (% isolates)	A (17.4) C (15.5)	A (63.0) B (9.7)	A (54.9) B (12.9)	D (56.8) E (15.3)	E (28.8) D (25.4)	D (91.9) E (5.6)

[a] Pattern A: resistance to cephalothin, streptomycin, gentamycin, and sulfathiazole; pattern B: resistance to chlortetracycline, cephalothin, streptomycin, gentamycin, and sulfathiazole; pattern C: resistance to sulfathiazole; pattern D: resistance to chlortetracycline, penicillin, ampicillin, cephalothin, and sulfathiazole; pattern E: resistance to penicillin, ampicillin, cephalothin, and sulfathiazole.

ces (Alderisio and DeLuca, 1999). Total FC counts are expected to exceed *E. coli* counts because FC counts include *Klebsiella* and *Enterobacter* species as well as *E. coli.*

Successful recovery of *E. coli* from goose feces varied with the sampling date. Kullas et al. (2002) isolated fecal *E. coli* from 46% of October samples, 8% of January samples, and 25% of March samples from Canada geese in Fort Collins, Colorado, USA. They suggested a direct correlation between the mean ambient temperature and the recovery of *E. coli,* and they reported mean temperatures of 10.7 C, −0.5 C, and 8.9 C for sampling dates. Our recovery rates for *E. coli* for the same respective sampling months were 100, 56, and 70%. Our respective sampling temperatures were 8.8 C, 0.3 C, and 8.8 C. Differences in recovery rates between studies may reflect variation in sample collection and culture techniques or differences in the fecal flora of the sampled populations, or the differences may indicate greater sensitivity of some strains of *E. coli* to low temperatures. Brittingham et al. (1988) postulated that the isolation rate of *E. coli* from waterfowl may vary according to the exposure of the birds to fecal matter from other vertebrate species. Fallacara et al. (2001) suggested waterfowl might acquire *E. coli* and other pathogens from water sources contaminated with human sewage or agricultural runoff. It is possible that in winter months when many surface water sources are frozen, migratory waterfowl may have limited access to environmental *E. coli* sources, thus decreasing the recovery rates during the coldest months.

The MAR value calculated for a given organism or sample depends on the specific panel of antibiotics chosen for the profile. As such, MAR values are primarily useful for comparing the resistance patterns of bacterial strains within a sample and for determining the range of antibiotic resistance determinants present within a sample population. We found a wide range

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of MAR patterns in both the enterococci and *E. coli* isolates of migratory birds, indicating great diversity in the microbial flora of these birds. The lower number of MAR patterns observed in the spring *E. coli* samples may be a reflection of a reduced sample size (12 birds).

MAR profiles have been used to differentiate point source from nonpoint source *E. coli* populations (Kaspar et al., 1990; Parveen et al., 1997). By analyzing MAR patterns using discriminant analysis, microbial source tracking has allowed the identification of the source of fecal pollution of surface waters (Wiggins, 1996; Harwood et al., 2000). However, *E. coli* isolates from goose feces were examined and found to be very poorly classified by microbial source tracking (0% correct classification) (Guan et al., 2002).

Enterococci have intrinsic resistance to the cephalosporins, often have high level resistance to aminoglycosides (streptomycin and gentamycin), and are developing widespread resistance to penicillin and ampicillin (Jeljaszewicz et al., 2000). All sampled geese harbored enterococci that were resistant to streptomycin and sulfathiazole, and more than 95% of geese carried enterococci resistant to cephalothin and gentamycin. The differences in the MAR profiles of the enterococci may, in part, reflect that several species comprise the enterococcal flora of migratory geese, with each species having different antibiotic resistance patterns.

Although the enterococci are pathogenic only under specific conditions, they are now among the most common causes of human nosocomial infections (Jeljaszewicz et al., 2000). The nonpigmented enterococci, *E. faecalis* and *E. faecium,* are the species most commonly associated with clinical infection. *Enterococcus faecalis* is considered the more pathogenic species because it is more likely to carry human virulence factors (Eaton and Gasson, 2001). Wheeler et al. (2002) proposed using *E. faecalis* as a human fecal indicator for microbial source tracking. They found that *E. faecalis* was present only in humans, dogs, and chickens. They examined three Canada geese and isolated *E. faecium* but not *E. faecalis.* We examined the feces of 40 birds and found that 15% of the geese carried *E. faecalis,* and 25% carried *E. faecium.* Geese defecate freely in ponds and coastal waters within their migration paths. Perhaps the suggestion that *E. faecalis* be used as an indicator of human fecal pollution may need to be reassessed for areas frequented by migratory Canada geese.

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