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Temporal Dynamics and Impact of Manure Storage on Antibiotic Resistance Patterns and Population Structure of *Escherichia coli* Isolates from a Commercial Swine Farm▽

Patrick Duriez and Edward Topp*

Agriculture and Agri-Food Canada, London, Ontario, Canada N5V 4T3

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Many confined-livestock farms store their wastes for several months prior to use as a fertilizer. Storing manure for extended periods could significantly bias the composition of enteric bacterial populations subsequently released into the environment. Here, we compared populations of *Escherichia coli* isolated from fresh feces and from the manure-holding tank (stored manure) of a commercial swine farm, each sampled monthly for 6 months. The 4,668 confirmed *E. coli* isolates were evaluated for resistance to amikacin, ampicillin, cephalothin, chloramphenicol, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, tetracycline, trimethoprim, and trimethoprim plus sulfamethoxazole. A subset of 1,687 isolates was fingerprinted by repetitive extragenic palindromic PCR (rep-PCR) with the BOXA1R primer to evaluate the diversity and the population structure of the collection. The population in the stored manure was generally more diverse than that in the fresh feces. Half of the genotypes detected in the stored manure were never detected in the fresh fecal material, and only 16% were detected only in the fresh feces. But the majority of the isolates (84%) were assigned to the 34% of genotypes shared between the two environments. The structure of the *E. coli* population showed important monthly variations both in the extent and distribution of the diversity of the observed genotypes. The frequency of detection of resistance to specific antibiotics was not significantly different between the two collections and varied importantly between monthly samples. Resistance to multiple antibiotics was much more temporally dynamic in the fresh feces than in the stored manure. There was no relationship between the distribution of rep-PCR fingerprints and the distribution of antibiotic resistance profiles, suggesting that specific antibiotic resistance determinants were dynamically distributed within the population.

Fecal contamination of surface water represents a threat to human and environmental health (16). This is particularly true when water resources are in proximity to land that is subject to increasing agricultural activity and burgeoning human populations, increasing the risk to adjacent waters from agricultural runoff, sewage effluent, leaking rural septic systems, and storm water discharge. *Escherichia coli* is a fecal indicator bacterium that has traditionally been used to evaluate the microbiological quality of surface and drinking water, using standard microbiological methods (10, 53). The presence of this organism is implicit evidence of fecal contamination and indicates a risk of contamination with viral, bacterial, or protozoan pathogens of enteric origin. Therefore, many jurisdictions mandate compliance with drinking and recreational water standards on the basis of contamination with *E. coli* (3, 14–16).

In most industrial countries, swine produced on commercial farms are raised confined in barns, and their waste is stored for several months as an anoxic slurry prior to being added as a fertilizer to the land when climate and crop conditions are suitable. In Canada, for example, about 85% of swine are produced on farms that use static liquid manure storage systems (49). These manure storage systems therefore represent on many farms the crucial secondary habitat that enteric bacteria must survive before they are released into the broader environment, where they could pose a threat to water quality. As well as reducing the abundance of enteric bacteria, storing waste for extended periods could significantly alter the composition of enteric bacterial populations subsequently released into the broader environment. The dynamism of bacterial populations during storage of anoxic manure slurry is somewhat unclear. The distribution of dominant bacteria in swine manure slurry is stable for at least several weeks (11, 30, 40). On the other hand, observed populations of *E. coli* distinguished by repetitive extragenic palindromic PCR (rep-PCR) were found to be consistently more diverse in stored manure slurry than in freshly shed feces from the corresponding swine (32). Changes in the distribution of attributes among populations of *E. coli* that are used to distinguish host source (e.g., antibiotic resistance and dominant host-specific genotypes detected by ribotyping, pulsed-field gel electrophoresis, or rep-PCR methods) could influence the ability of library-dependent microbial source tracking methods to correctly identify the porcine host (2, 4, 20, 26, 27, 35, 36, 38, 42, 50, 54).

In the study reported here, we examined the dynamics and characteristics of *E. coli* populations in fresh and in stored manure, both with respect to population structure and with respect to the frequency of multiple-antibiotic resistance. If resistance to specific antibiotic residues excreted by the animals conferred a fitness advantage to bacteria in the manure holding tank, the phenotype could be expected to be overrepresented in this habitat. Alternatively, if genes encoded resistance to antibiotics unnecessarily and imposed a fitness cost,

* Corresponding author. Mailing address: Southern Crop Protection and Food Research Centre, Agriculture and Agri-Food Canada, 1391 Sandford Street, London, Ontario, Canada N5V 4T3. Phone: (519) 457-1470, ext. 235. Fax: (519) 457-3997. E-mail: toppe@agr.gc.ca.

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bacteria carrying these determinants could be expected to be disadvantaged in the manure holding tank. We obtained from a single commercial farm on a monthly basis (March to August 2005) *E. coli* from freshly shed feces collected in the swine barn and from the farm's manure storage tank. Our specific objectives were to (i) compare the structure of *E. coli* populations obtained from stored and freshly shed manure by means of rep-PCR and determine how these varied with time, (ii) determine if the population from the storage lagoon differed from the population from the fresh manure with respect to the frequency and the profiles of antibiotic resistance, and (iii) determine if the distribution of antibiotic resistance profiles was associated with, or independent of, the population structure defined by rep-PCR.

MATERIALS AND METHODS

Husbandry practices and manure collection. Characteristics of the swine farm used in this study were described by Lu et al. (32). The farm used in this study is a farrow-to-finish operation consisting of approximately 2,000 animals. The animals received a feed mix consisting of corn and soybean meal. During the course of the study described here, the animals received the following antibiotics. Nursery pigs received a growth promotion level of lincomycin and spectinomycin (Linco-Spectrin), and finishing pigs received 40 g tonne^{-1} (40 ppm) of tylosin phosphate (Tylan) in their feed. Penicillin G was added to the water for 2 weeks after the animals were moved from the nursery to the finishing pens. Oxytetracycline was fed to the nursing sows (330 g tonne^{-1}; 330 ppm) in January and April and to the dry sows (550 g tonne^{-1}; 550 ppm) in April.

The farm was sampled on a monthly basis from March to August 2005. Sampling individual animals in the barn was not possible due to the number of individuals per pen and the type of pens used in the barn. In order to obtain a sample that was as representative as possible of the entire herd, about 100 g of feces was collected on the ground of one holding pen of each room ($n = 18$; there were six pens per room, and the number of pigs per pen varied approximately between 10 and 30) of the barn and mixed together. Then approximately 2.5 g of fecal material of each pen was pooled and thoroughly mixed in sterile bottles with sterile sodium metaphosphate buffer (pH 6.8; 2 g per liter) to yield a composite sample. Slurry from within the barn fell through slats to an open pit below. Material from the pit was pumped from below the barn every few days to the manure holding tank, a large concrete reservoir open to the air. The holding tank was emptied during the week before the May sampling, and only a thick layer of sludge at the bottom remained until August. Samples from the manure holding tank were collected from a depth of about 0.5 m below the surface and 0.5 m from the bottom of the tank (Sludge Judge Ultra sampler; NASCO Canada, Aurora, Ontario, Canada) and when possible (April, May, and August) in three different locations around the tank and pooled in 1-liter sterile bottles (Systems Plus, Woodstock, Ontario, Canada).

Regional climate data during the experiment were obtained from Environment Canada (http://www.climate.weatheroffice.ec.gc.ca/climateData/canada_e.html).

***E. coli* enumeration, isolation, and identification.** Isolation of *E. coli* was performed as previously described (32). Briefly, samples were serially diluted in sterile sodium metaphosphate buffer and spread plated on mFC-BCIG agar (8), made with mFC basal agar (Difco, Fisher Scientific, Ottawa, Ontario, Canada) and 100 μg 5-bromo-4-chloro-3-indolyl β-D-glucuronide cyclohexyl ammonium salt (Medox Diagnostics, Ottawa, Ontario, Canada) per liter and then restreaked twice on Luria-Bertani (LB) agar (Difco, Fisher Scientific) (8). Isolates were considered *E. coli* if they grew at 44.5°C, had a positive reaction for β-glucuronidase (blue color on mFC-BCIG agar), fermented lactose, and produced indole. Isolates confirmed to be *E. coli* were inoculated into sterile 96-well microtiter plates containing 100 μl well^{-1} of LB broth and incubated overnight at 37°C. Sterile glycerol (Sigma-Aldrich Canada Ltd., Mississauga, Ontario, Canada) was then added to each well at a final concentration of 15% (vol/vol), and the plates were stored at −70°C. Approximately 400 isolates were picked from each sample, when populations allowed it (see Table 2).

Determination of antibiotic resistance. Microtiter plates containing 100 μl of Mueller-Hinton broth (Difco, Fisher Scientific) were inoculated with bacteria from frozen glycerol stock and were grown statically for 16 to 24 h at 37°C. An aliquot of 4 μl was then transferred with a floating pin replicator (VP Scientific, San Diego, CA) into microtiter plates containing 200 μl of a sterile 0.02% solution of Tween 20 to improve the wetting of the replicator in sterile Milli-Q water. Using the 96-pin replicator, the cell suspension (experimentally adjusted to yield about 10^4 CFU per spot) was spotted onto a series of Mueller-Hinton agar plates containing one of the following antibiotics at the indicated breakpoint concentrations (μg ml^{-1}): amikacin (Ak), 64; ampicillin (Am), 32; cephalothin (Ce), 32; chloramphenicol (Cl), 32; kanamycin (Ka), 64; nalidixic acid (Na), 32; streptomycin (Sm), 64; sulfamethoxazole (Su), 512; trimethoprim (Tm), 16; trimethoprim-sulfamethoxazole (Ct), 4 to 76; or tetracycline (Te), 16. The breakpoint were specified by the National Antimicrobial Resistance Monitoring System (NARMS) (9) or the Société Française de Microbiologie Comité de l'antibiogramme (for trimethoprim) (48). The plates were incubated at 37°C for 20 to 24 h, and growth was scored by eye. Isolates were considered resistant to each antibiotic when growth at that antibiotic's breakpoint concentration was not limited to visibly isolated colonies. Isolates resistant to 4 or more antibiotics were considered multiresistant. The repeatability and validity of the method was evaluated using *E. coli* strain ATCC 25922.

Serotyping. Representative isolates were sent to the Laboratory for Foodborne Zoonoses, Public Health Agency of Canada (Guelph, Ontario, Canada), for serotyping by standard protocols (39).

BOX PCR fingerprinting. Cell suspensions of *E. coli* were prepared by inoculating 100 μl of fresh LB broth per well in a sterile 96-well microtiter plate with frozen stock cultures. Cells were grown statically at 37°C overnight and centrifuged at 710 × *g* for 25 min (Centra CL3 microplate centrifuge; Thermo IEC, Needham Heights, MA). The cells were resuspended in 100 μl of sterile Milli-Q H_2O and agitated at 1,000 rpm with a microplate shaker (Sarstedt, Montréal, QC, Canada) for 5 min. The resuspended cells were used directly as a template for the PCR or frozen at −20°C until required. Rep-PCR fingerprinting was done with the BOXA1R primer as described by Versalovic et al. (55). The final reaction mix (25 μl) consisted of 1× PCR buffer (Promega, Madison, WI), 1.5 mM $MgCl_2$, 1% dimethyl sulfoxide, 200 μM of each deoxynucleoside triphosphate (Invitrogen, Burlington, Ontario, Canada), 2 μM of the primer BOXA1R, 1 U of *Taq* polymerase (Promega), and 2 μl of suspended *E. coli* cells as the template. Amplification was performed with a Thermo MBS Satellite 0.2 Thermocycler instrument (VWR International, Mississauga, Ontario, Canada) as follows: after an initial denaturation at 94°C for 10 min, 34 cycles of denaturation (94°C, 3 s), (92°C, 30 s), annealing (50°C, 1 min), and extension (65°C, 8 min) were performed, followed by a final extension (65°C, 8 min). Six microliters of loading dye was added to 25 μl of PCR product, and 7 μl of this mixture was loaded into wells prepared with an 8-mm by 1-mm comb tooth size. Every eighth well received the MassRuler DNA ladder (Fermentas, Burlington, Ontario, Canada). PCR products were resolved by horizontal gel electrophoresis (2.5 V/cm for 16 h) in 1× Tris-borate-EDTA buffer. The gel was stained with 1 μg ml^{-1} ethidium bromide solution for 10 min and destained in Milli-Q water for 10 min. Gel images were captured as 16-bit TIFF images, using Alphaease FC software and an Alpha Innotech digital gel documentation system (Fisher Scientific, Ottawa, Ontario, Canada).

Computer-assisted image and data analysis. Normalization of gel images and assignment of fingerprints to isolates were done with the Bionumerics software package (version 4.5; Applied Maths, Kortrijk, Belgium) as published earlier (32). Filtering and background subtraction were optimized for each image independently according to methods available at http://www.ecolirep.umn.edu/addinggelimages.shtml. Positions of fingerprints on gels were normalized using the MassRuler DNA ladder as the external standard in the range of 400 bp to 4,000 bp. The assignment of strains to different clusters was performed by calculating the similarity coefficients with the curve-based Pearson similarity coefficient. Similarity trees were generated using the unweighted-pair group method using average linkage. Repeated experiments where the same isolate was amplified with BOX primers and run on different gels under similar conditions consistently showed an average similarity of 80% in our laboratory. Hence, clusters were initially assigned using the software on the basis of 80% similarity, and the final assignments were determined on the basis of careful visual inspection.

All data were grouped in an Excel database and used to perform basic statistical analyses. The chi-square test was used for the analysis of the distribution of antibiotic resistances in the different subsets of the collection. Associations were considered significant when P was <0.05. The diversity captured in the *E. coli* collections was estimated by rarefaction analysis using the analytical approximation algorithm of Hurlbert (23) and 95% confidence intervals estimated as described by Heck et al. (21). The calculations were carried out on a random subsample ($n = 84$) from each monthly sample to prevent sensitivity of the calculation to the size of the sample. The isolates were individually assigned a pseudorandom number between 1 and 10000 using Excel, and the 84 isolates with the lowest values were used for the calculation. Calculations were performed

TABLE 1. Estimates of genotypic diversity and total richness in *E. coli* populations

Origin	Month (no. of isolates)	Shannon-Wiener index[a]	Simpson index (1/*D*)[a]	$r^{2a,b}$	Genotypes			No. of isolates to capture 50% of predicted genotypes (mean ± SD)[a,d]
					Predicted no. (mean ± SD)[a,c]	Detected		
						No.[a]	% of predicted value	
Fresh feces	March (188)	1.93	4.34	0.97	30 ± 2	18	60	64 ± 8
	April (187)	2.84	11.06	0.97	50 ± 2	29	58	65 ± 4
	May (168)	2.71	10.50	0.95	36 ± 1	24	67	43 ± 2
	June (94)	1.11	1.94	0.92	11 ± 0.5	9	79	29 ± 3
	July (93)	2.92	13.36	0.95	41 ± 0.4	27	66	44 ± 1
	August (84)	2.64	10.86	0.92	26 ± 0.2	20	76	27 ± 0.5
Stored manure	March (188)	2.70	9.82	0.97	42 ± 1	26	62	57 ± 4
	April (237)	3.28	18.57	0.98	80 ± 3	39	49	94 ± 5
	May (127)	3.40	24.16	0.97	69 ± 0.5	38	55	69 ± 1
	June (139)	3.18	17.04	0.97	59 ± 1	34	57	65 ± 2
	July (91)	2.46	8.66	0.91	23 ± 0.3	18	78	26 ± 1
	August (91)	2.05	5.02	0.95	24 ± 1	17	70	43 ± 4

[a] Calculated on a randomly selected subsample of equal size ($n = 84$) from each collection.
[b] Coefficient of determination of goodness of fit to the Michaelis-Menten equation.
[c] The V_{max} parameter of the Michaelis-Menten equation.
[d] The K_D parameter of the Michaelis-Menten equation.

with the freeware program Analytical Rarefaction 1.3, available at http://www.uga.edu/strata/software/. Curves were plotted using SigmaPlot (version 9.1; SPSS Inc., Chicago, IL). The asymptotes of the rarefaction curves were estimated using the Michaelis-Menten equation, which is available in SigmaPlot as the one-site saturation ligand model (22). The asymptote is a measure of richness at sampling saturation and was used to estimate the fraction of total community diversity captured within the *E. coli* collections. The SigmaPlot curve fitter uses the Marquardt-Levenberg algorithm to find the coefficients that give the best fit between the equation and the data (33).

The Shannon-Wiener and Simpson diversity indices for populations of *E. coli* obtained from each manure sample were evaluated using randomly picked isolates ($n = 84$) from each monthly sample. The isolates were individually assigned a pseudorandom number between 1 and 1000 using Excel, and the 84 isolates with the lowest values were used for the calculation. The number of individuals sampled was normalized to match the smallest sample to account for the sensitivity to the sample size of both diversity indices. Diversity indices were determined with the software calculator available at http://www.changbioscience.com/genetics/shannon.html. Confidence intervals were calculated according to Grundmann et al. (19).

Significance of differences between distribution of genotypes in the aggregated populations were determined by the method described by Kropf et al. (29) using the abundance of all the genotypes in each sample as the unit of comparison.

RESULTS

In this study, the average population size of viable *E. coli* in fresh feces was $1.4 \times 10^7 \pm 1.0 \times 10^7$ cells g (wet weight)$^{-1}$ ($n = 4$, May to August). The average population size in the stored manure slurry was $1.2 \times 10^4 \pm 0.2 \times 10^4$ ml^{-1} in March, April, and May, which had average monthly air temperatures of −2.3°C, 7.8°C, and 11.8°C, respectively. The average population size in the stored manure slurry declined to $1.5 \times 10^3 \pm 1.8 \times 10^3$ ml^{-1} in June through August, when the average monthly air temperature was 21.8 ± 0.5°C.

E. coli isolates obtained from fresh feces and within the farm's manure holding tank (stored manure) were fingerprinted by means of rep-PCR, and rarefaction curves were used to estimate the abundance of genotypes within the collections (Table 1). The rarefaction data were fitted with the Michaelis-Menten equation and used to estimate the asymptote (saturation of richness) and the number of isolates required to capture half of the diversity. The Michaelis-Menten fit with the experimental data was excellent ($r^2 > 0.91$), and the estimated saturation of richness indicated that between 48% and 79% of the diversity of the collections were captured. Nine (June) to 35 (April) distinct genotypes were detected in the fresh feces, and 17 (August) to 63 (April) were detected in the stored manure. Diversity (expressed as the Shannon-Wiener or Simpson indices or by predicted number of genotypes) was consistently greater in the stored manure than in the fresh feces in March through June (differences were significant in March, May, and June). This was not the case in July and August, when the diversity in the stored manure declined dramatically to be significantly smaller in August. The diversity of *E. coli* in the fresh feces was much lower in June than in any other month.

One hundred fifty distinct genotypes were identified in the collection, with 51 (34%) detected in both fresh feces and stored manure, 24 (16%) detected in fresh feces only, and 75 (50%) detected in stored manure only. When individual isolates were considered, the genotypes detected both within the fresh feces and the stored manure comprised 84% ($n = 1{,}411$), the genotypes unique to the fresh feces comprised only 4% ($n = 69$), and the genotypes unique to the stored manure comprised 12% ($n = 208$) of the total *E. coli* collection. More than half of the isolates (59.8% of the total collection) shared one of 11 dominant genotypes, while 54 genotypes were detected in only one isolate, and 36 were shared by only two isolates. The distribution of the genotypes between the two aggregated samples (fresh feces versus stored manure) showed that there were no significant differences between the structures of the two populations (chi-square, underrepresented genotypes aggregated; $P = 0.08$). The greater diversity in the stored manure than in the fresh feces during March through to June is reflected in the relative abundance of underrepresented genotypes (defined as those detected in fewer than 2% of the total collection; aggregated in Fig. 1 as "others"). The transient profound decline in diversity in *E. coli* obtained from fresh

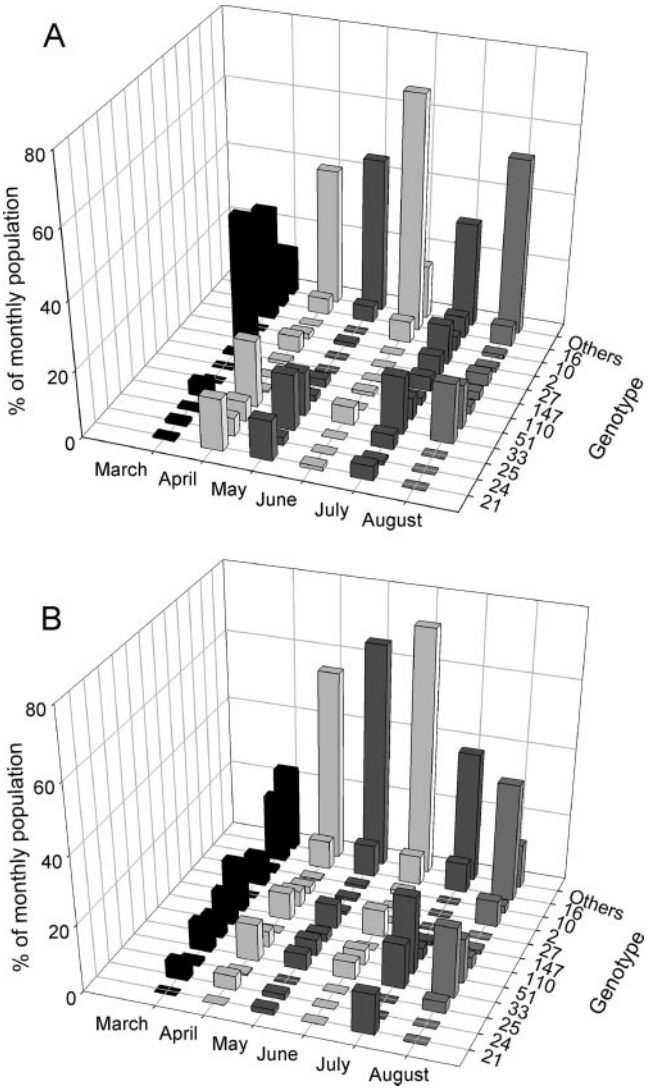

FIG. 1. Monthly variation in the distribution of BOX genotypes of *E. coli* obtained from fresh feces (A) (n = 873) and stored manure (B) (n = 730). "Others" denotes an aggregate of all fingerprints that were detected in less than 2% of all isolates in the collection.

feces in June was associated with the dominance of a single genotype (designated genotype 16). The precipitous decline in diversity in stored manure following the June sampling was associated with a decline in underrepresented genotypes. Genotypes (e.g., 16 and 33) that were consistently detected in fresh feces were also in the stored manure. Genotypes (e.g., 2 and 24) that were detected only sporadically in fresh feces were likewise sporadically detected in stored manure.

Four representative isolates of each of the 11 dominant genotypes were serotyped. These consisted of two isolates from the fresh feces and two isolates from the stored manure each chosen from a different month to reduce the likelihood of clonality. All four isolates from genotype 27 had the same serotype (O139:NM), whereas all the other genotypes exhibited little or no homogeneity in the expressed antigens. Other serotypes detected within the collection included O116:H30, O101:NM, O41:H32, O178:H32, OR:H48, O86:H51, O154:H48, O8:H49, O98:H39, OR:H12, O88:H12, O2:NM, O139:NM, O101:NM, O153:NM, O140:H32, O154:H25, O21:H25, O51:NM, O99:NM, O51:NM, and O8:H9. The serotypes of 11 isolates could not be determined.

Over the entire experiment, the frequencies of resistance to specific antibiotics in the fresh feces collection (n = 2,193) were not significantly different from those in the stored manure collection (n = 2,475), due to the very significant monthly (n = 6) variations. These frequencies (fresh feces and holding tank [mean ± standard deviation]) were as follows: Te, 99% ± 1% and 84% ± 12%; Su, 69% ± 32% and 52% ± 13%; Am, 73% ± 24% and 78% ± 18%; Sm, 53% ± 24% and 29% ± 15%; Tm, 40% ± 38% and 45% ± 7%; Cl, 41% ± 36% and 13% ± 25%; Ka, 20% ± 11% and 9% ± 10%; Ct, 15% ± 7% and 26% ± 12%; Na, 0.1% ± 0.2% and 0.5% ± 1.3%; Ce, 3% ± 3% and 4% ± 5%; and Ak, 0% and 0.2% ± 1.3%. However, when considered on a monthly basis, the resistance to a number of antibiotics varied widely and dynamically (Table 2). Perhaps most striking were the trends for Am, Sm, and Cl resistance frequency in the fresh feces collection. The frequencies increased from March through May, when almost all of the isolates were resistant to these antibiotics. Resistance decreased abruptly in June and thereafter increased through August. The June collection also had lower frequencies of resistance to Ka and Su, but trends for these antibiotics were

TABLE 2. Antibiotic resistance of *E. coli* isolates from fresh feces and stored manure

Antibiotic	% Resistant isolates (fresh feces/stored)[a]						
	March (396/1001)	April (383/352)	May (383/476)	June (378/172)	July (341/142)	August (312/350)	Total (2,193/2,475)
Am	73.0/86.2*	95.3/74.7*	100.0/88.2*	37.8/72.1*	52.5/91.1*	78.8/43.4*	73.2/78.2*
Ce	0.8/1.0	7.0/11.4*	6.5/8.2	0.3/1.2	0.0/5.6*	0.0/0.3	2.6/4.0*
Sm	42.2/13.4*	79.6/42.9*	84.3/45.4*	36.8/48.8*	26.7/46.0*	47.1/21.1*	53.4/28.9*
Te	98.5/66.9*	99.7/96.0*	100.0/92.9*	100.0/100.0	99.7/100.0	98.7/96.6	99.5/84.2*
Cl	37.9/4.7*	74.7/12.2*	90.6/10.3*	7.4/55.8*	9.4/56.5*	16.7/6.3*	40.8/13.2*
Na	0.0/0.0	0.0/0.3	0.5/1.5	0.3/0.0	0.0/3.2*	0.0/0.0	0.1/0.5
Ak	0.0/0.0	0.0/0.0	0.0/0.2	0.0/0.0	0.0/3.2*	0.0/0.0	0.0/0.2
Ka	26.3/5.7*	11.7/18.2*	43.7/3.8*	3.5/9.3*	3.5/29.8*	29.7/7.7*	19.8/8.9*
Tm	59.6/41.9*	36.9/43.8	39.4/56.5*	52.0/46.5	27.1/51.6*	17.1/37.4*	39.7/45.1*
Su	98.5/47.9*	76.8/50.0*	95.8/57.3*	48.8/64.5*	40.7/79.0*	42.3/44.6	68.7/52.2*
Ct	51.3/25.1*	5.5/30.7*	13.9/41.1*	3.7/26.7*	7.1/8.1	1.6/11.7*	14.7/26.3*

[a] Total numbers of isolates are in parentheses. *, values for fresh feces and the stored manure populations are significantly different.

TABLE 3. Monthly variation in the frequency of antibiotic resistance phenotypes in *E. coli*[a]

Resistance pattern	% of population (fresh feces/stored manure) with pattern in:					
	March	April	May	June	July	August
Am	0.0/14.0	0.0/0.0	0.0/1.9	0.0/0.0	0.0/0.0	0.3/0.0
Te	0.0/2.6	0.0/4.8	0.0/3.2	6.9/7.6	18.8/0.0	6.4/19.4
AmTe	0.3/13.1	2.1/7.7	0.5/10.9	4.0/1.7	14.1/3.2	15.7/7.4
TeSu	18.7/1.6	0.3/1.1	0.0/0.4	19.0/1.7	8.8/0.8	1.6/6.9
AmSmTe	0.0/1.4	1.6/9.7	0.0/5.9	0.3/1.2	3.5/0.0	11.2/1.1
AmSuTe	9.8/6.3	1.8/2.3	1.0/3.4	12.4/7.0	7.0/2.4	2.9/7.1
AmTeTm	0.3/7.8	1.8/2.8	0.0/4.4	4.0/1.7	8.2/5.6	5.1/7.7
SmTeTm	0.0/0.2	0.0/0.3	0.0/0.2	22.2/0.0	0.6/0.0	0.0/1.1
AmSmSuTe	0.5/2.3	6.0/2.6	2.1/3.2	1.6/4.1	3.5/0.8	3.5/4.3
AmClSmSuTe	1.3/1.2	32.9/0.6	28.7/1.7	0.5/5.2	4.4/8.1	5.1/1.1
AmCtSuTeTm	5.3/9.3	0.0/4.5	0.0/9.5	0.5/2.9	1.2/0.8	0.0/2.3
AmClSmSuTeTm	0.5/0.3	10.4/0.3	11.0/0.4	1.3/2.3	0.9/10.5	0.0/0.3
AmCtSmSuTeTm	5.8/1.7	0.0/5.4	0.0/18.5	0.0/2.3	2.9/0.0	0.3/2.0
AmClCtSmSuTeTm	15.4/0.2	3.1/1.1	2.1/2.1	0.3/8.1	0.6/0.8	0.0/0.3
Others[b]	42.2/38.1	39.9/56.8	54.6/34.5	27.0/54.1	25.5/66.9	47.8/38.9

[a] Sample sizes are identical to those used for Table 2.
[b] Aggregate of all isolates with phenotypes that were each detected in less than 2% of the total collection.

less coherent during the experiment. The lowest frequencies of resistance to the antibiotics in the holding tank populations were detected in March and August. Resistance to Ce, Na, and Ak remained low throughout the experiment, and Te resistance remained uniformly high. About half the isolates were resistant to Tm through the experiment. There were no consistent differences with respect to frequency of resistance in populations from the fresh feces and the holding tank. In the March-to-May period, in 17 (74%) of the 23 instances where there was a significant difference with respect to frequency of resistance to an antibiotic, it was higher in the fresh feces collection. In contrast, in the June-to-August period, in only 4 (19%) of the 21 instances of a significant difference was it higher in the fresh-feces collection.

One hundred eighty-eight resistance phenotypes representing combinations of resistance to up to nine antibiotics were found in the collection (Table 3). Eighty-seven distinct phenotypes (47% of the 188 resistance phenotypes) were detected in both the fresh feces and the stored manure. Only 22 phenotypes (12%) were found only in the fresh feces, and these represented only 35 isolates (0.75% of the collection). There were 79 (42%) profiles specific for the stored manure, representing 244 isolates (5.2% of all isolates). Fourteen (7.5%) phenotypes, each representing at least 2% of the total collection, accounted for 57% of the total collection. Very few isolates were resistant to no antibiotics or to more than eight antibiotics. There was no relationship between rep-PCR genotype and antibiotic resistance pattern: isolates from any one genotype had a wide variety of resistance phenotypes (data not shown). Trends in temporal variation were again highlighted by the June transition in the fresh feces population. Notably, in May, 28.7% of the population had the resistance phenotype AmClSmSuTe, and 11% were AmClSmSuTeTm. In June and thereafter, those phenotypes never represented more than 5.1% of the isolates. Phenotypes that were previously infrequently observed or undetected in the fresh feces population were obtained in June, namely, AmSuTe (12.4%), TeSu (19%), and SmTeTm (22.2%). In June, July, and August, the previously rare Te and AmTe phenotypes were prominent. There were no obvious trends or significant transitions in the holding tank population. The most consistently detected phenotypes in the holding tank were AmTe and AmTeTm.

DISCUSSION

It has been suggested that conditions outside the digestive tract can alter the genetic composition of *E. coli* populations once shed by the host, and there is some evidence to suggest that some *E. coli* strains may become adapted for survival in secondary habitats such as some soils and water (2, 7, 17, 24, 25, 51, 57). Were this to be the case in large-scale livestock manure slurry holding tanks, the population structure could be expected to be significantly skewed compared to the structures of populations shed by the animals, as less fit individuals perish and fitter genotypes become increasingly well represented. Swine manure slurry typically is at least 95% water, representing an approximately 50-fold dilution of freshly shed material. Recognizing that the holding tank is continuously inoculated with fresh material, and that the water content will vary with precipitation and with evaporation, there was significant attrition in the stored *E. coli* population, particularly in the warmer months. Throughout the experiment, the genotypes that dominated the *E. coli* population in freshly shed feces also dominated the community in the stored manure (Fig. 1). Fully 84% of all the isolates obtained from both the fresh feces and the holding tank shared common genotypes, and only 12% of the holding tank isolates had genotypes that were not detected in the fresh feces. Genotypes that were consistently detected in fresh feces (e.g., 16 and 33) were also similarly consistently present in the stored manure. Likewise, the same genotypes (e.g., 21 and 10) were sporadically and periodically detected in fresh feces and in the stored manure. The observed holding tank population was more diverse than the freshly shed population, except in the hot summer months of July and August (Table 1). The generally higher diversity in the holding tank is consistent with previous observations on this same farm, which had been investigated in the winter of 2003-2004 (32). Genotypes that were detected only in the manure (75 of them) could

be characteristic of isolates that have superior fitness in this secondary habitat, and likewise, genotypes (24 of them) detected only in the fresh feces could be characteristic of isolates with superior fitness in the primary habitat. These genotypes (all 99) were detected in only 16% of the total collection. Furthermore, none of the 99 genotypes that were detected in only one habitat or the other represented 2% or more of the overall collections. Taken together, these results indicate that the dominant genotypes were well represented in both habitats, suggesting that these did not have a particular fitness advantage in either.

Both the fresh feces and the holding tank collections exhibited important monthly variation in the population structure (Fig. 1). This was particularly evident with respect to the importance of genotypes that were poorly represented in the collection. For example, in the holding tank, the proportion of genotypes that were detected in less than 2% of the overall collection (aggregated as "other") steadily increased from March through to May and then gradually declined through to August. In the fresh feces, the "other" genotypes increased in frequency from March to May, were much less frequently observed in June, and then steadily increased through August. In contrast, the dynamics of the fresh feces population was the transient dominance of genotype 16 during the month of June, causing most of the underrepresented groups to drop below the detection level. The genetic composition of *E. coli* varies between individual animals, changes during the lifetime of the animal, and is influenced by feed composition (28, 43). We are unable to explain the May-to-June transition on the basis of any variation in husbandry (e.g., change in antibiotic regimen or feed composition), herd health (there were no clinical problems), herd composition (the proportions of animals of different ages and reproductive statuses were uniform throughout the study), or in-barn sanitation practices (e.g., an unusual use of disinfectant before sampling).

The manure holding tank was emptied during the week prior to the May sampling. The March and April samples represented waste that had accumulated since the previous autumn when the tank was last emptied, whereas in May and thereafter the material was much fresher. On this basis, we reasoned that the holding tank *E. coli* population in May and thereafter would more closely resemble the fresh feces population than in the previous months. This was not the case, with respect to either the population structure or the distribution of antibiotic resistance profiles. In fact, the tank is never completely emptied, and the sludge left at the bottom was undoubtedly carrying over an important preexisting population as the tank was subsequently refilled.

When the entire collection (fresh feces, $n = 2{,}193$, and holding tank, $n = 2{,}475$) was considered, there were no significant differences in the frequency of resistance to any antibiotic. However, when the collection was considered on the basis of specific rep-PCR-defined genotypes, in some cases there were significant differences in the frequency of antibiotic resistance. In 26 (21.5%) of 121 comparative observations (fresh feces versus holding tank; 11 antibiotics and 11 genotypes), the two populations differed in the frequency of resistance to an antibiotic. Of the 26 observations that showed significant differences, 21 (80.9%) revealed a lower frequency of resistance in the holding tank isolates than in the fresh-feces isolates. For example, the frequency of resistance to Cl and Sm was lower in the holding tank isolates of genotypes 16, 24, and 33 than in the fresh-feces isolates of these same genotypes (data not shown). However, the relative abundances of these three genotypes in the fresh feces and in the holding tank populations were similar (Fig. 1). Taken together, these results suggest that in some cases antibiotic resistance genes were lost in the holding tank, but this did not confer a selective advantage. Fitness in the holding tank was neutral with respect to resistance to any of the antibiotics; these attributes conferred neither a detectable advantage or disadvantage in this habitat.

Most of the antibiotics we evaluated for resistance were not used on this farm. The exceptions were oxytetracycline, which was briefly used during the experiment, and penicillin G, which was constantly administered to a portion of the herd and which could promote resistance to ampicillin. The frequency of ampicillin resistance measured on a monthly basis varied from 38% to 100% of the isolates. The very high frequency of Te resistance is consistent with what has been observed on other Ontario farms (6, 52). Overall, these results illustrate that there are factors beyond short-term on-farm antibiotic use that influence the frequency of antibiotic resistance and patterns of multiple antibiotic resistance in bacteria shed by livestock. In some cases, antibiotic resistance genes may be mobilized into environmental bacteria in soils receiving manure (18, 47). The role of environmental contamination from livestock wastes in promoting antibiotic resistance is difficult to evaluate against the background of the high frequency of resistance to antibiotics found in soil bacteria (12, 44, 45). Nevertheless, prudent use of antibiotics, particularly with respect to the chronic provision of growth-promoting agents and the use of antibiotics that are important for human and animal health, is advised (34).

The temporal flux of multiple antibiotic resistance phenotypes, particularly within the fresh feces collection, was striking, both in its tempo and in its temporal coherence (Table 3). There was no apparent relationship between resistance phenotype and rep-PCR-defined genotype. For example, during the course of the experiment, the AmSuTe phenotype was detected in organisms of 28 genotypes, the SmTeTm in 5, and the AmClSmSuTe in 28. Likewise, 65 distinct antibiotic resistance phenotypes were detected in genotype 16, 55 in genotype 33, 19 in genotype 27, and 44 in genotype 51. Clearly, variation in the frequency of various resistance phenotypes was not entirely due to the proliferation of distinct clonal populations that carried a specific complement of resistance genes. Rather, the results suggest that multiply resistant phenotypes varied in their nature and frequency of detection according to the accrual or the loss of resistance determinants. Conditions in the mammalian gastrointestinal tract are conducive to conjugal transfer of plasmid-borne antibiotic resistance genes (reviewed in reference 31). In our study, the frequencies of resistance to Cl, Su, Sm, Ka, and Am but not Te in the fresh feces collection all declined as of the June sampling. Linkage of these markers is consistent with what has previously been observed in swine isolates of *E. coli*, with chloramphenicol resistance being conferred by plasmid-borne *cmlA* (5). The *cmlA* gene was linked to *sul3* or *sul1* and to *aadA1* and *aadA2* in various configurations of class 1 integrons (5). Conjugative transfer of *cmlA* was accompanied by acquisition of resistance to Su, Te, and Ka.

The AmClSmSuTe phenotype has also been identified in *Salmonella* and been shown to be able to be transferred between serotypes (13, 41). Overall, the genetic elements underlying our observations remain to be determined, but the mechanisms underlying instability and horizontal transfer of antibiotic resistance genes in the porcine model are well established.

Both rep-PCR and antibiotic resistance profiling have been used to ascribe host source (e.g., human, livestock, wildlife) to environmental isolates of *E. coli* (20, 27, 35, 36, 38, 42, 50, 54). Typically, the host source is inferred on the basis of comparative analysis of the environmental isolates with a reference collection of *E. coli* strains obtained from the various potential fecal sources in the study area. One factor that will influence the accuracy of source identification is the temporal fidelity of the library with respect to the attributes being evaluated and compared. Results from this study suggest that rep-PCR fingerprints generated from a library constructed from a commercial swine manure storage facility would remain representative of the population structure over a period of at least several months. However, the frequency of resistance to specific antibiotics varied widely on a monthly basis, supporting previous findings that this temporal variability must be captured in the library construction (2, 4, 26, 58). These findings are consistent with the chromosomal location and apparent stability of repeated sequences detected by PCR with the BOXA1R primer and with the frequent association of antibiotic resistance determinants with potentially unstable plasmids, integrons, and transposons (1, 17, 37, 46, 56).

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