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Assessment of Phenotypic and Genotypic Diversity of *Escherichia coli* Shed by Healthy Lactating Dairy Cattle

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Abstract

A study was conducted to assess the diversity among fecal *Escherichia coli* from healthy lactating cattle. *E. coli* ($n = 100$) isolates from 10 healthy lactating dairy cows of a Pennsylvania dairy herd were examined for phenotypic and genotypic characteristics. Results revealed 26, 58, 10, and 6 *E. coli* isolates belonged to phylogenetic groups A, B1, B2, and D respectively. Overall, 63 serotypes, nine antibiotic resistance profiles, and 65 pulsed-field gel electrophoresis (PFGE) profiles were observed among the 100 isolates. Based on the combination of phenotypic and genotypic characteristics, the 100 *E. coli* isolates were classified into 76 clonal types. The numbers of different phenotypic and genotypic characteristics of *E. coli* were observed for each cow at ranges of 2–10, 1–4, 2–10, and 4–10 for serotypes, antibiograms, PFGE profiles, and clonal types, respectively. The Chao1 estimator was used to estimate diversity among fecal *E. coli*. It was estimated that a range of 3–55, 1–4, 2–55, and 8–55 fecal isolates from one cow would be required to include all possible types of *E. coli* based on serotype, antibiotic resistance profile, PFGE profile, and clonal type respectively. Based on the findings of the study it can be inferred that 1) dairy cattle should be considered as a significant reservoir of genotypically and phenotypically diverse *E. coli*, and 2) epidemiological investigations that focus on commensal bacteria should take into consideration the diversity within the species being investigated; if not addressed adequately, inappropriate sample size could lead to inaccurate study findings.

Introduction

The emergence of antibiotic resistance and pathotypes in *Escherichia coli* has raised considerable interest in understanding the diversity and epidemiology of *E. coli* infections in humans, animals, and their environment. The majority of studies reported thus far have focused on characterization of pathogenic *E. coli* isolated from ill humans and animals (Liebana *et al*., 2005; Akiba *et al*., 2000; Beutin *et al*., 1997; Mokady *et al*., 2005) while investigations on the diversity of the commensal bacterial flora of healthy humans and animals, including dairy cattle, are limited (Jarvis *et al*., 2000; Mokady *et al*., 2005; Nemeth *et al*., 1994). It has been suggested that commensal bacteria are able to protect the host from potential bacterial pathogen colonization of the gastrointestinal tract. While some pathogenic bacteria are not able to persist in the host because of competition with the commensal bacteria (van der Waaij, 1986), opportunistic pathogens capable of causing infection, including certain strains of *E. coli*, can be found among and classified as commensal flora when no signs of infection are present (Wray, 1986). Since adult ruminants have been identified as asymptomatic carriers of both pathogenic

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and nonpathogenic strains of *E. coli* it is very important that the *E. coli* population of the healthy bovine gut be investigated (Mainil and Daube, 2005).

In recent years, commensal bacteria have received considerable attention as potential reservoirs of antibiotic determinants and virulence factors. There are reports that suggest that monitoring commensal bacteria for antibiotic resistance would provide the early warning signs of emergence of antibiotic resistance in a given environment (Molbak, 2004; Sawant *et al*., 2007). In order to establish a rationale for the number of isolates needed to undertake monitoring for antibiotic resistance and pathotypes of bovine commensal *E. coli*, a study was conducted to estimate the diversity of *E. coli* populations harbored by healthy dairy cattle. The objectives of this study were to 1) explore the phenotypic and genotypic diversity of *E. coli* randomly isolated from the normal flora of healthy dairy cattle without the use of antibiotics for laboratory selection, and 2) estimate the proper sample size for future studies on *E. coli* shed by healthy dairy cattle.

Materials and Methods

Lactating cows

Ten cows were randomly selected from a Pennsylvania dairy to be included in the study. One cow included in the study was in early first lactation (73 days in milk), six cows (four, one, and one cows in first, second, and fifth lactations respectively) were in midlactation (120–190 days in milk), and three cows were in late first lactation (>400 days in milk). Cows were kept on pasture at the time of study were fed in the same tie stall barn potentially allowing for close contact of animals. Cows were fed corn silage and allowed to graze on grass.

According to farm treatment records, seven of the cows had not received any antibiotic treatment for at least 3 months prior to the sampling done for this study. Cow 304 was treated for mastitis with pirlimycin (one tube Pirsue, Pfizer Animal Health, Kalamazoo, MI) by intramuscular (IM) injection once a day for 3 days 1 month prior to being sampled. Cow 412 received the same treatment for mastitis 2 months prior to being sampled. Cow 466 was treated for metritis with 35 mL of IM penicillin once a day for 3 days 3 months prior to being sampled.

Sample collection

Five fecal samples were collected rectally in July 2004 from five lactating dairy cows (cow IDs 304, 440, 456, 468, and 474) described above. Five more fecal samples were collected from five different cows (cow IDs 412, 449, 454, 466, and 471) in August 2004 for a total of 10 fecal samples. All samples were transported to the lab on ice and were cultured on the same day they were collected.

One gram of each fecal sample was diluted in 9 mL of 0.85% sterile saline solution. Samples were then 10-fold serially diluted to 10^{-4}. Dilutions 10^{-4} and 10^{-3} were each plated on MacConkey's agar (MAC) (Oxoid, Ltd., Basingstoke, Hampshire, England, UK) and incubated at 37°C for 24 hours. For each sample, 10 to 15 isolated colonies ($n = 100$) with morphology characteristic of *E. coli* were subcultured and identified using API 20 E kits (bioMérieux, St. Louis, MO). The first 10 isolates from each cow showing a positive identity for *E. coli* (isolates identified as other species were discarded) were subcultured and stored in glycerol. These 100 isolates were later examined for their phenotypic and genotypic characteristics.

Phenotypic analysis

Serotypes and antibiograms were examined to determine phenotypic diversity. All isolates were serotyped by the Gastroenteric Disease Center at Pennsylvania State University, University Park, PA. O-groups were determined using procedures described by Orskov *et al*. (1977). H-types were determined based on restriction fragment length polymorphism analysis as described by Machado *et al*. (2000). Antibiograms were determined using the Kirby–Bauer disk diffusion assay in which all isolates were tested for resistance to 10 antimicrobials of veterinary significance including ampicillin (Amp) (10 μg/mL), chloramphenicol (Chl) (30 μg/mL), gentamicin (Gen) (10 μg/mL), enrofloxacin (Eno) (5 μg/mL), tetracycline (Tet) (30 μg/mL), spectinomycin (Spt) (100 μg/mL), ticarcillin (Tic) (75 μg/mL), ticarcillin/clavulanic acid (Tim) (75/10 μg/mL) (Remel; Lenexa, KS),

ceftiofur (Xnl) (30 μg/mL), and florfenicol (Ffc) (30 μg/mL) (Becton, Dickinson and Company; Sparks, MD). An *E. coli* reference strain (ATCC 25922) was used for the purpose of quality control as specified by the Clinical and Laboratory Standards Institute (CLSI). Antibiotic resistance or susceptibility was determined using the interpretive criteria defined by CLSI (previously National Committee for Clinical and Laboratory Standards [NCCLS], 2002). Isolates with zone diameters falling in the intermediate range were considered sensitive for the purposes of this study.

Genotypic analysis

Clermont *et al*. (2000) have identified genes and DNA fragments that can be used as markers for categorizing *E. coli* into four phylogenetic groups. They developed a polymerase chain reaction (PCR) assay for phylogenetic grouping of *E. coli* using *chuA* and *yjaA* genes and an anonymous DNA fragment, TspE4C2. The *E. coli* isolates ($n = 100$) were examined for their phylogenetic groups using this technique as described by Clermont *et al*. (2000). Isolates negative for *chuA* and TspE4C2 were designated phylogenetic group A. Isolates negative for *chuA* and positive for TspE4C2 were designated phylogenic group B1. Isolates positive for *chuA* and *yjaA* were designated phylogenetic group B2. Isolates positive for *chuA* and negative for *yjaA* were designated phylogenetic group D. All isolates were also screened for the presence of Shiga toxin 1 (stx_1) and Shiga toxin 2 (stx_2) genes as described by Meng *et al*. (1997).

Protocol for pulsed-field gel electrophoresis (PFGE) was carried out as previously described by Hegde *et al*. (2005). Briefly, a loopful of colony was suspended in 500 μL cell suspension buffer. Two hundred microliters of this solution was transferred to a 1.5-mL tube and 10 μL of proteinase K (20 mg/mL) was added along with 200 μL of 1% SeaKem Gold agarose (Cambrex, Rockland, ME) which was kept at 55°C. Two 200-μL aliquots of this mixture were transferred into plug molds and allowed to set for approximately 15 minutes. Plugs were then transferred to 5 mL cell lysis buffer containing 40 μL proteinase K (20 mg/mL) and incubated at 55°C for 45 minutes. The plugs were washed three times (15 minutes per wash) at 50°C and stored in plug wash buffer at 4°C until digestion. Plugs were digested using *Xba*1 restriction enzyme (New England Bio Labs, Beverley, MA) and run on a gel subjected to a pulsed field using the CHEF-mapper XA PFGE system (Bio-Rad, Hercules, CA). Fingerprint subtypes were analyzed using Gel Doc 2000 Molecular Analyst Fingerprinting Plus software, version 6.1 (Bio-Rad). The relatedness of restriction profiles was generated by the unweighted pair group method with arithmetic clusters based on Dice coefficients of each band pattern.

Clonal types

Isolates were sorted into clonal types based on phylogenetic group, PFGE profile, serotype, and antibiotic resistance profile. For the purposes of this study, the criteria used was that isolates must possess the exact same phenotypic and genotypic characteristics in order to be grouped into the same clonal type.

Quantification of diversity

To assess diversity among *E. coli* isolates shed by healthy lactating dairy cattle we examined differences in serotypes, PFGE, and antibiotic resistance profiles. Operational taxonomic units (OTU) refer to a grouping of interest based on a certain parameter such as species or subspecies. In this study we defined an OTU as a phenotypically or genotypically distinct *E. coli* isolate. Isolates exhibiting different serotypes and/or antibiotic resistance profiles were considered phenotypically distinct. We considered an *E. coli* isolate genotypically distinct when its PFGE profile showed a Dice coefficient $< 95\%$ when compared to PFGE profiles of other isolates as described by Krause *et al*. (1996). Overall diversity was assessed by examining differences in clonal types.

Diversity, or OTU richness, of *E. coli* shed by healthy lactating cows was estimated using the Chao estimator (Chao1) (Chao, 2004; Hughes *et al*., 2001, 2004). Chao1 is a nonparametric method used to estimate the number of OTUs in a microbial population. From a sample population, Chao1 estimates the number of missing OTUs that were not observed in the sample based on the number of rare OTUs observed

and therefore estimates the total number of OTUs in a population. This estimate can be used to determine the approximate sample size needed to account for all OTUs in a population. EstimateS version 7.5.1 software (Colwell, 2006) was used to calculate Chao1 values.

Results

Phenotypic characteristics

Serotyping results showed a high degree of variability among the *E. coli* isolated in this study (Table 1). A total of 63 serotypes comprising 38 O-groups (10 serotypes contain nontypeable O antigens) and 32 H-types were observed. Among these 63 serotypes, only 20 (32%) were observed in more than one isolate. The number of serotypes observed among *E. coli* isolated from each cow ranged from 2 to 10. The most predominant serotypes found in our study were O161:H7, O98:H49, and O15:H7 shared by six, six, and five isolates from three, one, and two cows respectively. Ninety percent of cows harbored *E. coli* isolates that were of six or more serotypes.

E. coli isolates were grouped into nine different antibiogram profiles which ranged from susceptibility to all 10 antibiotics to resistance to 8 out of 10 antibiotics (Table 1). The number of antibiograms observed among *E. coli* isolated from each cow ranged from one to four. All isolates from cows 466 and 468 along with the majority of isolates from cows 412, 449, 454, 456, and 474 (a total of 62 isolates) were susceptible to all antibiotics used in this study. Resistance only to Amp was the most frequently observed antibiogram (21 isolates from seven cows). The second most predominant antibiogram (10 isolates from two cows) consisted of isolates resistant to Amp, Chl, Ffc, Spt, Tet, Tic, Tim, and Xnl.

Genotypic characteristics

It was observed that 26, 58, 10, and 6 *E. coli* isolates belonged to phylogenetic groups A, B1, B2, and D, respectively (Table 1). *E. coli* belonging to phylogenetic groups A and B1 were carried by all 10 cows in the study. Two cows harbored *E. coli* belonging to phylogenetic group B2 while four cows harbored *E. coli* belonging to phylogenetic group D. Two of the 100 *E. coli* isolates were positive for Shiga toxin genes; one belonging to phylogenetic group A (positive for *stx1* and *stx2*) and one belonging to group B1 (positive only for *stx1*).

Sixty-five PFGE patterns were observed among 99 isolates (PFGE pattern could not be resolved for one isolate) included in this study (Fig. 1). Among these, 52 (80%) PFGE patterns were observed in only one *E. coli* isolate each (Table 1). The number of PFGE patterns observed among *E. coli* isolated from each cow ranged from 2 to 10. Nine out of ten of cows harbored *E. coli* isolates belonging to one of six or more PFGE profiles. Only 13 PFGE profiles were observed for more than one isolate.

Clonal types

Serotype, antibiogram, phylogenetic group, and PFGE profile were combined to assign each *E. coli* isolate to a clonal type. Based on this data set, the 100 isolates belonged to 76 clonal types (Table 1). The number of clonal types observed among *E. coli* isolated from each cow ranged from 4 to 10. Sixty-four (84%) clonal types were restricted to only one isolate. Of the 12 clonal types containing more than one isolate, only three clonal types (CT1, CT34, and CT41) were isolated from more than one cow.

Quantification of diversity

EstimateS version 7.5.1 software yielded Chao1 values of 145 and 9 for serotype and antibiogram data respectively (Table 2). This suggests, based on the samples size used in this study, that among the *E. coli* population it is estimated that there are 145 different serotypes and nine different antibiograms being shed by the 10 cows included in this study. Chao1 values for individual cows ranged from 3 to 55 and from 1 to 4 for serotype and antibiogram, respectively.

Krause *et al*. (1996) reported that PFGE profiles of isolates with a Dice coefficient $< 95\%$ were genotypically distinct. The two most closely related PFGE profiles observed in our study had a 78% Dice similarity coefficient. In our study, therefore, we considered each PFGE profile observed to be a distinct OTU. For our data, EstimateS version 7.5.1 software calculated

Table 1. Distribution of Phenotypic and Genotypic Characteristics Among *E. coli* Isolated from Healthy Lactating Dairy Cattle

Cow	*Clonal type*[a]	*Phylogenetic group*	*PFGE profile*	*Antibiogram*[b]	*Serotype*
304[c]	CT1	A	EC1	1	O161:H7
	CT2	A	EC5	1	O20:H30
	CT3	A	EC6	1	O127:H26
	CT21 (3)	B1	EC2	2	O8:H49
	CT22	B1	EC3	2	O96:H19
	CT23 (3)	B1	EC4	2	Ont:H36
Total	6	2	6	2	6
412[d]	CT1 (3)	A	EC1	1	O161:H7
	CT4	A	EC7	8	O13:H30
	CT5	A	EC11	7	O68:H12
	CT25	B1	EC8	1	Ont:H49
	CT26	B1	EC9	1	O6:H49
	CT27	B1	EC10	1	O161:H7
	CT29	B1	EC12	2	O147:H36
	CT71	D	EC13	2	O7:H15
Total	8	3	8	4	8
440[e]	CT6	A	EC15	3	O98:H23
	CT30 (6)	B1	EC14	3	O98:H49
	CT31 (2)	B1	EC14	3	O98:H23
	CT32	B1	EC14	4	O98:H23
Total	4	2	2	2	2
449[e]	CT7	A	EC20	1	Ont:H26
	CT8	A	EC23	1	O153:H25
	CT9	A	EC25	9	Ont:H16
	CT33	B1	EC16	1	O172:H36
	CT34	B1	EC18	1	O73:H27
	CT35	B1	EC22	1	O8:H19
	CT61	B2	EC17	2	O80:H45
	CT62	B2	EC19	1	O9:H12
	CT63	B2	EC21	1	O69:H−
	CT64	B2	EC24	1	O2:H5
Total	10	2	10	3	10
454[e]	CT10	A	EC28	2	O66:H9
	CT36	B1	EC26	2	O13:H2
	CT37	B1	EC29	1	O13:H11
	CT38	B1	EC33	6	O85:H42
	CT65	B2	EC27	1	O11:H25
	CT66	B2	EC30	1	O26:H+
	CT67	B2	EC31	1	O11:H36
	CT68	B2	EC31	1	O11:H25
	CT69	B2	EC32	1	O7:H48
	CT70	B2	EC34	1	O161:H26
Total	10	3	9	3	9
456[f]	CT11	A	EC39	1	O142:H34
	CT39 (3)	B1	EC36	1	O15:H7
	CT40	B1	EC37	2	O175:H16
	CT41	B1	EC37	2	O139:H19
	CT44	B1	EC38	1	O3:H36
	CT60	B1	—	3	O8:H19
	CT72	D	EC35	1	O7:H48
	CT73	D	EC36	1	O15:H7
Total	8	3	5	3	7

(*continued*)

TABLE 1. (*CONTINUED*)

Cow	*Clonal type*[a]	*Phylogenetic group*	*PFGE profile*	*Antibiogram*[b]	*Serotype*
466[g]	CT1	A	EC1	1	O161:H7
	CT12	A	EC40	1	O109:H27
	CT13	A	EC41	1	Ont:H32
	CT34	B1	EC18	1	O73:H27
	CT42 (2)[h]	B1	EC38	1	Ont:H42
	CT43	B1	EC38	1	Ont:H30
	CT45 (2)	B1	EC39	1	O76:H19
	CT46	B1	EC42	1	O171:H14
Total	8	2	7	1	8
468[f]	CT14[i]	A	EC43	1	O142:H34
	CT15	A	EC45	1	O127:H44
	CT16	A	EC48	1	O8:H30
	CT17	A	EC49	1	O134:H52
	CT18	A	EC50	1	O15:H7
	CT28	B1	EC12	1	O147:H36
	CT47	B1	EC46	1	O8:H19
	CT48	B1	EC47	1	O168:H38
	CT49	B1	EC51	1	O76:H19
	CT74	D	EC44	1	Ont:H28
Total	10	3	10	1	10
471[d]	CT19	A	EC53	2	O5:H30
	CT50	B1	EC52	5	O85:H42
	CT51	B1	EC54	2	O2:H8
	CT52	B1	EC55	6	O9:H48
	CT53	B1	EC56	2	O13:H2
	CT54	B1	EC57	1	O171:H14
	CT55	B1	EC58	1	O35:H2
	CT56	B1	EC59	1	O2:H8
	CT57	B1	EC60	2	O8:H7
	CT75	D	EC61	1	O120:H48
Total	10	3	10	4	9
474[j]	CT20 (3)	A	EC63	1	O107:H46
	CT24	B1	EC4	2	Ont:H21
	CT41	B1	EC37	2	O139:H19
	CT58 (3)	B1	EC62	1	Ont:H8
	CT59	B1	EC65	2	O64:H1
	CT76	D	EC64	1	O11:H25
Total	6	3	6	2	6

PFGE, pulsed-field gel electrophoresis; Amp, ampicillin; Chl, chloramphenicol; Gen, gentamicin; Eno, enrofloxacin; Tet, tetracycline; Spt, spectinomycin; Tic, ticarcillin; Tim, ticarcillin/clavulanic acid; Xnl, ceftiofur; Ffc, florfenicol.

[a]Number in parenthesis refers to number of isolates belonging to clonal type if $n > 1$.

[b]Number refers to antibiotic resistance pattern as follows: 1, no resistance; 2, Amp; 3, Amp-Chl-Ffc-Spt-Tet-Tic-Tim-Xnl; 4, Amp-Chl-Ffc-Spt-Tet-Tic-Xnl; 5, Amp-Gen; 6, Amp-Ffc; 7, Amp-Tet-Tic; 8, Gen-Tet; 9, Spt-Tet.

[c]Mid fifth lactation, pirlimycin treatment 1 month before sampling.

[d]Mid second lactation, pirlimycin treatment 1 month before sampling.

[e]Late first lactation.

[f]Mid first lactation.

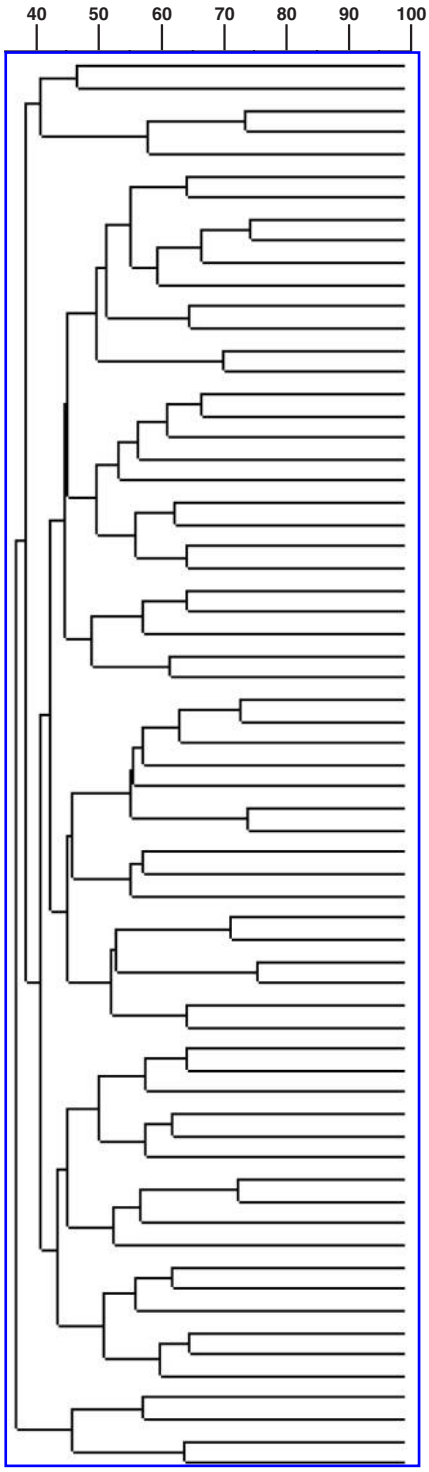
[g]Mid first lactation, penicillin treatment 2 months before sampling.

[h]One of these isolates tested positive for *stx1*.

[i]Isolate tested positive for *stx1* and *stx2*.

[j]Early first lactation.

Chao1 to be 403 PFGE profile OTUs for the entire 99 isolate sample set. This means it is estimated that among the *E. coli* populations shed by the 10 cows included in this study there are about 403 different PFGE patterns present based on the 65 PFGE profiles observed in our 99 isolate sample set. Estimates for the number of different PFGE profiles among *E. coli* populations



Cows	N	PFGE	Serotype	Ab	PG
304	1	EC5	O20:H30	1	A
440	1	EC15	O98:H23	3	A
466	1	EC41	Ont:H32	1	A
468	1	EC44	Ont:H28	1	D
449	1	EC16	O172:H36	1	B1
449	1	EC20	Ont:H26	1	A
449	1	EC25	Ont:H16	9	A
449	1	EC23	O153:H25	1	A
412	1	EC10	O161:H7	1	B1
440	9	EC14	O98:H49 (6), O98:H23 (3)	3(8), 4	B1
454	1	EC45	O127:H44	1	A
412	1	EC8	Ont:H49	1	B1
412	1	EC9	O6:H49	1	B1
471	1	EC61	O120:H48	1	D
474	3	EC63	O107:H46	1	A
468	1	EC47	O168:H38	1	B1
449	1	EC24	O2:H5	1	B2
454	1	EC26	O13:H2	2	B1
304, 474	4	EC4	Ont:H36 (3), Ont:H21	2	B1
454	1	EC27	O11:H25	1	B2
454	1	EC29	O13:H11	1	B1
456, 466	3	EC39	O76:19(2), O142:H34	1	A, B1(2)
466	1	EC40	O109:H27	1	A
449	1	EC21	O69:H-	1	B2
456, 466	4	EC38	Ont:H42(2), Ont:H30	1	B1
471	1	EC55	O9:H48	6	B1
454	1	EC32	O7:H48	1	B2
466	1	EC42	O171:H14	1	B1
412	1	EC7	O13:H30	8	A
454	1	EC33	O85:H42	6	B1
474	3	EC62	Ont:H8	1	B1
471	1	EC60	O8:H7	2	B1
474	1	EC65	O64:H1	2	B1
468	1	EC51	O76:H19	1	B1
412	2	EC12	O147:H36	8	B1
471	1	EC52	O85:H42	5	B1
468	1	EC49	O134:H52	1	A
468	1	EC50	O15:H7	1	A
471	1	EC54	O2:H8	2	B1
468	1	EC43	O142:H34	1	A
304, 412, 466	5	EC1	O161:H7	1	A
468	1	EC46	O8:H19	1	B1
468	1	EC48	O8:H30	1	A
304	3	EC2	O8:H49	2	B1
304	1	EC3	O96:H19	2	B1
449	1	EC17	O80:H45	2	B2
412	1	EC11	O68:H12	7	A
471	1	EC56	O13:H2	2	B1
454	2	EC31	O11:H36, O11:H25	1	B2
456	1	EC35	O7:H48	1	D
304	1	EC6	O127:H26	1	A
449	1	EC19	O9:H12	1	B2
474	1	EC64	O11:H25	1	D
412	1	EC13	O7:H15	2	D
449	1	EC22	O8:H19	1	B1
454	1	EC30	O26:H+	1	B2
456, 474	3	EC37	O175:H16, O139:H19(2)	2	B1
456	4	EC36	O15:H7	1	B1(3), D
454	1	EC34	O161:H26	1	B2
471	1	EC59	O2:H8	1	B1
454	1	EC28	O66:H9	2	A
471	1	EC57	O171:H14	1	B1
471	1	EC58	O35:H2	1	B1
412	2	EC18	O73:H27	7	B1
471	1	EC53	O5:H30	2	A

FIG. 1. Dendrogram of PFGE profiles from *E. coli* isolates shed by dairy cattle.

in individual cows showed a range in Chao1 values of 2 to 55 (Table 2).

The Chao1 value for clonal types shed by all cows in the study was calculated with and without genotypic data to illustrate the impact of genotype on clonal diversity (Table 2). Chao1 analysis estimates that 412 clonal types are being shed by the 10 cows included in this study when genotype is included in clonal types. When only phenotypes are included in clonal

Table 2. Chao1 Estimator[a] Values for *E. coli* Diversity Measured by Phenotypic and Genotypic Characteristics

				Clonal type[b]	
Cow	*PFGE*	*Antibiogram*	*Serotype*	*A*	*B*
304	12	2	12	12	12
412	29	4	12	22	29
440	2	2	3	3	8
449	55	4	55	55	55
454	23	3	23	23	55
456	7	4	22	22	22
466	12	1	13	13	13
468	55	1	55	55	55
471	55	4	23	55	55
474	12	2	12	12	12
All 10 cows	403	9	145	158	412

PFGE, pulsed-field gel electrophoresis.

[a]Chao1 is an estimate of OTUs found in a population based on the sample observations. Therefore it is estimated that there are 12 different PFGE profiles among *E. coli* isolates shed by cow 304 based on the six different PFGE profiles that were observed in 10 *E. coli* clones isolated from this cow and so on.

[b]A, clonal type including phenotypic characteristic only; B, clonal type including both phenotypic and genotypic characteristics.

type, Chao1 estimates that only 158 clonal types are being shed by all 10 cows. The Chao1 estimates for individual cows range from 8 to 55 and 3 to 55 for clonal types including genotype and clonal types only including phenotype, respectively.

Discussion

Phenotypic and genotypic characteristics

Of the 100 *E. coli* isolates characterized in this study 26, 58, 10, and 6 isolates belonged to phylogenetic groups A, B1, B2, and D respectively. Previous research has suggested that commensal and diarrheogenic *E. coli* strains mainly belong to groups A and B1, while most extraintestinal pathogenic strains usually belong to groups B2 or D (Clermont *et al*., 2000; Escobar-Páramo *et al*., 2004; Girardeau *et al*., 2005). Therefore, 84% of *E. coli* isolated in this study may be considered commensal or diarrheogenic strains as expected while 16%, despite being isolated from fecal samples, belonged to phylogenetic groups usually associated with extraintestinal pathogenic strains.

We observed 63 serotypes (38 O-groups and 32 H-types) among the 100 *E. coli* isolates included in this study. These results show more variability than found in a previous study by Bettelheim *et al*. (2005) who observed 52 different serotypes containing only 27 O-groups and 19 H-types among 474 *E. coli* isolates from 30 cows. Seventy-six percent ($n = 29$) of the O-groups observed in this study have been associated with human infections (Sussman, 1997). Our study found 27, 25, and 2 isolates with O-groups associated with enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), and enteroinvasive *E. coli* (EIEC) infections in humans respectively. According to Escobar-Páramo *et al*. (2004), ETEC strains are not found in phylogenetic groups B2 and D. Our data is in disagreement with these findings in that 22% (6 out of 27) of isolates included in this study with serotypes associated with ETEC belonged to groups B2 or D. We found that isolates with serotypes associates with EPEC strains were found in all four phylogeneic groups while those with serotypes associated with EIEC strains were only found in group B1. These findings agree with those reported by Escobar-Páramo *et al*. (2004). Twenty-one *E. coli* isolates belonged to O-groups that have been associated with human extraintestinal infections including urinary tract infections, neonatal meningitis, and septicemia. Escobar-Páramo *et al*. (2004) report that extraintestinal pathogenic *E. coli* are only found in phylogenetic groups B2 and D, while our data shows these strains are distributed throughout all four groups. Both of the isolates that tested positive for Shiga toxin genes belonged to phylogenetic group B1, which is in agreement with previous findings (Escobar-Páramo *et al*., 2004)

Antimicrobial resistance can occur as a result of directed or random genetic mutations in bacterial DNA, leading to variation in susceptibility within any bacterial population. Resistance is mainly due to the presence of extrachromosomal DNA, in the form of transposons and plasmids acquired from other bacteria (Catry *et al*., 2003). There are growing concerns about the acquisition of antimicrobial resistance by normal flora of the bovine intestine because dairy cattle may serve as a reservoir for antibiotic resistance that may be transferred to pathogenic organisms (Hoyle *et al*., 2004). In our study, 62% of the *E. coli* isolated from healthy lactating dairy cows were susceptible to all antibiotics used in the study, while 21% were resistant only to Amp (Table 1). Among the *E. coli*

strains resistant to more than one antibiotic isolated in this study, 11% were resistant to seven or more antibiotics, while 6% were resistant to two to three antibiotics.

Among isolates resistant only to Amp, 8 out of 21 (38%) belong to O-groups associated with *E. coli* strains that are pathogenic to humans, while two out of six (33%) isolates resistant to two to three antibiotics belong to O-groups associated with human infections. One *E. coli* isolate (CT60) resistant to 8 out of the 10 antibiotics tested in this study belonged to O-group 8 which has been associated with EPEC, ETEC, and urinary tract infections as well as septicemia. These results suggest dairy cattle may serve as a reservoir for multidrug-resistant pathogenic *E. coli*.

The greatest amount of diversity observed among *E. coli* isolates shed by healthy lactating dairy cattle was revealed in the PFGE data. Only 13 PFGE profiles were observed more than once. Among these profiles, phylogenetic groups were highly conserved. Isolates that shared the same PFGE profile also shared the same phylogenetic group except for one observation. For the three isolates that shared PFGE profile EC39, two belonged to phylogenetic group B1 while the other belonged to group A. Serotypes were conserved in 7 out of 13 PFGE profiles which were shared by more than one isolate.

Quantification of diversity

There are several different methods that may be used to estimate OTU richness in a given population (Chao, 2004; Hughes *et al*., 2001, 2004). In the past, these methods have been used by ecologists to examine plant and animal diversity in a given environment, although these methods are also applicable to examining microbial diversity (Hughes and Bohannan, 2004). All of these methods have certain advantages and limitations. In order to choose a method for estimating diversity which will yield the most accurate estimations researchers must have an understanding of OTU abundance (Chazdon *et al*., 1998). Since the diversity of *E. coli* in the bovine gut is not well characterized we chose methods based on previous statistical research. Smith and van Belle (1984) report that although the jackknife and boot strap methods have been shown to reduce bias, they tend to underestimate diversity when there is a large number of rare observations in the sample population. The abundance-based coverage estimator (ACE) separates the frequencies of abundant ($10 <$ individuals) and rare ($10 \geq$ individuals) observed in a sample to determine the diversity of a population. The diversity estimate calculated using ACE is based on the frequencies of rare individuals, therefore, the estimate heavily depends on the accuracy of these frequencies.

For the purposes of this study we chose to use the Chao1 estimator to estimate diversity. This method estimates diversity based on the number of OTUs observed only once (singletons) and the number of OTUs observed twice (doubletons) (Chao, 2004; Colwell and Coddington, 1994). In samples where no doubletons are observed a bias-corrected version of the Chao1 estimator was used (Chao, 2004). Although this estimator is a lower bound and may be an underestimation of diversity, it has been suggested that this method works very well in highly diverse populations (Chao, 1984; Colwell and Coddington, 1994).

Based on the Chao1 value calculated for serotype data, only 43% of the serotypes found among the *E. coli* population shed by healthy dairy cattle were accounted for. This means there may be other potential serotypes in the bovine gut that were not found in this study. The Chao1 value calculated for the antibiotic resistance profile data was 9 suggestive of approximately nine different antibiograms being shed by the 10 cows included in this study. Nine different antibiotic resistance profiles were observed in this study indicative of the fact that the sample size used was sufficient to account for antibiogram diversity of the *E. coli* population shed by the 10 healthy lactating dairy cattle included in the study.

We observed 65 distinct PFGE patterns in this study, 80% of these profiles were seen in only one isolate. Band differences among *E. coli* isolates are usually due to point mutations which create or delete chromosomal restriction sites resulting in differing numbers of bands in a PFGE profile (Tenover *et al*., 1995). Akiba *et al*. (2000) reported that mutations such as these can occur in the host in as little as 24 hours postinoculation. The Chao1 value calculated for PFGE profile data was 403 suggesting among *E. coli*

being shed by the 10 cows included in this study there are potentially 403 different PFGE profiles present.

Due to the degree of variability in PFGE profiles in our data set and previous observations of changes in PFGE profiles over short periods of time (Akiba *et al*., 2000), it is predicted that including genotypic data in clonal type analysis may result in an overestimation of diversity. In order to investigate this possibility, Chao1 values were calculated for clonal types including and excluding genotype. It was observed that when estimating the diversity of *E. coli* shed by the 10 cows included in this study, the Chao1 value more than doubled when genotype was included in clonal type as opposed to when the genotype data was excluded. This effect was seen in only three individuals (cows 412, 440, and 454) when estimating the diversity of *E. coli* shed by individual cows, suggesting that high genotypic diversity of isolates shed by these cows skewed the overall diversity estimate for total isolates. For the other seven cows, the exclusion of genotypic data from clonal type did not make a difference in diversity estimates.

Conclusions

It is important to recognize that in the span of this study we were not able to generate data that can fully describe the dynamics of the *E. coli* population in the fecal flora of dairy cattle. The effects of environmental perturbations (diet, antibiotics, or pathogens) on the normal gut microbiota can have a strong influence on the clonal types that are present at a given time of sampling. There is a constant turnover of bacterial species present in the gut flora. Even among species that are consistently present in the gut flora, many phenotypic and genotypic changes are occurring through gene transfer and genetic mutations in such a way that has allowed the rapid evolution of certain bacterial species. Our research provides a snapshot of the *E. coli* strains present in the gut flora at one point in time. We feel that our data will allow other researchers that plan to study commensal gut microflora a rationale for addressing issues related to diversity of *E. coli* in the gut of healthy lactating dairy cattle. Our results confirm that healthy lactating dairy cattle are a significant reservoir for a diverse array of nonpathogenic strains of *E. coli*. All of the cows included in this study were observed to be harboring four or more different strains of *E. coli*, though data analysis estimates it is likely that this number would be considerably higher if a larger sample of the *E. coli* population was examined. Also, our study only included isolates from cows on one farm. We can assume that there would be an even greater diversity observed among *E. coli* harbored by cows from different farms because of differences in diet and environmental factors which can impact fecal flora (Jarvis *et al*., 2000). Based on the results of this study we can conclude that 1) *E. coli* shed by dairy cattle are extremely diverse due to many different genetic profiles and serotypes while antibiotic resistance profiles are much less variable, and 2) due to genetic and phenotypic diversity, it is estimated that a sample size of > 55 isolates per cow is needed to properly represent the *E. coli* population shed by healthy dairy cattle in future studies.

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