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A newly developed *Escherichia coli* isolate panel from a cross section of U.S. animal production systems reveals geographic and commodity-based differences in antibiotic resistance gene carriage

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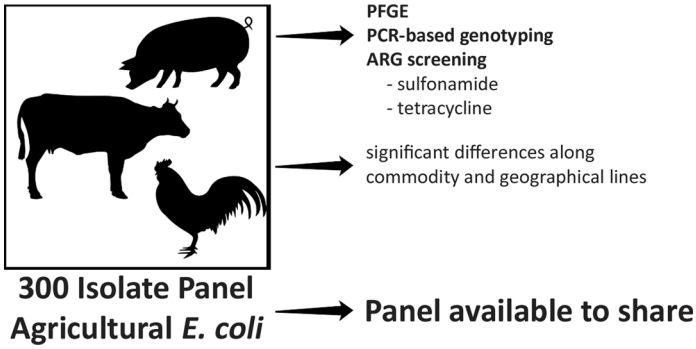
GRAPHICAL ABSTRACT



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ABSTRACT

There are limited numbers of *Escherichia coli* isolate panels that represent United States food animal production. The majority of existing *Escherichia coli* isolate panels are typically designed: (i) to optimize genetic and/or phenotypic diversity; or (ii) focus on human isolates. To address this shortfall in agriculturally-related resources, we have assembled a publicly-available isolate panel (AgEc) from the four major animal production commodities

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Animal production
Isolate panel

in the United States, including beef, dairy, poultry, and swine, as well as isolates from agriculturally-impacted environments, and other commodity groups. Diversity analyses by phylotyping and Pulsed-field Gel Electrophoresis revealed a highly diverse composition, with the 300 isolates clustered into 71 PFGE sub-types based upon an 80% similarity cutoff. To demonstrate the panel's utility, tetracycline and sulfonamide resistance genes were assayed, which identified 131 isolates harboring genes involved in tetracycline resistance, and 41 isolates containing sulfonamide resistance genes. There was strong overlap in the two pools of isolates, 38 of the 41 isolates harboring sulfonamide resistance genes also contained tetracycline resistance genes. Analysis of antimicrobial resistance gene patterns revealed significant differences along commodity and geographical lines. This panel therefore provides the research community an *E. coli* isolate panel for study of issues pertinent to U.S. food animal production.

1. Introduction

Species-specific bacterial isolate collections are a valuable resource for the scientific community, able to provide insight into genotypic and phenotypic variation within the target species. These panels are useful for studying population genetics and molecular evolution (Wiehlmann et al., 2007), and in the case of pathogens, can be used for developing highly-discriminatory molecular sub-typing methods for use in epidemiological investigation, outbreak detection, and source-tracking (Ducey et al., 2007). Such panels can also be used to examine host adaptation (Guard et al., 2016); determine rates of pathogenicity and antimicrobial resistance (AMR) (Trembizki et al., 2016); as well as determine other traits that may be of relevance (Price et al., 2012; Murphy et al., 2019). Multiple isolate collections exist for *Escherichia coli*, with the ECOR collection being the most identifiable (Ochman and Selander, 1984), and while multiple project-specific *E. coli* isolate libraries have been generated to answer region-specific questions relevant to animal production (Kozak et al., 2009a; Stocki et al., 2002; Apun et al., 2006), few have been designed as a resource for the broader scientific community. While there is great interest in issues of food safety and environmental quality related to agricultural production, limited access to working farms and commercial production facilities poses a barrier for many researchers.

E. coli is ubiquitous within all phases of animal production (including all aspects of preharvest and postharvest animal production, including waste treatment), and while it is predominantly a benign commensal organism, a subset is highly pathogenic. The relationship between *E. coli* and animal production is multifaceted and complex, and it is important that useful isolates – gathered from natural populations – be made available to the research community so that important linkages can be analyzed. One notable area of concern is that of AMR, with both pathogenic and non-pathogenic strains of *E. coli* capable of serving as a reservoir for antimicrobial resistance genes (ARGs) (Bailey et al., 2010). Coupled with the ability of *E. coli* to adapt and survive in a number of environments (Touchon et al., 2009) – for instance in water (Flint, 1987) and agricultural soils (Topp et al., 2003) – *E. coli* has established itself as a significant contributor in the dissemination of ARGs between ecosystems (Stokes and Gillings, 2011).

While there is substantial knowledge demonstrating the ability of *E. coli* to transfer ARG not only to other *E. coli* isolates, but to other organisms as well (Andremont, 2003), we are only now coming to understand how agricultural AMR may influence public health (Skurnik et al., 2016; Landers et al., 2012). The issue is further clouded by studies that have shown greater diversity and higher prevalence's of AMR in municipal waste streams as compared to animal production runoff found within close proximity of each other (Agga et al., 2015), greater diversity of ARG in prairie soils as opposed to farm soils (Durso et al., 2016; Cadena et al., 2018), the persistent nature of ARGs in the environment (Durso et al., 2012), and of naturally occurring antimicrobial resistant bacteria (ARB) populations indirectly influenced by agricultural practice (Udikovic-Kolic et al., 2014). These studies demonstrated that while agricultural environments have a potential role in the development and spread of AMR, it is hardly the only environment capable of doing so, and other environments may play a more critical role (Bengtsson-Palme et al., 2017). However, while the overall magnitude of the impact that agriculturally-derived ARB has on human health is still debated, there is little argument that reductions in ARB and ARG wherever possible would be beneficial for both the agricultural and the public health communities. To achieve such a goal, it is important to put into context the issue facing the agricultural community; to determine the proliferation of relevant ARGs within major animal production systems.

For this study, genes that confer, or were linked to, resistance to sulfonamides and tetracycline were targeted to assess the utility of the panel. Sulfonamides and tetracyclines are commonly administered for prophylaxis and/or therapeutic purposes in livestock operations in the U.S. (Sarmah et al., 2006), and the utilization of these antibiotics has resulted in performance benefits for the industry (Cromwell, 2006). Despite the positive economic impacts, the practice elicits significant public health concern, foremost of which is the occurrence of AMR concomitant to antimicrobial usage (Levy, 1982). These antimicrobials were selected because they are two commonly used antimicrobials in U.S. animal production with clinical utility (Economou and Gousia, 2015).

Given the complexities of the relationship between *E. coli* and animal production in the U.S., the objectives for this study were threefold: (i) develop a publicly accessible panel of agricultural *E. coli* isolates originating from the major food animal commodity production systems across the United States; (ii) evaluate the diversity of the isolates based on commodity and geography; and (iii) use the newly developed panel to determine the prevalence of tetracycline and sulfonamide resistance genes across animal production systems.

2. Materials and methods

2.1. Sample collection and E. coli isolation

Isolates were obtained from existing culture collections or were collected as part of ongoing research efforts at the participating laboratories. In all instances, manure and environmental samples were collected in sterile plastic bottles or clean Ziploc® bags, stored in coolers with icepacks for transport back to the laboratory, and processed immediately or transferred to a 4 °C refrigerator and processed within 48 h, according to protocols used in the participating laboratories.

Isolates from Georgia (Rothrock et al., 2016) and Illinois came from existing isolate collections. For isolates from Idaho and Nebraska, samples were diluted 1:10 in sterile phosphate buffered saline (PBS), plated directly onto CHROMagar *E. coli* (CHROMagar, Paris), and incubated at 37 °C for 48 h. Suspect blue colonies were passed onto 2xYT media (Sigma, St. Louis, MO) or CHROMagar plates. For isolates from California, samples were diluted and inoculated into IDEXX Colilert broth (IDEXX, Westport, Maine). Following 24 h incubation, 100 µL of positive enrichments were plated onto CHROMagar *E. coli*, incubated at 37 °C for 48 h, and re-streaked onto fresh CHROMagar plates before archiving. For isolates from Kentucky, after diluting in sterile PBS, samples were plated onto mFC agar (Sigma, St. Louis, MO). For swine

isolates from North Carolina, anaerobic swine wastewater lagoon samples, and environmental water samples were enriched in MacConkey broth for 35 °C for 6 h, from which 50 µL was spiral plated onto CHROMagar *E. coli* and incubated at 37 °C for 24 h. Presumptive *E. coli* colonies were passed to MacConkey agar and incubated at 37 °C for 24 h prior to phylotyping of lactose-fermenting isolates. Beef and dairy manure samples from North Carolina, and poultry litter from South Carolina, were processed similarly to the North Carolina liquid samples, however instead of an enrichment step, manure or litter was diluted 1:10 in sterile PBS prior to spiral plating on CHROMagar *E. coli* plates. Isolates were phylotyped as described below prior to selection for inclusion in the panel. If multiple isolates from the same farm/community had identical phylogroup results, only one was chosen for panel inclusion. No isolates were screened for ARGs prior to panel inclusion.

2.2. DNA extraction from E. coli isolates

DNA was extracted from all *E. coli* isolates for phylogenetic typing, and *tet* and *sul* ARG detection. Isolates were streaked for isolation onto individual Luria-Bertani (LB) agar plates for overnight growth at 37 °C. From each plate, a single colony was transferred to 5 mL LB broth in a sterile test tube and placed overnight in an orbital incubator at 37 °C and 180 rpm. A total of 1 mL of culture was used for DNA extraction using a Promega Wizard Genomic DNA Purification Kit (Promega, Madison, WI) according to manufacturer's instructions for the extraction of DNA from Gram-negative bacteria. DNA concentration and purity were determined via NanoDrop 2000c spectrophotometer (ThermoFisher, Wilmington, DE).

2.3. Phylogenetic typing

The Clermont phylotyping method (Clermont et al., 2000), as modified by Doumith et al. (Doumith et al. (2012)), was used to assign *E. coli* isolates into one of seven (A0, A1, B1, B2.2, B2.3, D1, and D2) phylotypes as described by Escobar-Paramo et al. (Escobar-Paramo et al. (2004)). Briefly, template DNA was amplified using a multiplex PCR assay, that utilized 1 µM of each primer and 10 ng of genomic DNA as template, and performed under the following conditions: (i) initial denaturation of 5 min at 94 °C; (ii) 30 amplification cycles consisting of 30 s at 94 °C, 30 s at 65 °C, 30 s at 72 °C; (iii) and a final extension of 5 min at 72 °C. Primer sets for the four amplification products are listed in Table S1, and target the following: a 373 bp fragment of *gadA*, which encodes glutamate decarboxylase-alpha, as an internal amplification control; a 281 bp fragment of *chuA*, which encodes an outer membrane hemin receptor, positive in B2.2, B2.3, D1, and D2 isolates; a 216 bp fragment of *yjaA*, which encodes an uncharacterized protein, positive in A1, B2.2 and B2.3 isolates; and a 152 bp fragment of DNA sequence TSPE4.C2, positive in B1, B2.3, and D2 isolates. *E. coli* strain ATCC 25922, which carries all four DNA targets (i.e., B2.3 phylotype), was used as a positive control.

2.4. Clonal relatedness

Clonal relatedness between *E. coli* panel isolates was determined using a 24 h *E. coli* pulsed-field gel electrophoresis (PFGE) procedure as previously described (Ribot et al., 2006). Briefly, cells from overnight culture were embedded into 1.0% Seakem Gold agarose (BioWhittaker Molecular Applications, Rockland, ME) and digested with 10 U of *XbaI* (Roche Molecular Biochemicals, Indianapolis, IN). DNA standards were prepared from *Salmonella enterica* serotype Braenderup H9812. Digested DNA was separated using the Bio-Rad CHEF-DRII PFGE system (Bio-Rad, Hercules, CA) per manufacturer's instructions. Electrophoresis was performed as follows: voltage of 6 V for 19 h; ramped pulse time of 2.16–54.17 s in 0.5 x Tris-borate EDTA (TBE) at 14 °C. Cluster analysis was performed using BioNumerics software (Applied Maths Scientific Software Development, Sint-Martens-Latem, Belgium) using Dice coefficient and unweighted pair-group method (UPGMA). Optimization settings for dendrograms were 1.5% with a position tolerance of 1.5%.

2.5. ARG identification

All 300 isolates were assayed for the presence of sulfonamide and tetracycline resistance genes. For sulfonamide resistance genes, individual PCR assays were performed as previously described (Lanz et al., 2003; Perreten and Boerlin, 2003; Sundstrom et al., 1988), whereas the multiplex PCR assays of Ng et al. (Ng et al. (2001)) were used for the identification of tetracycline resistance (*tet*A, B, C, D, E, G, K, L, M, O, A(P), Q, and S). Primer sets used for the identification of *sul* and *tet* resistance genes are found in Supplementary Table A.1. Given the association of sulfonamide resistance to class I integrons, all isolates that were positive for *sul*1, *sul*2, and/or *sul*3 were also checked for the presence of the *intl*1 gene according to Barraud et al. (Barraud et al. (2010)).

2.6. Antimicrobial susceptibility testing

Panel isolates positive for *tet* and/or *sul* resistance genes were confirmed for phenotype by testing for susceptibility to tetracycline and/or sulfisoxazole using the broth microdilution method recommended by NARMS. Isolates were considered resistant at breakpoints of ≥ 16 µg/mL for tetracycline, and ≥ 256 µg/mL for sulfonamide.

2.7. Statistical analysis

The following diversity indices were computed in PC-Ord v 6.0 (MjM Software Design, Gleneden Beach, OR): richness (*S*); evenness (*E*); Shannon-Wiener index (*H*); and Simpson's Index of Diversity (1 – *D*) (McCune et al., 2002). Pearson Chi-square tests, odds ratio calculations with determination of exact confidence limits, and binomial proportions with exact confidence limits were performed in SAS 9 using PROC FREQ (SAS, Cary, NC). Associations were considered significant when *P* values were < 0.05. To improve binomial proportion analysis, commodities and geographical locations with low frequencies were combined with others to produce an *n* of at least 10. For commodities, fish, horse, and lamb isolates were grouped together as "Other" ($n = 11$). For geographical locations, groups were assembled as follows: IL with KY ($n = 14$); ND, NE, and WA with ID ($n = 75$); and SC with NC ($n = 100$). Given the low frequency of sulfonamide resistance (*sul*) and integron 1 genes identified in the study, binomial proportion analysis differences were measured by Fishers Exact Test instead of Chi-square.

3. Results

3.1. Development and characterization of the E. coli agricultural panel

A total of 300 *E. coli* isolates were collected during this study. Isolates were cultured from 12 states, and included 6 agricultural commodities, as well as environmental areas closely associated with animal production (Table 1). The four major animal production systems in the United States are all well represented, with 273 of the 300 isolates directly associated with beef ($n = 38$), dairy ($n = 98$), poultry ($n = 37$), or swine ($n = 100$). The 16 environmental isolates were collected either from grab-samples collected from rivers downstream of swine production facilities in North Carolina, or sediment samples from irrigation return flows as described in Dungan et al. (Dungan et al. (2017)). To include geographical diversity within the four major animal production systems, isolates were collected from at least five different states.

Phylotyping revealed that all seven phylogroups were represented by at least seven isolates (Fig. 1). Phylogroup B1 (107 isolates) was the most abundant, while B2.2 (7 isolates) was the least abundant. Relative

Table 1
Geographic origin of isolates by commodity.

Beef (*n* = 38)	***n*** [a]	
California	21	(55.3)
Connecticut	3	(7.9)
North Carolina	6	(15.8)
North Dakota	1	(2.6)
Wisconsin	7	(18.4)
Dairy (*n* = 98)		
California	29	(29.6)
Connecticut	5	(5.1)
Idaho	42	(42.9)
North Carolina	4	(4.1)
Nebraska	3	(3.1)
Washington	3	(3.1)
Wisconsin	12	(12.2)
Environment (*n* = 16)		
Idaho	6	(37.5)
North Carolina	10	(62.5)
Fish (*n* = 4)		
Idaho	4	(100.0)
Horse (*n* = 5)		
Idaho	5	(100.0)
Lamb (*n* = 2)		
Idaho	2	(100.0)
Poultry (*n* = 37)		
Connecticut	3	(8.1)
Georgia	11	(29.7)
Kentucky	10	(27.0)
North Carolina	2	(5.4)
Nebraska	3	(8.1)
South Carolina	8	(21.6)
Swine (*n* = 100)		
California	12	(12.0)
Connecticut	4	(4.0)
Idaho	6	(6.0)
Illinois	4	(4.0)
North Carolina	70	(70.0)
Wisconsin	4	(4.0)

[a] Number of isolates by geographic location for each commodity. Bracketed value is percent contribution to each commodity from each state.

abundances of each phylogroup differed between commodities, and only dairy and swine had isolates from each of the seven phylogroups (Fig. 1). Both poultry and beef had isolates from six phylogroups, lacking only the rare B2.2 phylogroup (Carlos et al., 2010) (Fig. 1). Diversity (1 - *D*), based on phylotyping, was high for the four major agricultural production groups, with a range of 71.1% to 78.5% (Table 2), which is similar to other *E. coli* panels when using phylotyping (Wang et al., 2017).

All 300 isolates were typeable using PFGE, and 288 PFGE patterns were observed and assembled into 71 groups (based on 80% similarity). Thirteen groups contained a single isolate (Fig. 1; Supplementary Table A.2). Beef, dairy, poultry and swine were represented in 29, 47, 20, and 45 PFGE groups each, respectively (Fig. 2). PFGE groups that contained a beef isolate were equally likely to also contain dairy, poultry, or swine isolates, while the concurrence of commodity-specific isolates varied in PFGE groups containing dairy, poultry, or swine (Fig. 2). Of the 58 PFGE groups that contained more than one isolate, 5 contained 10 or more isolates, and only 6 consisted of isolates originating from a single commodity; 4 PFGE groups comprised a total of 12 swine isolates, and 2 PFGE groups comprised of 6 dairy isolates. Additionally, when examining the 58 PFGE groups, only three groups consisted of isolates originating from a single geographical location. Two of those are associated with single commodity groups, the first being 3 swine isolates from CA, and the other 2 dairy isolates from ID. The third is a pair of isolates (one horse, one dairy) also originating from ID. Otherwise, no discernable patterns appeared within PFGE groups regarding geographical location or commodity. Compared to phylotyping ("Total" in Table 2 - *S*, 7; *E*, 0.8593 ; 1 - *D*, 77.6%), PFGE (*S*, 71; *E*, 0.9256; 1 - *D*, 97.3%) proved to be the more discriminatory subtyping method, though it should be noted that of the 12 pairs of isolates that had identical PFGE profiles, three of those pairs (AgEc 175/176, AgEc 86/87, and AgEc 190/191) were able to be differentiated by phylotyping.

3.2. Prevalence of antibiotic resistance genes in E. coli isolates

After assembly of the panel, all 300 isolates were screened for 17 ARGs – 14 related to tetracycline resistance, and 3 related to sulfonamide resistance. Of the 131 isolates harboring tetracycline resistance genes, 122 carried a single *tet* gene (Table 3). Of the remaining nine isolates, seven harbored both *tet*(A) and *tet*(B), one isolate carried *tet*(B) and *tet*(C), and another *tet*(A) and *tet*(M). Overall, *tet*(B) was found in 22% of all isolates (*n* = 66), followed by *tet*(A) in 21.7% (*n* = 65), *tet*(C) in 2.7% (*n* = 8), and *tet*(M) in 0.3% (*n* = 1). Tetracycline resistance was identified in at least a third of all isolates from each phylogroup, except for phylogroup B2.3, in which 0/15 isolates carried the assayed genes (Table 3). The highest prevalence rates were for B2.2 (57.1%), A0 (53.7%), and A1 (53.1%) phylogroups. When examined by commodity, swine isolates demonstrated the highest prevalence rates, with 84% of isolates carrying one (*n* = 79) or multiple (*n* = 5) tetracycline resistance genes (Table 3). Additionally, examination by geography, for areas represented by ten or more isolates, isolates from North Carolina demonstrated the highest prevalence rates with 75% of isolates (*n* = 69) carrying one (*n* = 63) or multiple (*n* = 5) tetracycline resistance genes.

Sulfonamide resistance genes were found in isolates at a lower rate than tetracycline (Table 4), with 41 isolates positive for one (*n* = 35), two (*n* = 4), or all three (*n* = 2) genes. Overall, *sul*2 was found in 6.7% (*n* = 20) of all isolates, followed by *sul*3 in 5% (*n* = 15), and *sul*1 in 4.7% (*n* = 14). Additionally, 5.7% (*n* = 17) of isolates carried class 1

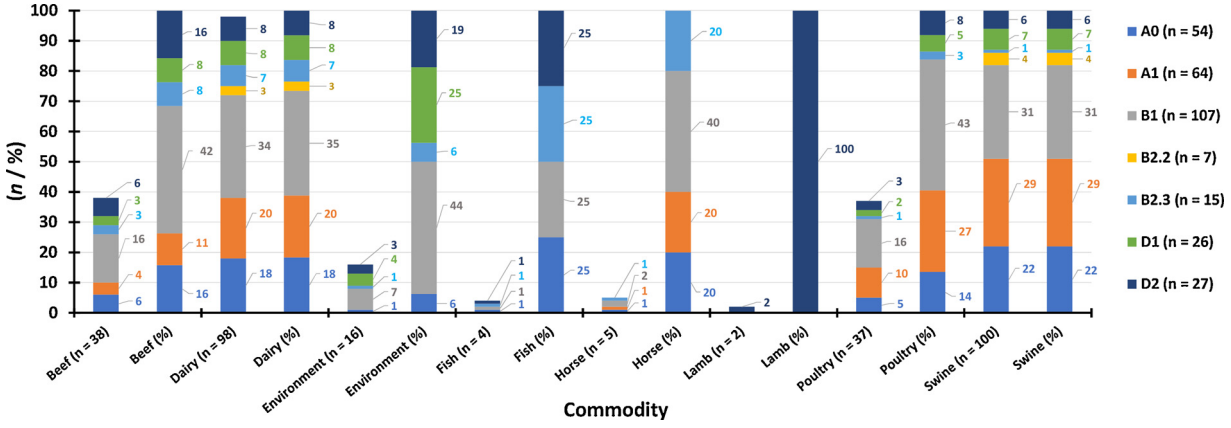

Fig. 1. Absolute counts (*n;* first column) and relative abundances (%; second column) of each phylogroup, by commodity.

Table 2
Diversity indices of commodities based on phylogroup.

Diversity Index	Beef	Dairy	Environment	Fish	Horse	Lamb	Poultry	Swine	Total
Richness (*S*)	6	7	5	4	4	1	6	7	7
Evenness (*E*)	0.8846	0.8773	0.8504	1.0000	0.9610	0.0000	0.8068	0.8145	0.8593
Shannon-Wiener (*H*)	1.5850	1.7072	1.3687	1.3863	1.3322	0.0000	1.4456	1.5849	1.6721
Simpson's Index of Diversity (1 - *D*)	74.9%	78.5%	70.3%	75.0%	72.0%	0.0%	71.2%	76.1%	77.6%

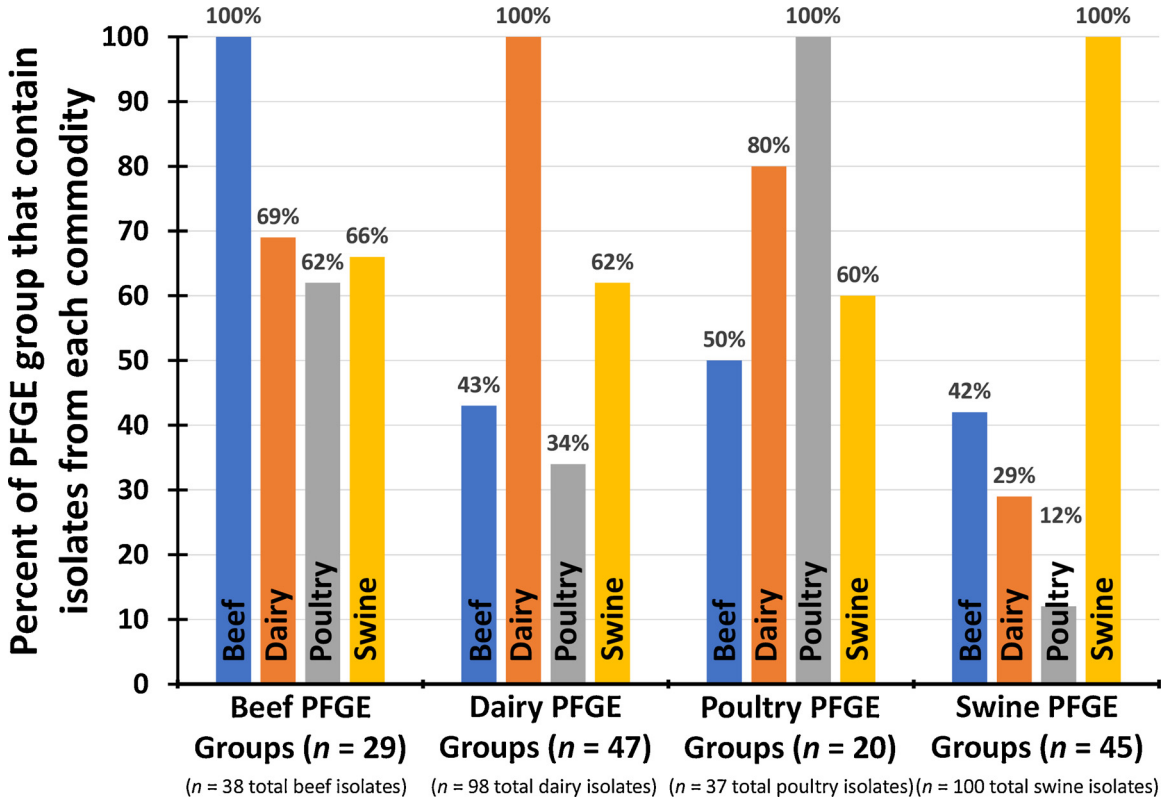

Fig. 2. Distribution and co-occurrence of isolates by commodity based on Pulsed Field Gel Electrophoresis (PFGE) groupings.

integrons, a majority of which also carried *sul*1 (n = 13; Table 5). Similar to tetracycline resistance, sulfonamide resistance genes were detected in isolates from each phylotype, with the exception of phylotype B2.3; phylotypes B2.2 (28.6%) and A0 (22.2%) continued to have the highest prevalence rates. When examined by commodity, the highest rate was for swine, with 23% of isolates positive for at least one sulfonamide resistance gene. For states represented by at least ten isolates, Georgia (36.4%; n = 4) and North Carolina (19.6%; n = 18) had the highest prevalence rates of isolates positive for sulfonamide resistance genes.

Over 44% (n = 134) of the screened *E. coli* isolates were found to carry at least one ARG, with 39 (13%), 5 (1.6%), and 2 (0.7%) isolates carrying 2, 3, or 4 ARGs respectively (Supplementary Information). Of the 17 ARGs screened, only 7 – 4 *tet* genes and 3 *sul* genes – were found to be present in the isolate panel. On a gene-by-gene basis, prevalence of tetracycline resistance genes was greater than that of sulfonamide, with 22.0% (n = 66) and 21.6% (n = 65) of isolates positive for *tet*(B) and *tet*(A), respectively, as compared to only 6.7% (n = 20) positive for *sul*2. The remaining four genes (*tet*(C), *tet*(M), *sul*3, and *sul*1) were all found in 5% or less of isolates. Examination of sulfonamide resistance also demonstrated that only 3 isolates (1 *sul*1 positive, and 2 *sul*3 positive) were positive for sulfonamide resistance genes without testing positive for tetracycline resistance genes (Table 5). Additionally, the two isolates that were positive for all three sulfonamide resistance genes, were also positive for *tet*(B).

The high rate of association between sulfonamide resistance genes and tetracycline resistance genes is outlined in Table 6. The odds for an isolate to be positive for *sul*1 were seven times greater when that isolate was also *tet*(A) positive ($P \leq$ 0.0001; OR, 7.39; CI, 2.38–22.92). While the presence of *tet*(A) did not impact the odds of *sul*2 or *sul*3, the presence of *tet*(B) raised the odds for an isolate to also contain either the *sul*2 ($P \leq$ 0.0001; OR, 10.23; CI, 3.75–27.88), or *sul*3 ($P =$ 0.02; OR, 3.35; CI, 1.17–9.62) gene.

A chi-squared test checked the association between tetracycline resistance and commodity groups (Table 7). Both poultry and swine had a significantly higher prevalence ($P <$ 0.0001) of *tetA* compared to other commodity groups, while only swine production demonstrated a significantly higher prevalence for *tetB* when compared to the other commodities ($P <$ 0.0001; Table 7). When examining the association of tetracycline resistance by state (Table 7) a significant ($P <$ 0.0001) geographical split between high frequency (CT, GA, IL and KY, and NC and SC) and low frequency (CA, ID with ND, NE, and WA, and WI) states was revealed for *tetA*. An almost identical pattern, also significant ($P <$ 0.0001) was seen with *tetB*, however CA grouped with the high frequency states, while CT grouped with the low frequency states.

A similar comparison using Fisher's Exact test (based on the low frequency of sulfonamide resistance genes identified amongst panel isolates) was performed to determine sulfonamide resistance associations amongst commodity groups and geographical locations. Significant associations are highlighted in Supplementary Tables A.3.

Table 3
Tetracycline resistance gene distribution in *Escherichia coli* isolates by phylogroup, commodity, and location.

		tetB		*tetC*		*tetA*		*tetB + tetC*		*tetA + tetM*		*tetA + tetB*		Total	
By phylogroup															
A0	*n* = 54	15	(27.8)[a]			12	(22.2)	1	(1.9)			1	(1.9)	29	(53.7)
A1	*n* = 64	18	(28.1)	1	(1.6)	13	(20.3)			1	(1.6)	1	(1.6)	34	(53.1)
B1	*n* = 107	19	(17.8)	2	(1.9)	22	(20.6)					3	(2.8)	46	(43.0)
B2.2	*n* = 7	1	(14.3)			3	(42.9)							4	(57.1)
B2.3	*n* = 15													0	(0.0)
D1	*n* = 26	2	(7.7)	2	(7.7)	4	(15.4)					1	(3.8)	9	(34.6)
D2	*n* = 27	3	(11.1)	2	(7.4)	3	(11.1)					1	(3.7)	9	(33.3)
By commodity															
Beef	*n* = 38	4	(10.5)	1	(2.6)	2	(5.3)							7	(18.4)
Dairy	*n* = 98	12	(12.2)	3	(3.1)	10	(10.2)	1	(1.0)					26	(26.5)
Environment	*n* = 16	1	(6.3)									1	(6.3)	2	(12.6)
Fish	*n* = 4													0	(0.0)
Horse	*n* = 5													0	(0.0)
Lamb	*n* = 2			2	(100.0)									2	(100.0)
Poultry	*n* = 37					8	(21.6)					2	(5.4)	10	(27.0)
Swine	*n* = 100	41	(41.0)	1	(1.0)	37	(37.0)			1	(1.0)	4	(4.0)	84	(84.0)
By location															
California	*n* = 62	11	(17.7)			10	(16.1)							21	(33.9)
Connecticut	*n* = 15					3	(20.0)							3	(20.0)
Georgia	*n* = 11					6	(54.5)					1	(9.1)	7	(63.6)
Idaho	*n* = 65	11	(16.9)	4	(6.2)	4	(6.2)	1	(1.5)					20	(30.8)
Illinois	*n* = 4	2	(50.0)			2	(50.0)							4	(100.0)
Kentucky	*n* = 10											1	(10.0)	1	(10.0)
Nebraska	*n* = 1			1	(16.7)									1	(16.7)
North Carolina	*n* = 92	33	(35.9)	1	(1.1)	29	(31.5)			1	(1.1)	5	(5.4)	69	(75.0)
North Dakota	*n* = 6	1	(100.0)											1	(100.0)
South Carolina	*n* = 8					1	(12.5)							1	(12.5)
Washington	*n* = 3					1	(33.3)							1	(33.3)
Wisconsin	*n* = 23			1	(4.3)	1	(4.3)							2	(8.6)
Total		**58**		**7**		**57**		**1**		**1**		**7**		**131**	

[a] Number and percentage (in brackets) of isolates (*n*) from individual phylogroup/commodity/geographical locations carrying the specific gene(s) in question.

Table 4
Sulfonamide resistance gene distribution in *Escherichia coli* isolates by phylogroup, commodity, and location.

		sul1		*sul2*		*sul3*		*sul1 + sul2*		*sul1 + sul2 + sul3*		Total	
By phylogroup													
A0	*n* = 54	1	(1.9)[a]	4	(7.4)	6	(11.1)	1	(1.9)			12	(22.2)
A1	*n* = 64			1	(1.6)	2	(3.1)	2	(3.1)	1	(1.6)	6	(9.4)
B1	*n* = 107	4	(3.7)	7	(6.5)	3	(2.8)	1	(0.9)			15	(14.0)
B2.2	*n* = 7	1	(14.3)	1	(14.3)							2	(28.6)
B2.3	*n* = 15											0	(0.0)
D1	*n* = 26	1	(3.8)			2	(7.7)					3	(11.5)
D2	*n* = 27	1	(3.7)	1	(3.7)					1	(3.7)	3	(11.1)
By commodity													
Beef	*n* = 38			4	(10.5)							4	(10.5)
Dairy	*n* = 98			6	(6.1)			1	(1.0)			7	(7.1)
Environment	*n* = 16	1	(6.3)	1	(6.3)							2	(12.5)
Fish	*n* = 4											0	(0.0)
Horse	*n* = 5											0	(0.0)
Lamb	*n* = 2											0	(0.0)
Poultry	*n* = 37	4	(10.8)					1	(2.7)			5	(13.5)
Swine	*n* = 100	3	(3.0)	3	(3.0)	13	(13.0)	2	(2.0)	2	(2.0)	23	(23.0)
By location													
California	*n* = 62			7	(11.3)			1	(1.6)			8	(12.9)
Connecticut	*n* = 15											0	(0.0)
Georgia	*n* = 11	3	(27.3)					1	(9.1)			4	(36.4)
Idaho	*n* = 65	2	(3.1)	4	(6.2)			1	(1.5)	1	(1.5)	8	(12.3)
Illinois	*n* = 4					2	(50.0)					2	(50.0)
Kentucky	*n* = 10											0	(0.0)
North Carolina	*n* = 92	2	(2.2)	3	(3.3)	11	(12.0)	1	(1.1)	1	(1.1)	18	(19.6)
North Dakota	*n* = 1											0	(0.0)
Nebraska	*n* = 6											0	(0.0)
South Carolina	*n* = 8	1	(12.5)									1	(12.5)
Washington	*n* = 3											0	(0.0)
Wisconsin	*n* = 23											0	(0.0)
Total		**8**		**14**		**13**		**4**		**2**		**41**	

[a] Number and percentage (in brackets) of isolates (*n*) from individual phylogroup/commodity/geographical locations carrying the specific gene(s) in question.

Table 5
Sulfonamide resistance gene isolates and their association with *intL*1 and tetracycline resistance gene isolates*.

		sul1[a] (n = 8)	sul2 (n = 14)	sul3[b] (n = 13)	sul1 + sul2 (n = 4)	sul1 + sul2 + sul3 (n = 2)
intL1	n = 17	7	1	3	4	2
tetA only isolates	n = 57	6	4	6	2	
tetB only isolates	n = 58		10	5	2	2
tetA + *tetB* isolates	n = 7	1				

[a] One *sul*1 positive isolate was not associated with tetracycline resistance.
[b] Two *sul*3 positive isolates were not associated with tetracycline resistance.
* No association between sulfonamide resistance genes and *tetC* genes.

Table 6
Odds ratios and 95% confidence intervals for associations between antibiotic resistance genes[a].

	tetB	*sul*1	*sul*2	*sul*3	*intL*1
tetA	0.36 (0.16 – 0.83†)	7.39 (2.38 – 22.92)	–	–	7.77 (2.753 – 21.95)
tetB		–	10.23 (3.75 – 27.88)	3.35 (1.17 – 9.62)	–
*sul*1			14.57 (4.45 – 47.75)	–	916.5 (99.57 – 8789.40)
*sul*2				–	14.54 (4.77 – 44.32)
*sul*3					11.37 (3.36 – 38.50)

[a] *tetC* and *tetM* showed no significant relationships and were therefore excluded from the table.
–, no statistically significant association detected.
† 95% confidence intervals shown in brackets.

Of particular note is that isolates from GA harbored *sul*1 at much higher rates than the remainder of the states included in the study. Other significant associations are sporadic, potentially due to the low number of sulfonamide resistance genes harbored by the panel isolates.

3.3. Confirmation of antibiotic resistance in E. coli isolates harboring tet and sul resistance genes

For isolates that demonstrated a positive PCR for one of the 17 tested ARGs in this study, we performed antimicrobial susceptibility testing according to NARMS standards. Results are shown in Supplementary Tables A5 (for tetracycline) and A6 (for sulfonamide). For the 131 isolates that were PCR positive for one of the 14 tetracycline resistance genes assayed for in this study, 129 (98.5%) of the isolates demonstrated an intermediate or resistant MIC. While isolates 86 (*tetB*) and 175 (*tetA*) displayed a positive PCR, both were sensitive to tetracycline. Additionally, isolates 39, 123, and 138 – all of which were *tetC* positive by PCR screen – had an intermediate MIC profile for tetracycline. For the 41 isolates that were positive for a sulfonamide resistance gene as determined by PCR, all 41 had a resistant MIC profile to sulfisoxazole.

4. Discussion

It is becoming clear that information on 'generic' *E. coli* can provide important context for food safety, microbiome investigations and whole genome sequencing studies. To address the growing awareness of the importance of commensal bacteria and the potential impact of agricultural production on human, animal, and environmental health, we assembled a panel of isolates that spanned the major food animal production systems of the United States. The collected isolates represent geographical locations across the U.S., and comprise randomly isolated, genetically diverse, strains from agricultural ecosystems. This collection of *E. coli* isolates is designed to be a resource available to the larger

Table 7
Associations of tetracycline resistance genes with commodity and geographical location.

		Beef	Dairy	Environ.	Other	Poultry	Swine
	Sample Size (n)	38	98	16	11	37	100
tetA	%	5.26%	10.20%	6.25%	0.00%	27.03%	42.00%
	95% Lower Conf Limit	0.64%	5.00%	0.16%	0.00%	13.79%	32.20%
	95% Upper Conf Limit	17.75%	17.97%	30.23%	28.49%	44.12%	52.29%
	Chi Square (Prob)	43.88	(< 0.0001)				
tetB	%	10.53%	13.27%	12.50%	0.00%	5.41%	45.00%
	95% Lower Conf Limit	2.94%	7.26%	1.55%	0.00%	0.66%	35.03%
	95% Upper Conf Limit	24.80%	21.62%	38.35%	28.49%	18.19%	55.27%
	Chi Square (Prob)	47.98	(< 0.0001)				

		CA	CT	GA	ID[a]	IL[a]	NC[a]	WI
	Sample Size (n)	62	15	11	75	14	100	23
tetA	%	16.13%	20.00%	63.64%	6.67%	21.42%	36.00%	4.34%
	95% Lower Conf Limit	8.02%	4.33%	30.79%	2.20%	4.66%	26.64%	0.11%
	95% Upper Conf Limit	27.67%	48.09%	89.07%	14.85%	50.80%	46.21%	21.95%
	Chi Square (Prob)	38.67	(< 0.0001)					
tetB	%	17.74%	0.00%	9.09%	17.33%	21.43%	38.00%	0.00%
	95% Lower Conf Limit	9.20%	0.00%	0.23%	9.57%	4.66%	28.48%	0.00%
	95% Upper Conf Limit	29.53%	21.80%	41.28%	27.81%	50.80%	48.25%	14.82%
	Chi Square (Prob)	28.31	(< 0.0001)					

[a] ID also includes ND, NE, and WA; IL also includes KY; NC also includes SC.

scientific community. *E. coli* is important as a pathogen of humans and animals, model organism for laboratory studies, and indicator organism used in monitoring and surveillance of water, soil, and air. As such, the utility of available *E. coli* culture collections, e.g., ECOR, has proven exceptionally valuable over the years (Bergthorsson and Ochman, 1998; Herzer et al., 1990; Mazel et al., 2000; Wirth et al., 2006; Picard et al., 1999). However, the ECOR standard reference collection has a limited ability to provide answers to agricultural-specific questions. Of the ECOR reference libraries 72 isolates, only 5 isolates originate from conventional food production animals (i.e., bovine and swine sources). Moreover, these isolates do not originate from the United States. Therefore, ECOR and other similar isolate panels, designed primarily to maximize genotypic variation, do not provide a natural sampling of genotypic and phenotypic attributes from individual agricultural ecosystems.

Although some early studies explored the structure of natural *E. coli* populations (Hartl and Dykhuizen, 1984; Selander and Levin, 1980; Selander et al., 1987), pathogenic organisms and pathogenic isolates have historically been the primary focus of most scientific inquiries. The need to understand the relationships between pathogens and their commensal counterparts has been acknowledged in the framework of food safety (Durso et al., 2004; Callaway et al., 2008), however recent advances in microbiome studies highlight importance of non-pathogens in maintaining the health of humans, animals and the environment. As an organism with many hosts that moves across and between these three ecosystem components, *E. coli* is a logical candidate to advance our understanding of the ecology of health and disease, particularly as it relates to food production systems. Tenaillon et al. (Tenaillon et al., 2010) discuss the utility of *E. coli* for exploring the dynamic nature of host-bacterium relationships, and the transitions between mutualism, commensalism, opportunistic pathogenesis, and specialized pathogenesis. They argue that an understanding of the ecology and evolution of the commensal strains is required to fully understand virulence and antibiotic resistance in pathogens. Additionally, due to the two-habitat nature of the *E. coli* life-cycle (Savageau, 1983), the selective forces that act on *E. coli* in the environment are postulated to be an important selective factor (Hartl and Dykhuizen, 1984; Durso et al., 2004; Tenaillon et al. (2010)) that have the potential to coincidentally select for traits such as antibiotic resistance (Tenaillon et al., 2010).

PFGE results demonstrated that a highly diverse panel of isolates has been assembled. PFGE identified a total of 71 isolate clusters at the 80% similarity level (Supplementary Figure A.1 and Supplementary Table A2). With few exceptions, these groups are not tied to a particular geographical location, phylogroup, or antibiotic resistance profile. The set was collected to represent the major US meat animal production systems, and we evaluated whether there was any grouping of *E. coli* PFGE profiles by commodity (Fig. 2). Beef isolates were equally likely to group with dairy, poultry and swine, however differences were observed for the other PFGE-commodity groups. Of note is that both dairy and swine were less likely to share a PFGE group with poultry, compared to beef/swine or beef/dairy, respectively. One possible explanation for this is the small number of total poultry isolates in the collection ($n = 37$). However there were approximately the same number of beef isolates ($n = 38$), and the same pattern was not observed with beef.

Within the panel of 300 isolates, nine pairs demonstrate indistinguishable PFGE patterns, based on the single XbaI enzyme used. It should be noted that of those 9 pairs, one pair has origins from different states (AgEc144 isolated in CT vs AgEc187 isolated in NC), and a second pair presents with different ARG profiles (AgEc86 harbors *tetB*, whereas AgEc87 does not). It remains possible that the remaining seven indistinguishable pairs are indeed clones, however all indistinguishable pairs come from different farms, and are epidemiologically unrelated. Due to the highly clonal nature of *E. coli* populations (Tenaillon et al., 2010), further study of these pairs, using additional enzymes for PFGE, genome sequencing, or other genotypic and phenotypic methodologies, would potentially resolve these pairs. We therefore suggest the continued inclusion of all 300 isolates, could potentially serve in assessing the discriminatory power of other subtyping assays.

Phylotyping, including the Clermont system used in this analysis, has been widely used to characterize *E. coli*, and explore their ecological relationships (Clermont et al., 2015). *E. coli* phylogeny has been linked with ecological niche and pathogenicity (Clermont et al., 2015; Kim et al., 1999), and numerous studies have explored species-specific distributions of *E. coli* phylotypes (Tenaillon et al., 2010; Johnson et al., 2017; Smati et al., 2015). The B2 subtype has been associated with human pathogens (Tenaillon et al., 2010; Johnson et al., 2017; Smati et al., 2015), and so it was not surprising to see that this was the least-frequently represented group in our strain set (Supplementary Table A.4). The phylotype distribution across the agricultural isolates collected in this study display patterns distinct from those of both humans and animals collected in a 2010 meta-analysis (Tenaillon et al., 2010), and further support the potential value of an *E. coli* strain set specifically representing U.S. agricultural production systems.

The assembled isolate panel was used to examine a subset of ARGs to determine if patterns would emerge from among the major animal production systems. As mentioned previously, sulfonamides and tetracyclines are commonly administered for prophylaxis and/or therapeutic purposes in livestock operations in the U.S. (Sarmah et al., 2006), are utilized for performance benefits for the industry (Cromwell, 2006), and are the two of the most commonly used antimicrobials in U.S. animal production (Economou and Gousia, 2015). Despite the frequency from which sulfonamide and tetracycline resistance genes are isolated from animal production systems, the determination of comprehensive rates of AMR are complicated by multiple variables including, but not limited to: target gene (Cadena et al., 2018); animal host (Kozak et al., 2009b); habitat (Knapp et al., 2011); seasonality (Beattie et al., 2018); and antibiotic usage strategy (Peak et al., 2007). In addition to the spatial, temporal, and management issues which can affect AMR dissemination, organism attributes also play a critical role.

Tetracycline resistance genes were identified in roughly half of the isolates (131 out of 300), a majority harboring *tetA* and/or *tetB*. Except for the 15 phylogroup B2.3 isolates, which harbored no resistance genes tested in this study, a third of the isolates from each phylogroup carried at least one tetracycline resistance gene. The A and B phylogroups had the highest frequencies (> 40%) of tetracycline resistance. These rates are lower than those reported by Hölzel et al. (Hölzel et al. (2012)), who analyzed the prevalence of *tet* resistance gene rates by phylogroup in porcine *E. coli* isolates. Association of tetracycline resistance with commodity revealed significant differences amongst animal production systems, with higher prevalence of tetracycline resistance in poultry (*tetA*) and swine (*tetA* and *tetB*) isolates. A study by Kozak et al. (Kozak et al. (2009a)), reported the presence of *tetA* and *tetB* genes in 27% and 59% of all tested swine isolates, respectively, numbers which are reflected in the swine isolates analyzed in this study. When viewed geographically, tetracycline associations were roughly split between the coasts, with the eastern-most states (CT, GA, IL and KY, NC and SC) having significantly higher rates of *tetA* than western-most states (CA, ID with ND, NE and WA, and WI). An almost identical pattern emerged for *tetB*, though CA associated with the higher frequency states, while CT grouped into the lower frequency category. Such geographical patterns to AMR have been reported before. A study by Berge (Berge et al., 2010) on cattle and dairy production along the Pacific coast demonstrated differences not only by state and commodity, but also by management (i.e., number of days on feed, conventional vs organic operation).

Two isolates were positive for tetracycline resistance genes, but negative on the phenotypic screen. One possible explanation for the discrepancy between genotype and phonotype for these two isolates is that although we detected the gene, it was not functional, or not expressed. One isolate was positive for *tetA*, the other for *tetB*. Both of these genes code for cytoplasmic membrane efflux pumps that confer

resistance by reducing intracellular concentrations of tetracycline once it enters the cell, before it can act upon the ribosomes. When transcribed, the N- and C-terminal halves of the *tetA* and *tetB* proteins are separate functional domains, and mutations in either half eliminate resistance (Chopra and Roberts, 2001). PCR assays, by design, detect only a fragment of each gene target. In this instance 251 and 659 base pairs for *tetA* and *tetB* respectively, compared to 1199 and 1736 base pairs, respectively, for the full gene. Additionally, expression of tetracycline resistance requires not only the *tetA* or *tetB* gene, but also a second gene product expressing a regulatory protein. The promoters for the tetracycline regulator gene overlap with the promoters for *tetA* or *tetB*, and both are regulated by tetracycline (Hillen and Berens, 1994). Thus, expression of tetracycline resistance is complex, and detection of a single fragment from a single gene may not always predict phenotypic expression profile.

Sulfonamide resistance genes were harbored in panel isolates at lower rates than reported elsewhere, all of which were phenotypically confirmed to be resistant to sulfonamide. For example, a study of swine farms in Ontario, CA reported a total detection rate in *E. coli* isolates of 44% for all three sulfonamide resistance genes (Kozak et al., 2009b), roughly double the rate observed in this study. Likewise, a study of Tunisian poultry production *E. coli* isolates demonstrated sulfonamide resistance rates of > 85%, with a majority of those isolates carrying *sul*2 and/or *sul*3 genes (Soufi et al., 2009), which is over 6 times higher than the rate in this studies poultry isolates. Lanz et al. (Lanz et al. (2003)), reported that *E. coli* isolated from swine and bovine production in Switzerland, demonstrated sulfonamide resistance rates of 81%, and 22%, respectively, rates significantly higher than those reported here. The low rates of sulfonamide resistance made finding relationships between commodity and geographical location difficult, though some associations were noted, particularly for poultry (*sul*1) and swine (*sul*3), which further highlight geographical associations. Interestingly, of the 41 isolates that harbored sulfonamide resistance genes, only three did not also possess tetracycline resistance. Strong positive associations of *tetA* with *sul*1, and *tetB* with *sul*2 were noted; relationships that were previously identified (Boerlin et al., 2005). Also, similar to Boerlin et al. (Boerlin et al., 2005), a negative relationship – most likely due to plasmid incompatibilities (Jones et al., 1992) – was observed between *tetA* and *tetB*.

Overall, the assembled panel of isolates captures a high degree of genetic diversity, while representing the major animal production groups from several geographical locations throughout the United States. Using this isolate panel, an assessment of tetracycline and sulfonamide resistance was performed to study relationships between ARGs, commodity groups, and geographical location. In many cases, similar or lower rates of resistance genes as compared to other studies was observed; lower observed rates of specific resistance genes may be a result of expanded range of geographical sampling in the assembled panel as compared to regional-specific studies that may represent greater homogeneity in management practices. The results demonstrated that the assembled isolate panel provides the research community with a genetically diverse resource, representative of *E. coli* isolates from U.S. animal production systems.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2019.120991.

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