Campylobacter coli in Swine Production: Antimicrobial Resistance Mechanisms and Molecular Epidemiology

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The aim of this study was to determine antimicrobial resistance, to evaluate and compare the use of two genotyping methods for molecular epidemiology purposes, and to determine the genotypic diversity of *Campylobacter coli* of porcine origin. A total of 100 *C. coli* isolates from swine were tested for susceptibility to six antimicrobials using the agar dilution method and genotyped using two high-resolution fingerprinting approaches: multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE). Evaluation of the methods was based on their resistance patterns, discriminatory indexes (DI), high test throughputs, costs, and turnaround times. Resistance to erythromycin and tetracycline was the most common. Both genotypic methods were found to have high discriminatory power, although MLST had a higher DI (0.936) than PFGE (DI = 0.889). It also had a higher throughput than PFGE. Isolates were clustered into 27 groups by MLST compared to 11 by PFGE. MLST was able to further discriminate the isolates grouped under the same cluster by PFGE. Out of the 65 MLST sequence types (STs) identified among the total isolates, 50 were reported for the first time. Most STs were found to be specific to the farm (*n* = 38) and to slaughter (*n* = 22). Resistance against tetracycline and erythromycin was encoded by the *tet*(O) gene and a A2075G point mutation in the 23S rRNA gene, respectively. A high ciprofloxacin MIC (>64 μg/liter) was conferred by a point mutation in the *gyrA* gene. The weak clonal structure of the *C. coli* population among swine was further highlighted by the index of association value of 0.293. The findings of this study indicate that multidrug-resistant diverse *C. coli* strains exhibiting resistance to ciprofloxacin and erythromycin are concerning, since these are the drugs of choice for treating invasive campylobacteriosis cases in humans.

Food-borne pathogens result in approximately 76 million illnesses in the United States every year (27). *Campylobacter* is the most common cause of bacterial gastroenteritis with an estimated 2.5 million cases annually in the United States and in 54,000 cases in the United Kingdom (1, 27). The preliminary data, as estimated by FoodNet, for diseases caused by enteric pathogens for the year 2004 show *Campylobacter* to have an incidence of 12.9 per 100,000 persons (2). *Campylobacter jejuni* in humans is considered to be the most important *Campylobacter* species, causing 95% of the food-borne infections. Data from the United Kingdom implicated *C. jejuni* as being responsible for 91.9% of the cases, followed by *Campylobacter coli* with 7.9% (6).

Poultry has been recognized as the primary reservoir of *C. jejuni*, while pigs are mostly implicated as reservoirs of *C. coli* (19, 20). Recent studies conducted in Spain and the United Kingdom have highlighted the importance of *C. coli* as an important human pathogen due to its ability to show increased resistance to greater number of antimicrobials and because it causes more indigenously acquired food-borne diseases than *Salmonella enterica* serovar Typhimurium (40, 44). *C. coli* has been suggested to be particularly suited to the swine production environment and has been isolated from pigs on farms in up to 100% of the samples collected (40). Epidemi-

ological evidence has suggested the zoonotic transmission of this pathogen from animals to humans (3, 25). Although most of the human cases are sporadic in nature, outbreaks caused by *Campylobacter* spp. have been reported in the past, caused by the consumption of raw milk and contaminated water (30, 39).

It is important to use typing methods that have high discriminatory power to identify and differentiate sources of this pathogen in animals, humans, and the environment. Many phenotypic and genotypic methods have been developed for *Campylobacter* (32). Multilocus sequence typing (MLST) is one such genotypic method that is based on indexing the genetic variation in housekeeping genes (7). This technique has been successfully employed for studying the longitudinal epidemiology of *C. jejuni* in human, animal, and environmental samples (5). Recently, the standardization of the MLST typing method was extended to *C. coli* (8). However, no molecular epidemiological study has been reported using MLST for *C. coli*. Pulsed-field gel electrophoresis (PFGE) is another genotyping method that has been used for investigating *C. jejuni* outbreaks and genotyping *C. coli* (21, 28, 34, 42). Although this method is highly discriminatory, interlaboratory comparisons could be difficult due to complex protocols and accessibility of equipment and software for analyzing the patterns in multiple laboratories.

Prompted by the importance of *C. coli* as a food-borne pathogen and the paucity of molecular epidemiological information on this species, we conducted this study. The aim of this study was to evaluate and compare the use of MLST and PFGE for genotyping *C. coli* isolates from swine based on discriminatory power, throughput, cost, and time. In this study,

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we also determined the resistance mechanisms coding for predominant antimicrobial resistance phenotypes and show evidence of the clonal structure of *C. coli* by measuring the index of association (I_A).

MATERIALS AND METHODS

Origin of *C. coli* strains. A total of 100 isolates were randomly selected from 1,459 *C. coli* isolates that were isolated as a part of a cross-sectional study conducted on swine farms and slaughter plants (46). The selected strains were from the conventional and antimicrobial-free (ABF) production systems representative of the processing stages at farm and slaughter, and the resistance patterns were observed during the entire study. Briefly, a total of 21 groups of pigs that belong to two distinct production systems (11 conventional and 10 ABF farms) were sampled at the nursery farms (6 weeks of age) and finishing farms (within 48 h of slaughter) followed by carcass swab sampling at the slaughter plant. Collection of swab samples at slaughter was done at three stages, including previsceration, postvisceration, and postchill. Prior to testing, the isolates were recovered from storage at −80°C and streaked on Mueller-Hinton agar supplemented with 5% sheep blood. All the incubations were done under microaerobic conditions at 42°C for 48 h. Antimicrobial susceptibility testing was done using the agar dilution method as described below. Genotyping of the *C. coli* isolates was done by MLST and PFGE as described later in this section.

Antimicrobial susceptibility testing. The agar dilution method was used as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) subcommittee on Veterinary Antimicrobial Susceptibility Testing for determining the susceptibility of *Campylobacter* isolates to different antimicrobials (31). We tested the isolates for their susceptibility against a panel of six antimicrobials. The following is a list of antimicrobials with their abbreviations and ranges of concentrations used: chloramphenicol (CHL; 0.25 to 128 μg/liter), ciprofloxacin (CIP; 0.008 to 4 μg/liter), erythromycin (ERY; 0.06 to 32 μg/liter), gentamicin (GEN; 0.06 to 32 μg/liter), nalidixic acid (NAL; 0.25 to 128 μg/liter), and tetracycline (TET; 0.06 to 32 μg/liter) (14). All the antimicrobials were procured from Sigma (Sigma, MO) except ciprofloxacin (Serologicals Proteins, Inc., IL). The NCCLS breakpoint interpretative criteria for the *Enterobacteriaceae* family were used for all the antimicrobials except erythromycin as recommended by NCCLS, as the interpretive standard breakpoint levels for the *Campylobacteriaceae* family are not yet available (14). For erythromycin (8 μg/liter), the breakpoint level used by the National Antimicrobial Resistance Monitoring System was adopted (12). *C. jejuni* ATCC 33560 was used as the quality control organism for this test (14). The MIC_{50} breakpoints used for each antimicrobial were as follows: chloramphenicol (32 μg/liter), ciprofloxacin (4 μg/liter), erythromycin (8 μg/liter), gentamicin (16 μg/liter), nalidixic acid (32 μg/liter), and tetracycline (16 μg/liter). *Campylobacter* isolates showing resistance against ciprofloxacin at the concentration of 4 μg/liter (the highest on the original panel) were further tested at higher concentrations of the antimicrobial up to 64 μg/liter. Multidrug resistance here is defined as isolates exhibiting resistance against three or more antimicrobials simultaneously.

MLST. Genomic DNA purification of the isolates for sequencing was done using a QIAGEN DNA purification kit (QIAGEN, Valencia, CA). MLST of the seven housekeeping genes (*aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *tkt*, and *uncA*) for *C. coli* was done following the method described previously (8). Briefly, all the housekeeping genes were amplified using PCR, and the products were run on agarose gel to confirm the correct amplicon size. Purification of the PCR products was done using a QiaAmp PCR purification kit following the manufacturer's instructions (QIAGEN, Valencia, CA). Sequencing reactions using the forward and reverse primers in separate wells was done using 2 μl of the BigDye Ready Reaction mix (version 3.1; Applied Biosystems, Foster City, California), 0.5 μl of 1:15 diluted primer, 5.5 μl of molecular grade deionized water, and 2 μl of the purified PCR product. The sequencing reaction was performed on an automated 3700 ABI capillary sequencer (Applied Biosystems, Foster City, California) with running conditions of 30 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. We used the ClustalW program for aligning the forward and reverse sequences (www.ebi.ac.uk/clustalw). The allelic profiles and the sequence types (STs) were then generated by blasting the correct sequence size on the MLST website from the *Campylobacter* database (accessible at http://www.pubmlst.org/campylobacter or http://www.mlst.net). A dendrogram for the MLST data was generated using the START software (23).

PFGE. PFGE was done following the rapid protocol for *Campylobacter* (38). Briefly, 400-ml overnight culture cells were lysed, and intact genomic DNA was digested in agarose-embedded plugs with SmaI restriction enzyme. The digested DNA was then separated by using a contour-clamped homogeneous electric field

(CHEF)-DRIII (Bio-Rad Laboratories, Hercules, CA) with the following conditions: 0.5× Tris–borate–EDTA, 1% SeaKem Gold agarose (FMC BioProducts, Rockland, ME), 14°C, 6 V/cm for 18 h with switch times ranging from 6.75 to 38.35 s with an included angle of 120°. *S. enterica* serovar Braenderup 17 Universal Marker (kindly provided by Leslie Wolf, North Carolina State Laboratory of Public Health, NC) was used as the reference marker. Gels were stained with ethidium bromide for 30 min, destained three times for 20 min each with distilled water, and photographed using an Alpha imager (Alpha 20 Innotech Corporation, San Leandro, CA). Analysis of PFGE data was performed using Bionumerics software (Applied Maths, Kortrijik, Belgium) using a "different bands" algorithm for clustering and the Ward algorithm of tree building approach with 0.47% optimization and 0.48% position tolerance determined by using procedures recommended by the manufacturer. Visual inspection of the patterns was performed as a final step for analysis.

Characterization of resistance genes. A total of 21 multidrug-resistant *C. coli* isolates were selected for further molecular characterization to determine the resistance genes coding for antimicrobial resistance against tetracycline and erythromycin and the class I integron. PCR was used to target the *tet*(O) and *tet*(M) genes for tetracycline resistance and a mutation at position 2075 in the 23S rRNA for erythromycin resistance, as explained previously (15, 16, 35). Class I integrons were targeted in these isolates following the method described by O'Halloran et al. (33). Targeting of the threonine-to-isoleucine point mutation in the quinolone resistance-determining region (QRDR) of the *gyrA* gene conferring resistance to ciprofloxacin resistance was done using the mismatch mutation amplification assay (MAMA) PCR (49). Amplification reactions were carried out with 1 μl of purified DNA (QIAGEN DNAeasy tissue kit; QIAGEN, Valencia, CA), 300 μM deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 50 pmol of primers, and 0.5 U of Gold *Taq* polymerase (Perkin-Elmer, Foster City, CA). Distilled water was added to bring the final volume to 20 μl. The PCR cycle included initial denaturation at 95°C for 5 min and 30 cycles of denaturation for 1 min at 95°C, primer annealing for 1 min at 54°C, and extension for 1 min at 72°C.

Data analysis. Simpson's index of diversity was calculated to compare the discriminatory power of the two genotyping methods used in this study (22). The I_A was determined using the START program to assess the clonality of the population (26). An absolute value of zero ($I_A = 0$) indicates that the population is freely recombining and is not clonal. A value of 1.0 indicates the high genetic diversity of isolates.

RESULTS

Diversity of sequence types across the two production systems based on MLST. Assignment of the allele frequencies and STs was done using algorithms described in the *Campylobacter* MLST database (accessible at http://www.pubmlst.org/campylobacter or http://www.mlst.net). A total of 65 STs were generated from sequence typing of 100 *C. coli* isolates with 47 STs occurring singly and ST-1413 being the most common seen in seven isolates from the carcasses of ABF pigs. Based on the allelic profiles of the housekeeping genes, 50 new STs were assigned for the first time after submitting the information to the MLST database. Twenty-four out of the 50 new STs originated from the ABF swine production units. The remaining 26 new STs were from *C. coli* isolates from the conventional production system. Within individual production systems, we observed STs that were found specific to the processing stages either at the farm or slaughter, with 38 out of the 65 STs found to be specific to the farm and another 22 found only at the slaughter stage (Table 1). The three most predominant STs occurring in the database included ST-1413 (7 isolates), ST-854 (6 isolates), and ST-1123 (5 isolates), representing 18% of the isolates. Multiple STs were generated for individual antimicrobial resistance patterns, with the majority being specific either to the farm or the slaughter stage (Table 1). For instance, ST-1413 ($n = 7$) was observed only among the *C. coli* ciprofloxacin-resistant strains (resistance pattern: CIP ERY NAL TET) isolated from the conventional nursery pigs. How-

TABLE 1. Total number of *C. coli* isolates under each production system including the MLST sequence types and PFGE clusters

Production type (isolates tested)	Processing stage	No. of strains	No. of STs (% diversity)	No. of new STs (%)[a]	No. of unique STs (%)[b]	PFGE cluster(s) (n)[c]
ABF (50)	Nursery	9	8 (89)	4 (50)	4 (50)	1, 3, 4, 5, 10, 11 (6)
	Finishing	27	20 (74)	11 (55)	11 (55)	1, 2, 3, 4, 5, 7, 8, 9, 11 (9)
	Preevisceration	5	5 (100)	5 (100)	3 (60)	1, 8, 9 (3)
	Postevisceration	7	6 (86)	3 (50)	3 (50)	1, 5, 8 (3)
	Postchill	2	2 (100)	1 (50)	1 (50)	8 (1)
Conventional (50)	Nursery	14	8 (57)	7 (88)	6 (75)	1, 5, 8, 9, 10 (5)
	Finishing	21	18 (86)	12 (67)	13 (72)	1, 2, 3, 4, 9, 11 (6)
	Preevisceration	5	5 (100)	4 (80)	4 (80)	2, 3 (2)
	Postevisceration	9	9 (100)	2 (22)	7 (78)	3, 6, 8, 9 (4)
	Postchill	1	1 (100)	1 (100)	1 (100)	2 (1)

[a] Sequence types that have been reported for the first time.
[b] Sequence types that were found only in the specific processing stage.
[c] Total PFGE clusters and the cluster numbers where the sequence types were grouped.

ever, two additional isolates from the same farm and sharing the same resistance pattern as above had different STs (ST-1096, cluster 9; and ST-19, cluster 19).

Based on the MLST dendrogram generated by the START program (data not shown), we observed a total of 27 clusters, with cluster 9 being the largest ($n = 18$) (Table 2). Seven isolates were represented by single branches and occurred independently without being a part of any group. A majority of the isolates ($n = 16$) in cluster 9 were from the nursery and finishing farms and included 8 out of the 15 ciprofloxacin-resistant *C. coli* isolates. Clusters 2, 5, 6, 19, and 21 were comprised of isolates from the farm, while clusters 7, 8, and 24 included slaughter isolates only. Multiple STs were found among isolates from a single farm at different stages of processing both at farm and slaughter. For example, isolates 2003 and 2032 with the tetracycline resistance pattern (TET) were isolated from the same ABF farm but were associated with ST-890 and -825, respectively. Similarly, isolates 3490 and 3491 were from the same pig (TET resistant) reared in the conventional system but were associated with two different STs, ST-1130 and ST-854, respectively.

The I_A for the whole population was 0.293, indicating a weak clonal structure. However, the I_A values for the ABF and the conventional populations were 0.279 and 0.535, respectively, indicating a higher clonality of *C. coli* isolates from the conventional system. Simpson's index of diversity calculated for the MLST method was found to be 0.936. Such a high DI value indicates that MLST has a very high discriminatory power.

Genotypic diversity based on PFGE fingerprinting. The SmaI-digested genome of *C. coli* resulted in the generation of an average of 6 to 10 bands. Using 70% genetic similarity as the cutoff, a total of 11 clusters were observed, with clusters 1 (17 isolates), 11 (16 isolates), and 3 (15 isolates) being the predominant ones comprising 48% of the total isolates (Fig. 1). We followed the recommended criteria for interpreting PFGE banding patterns, with isolates differing by one to three bands being most likely clonal (45).

Three clusters had isolates grouped together based on their antimicrobial resistance patterns. These included cluster 4 ($n = 4$; TET), cluster 6 ($n = 3$; TET), and cluster 10 ($n = 6$; CIP ERY NAL TET). All the isolates from cluster 6 and five out of six isolates in cluster 10 were epidemiologically related,

being isolated from the same slaughter and farm groups. One group of isolates each in clusters 1 (isolates 762, 3995, and 4037) and 11 (isolates 26, 793, and 794) were epidemiologically related, representing the nursery, finishing, and slaughter stages of two different ABF farms. However, other isolates from these farms and slaughter stages were found to be unrelated and clustered separately. Overall, the population exhibited considerable genotypic diversity. The remaining clusters were very diverse based on the location and time of isolation and the resistance patterns of these isolates. This indicates the diverse nature of the *C. coli* isolates. The PFGE method had a discriminatory index of 0.889.

Comparison of clustering by MLST and PFGE. We identified a total of 27 and 11 clusters for the 100 *C. coli* isolates genotyped using MLST and PFGE, respectively. The association of MLST STs with PFGE clusters is shown in Fig. 1 and Table 2. Out of the 65 MLST STs observed for the complete data set, 53 STs (81.5%) representing 62 *C. coli* isolates were represented by a single PFGE cluster, indicating that these isolates were not distinguished by the PFGE method. The remaining 12 STs were clustered in two to three PFGE clusters. A single PFGE cluster (cluster 4) was found to be associated with a single ST (ST-854). The remaining 10 PFGE clusters were associated with multiple STs. PFGE type 1 was the most heterogeneous cluster and included 16 STs representing the farm and slaughter stages of both production systems. This indicates the poor correlation in polymorphism between MLST and PFGE findings.

Both of the methods were able to further differentiate the isolates clustered together in a single group by either MLST or PFGE. For example, PFGE cluster types 1 ($n = 17$ isolates) and 11 ($n = 16$ isolates) were differentiated into 10 and 8 MLST clusters, respectively. Similarly, MLST cluster 9 ($n = 18$ isolates) was represented by six PFGE clusters when typed by PFGE. However, we found that MLST had a better discriminatory power of 0.936 compared to 0.889 for PFGE. Visual comparison of the two genotyping approaches as shown in Table 2 reflected the ability of MLST to discriminate better between isolates that were clustered in the same group by PFGE. Overall, MLST was able to discriminate better between the *C. coli* isolates from the conventional and the ABF systems. MLST had a higher test throughput, as reactions can be carried

TABLE 2. MLST and PFGE data for the 100 *C. coli* isolates

Production system	Processing stage	Resistance pattern[b]	Isolate ID	aspA	glnA	gltA	glyA	pgm	tkt	uncA	ST[c]	MLST cluster	PFGE cluster
ABF	Nursery	NAL	53	33	39	30	78	104	43	17	1068	4	5
ABF	Nursery	Pansusceptible	183	33	39	30	82	104	43	68	1112	22	11
ABF	Nursery	Pansusceptible	752	33	38	30	82	104	173	68	1134	18	11
ABF	Nursery	ERY TET	26	33	39	37	82	104	43	68	1414	21	11
ABF	Nursery	Pansusceptible	252	33	39	37	82	104	43	68	1414	21	3
ABF	Nursery	TET	762	33	39	30	174	118	35	17	1431	6	1
ABF	Nursery	ERY NAL TET	7	33	39	46	82	104	43	68	1437	21	1
ABF	Nursery	CIP NAL TET	260	33	153	30	82	104	44	17	1438	15	10
ABF	Nursery	TET	206	33	38	30	82	104	43	17	854	4	4
ABF	Finishing	TET	4010	33	38	30	82	104	43	17	854	4	4
ABF	Finishing	TET	4082	33	38	30	82	104	43	17	854	4	4
ABF	Finishing	TET	2003	33	38	30	82	104	35	36	890	9	3
ABF	Finishing	ERY NAL TET	2112	33	38	30	82	104	35	17	1096	9	3
ABF	Finishing	ERY	793	33	38	30	82	152	173	68	1102	18	11
ABF	Finishing	ERY TET	786	33	39	30	82	104	43	68	1112	22	2
ABF	Finishing	Pansusceptible	3518	53	38	44	82	118	35	36	1123	13	2
ABF	Finishing	Pansusceptible	3978	53	38	44	82	118	35	36	1123	13	8
ABF	Finishing	NAL TET	953	33	38	30	82	104	173	68	1134	18	11
ABF	Finishing	Pansusceptible	2059	33	38	30	82	104	173	68	1134	18	11
ABF	Finishing	Pansusceptible	3819	33	38	30	82	104	173	68	1134	18	11
ABF	Finishing	TET	4115	53	38	30	82	118	35	36	1200	13	8
ABF	Finishing	CIP NAL TET	4076	33	39	132	82	104	44	17	1424	3	7
ABF	Finishing	Pansusceptible	3969	53	38	30	81	118	85	36	1426	1	11
ABF	Finishing	ERY TET	3995	33	39	30	79	104	56	17	1427	5	1
ABF	Finishing	ERY	1987	33	39	30	82	118	35	36	1432	12	5
ABF	Finishing	ERY NAL TET	952	33	39	134	82	104	56	17	1433	5	1
ABF	Finishing	TET	4077	33	39	44	82	104	44	17	1436	3	7
ABF	Finishing	ERY TET	1010	33	38	30	82	118	35	17	1446	9	3
ABF	Finishing	ERY TET	4017	33	38	30	82	118	35	17	1446	9	3
ABF	Finishing	Pansusceptible	1038	33	38	32	82	104	35	68	1447	18	11
ABF	Finishing	ERY TET	2164	53	39	44	82	118	35	17	1448	11	8
ABF	Finishing	ERY TET	2191	53	39	44	82	104	35	36	1450	10	1
ABF	Finishing	NAL	3552	53	39	44	82	104	35	36	1450	10	8
ABF	Finishing	CIP NAL TET	4075	33	39	46	82	104	44	17	1452	3	7
ABF	Finishing	Pansusceptible	4100	33	39	46	82	104	44	17	1452	3	7
ABF	Finishing	TET	2032	33	39	30	82	113	47	17	825	2	9
ABF	Preevisceration	ERY	794	33	39	47	82	104	43	36	1415	22	11
ABF	Preevisceration	Pansusceptible	4029	33	39	30	82	118	35	36	1432	12	8
ABF	Preevisceration	Pansusceptible	843	33	39	30	82	104	85	17	1445	4	1
ABF	Preevisceration	Pansusceptible	3580	53	39	44	82	104	35	36	1450	10	8
ABF	Preevisceration	Pansusceptible	3600	53	39	37	82	104	85	17	1451	25	1
ABF	Postevisceration	ERY TET	4037	33	38	30	82	104	35	36	890	9	1
ABF	Postevisceration	TET	2205	33	38	30	78	104	35	17	1113	8	5
ABF	Postevisceration	TET	4042	53	38	44	82	118	35	36	1123	13	8
ABF	Postevisceration	TET	4049	53	38	44	82	118	35	36	1123	13	8
ABF	Postevisceration	ERY	817	33	153	30	82	118	43	17	1416	15	1
ABF	Postevisceration	ERY	4055	33	39	30	82	118	35	36	1432	12	8
ABF	Postevisceration	TET	2207	33	38	37	78	104	35	17	1449	8	5
ABF	Postchill	Pansusceptible	3635	33	39	30	82	104	43	17	828	4	8
ABF	Postchill	ERY	3630	53	38	30	81	118	35	36	1469	1	8
Conv[a]	Nursery	CIP ERY NAL TET	475	33	38	30	82	104	35	17	1096	9	5
Conv	Nursery	CIP ERY NAL TET	524	33	38	30	82	104	117	17	1413	9	10
Conv	Nursery	CIP ERY NAL TET	525	33	38	30	82	104	117	17	1413	9	1
Conv	Nursery	CIP ERY NAL TET	549	33	38	30	82	104	117	17	1413	9	9
Conv	Nursery	CIP ERY NAL TET	552	33	38	30	82	104	117	17	1413	9	9
Conv	Nursery	CIP ERY NAL TET	554	33	38	30	82	104	117	17	1413	9	10
Conv	Nursery	CIP ERY NAL TET	555	33	38	30	82	104	117	17	1413	9	10
Conv	Nursery	CIP ERY NAL TET	556	33	38	30	82	104	117	17	1413	9	10
Conv	Nursery	ERY	84	33	39	44	82	104	35	36	1417	10	8
Conv	Nursery	ERY TET	503	33	39	46	174	104	35	17	1418	6	1
Conv	Nursery	CIP ERY GEN NAL TET	548	33	38	46	82	104	117	17	1419	19	9
Conv	Nursery	CIP ERY GEN NAL TET	461	33	39	46	82	113	35	17	1440	2	1
Conv	Nursery	CIP ERY NAL TET	526	33	38	37	82	104	117	17	1441	19	11
Conv	Nursery	ERY GEN TET	533	33	39	30	82	113	117	17	1465	2	1
Conv	Finishing	ERY TET	3441	33	38	30	82	104	43	17	854	4	2
Conv	Finishing	TET	3736	33	38	30	82	104	43	17	854	4	4
Conv	Finishing	ERY TET	4246	33	39	30	82	104	43	36	1056	22	2

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TABLE 2—*Continued*

Production system	Processing stage	Resistance pattern[b]	Isolate ID	Allelic profile							ST[c]	MLST cluster	PFGE cluster
				aspA	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*			
Conv	Finishing	ERY TET	827	33	153	30	82	104	35	17	1059	15	3
Conv	Finishing	CHL ERY TET	4511	33	38	30	82	104	35	17	1096	9	3
Conv	Finishing	ERY	872	33	38	30	82	104	85	17	1177	9	3
Conv	Finishing	ERY TET	4489	33	39	44	82	104	35	36	1417	10	2
Conv	Finishing	TET	648	32	39	115	115	104	85	17	1420	26	1
Conv	Finishing	ERY TET	423	33	38	46	82	104	173	17	1422	19	11
Conv	Finishing	ERY TET	429	33	39	46	82	104	47	17	1423	3	1
Conv	Finishing	CHL CIP ERY NAL TET	4516	33	39	134	174	104	43	68	1425	21	1
Conv	Finishing	CHL CIP ERY NAL TET	4517	33	39	134	174	104	43	68	1425	21	1
Conv	Finishing	ERY TET	841	33	39	44	82	189	35	36	1429	10	2
Conv	Finishing	TET	4396	32	153	30	82	104	44	36	1430	16	2
Conv	Finishing	TET	448	32	38	44	82	104	43	17	1434	23	2
Conv	Finishing	ERY TET	4490	33	153	30	82	104	44	17	1438	15	9
Conv	Finishing	ERY NAL TET	458	33	38	30	82	104	173	17	1439	9	11
Conv	Finishing	ERY NAL TET	459	33	38	30	82	104	173	17	1439	9	11
Conv	Finishing	ERY TET	623	33	38	132	82	104	85	17	1442	19	3
Conv	Finishing	TET	653	33	153	30	173	217	44	68	1466	14	11
Conv	Finishing	ERY	3451	33	39	30	82	113	47	17	825	2	9
Conv	Preevisceration	ERY TET	3480	33	38	30	82	104	35	36	890	18	3
Conv	Preevisceration	ERY	690	33	38	44	82	104	117	36	1421	20	2
Conv	Preevisceration	ERY	694	33	39	134	161	118	43	17	1435	24	3
Conv	Preevisceration	ERY	699	32	38	44	82	113	43	36	1443	23	2
Conv	Preevisceration	ERY TET	4324	32	38	30	167	104	35	17	1453	7	3
Conv	Postevisceration	TET	3491	33	38	30	82	104	43	17	854	4	11
Conv	Postevisceration	TET	4559	33	38	30	82	104	85	68	887	18	6
Conv	Postevisceration	ERY TET	3507	53	38	30	81	104	44	36	1097	1	3
Conv	Postevisceration	ERY TET	4276	53	38	44	82	118	35	36	1123	13	8
Conv	Postevisceration	ERY TET	892	33	38	134	161	104	43	17	1125	24	3
Conv	Postevisceration	TET	3490	33	38	30	82	104	43	68	1130	18	11
Conv	Postevisceration	TET	4549	33	153	30	82	104	43	36	1142	22	6
Conv	Postevisceration	ERY TET	4338	58	38	30	167	118	35	17	1454	7	3
Conv	Postevisceration	TET	4560	33	38	37	82	104	85	68	1455	17	6
Conv	Postchill	ERY TET	4445	53	38	30	81	118	43	36	1428	1	2

[a] Conventional farms.

[b] CHL, chloramphenicol; CIP, ciprofloxacin; ERY, erythromycin; GEN, gentamicin; NAL, nalidixic acid; TET, tetracycline.

[c] Sequence type indicates the unique number assigned on the basis of the allelic profile generated based on the allele nucleotide sequence number in the MLST database (www.pubmlst.org/campylobacter).

out in 96-well plates. However, PFGE was more cost-effective, costing approximately $7.00 per reaction compared to $42 for MLST.

Identification of antimicrobial resistance genes. All of the 21 *C. coli* isolates were positive for the *tet*(O) gene as shown in Table 3. These isolates were resistant at the highest concentration of tetracycline tested (32 µg/liter). A point mutation from adenine to guanine at position A2075G in the peptidyl transferase region of 23S rRNA was detected in all *C. coli* isolates that were resistant to erythromycin. Sixteen of the 21 isolates were resistant at the highest concentration of erythromycin tested (32 µg/liter). A single isolate for which the MIC was 16 µg/liter also carried the point mutation in the 23S rRNA gene. Ten of the 17 ciprofloxacin-resistant *C. coli* isolates were positive by MAMA PCR, indicating the point mutation at amino acid position 86 with threonine (Thr) replaced by isoleucine (Ile) (Table 3).
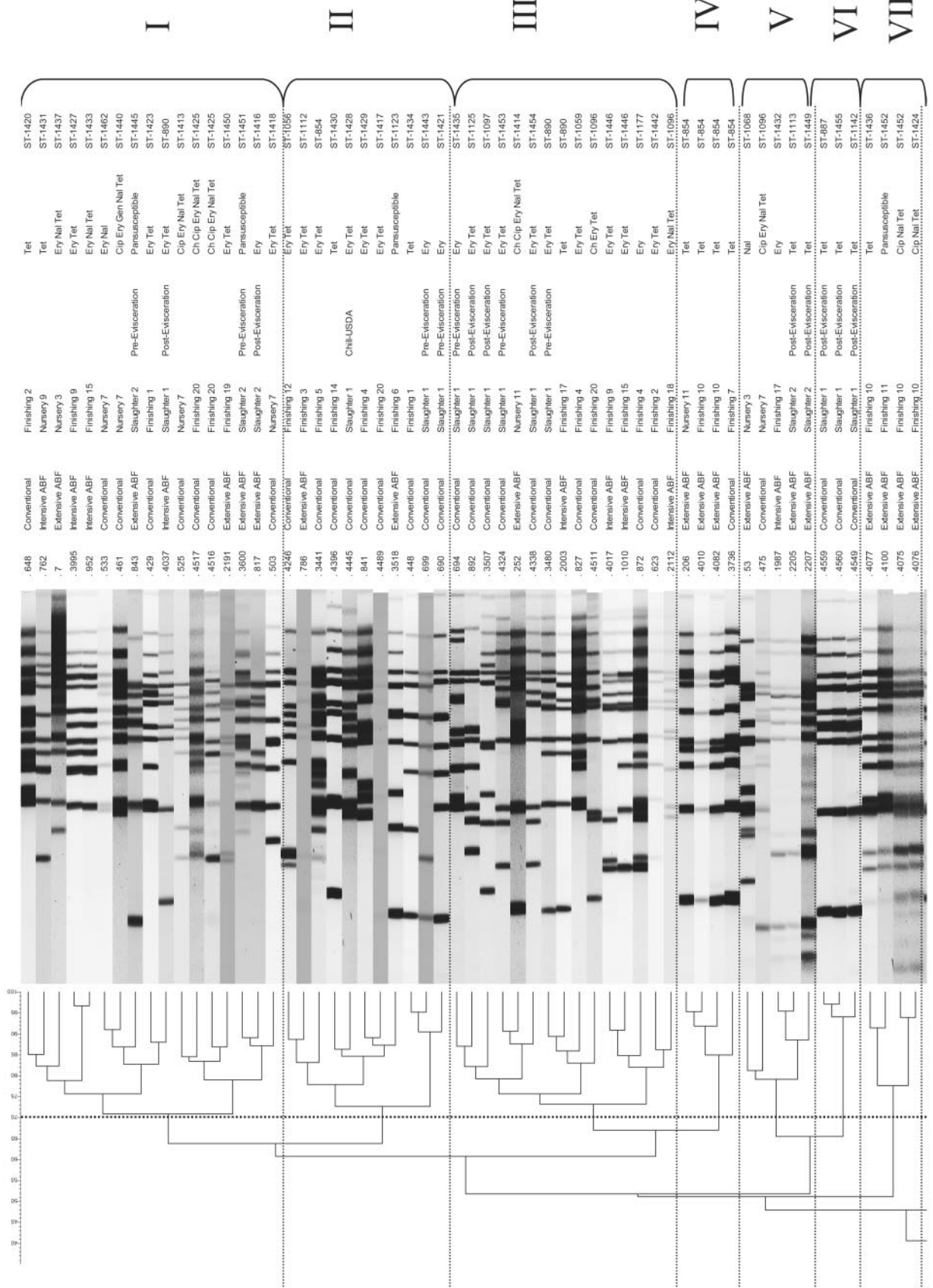
Ciprofloxacin-resistant *C. coli* isolates that were resistant at the concentration of 4 µg/liter were further tested by the same method at concentrations up to 64 µg/liter to determine the MIC. Five out of the 10 *C. coli* isolates were found resistant at the highest concentration tested, while an MIC of 64 µg/liter was found for two isolates. Overall, MICs for ciprofloxacin

were higher for isolates from the conventional system than for those from the ABF system. Sequencing of the QRDR of the *gyrA* region for all of the 17 isolates revealed a point mutation at amino acid position 147 with glutamic acid replaced by aspartic acid (GAA replaced by GAC) and two silent mutations in isolates 554 and 556 at positions 116 (alanine; GCA replaced by GCT) and 108 (glycine; GGA replaced by GGC), respectively (Table 3). The remaining isolates, including the MAMA PCR-negative isolates, did not reveal any mutation in their QRDRs.

DISCUSSION

There are a multitude of studies that have reported the weak clonal population structure and the hypervariable genome of *Campylobacter* (7, 8, 34, 47). This makes the choice of using a genotyping method for determining the source of an outbreak or comparing isolates from different sources more complex and difficult to interpret. To circumvent this problem, an MLST scheme was developed for *C. jejuni* and has been shown to be a reliable method for typing human, animal, and environmental strains of this pathogen (5, 7, 24, 25, 42). Recently, the MLST scheme was extended for typing *C. coli*, another impor-

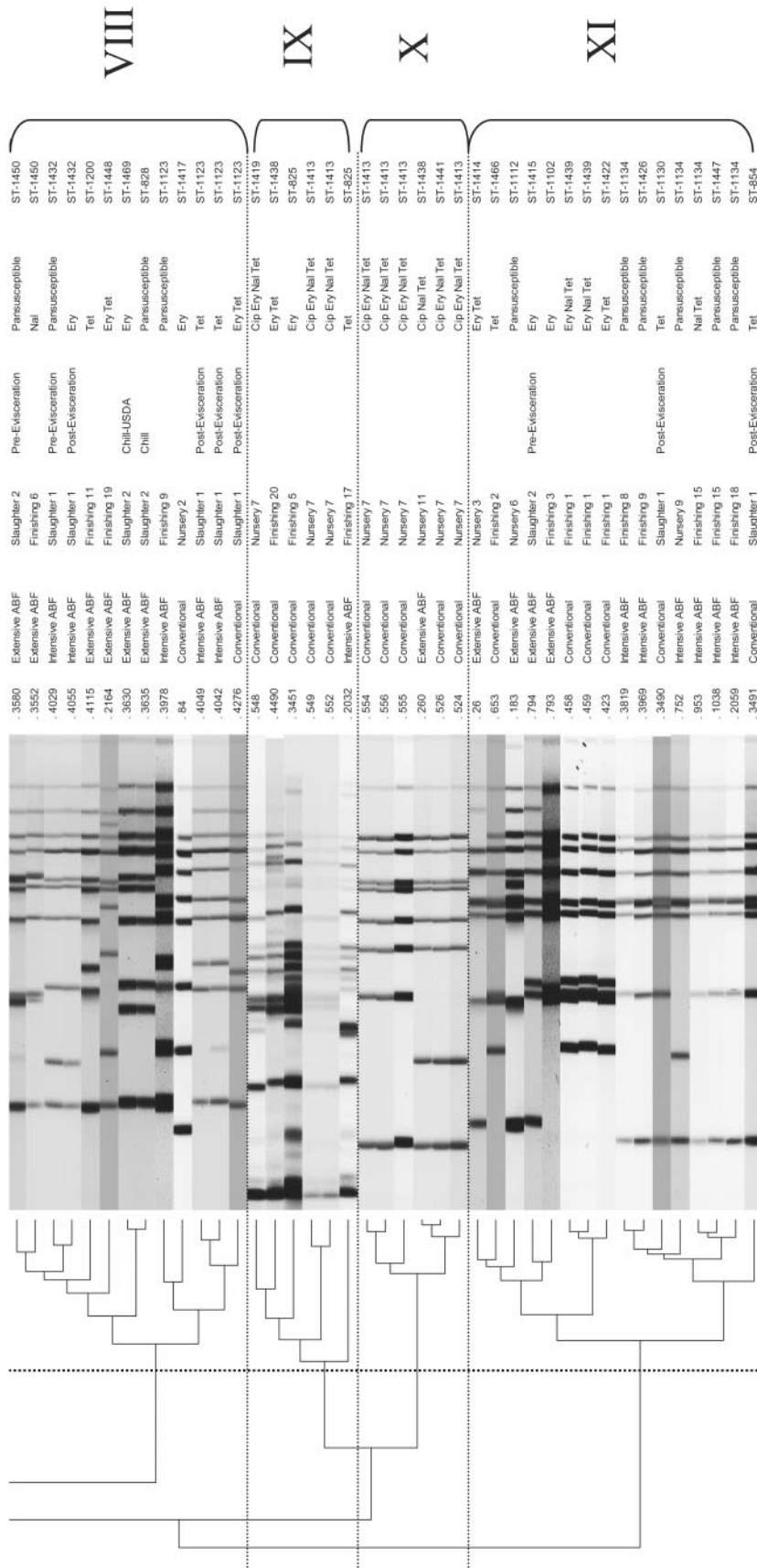| PFGE Clusters	Sequence Type	Resistance Pattern	Slaughter Stage	Farm/ Slaughter	Production System	Isolate ID
I	ST-1420	Tet		Finishing 2	Conventional	648
	ST-1431	Tet		Nursery 9	Intensive ABF	762
	ST-1437	Ery Nal Tet		Nursery 3	Extensive ABF	7
	ST-1427	Ery Tet		Finishing 9	Intensive ABF	3995
	ST-1433	Ery Nal Tet		Finishing 15	Intensive ABF	952
	ST-1462	Ery Nal		Nursery 7	Conventional	533
	ST-1440	Cip Ery Gen Nal Tet		Nursery 7	Conventional	461
	ST-1445	Pansusceptible	Pre-Evisceration	Slaughter 2	Extensive ABF	843
	ST-1423	Ery Tet		Finishing 1	Conventional	429
	ST-890	Ery Tet	Post-Evisceration	Slaughter 1	Intensive ABF	4037
	ST-1413	Cip Ery Nal Tet		Nursery 7	Conventional	525
	ST-1425	Ch Cip Ery Nal Tet		Finishing 20	Conventional	4517
	ST-1425	Ch Cip Ery Nal Tet		Finishing 20	Conventional	4516
	ST-1450	Ery Tet	Pre-Evisceration	Finishing 19	Extensive ABF	2191
	ST-1451	Pansusceptible	Post-Evisceration	Slaughter 2	Extensive ABF	3600
	ST-1416	Ery	Post-Evisceration	Slaughter 2	Extensive ABF	817
	ST-1418	Ery Tet		Nursery 7	Conventional	503
II	ST-1056	Ery Tet		Finishing 12	Conventional	4246
	ST-1112	Ery Tet		Finishing 3	Extensive ABF	786
	ST-854	Ery Tet		Finishing 5	Conventional	3441
	ST-1430	Tet		Finishing 14	Conventional	4396
	ST-1428	Ery Tet	Chili-USDA	Slaughter 1	Conventional	4445
	ST-1429	Ery Tet		Finishing 4	Conventional	841
	ST-1417	Ery Tet		Finishing 20	Conventional	4489
	ST-1123	Pansusceptible		Finishing 6	Extensive ABF	3518
	ST-1434	Tet	Pre-Evisceration	Finishing 1	Conventional	448
	ST-1443	Ery	Pre-Evisceration	Slaughter 1	Conventional	699
III	ST-1421	Ery	Pre-Evisceration	Slaughter 1	Conventional	694
	ST-1435	Ery Tet	Post-Evisceration	Slaughter 1	Conventional	892
	ST-1125	Ery Tet	Pre-Evisceration	Slaughter 1	Conventional	3507
	ST-1097	Ery Tet		Slaughter 1	Conventional	4324
	ST-1414	Ch Cip Ery Nal Tet	Post-Evisceration	Nursery 11	Extensive ABF	252
	ST-1454	Ery Tet	Pre-Evisceration	Slaughter 1	Conventional	4338
	ST-1417	Ery Tet		Slaughter 1	Conventional	3480
	ST-890	Tet		Finishing 17	Intensive ABF	2003
	ST-1059	Ery Tet		Finishing 4	Conventional	827
	ST-1096	Ch Ery Tet		Finishing 20	Conventional	4511
	ST-1446	Ery Tet		Finishing 9	Intensive ABF	4017
	ST-1177	Ery Tet		Finishing 15	Intensive ABF	1010
	ST-1442	Ery		Finishing 4	Conventional	872
	ST-1096	Ery Nal Tet		Finishing 2	Conventional	623
IV	ST-1096	Ery Nal Tet		Finishing 18	Intensive ABF	2112
	ST-854	Tet		Nursery 11	Extensive ABF	206
	ST-854	Tet		Finishing 10	Conventional	4010
	ST-854	Tet		Finishing 10	Extensive ABF	4082
V	ST-1068	Nal		Finishing 7	Conventional	3736
	ST-1432	Cip Ery Nal Tet		Nursery 3	Extensive ABF	53
	ST-1113	Ery		Nursery 7	Conventional	475
	ST-1449	Tet	Post-Evisceration	Finishing 17	Intensive ABF	1987
VI	ST-1455	Tet	Post-Evisceration	Slaughter 2	Extensive ABF	2205
	ST-1142	Tet	Post-Evisceration	Slaughter 1	Conventional	4559
	ST-1436	Tet	Post-Evisceration	Slaughter 1	Conventional	4560
VII		Pansusceptible		Slaughter 1	Conventional	4549
	ST-887	Pansusceptible		Finishing 10	Extensive ABF	4077
	ST-1452	Cip Nal Tet		Finishing 11	Extensive ABF	4100
	ST-1452	Cip Nal Tet		Finishing 10	Extensive ABF	4075
	ST-1424			Finishing 10	Extensive ABF	4076

FIG. 1. PFGE dendrogram for the 100 *C. coli* isolates showing the 11 clusters at 70% genetic similarity among the band profiles.

TABLE 3. Antimicrobial resistance and molecular characterization of *C. coli* isolates isolated from swine in this study

Isolate ID	Production system	Processing stage	MIC (µg/ml)						Resistance pattern[b]	Mutation[c]	
			CHL (32)[a]	CIP (4)	ERY (8)	GEN (16)	NAL (32)	TET (16)		Thr 86 Ile	23S rRNA gene
49	ABF	Nursery	4	0.25	8	2	32	>32	ERY NAL TET	−	−
260	ABF	Nursery	8	8	4	8	>128	>32	CIP NAL TET	−	−
952	ABF	Finishing	16	2	>32	2	>128	>32	ERY NAL TET	−	−
966	ABF	Finishing	8	2	16	2	>128	>32	ERY NAL TET	−	+
1005	ABF	Finishing	4	1	>32	1	>128	>32	ERY NAL TET	−	+
4075	ABF	Finishing	8	8	4	8	>128	>32	CIP NAL TET	−	−
4076	ABF	Finishing	8	8	4	8	>128	>32	CIP NAL TET	−	−
3804	ABF	Finishing	8	4	>32	2	64	>32	CIP ERY NAL TET	−	−
461	Conventional	Nursery	4	4	>32	16	>128	>32	CIP ERY GEN NAL TET	−	+
475	Conventional	Nursery	8	>4	>32	2	128	>32	CIP ERY NAL TET	+	+
524	Conventional	Nursery	8	>4	>32	8	>128	>32	CIP ERY NAL TET	+	+
525	Conventional	Nursery	8	64	>32	1	128	>32	CIP ERY NAL TET	+	+
526	Conventional	Nursery	8	64	>32	1	128	>32	CIP ERY NAL TET	+	+
548	Conventional	Nursery	2	>64	>32	1	128	>32	CIP ERY NAL TET	+	+
549	Conventional	Nursery	2	>64	>32	2	128	>32	CIP ERY NAL TET	+	+
552	Conventional	Nursery	4	>64	>32	2	128	>32	CIP ERY NAL TET	+	+
554	Conventional	Nursery	4	>64	>32	2	128	>32	CIP ERY NAL TET	+	+
555	Conventional	Nursery	4	>64	>32	2	128	>32	CIP ERY NAL TET	+	+
459	Conventional	Finishing	8	0.5	>32	1	32	>32	ERY NAL TET	−	+
4516	Conventional	Finishing	32	4	>32	1	128	>32	CHL CIP ERY NAL TET	−	+
4517	Conventional	Finishing	32	4	>32	1	128	>32	CHL CIP ERY NAL TET	−	+

[a] Breakpoint level based on *C. jejuni* ATCC 33560 in µg/ml antimicrobial concentration.
[b] CHL, chloramphenicol; CIP, ciprofloxacin; ERY, erythromycin; GEN, gentamicin; NAL, nalidixic acid; TET, tetracycline.
[c] Point mutations in *gyrA* and the 23S rRNA genes coding for ciprofloxacin and erythromycin resistance, respectively. All isolates were positive for the *tet*(O) gene.

tant species of *Campylobacter* besides *C. jejuni* that causes food-borne gastroenteritis (6, 8, 44). Another technique used routinely for typing both *C. jejuni* and *C. coli* is PFGE (3, 4, 13, 22, 41, 42). This method is being used by PulseNet within the United States for the nationwide surveillance of this pathogen along with other food-borne pathogens like *Salmonella* and *Shigella* (43). It has been used both for typing this pathogen and for discriminating between *C. jejuni* isolates responsible for 12 outbreaks in the United States (42). These two methods have not been used together to investigate the genetic diversity of *C. coli* strains isolated from swine. The utility of both the methods as tools for understanding the epidemiology of this pathogen in the swine production environment is important. Therefore, in this study, we used these two methods for genotyping 100 phenotypically diverse *C. coli* isolated from swine reared under conventional and antimicrobial-free production systems and compared their discriminatory powers, throughputs, and group associations.

MLST and PFGE differentiated the isolates into 26 and 11 clusters, respectively, exhibiting high levels of discrimination depending on the farm type and the antimicrobial resistance pattern. Our results were consistent with other studies where both these methods have been shown to differentiate between closely related strains of *Campylobacter* (24, 41, 42). The distribution of specific STs among the isolates at different stages of production and with particular resistance patterns indicated that certain STs were adapted to a specific stage of production. Specific STs were found either at the farm or at the slaughter stage (Tables 1 and 2). For example, ST-1413 was seen in *C. coli* isolates with a CIP ERY NAL TET resistance pattern and only in isolates from pigs at a nursery farm in the conventional production system. Similar results have been reported by

other studies where specific clones of *C. jejuni* have been found associated with particular niches (5). A study in the United Kingdom reported a *C. coli* strain that may have become adapted to persist in water and act as a source of infection to humans (24).

The results observed in our study of specific STs being associated with a specific production or processing stage such as slaughter may imply that not all strains detected at slaughter originated from the farm, and other factors such as cross-contamination during trucking and in holding pens remain a concern. The genotyping results provided evidence of multiple *Campylobacter* genotypes grouped together in different clusters. We observed clusters with isolates that were not related either temporally or spatially, indicating significant genotypic diversity. Hume et al., reported the absence of shared genotype from isolates that were isolated from the sow, its respective piglets and the littermates highlighting the diverse genome of this pathogen (21). Similar observations were made when we analyzed the clusters with respect to the resistance patterns. Barring a few STs that were restricted to specific resistance patterns (ST-1413 was associated with isolates with a CIP ERY NAL TET resistance pattern), most of the STs were found to be associated with multiple resistance patterns. *C. jejuni* isolates with similar PFGE patterns but with different resistance patterns have been reported before (3).

We found MLST to have a better discriminatory power and test throughput than PFGE. Previous studies have reported the better discriminatory power of PFGE compared to MLST when used for typing *C. jejuni* (41, 42). However, MLST has been found to be as discriminatory as PFGE for distinguishing between temporally related isolates and the epidemic-causing isolates in different outbreaks caused by *C. jejuni* (42). The

ability of MLST to have the same value for epidemiological typing as that of PFGE, AFLP, and ribotyping has also been demonstrated before (10). In the current study, PFGE resulted in good discrimination, but it did not discriminate as well as MLST. These findings imply that both genotyping methods are very good, and the use of both methods would ideally be a preferred way for tracking clones. It should also be emphasized that the outcome of PFGE typing is susceptible to changes in the chromosome and inter- and intragenomic recombinations, making this method unsuitable for studying the epidemiology of pathogens with hyperplastic genome including *Campylobacter* (18, 34, 47, 48).

Analysis of clusters generated by the two methods revealed the high level of genotypic diversity present in *C. coli*. We detected clustering of isolates based on the processing stage or sample type rather than the production system. However, in a study conducted in a dairy cattle environment, no clustering of *C. coli* isolates based on sample type was observed (24). This difference could be attributed to the different environments for dairy cattle and pigs. Seventeen clonal complexes have been defined so far for *C. jejuni*, and association of these clonal complexes with the given host has been shown by MLST and PFGE (5, 7). There are only two clonal complexes that have been defined for *C. coli* so far (29). Our lab is currently involved in defining the clonal complex for this species of *Campylobacter* in swine. This will help us in understanding its epidemiology in different hosts and environments.

The main mechanism of resistance against ciprofloxacin was the threonine-to-isoleucine point mutation at amino acid position 86 as confirmed by both MAMA PCR and sequencing of the QRDR. Mutation at amino acid position 86 has been shown to confer high resistance to this antimicrobial at concentrations ranging from 32 µg/liter up to 128 µg/liter (17, 36). A single isolate carried another mutation at position 147 in addition to the mutation at position 86. We do not know at this stage whether the additional mutation has potentiated the ability of this isolate to become resistant to even higher concentration of ciprofloxacin than isolates that carry a mutation only at position 86. Other mutations in the QRDR that have been linked to ciprofloxacin resistance in *Campylobacter* include Asp-90, Ala-70, and Pro-104 mutations (11, 36). Recently, high resistance to moxifloxacin, a fluoroquinolone, was shown in *Campylobacter* isolates that had a double mutation in the QRDR of the *gyrA* gene.

Resistance against tetracycline was mediated through the *tet*(O) gene in 100% of the isolates that showed the tetracycline resistance phenotype and tested with PCR. The *tet*(O) gene, which can be both plasmid and chromosomally located, has been shown to confer high resistance against tetracycline ranging from >256 to 512 µg/liter concentrations in *C. coli* and *C. jejuni*, respectively (16, 37). All of the 21 isolates tested were highly resistant to tetracycline (MIC > 32 µg/liter). The ability of this gene to be transferred both intraspecies and interspecies in *Campylobacter* through conjugation could explain the high frequency of resistance seen against this antimicrobial in isolates in this study. *C. coli* isolates with an A2075G point mutation in their 23S rRNA gene were responsible for resistance against the macrolide erythromycin. In *C. coli*, resistance against erythromycin has been shown before to be mediated through the A2075G point mutation (16, 35). Although the

highest concentration of erythromycin tested in our study was 32 µg/liter, *C. coli* isolates with the above point mutation have been shown to exhibit resistance to as high as >1,024 µg/liter of the antimicrobial (16). The simultaneous resistance to important classes of antimicrobials, including fluoroquinolones and macrolides, shown by the 21 characterized *C. coli* isolates is very concerning.

The overall *C. coli* population had a weaker clonal structure ($I_A = 0.293$) compared to the I_A value of 0.57 for *C. jejuni* (7). Our findings show that the *C. coli* population has low clonal structure, and widespread genotypic diversity was seen. In *C. jejuni*, 17 clonal complexes have been defined so far, and association of these clonal complexes with the given host has been shown by MLST and PFGE (5, 7). Our lab is currently involved in defining a clonal complex for this species of *Campylobacter*. We found the *C. coli* population isolated from the conventional production system to be slightly more clonal than that of the ABF system. This could be attributed to the presence of a different lineage of this species circulating in these production systems. Our results differ from other studies that have reported *C. coli* to be less diverse than *C. jejuni* by using MLST and AFLP (8, 9). However, it should also be noted that the isolates in the current study are not representative of the *C. coli* population existing in these systems, and we are cautious that no generalization is deduced from the study. We emphasize that MLST has the potential to be used for studying the molecular epidemiology of *Campylobacter* due to its high discriminatory power, the simplicity of data handling and analysis, reproducibility of sequence data, high test throughput, and the ease with which data can be exchanged between different laboratories via the internet. This study highlights the high genotypic diversity of antimicrobial-resistant *C. coli* in the swine production systems.

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