

Characterization of Chloramphenicol Resistance in Beta-Hemolytic *Escherichia coli* Associated with Diarrhea in Neonatal Swine

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Ninety beta-hemolytic *Escherichia coli* isolates associated with diarrhea in neonatal pigs from multiple farms in Oklahoma were investigated for known associated disease serotypes, virulence factors, ribotypes, and antimicrobial susceptibility phenotypes. Fifteen different serotypes were observed, with 58% of isolates belonging to groups that produce one of three major enterotoxins: O149, O147, and O139. Thirty percent of the swine *E. coli* isolates possessed a combination of F4 fimbriae and the heat-labile toxin and heat-stable toxin B enterotoxins. Seventy-three percent of the *E. coli* isolates were resistant to five or more antibiotics. Interestingly, 53% of swine *E. coli* isolates exhibited resistance to chloramphenicol (CHL), an antibiotic whose use in food animals has been prohibited in the United States since the mid-1980s. The *cmlA* gene, which encodes a putative CHL efflux pump, was detected by PCR in 47 of the 48 CHL-resistant isolates, and 4 of these also possessed the *cat2* gene, which encodes a chloramphenicol acetyltransferase. The one CHL-resistant isolate that did not contain either *cmlA* or *cat-2* possessed the *flo* gene, which confers resistance to both florfenicol and CHL. To determine whether CHL-resistant swine *E. coli* isolates represented dissemination of a clonal strain, all 90 isolates were analyzed by ribotyping. Seventeen distinct *E. coli* ribogroups were identified, with CHL resistance observed among the isolates in all except one of the major ribogroups. The identification of the *cmlA* gene among diverse hemolytic enterotoxigenic *E. coli* strains demonstrates its broad dissemination in the swine production environment and its persistence even in the absence of CHL selection pressure.

Antimicrobials are valuable tools that animal producers use to quickly address clinical disease and to maintain healthy and productive animals, but the treatment of whole herds and flocks with antimicrobials for disease prevention and growth promotion is a controversial practice (13, 16, 20, 23, 28). Broad use of antimicrobials in agriculture selects for resistant bacteria that may enter the food chain and potentially result in food-borne illness in humans that is less responsive to treatment with conventional antibiotics. In addition to the human health concerns, antimicrobial-resistant pathogens also pose a severe and costly animal health problem, as they prolong illness and decrease productivity through higher morbidity and mortality rates.

Escherichia coli is the most common etiologic agent of neonatal diarrhea in pigs aged 0 to 4 days (7, 13). Causative strains are usually enterotoxigenic *E. coli* (ETEC) isolates that colonize the small intestine and that produce one or more enterotoxins. Clinical signs of ETEC infection may first be observed within hours after birth, resulting in increased rates of mortality during the first few days of life. Treatment typically consists of a broad-spectrum antimicrobial, although resistance to such drugs has greatly increased over the last several years (1, 4, 11, 17, 22, 25).

Chloramphenicol (CHL) is a broad-spectrum antibiotic that was used extensively in veterinary medicine until concerns over

its toxicity emerged (26). Human exposure to CHL has been linked to aplastic anemia, a type of bone marrow suppression that is usually irreversible and often fatal. Interestingly, development of the disease does not appear to be dependent on the dose or duration of exposure to CHL. The possibility that trace residues of CHL in food products may induce the disease led the U.S. Food and Drug Administration to ban its use in food animals in the 1980s (14). Currently, only a fluorinated derivative of CHL, florfenicol (FFN), is approved for veterinary use in food animals, but FFN is not approved for use in swine in the United States.

Resistance to CHL may be mediated either enzymatically through the chemical inactivation of the drug or nonenzymatically through drug efflux. Chloramphenicol acetyltransferase catalyzes the acetylation of the 3'-OH of CHL and is responsible for most enzymatic resistance to CHL (27, 29). The *cmlA* gene confers nonenzymatic resistance to CHL. Although its mechanism has yet to be characterized, the similarity of the primary structure of the *cmlA* protein to those of bacterial transport proteins suggests that it functions as a drug efflux pump (6, 32). The *flo* gene, whose product shares 57% amino acid sequence identity to the product of *cmlA*, also encodes a putative efflux pump that confers resistance to both CHL and FFN (8, 9, 12, 21). Additionally, Cloeckert et al. (10) recently reported on a new *flo* gene variant that was identified on the R55 IncC plasmid isolated from *Klebsiella pneumoniae* and that also confers nonenzymatic CHL resistance.

The present study examined the antimicrobial susceptibility patterns and genetic relatedness of beta-hemolytic *E. coli* strains isolated from neonatal swine with diarrhea. We hypoth-

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esized that a high percentage of isolates would be clonal in nature and resistant to antimicrobials commonly used in swine production. Although we do report a high rate of multiple-drug-resistant phenotypes, a surprisingly high incidence of resistance to CHL and FFN was observed, despite the lack of an obvious selection pressure in swine production. We further investigated the mechanisms of CHL resistance since there is limited information regarding the molecular mechanisms of resistance to this drug among hemolytic swine ETEC isolates.

MATERIALS AND METHODS

Materials. Materials purchased from commercial sources included antimicrobial susceptibility plates and JustOne strips (Trek Diagnostic Systems, Westlake, Ohio), AmpliTaq Gold DNA polymerase and deoxynucleoside triphosphates (Applied Biosystems, Foster City, Calif.), and RiboPrinter reagents (Qualicon, Inc., Wilmington, Del.). The oligonucleotides used as primers in the PCR were synthesized by Biosynthesis, Inc. (Lewisville, Tex.). PCR products were submitted for DNA sequencing at the DNA Core Facility in the Department of Veterinary Pathobiology, Texas A&M University, College Station.

Bacterial strains. The present study focuses on 90 beta-hemolytic *E. coli* isolates recovered from neonatal pigs with diarrhea from multiple farms in Oklahoma from 1998 to 1999. *E. coli* was isolated from swine intestines upon necropsy by spread plating on blood and MacConkey agar plates. Indole and oxidase tests were performed for lactose-positive colonies. API 20E test strips (bioMérieux Vitek, Hazelwood, Mo.) were also used to confirm the identification of the isolate as *E. coli*. Swine *E. coli* isolates displaying decreased susceptibilities to CHL were subsequently collected for further analysis to determine the mechanism of resistance. Isolates were stored as 10% glycerol stocks at -80°C until analysis. Isolates were submitted to the *E. coli* Reference Center located at the Pennsylvania State University for O-antigen serotyping and virulence factor analysis.

Determination of antimicrobial susceptibility. The MICs of the antimicrobials were determined by broth microdilution according to the methods described by the National Committee for Clinical Laboratory Standards (NCCLS) (24). Susceptibility testing was performed with the Sensititre automated antimicrobial susceptibility system according to the manufacturer's instructions (Trek Diagnostic Systems). The following antimicrobials were assayed: amikacin, amoxicillin-clavulanic acid, ampicillin, apramycin, ceftiofur, ceftriaxone, cephalothin, CHL, ciprofloxacin, FFN, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim-sulfamethoxazole. *E. coli* ATCC 25922, *E. coli* ATCC 35218, and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains for broth microdilution susceptibility testing.

PCR. Genes encoding antimicrobial resistance determinants were detected by PCR. The primer sets used for amplification of *cmlA*, *flo*, *cat-1*, *cat-2*, and *cat-3* were the same as those described previously (19, 29). Templates of total DNA from each isolate were prepared as follows. Bacteria were streak plated onto tryptic soy agar plates containing 5% sheep's blood, and the plates were incubated overnight at 35°C . Three to five bacterial colonies were lifted from the plate and resuspended in 0.5 ml of sterile water. The suspension was heated to 95°C for 10 min, and then the cell debris was removed by centrifugation. The supernatant (10 μl) was used as the template in the PCRs. Each reaction mixture (50 μl) also contained $1\times$ AmpliTaq Gold DNA polymerase reaction buffer, 2.5 mM MgCl_2 , 1 mM deoxynucleoside triphosphates, 1 pmol of each respective oligonucleotide primer per μl , and 1 U of AmpliTaq Gold DNA polymerase. All reaction mixtures were first heated to 95°C for 10 min to activate the AmpliTaq Gold polymerase. The denaturation, annealing, and extension conditions for PCR were the same as those described previously for each primer set (19, 29). Sequence comparisons were made with the BLAST program of the National Center for Biotechnology Information (3).

Ribotyping. Ribotyping was performed with the RiboPrinter Microbial Characterization System (Qualicon, Inc.) and the standard *EcoRI* DNA preparation kit, as described in the manufacturer's operations and analytical guides. Bacterial DNA was digested with *EcoRI*, and gel electrophoresis was used to separate the restriction fragments into distinctive patterns. DNA from isolates with the different DNA patterns was hybridized with a chemiluminescent *E. coli* rRNA probe. The characterization system automatically placed the pattern for each isolate into common ribogroups (clusters) on the basis of the similarity of the band positions to the band positions and the intensities of patterns in the RiboPrinter's database. The ribogroups were analyzed and visually refined by the manufacturer's standard procedure. A dendrogram was constructed from

TABLE 1. Antimicrobial resistance phenotypes of swine *E. coli* isolates

Class and antimicrobial	% Resistant strains ^a (n = 90)
Phenicol	
FFN ^b	64
CHL.....	53
Penicillins	
Ampicillin	34
Amoxicillin-clavulanic acid	4
Cephalosporins	
Cephalothin	13
Ceftiofur.....	1
Ceftriaxone	0
Tetracycline	96
Aminoglycosides	
Amikacin	0
Apramycin.....	14
Gentamicin	14
Kanamycin	84
Streptomycin.....	82
Sulfonamides and potentiated sulfonamides	
Sulfamethoxazole	89
Trimethoprim-sulfamethoxazole	22
Quinolones or fluoroquinolones	
Nalidixic acid.....	0
Ciprofloxacin	0

^a MICs were determined by microdilution methods according to NCCLS standards.

^b Resistance was based on the NCCLS breakpoint for bovine respiratory pathogens, ≥ 8 $\mu\text{g}/\text{ml}$.

the ribogroup patterns on the basis of the Pearson correlation coefficient by using an optimization coefficient of 1.56%. Similarity coefficients were calculated on the basis of both band positions and relative intensity.

RESULTS AND DISCUSSION

Antimicrobial susceptibility patterns of swine *E. coli* isolates. Beta-hemolytic *E. coli* is the most common bacterial etiologic agent of diarrhea in neonatal and postweaning pigs. Treatment of enteric *E. coli* infection in swine commonly includes the use of broad-spectrum antibiotics (13, 16, 23). We characterized the patterns of susceptibility of 90 *E. coli* isolates from diarrheic neonatal pigs to 17 antimicrobial agents of human and veterinary therapeutic significance. The rates of resistance, as determined by measuring the MICs and comparing them to the resistance breakpoints established by NCCLS, are listed in Table 1. The highest rates of resistance were to tetracycline (96%), sulfamethoxazole (89%), kanamycin (84%), streptomycin (82%), FFN (64%), and CHL (53%). All isolates were susceptible to nalidixic acid, ciprofloxacin, amikacin, and ceftriaxone. Resistance to multiple drugs was frequently observed, with 66 of 90 (73%) of the *E. coli* isolates resistant to five or more antibiotics (data not shown). The swine *E. coli* isolates were similar to other clinical veterinary *E. coli* strains in terms of their decreased susceptibilities to tetracycline, gentamicin, streptomycin, and sulfamethoxazole (1, 11, 17, 19, 22,

TABLE 2. Prevalence of *cmlA*, *cat-2*, and *flo* genes in CHL-resistant swine *E. coli*

Resistance genotype	MIC ($\mu\text{g/ml}$)		No. of isolates positive for resistance gene/no. of isolates tested
	CHL	FFN	
<i>cmlA</i>	≥ 32	8– ≥ 16	43/48
<i>cmlA</i> , <i>cat-2</i>	32	8–16	4/48
<i>flo</i>	256	256	1/48

25, 30, 31). These bacterial isolates also exhibited a rate of resistance to kanamycin similar to that reported previously for bovine *E. coli* isolates (30), a rate of resistance to ampicillin similar to that seen for avian *E. coli* isolates (5), levels of susceptibility to cephalosporins similar to those seen for avian *E. coli* isolates (5), and levels of susceptibility to fluoroquinolones similar to those seen for bovine *E. coli* isolates (30). These similarities and differences in antibiotic resistance exhibited by these three distinct veterinary groups of *E. coli* may reflect therapeutic use or the availability of certain antimicrobial agents for the treatment of infections in poultry, cattle, and swine as well as a shared ecology of drug resistance genes among the farm microbiota (5, 15, 20, 30).

Tetracyclines, aminoglycosides, and sulfonamides are widely used in swine production for the treatment and prevention of disease and for growth promotion, and therefore, a high rate of resistance to drugs in these antimicrobial classes was not unexpected. The phenicols, however, are not approved for use in swine in the United States. CHL has been banned from use in food animals since the mid-1980s, and FFN is approved for use only in cattle (14). Despite the apparent lack of selection pressure, high rates of resistance to these two antibiotics were identified in swine *E. coli* isolates, with 64% of isolates resistant to FFN and 53% of isolates resistant to CHL. For the CHL-resistant *E. coli* isolates, the CHL MIC ranged from 32 to 256 $\mu\text{g/ml}$ and the FFN MIC ranged from 8 to 256 $\mu\text{g/ml}$ (Table 2). Most isolates (47 of 48) were resistant to CHL at 32 $\mu\text{g/ml}$ and FFN at 8 to 16 $\mu\text{g/ml}$. For one isolate the MICs of both CHL and FFN were 256 $\mu\text{g/ml}$.

The *cmlA* gene is widely disseminated among swine *E. coli* isolates. We next investigated the genetic mechanisms for resistance to CHL and FFN by assaying the swine *E. coli* isolates for the presence of five genes known to confer resistance to these antimicrobials: *cmlA*, *cat-1*, *cat-2*, *cat-3*, and *flo*. Using total genomic DNA from each of the 48 CHL-resistant isolates as the template in a PCR, we found that 47 were positive for the *cmlA* gene, with 4 of these isolates also possessing one of the chloramphenicol acetyltransferase genes (Table 2). CHL MICs were not higher for the isolates with a *cmlA*⁺ *cat-2*⁺ genotype than for those with the *cmlA* gene alone, suggesting that there is no additive effect from the two resistance mechanisms.

The one CHL-resistant isolate (isolate CVM873) that did not possess either *cmlA* or *cat-2* was positive for the *flo* gene and was identified as belonging to the O147 serogroup. The *flo* gene has been described previously and confers resistance to both FFN and CHL. The FFN and CHL MICs for this swine isolate were 256 $\mu\text{g/ml}$. The high level of resistance to the phenicols in this *E. coli* isolate is similar to the levels of resistance exhibited by bovine *E. coli* isolates that possess the *flo*

TABLE 3. Pathotypes and serotypes of swine *E. coli*

Pathotype ^a	No. of isolates	No. of CHL ^r isolates ^b	Serotype(s) ^c
None	2	1	O35 (1), O91
Stx2	1	0	O2
EAE	1	0	NT
F4	2	2	O149 (1), NT (1)
CNF1	9	0	O4, O11, O75, O114, O127
F107	12	6	O138, O139 (4), O147 (1), NT (1)
Stx2, F107	4	1	O2, O121, O147 (1), NT
STb, F4	1	0	O98
LT, F4	1	1	O149 (1)
STa, STb, F4	1	0	O8
STa, STb, Stx2	1	0	NT
STa, STb, F107	1	0	O147
STb, Stx2, F107	4	0	O147, NT
LT, STb, F4	27	17	O8, O149 (17), NT
STa, STb, Stx2, F107	21	19	O147 (13), NT (6)
LT, STb, F4, F6	1	0	O8
STa, STb, Stx2, F5, F107	1	1	NT (1)

^a Isolates were positive for the indicated virulence factors: heat-labile toxin (LT), heat-stable toxin A (STa), heat-stable toxin B (STb), Shiga-like toxin II (Stx2), cytotoxic necrotizing factor 1 (CNF1), K88(F4) fimbriae (F4), K99(F5) fimbriae (F5), 987P fimbriae (F6), F107 fimbriae (F107), and the *E. coli* attaching-and-effacing factor (EAE).

^b The number of CHL-resistant (CHL) isolates with the indicated pathotype.

^c Serotypes associated with the corresponding pathotype are listed with the number of CHL-resistant isolates of each serotype (in parentheses). NT, not typeable.

gene (30). Although FFN is approved for use only in cattle in the United States, the presence of the *flo* gene has previously been reported in CHL-resistant *E. coli* strains isolated from chickens (19). Only one isolate possessed the *flo* genotype, yet 47 of 48 isolates that were negative for *flo* were resistant to FFN (MICs, $\geq 8 \mu\text{g/ml}$). The CHL resistance gene, *cmlA*, does not confer resistance to FFN (12), suggesting that a gene reservoir for FFN resistance already exists in swine *E. coli* isolates and involves a gene(s) other than *flo* and *cmlA*. This may present a clinical obstacle for expanded veterinary use of this drug in the treatment of *E. coli*-related swine enteric diseases.

CHL-resistant swine *E. coli* isolates do not represent expansion of a single clone. The persistence of CHL resistance in swine *E. coli* isolates may have resulted from continual colonization with one or a few clonal strains from a common environmental reservoir that was selected earlier when CHL was used therapeutically in the 1980s. We therefore examined the relatedness of all 90 strains according to their serotypes, ribotypes, and the presence of five virulence factors (pathotypes). The *E. coli* pathotypes and O serotypes are listed in Table 3. Fifteen different serotypes were observed, with 58% of the isolates belonging to groups that produce one of three major enterotoxins, O149, O147, and O139 (7, 13). Seventy-nine percent (15 of 19) of O147 *E. coli* isolates and 72% (18 of 25) of O149 *E. coli* isolates were CHL resistant. Fifty-three percent (48 of 90) of swine *E. coli* isolates, of which 75% (36 of 48) were CHL resistant, belonged to one of two *E. coli* pathotypes that possessed either heat-labile toxin, heat-stable toxin B (STb), and F4 fimbriae or heat-stable toxin A, STb, Shiga-like toxin 2, and F107 pili (Table 3). Sixty-four percent (58 of

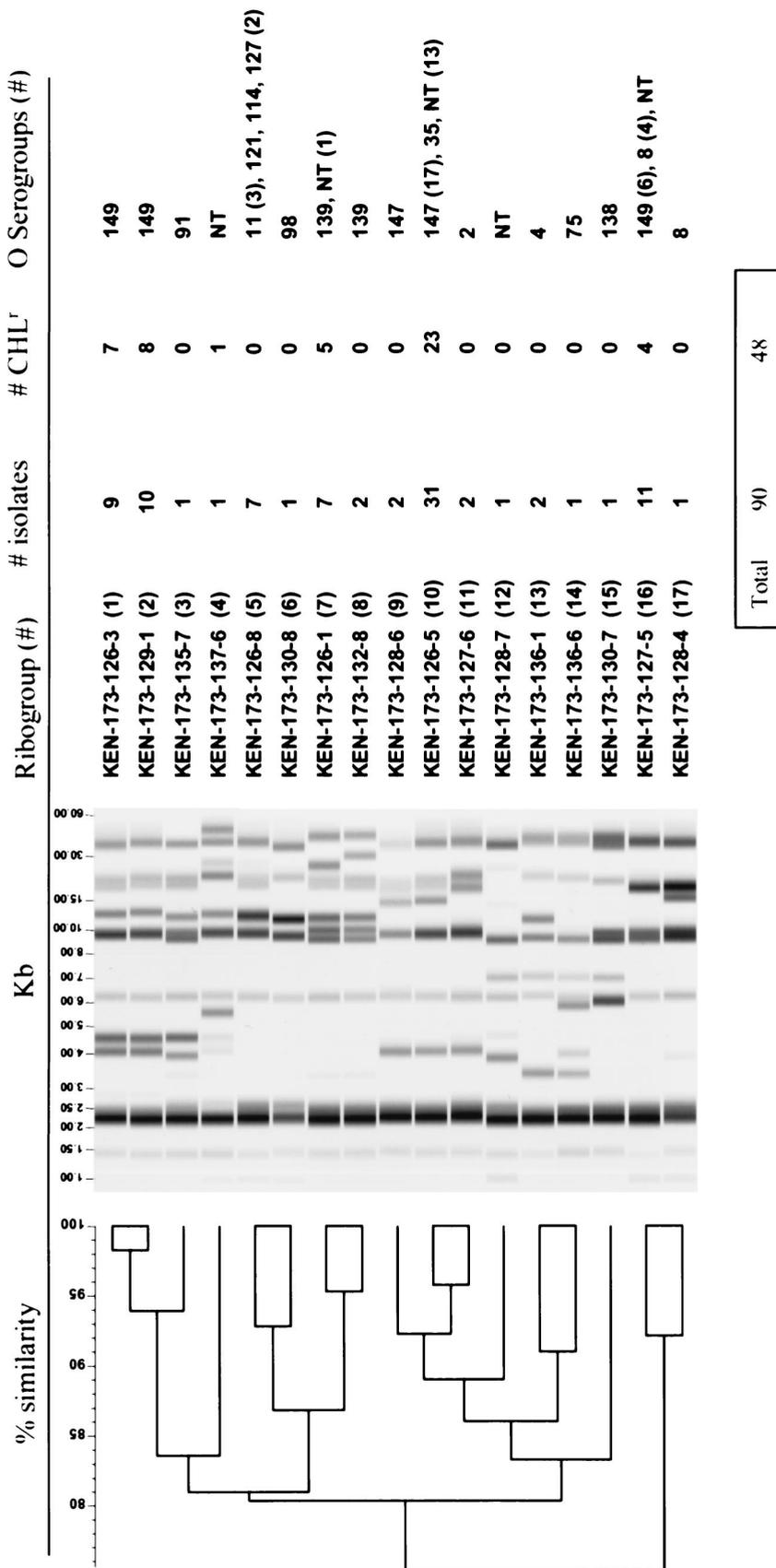


FIG. 1. Ribogroup patterns of swine *E. coli* isolates. The image data for each lane were processed to normalize the band positions relative to the positions of the standards, to reduce background, and to scale the band intensity. The ribogroup name, the total number of isolates per *E. coli* ribogroup, the number of CHL-resistant isolates per *E. coli* ribogroup, and serotypes are shown on the right. The molecular mass scale (in kilobase pairs) and the similarity coefficient are shown at the top of the figure. NT, nontypeable.

90) of the swine *E. coli* isolates possessed the gene for STb, and 36% (32 of 90) possessed the *stx-2* gene. The most common fimbrial antigens detected were F107 (43 of 90 isolates) and F4 (33 of 90 isolates). All isolates were negative for the *stx-1* or the *cnf-2* gene (data not shown). These data demonstrate that CHL resistance does not exclusively belong to any one swine *E. coli* pathotype or O serogroup.

To determine whether CHL-resistant swine *E. coli* isolates represented dissemination of a clonal strain, all 90 isolates were analyzed by ribotyping. Seventeen distinct *E. coli* ribogroups were identified, with 83% of the isolates clustering into six major ribogroups (Fig. 1). CHL resistance was observed among the isolates in all except one of the major ribogroups, with the largest group containing 23 of 31 isolates resistant to CHL. Seventy-nine percent of the CHL-resistant *E. coli* isolates were found in ribogroups 1 ($n = 7$), 2 ($n = 8$), and 10 ($n = 23$). The majority of ribogroups contained *E. coli* isolates of one serotype; however, three ribogroups (ribogroups 5, 10, and 16) contained multiple serotypes. Ribogroup 10 was the largest cluster identified ($n = 31$) and was composed of isolates comprising primarily serotype O147 and 13 nontypeable isolates. Ribogroup 16 was the second largest cluster ($n = 11$) and was composed of isolates of either serogroup O149 or serogroup O8, suggesting a close evolutionary relationship between these two serotypes. Ribogroups 1 and 2 contained isolates that were only of the O149 serogroup and that were genetically similar to each other; however, they vastly differed from the other ribogroup with serogroup O149 strains (ribogroup 16) (Fig. 1). All ribogroups that possessed multiple strains (ribogroups 1, 2, 5, 7, 8, 9, 10, 11, 13, and 16) included isolates recovered from diseased swine in both 1998 and 1999 (data not shown), indicating the persistence of virulent bacterial clones. The potential for the use of ribotyping as a tool for subtyping within certain serotypes is supported by the clustering of *E. coli* isolates with common serotypes in different ribogroups. The different ribogroups of *E. coli* isolates are shown in Fig. 1 to share serotype O149 (ribogroups 1, 2, and 16), serotype O139 (ribogroups 7 and 8), and serotype O8 (ribogroups 16 and 17). Confirmation of the appropriateness of ribotyping as a subtyping tool for pathogenic swine *E. coli* will require testing of additional isolates from multiple sources, time periods, and geographical locations.

Taken together with the serotyping and virulence gene data, we conclude that, unlike *Salmonella enterica* serovar Typhimurium DT104 (8), the high rate of CHL resistance in our isolates does not represent the clonal expansion of a single resistant strain but the dissemination of *cmlA* among genetically diverse *E. coli* isolates. We have made similar observations with regard to the FFN resistance gene, *flo*, in bovine and avian *E. coli* isolates (19, 30). Another possibility for the prevalence of the CHL resistance phenotype in swine *E. coli* isolates is that a plasmid carrying the *cmlA* gene is widely disseminated among these isolates. Bovine and avian *E. coli* isolates that are CHL resistant have been found to carry the *flo* gene on large plasmids that also contain genes for multiple drug resistance (19, 30).

All 48 of our CHL-resistant isolates from swine were also resistant to at least four other drugs (data not shown). CHL resistance may be coselected with other antimicrobial resistance phenotypes if a linkage exists between their respective

genes. We examined the resistance phenotypes to determine whether CHL resistance can be coselected by other antimicrobials. There was no preferential selection of CHL-resistant strains by most antimicrobials. A statistically high level of significance for coselection, however, was observed with kanamycin ($P = 0.0001$), sulfamethoxazole ($P = 0.0002$), and tetracycline ($P = 0.018$), agents commonly used in swine in the United States. The use of these agents may serve to maintain plasmids on which CHL resistance determinants reside with other resistance genes. Also, *cmlA* may be linked to ETEC virulence plasmids (17, 18), creating a physical linkage that would ensure the persistence of the CHL resistance phenotype among swine ETEC isolates. Further analysis of the genetic location of *cmlA* is needed to address this issue.

In the early 1980s, studies reported rates of CHL resistance among *E. coli* isolates from commercial swine herds in South Dakota and Utah of 20 and 11%, respectively (11, 22). A survey of veterinarians at that time found that CHL was the preferred drug for the treatment of neonatal colibacillosis, a practice that presumably selected for CHL-resistant strains. The U.S. Food and Drug Administration has banned the use of CHL as a therapeutic agent in food animals since the mid-1980s. Since this ban has been actively enforced, the apparent selection pressure for resistance to this drug should have been removed. Thus, our report of a 53% rate of resistance to CHL is an unexpected, but not an unprecedented, finding. Several studies in Europe have also reported persistent rates of resistance years after withdrawal of CHL as a therapeutic drug for farm animals (1, 25). A recent report by Aarestrup et al. (2) indicated that it was possible to reduce the occurrence of antimicrobial resistance in enterococci isolated from food animals when the antimicrobial selection pressures were removed. They also demonstrated that antimicrobial resistance can persist, most likely as a consequence of coselection with other antimicrobials. The identification of the *cmlA* gene among diverse beta-hemolytic ETEC strains suggests the broad dissemination of this genotype in the swine production environment and suggests that CHL resistance can persist even in the absence of CHL selection pressure. This is most likely due to coselection of CHL resistance with either common swine ETEC virulence genes or other antimicrobial resistance phenotypes. Regardless, our data suggest that the withdrawal of antimicrobials from use in response to increased rates of resistance may not be an effective strategy for restoration of the therapeutic effectiveness of a specific drug. Simultaneous reductions in the selection pressures of coselecting agents may be required to reverse the emergence, spread, and persistence of antimicrobial resistance in the animal production environment.

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